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Fundamentals of Pharmaceutical Nanoscience

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Preface

Comments on the Early History of the Controlled Drug Delivery Field, and the Development of "Nanopharmaceuticals"

The field of drug delivery is as old as man. Ancient man chewed natural products, or cooked them in water and drank their extracts, all to alleviate a variety of pains or other body problems. On the other hand, the field of *controlled* drug delivery systems (DDS) really began only about 60 years ago. The earliest controlled DDS devices appeared in the 1950s. They were called "Spansules" and were oral drug tablets with coatings that dissolved at various rates and then released the drug all at once after the coating was gone. The composition and/or thickness of the coating could be varied to control the time before drug release.

In the mid-1960s, Judah Folkman at Harvard Medical School discovered that when he circulated rabbit blood through a silicone rubber tube and exposed the tubing to anaesthetic gases on the outside, the rabbits went to sleep. So he proposed a new way to administer a drug at a controlled rate by encapsulating a drug in a sealed silicone capsule and implanting it. The wall thickness and surface area of the capsule then determined a constant rate of drug release, as long as saturated drug conditions existed within the capsule. This was really the beginning of the controlled release field.

At the same time, a pharmaceutical chemist-entrepreneur named Alejandro Zaffaroni had been thinking about starting a company around the concept of controlled DD, and in 1968 he founded Alza Corp. in Palo Alto, CA. Folkman was invited to be the Chairman of his Scientific Advisory Board. Alza was the first company dedicated to the field of controlled DD, and it led the way by patenting and getting FDA approval for a wide range of drug delivery devices. Many others soon followed with their own compositions and concepts. In this very productive period of the 1970s and 1980s, a number of new controlled DD devices and compositions were developed and clinically approved. They included a contraceptive drug-loaded poly(ethylene-co-vinyl acetate) or poly(EVA) IUD for insertion into the uterus, six contraceptive drug-loaded silicone rubber tubes for subcutaneous implantation, a drug-loaded skin patch for topical application (seven different drug skin patches

were developed and approved), a glaucoma drug-loaded poly(EVA) sandwich wafer for insertion into the eye, a drug-loaded acrylic sandwich hydrogel for oral ingestion and so on. All of these controlled DDS were macroscopic in scale.

In the early to mid-1980s, drug-loaded, degradable microparticles composed of poly[lactic-co-glycolic]acid (PLGA) were developed for subcutaneous implantation and delivery; several were approved for clinical use. Early work on PLGA microparticles took place at the University of AL and Southern Research Institute in Alabama by Tice, Lewis, Kent, Lacey, Sanders and others. All of these DDS were microscopic in scale.

The development of nano-scale DDS began in parallel in the 1970s with the much larger size macroscopic DDS but it has taken a much longer time for nanocarrier DDS to reach the clinic. This is in part due to the fact that nanocarrier DDS are designed to be injected into the blood, and they are usually composed of both drug molecules and polymer molecules. Thus, control of the molecular weight and compatibility of the carrier molecules themselves are important regulatory issues, along with the drug itself. The injection of drug-loaded degradable polymer microspheres such as PLGA can raise similar regulatory issues.

In the early 1970s, the concept of PEGylation of protein drugs was introduced by Davis at Rutgers University. In 1976 Langer and Folkman published the delivery of active protein drugs from a protein-loaded hydrophobic polymer matrix, where the proteins dissolved from the surface into the surroundings, and created nano-sized pores in the matrix for further drug delivery. In the mid-1970s in Prague, Kopecek was developing a new polymer carrier (poly[hydroxypropyl methacrylamide], or PHPMA), and they covalently conjugated drugs to the backbone. Kopecek also introduced the concept of attaching cell-targeting ligands to the PHPMA, and along with Duncan in the UK, conjugated the drug via a lysosomal enzyme-degradable peptide linkage to the polymer backbone. Duncan has published extensively on nanocarriers for intracellular drug delivery. Other important nanocarrier DDS were subsequently developed in the 1980s and 1990s and included liposomes (Bangham "discovered" liposomes in 1965 and 30 years later, in 1995, the liposome-doxorubicin product called "Doxil®" became the first nanocarrier-drug DDS approved for clinical use). A-B diblock copolymer micelles were developed as drug carriers in Japan around 1989, especially with PEG as one of the blocks, by Kataoka and Okano. Kabanov in the USA also developed A-B-A triblock Pluronic® micelles as drug carriers. Many different block copolymer micellar drug carriers are in clinical trials today. This text brings the reader up to date with this rapidly expanding and exciting field of drug nanocarriers.

Comments on This Volume, "Fundamentals of Pharmaceutical Nanoscience"

This book is both unusual and special. It is unusual in that it covers such a broad range of topics and their sub-specialities that it is like several books combined into one. It is special because it is both comprehensive and detailed. The particular topics

and sub-specialities that are included represent the most important fundamental and practical aspects of drug delivery nanoscience. This book will be extremely useful for the beginning undergraduate students in pharmaceutical sciences, and for the graduate researchers in drug delivery technology, as well as for the research directors and leaders in this field. It is a comprehensive book that each of these scientists will want to have on his or her bookshelf.

The book is divided into three major parts: Part I—Nanomaterials Fabrication, Characterization and Use; Part II—Concepts Underpinning the Application of Biomedical Nanomaterials; and Part III—Therapeutic and Diagnostic Applications.

Part I includes chapters describing the many different and diverse drug nanocarriers that have been developed. These nanocarriers include those based on natural surfactants and carriers (e.g. liposomes and solid lipid nanoparticles), synthetic polymeric carriers (e.g. micelles, polymersomes, polymeric nanoparticles and polymer–drug conjugates), nano-scale dispersed drugs (e.g. drug nanocrystals and nanoemulsions) and inert "porous" nanocarriers (e.g. porous Si nanoparticles and carbon nanotubes).

Part II includes chapters that cover the three major delivery issues: targeting, drug loading and dissolution and biologic barriers. The last topic area includes barriers encountered in the three major drug delivery routes (e.g. transdermal, oral and mucosal) and in two important blood-related barriers (e.g. blood–brain barrier and blood–eye barrier).

Part III is a very diverse and important part of this comprehensive book. It covers applications and analytical tools of nanomedicine. The first five chapters in this part cover key drug delivery applications that are the cutting edge of the drug delivery field today: cancer chemotherapy, vaccines, anti-infectives, gene and siRNA therapy, and biomolecular drugs and ligands (peptides, proteins and antibodies). The next three chapters of Part III cover topics that are not as directly related to drug delivery as earlier chapters have been; nevertheless, these topics are growing in importance in the pharmaceutical field. They cover the emerging application areas of: tissue engineering (also called regenerative medicine); imaging, which is critical to targeted delivery of the drug, as well as to evaluation of drug efficacy; biosensors and diagnostics which are also vital to optimizing the dose and evaluating drug efficacy. The last chapter of this book is a practical discussion of the various commercialization aspects of pharmaceutical nanoscience.

In summary, this book is one of the most comprehensive books available that combines both the fundamental pharmaceutical principles of nanocarrier drug delivery plus the most important practical applications of that nanotechnology.

Seattle, WA Allan S. Hoffman

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Chapter 1
Introduction

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Abstract Nanoscience is the study of properties and characteristics that are unique to the nanoscale and pharmaceutical nanoscience is the application of the ensuing knowledge to the development of pharmaceuticals. Materials properties residing at the nanoscale have been found to offer a number of pharmaceutical products advanced functionality. Nanoparticles are fabricated from various chemistries, e.g. polymer-drug conjugates, self-assembled polymers, self-assembled low molecular weight amphiphiles, silica, crystalline iron salts, hydrophobic polymer nanoprecipitates and drug nanocrystals. Principally, with nano-enabled entities, there is unprecedented control of the in vivo fate of the drug compound and the science has subsequently already led to a number of approved products, e.g. Doxil, Ambisome and Abraxane with others in clinical development. As well as being able to control the biodistribution of drug compounds, emerging applications of pharmaceutical nanoscience include the use of nanofabricated materials for regenerative medicine and their application in diagnostic imaging.

1.1 Pharmaceutical Nanoscience

Pharmaceutical nanoscience is not a new discipline but activity in this area has grown considerably over the past 40 years. In the past the formulation of medicines was limited to the presentation of such compounds as solutions, compressed powders (tablets), encapsulated powders and oils (capsules), coarse suspensions and emulsions. Scientific and technological advances have moved beyond these conventional dosage forms to dosage forms firmly underpinned by nanoscience—the nano-enabled dosage forms or nanomedicines (Fig. 1.1).

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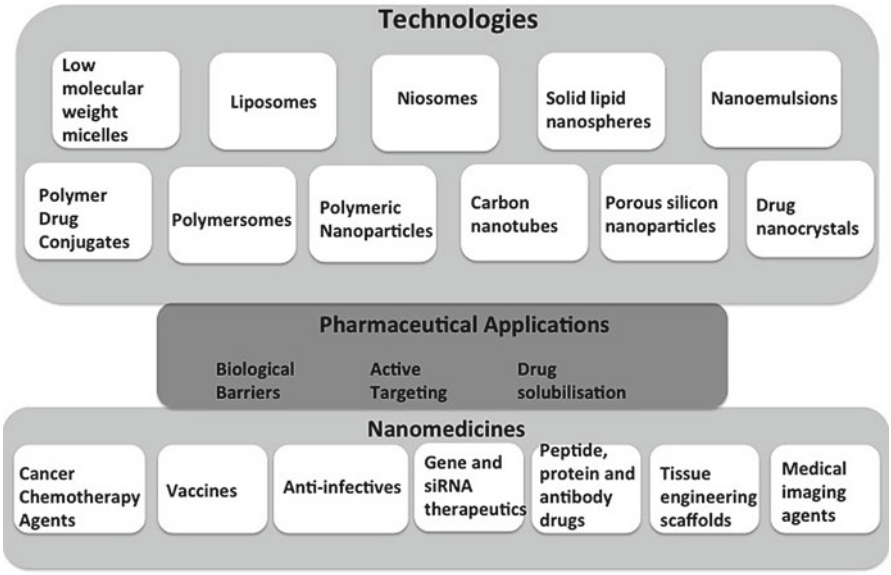

Fig. 1.1 Pharmaceutical nanoscience technologies, applications and the resulting nanomedicines

Nanomedicines consist of functional nanoparticles presented in a liquid or solid matrix. Nanomedicines have been launched in which nanoscale properties have ensured unprecedented control of the molecule's biological destination when administered for the treatment of disease, e.g. Doxil — liposomal doxorubicin (Gabizon 2001) and Abraxane — albumin stabilised crystalline paclitaxel (Cortes and Saura 2010), both cancer nanomedicines. Additionally nanoparticulate imaging agents have now been commercialised (Uchegbu and Siew 2013). At the heart of these nanosystems lies a basic scientific phenomenon — self-assembly. Most of the nanosystems discussed in this volume are the product of molecular design, which predisposes towards classical self-assembly or a form of self-assembly e.g. controlled precipitation in aqueous media. The resulting self-assemblies/nanoprecipitates present as nanoparticles of 10–800 nm in diameter. Some nanosystems, such as the polymer-drug conjugates (Duncan 2003), present as molecular entities in solution but on administration behave like a nanoparticle. Inorganic silica and carbon nanoparticles are also being increasingly studied. However, classical self-assembly and controlled and stabilised nanoprecipitation lie at the heart of most nanomedicines. As such polar lipids assemble into micelles and liposomes, amphiphilic polymers self-assemble into polymeric micelles, polymeric vesicles and dense amorphous nanoprecipitates and both drug particles and hydrophobic polymers form nanoprecipitates in the presence of a polar amphiphile. The science underpinning these pharmaceutical nanoparticles is of course noteworthy, but how do these nanosystems assist us in drug development. In other words, how is pharmaceutical nanoscience relevant to the treatment of disease?

1.2 Pharmaceutical Innovation

Pharmaceutical nanoscience has led to real pharmaceutical innovation. This innovation is exemplified by the development of nanotechnologies that are capable of fundamentally changing the way in which drugs work, with a reduction in side effects and an improvement in therapeutic indices documented for some of the early exponents such as Doxil (a reduction in cardiac toxicities (Gabizon et al. 2003)) and Ambisome (a reduction in renal toxicities (Lee et al. 1994; Walsh et al. 1998)). These nanotechnologies have made drugs active by directing them to the site of pathology; such as with the peptide analgesic nanofibres, which enable peptide delivery to the brain (Mazza et al. 2013). Nanotechnologies have also been effective at increasing the overall systemic exposure, e.g. by promoting drug absorption via the oral route for example (Siew et al. 2012). It is clear that by using nanoparticles, drug delivery across important biological barriers such as the blood brain barrier is achievable (Fig. 1.2).

In the 1970s it was realised that conjugating a hydrophilic polymer to a protein drug resulted in an enlargement in its hydrodynamic diameter, a degree of metabolic stability and as such a resistance to both glomerular filtration and liver degradation (Veronese 2009). By the 1990s, liposomes, which until then had been an academic curiosity, demonstrated that the encapsulation of a hydrophilic drug within the liposome's hydrophilic core could achieve a change in the drug's biodistribution, causing it to accumulate in tumour areas, extravasate the vasculature and avoid heart tissue, the site of drug's toxicity (Gabizon et al. 2003). The net result was the launch of Doxil (liposomal doxorubicin). Finally in 2001, it was discovered that the

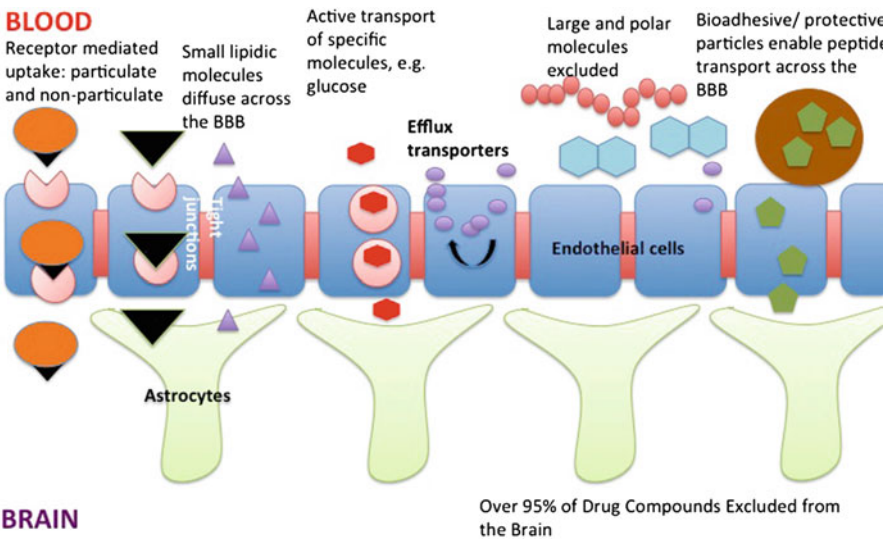

Fig. 1.2 Drug delivery across the challenging biological barrier: the blood brain barrier

hydrophobic drug paclitaxel could be stabilised in aqueous media by binding non-covalently to albumin (Paal et al. 2001). Further studies revealed that the albumin coating on nanocrystalline paclitaxel could direct drug to secreted amino acids rich in cysteine (SPARC) residues (Cortes and Saura 2010). SPARC residues are associated with malignant tissue. In essence, these data underpinned the launch of Abraxane.

Currently researchers are using functionality at the nanoscale to develop drugs to treat neurological disorders (Lalatsa et al. 2012a, b; Mazza et al. 2013) and malignancies (Davis et al. 2010; Hrkach et al. 2012) and to develop diagnostics for use in cancer patients (Mayes et al. 2012).

It is interesting to note the current move away from merely investigating the impact of particle size on biodistribution and biological properties to a concerted and international effort to investigate the impact of shape on biological properties. This could lead to a new field of nanogeometry. The work of Discher's group, documented in this volume elegantly, demonstrates that high axial ratio structures, the worm-like micelles give rise to fundamentally different biological behaviours. Mitragotri (Mitragotri 2009) and DeSimone (Gratton et al. 2008) have shown that the shape of a particle alters the way in which a particle interacts with cells, with rod-like particles being favourably internalised by HeLa cells (Gratton et al. 2008) and rod-like particles interacting favourably with macrophages (Mitragotri 2009; Doshi and Mitragotri 2010). Furthermore our own work has shown that peptide nanofibres are excellent systems for the delivery of peptides to the brain (Mazza et al. 2013). With data from these early studies now firmly presented, there are now ample opportunities for scientists to study how nanoparticle shape (e.g. discs, fibres, spheres (Lalatsa et al. 2012a, b) quantitatively alters drug biodistribution, pharmacological activity and toxicokinetics; i.e. leading to the new discipline of nanogeometry (Ferrari 2008).

In essence the last 40 years have seen nanoscience grow up to yield nanotechnology and eventually nanomedicines. This volume serves as an informative introduction to this pharmaceutical innovation.

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Part I
Nanomaterials Fabrication, Characterisation and Use

Chapter 2
Low Molecular Weight Micelles

Ijeoma F. Uchegbu

Abstract Low molecular weight amphiphile micelles are formed from the self-assembly of comparatively hydrophilic amphiphiles (molecular weight <1,500 Da). These structures may be spherical or present as nanofibres in the case of peptide amphiphiles; the latter with one axis in the 5–20 nm size range. Micelles are formed from amphiphiles in aqueous media and micelle formation is driven by the need to reduce the energetically unfavourable interactions between the hydrophobic regions of the amphiphilic molecule and the bulk water molecules. Micelles are used for the delivery of hydrophobic drugs and are usually used in intravenous formulations. Hydrophobic drugs may be encapsulated within the hydrophobic micelle core, increasing the level of hydrophobic drug that may be incorporated within aqueous media by over 1,000-fold in some cases. The characterisation of micelles for pharmaceutical use involves a determination of: the critical micellar concentration (the concentration at which micellisation starts), the colloidal stability of the dispersion, micelle particle size, micelle morphology and the drug encapsulation or drug solubilisation capacity of the micellar dispersion. Micelles formed from low molecular weight amphiphiles are dynamic structures and there is continuous exchange of material between the micellar aggregate and the bulk medium; this dynamic exchange has a negative effect on the stability and biocompatibility of micellar formulations.

2.1 Introduction

Micelles are colloidal aggregates formed by the self-assembly of amphiphiles (Fig. 2.1) and the use of micelles in various industrial and domestic applications is not new. The solubilisation of fats for example, which essentially involves the

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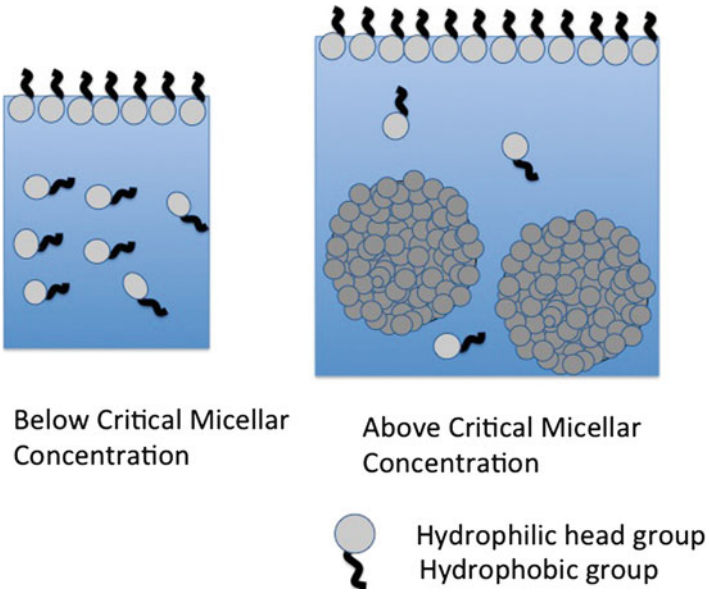

Fig. 2.1 The self-assembly of amphiphiles into micelles. Below the critical micellar concentration, the molecules exist as monomers and above the critical micellar concentration, the monomers exist as micelles with a hydrophobic core and a hydrophilic surface. Drug solubilisation in aqueous media is achieved by the incorporation of hydrophobic drugs within the apolar micellar core

encapsulation of fats within a micellar core by amphiphiles (soaps), is many thousands of years old. However, it was at the start of the twentieth century that the first formal identification of micellar systems was made by McBain and colleagues (Laing and McBain 1920). The colloidal aggregates formed when fatty acids are added to alkalis were termed micelles and described as molecular aggregates. The micelle has since taken its place as a fundamental unit involved in the dispersion of hydrophobic drugs within aqueous media (Florence and Attwood 2006) and the dispersion of dietary fats within the small intestine (St-Pierre et al. 2001).

2.2 Materials Chemistry

Micelles are formed from amphiphilic compounds in which the hydrophilic and hydrophobic chemical moieties lie in geometrically distinct regions of the molecule. The low molecular weight amphiphiles, which form micelles, are relatively hydrophilic (e.g. Fig. 2.2). Israelachvili's Critical Packing Parameter (CPP) has been used to categorise the types of self-assemblies that will arise from a range of amphiphilic molecules (Israelachvili 2011). Essentially the resulting self-assembly

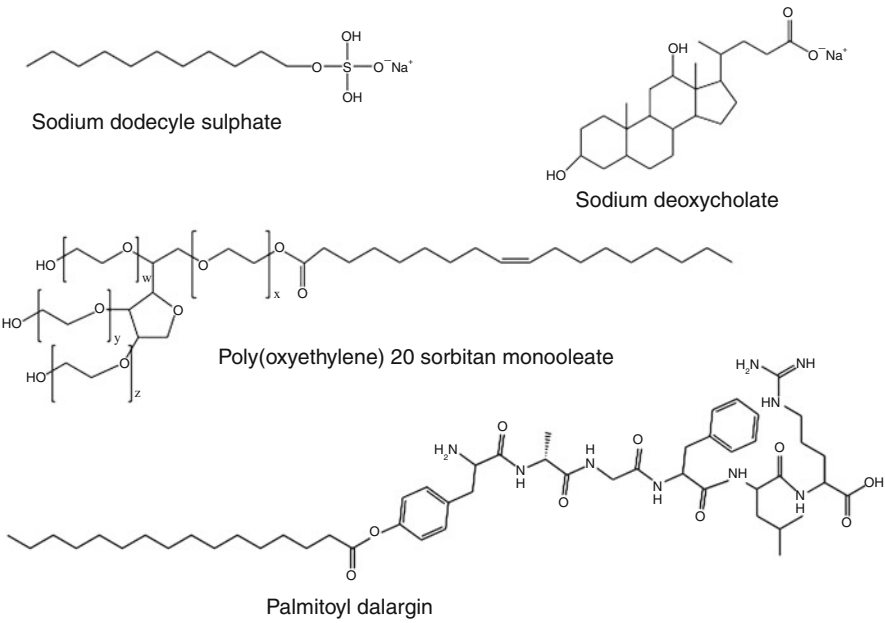

Fig. 2.2 Micelle forming amphiphiles used in pharmaceutical formulations. Sodium dodecyl sulphate, sodium deoxycholate and poly(oxyethylene) 20 sorbitan monooleate form spherical micelles while palmitoyl dalargin forms nanofibres

is defined by the relative sizes of the hydrophobic and hydrophilic regions of the molecule (2.1).

$$CPP = \frac{v}{a_0 l_c} \tag{2.1}$$

where v = the volume of the hydrocarbon, a_0 = the hydrophilic head group area and l_c = chain length. A CPP of less than 0.33 (symptomatic of relatively hydrophilic molecules) leads to the formation of spherical micelles within aqueous media, while a CPP of between 0.5 and 1 (applicable to relatively hydrophobic molecules) leads to the formation of closed bilayers — ultimately vesicles, such as liposomes and niosomes in aqueous media. A CPP of above 1 (indicative of extremely hydrophobic molecules) results in the formation of reverse micelles in non-aqueous media. In reverse micelles, which form in non-aqueous media, the core of the micelle is hydrophilic and the surface hydrophobic and thus the hydrophilic head groups are shielded from the non-aqueous bulk phase in the self-assembly. The majority of pharmaceutical applications of micelles involve the use of spherical micelles in aqueous media. Essentially micelles are characterised by a fluid interior and form in aqueous media when the optimum hydrophilic head group area is large enough to support a high radius of curvature in the self-assembly and yet still permit the

hydrocarbon chain to be in a fluid state (Israelachvili 2011). As well as spherical micelles, amphiphilic molecules may self-assemble into fibrous (Cui et al. 2010) micellar structures.

2.3 Micellisation

Micellisation takes place when the mean activity of the monomer in the aggregate (μ_N^0) is less than the activity of the unaggregated monomer (μ_1^0) (2.2) (Israelachvili 2011).

$$\mu_N^0 < \mu_1^0 \tag{2.2}$$

Micelles may form spontaneously in aqueous media or with the input of a little energy, either by shaking or vortexing or may be formed with the input of significant energy such as that resulting from probe or bath sonication.

At low concentrations in water (below the critical micellar concentration — CMC) amphiphilic molecules, with a hydrophobic moiety and a hydrophilic moiety, orient themselves at the air–water interface and in the bulk disperse phase they exist as monomers (Fig. 2.1). As the concentration of the amphiphiles increases, the amphiphilic molecules aggregate, such that the hydrophobic moiety is shielded from the aqueous medium. The driving force for the micellisation at room temperature is usually the release of water molecules adjacent to the hydrophobic moiety, which are now free to hydrogen bond with one another and thus experience an entropy gain (Tanford 1980). Micellisation occurs at a critical concentration, the CMC and a critical temperature, the critical micellar temperature, and is the point at which the amphiphile is no longer soluble in the aqueous medium as a monomer. Once the monomer's intrinsic solubility is exceeded, micelle forming molecules then proceed to aggregate and shield the hydrophobic moieties in the molecule from the aqueous disperse phase. The micelles thus consist of a hydrophobic core and a hydrophilic surface and the hydrophobic core may be used to encapsulate drug molecules. Spherical micelles are typically 5–20 nm in diameter and so are at the smaller end of the nanoparticle spectrum (Florence and Attwood 2006). The final size of the micelle is ultimately controlled by a desire to minimise the entropy deficit associated with the formation of a large-sized structure (Israelachvili 2011). Micelles may either be produced in aqueous media or in non-aqueous media and in the latter case, they are then known as reverse or inverted micelles. Although the CPP appears to define the molecules which form micelles within a relatively narrow range (2.1), micelles may be formed from a number of chemical entities, e.g. molecules with the same hydrophilic head group but with different hydrophobic groups. Micelles formed by a homologous series of alkyl substituents sharing a common hydrophilic head group exhibit CMCs which vary linearly with the number of carbon atoms (m) in the alkyl or acyl unit (2.3) (Florence and Attwood 2006).

$$\log[CMC] = A - Bm \tag{2.3}$$

Where *A* and *B* are constants specific for the particular homologous series. As becomes immediately apparent, the more hydrophobic an amphiphile the lower, of course, is its CMC. The formation of micelles from amphiphiles is not limited to low molecular weight compounds and indeed polymers also form micelles (Wang et al. 2004; Siew et al. 2012); however, the current chapter is limited to a discussion of micelles formed from low molecular weight amphiphiles (Molecular weight <1,500 Da). One notable thing about low molecular weight micelles is that since they are formed from relatively hydrophilic compounds (Fig. 2.2), the structures are relatively dynamic. The aggregate residence time of the monomers (t_R) is comparatively low ($t_R = 10^{-4}$ s) when compared to the residence time of monomers within a bilayer ($t_R = 10^4$ s) (Israelachvili 2011). This dynamic nature has a negative impact on micelle stability and also contributes to the toxic effects encountered with micellar formulations. The dynamic nature of the aggregate and relatively high monomer aqueous solubility means that micelle monomers will be present in relative abundance in the aqueous bulk and thus may easily integrate within membranes and cause haemolysis for example; integrating within membranes by simple diffusion in response to a concentration gradient. A study of poly(oxyethylene) 20 sorbitan monooleate micelles compared to polymeric micelles formed from *N*,*N*,*N*-trimethyl, *N*,*N*-dimethyl, *N*-monomethyl, *N*-palmitoyl, 6-*O*-glycol chitosan (quaternary ammonium palmitoyl glycol chitosan—GCPQ) revealed that while poly(oxyethylene) 20 sorbitan monooleate micelles caused 80 % haemolysis at a concentration of 1 mg mL^{-1}, GCPQ micelles caused no haemolysis at a concentration of 1 mg mL^{-1} and less than 20 % haemolysis at a concentration of 10 mg mL^{-1} (Siew et al. 2012). Both poly(oxyethylene) 20 sorbitan monooleate and GCPQ have CMCs in the micromolar range (Patist et al. 2000; Siew et al. 2012), indicating that the toxicity observed with poly(oxyethylene) 20 sorbitan monooleate may be attributed to the dynamic nature of the low molecular weight amphiphile micelles. Additionally when the micelle forming amphiphile Solulan C24 is contained within a non-ionic surfactant bilayer, it is less toxic to Caco-2 cell monolayers when compared to Solulan C24 micelles or Solulan monomers (Dimitrijevic et al. 1997).

2.4 Preparation of Micellar Formulations

At the laboratory scale, micellar formulations are prepared by dissolving the amphiphile and drug in organic solvents, evaporating the organic solvent to form a film and then hydrating the film with agitation (Brajtburg et al. 1994a, b; Javali et al. 2012). In a minor variation of this more commonly used technique, the drug may be added to the preformed micelles (formed by hydrating an amphiphile film subsequent to evaporation of an organic solution of the amphiphile), followed by filtration to remove unentrapped drug (Li et al. 2011). To avoid the use of organic solvents, however, micelle forming drugs may simply be added to aqueous media and the mixture probe sonicated and filtered to yield the micellar formulation (Cheng et al. 1999). Likewise peptide nanofibres may be produced by probe sonication of the

peptide amphiphile in aqueous media (Mazza et al. 2013). Additionally at the industrial scale, the methods used to make liposomes (Gregoriadis 2006) may also be employed for the preparation of micelles; namely the size reduction of a coarse dispersion resulting from the hydration of a thin film using high pressure homogenisation, microfluidisation and high pressure extrusion.

2.5 Characterisation of Micellar Formulations

To characterise micellar formulations, it is necessary to first determine the CMC of the amphiphilic molecules. This provides a contextual basis for most, if not all of the relevant properties. Generally a lower CMC is preferable as this will ensure that the micelles are not destabilised on dilution. There are various methods that are used to measure the CMC; these methods exploit the fact that the macroscopic properties of an aqueous dispersion of micellar aggregates changes profoundly at the CMC. For example the surface tension progressively decreases as molecules associate at the air aqueous interface (Fig. 2.1); this continues until the surface tension reaches a limiting value and a point at which the addition of further molecules to the aqueous dispersion does not result in the molecules associating at the surface but results in the molecules aggregating into micelles (Fig. 2.1). Surface tension measurements as a function of concentration thus provide a means of determining the CMC (Nagadome et al. 1992). Additionally fluorescent and colorimetric probes may be used to determine the CMC. These probes alter their spectroscopic characteristics on being solubilised within the micelle. For example the hypsochromic shift experienced by methyl orange, as it is solubilised within the micellar apolar core, may be monitored (Karukstis et al. 1998) as the concentration of the amphiphile increases (Fig. 2.3) (Wang et al. 2004). Furthermore isothermal calorimetry may be used to determine the CMC by preparing demicellisation enthalpograms. With this method, a dispersion of the micelles is progressively added to the calorimetry cell and the enthalpy change of micellisation monitored; once the CMC is reached inside the calorimetry cell, demicellisation is halted and micelles begin to form, causing a break point in the enthalpy change with concentration data. Table 2.1 details the various methods which may be used to determine the CMC of a pharmaceutical amphiphile.

The CMCs of the most commonly used pharmaceutical low molecular weight amphiphiles are shown in Table 2.2.

Following determination of the CMC, methods such as light scattering to measure molecular weight may be used to determine the aggregation number of micelles (Okano et al. 2000; Wang et al. 2004). Micelle size may be determined by photon correlation spectroscopy (Dal Bo et al. 2012) or electron microscopy (Mclean et al. 1989). The zeta potential of a micellar dispersion may also be determined by measuring the electrophoretic mobility of the micelles in the dispersion. The solubilisation capacity of the micelles must also be determined, i.e. number of moles of drug solubilised per mole of micelle forming amphiphile. The micellar solubilisation capacity depends on the nature of the drug, nature of the micelle forming surfactant and other environmental factors such as temperature, and pH

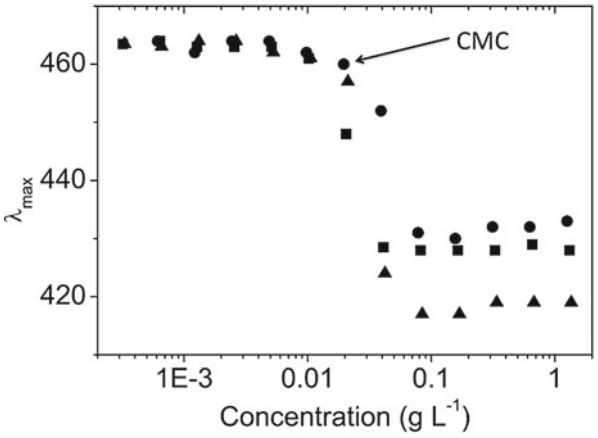

Fig. 2.3 Determination of the critical micellar concentration of low molecular weight cetyl poly(ethylenimine) by measuring the hypsochromic shift in methyl orange dispersions: *filled circle* = LCPEI14 [linear *N*-cetyl poly(ethylenimine), 14 mol% cetylation, molecular weight = 740 Da], *filled square* = LCPEI23 [linear *N*-cetyl poly(ethylenimine), 23 mol% cetylation, molecular weight = 870 Da], *filled triangle* = LCPEI42 [linear *N*-cetyl poly(ethylenimine), 42 mol% cetylation, molecular weight = 1,320 Da]

(with ionic amphiphiles or ionic drugs) (Florence and Attwood 2006). In essence the more hydrophobic the amphiphile, and hence the lower the CMC, the more drug that is encapsulated within the micelle.

As micellar dispersions are metastable structures, data on the colloidal stability of the micellar drug formulation must also be collected. This may be achieved by monitoring the micelle particle size, drug encapsulation and micellar morphology over time.

The European Medicines Agency (EMA) recently outlined the relevant factors to consider when developing a micelle formulation for intravenous administration (European Medicines Agency 2012). As well as requiring applicants for a product licence to provide information on the CMC, solubilising capacity and stability of the micelles/ micelle formulations; the EMA also requires applicants to establish the ratio of free and encapsulated drug present in the formulation and the fate of encapsulated drug and micelles in vivo, with a requirement to quantify encapsulated vs. non-encapsulated drug in vivo or at least model the trajectories of these formulation components.

2.6 The Application of Micellar Formulations to Pharmacy

2.6.1 Established Formulations

Micelle forming low molecular weight amphiphiles such as the poly(oxyethylene) sorbitan esters, bile salts and sodium dodecyl sulphate (Fig. 2.2) are used in pharmaceutical formulations as tablet lubricants, in topical formulations as emulsifiers

Table 2.1 Methods for the determination of the critical micelle concentration

Method	Principle	Reference	Advantages	Comments
Surface tension	Measures the change in surface tension caused by progressively more molecules associating at the air—aqueous interface; a limiting value signals the onset of micellisation	Nagadome et al. (1992)	Simple methodology	Large quantities of sample required (>100 mg)
Conductivity	Measures the change in conductivity as ionic surfactants aggregate into micelles	Mehrotra and Jain (1992); Okano et al. (2000)	Simple methodology	Only suitable for ionic amphiphiles
Capillary electrophoresis	Measures the conductivity change when ionic amphiphiles aggregate into micelles	Cifuentes et al. (1997)	Low sample requirements <10 mg	Only suitable for ionic amphiphiles
Cyclic voltammetry	Measures the change in current associated with an electrochemical probe, at varying potential and in the presence of a micelle forming amphiphile	Mandal et al. (1988)	Relatively simple experimentation	Only suitable for ionic amphiphiles
Isothermal calorimetry	Measures the enthalpy of demicellisation as the micelles are diluted	Hildebrand et al. (2004)	Accurate method provides additional information on other thermodynamic parameters (e.g. the entropy and free energy change of micellisation)	Expensive instrumentation
Nuclear magnetic resonance spectroscopy	Measures the chemical shift changes of a relevant proton as the amphiphile self assembles into micelles	Zhao and Fung (1993)	Small sample requirements — mg	Expensive instrumentation
Colorimetry—methyl orange	Measures the change in the absorption spectrum, the hypsochromic shift, experienced by methyl orange as it associates with the hydrophobic micelle core	Karukstis et al. (1998); Buwalda and Engberts (2001); Wang et al. (2004)	Rapid analysis	Tends to overestimate the CMC value (Siew et al. 2012)
Flourescence spectroscopy—pyrene	Measures the change in the emission spectra of pyrene as it associates with the hydrophobic core of the micelle	Kalyanasundaram and Thomas (1977); Chooi et al. (2010)	Rapid analysis	Errors may arise if pyrene associates with the monomers in solution, leading to an underestimation of the CMC (Chooi et al. 2010)

Table 2.2 The critical micellar concentration of pharmaceutical low molecular weight amphiphiles

Compound	CMC (mM)	Temperature of determination (°C)	CMC methodology	Reference
Sodium dodecyl sulphate	8.2	20	1. Conductivity 2. Dye solubilisation	Williams et al. (1955)
Poly(oxyethylene) 20 sorbitan monooleate	0.018	22	Surface tension	Patist et al. (2000)
Sodium deoxycholate	6.5[a]	25	Pyrene fluorescence	Matsuoka and Moroi (2002)

[a]This is the second CMC when stable micelles begin to form. Sub-micellar aggregation is seen at 2.5 mM (Matsuoka and Moroi 2002)

(sodium dodecyl sulphate) or as emulsifiers, solubilisers and suspending agents in topical, oral or parenteral formulations [poly(oxyethylene) sorbitan esters and sodium deoxycholate] (Rowe et al. 2009). Parenteral formulations provide the best examples of the use of low molecular weight amphiphiles as micellar dispersions (Strickley 2004). Poly(oxyethylene) 20 sorbitan monooleate (Molecular Weight = 1,310 Da) (Rowe et al. 2009) is the most widely used micelle forming low molecular weight amphiphile in pharmaceutical formulations (Nema et al. 1997; Powell et al. 1998). An example of a micellar dispersion comprising poly(oxyethylene) 20 sorbitan monooleate as the micelle forming amphiphile is Hectorol, a doxercalciferol intravenous formulation (Table 2.3). Poly(oxyethylene) 20 sorbitan monooleate has a comparatively low CMC of 18 μM (Table 2.2), making it an ideal agent for parenteral formulations in particular, where stability to dilution in the plasma is critical. Only aqueous formulations may be administered intravenously and poly(oxyethylene) 20 sorbitan monooleate is the excipient of choice for formulation of drugs with poor aqueous solubility for intravenous use. Although there are numerous experimental studies outlining the use of low molecular weight amphiphiles to solubilise hydrophobic drugs (Lasic 1992; Alvarez-Nunez and Yalkowsky 2000; Jain et al. 2010), the actual commercial exploitation of micellar systems is not particularly widespread, despite the fact that most new chemical entities originating from drug discovery programmes are hydrophobic molecules (Kirkpatrick 2003). Examples of some commercial micelle formulations are given in Table 2.3.

2.6.2 Experimental Formulations

Micelles are dynamic structures in which there is a fairly vigorous movement of the amphiphilic molecules from the micelle to the bulk liquid as outlined above; this dynamic nature is often associated with an inherent instability. In an effort to produce more stable delivery systems, a number of researchers have prepared mixed

Table 2.3 Commercial micellar formulations

Drug	Trade name	Indication(s)	Manufacturer	Formulation and administration	Reference
Calcitriol	Calcijex	Hypocalcemia associated with chronic renal dialysis	Abbott	A micellar dispersion containing: calcitriol (1 μg mL^{-1}), poly(oxyethylene) 20 sorbitan monolaurate (4 mg mL^{-1}), sodium ascorbate (10 mg mL^{-1}), sodium chloride (1 mg mL^{-1}), ethylene diamine tetraacetic acid (1.1 mg mL^{-1}), sodium phosphate (9.2 mg mL^{-1}), pH = 6.5–8.0 Administered as an intravenous bolus	Strickley (2004)
Doxercalciferol	Hectorol	Secondary hyperparathyroidism associated with chronic renal dialysis	Bone care	A micellar dispersion containing: doxercalciferol (2 μg mL^{-1}), poly(oxyethylene) 20 sorbitan monooleate (4 mg mL^{-1}), sodium ascorbate (10 mg mL^{-1}), sodium chloride (1.5 mg mL^{-1}), disodium ethylene diamine tetraacetic acid (1.1 mg mL^{-1}), sodium phosphates (9.2 mg mL^{-1}) Administered as an intravenous bolus	Strickley (2004)
Amphotericin B	Fungizone	Life-threatening fungal infections, mucocutaneous leishmaniasis	Bristol Myers Squibb	A lyophilised solid containing: amphotericin B (50 mg), sodium desoxycholate (41 mg), sodium phosphate (20.2 mg) Reconstituted in 10 mL water for injection and further diluted to an amphotericin B concentration of 0.1 mg mL^{-1} in dextrose (5 % w/v) injection. Administered as a slow infusion	Dailymed (2012)

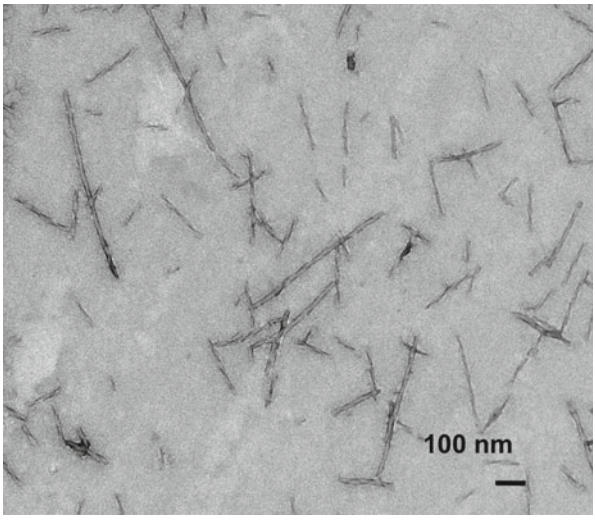

Fig. 2.4 Palmitoyl dalargin peptide nanofibres [reproduced with permission from (Lalatsa et al. 2012)]

micelles in which the micelle is composed of hydrophilic micelle forming amphiphiles and the less hydrophilic bilayer forming phospholipids.

Mixed micellar formulations composed of sodium cholate, an undisclosed phospholipid and the poorly soluble compound silybin (solubility in water < 50 μg mL^{-1}), produced a 150 % increase in the oral bioavailability of the drug in dogs, when compared to silybin *N*-methyl glucamide (Yu et al. 2010). Silybin is an extract of milk thistle (*Silybum marianum*), with antifibrotic and anti-inflammatory properties (Loguercio and Festi 2011). A further example of the application of mixed micelles is in the transdermal delivery of diclofenac in sodium deoxycholate — lecithin mixed micelles (Hendradi et al. 2003). This micellar formulation was an effective anti-inflammatory agent in preclinical studies and showed a reduction in swelling when applied to the skin of a carrageenan-induced hind paw oedema rat model. The reduction in oedema was increased by the incorporation of cyclic terpenes such as D-limonene and L-menthol. The positive effects on the inflammation model were attributed to the solubilisation of diclofenac within the mixed micelles and the greater flux of diclofenac through the skin (Hendradi et al. 2003).

As well as spherical micelles, there have been recent reports of drug delivery using nanofibres—essentially micelles with a long axial ratio. Amphiphilic poly(amino acids) form a wide range of structures on self-assembly in aqueous media: micelles, bilayer vesicles and nanoparticles; the specific structure formed is driven by the amphiphile's chemistry (Lalatsa et al. 2012). Amphiphilic peptides, comprising a peptide attached to a fatty acid moiety at one end (e.g. palmitoyl dalargin—Fig. 2.1), form peptide nanofibres (Cui et al. 2010; Mazza et al. 2013) of ~20 nm in diameter and up to 1 μm in length (Fig. 2.4). The nanofibre morphology consists of a hydrophobic shaft with the peptide beta sheet wrapped tightly around

this central shaft (Mazza et al. 2013). The lipidised peptides adopt a cylindrical morphology as opposed to a spherical morphology, as the hydrophobic association of the lipidic moieties on the molecules is balanced, not by electrostatic or steric repulsion by the molecules' hydrophilic head groups as would be seen with a spherical morphology, but by the formation of a peptide beta sheet; the beta sheet prevents the formation of a spherical morphology as the hydrated amphiphiles are not cone-shaped (as is seen with spherical micelle forming molecules), but are cylindrical in shape and unable to support the radius of curvature required for a spherical morphology. The arrangement of molecules at the tip of the nanofibre is not entirely clear but it is possible that the molecules are more loosely associated at the end of the fibres. It can be envisaged that the molecules at the end of the fibres could adopt a different intermolecular arrangement in an effort to escape the entropy penalty associated with uncontrolled fibre elongation.

Recently we reported that peptide nanofibres, such as palmitoyl dalargin, enable the delivery of peptides to the brain (Mazza et al. 2013). When the analgesic peptide dalargin was intravenously administered to mice, the peptide was not detected in the brain and there was no pharmacological activity observed as a result. On the contrary, when palmitoyl dalargin peptide nanofibres (20 nm in diameter and 500 nm in length) were administered intravenously, the peptide was delivered to the brain and was pharmacologically active (Mazza et al. 2013). The plain peptide, dalargin, was rapidly degraded in the plasma, whereas the peptide nanofibres, by virtue of the fact that the beta sheet is wrapped tightly around the nanofibre core, were not degraded rapidly in the plasma and were able to cross the blood brain barrier, leading to activity from the peptide (dalargin) once it was cleaved from its peptide prodrug (palmitoyl dalargin).

Peptide nanofibres are also useful for the delivery of hydrophobic drugs. Peptide nanofibres prepared from the amphiphilic peptide: palmitoyl-$A_4G_3E_3$ (12 nm in diameter and several microns in length) increased the level of camptothecin present in aqueous media by 50-fold by encapsulating the hydrophobic drug within the hydrophobic peptide nanofibre core (Soukasene et al. 2011). The peptide nanofibre camptothecin formulation also inhibited tumour growth in a mouse orthotopic breast cancer model (Soukasene et al. 2011). This peptide nanofibre camptothecin formulation was just as efficacious as a solution of camptothecin in PEG 400, propylene glycol and polysorbate 80.

Peptide nanofibres are just emerging as biomaterials (Cui et al. 2010) and no doubt additional applications will be forthcoming in the very near future.

2.6.3 Toxicology

The short aggregate residence times (t_R) enjoyed by micelle forming molecules (Israelachvili 2011) and the presence of a relatively high level of solubilised monomers in the bulk medium enable micelle monomers to interact deleteriously with

cell membranes. The short micelle t_R also contributes to the instability of micellar formulations. The haemolysis caused by micellar amphiphilic molecules such as poly(oxyethylene) 20 sorbitan monooleate (Siew et al. 2012) is one of the reasons why the levels of micelle forming amphiphiles injected intravenously are controlled; acceptable levels are typically less than 10 % w/v (Rowe et al. 2009). As would be expected from the foregoing, haemolytic potential varies inversely with the CMC and hence haemolysis follows this trend with the commonly used amphiphiles of decreasing CMC: sodium dodecyl sulphate > sodium deoxycholate (Ross et al. 2004). In essence the more hydrophilic micelle forming amphiphiles have a higher CMC and so there is a relatively high concentration of monomers external to the micelle in equilibrium with the micelle; such monomers are able to incorporate within and lyse membranes

The instability of micellar formulations, as a result of the high t_R, also contributes to their toxicological profile. For example the intravitreal injection of 1-*O*-octadecyl-sn-glycerol-3-phosphonoformate, indicated for the treatment of cytomegalovirus infections, as a self-assembled micelle results in retinal damage in rabbits with the drug micelles (Cheng et al. 1999). No retinal damage was observed when 1-*O*-octadecyl-sn-glycerol-3-phosphonoformate was formulated as a dioleyl phosphatidyl choline and cholesterol liposome. In the case of the micelles however, 1-*O*-octadecyl-sn-glycerol-3-phosphonoformate precipitated from the micellar formulation resulting in local opacities. This opacity lasted for up to 8 weeks with higher drug doses (0.1 mL of an 885 mM formulation) and resulted in optic nerve oedema in the high-dose animals. One case of retinal detachment was recorded out of four animals.

Additionally micellar Fungizone formulations of amphotericin B (Table 2.3) are more toxic to mammalian cells than liposomal formulations of amphotericin B as there is more self-associated amphotericin B unbound to the micelles in the micellar formulations and this self-associated and non-micellar amphotericin B is toxic to mammalian cells (Brajtburg et al. 1994a, b). The toxic effects of amphotericin B are caused by the extraction of membrane cholesterol and subsequent K^+ leakage. These micellar toxicities were not limited to in vitro situations as the intravenous injection of Fungizone caused more kidney toxicity (increase in plasma creatinine levels) when compared to the intravenous injection of liposomal and polycarprolactone/polaxamine 188 particulate amphotericin B formulations (Echevarria et al. 2000) even though there was less exposure to total amphotericin B with the Fungizone formulation. Interestingly, heating amphotericin B — deoxycholate micelles (4 nm in diameter and tubules) at 70 °C for 20 min reduced its toxicity by about tenfold (van Etten et al. 2000). Mice died when given an intravenous dose of 0.8 mg/kg of the standard formulation, but only died when given a 7 mg/kg intravenous dose of the heated formulation. Ultimately this reduced toxicity leads to the ability to administer higher doses and ultimately improves the efficacy of the formulation. The heating altered the micelles and produced larger 300 nm aggregates, which presumably were an entirely different arrangement of the molecules.

2.7 Conclusions

Low molecular weight amphiphile spherical micelles are 5–20 nm in diameter and formed from relatively hydrophilic amphiphiles. Low molecular weight amphiphile micelles are generally used in pharmacy for the delivery of hydrophobic drugs within an aqueous disperse phase, with the drug residing within the hydrophobic core of the micelle. The vast majority of micellar formulations on the market are the result of a need to administer a hydrophobic drug into the aqueous blood compartment. The level of micelle forming amphiphile must be controlled to limit the toxicity that arises as a result of the dynamic nature of the micellar self-assembly. A new type of pharmaceutical micelle has emerged recently, i.e. the peptide nanofibre. Peptide nanofibres are cylindrical micelles of 10–20 nm in diameter and 500 nm to a few microns in length. They are formed from short chain amphiphilic peptides in which the peptide chain is attached to a lipidic group. Peptide nanofibres, prepared from a lipidic prodrug of the peptide, enable the delivery of peptides to the brain and peptide nanofibres may also be used for the intravenous administration of hydrophobic drugs encapsulated within the peptide nanofibre core.

Problem Box

Q1: What are the main molecular attributes of a micelle forming low molecular weight amphiphile?

Answer

Low molecular weight micelle forming amphiphiles have a molecular weight of less than approximately 1,500 Da and contain geometrically distinct hydrophobic and hydrophilic regions. Generally molecules that form micelles in aqueous media are hydrophilic and are usually miscible with water while molecules that form reverse micelles in non-aqueous media are usually relatively hydrophobic.

Q2: How many low molecular weight amphiphile micelles be used in pharmacy?

Answer

Low molecular weight amphiphile micelles may be used for the incorporation of hydrophobic drug compounds in aqueous media. An example of a pharmaceutical product relying on micellar drug delivery is Hectoral, a doxercalciferol intravenous injection formulated with poly(oxyethylene) 20 sorbitan monooleate micelles.

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Chapter 3
Liposomes

Jessica Kalra and Marcel B. Bally

Abstract Lipid-based nanoparticles such as liposomes have been at the forefront of drug delivery science since their discovery in the 1960s. Although liposomes were used initially as model membrane systems, creative scientists quickly saw the potential for liposomes as drug carriers. Since then, liposomes have been used as small-scale carrier systems capable of delivering low molecular weight drugs as well as large proteins and even therapeutic nucleic acid sequences. These formulations can be designed to passively target areas of disease. Additionally, lipid composition may be used to control when and where a liposome-associated therapeutic agent is released. In this chapter, the general makeup of lipid carriers, the manufacturing processes used to generate them, important physical characteristics, and their behavior in vivo are discussed. Examples of biomedical applications are given, including a review of some liposomal drug formulations that have already been approved for clinical use.

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List of Abbreviations

AAT	Alpha-1antitrypsin
ASON	Antisense oligonucleotides
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CTL	Cytotoxic T lymphocytes
DDS	Drug delivery system
dLOS	Deacylated lipooligosaccharide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium- propane (chloride salt)
DOTMA	*N*-[1-(2, 3-dioleyloyx) propyl]-*N*-*N*-*N*-trimethyl ammonia chloride
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPC	Distearoylphosphatidylcholine
EM	Electron microscopy
EPR	Enhanced permeability and retention
GC	Glucocorticoid
HAV	Hepatitis A virus
hdls	High-density lipoproteins
i.v.	An intravenous
IBD	Inflammatory bowel disease
LUV	Large unilamellar vesicle
MHCII	Major histocompatibility complex class II
MLV	Multilaminar vesicles
MPS	Mononuclear phagocytic system
MRI	Magnetic resonance imaging
NMR	Nuclear magnetic resonance
OMP	Purified outer membrane proteins
PEG	Polyethylene glycol
PKN3	Protein kinase N3
RES	Reticuloendothelial system
SAXS	Small angle X-ray scattering
SDBS	Sodium dodecylbenzene sulfonate
SEC	Size exclusion chromatography
siRNA	Small interfering ribonucleic acids
SUV	Small unilamellar vesicle
T_c	Transition temperature
VEGF	Vascular endothelial growth factor

3.1 Introduction

A *nanoparticle* is a microscopic polymer, protein, or lipid-based structure between 1 and 200 nm in size. Examples of nanoparticles include liposomes, polymeric micelles, capsules, colloids, and dendrimers. Nanoparticles are being investigated as tools in biomedical, optical, and electronic research. *Nanomedicine* is the application of nanoparticles and nanotechnology in medicine. The rationale behind employing nanoparticles in medicine revolves around their utility as a delivery vehicle. Drug delivery system (DDS) has been shown to improve the pharmacological and therapeutic properties of the associated agent, while at the same time affording the agent with protection against premature degradation. In some circumstances DDS are designed for intelligent delivery, where nanoparticles distribute to specific targets. The biomedical applications of nanoparticles are only beginning to be realized. For example, nanoparticles have been used in the delivery of radioactive and contrast agents for diagnostic imaging such as Ultrasound and Magnetic Resonance Imaging (MRI). Therapeutic agents that have been incorporated into nanoparticles include cytotoxic agents in the treatment of cancer, immunogenic agents in the production of vaccines, monomers and macromolecules, and nucleic acids for the purpose of gene delivery.

Of the nanocarriers, *liposomes* have been widely studied and a substantial amount of research has focused on characterization of these structures since their discovery 5 decades ago. Lipid-based vesicles were first described in 1965 by a British hematologist, Dr. Alec D Bangham, from the Babraham Institute near Cambridge (Bangham 1993). Dr. Bangham discovered that when phospholipid molecules (Fig. 3.1) from egg lecithin are introduced to an aqueous solution, they spontaneously organize into three dimensional bilayers (Fig. 3.2a). Each bilayer is able to compartmentalize the aqueous solution including any solutes dissolved within that solution (Fig. 3.2b) (Bangham 1978).

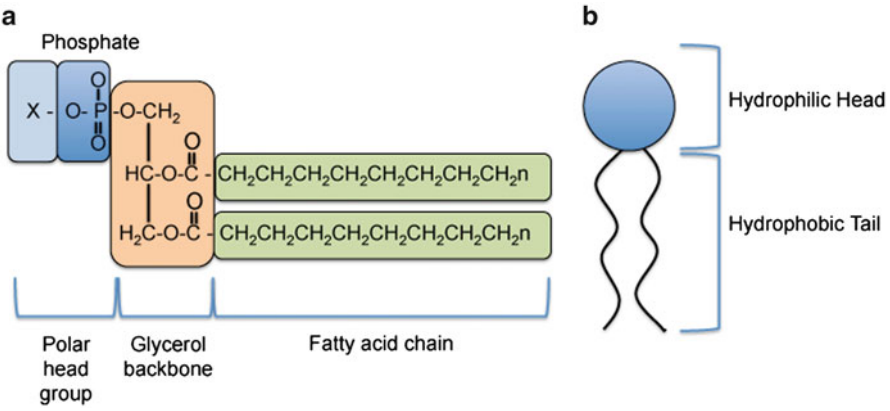

Fig. 3.1 The molecular structure of a generic phospholipid (**a**), and its symbolic representation (**b**)

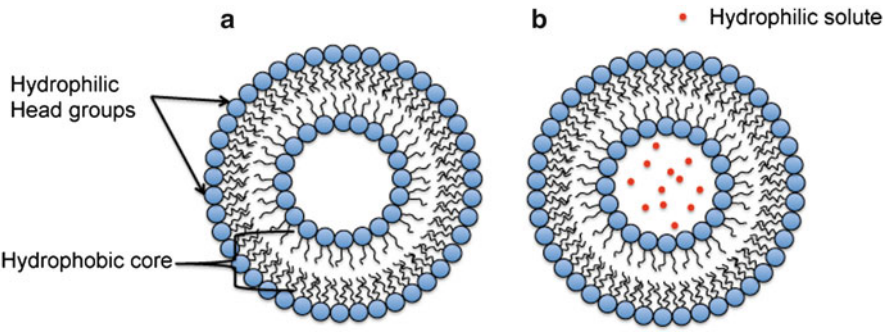

Fig. 3.2 A two-dimensional illustration of the lipid bilayer surrounding a lipid-derived vesicle (**a**), and a liposome showing encapsulated solute (*red circles*) (**b**)

As seen in the cell membrane, the lipid membrane that forms a liposome organizes into a bilayer due to the orientation of the phospholipids in water. Recall that phospholipids are *amphipathic* in nature, which means phospholipids have both hydrophobic and hydrophilic properties (Fig. 3.1). In water, the hydrophobic fatty acid chains of the phospholipids aggregate together to exclude water creating the hydrophobic inner core of the bilayer, while the hydrophillic head groups orient themselves towards the aqueous solution (Fig. 3.2a).

Dr. Bangham and his colleague R. W. Horne stumbled upon their discovery when they were experimenting with a new electron microscope. They noted that by adding a negative stain in aqueous solution to dry phospholipids isolated from egg lecithin, vesicular structures they termed smectic mesophases would spontaneously form (Bangham and Horne 1964). The resemblance to the plasma membrane was appreciated immediately and the resulting electron microscope images served as the first real evidence of the lipid-based bilayered nature of the cell membrane (Fig. 3.3). At the outset, the utility of these lipid structures as a model to study membrane structure and function was fully recognized and studies investigating membrane transport, permeability, adhesion, and fusion quickly ensued. Additionally, lipid vesicles were used to study the evolution of the plasma membrane of early cells.

In 1968, Sessa and Weissmann coined the term *liposome* (Sessa and Weissmann 1968), which is derived from the Greek words: *lipo* (“fat”) and *soma* (“body”). A liposome is a synthetic, small (30 nm to 5 μm), spherical, tubular, or ovoid particle, composed of amphipathic lipids such as phospholipids (Fig. 3.1, Table 3.1) which organize into lipid bilayers that surround an aqueous solution (Fig. 3.2). As a result of their spontaneous organization, liposomes placed in a solution containing polar solutes are able to compartmentalize these solutes (Fig. 3.2b).

Many types of lipid-based nanoparticle structures can form when amphipathic lipids are immersed in different solutions. These structures include liposomes (Fig. 3.4a), micelles (Fig. 3.4b), and reverse micelles (Fig. 3.4c). Liposomes differ from micelles in that the latter are composed of monolayers rather then bilayers.

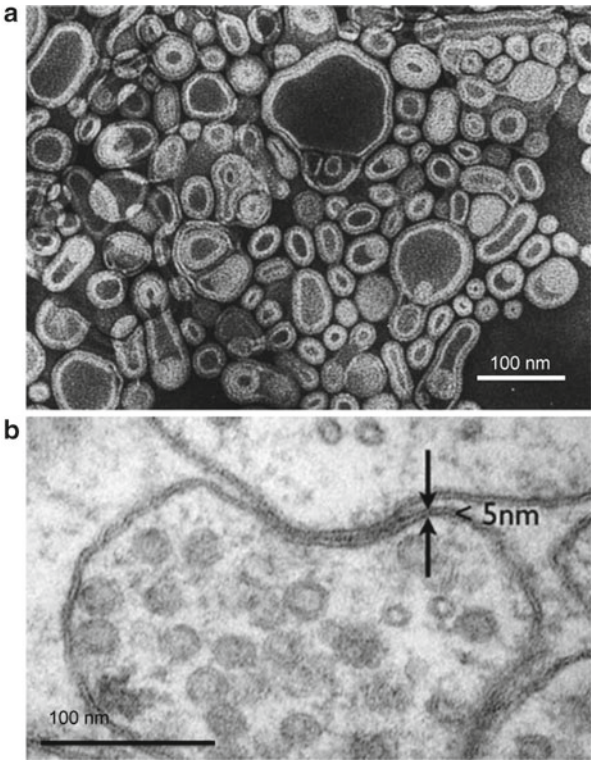

Fig. 3.3 Electronmicrograph of highly sonicated 4 % phosphatidic acid-phosphatidyl choline liposomes negativley stained with ammonium molybdate in the presence of 300 mM *n*-butanol (**a**) (Johnson and Bangham 1969), and a neuromuscular junction in *C. elegans* (**b**) (Jorgensen, S.W.a.E. http://www.research.utah.edu/advanced-microscopy/education/electron-micro/index.html)

However, the phospholipid organization of a micelle is similar to that of liposomes where the hydrophobic tails organize interiorly to exclude water, and the phospholipid head groups organize exteriorly facing the aqueous solution. A reverse micelle forms when amphipathic lipids are placed in a nonpolar solution. In this case, the hydrophobic tails are exteriorized facing the nonpolar solution, while the hydrophilic head groups are interiorized potentially interacting with and encapsulating polar structures.

It was Gregory Gregoriadis who first proposed the drug carrier utility of these lipid vesicles, demonstrating that liposomes can be used as carriers of enzymes and suggesting the application to other agents in biology and medicine (Gregoriadis 1976, 1978; Gregoriadis et al. 1971; Gregoriadis and Ryman 1972). The bilayered nature of liposomes makes them an attractive delivery system for both hydrophilic and hydrophobic substances. Hydrophilic molecules dissolved in the aqueous solution used to prepare the liposomes can be trapped in the water compartments,

Table 3.1 Lipids used in the formation of liposomes and their properties

Name	Abbreviation	Fatty acid	Transition temperature	Net charge at pH 7.4
Egg phosphatidylcholine	EPC		–15–17	0
Dilauryloylphosphatidylcholine	DLPC	12:0	–1	0
Dimyristoylphosphatidylcholine	DMPC	14:0	23	0
Dipalmitoylphosphatidylcholine	DPPC	16:0	41	0
Distearoylphosphatidylcholine	DSPC	18:0	55	0
I-Myristoyl-2-palmitoylphosphatidylcholine	MPPC	14:0,16:0	27	0
I-Palmitoyl-2-myristoyl phosphatidylcholine	PMPC	16:0, 14:0	35	0
I-Palmitoyl-2-stearoyl phosphatidylcholine	PSPC	16:0,18:0	44	0
I-Stearoyl-2-palmitoyl phosphatidylcholine	SPPC	18:0, 16:0	47	0
Dioleoylphosphatidylcholine	DOPC	18:1	–20	0
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	DOPE	18:1	–16	0
1,2-dioleoyl-sn-glycero-3-phosphate	DOPA	18:1	–8	–1.3
Dilauryloylphosphatidylglycerol	DLPG		4	–1
Dimyristoylphosphatidylglycerol	DMPG	14:0	23	–1
Dipalmitoylphosphatidylglycerol	DPPG	16:0	41	–1
Distearoylphosphatidylglycerol	DSPG		55	–1
Dioleoylphosphatidylglycerol	DOPG	18:1	–18	–1
Dimyristoyl phosphatidic acid	DMPA	14:0	50	–1.3
Dipalmitoyl phosphatidic acid	DPPA	16:0	67	–1.3
Dimyristoyl phosphatidylethanolamine	DMPE		50	0
Dipalmitoyl phosphatidylethanolamine	DPPE		63	0
Dimyristoyl phosphatidylserine	DMPS	14:0	35	–1
Dipalmitoyl phosphatidylserine	DPPS	16:0	54	–1
	DOPS	18:1	–11	–1
Brain phosphatidylserine	PS		6–8	–
Brain sphingomyelin	BSP		32	0
Dipalmitoyl sphingomyelin	DPSP		41	0
Distearoyl sphingomyelin	DSSP		57	0

Adapted from (Szoka and Papahadjopoulos 1980) (http://avantilipids.com)

whereas hydrophobic molecules can associate with the hydrophobic core of the phospholipid bilayers (Fig. 3.5).

Liposomes are being used by the food, cosmetic, agricultural, and pharmaceutical industries as carrier systems for the protection and delivery of vaccines, drugs, enzymes, macromolecules, or other substances. Since the early days of liposome research, numerous studies have been published as well as entire volumes on the manufacture and utility of these vesicles in medicine. In this chapter the data generated over the last 50 years is synthesized and topics including the general makeup of lipid carriers, manufacturing processes, physical characteristics, behavior in the human body, and biomedical applications are considered.

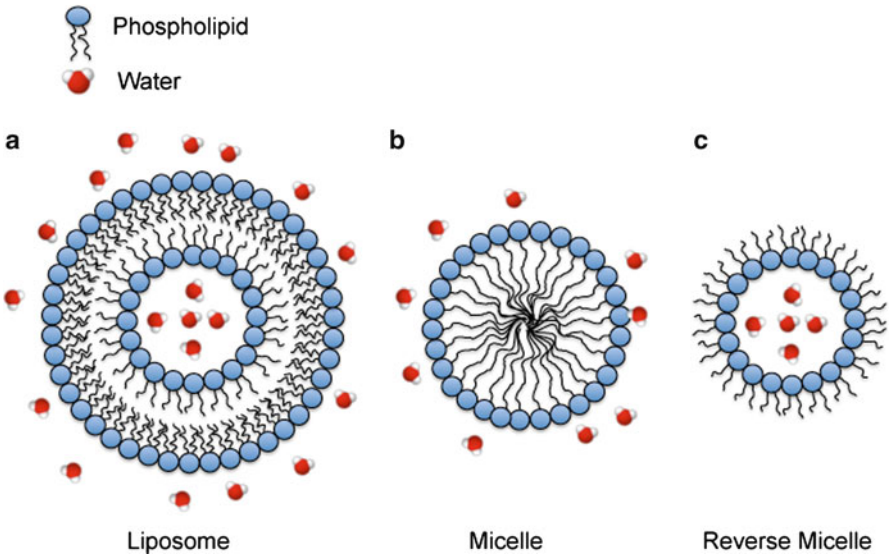

Fig. 3.4 A two-dimensional illustration of the phospholipid organization of a liposome (**a**), micelle (**b**), and reverse micelle (**c**)

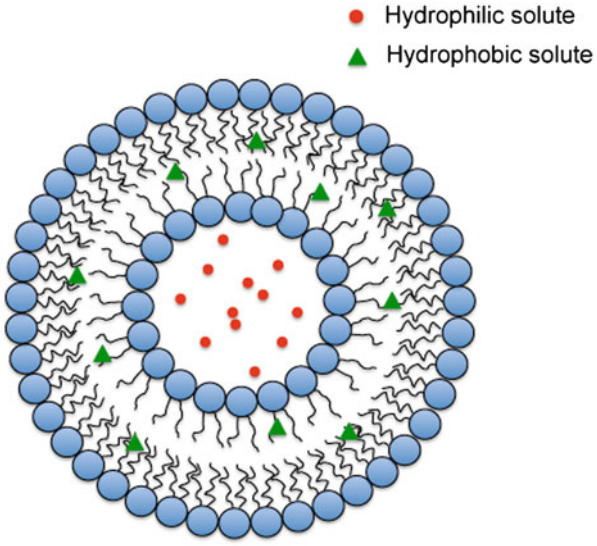

Fig. 3.5 The association of a liposome with hydrophillic (*red circle*) and hydrophobic (*green triangles*) solutes

3.2 Materials Chemistry

Liposomes can be prepared using many different types of lipids. As the liposome is a synthetic analog of the cell membrane, the two types of lipids commonly used to construct the basic structure of the vesicle are also found in the cell membrane: phospholipids and cholesterol. Common phospholipids used for vesicle preparation include naturally derived lipids such as Egg phosphatidylcholine (Egg PC) or Soya phosphatidylcholine (Soya PC). These lipids comprise a mixture of phosphatidylcholine species with differences in fatty acyl chain composition. For medical applications it is now considered better to use synthetic or semisynthetic lipids that represent only one lipid species as these lipids can be chemically defined and their behavior when incorporated into liposomes is predictable. Some examples of synthetic lipids include 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Distearoylphosphatidylcholine (DSPC), or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). Synthetic or semisynthetic nonionic, anionic, or cationic surfactant can also be included in a liposome formulation. There are currently thousands of synthetic lipids that have modified chemical head groups designed to exhibit anionic charges or cationic charges and to promote unique functions when included in the liposomes; functions such as temperature sensitivity, pH sensitivity, and light sensitivity. Semisynthetic and synthetic lipids are cost-effective and analytically definable. The choice of lipid(s) will depend on the desired function and properties of the formulation being made. Table 3.1 lists commercially available phospholipids for liposome manufacturing and some of their key properties that may influence liposome formulation, such as, fatty acid chain length and saturation, charge, and *transition temperature* (T_c), the temperature at which lipids undergo a gel-liquid crystalline phase transition.

Liposomal structures composed of phospholipids alone can be unstable in biological systems such as serum or plasma. To minimize instability, cholesterol (Fig. 3.6a)

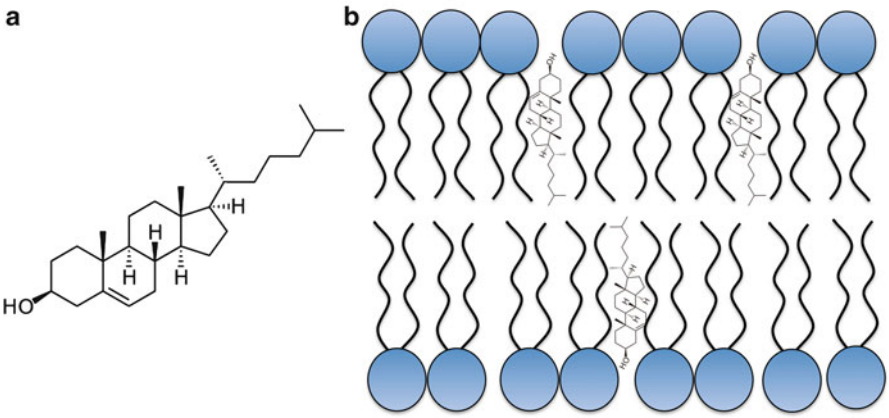

Fig. 3.6 The molecular structure of cholesterol (**a**), and an illustration of the incorporation of cholesterol into the phospholipid bilayer (**b**)

can be incorporated into the lipid mixture used to manufacture liposomes (Kirby et al. 1980). Cholesterol intercalates with the phospholipid bilayer, oriented so that the nonpolar rings are embedded in the hydrophobic core, and the hydroxyl functional group interacts with the phospholipid head groups (Fig. 3.6b). The bulky steroid rings of cholesterol enable the molecule to modulate membrane rigidity, and therefore stability, by restricting mobility of the phospholipids at higher temperatures, while increasing fluidity by preventing packing at lower temperature. In this way cholesterol is able to enhance the permeability barrier function of the bilayer, playing a role in the encapsulation efficiency of drugs when incorporated into liposomal formulations (Kirby et al. 1980; Butler and Smith 1978; LaBelle and Racker 1977).

Aggregation is a common problem that can occur after manufacturing of liposomes or after administration. Certain lipids can be used to prevent liposome-liposome aggregation or liposome-cell interaction including poly(ethyleneglycol) (PEG)-modified lipids or phosphatidylglycerols (Allen et al. 2002; Yoshioka 1991). Cholesterol influences the binding of serum proteins to the liposome surface, which may also help to prevent liposome aggregation (Xu and Anchordoquy 2010; Zhang and Anchordoquy 2004).

The choice of lipids in the design of liposome is critical to the desired characteristics of the end product. Properties of lipid vesicles such as size and homogeneity can vary depending on the lipid composition. For example, vesicle size is influenced by the lipid charge where charged lipids form smaller liposomes with less lamellae. Furthermore, size can have an impact on the distribution and pharmacokinetics of the liposome in situ (Mirahmadi et al. 2010). In many cases the liposome is constructed using mixed lipid species in order to obtain the exact lipid properties needed for the desired liposome. The following sections discuss how T_c, head group and surface charge, degree of saturation, and cholesterol may influence liposome behavior.

3.2.1 Phase Transition Temperature (T_c)

The T_c is the temperature at which lipids move from a gel phase to a liquid crystalline phase. The molecular organization of lipids accounts for the differences between the two phases. In the gel phase the hydrocarbon chains are fully extended and closely packed, while in the crystalline phase the hydrocarbon chains are disordered and more fluid. Transition temperature is a function of fatty acid chain length, saturation, charge, and head group species. The T_c increases by 14–17° with the addition of two methylene units in the fatty acid chain (Fig. 3.7a) (Hardin et al. 2011). On the other hand, the presence of carbon-carbon double bonds, branching, or bulky side groups within the hydrocarbon chains decreases T_c. A significant decrease in T_c is seen with the presence of even one carbon-carbon double bond (Fig. 3.7b) (Szoka and Papahadjopoulos 1980). The head group of the phospholipid, such as phosphatidylserine, phosphatidylglycerol, or phosphatidic acid, can also have a considerable influence on the T_c. Finally, the T_c of phospholipids may be modulated by

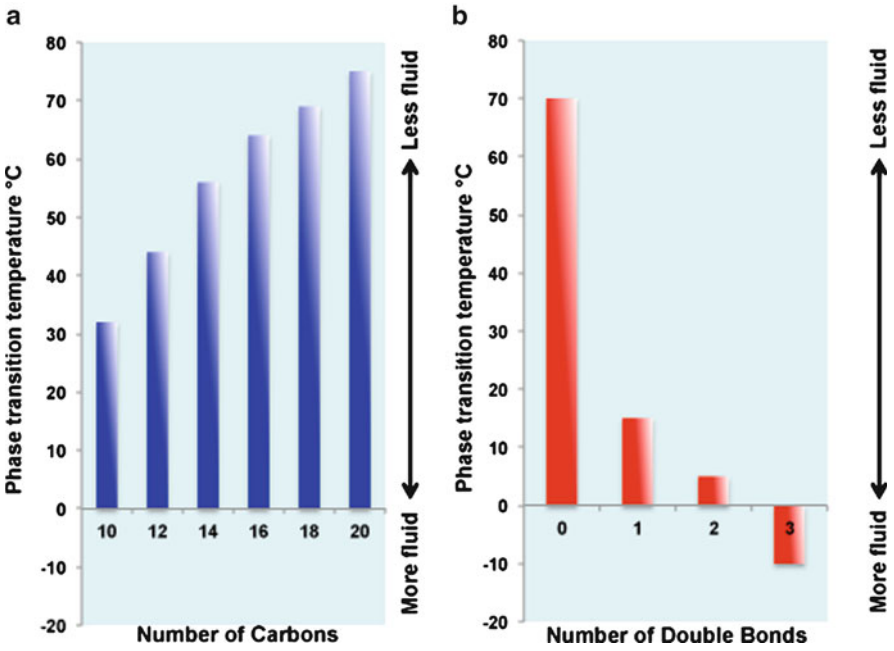

Fig. 3.7 Effect of fatty acid chain length (**a**) and saturation (**b**) on T_c

interactions with Ca^{2+}, Mg^{2+}, or by H^+ ion titration of the head group as well as alterations in pH (Cevc et al. 1981; Tari et al. 1994). T_c is an important consideration when selecting the method used to manufacture liposomes, as it is preferable to maintain the temperature of the solution of hydrated lipids above the T_c throughout the manufacturing process. Moreover, the ability to prepare vesicles using lipids with defined transition temperatures is an important parameter for drug delivery since the permeability of vesicles to entrapped compounds is related to the T_c of the bulk phospholipid used. Encapsulated agents within liposomes prepared with lipids that have a high T_c tend to be retained better when compared to liposomes prepared with lipids that have a low T_c (Anderson and Omri 2004). Importantly, there are no hard and fast rules about how to choose a lipid composition for a particular agent of interest and the release of encapsulated agents should be determined empirically in assays conducted in vitro as well as in vivo.

3.2.2 Head Group and Surface Charge

Neutral, anionic, and cationic liposomes may be formed depending on the nature of the head group on the chosen lipid(s). As noted in Tables 3.1 and 3.2, there are many options for head groups including but not limited to; neutral head groups such

Table 3.2 Molecular formula of neutral and negative head groups

Neutral	Negative
Phosphatidylcholine	Phosphatidylglycerol
$R–PO_4–CH_2CH_2N(CH_3)_3$	$R–PO_4–H_2CHOHCH_2OH$
Phosphatidylethanolamine	Phosphatidylserine
$R–PO_4–CH_2CH_2NH_3$	$R–PO_4–CH_2CHCOONH_3$
	Phosphatidic acid
	$R–PO_2–(OH)_2$

as phosphatidylcholine and phosphoethanolamine and negatively charged head groups such as phosphatidylglycerol, phosphatidylserine, and phosphatidic acid (Table 3.2). Anionic lipids in biological membranes play important functional roles including maintenance of ion gradients and participation in membrane fusion events. In biological membranes, charged lipids are often distributed asymmetrically where the anionic lipid phosphatidylserine is primarily found on the inner leaflet of the lipid bilayer (Leventis and Grinstein 2010). When something disturbs the distribution of phosphatidylserine in the plasma membrane of cells, reactions are initiated. For example, the assembly of protein aggregates on the surface of platelets requires exposure of phosphatidylserine (Leventis and Grinstein 2010; Kay and Grinstein 2011). Exposure of phosphatidylserine may also mark dead or dying cells for elimination by phagocytic cells called macrophages (Leventis and Grinstein 2010; Kay and Grinstein 2011). Liposome prepared using phosphatidylserine may experience similar reactions in situ such as, the binding of serum proteins (Kiwada et al. 1998; Miller et al. 1998), and recognition by the phagocytic cells of the mononuclear phagocytic system (MPS) (Aramaki 2000; Wu and Nakanishi 2011). For this reason, when anionic liposomes are injected intravenously they are readily removed from the circulation (Ishida et al. 2002). Rapid removal by cells of the MPS can lead to immune-mediated side effects such as vasoconstriction, pulmonary hypertension, dyspnea, and a drop in circulating platelets and leukocytes (Awasthi et al. 2004). Use of phosphatidylserine in liposomes can, however, be a desirable feature when considering the potential of a liposome to deliver a macrophage-activating agent, when trying to manipulate the macrophage population, or when trying to promote clotting reactions. It is important to remember that not all anionic lipids cause reactions comparable to those caused by phosphatidylserine. As already mentioned above, phosphatidylglycerol can prevent liposome aggregation and can be used as a lipid component that increases the circulation longevity of intravenously injected liposomes. The use of anionic lipids can also be an advantage when considering the agent that is being associated with the liposomes. For example, cationic peptides can form complexes with anionic lipids such as phosphatidylserine and the resulting complex can be used in the preparation of liposomes. Negatively charged liposomes have also been used to bind negatively charged molecules such as DNA through divalently charged cations.

Although cationic lipids do not occur in nature, lipids with cationic head groups have been synthesized for delivery of plasmid expression vectors, antisense oligonucleotides (ASON), and small interfering ribonucleic acids (siRNA) (Dass 2004;

Ewert et al. 2010; Tranchant et al. 2004). Cationic liposomes are reported to be better gene delivery vehicles than either neutral or anionic liposomes. For example, positively charged liposomes such as lipofectin are prepared using a mixture of *N*-[1-(2, 3-dioleyloyx) propyl]-*N*-*N*-*N*-trimethyl ammonia chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE). DOTMA is one example of a positively charged lipid that can form complexes with anionic polymers such as DNA, ASON, and siRNA (Wong et al. 1996, 2002; Reimer et al. 1995). DOPE is a lipid that is known to play a functional role in mediating membrane fusion events (Bailey and Cullis 1997). The resulting formulation is thought to fuse with the cell membrane or with an endosomal membrane after internalization into a cell and this fusion reaction is associated with release of the associated DNA, ASON, or siRNA. As with anionic liposomes, cationic liposomes are known to engender toxicities. Further, cationic liposomes, like anionic liposomes, bind serum proteins and are rapidly removed following administration by cells of the reticuloendothelial system (RES) (Maurer et al. 1999). It should be noted that the biological reactions triggered when using certain anionic liposomes (those containing phosphatidylserine) and cationic liposomes can be avoided through the use of PEG-modified lipids; where the PEG modification may effectively shield the cationic or anionic charge that stimulates the biological response (Chiu et al. 2001, 2003; Ho et al. 2010). In general, most liposomal formulations are prepared using neutral lipids, cholesterol, and in some cases phosphatidylglycerol or PEG-modified lipids, as these liposomes are generally nontoxic and relatively stable.

3.2.3 Degree of Saturation

As already discussed, the saturation of the fatty acid chain influences the T_c, where the presence of one or more double bond reduces T_c. Additionally, the long-term stability of a liposome is determined by the lipid species used and more specifically the degree of fatty acid saturation. While saturated lipids (Fig. 3.8a) offer the greatest stability, they tend to have higher transition temperatures. Although the introduction of carbon-carbon double bonds reduces the T_c, double bonds increase the risk of oxidation and thus instability. Lipids from biological sources (egg, bovine, or soybean) are polyunsaturated (Fig. 3.8c) and less stable than saturated or monounsaturated fatty acids (Fig. 3.8b).

3.2.4 Cholesterol

Cholesterol is an important component of the cell membranes comprising up to 50 % of the lipid mass. The presence of cholesterol in a biological membrane enables a buffering of fluidity and rigidity and thus may help to stabilize the bilayer.

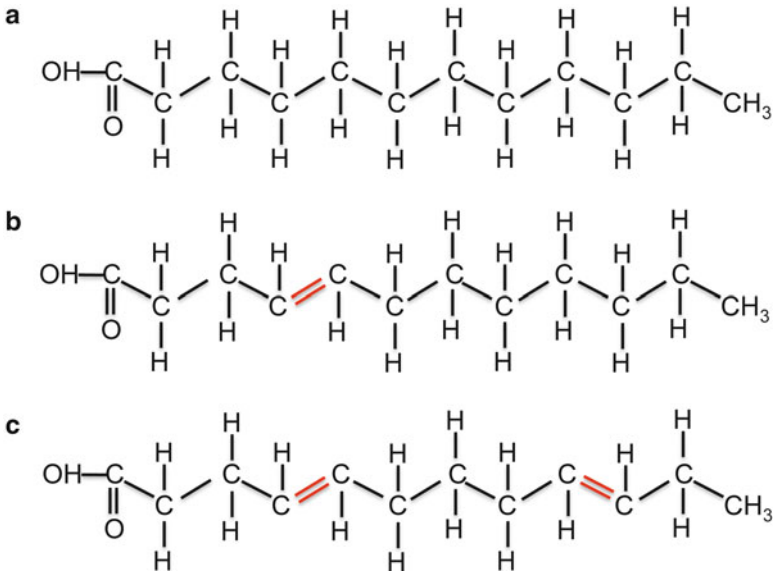

Fig. 3.8 Structural formula of a saturated (**a**), monounsaturated (**b**), and polyunsaturated (**c**) fatty acid

Cholesterol may also play important functional roles in biological membranes by regulating formation of lipid domains or lipid rafts. In liposomes, cholesterol is used to improve liposome stability. As summarized in an excellent review by Szoka et al., the effects of cholesterol on the properties of phospholipid membranes include elimination of the T_c at a 33 % mole ratio of cholesterol to phospholipids, the condensation of the area of phospholipid molecules in monolayers, the inhibition of motion within the outer segment of the phospholipid acyl chains in bilayers, the increase in width of bilayers composed of short chain phospholipids, and the increased perpendicular orientation of the acyl chains (Szoka and Papahadjopoulos 1980). Of greatest importance to the preparation of liposomes as a drug carrier system is the ability of cholesterol to decrease the permeability of phospholipid bilayers to ions and small polar molecules and to reduce the ability of proteins to penetrate and increase disorder within the bilayer. Cholesterol can be incorporated into a liposome at molar ratios of up to 1:1. Some reports have used higher amounts of cholesterol; however, higher levels may lead to precipitation of cholesterol crystals. Crystalline cholesterol is difficult to separate from liposomes and can trigger undesirable properties (Alving et al. 1996). Finally, the amount of cholesterol used to construct a liposome will depend on the application for which the liposomes are being used. For example, Mozafari et al. have found that greater 40 % cholesterol is not useful in liposomes used for gene and drug delivery (Mozafari 2010). Importantly, there may be examples where the preparation of liposomes which lack cholesterol provide the best properties (Dos Santos et al. 2005, 2007).

3.3 Liposome Manufacturing

The general steps of the manufacturing procedure are; (1) Preparation of the lipids for hydration; (2) Hydration; (3) Sizing to a homogeneous distribution of vesicles; and (4) Encapsulation (in cases where the agent of interest is encapsulated/associated passively the agent is added while the lipids are being hydrated). In this section, the most commonly used methods for liposome manufacturing are briefly described below and outlined in Fig. 3.9.

3.3.1 Lipid Preparation

The lipid preparation step can vary depending on the organic solvents used for lipid solubilization and the method of lipid drying. The choice of liposome preparation method depends on the final characteristics of the desired liposome. According to

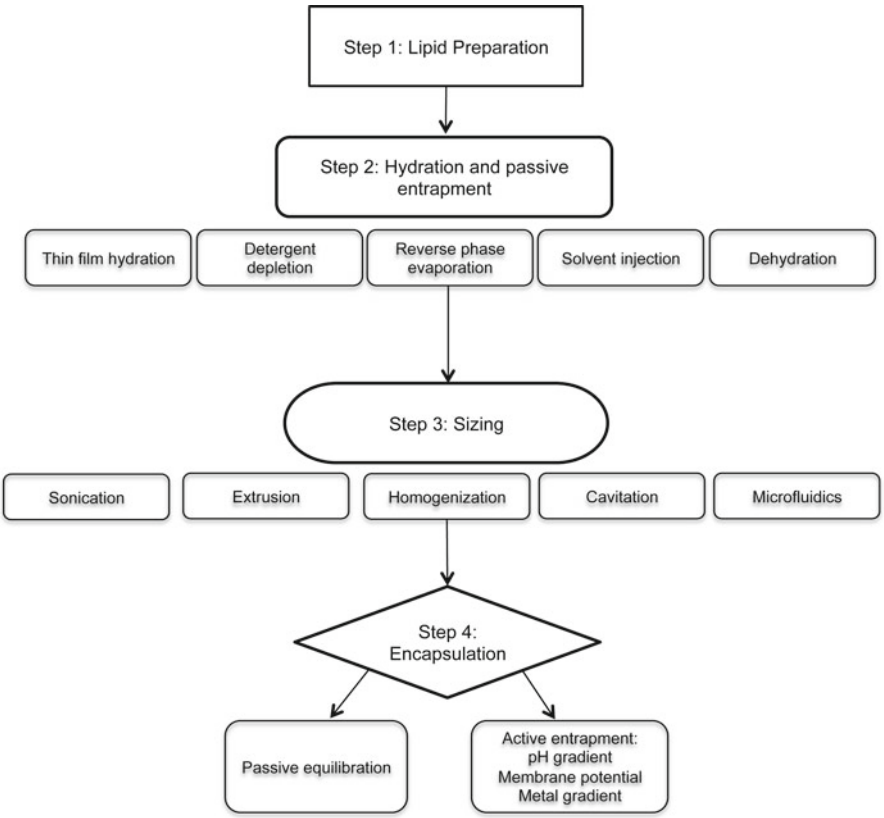

Fig. 3.9 Steps in liposome manufacturing

Mozafari et al., optimum size, size distribution, shelf-life of the vesicles for the intended application, the medium in which the lipid vesicles are dispersed, the physicochemical characteristics of the material to be entrapped, and batch-to- batch reproducibility all play an important role in determining the method of preparation (Mozafari 2005, 2010). If the preparation is destined for use in humans it is also important to use solvents that are considered acceptable in trace quantities. A typical procedure for liposome preparation involves the solubilization of dried lipid(s) in an organic solvent such as chloroform or cyclohexane. This step is performed to ensure that the lipids are completely mixed. In some cases, small amounts of ethanol or methanol are needed to help solubilize all the lipid components. Once a clear solution is achieved, the solvent is removed by evaporation under nitrogen or argon gas or by rotary evaporation. The resulting lipid film is ready for the hydration step. There are other preparation methods that take advantage of the fact that lipids can be solubilized in ethanol and when the ethanol solution is mixed with the hydrating buffer lipid structures form spontaneously. This method is dependent on a number of factors including the final ethanol to hydrating solution ratio, the solubility of the lipids in ethanol, temperature, and mixing conditions. Yet another way of preparing or "solubilizing" lipids involves dissolving lipids in detergent solutions. When the detergent-dissolved lipids are diluted into non-detergent solutions lipid structures spontaneously form. The latter method is used at times for reconstituting membrane-associated proteins; however, it is not a method of choice as it is very difficult to remove detergents and the presence of residual detergents can influence the permeability of the liposomes after they are formed.

3.3.2 Hydration of Lipids

Methods of lipid hydration can vary and include thin film hydration as described below, detergent depletion, reverse-phase evaporation, solvent injection, and dehydration/rehydration. Liposomes form spontaneously when a hydrating buffer is added to dried lipids and mixed. The hydration step should consider the phase transition temperature of the component lipids, mixing rate and time required to achieve complete hydration. Maintaining the temperature of the hydration buffer above the T_c of the bulk phospholipid component can facilitate hydration. The hydration buffer can come in the form of distilled water or buffered salt solutions and/or sugar solutions. The choice of hydration media will depend on the end application being considered and the agent that is being associated with the liposome. It is important to note that the morphology of the liposome may be influenced by parameters such as temperature, pH, ionic strength, and even the concentration of lipids used. The salts used must be compatible with the lipids that have been selected. For example, divalent metals will interact with anionic lipids forming complexes that are very difficult to hydrate. Some lipid compositions are selected to promote fusion in low pH environments and these lipids can be hydrated well in neutral solutions but not low pH solutions.

When a dried lipid film is hydrated the liposomes produced are heterogeneous in size and multilamellar (Fig. 3.8). *Multilaminar vesicles* (*MLV*) have many concentric lipid bilayers that can vary remarkably in size. The sizes obtained will depend on the hydration process used and lipid composition. When using charged lipids the MLVs that are formed are typically smaller with fewer lamellae. MLV can be processed further to achieve smaller structures with only a single bilayer. This is accomplished using sonic energy (*sonication*), cavitation (e.g., French press methodology (Barenholzt et al. 1979)), or by *extrusion* (Hope et al. 1985; Olson et al. 1979). Sonication and cavitation produce very small liposomes (<50 nm), but these small liposomes are often "contaminated" with a proportion of large structures and this can limit their utility in applications involving parenteral administration. Extrusion methods provide the opportunity to generate uniform liposomes with different mean diameters and are currently the method of choice for manufacturing liposomes.

The size and structure of liposomes when prepared by use of detergent, reverse-phase methods, solvent injection, and solvent dilution will depend a great deal on the manufacturing process and quality by design concepts can be used to produce uniform-sized single bilayer liposomes without extrusion (Xu et al. 2011). Further, new methods involving the application of microfluidic chips can be used to generate well-defined small lipid structures by rapid mixing of ethanol solubilized lipids and hydration buffers (Belliveau et al. 2012; van Swaay and deMello 2013). These methods will be discussed further below.

As indicated in Fig. 3.9, there are three points in the manufacturing process where drugs, proteins, peptides, or nucleic acids can be added. If one is contemplating the use of hydrophobic drugs or therapeutic lipids, it is possible to add these at the time when the lipids are being mixed in solvents or detergents. The agents, if reasonably water-soluble, can be added to the solution used to hydrate the liposomes. When associating agents with liposomes at these stages, it is referred to as *passive trapping* as the drug is captured in the liposome during the manufacturing process. As indicated below, *active trapping* is done when the agent of interest is added after the liposome has been manufactured.

When using passive trapping methods where the agent is contained in the hydration buffer there are two important factors that should be considered. First, one cannot assume that the concentration of the agent in the hydrating buffer is going to equal the concentration of drug in the aqueous core of the liposome. This is due to nonequilibrium solute distribution effects (Perkins et al. 1988), where water permeates across forming membrane sheets at a rate that is faster than the solute permeates. This phenomenon effectively limits the amount of the agent associated with the liposome. Improvements in lipid hydration while attaining better solute distribution can be achieved through use of reverse-phase evaporation methods or by repeated freeze-thaw cycles, particularly when the thawing is done at temperatures above the T_c of the bulk phospholipid component (Mayer et al. 1985; Pidgeon et al. 1987). Second, the trapping efficiency achieved for an agent that is encapsulated through passive trapping methods is dependent on lipid concentration and the "trapped volume" within the liposome typically reported as μL trapped volume per μmol lipid.

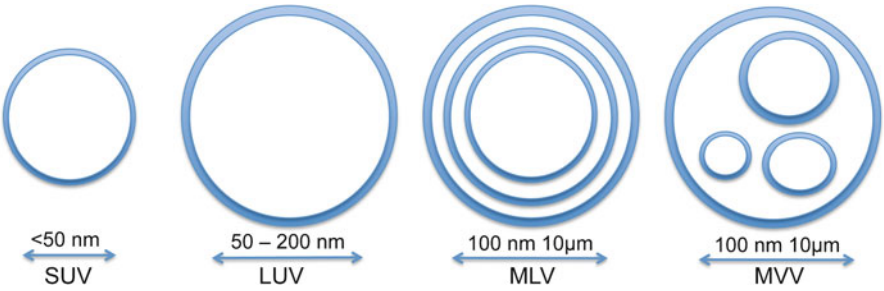

Fig. 3.10 Illustration of the four types of lipid vesicles formed after hydration

The trapped volume for a solution of liposomes could be calculated simply by relating the diameter of the spherical liposome to the number of liposomes per μmol lipid. Based on a number of assumption it can be estimated that a 100 nm liposomal formulation will have an ideal trapped volume of 2.2 μL/μmol lipid. As suggested above, if the solute being used is also the marker used to determine the trapped volume, then measured trapped volumes may be considerably less than calculated ideal trapped volume. This is a result of nonequilibrium solute distribution. Given what is known about trapped volume, individuals will recognize that lipid concentrations will affect the amount of trapped volume in a solution. Assuming a trapped volume of 2.2 μL/μmol lipid, a 100 mM dispersion of liposomal lipid will encapsulate about 220 μL in a 1 mL volume. If the lipid concentration is increased to 400 mM, about 880 μL in a 1 mL volume is contained inside the liposome. Such solutions can be manufactured, but the solutions are very viscous and difficult to work with.

3.3.3 Sizing of the MLV

Large and heterogeneous liposomal formulations such as MLVs need to be processed further. If they are to be used pharmaceutically and for parenteral applications, the goal is to generate unilamellar vesicles. Unilamelar vesicles can be categorized into two classes on the basis of size; vesicles under 50 nm are considered the small unilamellar vesicle (SUV), whereas those with a greater diameter are large unilamellar vesicle (LUV) (Fig. 3.10). For research purposes, some methods have been described to generate giant unilamellar liposomes, but these will not be discussed here. For a variety of reasons, liposomes that are designed for use in therapeutic applications are typically between 50 nm to 2 μm, but more preferably between 80 and 200 nm.

Several downsizing techniques have been established in order to make the heterogeneous MLVs uniform. The first published downsizing method was sonication. The extrusion technique whereby liposomes are forced through filters with well-defined pores is the most widely used method of downsizing. As already indicated other methods which help to downsize the large MLV are cavitation and

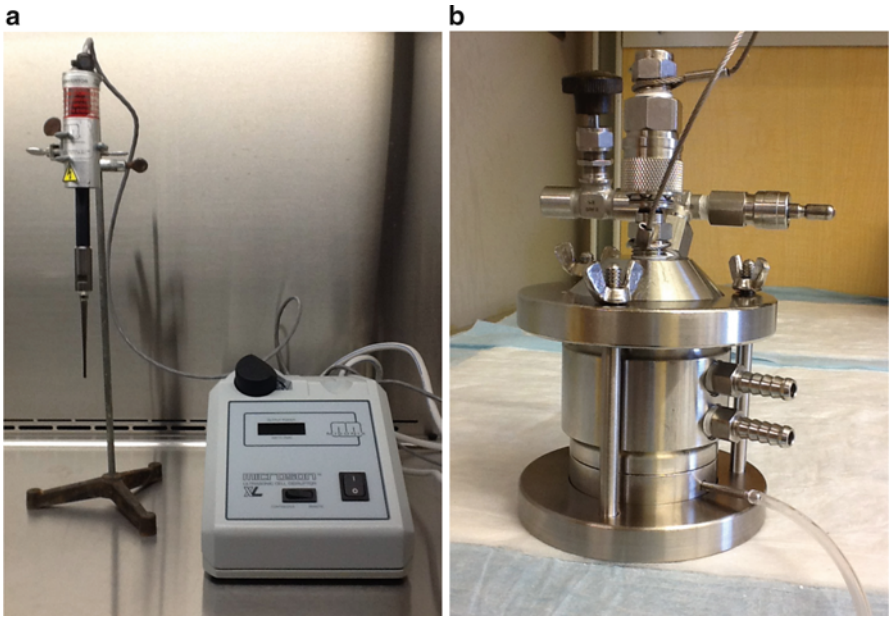

Fig. 3.11 Photographs of a probe tip sonicator (**a**), and an extruder (**b**)

homogenization techniques including microfluidization, high-pressure homogenization, and shear force-induced homogenization techniques. In the following section the two most common procedures for downsizing are briefly described.

Sonication: The disruption of MLV using sonic energy produces SUV with diameters that range from 15 to 50 nm. In this method, high intensity sound energy is applied to the liposomal dispersion either directly with a probe tip (Fig. 3.11a) or indirectly using a bath sonicator. Bath sonicators are used more frequently as they do not introduce metal contaminates (e.g., titanium) that may be introduced when using a probe sonicator. MLV dispersion is accomplished by placing a test tube containing the sample in a bath sonicator and sonicating for 5–10 min. The end size of the vesicles is influenced by lipid composition and concentration, temperature, volume of the sample, and sonication time. It is important to note that the SUV are generally unstable and may spontaneously fuse to form larger vesicles when stored below their phase transition temperature. A main drawback to a sonication procedure is the batch-to-batch variability in size that is the result of a difficulty in controlling the conditions of sonication.

Extrusion: The most popular downsizing method is extrusion. The equipment used for extrusion is shown in Fig. 3.11b. Extrusion involves forcing the MLV suspension through a membrane with a specified pore size. These filters can be purchased with pore sizes of 30 nm, 50 nm, 80 nm, 100 nm, 200 nm, and larger. The MLVs are pushed through these filters under medium pressures (up to 800 psi). The pressure used will depend on the pore size of the filter, lipid concentration, and lipid composition.

For a dispersion of 100 mM lipid it is possible to generate 100 nm liposomes by passing the dispersion through the filter 10 or more times and this can be accomplished in less than 30 min. As suggested already, all downsizing methods should have the lipid solution maintained at a temperature above the T_c of the bulk phospholipid component. The extrusion method is fast, very reproducible, and is fully scalable. A company called Northern Lipids Inc. markets extrusion devices capable of rapidly generating hundreds of liters of liposome solutions.

3.3.3.1 Encapsulation

The manufacturing of a liposome destined to be a carrier vesicle must consider the entrapment of materials within the structure. Materials that are encapsulated within liposomes are diverse with diverse physical and chemical properties. They can range from small contrast agents, biogenic amines, nucleic acids, peptides to large proteins. These compounds can be hydrophobic or hydrophilic.

As indicated above, agents can be trapped or associated with liposomes using passive trapping methods during preparation or after the liposomes have been manufactured. The spontaneous organization of phospholipids into a bilayer structure in the presence of water is coupled with the entrapment of a portion of the aqueous volume within a closed bilayer. As discussed already the entrapment of water-soluble molecules depends on the entrapped aqueous volume. The size and lamellarity of vesicles as well as lipid concentrations will influence this volume. The successful encapsulation of water-soluble compounds can be measured by two parameters: (a) the efficiency of encapsulation, which is the percentage of the compound initially added to the preparation that becomes entrapped in the aqueous space; and (b) the volume (liters) of aqueous space encapsulated per mole of phospholipid. For water-soluble compounds of relatively low molecular weight, the former will depend upon the relative amounts of lipid and water present in the preparation, whereas the latter will be a function of liposome size and number of lamellae. Unilamellar vesicles are preferred to multilameller ones due to their larger entrapped aqueous volume. Additionally, LUVs are most effective at passive loading as the volume of the encapsulated water is higher when compared to SUVs. It is important to note that circulation longevity is inversely proportional to vesicle size, thus a balance exists between encapsulation efficiency and circulation half-life. In addition, under situations where the trapping efficiency is low or the dose of encapsulated agent required is high, the dose of liposomal lipid may be large; in some examples beyond what can be achieved practically.

The incorporation of lipid-soluble active molecules which have been added to the lipids prior to hydration is another form of passive trapping. In this case, hydrophobic materials should be co-dissolved with the lipid in an organic solvent to produce a homogeneous mixture. The organic solvent is removed and the lipid/drug residue processed as in a typical liposome preparation. The efficiency of this method is dependent on lipid concentration and lipid composition. The lipophilic/hydrophobic agent can intercalate into the bilayer; however, it is known that the bilayer can

only accommodate limited amounts of these agents without having substantial effects on the liposome structure and/or properties such as permeability, size, and stability of the bilayer.

For active trapping, preformed vesicles are used and molecules are driven across the lipid bilayer in response to an ion gradient. For such a procedure liposomes with a transmembrane pH gradient or a membrane potential are generated and incubation with cationic molecules leads to the accumulation of the agent within the interior of the lipid structure. When a pH gradient is used, liposomes are made in a low pH buffer. Subsequently the pH of the buffer is adjusted by addition of a base. The net result is a neutral external environment and an acidic internal environment. When such liposomes are incubated in a solution containing a cationic structure, these molecules readily equilibrate across the membrane. Besides a pH gradient, other transmembrane gradients such as a metal ion gradient could also be employed to load drugs into liposomes. Copper (Cu^{2+}) has frequently been used for this purpose. Encapsulation efficiency depends on a number of features of both the agent being entrapped as well as characteristics of the liposome. Active trapping tends to provide higher encapsulation efficiencies; however, this is at a cost of more laborious and complicated protocols.

3.4 Liposome Physical Characterization

Once prepared, liposomes must be fully characterized to ensure the integrity of the manufacturing process and that the quality of the product has been maintained. Additionally, performance of the vesicle, its stability, drug release rates in vitro and in vivo, free versus liposome-associated drug for in vitro and in vivo samples, pharmacokinetics, and biodistribution behavior can all be affected by the structural and chemical integrity of the liposome. For research purposes the most important parameters to characterize for a given liposomal preparation include visual appearance, size distribution, lamellarity, stability, surface potential (zeta potential), and encapsulation efficiency (drug to lipid ratio). For pharmaceutical applications, experts helped the FDA develop guidelines for liposomal drug products and these are worth reviewing in detail even when working in an academic laboratory (Guidance for industry liposome drug products. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070570.pdf).

3.4.1 Appearance

Liposome suspensions can range from translucent to milky, depending on the composition and particle size. If the liposome solution has a bluish shade and is slightly translucent, this is a good indicator that the liposome size is small and homogeneous. These formulations should contain no visible aggregates. When preparing liposomes using a probe sonicator, a flat, gray color indicates the presence of non-liposomal

by-products. MLVs and larger liposomal formulations, depending on lipid concentration and composition, can appear milky. To aid in morphological characterization, a phase contrast microscope can be used to detect liposomes that are >300 nm and is very helpful in the detection of aggregates and crystals. The latter structures may occur when cholesterol is used at too high concentrations. A great deal can be learned about the liposomal product through the use of electron microscopy (EM) methods. More complex information about structure, size, and lamellarity can be gleaned by using negative staining, freeze-fracture, and cryo-electron microscopy methods. These images can also help to determine the location of the encapsulated drug and whether it is in a soluble or in soluble form. For hydrophobic agents, EM methods can help to determine whether the agent has introduced defects in the lipid bilayer. A polarizing microscope can also be used to assess liposome lamellarity. Finally, attached proteins such as monoclonal antibodies can be visualized using high resolution spanning probe microscopy such as atomic force microscopy.

3.4.2 Size and Size Distribution

For many physical and biological studies, the average size and size distribution (*polydispersity*) of the liposomes used is an important parameter for determining liposome stability, circulation longevity, biodistribution, and drug release. For parenteral applications the optimal size range appears to be between 50 and 400 nm and more preferably between 80 and 200 nm (Nagayasu et al. 1999). Size can be assessed using electron microscopy, but the more common methods involve dynamic light scattering. Some other methods for sizing liposomes have been developed such as size exclusion chromatography (SEC), spectroscopy, and centrifugation, but these methods must be calibrated using liposomes with defined size distributions either determined by light scattering methods or EM methods. Drawbacks to EM-based methods include the presence of artifacts owing to changes that occur during sample preparation and the amount of time required to analyze a large number of vesicles. Practically speaking light scattering is the method of choice. Dynamic light scattering quantitatively measures the size of vesicles in an aqueous medium. This method is reliable for liposomes with a relatively homogeneous size distribution. Light scattering provides an average of the size of a large number of liposomes simultaneously. However, it does not provide information on the shape of the vesicle and the technique can be influenced by many factors including solution density, lipid concentration, and presence of certain salts.

3.4.3 Determination of Lamillarity

As indicated above, liposome lamellarity can be qualitatively assessed using EM methods. One of the best methods, however, involves use of Nuclear Magnetic

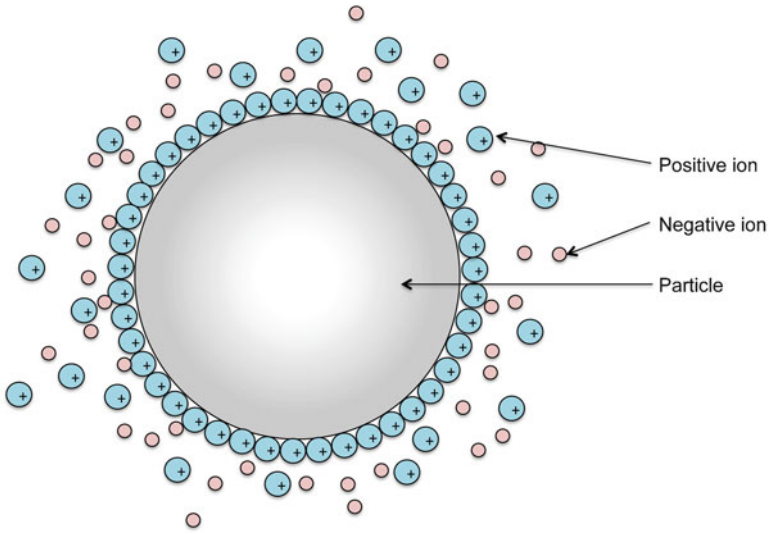

Fig. 3.12 Organization of ions around a charged particle

Resonance (NMR) spectroscopy. Briefly, liposomes are prepared with phospholipids containing ^{31}P which can be detected by phosphorus NMR. The ^{31}P NMR signal from phospholipids can be quenched/shifted by the addition of Mn^{2+} which binds to the membrane. Since Mn^{2+} is not permeable across the liposomal membrane, it can only shift the ^{31}P NMR signal from phospholipids on the exterior face of the liposomes. Thus, for a unilamellar liposome one would anticipate that the ^{31}P NMR signal would be reduced by a factor of 2. The loss of signal would be less than 2 if the liposomes contained multiple lamella. When using small unilamellar liposomes that are 50 nm or smaller, greater than a twofold loss of the ^{31}P signal would be observed as more than 50 % of the phospholipids are in the outer leaflet of the liposomal membrane. Other techniques for lamellarity determination include small angle X-ray scattering (SAXS) and methods that are based on the change in the fluorescence signal of marker lipids upon the addition of reagents that can quench the signal.

3.4.4 Surface (Zeta) Potential

Particles in an aqueous medium tend to adopt a surface charge when ionized or when surrounding charged species are adsorbed to the surface. As a result the surface charge can modify the surrounding ion distribution (Fig. 3.12), which can further influence the movement of the particle in solution. The *zeta potential* is a measure of the magnitude of repulsion or attraction between particles that results from this organization of ions around the structure and in this way is a measure of the particles surface charge. Zeta potential is measured by applying a voltage across

a pair of electrodes at either end of a system containing the particle dispersion. Particles will migrate toward the oppositely charged electrode with a velocity that is proportional to the magnitude of their surface charge. Evaluation of the zeta potential of a liposome preparation can help to predict the stability and in vivo fate of the formulation. Liposomes with high zeta potential (>30 mV negative or positive) are often very stable in solution, but are rapidly eliminated following intravenous administration. Liposomes that have no surface potentials tend to aggregate and can coalesce out of solution. Any modification of the liposome surface, such as surface covering by polymer addition to eliminate surface–surface interactions, can influence zeta potential.

3.4.5 Stability

The methods discussed above measure parameters that can reflect changes in liposomes over time and thus can be used to measure liposome stability. However, characterization of stability also includes an assessment of (a) the chemical stability of the lipids; (b) retention of entrapped contents; and (c) chemical stability of the entrapped contents. For pharmaceuticals approved for use in patients, a 2-year stability is highly desirable. Ideally, this length of stability can be achieved when the product is stored at room temperature. When considering physical stability one is assessing the appearance of the solution, liposome size, and free versus liposome-associated drug as described above. Chemical stability is a measure of the potential for hydrolysis and oxidation of lipids. Hydrolysis can lead to the loss of one or both of the fatty acid chains to create *lyso-phospholipids* (a phospholipid with one acyl chain rather than two) and free fatty acids. The free fatty acids can act like a detergent and will disrupt the bilayer stability leading to loss of associated contents and generation of larger structures. Oxidation will occur in the presence of lipids that contain unsaturated fatty acyl chains. An antioxidant, such as butylated hydroxytoluene, and storing the liposome solutions under inert gases (argon or Nitrogen) can limit oxidation. Highly charged cationic liposomes can be stable in liquid form in the presence of low salt solutions (at optimal pH) and antioxidants. The addition of cryprotectants such as trehalose, dextrose, or sucrose significantly increases the stability by providing the opportunity to maintain liposome formulations as freeze-dried powders.

3.4.6 Free vs. Liposome-Associated Assays

As indicated already, encapsulation efficiency is commonly determined by measuring lipid concentration and the concentration of the associated agent. When using active trapping methods, conditions are typically defined to achieve greater than 98 % encapsulation efficiency. When these formulations are stored for any length of time,

it is important to make sure that the amount of encapsulated drug remains the same over time. Methods for determining the amount of encapsulated/entrapped materials within vesicles involve separation of the nonencapsulated material from the loaded vesicles followed by disruption of the liposomal membrane for the release of the entrapped material and quantification of the released material. Different methods can be used for the separation of loaded vesicles from the nonencapsulated material such as centrifugation, SEC, dialysis, and other more specific methods that depend on the characteristics of the material being encapsulated. Once separated, the disruption of the loaded vesicles is normally performed by diluting the aqueous vesicular suspension with alcohols (ethanol or methanol), extraction of lipids into chloroform, methanol, water mixtures (Bligh and Dyer (1959) extraction methods), or detergent-induced bilayer disruption. After release of the entrapped material, the techniques used for quantification depend on the nature of this material and include spectrophotometry, fluorescence spectroscopy enzyme-based methods, and electrochemical techniques. Since techniques used to separate free from vesicle-encapsulated contents can potentially cause their leakage and, in some cases, ambiguity in the extent of separation, methods that do not rely on separation are of interest. Reported methods include ^{1}H NMR fluorescence methods and electron spin resonance.

3.5 Interactions with Biological Systems

The biodistribution, plasma elimination, and therapeutic/toxic activity of a liposomal agent depend on the physiochemical characteristics of the liposome as well as the associated agent. Size, surface charge, bilayer packing, dose, and route of administration all play a role in how liposomes interact with biological systems. Neutral liposomes, in the absence of an associated agent, are considered to be nontoxic. Some anionic (in particular phosphatidylserine) and almost all cationic lipids are toxic and when incorporated into a liposome without an associated agent, these lipids can exert biological effects. Following the parenteral administration of liposomes, proteins will interact with it and this changes the properties of the formulation. The interaction between proteins in plasma containing tissue culture media, in interstitial fluid fluids, and in blood (serum or plasma) is not well understood. It is worth noting that much effort has gone into characterizing the properties of liposomes prior to administration (see above sections), while understanding that those properties change the instant the formulation has been administered. It is known that for some formulations protein-binding can lead to instability. For example, apolipoprotein binding can compromise the integrity of the lipid bilayer causing rapid leakage of liposome contents. Other proteins known as opsonins (C3b, IgG, and fibronectin) can mark liposomes as foreign matter and instigate their elimination by phagocytic cells of the MPS. In order to achieve extended circulation longevity and a potential for intravenous-injected liposomes to reach the site of disease, liposomes must be prepared using materials that limit MPS uptake or at the very least reduce the rate of MPS uptake. Some have argued that this can be achieved

through use of lipids that block protein-binding, but it is more likely that formulations prepared with materials that prevent or reduce surface–surface interactions are key (Allen et al. 2002). If an intravenous liposome formulation is successful at evading the bodies' defense system, it must be able to move from the blood compartment to extravascular sites. This extravasation event can happen in diseased states as well as normal tissue, but it is most efficient in regions where the disease has a compromised blood vessel structure and function (see below). Once a liposomal formulation has crossed into the interstitial space, it can remain there and slowly release its contents. Alternatively, the liposomes can interact nonspecifically with active phagocytic cells that may reside within the site, or they can interact in a more target specific fashion due to proteins that are nonspecifically associated with the liposomes following administration. Finally liposomes may interact with a specific target through the attachment of targeting ligands on the surface of the liposomes prior to administration.

As mentioned above SUVs which are <50 nm in size exhibit shorter circulation lifetimes in part because they can extravasate in normal tissues that exhibit natural fenestrations in the associated blood vessels. The liver, for example, has *fenestrated* endothelial cells. These fenestrations are approximately 100 nm diameter and allow small formulations to move readily into the liver tissue. LUV formulations that exhibit diameters of between 80 and 200 nm do not extravasate across normal fenestrated endothelial cells and therefore remain in the plasma compartment for longer time periods. Liposomal formulations that exhibit long circulation lifetimes tend to accumulate at sites of disease such as tumors and sites of infection or inflammation because these sites have poorly formed or damaged blood vessels. This accumulation is a consequence of the structure of the microvasculature in these diseased regions. Capillaries in diseased areas tend to exhibit large openings up to 500 nm between endothelial cells through which liposomes can permeate. Additionally, in tumor tissues there is a lack of an effective lymphatic drainage system so the liposomes that have extravasated in tumors essentially become trapped in the tumor tissue. Together the increased uptake and reduced lymphatic drainage is the basis for the *enhanced permeability and retention* (*EPR*) *effect* (Matsumura and Maeda 1986). The EPR effect is a guiding principle through which many nanoparticulate formulations have been developed. It should be noted that the ability of nanoparticles to move from the blood compartment and be retained in regions where lymphatic drainage is compromised was also recognized by scientists studying a protein called Vascular Permeability Factor (VPF) (Nagy et al. 1995). Eventually it was shown that VPF was identical to vascular endothelial growth factor (VEGF), a known angiogenesis promoter, and an important therapeutic target for the treatment of cancer.

As already mentioned, the circulation lifetime of liposomes can be increased by inclusion of lipids that prevent surface–surface interactions. Lipids that have been modified with the polymer *polyethylene glycol* (*PEG*) (Fig. 3.13) are the best recognized example. It has been proposed that the polymer acts as a steric barrier reducing the level of protein-binding by liposomes after administration; however, some reports dispute this (Allen et al. 2002). Regardless, liposomes prepared with PEG-modified lipids exhibit dose-independent pharmacokinetic behavior and long

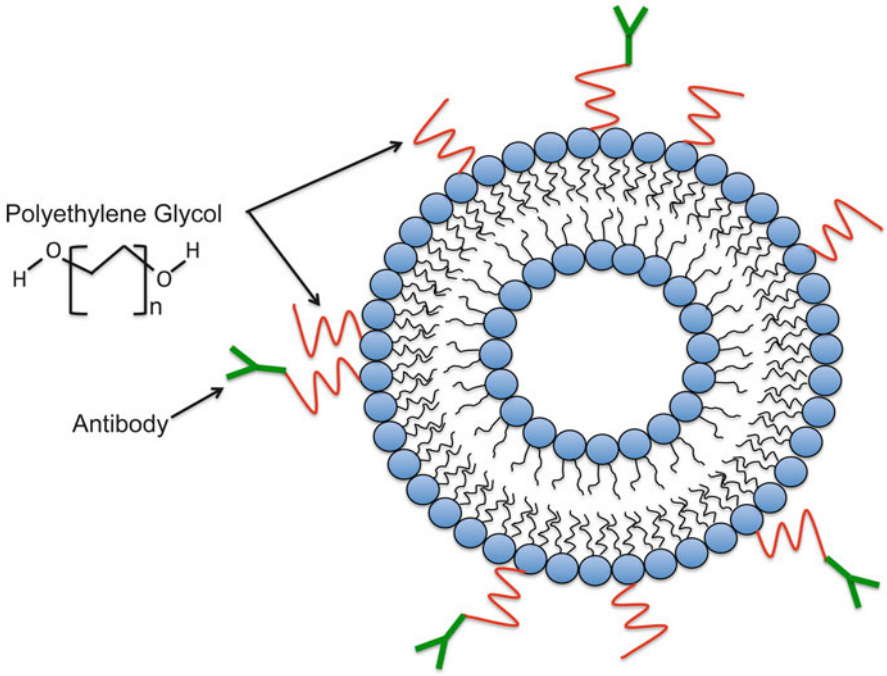

Fig. 3.13 An illustration of a pegylated immunoliposome

circulation lifetimes. Liposomes that lack cholesterol or contain cationic or anionic lipids can be designed to exhibit long circulation lifetimes if those formulations incorporate the appropriate amount of PEG-modified lipids. In general, liposomes that include PEG-modified lipids have been referred to as *sterically stabilized* or *stealth liposomes*. In addition to a PEG coating, liposomes may be modified to through the attachment of a ligand on the membrane surface. These molecules could be selected to enable the targeted delivery of encapsulated materials to specific cells through the binding of ligand to receptor. Targeting ligands include monoclonal antibodies (in which case the resulting liposome is known as an *immunoliposome*), vitamins, or specific antigens. Other modifications to the surface of liposomes include cell-penetrating peptide sequences; peptides that can facilitate the transport of proteins and larger structures such as liposomes across cell membranes (Koren and Torchilin 2012).

3.6 Biomedical Application of Liposomes

The use of liposomes in medicine has been realized in many ways. Liposomes were first used in early 1980s as delivery vehicles for contrast agents and imaging agents for detection of infection and sites of inflammation (Mauk and Gamble 1979;

Morgan et al. 1981; Oyen et al. 1996). As already noted, Dr. Gregoriadis envisioned the use of liposomes for delivery of therapeutic agents in the mid-1970s (Gregoriadis and Buckland 1973). Post and Fidler started to explore the use of liposomal formulations to activate the immune system in the 1979 (Poste et al. 1979). The use of liposomes to deliver nucleic acids was first recognized in the laboratory of Demtri Papahadjopolous in the early 1980s (Straubinger and Papahadjopoulos 1983). Phil Felgner was the first to explore the possibility that cationic liposomes could be used for delivery of therapeutic nucleic acids in the early 1990s (Felgner 1991). The race to develop therapeutically effective liposomal drug formulations for the treatment of cancer or infections has involved thousands of scientists around the world. Some might say that this effort has been less than successful, but it should be noted that it typically takes 10–15 years to develop regulatory-approved agents for human use. In the context of liposomal formulations, efforts over the past 40 years have resulted in many approved liposomal drugs for treatment of cancer, infections, and macular degeneration. In addition, there are numerous product candidates in advanced clinical studies. Thus, the therapeutic potential for liposomal formulations is only just starting to be realized. In this section, some of the biomedical applications of liposomal research are summarized.

3.6.1 Liposomes in Drug Delivery

Liposomes are the most clinically established nanopharmaceutical formulation technology for drug delivery applications and the development of effective nanomedicines. The rationale for using liposomes as a carrier system for drugs has already been provided above, but these advantages are worth repeating here. Liposomes can: (1) protect an associated agent against degradation; (2) prolong the half-life of an associated drug in circulation; (3) change the biodistribution of a liposome-associated drug resulting in reduced toxicities and enhanced therapeutic activity; and (4) be used to target specific cell populations, using targeting ligands that have been associated to the carrier prior to administration or through serum proteins that bind the formulation after administration.

3.6.1.1 Cancer

Liposomal formulations of anticancer agents have been on the market since the early 1990s. These include Doxil, DaunoXome, and Myocet. Cytotoxic agents when delivered systemically distribute nonspecifically throughout the body leading to major toxicities. For the anthracycline doxorubicin, the active agent in Doxil and Myocet, the toxicity of major concern was dose-limiting cardiotoxicity. The use of liposomal formulations to trap doxorubicin resulted in reduced drug delivery to cardiac tissue and a reduction in risks for congestive cardiomyopathy. Two commercially available liposomal formulations of anthracyclines are pegylated liposomal doxorubicin (Doxil also known as Caelyx) and a non-pegylated liposomal

doxorubicin (Myocet). Myocet is approved in Europe and Canada for use in metastatic breast cancer patients in combination with cyclophosphomide. A detailed comparison of these two formulations has already been published (Waterhouse et al. 2001). Marqibo (vincristine sulfate injection, OPTISOME), a novel targeted anticancer compound for Non-Hodgkin's Lymphoma and Acute Lymphoblastic Leukemia, has recently been approved by the FDA. Liposomal cisplatin (Lipoplatin) is a nontoxic alternative agent to cisplatin currently undergoing phase III clinical trials. A phase II clinical trial of Lipoplatin in combination with vinorelbine used in first-line treatment of patients with metastatic breast cancer resulted in a 53.1 % response rate with limited toxicity (Farhat et al. 2011). CPX-1 is a fixed ratio liposomal formulation of Irinotecan (CPT-11)/Floxuridine (FUDR) which completed phase I clinical assessments and is currently awaiting further clinical development. CPX-351 is another fixed ratio liposomal formulation of cytarabine and daunorubicin which is being evaluated in phase III clinical studies in patients with acute myeloid leukemia. A thermosensitive liposomal formulation of doxorubicin (Thermodox) was being developed in combination with radiofrequency ablation. The phase III study in patients with hepatocellular carcinoma (HCC) was halted in early 2013 because the study did not achieve its primary therapeutic endpoint. However, the formulation is still being studied in breast cancer patients with locally recurring disease. A liposomal irinotecan formulation (MM-398) is being tested in a phase III study for the treatment of patients with pancreatic cancer who failed previous treatment with gemcitabine. Other liposomal formulations of antineoplastic agents currently being evaluated in vivo include topotecan and vinorelbine. Liposomal formulations are also being studied for the efficient delivery of targeted therapies and siRNA designed to silence gene expression in target cell populations of particular liver cancer cells.

3.6.1.2 Infectious Disease

There are some antibiotics that have limited clinical potential because of toxicities and or poor pharmacokinetic/biodistribution behavior. Despite very efficient antibacterial/antifungal activity, these drugs can only be used as a last resort when the risk of mortality is high. There are many advantages of using liposomal formulations of antimicrobial agents such as improved pharmacokinetics and biodistribution, decreased toxicity, enhanced activity against intracellular pathogens, target selectivity, and the possibility to overcome bacterial drug resistance. The trademark example of a lipid-based antimicrobial formulation is the clinically antifungal agent amphotericin B (AmBisome, Abelcet, Amphocil, Amphotec). It is interesting that some of these formulations were initially being developed as liposomal formulations, but during the development phase it was recognized that Amphotercin B naturally formed a lipid complex and the resulting product (Abelcet) was a lipid formulation rather than a liposomal formulation. Free Amphotericin B is known for serious side effects and multiple organ damage especially nephrotoxicity. Delivery of this drug in a lipid-based formulation reduces the drugs' toxicity. As another example, the liposomal formulation of amikacin has been successfully administered

by inhalation to cystic fibrosis patients with chronic pseudomonas infection (Okusanya et al. 2009). A number of other antibiotics have been evaluated preclinically in liposome formulations including aminoglycosides, quinolones, polypeptides, and betalactames.

3.6.1.3 Inflammatory Disease

Liposomes have been extensively investigated as DDSs in the treatment of inflammatory diseases such as rheumatoid arthritis (RA). As with cancer, inflammatory conditions are associated with local changes in the blood vessels that promote the EPR effect. Pro-inflammatory cytokines, for example, are known to increase capillary permeability. Therefore, liposomes tend to accumulate in areas of active inflammation. Low bioavailability, high clearance rates, and limited selectivity of several important drugs used for the treatment of inflammatory disease are reasons for why these drugs are used in high doses and often for prolonged periods of time. The dosing regimens increase the risk for systemic side effects, as is observed with the standard of care glucocorticoid (GC) analogs such as Prednisone, Prednisolone, Betamethasone, and Dexamethasone. Systemic side effects of these GC analogs include weight gain, secondary Diabetes Mellitus, peptic ulcer disease, psychiatric syndromes, secondary osteoporosis, and loss of visual and auditory acuity. Free GCs are rapidly cleared from circulation and accumulate at sites of disease to a very limited extent. The use of liposomes as drug carriers may increase the therapeutic efficacy of glucocorticoids like prednisolone while reducing the systemic toxicity. In arthritis, the efficacy of prednisolone-loaded long-circulating liposomes is currently being evaluated in a phase II clinical trial (van den Hoven et al. 2011). Liposomal methylprednisolone formulations display enhanced efficacy in acute inflammatory and chronic demyelinating models of multiple sclerosis and it is this formulation, over the free drug, that may be able to provide a method of reducing disease progression. It has been shown that liposomes introduced intravenously tend to accumulate in the inflamed colonic tissue of Inflammatory Bowel Disease (IBD) and anionic liposomes adhere better to inflamed mucosa compared to normal mucosa. Therefore, anionic liposomes may be suitable for the delivery of drugs to sites of inflammation in diseases such as ulcerative colitis and Chron's disease (Schroeder et al. 2010).

3.6.1.4 Pain

Analgesics such as opioids are effective pain relievers that come with a high side effect profile including nausea, vomiting, sedation, sleep disturbances, and respiratory depression. Liposomal formulations of opioids have been shown to target the site of pain, trauma, surgery, or injury better than when the same opioids are given in the absence of a carrier. Liposomal bupivicaine was recently approved for use as a postsurgical analgesic. Most recently a study performed by Dasta et al. showed

that liposome bupivacaine was associated with significantly lower cumulative pain scores, delayed and less consumption of opioids, and fewer opioid-related side effects when compared to free bupivacaine (Dasta et al. 2012). Other liposomal-based analgesics currently undergoing clinical investigations include mepivicaine, liposomal lidocaine, and liposomal morphine (DepoDur).

3.6.2 Liposomal Vaccines

The rationale for using liposomes to deliver vaccination products includes: (1) the potential for liposomes to present soluble antigens in particulate form that extends their half-life in vivo, protecting them from enzymatic breakdown and promoting delivery of the antigen to the appropriate antigen-processing cells; and (2) the fact that lipids can be selected/designed to be immunostimulatory. Liposomes with encapsulated protein or peptide antigen are known to be internalized by antigen-processing cells. Once localized to the endosomal compartments and the lysosomes, the liposome-associated antigen can be degraded and the resultant peptides complexed with the major histocompatibility complex class II (MHCII) and presented to T lymphocytes. This results in the stimulation of specific T-helper cells and, ultimately, stimulation of specific B cells. Taken together, liposomal delivery can promote cellular and humoral immunity. The Hepatitis A vaccine Epaxal and influenza vaccine Inflexal are two examples of liposome-based vaccines that are currently approved for use in North America. As an example, Epaxal is a formalin-inactivated Hepatitis A Virus (HAV) adsorbed to the surface of liposomes. This formulation was able to provide 88–97 % seroprotection 2 weeks after a single injection and was shown to be well-tolerated (Bovier 2008). Recently, a phase I study of a *Neisseria meningitiis* liposomal vaccine was performed by Zollinger et al. where the purified outer membrane proteins (OMP) and purified deacylated lipooligosaccharide (dLOS) from the pathogen were formulated for use as a vaccine (Zollinger et al. 2012). Other examples that have been investigated include synthetic human MUC1 peptides (Agrawal et al. 1998), which are considered candidates for therapeutic cancer vaccines and formaldehyde-inactivated ricin toxoid for protection against inhaled ricin (Griffiths et al. 1997, 1998, 1999).

3.6.3 Liposomes in Diagnostic Imaging

The use of liposomes as a carrier of imaging agents has been accomplished in all imaging modalities. These encapsulated agents exhibit better biodistribution and enhanced contrast compared with free agents. Definity™ (octafluoropropane) is an example of an approved diagnostic contrast enhancement formulation used in ultrasound procedures. Liposomes for ultrasonography are also being developed where trapped carbon dioxide gas bubbles provide good sound reflection (Torchilin 2005).

For MRI, metal atoms included in PEGylated liposomes have been shown to enhance signal intensity (Hossann et al. 2013). Computerized tomography (CT) contrast agents such as ioporomide have been trapped within PEgylated liposomes improving biodistribution and contrast in animals (Krause et al. 2011).

3.6.4 Liposomes in Nucleic Acid Delivery

The rationale for delivering DNA and RNA in liposomes is different from the drugs and contrast agents discussed above. Unlike small molecules that can diffuse throughout tissues and into cells, nucleic acids are macromolecules that are not prone to rapid cellular uptake. Rather, without the protection afforded by nanoparticles such as liposomes, nucleic acids are susceptible to degradation by nucleases and because they are highly charged they are unable to cross cell membranes. In addition, nonviral vectors such as liposomes are considered non-immunogenic, safe, easy to produce, and have the ability to encapsulate large amounts of nucleic acid. The story however is not so simple. Although liposomes can be designed to be non-immunogenic, the associated nucleic acids can be highly immunogenic through interactions with toll-like receptors and even more immunogenic when delivered in a particulate form. Furthermore, although liposomes can be designed to be non-toxic, optimal nucleic acid association is often achieved using cationic lipids and cationic lipids are known to be toxic. Regardless, formulations have now been created that allow for the efficient delivery of therapeutic nucleic acids to certain cell populations and therefore these formulations will have a significant role in the development of new classes of therapeutic agents including siRNA, microRNA, ASON, and maybe even plasmid expression vectors.

As discussed above, cationic lipids are able to interact spontaneously with negatively charged DNA. Subsequently, the DNA may become encapsulated within a lipid structure. The resulting structure, which may or may not comprise a surrounding lipid bilayer, is also able to interact with negatively charged membranes within the cell. Fusion between the formulation and the cells plasma membrane results in delivery of nucleic acids directly across the plasma membrane and into the cytoplasm. Alternatively, internalized formulations can interact with the inner leaflet of the endosomal or lysosomal compartment in a manner that causes dissociation of the nucleic acid and delivery into the cytoplasm. As already indicated liposomal formulations can be prepared with cell-penetrating peptides that could facilitate intracellular delivery as well. When discussing biomedical applications of liposomes used to deliver therapeutic nucleic acids, the possible therapeutic applications are almost endless. Delivery of DNA encoding a functional gene to replace a mutated gene causing disease could correct dysfunctions specifically linked to the mutation. siRNA can be designed to silence the expression of genes that are known to play a role in disease processes.

There have been many clinical trials of lipid-based DNA formulations designed to transfer selected genes to target cells in patients with cancer, cystic fibrosis (CF),

alpha-1antitrypsin (AAT) deficiency, and coronary artery disease. Many clinical trials have employed lipid formulations of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA administered to the nasal epithelium of patients (Knowles et al. 1998; Porteous et al. 1997). Similar studies were performed with the AAT gene in patients with AAT deficiency (Brigham et al. 2000). In both circumstances, clinical studies found no adverse reactions to the therapy and they produced data showing effective gene delivery. In 2000 several clinical studies were conducted with CFTR-based formulations delivered via nebulization to the lungs. Many of these patients exhibited poor gene expression, immunogenic side effects, and only limited efficacy in the normalization of the lung function (Cao et al. 2011). Other formulations tested in clinical trials include an expression vector for IL-2 designed for intra-tumoral injection in patients with head and neck cancer (Morse 2000). A similar approach was used for an EIA expression vector for patients with breast cancer (Yoo et al. 2001). In 2000 Laitinen et al. studied the delivery of a VEGF expression vector for use in treating human coronary arteries post angioplasty. This group demonstrated successful gene transfer as well as an acceptable safety profile (Laitinen et al. 2000). The roadblocks for use of lipid-based gene delivery systems in the clinic involve transfection efficiency and limited sustained transgene expression in target tissues (Cao et al. 2011).

Lipid-based formulations of siRNA and ASON designed to silence genes in vivo are now being rigorously investigated. Phase I clinical trials of a liposomal c-raf-1 antisense oligonucleotide in patients with advanced solid tumors were shown to successfully decrease mRNA and protein levels with little to no toxicity (Dritschilo et al. 2006; Rudin et al. 2004). A phase I clinical trial is ongoing for a siRNA-formulation directed against protein kinase N3 (PKN3) (Atu027). In vivo results showed that systemic administration of this formulation in mice, rats, and nonhuman primates resulted in silencing of PKN3 expression. Additionally this group showed that use of Atu027 in orthotopic mouse models for prostate and pancreatic cancers led to an inhibition of tumor growth and reduced lymph node metastases (Aleku et al. 2008). There is not sufficient space to discuss the many projects evaluating liposomal/siRNA delivery systems; however, a recent review by Kapoor et al. examines both characterization and current research (Kapoor et al. 2012). In the context of liposomal-based delivery systems of siRNA, there is ample evidence to indicate future success.

3.7 Conclusion

Liposomal formulations of a variety of drugs and the associated benefits achieved in terms of reduced toxicity, increased efficacy, and ability to use therapeutic agents that are prone to degradation have helped to spur the excitement currently felt by those developing a wide range of nanomedicines using materials other than lipids. Continued basic research efforts relating how lipid structure and lipid–drug interactions influence the formation and biological behavior of liposomes have helped

define a new generation of technologies with greater therapeutic potential. The future of liposomes in the development of pharmaceuticals for use in patients is only growing. Areas of future research will concern development of increasingly stable structures with an engineered ability to trigger release of associated contents through external factors such as radiation, radioisotopes, or heat. Multifunctional formulations will incorporate multiple therapeutic agents to treat complex diseases where there are multiple dysregulated pathways or processes requiring intervention within the same time frame. There is an expectation in the nanomedicine community, including those developing liposomal formulations, that the future of personalized medicine will be dependent on the use of nanoformulation technologies. Those healthcare professionals trying to define better treatment solutions for patients with complex diseases are looking to the nanomedicine community for these new treatment solutions.

Problem Box

1. Liposomes constructed using 80 % DOPC and 20 % cholesterol were shown to have poor encapsulation efficiency. Explain the possible reasons for this issue.
2. Describe the design of a lipid-based delivery system to be used in targeting an anticancer agent to tumor cells that express the antigen Her2/neu on the surface.

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Chapter 4
Niosomes

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Abstract During the past decades the use of vesicles for drug delivery has been established. One of these vesicular systems is the niosomes; these may be unilamellar or multilamellar. The niosome bilayer is different from the liposome bilayer in that niosomes are prepared from uncharged single-chain surfactants with the incorporation of cholesterol or other amphiphilic molecules, whereas liposomes are prepared from double-chain phospholipids (neutral or charged) and cholesterol. As with liposomes, the in vitro/in vivo properties of niosomes depend both on the composition of the bilayer and on method of their production. Niosomes may be used as carriers of both hydrophilic and lipophilic drugs.

In this chapter, niosome formation, composition, preparation, characterization, evaluation, advantages, disadvantages, and the more recent applications of niosomes are extensively discussed.

4.1 Introduction

The increasing interest in designing new drug delivery vehicles stems from the necessity to overcome the barriers to drug action, such as limited circulation half-life, reduced solubility, and undesirable side-effects associated with a given therapeutic agent. Some candidate drugs are not bioavailable in parenteral formulations or systemic doses, making the drug useless, regardless of its therapeutic potential. The use of pharmaceutical carriers to enhance the in vivo efficiency of many drugs

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is well established in pharmaceutical research and in the clinical setting (Alonso 2004; Gregoriadis 1988; Müller 1991; Rolland 1993) and thousands of scientists are involved in the study of liposomes and other colloidal structures as carriers for drug delivery.

Liposomes, described by Bangham in 1965 (Bangham et al. 1965), have been prepared with a variety of phospholipids and have been extensively studied as drug carriers (Alberts and Garcia 1997; Bandak et al. 1999; Berry et al. 1998; Boswell et al. 1998; Gabizon et al. 1998; Manosroi and Manosroi 1997; Tsuchihashi et al. 1999). However, problems arise with the general application of liposomes; problems include their low physical and chemical stability. One alternative to the use of phospholipids is the use of non-ionic surfactants (Bouwstra et al. 1997; Horiuchi and Tajima 2000; Uchegbu and Florence 1995; van Hal et al. 1996). Vesicles formed by surfactants are known as niosomes or non-ionic surfactant vesicles (NSVs). Niosomes are similar in terms of structure and certain physical properties to liposomes (Uchegbu and Florence 1995). The self-assembly of non-ionic surfactants into vesicles was first reported in the 70s by researchers in the cosmetic industry (Handjani-Vila et al. 1979).

4.2 Niosomal Components

4.2.1 Surfactants

Several non-ionic surfactants are known to form vesicles. These amphiphiles have two portions: a hydrophilic head group and a hydrophobic tail. Ether, amide, or ester bonds may link the two portions of the molecule. Surfactant vesicles may be prepared from amino acids (Mohanty and Dey 2006; Roy and Dey 2007), fatty acids (Morigaki and Walde 2007; Namani et al. 2007), amides (Wieprecht et al. 2002), alkyl esters (van Hal et al. 1996), and alkyl ether surfactants (Harvey et al. 2005). Alkyl ether surfactants may be broadly divided into two classes based on the nature of the hydrophilic head group: alkyl ethers in which the hydrophilic head group consists of repeat glycerol subunits, related isomers, or larger sugar molecules and those in which the hydrophilic head group consists of repeat ethylene oxide subunits.

The synthesis and characterization of new surfactants with specific physical and chemical properties have been reported in which the surfactants are useful to pharmacy and colloid science (Dipti and Tyagi 2006; Menger and Littau 1993; Muzzalupo et al. 2008; Renouf et al. 1998, 1999; Zana 1997).

New non-ionic surfactant molecules, in which the hydrophilic region consists of azacrown ether units, have been synthesized by Muzzalupo et al. (2005). One such compound is a bola-amphiphile and is composed of two identical azacrown ether units, as polar heads, linked by a long alkyl chain (Muzzalupo et al. 1996).

It has been demonstrated that the bola-amphiphiles is able to assemble into colloidal structures, if associated with cholesterol or other amphiphilic molecules (Muzzalupo et al. 2005).

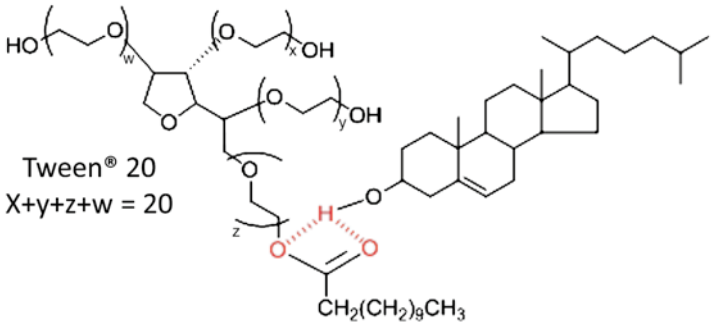

Fig. 4.1 Possible hydrogen bonding interaction between the 3-OH group of the cholesterol and the carbonyl oxygen and also weaker interaction at oxygen of the ester bond

4.2.2 Cholesterol

Cholesterol provides rigidity, fluidity, and permeability to cell membranes and vesicle membranes, which is not provided by the surfactants alone (they are relatively brittle otherwise) (Simons and Ikonen 2000); cholesterol is thus incorporated into the artificial vesicle bilayers in order to increase their ordered state.

Some non-ionic surfactants only form vesicles when cholesterol is included in the bilayer to the level of 30–50 mol% and so cholesterol is usually included at a 1:1 molar ratio in most vesicular formulations. In fact, the water-soluble detergent polysorbate 20 [Hydrophobic Lipophilic Balance (HLB) value = 16.7] is not able to form niosomes in the absence of cholesterol, whereas it forms stable NSVs in the presence of equimolar cholesterol concentrations (Carafa et al. 1998; Saettone et al. 1996; Santucci et al. 1996). It has been suggested (Nasseri 2005) that an interaction occurs between the ester bond of the amphiphilic molecule (phospholipid/surfactant) and the 3-OH group of cholesterol in vesicular systems (Fig. 4.1).

Furthermore, cholesterol stabilizes niosomes against the destabilizing effects of plasma and serum proteins and decreases the permeability of vesicles to entrapped solute, preventing leakage (Baillie et al. 1985; Rogerson et al. 1988).

4.2.3 Charged Molecules

Charged molecules (e.g. negative charged molecules such as dicetyl phosphate and phosphatidic acid) are added to overcome the aggregation of vesicles by creating electrostatic repulsions between the individual vesicles (Junyaprasert et al. 2008; Müller et al. 2001). A charge on the niosomes (e.g. a positive charge from stearylamine or cetylpyridinium chloride) can also alter the in vivo distribution pattern. It is currently admitted that zeta potentials over ±30 mV are required for full

electrostatic stabilization; potentials between ±5 and 15 mV are in the region where there is limited flocculation and a zeta potential of between ±3 and 5 mV is associated with maximum flocculation. Thus, particle aggregation is less likely to occur for charged particles (high zeta potential) due to electrostatic repulsion. However, this rule cannot strictly be applied to systems, which contain steric stabilizers, because the adsorption of steric stabilizers will decrease the zeta potential due to the shift in the shear plane of the particle. It should be noted that the zeta potential depends on the ions present in the medium (Heurtault et al. 2003).

4.3 Factors Governing the Niosome Formation

4.3.1 Thermodynamic Features

The assembly into closed bilayers, both in the case of liposomes (Mozafari and Mortazavi 2005) and niosomes, is not spontaneous. Thermodynamically stable vesicles are formed only in presence of specific mixtures of surfactants and charge-inducing agents. Niosome formation involves the input of energy, for instance by means of physical agitation, e.g. by using the hand-shaking method (Baillie et al. 1985), or heat, e.g. by using the heating method (Mozafari et al. 2002, 2007; Mozafari 2005a). In the resulting closed bilayer structure, hydrophobic parts of the molecule are oriented away from the aqueous solvent, whereas the hydrophilic head group comes in contact with the aqueous solvent. The parameters, which play a role in vesicle formation, are the thermodynamic and physicochemical parameters such as the hydrophilic lipophilic balance and geometric features of amphiphilic molecule

The energy required to form vesicles with amphiphilic molecules has three contributors related to the surface energy, the mechanical energy, and the chemical potential excess energy.

The association of non-ionic surfactant monomers into vesicles on hydration is a result of the fact that there exists a high interfacial tension between water and the hydrocarbon portion (or any other hydrophobic group) of the amphiphile; this high interfacial energy causes these groups to associate. Simultaneously, the steric, hydrophilic, and/or ionic repulsion between the head groups ensure that these groups are in contact with water. These two opposing forces result in a supramolecular assembly (Uchegbu and Florence 1995). Surfactants form anisometric micelles, when dissolved in water at low concentrations, while at higher concentrations (more than 70 %) they exist only in lamellar liquid-crystalline phases (Balzer 1991; Platz et al. 1994). Thermodynamically the energy associated with the formation of vesicles (Schukin and Amelina 1979) or the aggregation energy (Hunter 1991) is the sum of three contributors, namely the surface energy (E_γ), the mechanical energy ($E_{\Delta P}$) due to the curvature effect, and the variation of the chemical potential excess ($E_{\Delta\mu'}$):

$$E = E_\gamma + E_{\Delta P} - E_{\Delta\mu'} \tag{4.1}$$

In order to find the values for the formation energy, E, using the differential geometry results (Do Carmo 1976), the three terms may be globally considered and the energy balance (δ) defined as:

$$\delta = \frac{2.\Delta\mu'}{\gamma.S_{\mathrm{m}}} \tag{4.2}$$

where S_{m} is the molar surface area corresponding to the amphiphile head group, γ is the surface tension, and $\Delta\mu'$ is the chemical potential. The dimensionless character of δ allows different systems to be compared over several experimental conditions.

Thermodynamically the self-assembly must contend with a negative entropy component ($-\Delta S$) and reduction in free energy ($-E^{\circ}$) which is only achieved by the favourable enthalpy ($-\Delta H$) contribution arising from the establishment of van der Waals attractions, hydrophobic forces, hydrogen bond formation, and the screening of electrostatic interactions (Israelachvili 1992).

The formation of small niosomes requires the input of considerable energy and creates a thermodynamically unfavourable packing status. Aggregation (and/or fusion) of these vesicles is a mechanism by which the system dissipates the excess surface energy originating from the distorted molecular packing (Lentz et al. 1985).

Vesicles are generally designed to be incorporated in complex mixtures. Therefore, it is very important to understand their behaviour in the presence of various agents. Many organic substances are more or less surface-active, thus it is reasonable to study the properties of surfactants in the presence of vesicle preparations. Previous studies have shown that niosomes have a greater resistance to micellar solubilization than phospholipid vesicles (Lesieur et al. 1990; Vanlerberghe and Morancais 1996).

4.3.2 Hydrophile-Lipophile Balance

Analysis of vesicle structures indicates that vesicle formation may depend on the balance between hydrophobicity and hydrophilicity (generally expressed as an HLB value). The guidance offered by the HLB number is useful in the evaluation of new classes of compounds for their vesicle-forming ability.

4.3.3 Geometric Features of Amphiphilic Molecule

The geometric feature may be another crucial factor in determining the type of aggregate formed in aqueous environments (Israelachvili 1992). To explain the aqueous behaviour of non-ionic surfactants, a hydrophobic effect was proposed many years ago, as the essential driver for self-assembly (Tanford 1980). The morphologies of the spontaneously formed association colloids may be predicted with

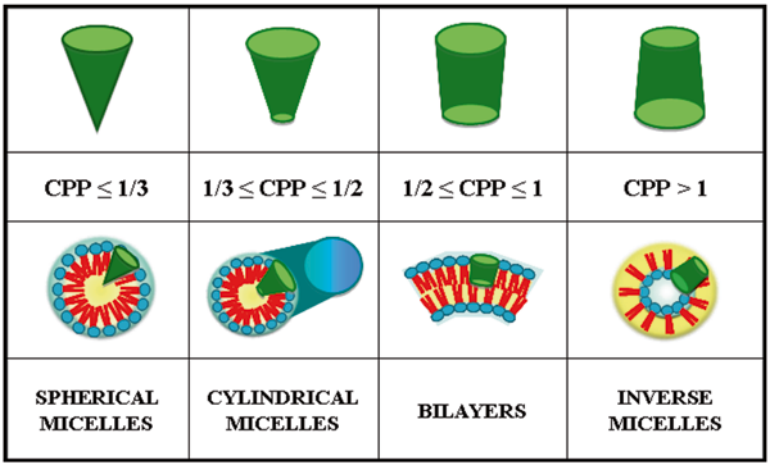

Fig. 4.2 The relationship between the CPP and the morphology of self-assembled amphiphilic molecules

considerable certainty using nominal geometric parameters of the surfactant molecule: the polar head surface area (a_0), the hydrophobic tail volume (V), and the critical hydrophobic tail length (l_c).

The critical packing parameter (CPP) has been defined by Israelachvili (1992):

$$CPP = \frac{V}{a_0 l_c} \tag{4.3}$$

According to the CPP value, the shape and size of the equilibrium aggregate would evolve from spherical micelles ($CPP \leq 0.33$) to cylindrical micelles ($1/3 \leq CPP \leq 0.5$), bilayers ($0.5 \leq CPP \leq 1$) or inverse micelles ($CPP > 1$) (Fig. 4.2).

4.4 Niosome Preparation

Many approaches exist to prepare niosomes; however, some of these methods are used to manufacture liposomes and have been adapted/used as such. Differently from liposomes, the niosome preparation is carried out in the absence of nitrogen as the surfactant is resistant to air oxidation. Moreover, the elevated temperature used does not represent an issue, because non-ionic surfactants are more stable than phospholipids. Many techniques such as the thin film hydration, the organic solvent injection method, the reverse phase evaporation method, and the bubble method have been previously described for the preparation of niosomes (Apoorva et al. 2012; Uchegbu and Vyas 1998).

Recently a few innovative methods of niosome preparation have been reported in the literature and these will be described in this section.

4.4.1 Proniosome

A technique of producing niosomes involves coating a water-soluble carrier with non-ionic surfactants (e.g. sucrose stearate and the Span surfactants). The water-soluble carrier consists of a sugar such as maltodextrin, glucose, sorbitol, or lactose monohydrate. The result of the coating process is a dry formulation in which each water-soluble particle is covered with a thin film of dry surfactant. This preparation is known as a "proniosome" preparation (Hu and Rhodes 2000).

Proniosome powders may be stored in sealed containers at 4 °C and may be hydrated immediately before use to yield surfactant vesicles. The niosome dispersions are obtained by hydrating the proniosome preparation with a warm aqueous phase at a temperature above the phase transition temperature and brief agitation. However, in this method a thin surfactant film on the carrier is desirable to facilitate the hydration of the proniosome powder. In fact, the rate of surfactant application is controlled so that the powder bed of spray-dried sugar does not become overly wet such that a slurry would form. An important advantage of proniosome-derived niosomes is the reduction of niosome' physical instability such as aggregation, fusion, and leakage. In addition the proniosomes are obtained as a dry powder, so further processing is possible. To provide convenient unit dosing, the proniosome powder may be processed to make beads, tablets, or capsules.

4.4.2 Heating Method (HM)

The heating method developed by Mozafari to produce vesicles involves the hydration of an amphipathic molecule in an aqueous solution containing 3 vol% polyol at high temperature (Mozafari 2005b). HM-niosomes are prepared as follows: mixtures of non-ionic surfactant, cholesterol, and/or charge-inducing molecules are added to an aqueous medium (e.g. buffer or distilled H_2O) in the presence of a glycerol as the polyol. Glycerol is utilized since it is a water-soluble and a physiologically acceptable chemical with the ability to act as a tonicity agent and increase the vesicle stability by preventing coagulation and sedimentation. The mixture is heated while stirring (at low shear forces) until vesicles are formed. This is a non-toxic, scalable, and one-step method.

4.4.3 Supercritical Carbon Dioxide Fluid ($scCO_2$)

In the last decade a new method, the supercritical reverse phase evaporation (scRPE) method, for the preparation of several bilayer vesicles using supercritical fluids has been developed (Imura et al. 2003; Otake et al. 2001). Supercritical fluids are those fluids which are non-condensable and highly dense at temperatures and pressures

beyond the critical values. At the critical point, supercritical fluids have the density of a liquid and low viscosity but with the flow property of a gas. Carbon dioxide is a widely used gas to produce supercritical fluid because of its low critical temperature ($T_c = 31.1$ °C) and pressure ($P_c = 73.8$ bar). Similar to non-polar solvents, the flow property of CO_2 may be adjusted by changing the pressure or temperature. Therefore supercritical carbon dioxide ($scCO_2$) may be used to substitute organic solvents in the formation of bilayer vesicles with the advantages of being environmentally friendly, non-toxic, non-inflammable, and inexpensive (Bunker et al. 1997; Cooper 2000; Heo et al. 2000). The $scCO_2$ method efficiently entraps water-soluble and thermolabile substances in niosomes without using highly toxic organic solvents.

Manosroi et al. (2008a) report that niosomes prepared with the $scCO_2$ method are large unilamellar structures with a size range of 100–440 nm. A reduced size distribution may be obtained by the combination of the $scCO_2$ method with sonication or polycarbonate filter extrusion methods.

Because the method is a one-step method and uses no inflammable, volatile or toxic organic solvents, scale-up of niosome production would be less problematic.

4.4.4 Membrane Contactor

The scale-up of niosome production, from laboratory scale using a syringe-pump device to a pilot scale using the membrane contactor module, has been reported by Pham et al. (2012).

An ethanol injection-based method was applied for niosome preparation using the Shirasu Porous Glass (SPG) membrane contactor.

For the niosome preparation, the required amounts of excipients and active principle ingredients were dissolved in ethanol. The ethanolic phase was placed in the pressurized vessel, under N_2, the circulation of both aqueous and organic phases at the same time was allowed, and the organic phase permeated through the pores into the aqueous phase. Spontaneous niosome formation occurred as soon as the organic solution was in contact with the aqueous phase. Then, the suspension was stabilized for 15 min with magnetic stirring. The ethanol was removed by rotary evaporation under reduced pressure.

4.4.5 Vesicle Purification

The hydration of niosome mixtures rarely leads to complete drug encapsulation, regardless of the drug loading optimization steps taken. It is thus often a requirement that unencapsulated drug must be removed. Although it may be argued that the use of systems in which the drug is partially encapsulated in niosomes may eventually yield systems with a beneficial biphasic biodistribution profile. This drug

delivery system would give an initial burst to initiate therapy followed by a sustained maintenance dose. The methods used for the removal of unentrapped material include different techniques:

4.4.6 Dialysis

Dialysis is based on the principles of the diffusion and osmosis of solutes and fluid across a semi-permeable membrane. Several authors (Hao et al. 2002; Manconi et al. 2005; Muzzalupo et al. 2005, 2007) have used this technique to purify their vesicle dispersion from unentrapped materials: the prepared niosomes are filled into dialysis bags and the free drug is dialyzed against buffered saline.

4.4.7 Gel Filtration

In order to separate loaded niosomes from unentrapped substances purified vesicle dispersion may be obtained by means of gel filtration on Sephadex G75 (Carafa et al. 2002, 2004, 2006; Terzano et al. 2005), G50 (Manconi et al. 2002, 2003), or G25 (Tabbakhian et al. 2006) columns.

4.4.8 Centrifugation/Ultracentrifugation

To purify niosomes, several authors (Alsarra et al. 2005; Nasseri 2005; Türker et al. 2005) report the use of centrifugation. This process is based on centrifugal force (*g*-force) to remove the unentrapped drug from niosomes and to obtain the largest fraction of vesicles residing in the pellet according to their size, shape, and density. However, some particles, extremely small in size, will not sediment unless subjected to high centrifugal force (ultracentrifugation). The ultracentrifuge is a special type of centrifuge that is capable of generating accelerations of $1{,}000{,}000 \times g$ (Dufes et al. 2004; Erdoğan et al. 2005; Guinedi et al. 2005). In this type of separation repeated washing of the pellets by resuspending in isotonic solvents and re-pelleting may result in complete removal of unentrapped drug. At the end of the centrifugation/ultracentrifugation steps, the pelleted vesicles are resuspended in appropriate buffered solutions.

Several authors combine the aforementioned centrifugation processes with Sephadex chromatography mini-columns (Minicolumn centrifugation method). In the minicolumn centrifugation method, mixtures of vesicle-entrapped and free low molecular weight solutes are applied to the column bed and inserted in a spin tube. During the centrifugation the vesicles run through the column under *g*-force, while the free solute is quantitatively retained in the Sephadex bed (Gupta et al. 2005; Singh et al. 2004).

This procedure, compared to other purification methods, is applicable to a variety of solutes and 92 to 100 % recovery is achieved for the niosomes with no dilution of the niosomal preparation.

Specific centrifugation methods have been used by several authors to remove the unentrapped genetic material from niosomes, such as density gradient centrifugation (Jain et al. 2005; Vyas et al. 2005). As the name implies, density gradient centrifugation utilizes a specific medium that gradually increases in density from the top to the bottom of a centrifuge tube. This means that under centrifugal force, particles will move through the density gradient and stop when the density of the particle equals the density of the surrounding medium. Ficoll is used to create the density gradient and Ficoll is an inert, high molecular weight, densely branched polysaccharide that more nearly approximates to a compact sphere. It is especially used for the isolation of leukocytes from peripheral blood (erythrocytes), and for the separation of leukocyte types (New 1990).

All of these methods have their inherent advantages and disadvantages. The choice of the method must take different factors into account and for industrial purposes it may be more worthwhile to concentrate efforts and resources on the achievement of high levels of drug loading so as to avoid these separation steps altogether or to consider systems in which the unentrapped drug may be useful as a specific priming dose.

4.5 Niosome Characterization

A rational characterization of nanosystems is needed for the control of the quality of the product, which is a prerequisite for the development and clinical applications (Khosravi-Darani et al. 2007). Having direct impact on the stability and critical importance on their in vivo performance, parameters such as morphology, size, polydispersity index, number of lamellae, zeta potential, bilayer fluidity, composition, encapsulation efficiency, carrier-bioactive interaction, and chemical stability must be evaluated.

4.5.1 Vesicle Size

Determination of nanocarrier size distribution is a necessary quality control assay because of the importance of this parameter on the physical properties and stability of the formulations (Goren et al. 1990). Size distribution, along with composition, defines plasma pharmacokinetics, biodistribution, and the stability of the nanocarriers and their associated/entrapped substances in plasma and other organs (Barenholz and Amselem 1993).

Niosomes are usually spherical and so their diameter may be determined using microscopy or dynamic light scattering (DLS or photon correlation microscopy, PCS).

DLS provides cumulative average information of the size of a large number of particles simultaneously and it is useful for the determination of particle size distribution (Manosroi et al. 2008a; Vangala et al. 2006). Moreover, DLS provides valuable information on the homogeneity of the vesicular suspension (Girigoswami et al. 2006; Muzzalupo et al. 2008). A single sharp peak in the DLS profile implies the existence of a single population of vesicles. The polydispersity index (PI) is of critical importance to in vivo performance: PI values of less than 0.4 are indicative of a monodisperse sample population. One problem in size determination with DLS is that it does not provide information on the shape of the nanosystem and it assumes any aggregation of more than one particle as one single particle. The microscopic approach is commonly used to characterize the structure/morphology/geometry of the nanocarriers. The microscope studies give us a qualitative idea about the size and shape of the particles formed in various surfactant/cholesterol systems.

Electron microscopy techniques have been widely used to measure the size and the size distribution of the particles. Several electron microscopy techniques may be employed for nanocarrier research. These include: scanning electron microscopy (SEM), preferentially for solid products (Abd-Elbary et al. 2008; Blazek-Welsh and Rhodes 2001), transmission electron microscopy (TEM), for liquid state samples (Abd-Elbary et al. 2008; Manconi et al. 2005; Manosroi et al. 2008b; Muzzalupo et al. 2005, 2008; Vangala et al. 2006), negative-stained transmission electron microscopy (NS-TEM) (Liu and Guo 2007a, b), freeze fracture transmission electron microscopy (FF-TEM) (Carafa et al. 2004, 2006; Manosroi et al. 2008a), and cryo-transmission electron microscopy (cryo-TEM). Atomic force microscopy (AFM) (Cortesi et al. 2007) is also a useful technique.

The scanning tunnelling microscope (STM), used in 1982 by Binnig et al. (1982), is also a powerful tool for the study of micro- and nanoscale structures. Compared with the other types of microscopy, STM has unique characteristics, including: (1) ultra-high resolution down to atomic dimensions, (2) three-dimensional images with very high resolution especially in the vertical direction, (3) a variety of operating conditions, such as in vacuum, air, and liquids, (4) an observation range spanning from micrometres to angstroms, (5) the ability to do tunnelling spectroscopy. Furthermore, STM in particular is very useful in determining the bilayer thickness of liposomes and niosomes (Zareie et al. 1997).

Microscopy techniques sometimes produce artefacts and hence it will be useful to combine different techniques to obtain reliable results.

4.5.2 Zeta Potential and Surface Properties

The zeta potential value is important to vesicle stability and vesicle in vivo fate. Several authors confirmed the importance of zeta potential measurements to assess vesicle formation, to study drug/vesicle interaction and formulation stability (del Burgo et al. 2007; Carafa et al. 2004, 2006; Liu et al. 2007).

The zeta potential is a critical parameter to be taken carefully into account in the preparation of niosomes (Sennato et al. 2008; Agrati et al. 2011).

4.5.3 Bilayer Characterization

Niosomes may be classified morphologically, according to the number of membrane bilayers (lamellae) as unilamellar and multilamellar vesicles. Unilamellar vesicles are characterized by the presence of one surfactant bilayer, while multilamellar vesicles by several concentric surfactant bilayers in an onion-skin arrangement. The number of lamellae is determined by using AFM (Di Marzio et al. 2011) microscopy techniques, NMR spectroscopy (Kreuter 1994), and small angle X-ray scattering (SAXS), (Carafa et al. 2006; Liu and Guo 2007a, b). The latter method together with the in situ energy-dispersive X-ray diffraction (EDXD) has been also used to characterize the bilayer thickness (Caracciolo et al. 2008; Pozzi et al. 2010).

Blazek-Welsh and Rhodes (2001) used SEM images to estimate the thickness of proniosomes at various concentrations of surfactants.

Another feature of the niosomes is the fluidity of their membranes, which allows membrane deformation without disrupting bilayer integrity (vesicle stability) and drug release ability.

These parameters may be measured by means of fluorescence probes (e.g. DPH and pyrene) as function of temperature and/or time (Girigoswami et al. 2006; Lentz 1993; Liu and Guo 2007b; Macdonald et al. 1988; Manosroi et al. 2003; Sarkar et al. 2002; Zhai et al. 2004).

Manosroi et al. determined the microviscosity of niosomal membranes by fluorescence polarization in order to study packing structure of the vesicular membrane (Manosroi et al. 2003, 2008a).

4.5.4 Vesicle Stability

Vesicle stability is a complex issue and involves chemical stability, physical stability, and biological stability, which are all inter-related. The evaluation of these parameters is fundamental when determining the potential in vitro/in vivo applications in nanomedicine. Biological stability, however, depends on the presence of agents that interact with the vesicular structure after patient administration and it therefore also depends on the administration route. Generally, stability is determined by means of size and zeta potential variations (DLS or microscopy techniques) or by the evaluation of the release rate of different probes as a function of time and/or temperature and in absence or in presence of biological fluids.

4.5.5 Entrapment Efficiency

The entrapment efficiency may be defined as the amount of active substances delivered by niosomes. The entrapment efficiency (EE) of vesicular systems may be expressed as:

$$EE = \frac{amount\ \ entrapped}{total\,amount} \times 100 \tag{4.4}$$

The entrapment efficiency may be determined by spectrophotometry (Manconi et al. 2005; Muzzalupo et al. 2005) or in the case of genetic material (luciferase plasmid) by gel electrophoresis followed by UV densitometry (Manosroi et al. 2008b). In addition the entrapment efficiency may be evaluated fluorometrically using a hydrophilic fluorescent marker (e.g. calcein) (Santucci et al. 1996; Manosroi et al. 2003) and one of following equations:

$$EE = \frac{Mole_{probe\,entrapped}}{Mole_{surfactant}} \times 100 \tag{4.5}$$

$$EE = \frac{I_{in} - I_{tx} \times r \times 100}{I_{total} - I_{tx} \times r} \times 100 \tag{4.6}$$

where I_{total} and I_{in} are fluorescence intensity before (I_{total}) and after (I_{in}) the addition cobalt chloride ($CoCl_2$), I_{tx} is fluorescence intensity after addition of organic solvent, such as *n*-propanol or detergent, such as Triton X-100, r is the volume correction factor (~1.04). The second equation does not require vesicle purification.

4.5.6 In Vitro Release

The release of drugs from niosomal suspensions may be affected by various parameters such as hydration volume, drug concentration, and location within the niosomes. These parameters may be monitored using fluorescent probes. Girigoswami et al. (2006) used Fluorescence Resonance Energy Transfer (FRET) to monitor release of encapsulated substances in niosomes, monitoring the entry/exit dynamics of donor/acceptor dyes.

Another method used to evaluate in vitro release rates includes the use of dialysis tubes placed in buffer solution with constant shaking at 25, 32, or 37 °C. At various time intervals, the buffer is analyzed for the drug content by an appropriate assay method.

The release kinetics in vitro may be studied by a simple equation (Sinclair and Peppas 1984):

$$\frac{M_t}{M_\infty} = Kt^n \tag{4.7}$$

where M_t is the amount of drug released at time t, M_∞ is the loaded drug amount, K is the kinetic constant and n is the exponent, related to the release mechanism (Carafa et al. 2004).

4.5.7 pH Sensitivity Assessment

Stimuli-sensitive niosomes, which release their cargo in response to an external stimuli, constitute interesting alternatives for therapies directed towards solid tumours and other spatially well-defined targets. The gradual decrease in pH experienced by niosomes that are internalized via endocytosis constitutes a potentially very useful intrinsic stimulus and several pH-sensitive niosome formulations based on this strategy have been developed and evaluated biologically (Bergstrand et al. 2003; Carafa et al. 2006; Di Marzio et al. 2008, 2011; Masotti et al. 2010).

Steady-state fluorescence spectroscopy has also been used to characterize the pH sensitivity of the niosomes. The fluorescent probes chosen for such experiments were 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS) and Nile Red. HPTS is a hydrophilic molecule used as a pH-sensitive fluorescence probe for measurements of the aqueous interior of artificial vesicular systems. The fluorescence intensity (at 510 nm) of HPTS is strongly dependent upon the degree of ionization of the 8-hydroxyl group (pK = 7.2) and hence upon the environmental pH. The hydrophobic probe Nile Red has a low solubility and fluorescence in water and hence its fluorescence emission properties are strongly medium-dependent. This probe, when integrated into the vesicle bilayers, is monitored for its shift in emission maximum, which occurs during the transition from lamellar to micellar phase (Di Marzio et al. 2011).

To study the effect of pH on physical stability, niosomes must be diluted with buffers at pH 7.4 or at pH 5.5. The effect of pH on colloidal stability may be evaluated by DLS and by turbidity measurements.

The effect of pH on vesicle bilayers may also be evaluated by SAXS. This technique is able to measure bilayer thickness changes at different pH values. The maintenance of pH sensitivity in serum must also be defined by monitoring fluorescent probe release at different pH values and in the presence of serum (Carafa et al. 2006; Di Marzio et al. 2008).

An assay, performed at different pH values, for vesicle-vesicle fusion is based on the non radioactive resonance energy transfer (NRET) between two fluorescent lipids *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl) phosphatidylethanolamine (N-NBD-PE, energy donor) and *N*-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (N-Rh-PE, energy acceptor). This technique provides a means by which lipid mixing during vesicle fusion may be followed, since fusion of the vesicles will result in intermixing of the membrane lipids and energy transfer between the probes (Francis et al. 2001).

4.6 Niosome Applications

Niosomal drug delivery is potentially applicable to many pharmacological agents. There is a growing number of publications on potential niosome applications in therapy. Some examples of these numerous studies are reported below.

4.6.1 Oral Delivery

The oral route is the most widely accepted means of drug administration. To overcome the gastrointestinal barriers various types of colloidal systems such as niosomes have been investigated in order to improve the poor and variable oral bioavailability of some drugs. With respect to hydrophobic drugs, evidence exists that the in vitro gastrointestinal stability of paclitaxel is well preserved with Span 40 niosomes (Bayindir and Yuksel 2010); moreover, Span 20, Span 40, and Span 60 vesicles significantly improved the oral bioavailability of griseofulvin in albino rats after a single oral dose (Jadon et al. 2009). Recently, niosomes prepared from Span 40, Span 60, and cholesterol were proposed as potential oral delivery systems for ganciclovir. In vivo studies in rats revealed a fivefold increase in the bioavailability of the drug after oral administration of an optimized formulation when compared to a standard tablet dosage form (Akhter et al. 2012).

Also a proniosome formulation (Span 60: cholesterol: dicetyl phosphate, 1:1: 0.1) was studied as a means of improving the extent of celecoxib absorption, compared to a conventional marketed celecoxib capsule in human volunteers (Nasr 2010).

Furthermore, due to great progress in biotechnology, the pharmaceutical industry is capable of producing a large number of potential therapeutic proteins and peptides in high amounts. The main features of many therapeutic molecules are hydrophilicity and susceptibility to degradation by gastrointestinal enzymes and the acidic pH of the stomach. Those features are responsible for the low oral bioavailability experienced with these products. An investigation was initiated to determine if niosomes might stabilize these molecules in the gastrointestinal tract and improve their permeation through the intestinal mucosa. In an in vitro study conducted by Yoshida et al. (1992) on a vasopressin derivative entrapped in niosomes, it was shown that the entrapment of the drug in niosomes significantly increased the stability of the peptide, in an intestinal loop model.

4.6.2 Ocular Delivery

Topical ocular drug delivery is one of the commonly used and preferred routes for treating conditions that affect the anterior segment of the eye (cornea, conjunctiva,

sclera, iris, and lens). However, there are many anatomical and physiological barriers and a strategy to cross these barriers is required.

Mucoadhesive niosomal ophthalmic formulations of acetazolamide (Aggarwal et al. 2007) and timolol (Aggarwal and Kaur 2005; Kaur et al. 2010) have been studied and compared to marketed formulations, in order to reduce intraocular pressure. Furthermore recently, Span 60-based giant oval niosomes were reported to be capable of enhancing the corneal permeation of naltrexone hydrochloride, while also being practically non-irritant (Abdelkader et al. 2011). Finally niosomal formulations, prepared using various surfactants (Tween 60, Tween 80, or Brij 35), showed a controlled in vitro release of gentamicin (Abdelbary and El-gendy 2008).

4.6.3 Dermal and Transdermal Delivery

The slow penetration of drugs through the skin is the major drawback of the transdermal route of delivery. In the last decade several studies have been performed to evaluate the effect of niosomes on the dermal and transdermal delivery of different active substances. The transdermal field has seen some promising data emerge. Some examples of recent applications reported include the transdermal or dermal delivery of: gallidermin (Manosroi et al. 2010), terbinafine (Sathali and Rajalakshmi 2010), curcumin (Gupta and Dixit 2011), vinpocetine (El-Laithy et al. 2011), and elagic acid (Junyaprasert et al. 2012). Additionally pH-sensitive niosomes composed of Tween 20 or Span 60 mixed with cholesterol and cholesteryl hemisuccinate (CHEMS) have been used for the topical delivery of ibuprofen. When niosomes with Span 60 and CHEMS were prepared, there was a statistically significant increase in the in vitro skin permeation of the drug (Carafa et al. 2009).

To enhance the transdermal adsorption of drugs, elastic niosomes (Di Marzio et al. 2012; Manosroi et al. 2008c, 2009, 2011) and niosome-loaded hydrogel systems (Antunes et al. 2011; Lakshmi et al. 2007; Marianecci et al. 2011) have also been used.

Topical immunization is novel and such needle-free strategies involving vaccine delivery through topical application of the antigen and adjuvants directly, or via a suitable carrier system, to intact skin have been studied, with niosomes exploited for topical immunization (Maheshwari et al. 2011; Mahor et al. 2007).

4.6.4 Pulmonary Delivery

In inflammatory diseases, infections, or cancer of the respiratory tract, the therapeutic value of pulmonary administration may exceed that of oral or parenteral administration, since the area affected is directly reached by the drug. In diseases characterized by bronchial mucus hypersecretion, lipophilic substances, such as corticosteroids, are remarkably impeded from reaching their receptors, which are

localized within the cytoplasm of bronchial epithelial cells. Niosomes have been used to circumvent this problem (Marianecci et al. 2010; Terzano et al. 2005).

Recently Span and Tween niosomes were studied for the pulmonary delivery of anti-tuberculosis drugs (Jatav et al. 2011; Mehta et al. 2011; Moazeni et al. 2010; El-Ridy et al. 2011).

4.6.5 Anti-neoplastic Therapy

One of the advantages of the drug loading in a niosome is the selective delivery of cytotoxic agents to the tumour site; this has been associated with a decrease in toxic effects. Drug-loaded nanovesicles have been shown to improve the therapeutic outcome of traditional chemotherapeutics by altering drug pharmacokinetics and biodistribution. For example, the intravenous administration of hydroxycamptothecin (HCPT), entrapped in niosomes, to S-180 tumour-bearing mice resulted in total regression of tumours, higher plasma drug levels, and slower drug elimination when compared to the drug in solution (Shi et al. 2006).

Niosomes have also been used in melanoma therapies (Cosco et al. 2009; Gude et al. 2002; Paolino et al. 2008).

New nanohybrid systems composed of non-ionic surfactants inserted within liposomes, which were then loaded with paclitaxel (PTX), were prepared to overcome multidrug resistance in PTX-resistant human lung cancer cell lines. Three non-ionic surfactants, Solutol® HS 15, Pluronic F68, and Cremophor EL, were inserted into liposomes. The apoptotic assay and the cell cycle analysis showed that the nanohybrid systems could induce more apoptotic cells in drug-resistant cells when compared with liposomes (Ji et al. 2012).

4.6.6 Drug Targeting

4.6.6.1 The Reticulo-Endothelial System (RES)

After intravenous administration, the lipid and surfactant vesicles are surrounded by blood components including many types of circulating serum factors known as opsonins which mark them for clearance by RES macrophages.

Niosomes may be used to target drugs in the treatment of diseases in which the infecting organism reside in the organs of RES; diseases such as leishmaniasis. The study of antimony distribution in mice, performed by Hunter et al. (1988), showed high liver levels of the drug after intravenous administration of the carrier forms of the drug, without damage to the heart and kidney.

More recently, Span 60/Tween 61 niosomes conjugated with a purified monoclonal antibody against the CD44 receptor were proposed for immuno-targeting (Hood et al. 2007) and "hydrid" Tween 20 niosomes were reported to show intrinsic selectivity towards macrophages (Agrati et al. 2011).

4.6.6.2 Organs Other Than RES

Glucose and transferrin conjugation to niosomes enhance vesicle distribution to the brain (Dufes et al. 2004) and solid tumours (Hong et al. 2009).

To obtain a potential brain-targeted delivery system for the anticancer agent doxorubicin, a niosomal formulation was functionalized by *N*-palmitoyl glucosamine. The intravenous administration to rats of these NPG-niosomal formulations reduced drug accumulation in the heart, prolonged drug residence time in the blood, and enhanced the concentration in the brain, when compared to the commercial formulation (Bragagni et al. 2012).

4.6.7 Immunological Applications

Niosomes have been used to study the nature of the immune response to antigens. Brewer and Alexander (1992) have reported niosomes as potent adjuvants as niosomes were shown to demonstrate immunological selectivity and low toxicity.

Span niosomes have also been studied as DNA vaccine carriers, after conjugation with *o*-palmitoyl mannan (Jain et al. 2005; Vyas et al. 2005).

4.6.8 Gene Delivery

Gene therapy has been studied intensively as a possible treatment for a variety of inherited or acquired disorders. The success of gene therapy is highly dependent on the delivery vector, used for the gene. Although niosomes have been applied in pharmaceutics since the 1980s, only a few reports have focused on their application as vectors for gene delivery. To be used as vectors the niosomes need to incorporate charged moieties into the bilayer. For example cationic niosomes have been used as carriers for plasmid (Manosroi et al. 2008b) and oligonucleotide delivery (Huang et al. 2008, 2011).

4.6.9 Diagnostic Agents

Nanocarrier-mediated delivery has emerged as a means of enhancing the delivery of imaging agents to tumours, thereby increasing the potential for diagnosis at an earlier stage. It has been shown that the combination of PEG and glucose conjugates to the surface of niosomes significantly improved tumour targeting of an encapsulated paramagnetic nanoparticles for magnetic resonance imaging in a human carcinoma xenograft model (Luciani et al. 2004).

4.7 Final Considerations

Niosomes have been extensively studied as an alternative to liposomes. Niosomes have some advantages over liposomes, such as their relatively higher chemical stability, improved purity, and relatively lower cost in comparison with liposomes. The niosome bilayer is different from the liposome bilayer in that niosomes are prepared from uncharged single-chain surfactants and cholesterol, whereas liposomes are prepared from double-chain phospholipids (neutral or charged) and cholesterol. As with liposomes, the in vitro/in vivo properties of niosomes depend both on the composition of the bilayer and on method of their production. Niosomal drug delivery has yet to make its clinical debut even though these systems have been demonstrated to yield promising therapeutics. Niosomes alter the plasma clearance kinetics, tissue distribution, metabolism, and cellular interaction of the drug. Despite the many promising proof of concept studies there is still a long road ahead to becoming a clinical reality. This means there are still many challenges that need to be overcome and there are still opportunities for academic and industrial scientists to make a decisive impact.

Problem Box

How does the addition of cholesterol to the niosome bilayer influence their in vitro/in vivo behaviour?

Cholesterol provides rigidity to cell membranes and vesicle membranes, which is not provided by the surfactants alone. Cholesterol is thus incorporated into the artificial vesicle bilayers in order to increase their ordered state. Furthermore, cholesterol stabilizes niosomes against the destabilizing effects of plasma and serum proteins and decreases the permeability of vesicles to entrapped solute, preventing leakage.

Which niosome preparation method may be amenable to industrial scale-up?

The supercritical carbon dioxide reverse phase evaporation ($scCO_2$-RPE) method is a one-step method and uses no inflammable, volatile or toxic organic solvents; for these reasons it would be less problematic to use this process for the industrial scale preparation of niosomes.

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Chapter 5
Solid Lipid Nanoparticles (SLN™)

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Abstract Solid lipid nanoparticles (SLN™) are a new generation of drug delivery systems being exploited for several drugs since the nineties. These particles can be composed of different types of solid lipids, such as glycerides, waxes, and fatty acids, and stabilized by a wide range of surfactants. In the present chapter, the chemical structure, production methodology, and physicochemical characterization are systematically discussed. Parameters such as particle size, distribution, polymorphic behaviour, and crystallization are required to characterize SLN and may predict their in vitro stability and in vivo profile, therefore structural parameters can influence the biopharmaceutical properties. The use of SLN for drug delivery is also dependent on their toxicological profile in vitro. Nanotoxicology is also discussed addressing the key points that may limit the clinical use of SLN.

5.1 Introduction

Nanotechnology applied to drug delivery reached the clinic within the last few decades. Nanosciences and Nanotechnologies were defined by the Royal Society and Royal Academy of Engineering *as the study of phenomena and manipulation of materials at atomic*, *molecular and macromolecular scales*, *and the design*,

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characterization, *production and application of structures*, *devices and systems by controlling shape and size at nanometer scale* (Ann 2004; Dowling et al. 2004). Lipid nanoparticles are being developed for drug delivery to overcome the limitations of traditional therapies and are providing endless opportunities in this area. Since the nineties that Solid Lipid Nanoparticles (SLN™) have captured the attention of several researchers because they combine innovation and versatility and also show major advantages for various application routes (parenteral, oral, dermal, ocular, pulmonary, rectal). The main features of SLN include their suitability to optimize drug loading and release profiles, long shelf life and low chronic toxicity, protection of incorporated labile drugs, site-specific targeting, and excellent physical stability (Souto and Muller 2010; Fangueiro et al. 2013; Wissing et al. 2004). However, some limitations may also be pointed out, such as the insufficient loading capacity, the risk of drug expulsion after polymorphic transitions during storage, and the high amount of water (70–99.9 %) required in their composition. SLN typically consist of three essential components, i.e. solid lipid, surfactant, and water. These lipid particles are composed only of solid lipids at body and room temperature, having a mean particle size of between 50 and 1,000 nm (Souto and Doktorovova 2009; Souto and Muller 2011; Souto et al. 2011a). They have the potential to load both hydrophilic and hydrophobic drugs. Depending on the location of the drug, i.e. if the drug is entrapped inside the lipid matrix, encapsulated or adsorbed onto the surface of particles, they can exist in three different morphological types. The SLN Type I is composed of a homogenous matrix in which the drug is molecularly dispersed within the lipid core or it is present in the form of amorphous clusters, also homogenously dispersed in the lipid core. The SLN Type II or drug-enriched shell model occurs when the lipid concentration is low relatively to the drug, and a drug-free (or drug-reduced) lipid core is formed when the drug reaches its saturation solubility in the remaining melt. An outer shell will solidify containing both drug and lipid with mainly drug on the surface of the particles. The SLN Type III or drug-enriched core model is formed when the drug concentration is relatively close to or at its saturation solubility in the lipid melt. The drug forms a core surrounded by a lipid-enriched shell. Types I and III are those reported to provide prolonged or/and controlled release profiles. The present chapter reviews the lipid nanoparticles chemistry, production techniques, physicochemical characterization, and their developments in drug delivery with a special focus on nanotoxicology of lipid particles.

5.2 Nanomaterials

The specific characteristic of SLN is their size, which falls in the transitional zone between individual atoms or molecules and the corresponding bulk materials. Size reduction can modify the physical and chemical properties of nanomaterials distinctively from their bulk and molecular counterparts. Independently of the particle size, the materials present in the nanoparticles in contact with cell membranes and the chemical reactivity of those materials play a dominant role when the particles react with other substances or tissues (Yang et al. 2008). Colloidally dispersed lipids

are interesting carrier systems for bioactive substances, particularly for lipophilic drugs. By definition, SLN are colloidal dispersions, since they are neither a suspension nor an emulsion (Heike 2011). Lipid compounds (of animal or vegetable resources) are generally regarded as safe (GRAS) and have proved their biocompatibility and biodegradability, since they are physiological lipids that occur naturally in the organism. The most commonly used lipids are listed in Table 5.1. The general lipid composition (e.g. fatty acids, waxes) will have a different crystallinity and capacity for accommodation of drug molecules. In addition, the organization of lipid molecules after recrystallization could result in three possible forms, mainly, an amorphous α form, an orthorhombic perpendicular β′ form, and a triclinic parallel β polymorphic form. The degree of crystallinity of the lipid matrix increases from the α form, to β′ form, and finally to β form (Westesen et al. 1993). Lipids usually show high crystallinity during storage, and not immediately after SLN production. This could lead to a problem related to drug expulsion from the lipid matrix, since less space is left for the accommodation of drug molecules with the creation of a perfect crystal lattice. The metastable form β′ is usually related to a controlled drug release profile (Wiechers and Souto 2010).

Triacylglycerols (TAGs) are widely used in pharmaceuticals as matrix materials. Physical properties, such as polymorphism, melting and solidification, density, and molecular flexibility, are influenced by the crystalline phases of TAGs. The structural properties of TAG crystals are influenced by their molecular properties, such as saturation/unsaturation of the fatty acid moieties, glycerol conformations, symmetry/asymmetry of the fatty acid compositions connected to the glycerol groups. TAGs present as fats as do diacyl- and monoacylglycerols. The physical properties of TAGs depend on their fatty acid compositions (Kaneko 2001). TAGs mostly used in SLN are trilaurin, trimyristin, tripalmitin, and tristearin (Souto et al. 2011b). These types of lipids are reported to be safe for use as colloidal carriers. Tripalmitin and tristearin are biodegradable by lipases (Olbrich et al. 2002). However, some disadvantages are associated with the use of pure TAGs, since immediately after recrystallization the α form is formed and more drug is incorporated due to the less-ordered crystal structure. During storage, other polymorphic modifications may occur (β and β′) and the reorganization does not leave space enough for the loaded drug molecules, leading to their expulsion from the lipid matrix (Souto et al. 2011b). Nevertheless, their use should not be discarded, since studies of long-term stability of SLN-based TAGs have also been reported (Souto and Muller 2006). Mono- and diacylglycerols are also broadly used in SLN production. Despite having higher solubilization capacity than TAGs, their use in lipid matrices has the advantage of forming less-ordered crystal structures leaving more space for drug accommodation (Souto et al. 2011b).

Fatty acids are also used for SLN production since these also figure in the membranes and fat tissues (Kaneko 2001). The main advantages of their applications are the acyl chains that provide flexibility and consequently lead to a molecular conformation that results in lateral packing. The crystal structure mainly depends on the physical properties, such as the presence of long chains, melting point, heat capacity, and elasticity (Kaneko 2001). Depending on the chain length of the fatty acid, fatty alcohols are suitable to be used in lipid matrices, since they show good biotolerability as the other lipids mentioned above for dermal delivery. They could

Table 5.1 Examples of common solid lipids for the production of SLN

	Trade name	Chemical terminology	Reference
TAGs	Precirol®ATO5	Mixture of monoglycerides (8–22 %), diglycerides (40–60 %), and triglycerides (25–35 %) of palmitic and stearic acid	Das et al. (2011); Sivaramakrishnan et al. (2004)
	Compritol®888ATO	Glyceryl dibehenate	Das et al. (2011); Sivaramakrishnan et al. (2004); Blasi et al. (2011); Kuo and Chung (2011); Rahman et al. (2010); Doktorovova et al. (2011); Kuo and Chen (2009)
	Dynasan®114	Glyceryl trimyristate	Martins et al. (2012); Aditya et al. (2010); Petersen et al. (2011); Noack et al. (2012)
	Dynasan®116	Glyceryl tripalmitate	Kuo and Chung (2011); Kuo and Lin (2009); Cengiz et al. (2006)
	Dynasan®118	Glyceryl tristearate	Petersen et al. (2011); Noack et al. (2012)
	Imwitor®900 K	Mono- and diglycerides based or hydrogenated fats with a glyceryl monostearate content of 40–55 %	Sivaramakrishnan et al. (2004); Doktorovova et al. (2011)
	Softisan®100	Blends of TAGs Composed by fatty acids with a chain length of C_{10}–C_{18}	Fangueiro et al. (2013); Zhang et al. (2008)
	Softisan®142	Blends of TAGs Composed by fatty acids with a chain length of C_{10}–C_{18}	Blasi et al. (2011); Nassimi et al. (2010)
Fatty acids	Stearic acid	Saturated C_{18} fatty acid	Ghadiri et al. (2012); Severino et al. (2011); Zhang et al. (2000)
Fatty alcohols	Cetyl alcohol		Sanna et al. (2010)
	Stearyl alcohol		Sanna et al. (2010); Souto et al. (2004a)
	Witepsol®E85	Mixtures of hard fats	Sarmento et al. (2011); Kuo and Chen (2009); Martins et al. (2012)
Waxes	Cacao butter		Kuo and Chung (2011); Kuo and Lin (2009); Kim et al. (2005)
	Carnauba wax		Kheradmandnia et al. (2010)
	Beeswax		Kheradmandnia et al. (2010); Attama and Müller-Goymann (2008)
	Cetyl palmitate	Ester of palmtic acid and cetyl alcohol	Carbone et al. (2012); Blasi et al. (2011); Martins et al. (2012); Ghadiri et al. (2012); Fangueiro et al. (2012)
Cationic lipids	CTAB	Cetyl trimethylammonium bromide	Carbone et al. (2012); Doktorovova et al. (2012)
	DDAB	Dimethyldioctadecylammonium bromide	Carbone et al. (2012)
	DOTAP	1,2-dioleoyl-3-trimethylammonium-propane	Tabatt et al. (2004); Carbone et al. (2012); del Pozo-Rodríguez et al. (2010)
	DODAB	Dioctadecyldimethyl ammonium bromide	Kuo and Chen (2009); Kuo and Wang (2010)

be useful as skin permeation enhancers due to the disturbance in the lipid packing order (Sanna et al. 2010). For parenteral administration, these types of lipids are also shown to be biodegradable and safe, since they are metabolized in the body via endogenous alcohol dehydrogenase enzyme systems (Dong and Mumper 2006).

Hard fats could compromise the feasibility of several types of SLN. They can be a mixture of acylglycerols based on saturated fatty acids. Their use in SLN led to a lipid matrix able to load proteins and peptides safely for parenteral administration (Fangueiro et al. 2013; Almeida et al. 1997; Sarmento et al. 2011).

Waxes are a group of lipids comprising esters of fatty acids and fatty alcohols. In opposition to acylglycerols, the alcohol represented is not glycerol. Waxes may contain free hydroxyl groups within the molecule (e.g. hydroxyoctanosyl, hydroxystearate) or free fatty acid functions (e.g. beeswax). Their polymorphism is mainly an orthorhombic form that prevails, and the polymorphic transition rate is low (Jenning and Gohla 2000). They have been proven to be able to accommodate drugs with limited solubility and also to provide a release profile depending on the amount of wax in the lipid matrix (Kheradmandnia et al. 2010).

Cationic lipids have been recently applied mainly for gene therapy (Tabatt et al. 2004), and in particular for application to negatively charged mucosal surfaces, such as the ocular mucosa (del Pozo-Rodríguez et al. 2008). Their chemical composition is mainly quaternary ammonium salts. In SLN, they are reported to be useful for monoclonal antibody adsorption and/or the absorption of DNA onto the surface of the particles and for specific targeting (Carbone et al. 2012). These lipids may be used as lipid matrices along with the other lipids already described. The anti-inflammatory effects of cationic lipids have also been reported (Filion and Phillips 1997) and they may be useful for some administration routes. A list of most used solid lipids for the production of SLN is shown in Table 5.1.

Emulsifiers have amphiphilic structure which are used to reduce the surface tension and facilitate the particle partition, i.e. with their hydrophilic groups oriented towards the aqueous phase and the hydrophobic groups oriented to the lipid (Rosen 2004). The selection of the emulsifiers mainly depends on the chosen lipid, since they need to be qualitatively and quantitatively compatible (Severino et al. 2012). The hydrophilic-lipophilic balance (HLB) is directly related to the solubility, i.e. it is the balance of the size and strength of the lipophilic and hydrophilic groups of the emulsifier and it is mandatory to form an emulsion. Thus, the required HLB (rHLB) of a final dispersion should be calculated according to the HLB of the lipid and HLB of the emulsifier (and co-emulsifier, if needed), using the following equation (Souza et al. 2012; Vieira et al. 2012):

$$rHLB = \left[\%_{Lipid} \times HLB_{Lipid}\right] = \left[\%_{Emulsifier} \times HLB_{Emulsifier}\right] + \left[\%_{Co\ \ Emulsifier} \times HLB_{Co\ \ Emulsifier}\right]$$

This equation could be useful to predict the quantities required to form an emulsion, and it is highly applied in fluid emulsions, such as SLN. However, for cream type emulsions and w/o/w emulsions it is not sufficient. Nevertheless, calculating the

rHLB is extremely relevant, since the selection of the ideal chemical emulsifier is vital to the stability of the entire formulation. The blend of the emulsifier should adjust to the tails of the lipid chains in the interface to allow the coexistence of the oil droplets in a continuous aqueous phase. The interactions between lipids and emulsifiers are mainly electrostatic, since hydrophobic moieties have attractive forces to lipid molecules. Emulsifiers are physically adsorbed onto the surface or loaded within the lipid matrix. The hydrophilic groups of the emulsifier give rise to repulsive forces, depending on the volume size and chemical nature of the hydrophilic moieties of emulsifiers (Rosen 2004).

The emulsifiers typically used in the production of SLN could be selected according to their nature (i.e. HLB) and also to the nature of the hydrophilic group. Apparently, the non-ionic emulsifiers do not show ionic charge (e.g. $RCOOCH_2CHOHCH_2OH$, monoglyceride of long-chain fatty acid). The most used are the polyoxyethylene sorbitan fatty acid esters (Tween® 20, 40, 60, 80), sorbitan fatty acid esters (Span®20, 40, 60, 80), polyoxyethylene sorbitol esters (Mirj® 45, 52, 53, 59), alkyl aryl polyether alcohols (Tyloxapol), and the triblock copolymers composed of polyoxypropylene (poloxamer, pluronic or Lutrol®F68, F127). Sugar esters could also be employed, e.g. esters of stearic, palmitic, oleic, and lauric acids.

Polyoxyethylene sorbitan fatty acid esters and triblock copolymers composed of polyoxypropylene are hydrophilic amphiphilic molecules that are dissolved in the aqueous phase of emulsions, and their lipophilic moiety is adsorbed onto the particles surface and the long polyoxypropylene chains allow the stabilization and aggregation with other particles. Sorbitan fatty acid esters are lipophilic amphiphilic molecules that are better adsorbed onto the particle surface and easily dissolved within melted lipids (Kaneko 2001).

The use of anionic or cationic emulsifiers could be important to improve the zeta potential (ZP), and thus input an electrostatic charge to avoid particle aggregation/sedimentation. The cationic emulsifiers have a molecule moiety with a positive charge (e.g. salt of a long-chain amine $RNH_3^+Cl^-$, or a quaternary ammonium salt, $RN(CH_3)_3^+Cl^-$). Stearylamine (Kuo and Chen 2009; Vighi et al. 2010; Pedersen et al. 2006), cationic lipids (quaternary ammonium salts) (Tabatt et al. 2004; Doktorovova et al. 2011, 2012), and Esterquat 1 (*N*,*N*-di-(b-stearoylethyl)-*N*,*N*-*dimethyl-ammonium* chloride) (Vighi et al. 2007, 2010) are examples used in SLN. Anionic emulsifiers have a negative moiety in the molecule (e.g. $RCOO^-Na^+$, sodium salt) and the most applied are bile salts (e.g. sodium cholate and sodium taurocholate), which improve the absorption of particles in the gastrointestinal tract (GIT). Other types of emulsifiers often applied are the phospholipids. Phospholipids derived from soy or egg phosphatidyl choline have a variable fatty chain composition. Generally, soybean phosphatidyl choline contains more saturated fatty acyl chains than egg phosphatidyl choline (Souto et al. 2011b). Its use is reported to improve the emulsions stability and it may also be applied as a permeation enhancer for topical administration (Dreher et al. 1997; Cui et al. 2006). Also, its use has resulted in a decrease in particle size due to the amphiphilic properties of phosphatidyl choline (Schubert and Muller-Goymann 2005; Schubert et al. 2006).

5.3 Production Methods of SLN

The classical production methods of SLN are very well described in the literature (Souto and Muller 2011; Souto et al. 2011a; Müller et al. 2000) and include those not requiring organic solvents and those requiring the use of solvents to solubilize the lipid materials. The selection of the ideal method mainly depends on the properties of the required drug, such as solubility, chemistry, molecular weight, and thermal stability. The most applied and simple method is the High Pressure Homogenization (HPH), either hot HPH or/and cold HPH (Müller et al. 2000). For both, the drug is dispersed or solubilized in the melted lipids. In hot HPH, an aqueous surfactant solution at the same temperature is added to the lipid phase and homogenized by high shear homogenization for 1 min at 8,000 rpm. Then, this pre-emulsion is processed by HPH to obtain a desirable particle size. Usually, 3–5 homogenization cycles at 500 bar are sufficient. After this process a nanoemulsion is obtained and following a temperature decrease there is lipid recrystallization, and consequently the formation of SLN. The cold HPH method is slightly different from the hot HPH method. After drug dispersion or solubilization in the melted lipid, this mixture is cooled using nitrogen liquid or dry ice. The obtained solid lipid mixture is ground using a mortar to produce microparticles which are suspended in an aqueous surfactant solution. Nanoparticles are formed when this microparticle suspension is homogenized by HPH at room temperature or below. The shear forces and cavitation forces in the homogenizer are able to break the microparticles into nanoparticles (Mishra et al. 2010). The main difference of these two techniques is the temperature, because cold HPH is applied when the drug is labile or heat-sensitive. The cold HPH was also developed to overcome some failures of hot HPH, such as drug distribution in the aqueous phase during homogenization and complexity of the crystallization step, which could lead to supercooled melts. However, hot HPH is the most applied process due to ease of scale-up.

Production of SLN by sonication or by high shear homogenization is applied less frequently. The lipid phase and aqueous phase are heated up to the same temperature and emulsified by mechanical stirring (high shear homogenization) or sonication. The main disadvantage of these techniques is the presence of both microparticles and nanoparticles in the final dispersion (Sinha et al. 2011).

The microemulsion technique was developed by Gasco (1997) and since then, it is being developed and modified by different research groups (Pedersen et al. 2006; Fontana et al. 2005; Patel and Patravale 2011; Ghadiri et al. 2012). The lipid and aqueous phase are heated and homogenized at a temperature above the melting point of lipid. This microemulsion is diluted with cooled water, which leads to the breaking of the microemulsion into a nanoemulsion. The obtained nanoemulsion is cooled leading to the formation of SLN. During stirring, the control of the temperature is an important factor to keep the lipid in the melted state (Sinha et al. 2011).

The double emulsion method is an alternative method for producing SLN described by Garcia-Fuentes et al. (2002). This method is appropriate for the incorporation of hydrophilic or/and labile drugs, such as proteins and peptides, since these are loaded in an inner aqueous phase avoiding chemical or enzymatic degradation. Briefly, the hydrophilic drug is solubilized in an inner aqueous phase and

added to the lipid phase containing a suitable emulsifier to form a primary w/o emulsion under high shear homogenization. The second step involves the dispersion of this primary w/o emulsion in an external aqueous surfactant phase to form the final w/o/w emulsion (Fangueiro et al. 2013).

The solvent emulsification-evaporation method described by Sjöström and Bergensåhl (1992) has been applied when the drug is not soluble in the lipid. In this case, an organic solvent, non-miscible with water (e.g. cyclohexane or chloroform) but soluble in the lipid and the lipid are dispersed in an aqueous surfactant solution to produce an o/w emulsion. The organic solvent is evaporated during stirring. This method is useful for hydrophilic drugs, such as proteins and peptides.

The solvent displacement method (also named as the nanoprecipitation method) was initially described for the production of polymeric nanoparticles (Quintanar-Guerrero et al. 1999) and has been adapted for lipid nanoparticles (Videira et al. 2002; Hu et al. 2002; Dong et al. 2012). This method requires a water-miscible organic solvent (e.g. acetone, ethanol, or methanol) that is used to dissolve the lipid phase. This lipid phase (containing the lipid and drug) is added by injection to an aqueous surfactant solution. Lipid particles are formed after complete removal of the solvent by diffusion or by distillation leading to nanoparticles precipitation (Souto and Müller 2007).

In the emulsification-diffusion method, a solution of a semi-polar organic solvent previously saturated with water to ensure thermodynamic equilibrium is used to dissolve the lipid. This solution is added to an aqueous surfactant solution obtaining a w/o emulsion. The saturated solution prevents the diffusion of the solvent from the droplets into the water phase. Formation of SLN is obtained by adding an excess of water to the emulsion, which facilitates the diffusion of solvent from the droplets (Wissing et al. 2004; Sinha et al. 2011).

The phase inversion-based method has been described by Heurtault et al. (2002). This method involves two steps. First, all components are melted and magnetically stirred using a temperature program (e.g. 25–85 °C) and cooled down to lower temperature (e.g. 60 °C). Three temperature cycles (85-60-85-60-85 °C) are applied to reach the inversion process defined by a temperature range. In the second step, an irreversible shock is applied by adding cold water. This process leads to the formation of stable nanoparticles.

More recently a new method has been described for the production of SLN. This method developed by Battaglia et al. (2010) produces SLN by coacervation in a controlled way, starting from fatty acid alkaline salts. The basis of the method is the interaction of micellar solution of sodium salts of fatty acids (e.g. sodium stearate, sodium palmitate, sodium myristate, sodium behenate) and an acid solution (coacervating solution) in the presence of an amphiphilic polymeric stabilizing agent (Battaglia et al. 2010; Corrias and Lai 2011). With the decrease of pH, the SLN can be precipitated. It is a very simple, inexpensive, and thermosensitive method that allows the incorporation of drugs and also can be applied from the laboratorial scale to the industrial scale (Battaglia and Gallarate 2012).

The different methods described here have been classified as those not using organic solvents (Fig. 5.1) and those using organic solvents (Fig. 5.2).

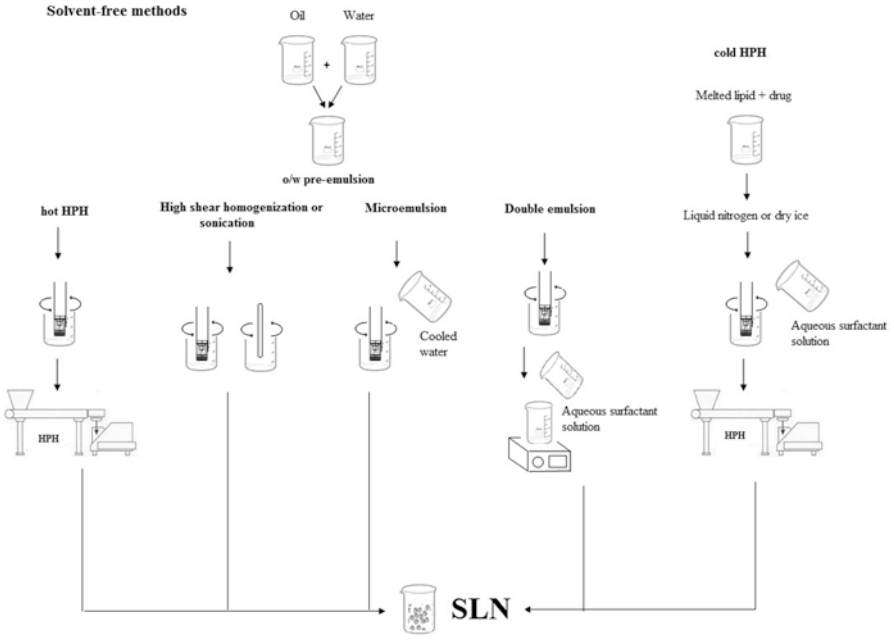

Fig. 5.1 Schematic representation of the production of SLN by solvent-free methods

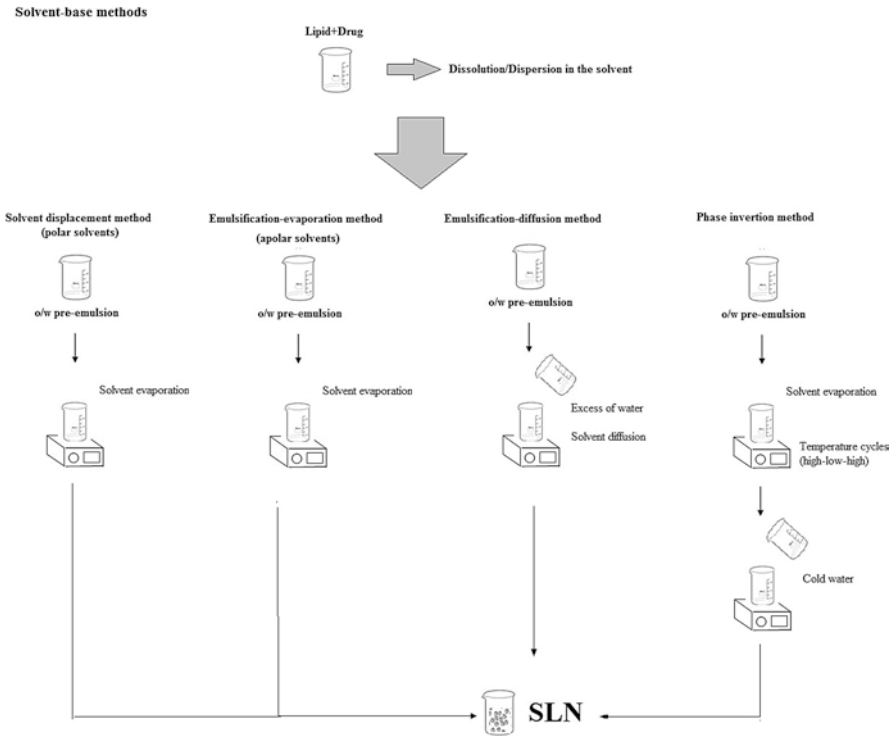

Fig. 5.2 Schematic representation of the production of SLN by solvent-based methods

5.4 Characterization and Evaluation of SLN

The physicochemical characterization of SLN is crucial to evaluate their stability, safety, and suitability for drug delivery. The physicochemical properties of SLN may compromise the administration route and the toxicological profile. A major parameter is the colloidal stability of SLN in aqueous dispersions and also the crystallization and polymorphic behaviour of the lipid matrix. The most commonly applied methods for the characterization of SLN are described in this section.

5.4.1 Mean Particle Size, Distribution, and Electrical Charge

After SLN production, the first parameter to assess should be the mean particle size and size distribution, since it is desirable to have monodispersed populations in the nanometer range. The most common techniques are the dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), and the laser diffraction (LD). DLS is capable of determining the particle size in the submicron range based on the Brownian movement of the spherical particles in suspension. A monochromatic light beam, such as a laser, hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle (Berne and Pecora 2000). The frequency shifts, the angular distribution, the polarization, and the intensity of the scattered light, are determined by the size, shape, and molecular interactions in the scattering material (Gethner and Gaskin 1978). It is possible to compute the spherical size distribution and give a description of the particle's motion in the medium, measuring its diffusion coefficient and using the autocorrelation function (Berne and Pecora 2000).

LD is another technique that could be applied based on the principle that particles passing through a laser beam will scatter light at an angle that it is directly related to their size. The observed scattering angle is dependent on the shape and size of the particles and increases logarithmically with the increase of the particle size. The scattering intensity is high for larger particles and low for smaller particles (Eshel et al. 1991; Ma et al. 2000). Both techniques are broadly applied because of the advantages they show, e.g. fast data collection, relatively inexpensive, good reproducibility, low volume of samples, and automated for routine measurements. In addition, extensive experience in the technique is not required. The main differences between DLS and LD are the collected data. DLS reports the mean particle size of the entire population, whereas LD usually reports the mean particle size of 10 % (LD_{10}), 50 % (LD_{50}), and 90 % (LD_{090}) of the population of the particles. Another difference is the detection limit; DLS is not able to detect particles above 3 μm, while LD is.

Each particle develops a net charge at the surface affecting the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions (ions of opposite charge to that of the particle) close to the surface. Thus, an electrical double layer is formed. In the inner region, there is the Stern layer, the ions are strongly bound, and in the outer diffuse region they are less firmly attached. The zeta potential (ZP) refers the electrical charge at the surface of the

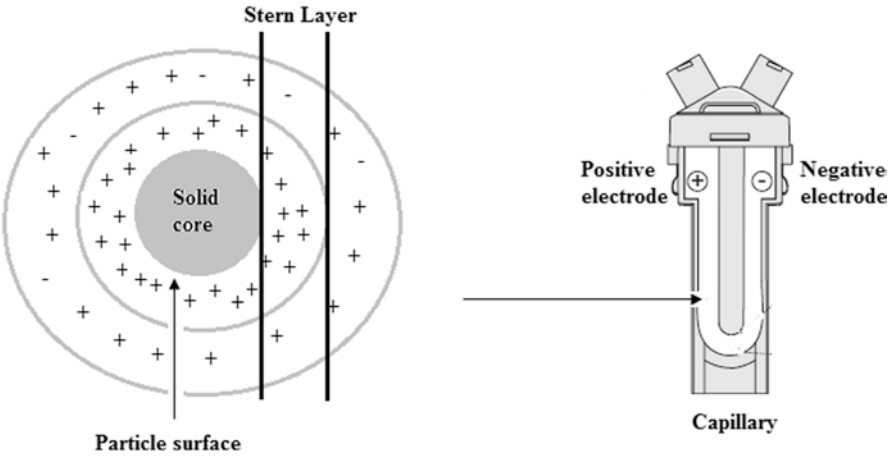

Fig. 5.3 Illustration of the ZP in the surface of particles and the esquematic representation of a capillary and the electrodes

hydrodynamic shear surrounding the colloidal particles. The magnitude of the ZP gives an indication about the long-term stability of the colloidal system (Hiemenz and Rajagopalan 1997). Thus, if all the particles in suspension have a high negative or positive value of ZP, then they will tend to repulse each other and there is no tendency to flocculate. However, if the particles have low ZP values, then there is no force to prevent the particles coming together and flocculating (Dhont 2001). The limiting values between stable and unstable suspensions are generally taken at either +30 mV or −30 mV. DLS is a technique that is used to determine the ZP, however other techniques exist, such as electrophoretic light scattering (ELS), acoustic, and electroacoustic methods. However, DLS is the most sensitive and versatile technique, but it does not directly measure the ZP. The analysis is carried out in a cell with electrodes in each end to which a potential is applied. When the voltage is applied, the particles move towards the electrode of opposite charge, and their velocity is measured and expressed as ZP (Fig. 5.3). The mobility of the particles is commonly determined by laser Doppler anemometry, which is based on the evaluation of a frequency (Doppler) shift that is observed for the light scattered from the particles motion in the electric field. DLS converts this signal directly in ZP through the Helmholtz-Swoluchowski equation (Bunjes 2005):

$$\mu = \frac{\varepsilon\zeta}{\eta\zeta}$$

where μ is the electrophoretic mobility, ε is the permittivity, ζ is the zeta potential, and η is the viscosity of the dispersion medium.

Unlike other parameters, such as particle size, ZP is affected by the surrounding environment, e.g. pH, ionic strength, and consequently the type of ions in the suspension. Therefore, the measurements should be conducted after dilution to avoid multiple scattering and for better resolution (Renliang 2008).

5.4.2 Microscopy

Microscopy is a technique used to verify the surface, morphology of the particles, their size, distribution, and shape, and other potential species that may have been produced simultaneously. Particle shape is a very important factor, since particles are preferentially required to be spherical. For controlled release, the particles should provide protection for the loaded drugs, where the contact with the aqueous environment should be minimal (Bunjes 2005). The most applied techniques are the scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM). Usually, these sophisticated techniques require sample treatment, (e.g. freeze-drying) prior to imaging, thus particles are not observed in their native state (i.e. as liquid dispersions). The major difference between TEM and SEM is the electron incidence in the sample. In SEM, electrons are scanned over the surface of the sample and can produce very high-resolution images of a sample surface, revealing details less than 1 nm in size. In TEM, electrons are transmitted through the sample providing information about the thickness and composition (crystal structure) of samples (Dubes et al. 2003). In SEM, a chemical fixation to preserve and stabilize particle structure is required. Fixation is usually performed by incubation in a buffer solution, such as glutaraldehyde, sometimes in combination with formaldehyde and other fixatives, and optionally followed by post-fixation with osmium tetroxide (Muller-Goymann 2004).

TEM is relatively fast and simple with negative staining (Friedrich et al. 2010). Samples for TEM need to be as thin as possible to allow the electron beams to travel through the sample. This technique requires a drying process and yields additional information about the internal structure of the nanoparticles. The samples are fixed with a solution containing heavy metal salts which provide high contrast in the electron microscope (e.g. uranyl acetate or phosphotungstic acid) (Kuntsche et al. 2011). Unfortunately, the resulting images have low resolution and since the freeze-drying of samples is required, they are not observed in the original state, leading to artefacts in the images (Bunjes 2005). Cryogenic microscopy is very useful for lipids, requiring low temperatures. Cryofixation may be used and low-temperature SEM/TEM performed on the cryogenically fixed samples.

AFM utilizes the force acting between a surface and a probing tip resulting in a spatial resolution of up to 0.01 nm for imaging (Mühlen et al. 1996). This technique allows imaging under hydrated conditions without pre-treatment of the samples; however samples need to be fixed, e.g. by adsorption (Muller-Goymann 2004). AFM provides a three-dimensional profile surface, which leads to a higher resolution and gives more information about the particles` surface. Also, the pre-treatment of the samples is not required. However, AFM requires longer time for analysis than SEM or TEM (Mühlen et al. 1996; Geisse 2009; Olbrich et al. 2001; Shahgaldian et al. 2003).

5.4.3 Thermal Analysis

During the recrystallization process, lipid materials could exist in a well-defined crystal, or as a mixture of different internal lattice structures. Thermal analysis could

provide information about the polymorphic modifications of lipid materials, crystallization, and thermal behaviour. The polymorphic behaviour and crystallinity of SLN can be checked by differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), thermomechanical analysis (TMA), and the differential thermal analysis (DTA). Phase transitions are followed by free energy changes, associated with changes in the enthalpy or entropy of the system. Enthalpy changes of samples are the result of an endothermic or exothermic reaction that it is translated in a signal, depending on the consumption of energy (e.g. melting of a solid), or a release of energy (e.g. recrystallization of an isotropic melt). The transition from the crystalline to amorphous phase usually requires a high input of energy. Therefore, care has to be taken to ensure that the measuring device is sensitive enough to give a sufficiently low detection limit. Entropy changes can be recognized by a change in the baseline slope, due to a change in the specific heat capacity (Ford and Mann 2012).

DSC is the most applied thermal analysis in SLN that evaluates the physical and chemical properties of the lipids as a function of time and temperature (McElhaney 1982). Two samples are usually analyzed, namely the SLN sample and the bulk material (i.e. the solid lipid used), and the temperature of both samples is raised identically over time in different containers (Gill et al. 2010). The difference in the input energy required to match both temperatures would be the amount of excess heat absorbed (endothermic) or released (exothermic) by the lipid in the SLN. During a change in temperature, DSC measures the heat quantity needed for the transition to occur, which is released or absorbed excessively by the sample on the basis of a temperature difference between the sample and the bulk material. In DSC, the amount of heat put into the systems is exactly equivalent to the amount of heat absorbed or released during a transition (Christian and O'Reilly 1986). The main advantages of this technique are the detection of polymorphism, the small sample size (e.g. 2–3 mg (solid) or ml (liquid)), relatively fast analysis times, and a sensitive and versatile procedure. TGA is also very similar to DSC; the major difference is the continuous analysis of sample and bulk material, and it is based on the thermobalance. It measures the mass variation of a sample when subjected to temperature. The source of heat is an infra-red lamp, and the atmosphere is controlled by the addition of inert gases (e.g. nitrogen, helium) or reactive gases (e.g. oxygen, hydrogen). The decomposition of effluent material can be characterized also, by coupling a gas chromatography or mass spectrometery system (McCauley and Brittain 1995). DTA is similar to DSC; however it is the more accurate of all thermal analyses procedures, because the thermocouple is inserted into the sample (McCauley and Brittain 1995). TMA measures the deformation of the sample under non-oscillating stress subjected to temperature. The sample usually is placed in a small tube connected by a quartz probe to a differential exchanger. The movements of the sample are monitored by the displacement of the exchanger. This technique enables the measurement of several characteristics, such as tensile strength, volume expansion, penetration, or elasticity (McCauley and Brittain 1995).

Figure 5.4 shows the typical DSC profiles of a solid bulk lipid, analyzed before tempering the material for 1 h at 100 °C. The differences between the two heating runs (A and B) allow the detection between different polymorphic forms in the same material. In Fig. 5.4a, the difffractogram clearly shows a sharp peak representing the β-form

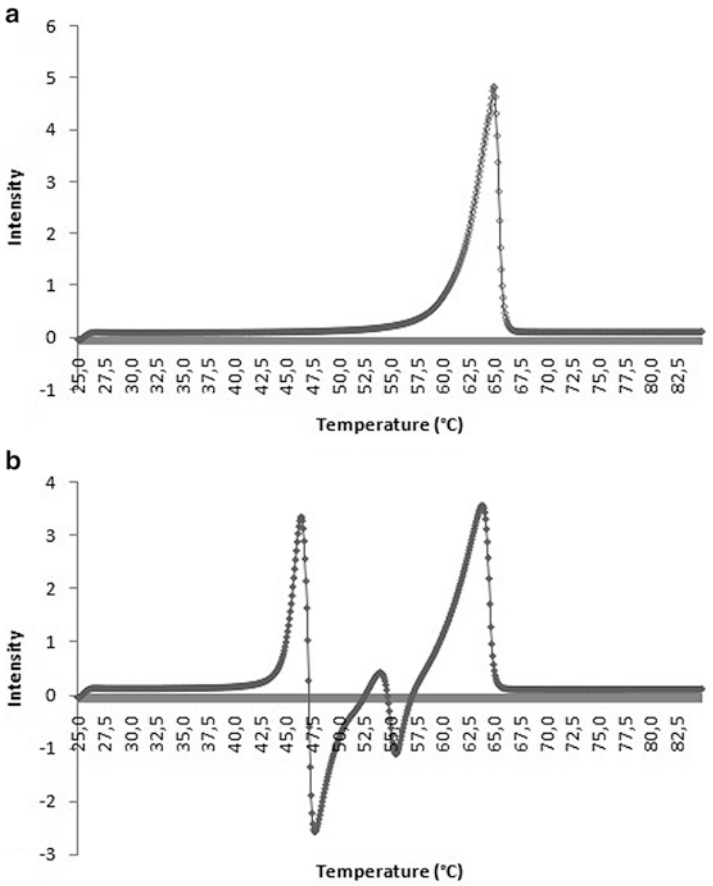

Fig. 5.4 DSC analysis of bulk lipid. (**a**) First heating run before tempering and (**b**) second heating run before tempering (modified after Souto and Müller (2006))

of the tested bulk lipid (i.e. Dynasan®116) (Souto and Muller 2006). In the second heating run (Fig. 5.4b) it is also possible to detect the metastable α-form. This type of analysis mimics the thermal stress of the lipid, since for production of SLN it is necessary to melt the lipid first and then cool the pre-emulsion for SLN formation. Analysis of SLN represents the second heating run. This explains the different polymorphic forms between bulk materials and the lipid particles (Souto and Muller 2006).

5.4.4 Crystallinity and Polymorphism

X-ray is a widely used method for the evaluation of crystallinity, since it applies wavelengths of the same magnitude as the distance between the atoms or molecules of crystal. Thus, it allows the determination of the arrangement of molecules within a crystal, i.e. the macromolecular structure of the particles (Heurtault et al. 2003). This method

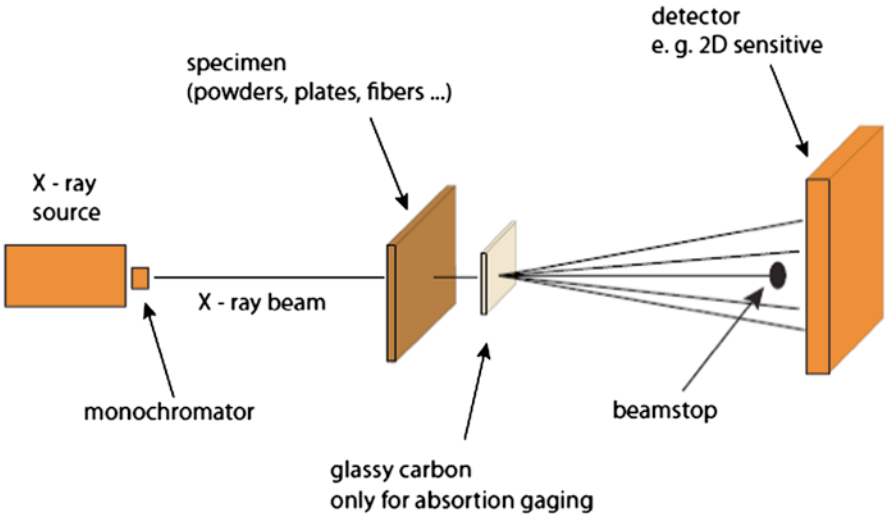

Fig. 5.5 Illustration of the X-ray instrumentation and methodology

is based on a beam of X-rays that reaches a crystal and causes the spread of light into many specific directions. This energetic radiation may arise from the removal of inner orbital electrons. These transitions are followed by the emission of an X-ray photon having energy equal to the energy difference between the two states (Krakty 1982). From the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal, as shown in Fig. 5.5. From this electron density, the mean positions of the atoms in the crystal can be determined, as well as their chemical bonds, their disorder, and various other factors (Jenknins 2000). This technique can be carried out using the small angle X-ray scattering (SAXS) or the wide angle X-ray scattering (WAXS), both widely applied in the evaluation of SLN crystallinity (Schubert et al. 2006; Heurtault et al. 2003; Müller et al. 2008; Aji Alex et al. 2011). The only difference between both methods is the range of scattering angles 2θ. While standard diffractometers cover angles between about 5° and 180°, the range between 0.01° and 3° is typical for small angle instruments. These techniques are specific for studying structural features of colloidal size in bulk materials and particles loaded with drug, determining their polymorphic transitions and the quantitative determination of crystalline components in the formulation (Bunjes and Unruh 2007). It is also important to confirm the presence of the drug in the systems, i.e. if the drug is molecularly dispersed in the lipid matrix, or if it is in the amorphous state. Studies with this technique revealed the polymorphism of particles composed of acylglycerols (Westesen et al. 1997; Bunjes et al. 2003) and differences when compared to the bulk materials (Souto et al. 2006).

This technique is applied to differentiate between the crystalline and amorphous material. A typical example is shown in Fig. 5.6. The X-ray study confirms the transition rates of both liquid and solid lipids used in the lipid particles. The use of higher amounts of oil in the particles (Fig. 5.6b) decreases the crystallinity, and consequently decreases the peaks intensity in the X-ray diffractogram.

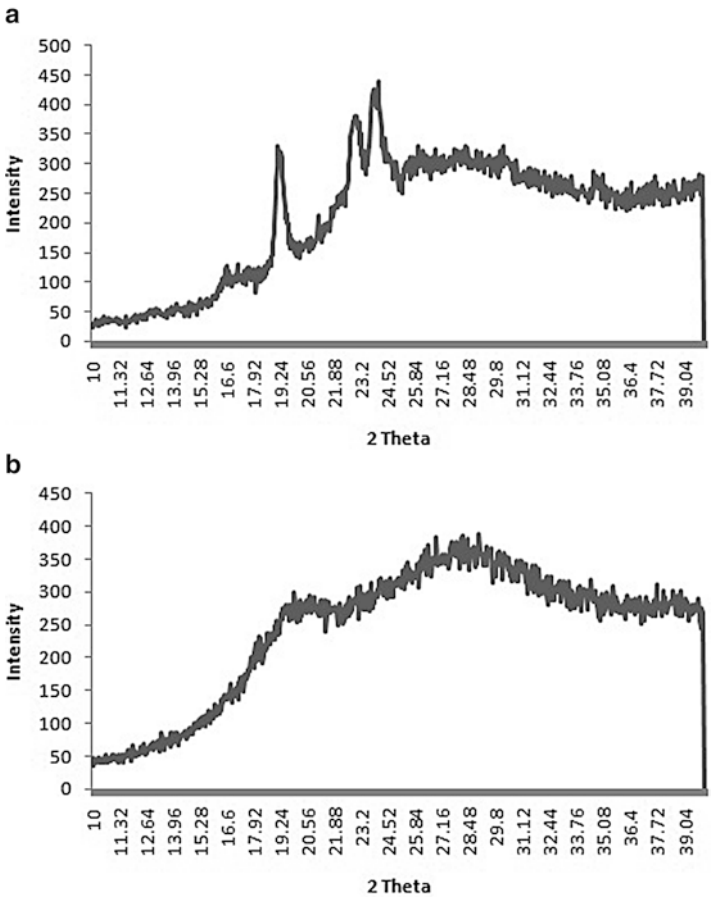

Fig. 5.6 X-ray analysis of lipid particles. (**a**) Lipid particles made of solid lipid and (**b**) lipid particles made of liquid and solid lipids (modified after Souto et al. (2004b))

5.4.5 Infra-Red Spectroscopy

Infra-red spectroscopy is applied to characterize SLN at molecular level. Each molecule absorbs at a specific wavelength that is related to its molecular and chemical structure. The usefulness of Fourier transform infra-red (FTIR) imaging in the characterization of drug delivery systems was demonstrated by Coutts-Lendon et al. (2003). FTIR could be useful to identify the lipid composition and also the presence of drug contaminants and degradants in the lipid matrix. For bulk materials, FTIR could also provide information about the functional groups. In general, this technique is very sensitive, non-destructive, and the amount of sample required for analysis is about a few nanograms. The particles are isolated by microneedle and transferred to a circular salt plate placed on the instrument. The recorded data is a three-dimensional spectral image, with spatial information in the *x*- and *y*- directions and spectral information in the *z*- direction (Barber 1993; Bhargava and Levin 2001).

5.5 Applications of SLN in Drug Delivery

SLN play an important role in the delivery of a variety of drugs, poorly water-soluble drugs, hydrophilic drugs, such as peptides and proteins (Almeida and Souto 2007). The many advantages already mentioned are mainly related with their nanometer size, but also to the protection of the drug from chemical and enzymatic degradation. In addition, the improvement on the bioavailability is also a reason for selecting SLN, independently of the administration route. SLN changes the pharmacokinetic and biodistribution profiles of the loaded drug and may offer the possibility of targeted delivery.

Depending on the administration route, SLN can overcome the many barriers for the absorption of the majority of drugs.

For oral delivery, the GIT could compromise the activity of several drugs (e.g. peptides and proteins) due to the harsh environment, as well as the transcellular pathway that particles take that leads to the uptake via M cells to the transport of the particles to the lymph (Mrsny 2012). However, some strategies to overcome these barriers are being exploited, such as the use of permeation enhancers, modification of drug solubilization/permeabilization, and the use of protease inhibitors to avoid proteolytic degradation (Sant et al. 2012).

In ocular delivery, the anatomy and physiology of the eye is an interesting challenge for drug delivery. From the anatomical point of view, the blood-retina barrier for delivering drugs into the post-segment of the eye is one of the most difficult barriers to overcome. In addition, the physiologic characteristics of the eye also limit the drug delivery, due to the short drug residence time in the eye (Araújo et al. 2009). For these reasons, new drug delivery systems based on solid lipids are required for ocular purposes to facilitate drug penetration through the corneal epithelium. New strategies report the development of positively charged SLN since the slightly anionic characteristics of the ocular mucosa above its isoelectric point (Araújo et al. 2009; Sandri et al. 2010; Başaran et al. 2010) contribute to particles' adhesion onto the ocular surface increasing drug residence time (Araújo et al. 2009).

For intravenous delivery, there are also some features, e.g. the particle size and size distribution need to be optimized to allow the circulation of particles through the vasculature. The main problem is the activation of the reticulo-endothelial system (RES), responsible for the uptake of the particles to the spleen and kidney (Yoo et al. 2011). Many strategies to overcome the particles recognition by the immune system include the use of polyethylene (PEGs) recovering the particles' surface to extend their residence time circulation in the bloodstream. This strategy also named as "stealth technology" results in long circulating particles loading active molecules with high target efficiency and activity (Moghimi et al. 2001; Cavadas et al. 2011). For brain delivery and targeting the particles have been tested, where the blood–brain barrier limits the passage of drugs. Furthermore, the surface of SLN can easily be modified offering drug targeting to tumours, the liver, and the brain with many ligands (e.g. monoclonal antibodies, DNA) (Harms and Müller-Goymann 2011; Blasi et al. 2007).

Dermal and transdermal delivery are an area of great interest for delivering drugs. The main advantages that SLN offers by this route are (1) chemical protection of drugs, (2) modulation of release profile, and (3) occlusive effect provided by SLN due a formation of an occlusive adhesive lipid film (Battaglia and Gallarate 2012).

One the major barriers of the skin is the stratum corneum, which offers a protective and effective barrier to microorganisms (Neubert 2011) and also avoids SLN permeation. SLNs show good biocompatibility and biodegradability, since they are composed of lipids that are present in the skin's structure (Souto et al. 2011b).

Table 5.2 summarizes examples of SLN formulations with respect to their therapeutic effect and administration route.

5.6 Conclusion

SLN emerged in the last decade as innovative and versatile drug delivery systems due to the many advantages they show relative to other traditional therapies. From a physicochemical point of view, SLN are simple delivery systems based on solid lipids that may be used for the delivery of many problematic drugs, such as peptides, proteins, and poorly water-soluble drugs. Knowledge about the physicochemical parameters of SLN is important due to the influence of these physicochemical parameters on biopharmaceutical behaviour. Even though this knowledge has increased considerably, over the years, it is very important to evaluate the interactions of drugs with SLN as this will aid in the prediction of the SLN in vivo pharmaceutical profile. In conclusion, intensive structural investigations are required if SLN are to be accepted as the new generation of drug carriers.

Problem Box

Question 1

(a) What are the main excipient components used in development of solid lipid nanoparticles (SLN) and how are drugs loaded within the lipid matrix?
(b) Why the determination of hydrophilic lipophilic balance (HLB) of the separate components is so important?

Answers

(a) The main components required for the development of SLN are the solid lipid, emulsifier, co-emulsifier (optional), and water. Lipophilic drugs are loaded within the lipid matrix, i.e. they need to be soluble in the solid lipid chosen in order to be molecularly dispersed and form a thermodynamically stable lipid matrix. For hydrophilic drug encapsulation, SLN are prepared using a water-in-oil in water emulsion technique, where hydrophilic drugs are loaded within the inner aqueous phase surrounded by the lipid matrix.

(continued)

Table 5.2 Examples of drugs loaded in SLN and their therapeutic effect, type of lipid used in the lipid matrix, surfactants and respective administration route

Drug	Therapeutic applications	Solid lipid	Surfactant(s)	Administration route	References
Artemether	Antimalaria	Dynasan®114	Tween® 80	Oral	Aditya et al. (2010)
Carvedilol	Antihypertensive	Imwitor®900 K	Pluronic® F 68 and, soybean phospatidylcholine	Intraduodenal	Venishetty et al. (2012)
Clotrimazole	Antifungic	Dynasan®116	Tyloxapol	Dermal	Souto and Müller (2005)
Clozapine	Antipsychotic	Dynasan®114, 116, and 118	Pluronic® F 68, soybean phospatidylcholine, and stearylamine	Intravenous Intraduodenal	Manjunath and Venkateswarlu (2005)
Cyclosporine A	Immune suppressor	Imwitor®900	Tagat S and sodium cholate	Oral	Müller et al. (2008)
		Compritol® 888 ATO	Pluronic® F 68 and Tween® 80	Ocular	Gokce et al. (2008)
Diazepam	Sedative, anticonvulsivant	Cetyl palmitate	Plantacare	Rectal	Sznitowska et al. (2001)
Fluticasone	Corticosteroid anti-inflammatory	Precirol® ATO 5	Tween® 80 and soybean phospatidylcholine	Dermal	Doktorovova et al. (2010)
Gatifloxacin	Antibiotic	Stearic acid and Compritol® 888 ATO	Pluronic® F 68	Ocular	Kalam et al. (2010)
Insulin	Diabetes mellitus	Softisan®100	Lipoid® S75 Pluronic® F 68	Oral	Fangueiro et al. (2013)
		Stearic acid and palmitic acid	Soybean phospatidylcholine and sodium cholate	Pulmonary	Liu et al. (2008)
Ketoconazole	Antifungic	Compritol® 888 ATO	Pluronic® F 68 and sodium deoxycholate	Dermal	Souto and Müller (2005)
Ketoprofen	Nonsteroidal anti-inflammatory	Beeswax and carnauba wax	Tween® 80 and egg phospatidylcholine	n.a.	Kheradmandnia et al. (2010)
Lopinavir	HIV infection	Compritol® 888 ATO	Pluronic® F 127	Intravenous	Aji Alex et al. (2011)
Paclitaxel	Chemotherapic	Precirol® ATO 5	Tween® 80	Pulmonar	Videira et al. (2012)
Paromomycin	Antimicrobial	Cetyl palmitate and stearic acid	Tween® 80, Span® 85	Dermal	Ghadiri et al. (2012)

(continued)

Table 5.2 (continued)

Drug	Therapeutic applications	Solid lipid	Surfactant(s)	Administration route	References
Rifampicin, isoniazid and pyrazinamide	Antitubercular	Stearic acid	PVA	Pulmonar	Pandey and Khuller (2005)
Saquinavir, Stavudine, and Delavirdine	HIV infection	Compritol® 888 ATO, Dynasan® 116, and cacao butter	Phospatidylcholine, cholesteryl hemisuccinate, and taurocholate	n.a.	Kuo and Chung (2011)
Taspine	Acetylcholinesterase inhibitor	Compritol® 888	Pluronic® F 68	Intravenous	Lu et al. (2008)
Timolol	Non-selective beta-adrenergic receptor blocker	Phospholipon 90G®	Tween®80	Ocular	Attama et al. (2009)
Titanium dioxide	Sunscreen	Dynasan® 116	Tyloxapol®	Dermal	Cengiz et al. (2006)
Tobramycin	Antibiotic	Stearic acid	Sodium taurocholate and Cremophor® EL	Ocular	Cavalli et al. (2002)
Tretinoin	Potential anti-cancer drug	Precirol® ATO 5 Compritol® 888 ATO Dynasan® 114 Dynasan® 118	Tween® 80	Oral	Das et al. (2011)
Vitamin E or α-tocopherol	Anti-aging	Cetyl Palmitate	Tego Care® 450	Dermal	Fangueiro et al. (2012)

Problem Box (continued)

(b) The determination of HLB is very important if one wishes to develop a long-term stable system in which the solid lipid and emulsifiers used are in thermodynamic equilibrium. The HLB value may also be useful when predicting the amount of each component to be used in the final dispersions. Since emulsifiers and solid lipids should be chemically and physically compatible, the blend of the emulsifiers should be adjusted to take account of the length of the lipid chains at the interface. This will guarantee the stability of the oil droplets in the continuous aqueous phase.

Question 2

(a) What are the main advantages associated with the use of SLN for oral drug delivery?
(b) What are the routes of administration already exploited in the use of SLN in drug delivery?

Answers

(a) The main advantages of SLN for oral drug delivery are the protection of the drug from chemical and enzymatic degradation, the improvement in drug bioavailability by optimizing drug loading and release profiles, the possibility of targeted delivery of the loaded drug, the long shelf life and low chronic toxicity of the dosage form, and the excellent physical stability of the final product.
(b) The routes of administration already exploited in the use of drug delivery SLN are the oral, pulmonary, dermal, transdermal, ocular, intravenous, and rectal routes.

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Chapter 6
The Potential of Nanoemulsions in Biomedicine

M. Mazza, M. Alonso-Sande, M.-C. Jones, and M. de la Fuente

Abstract Nanoemulsions are nano-sized oil-in-water or water-in-oil emulsions with a number of applications in biomedicine. Nanoemulsions are highly versatile systems, in terms of composition and physicochemical properties, which can be tailor-made using simple and mild technologies to associate a great variety of drugs and fulfil the requirements for a wide range of pharmaceutical applications. This chapter aims to provide the reader with an overview on compositions and manufacturing methodologies and covers the most recent applications that have been reported in the field of drug delivery.

Abbreviations

BCS	Biopharmaceutical classification system
CFC	Chlorofluorocarbon
CPI	Catastrophic phase inversion

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GRAS	Generally Recognised As Safe by United States' Food and Drug Administration agency
HAMPA 1	Azobenzene substituted poly(sodium acrylate)
HIV	Human immunodeficiency virus
HLB	Hydrophilic–lipophilic balance
LCT	Long chain triglycerides
LSW	Lifshitz-Slezov-Wagner
MCT	Medium chain triglycerides
NIRF	Near-Infrared Fluorescent (Imaging)
O/W nanoemulsion	Oil-in-water nanoemulsion
PARG	Polyarginine
PCL	Poly(ε-caprolactone)
PCL-PEG	Poly(ε-caprolactone)-poly(ethylene oxide)
PEG	Poly(ethylene glycol)
PELC	Poly(ethylene oxide)-block-poly(lactide-co-ε-caprolactone)
PIC	Phase inversion composition
PIT	Phase inversion temperature
PLA	Poly(lactic acid)
PLA-PEG	Poly(lactic acid)-poly(ethylene oxide)
PLGA	Poly(lactic-co-glycolic) acid
PLGA-PEG	Poly(lactic-co-glycolic)-poly(ethylene oxide)
S:O ratio	Surfactant-to-oil ratio
SANS	Small Angle Neutron Scattering
SAXS	Small Angle X-ray Scattering
SEM	Scanning Electron Microscopy
Smix	Mixing ratio of surfactant and co-surfactant
TEM	Transmission Electron Microscopy
W/O nanoemulsion	Water-in-oil nanoemulsion

6.1 Nanoemulsions: Versatile Drug Carriers for Pharmaceutical Applications

Emulsions are mixtures of two immiscible phases, wherein an emulsifier (surfactant) is added in the continuous or external phase to stabilise the dispersed droplets (internal phase). Emulsions are classified as oil-in-water (O/W), when oil droplets are dispersed in aqueous medium, or water-in-oil (W/O), when the internal phase is formed by water.

Emulsions can be further classified depending on droplet size into coarse emulsions and nanoemulsions. The latter are colloidal systems that contain droplets in the nanometer range (typically 10–300 nm). Microemulsions, which also fit in that definition, will not be covered here in detail. In this review, nanoemulsions will be taken to mean kinetically, but not thermodynamically stable, spherical nano-sized emulsions.

Aqueous O/W nanoemulsions constitute the most common type (see Sect. 6.2). Their potential in biomedicine is enormous. They are particularly suited to entrap

hydrophobic drugs in their oily core hence increasing their aqueous levels and absorption. A number of formulations are currently marketed, as for example Novasorb® and Restasis®, or undergoing clinical trials, e.g. CS-1000.

In comparison, research involving W/O nanoemulsions in biomedicine is less extensive (see Sect. 6.3). This is mainly related to the oily nature of the external phase, which restricts administration to non-intravenous routes and limits oral use due to palatability. In spite of this, W/O nanoemulsions remain theoretically suited to the encapsulation and delivery of hydrophilic drugs and macromolecules therefore holding a great potential in drug delivery.

Polymer-coated nanoemulsions, otherwise known as nanocapsules, differ from conventional nanoemulsions in terms of stability, drug loading, and kinetics of drug release (see Sect. 6.4). Nanocapsules are highly versatile systems that can accommodate hydrophilic and hydrophobic agents either dissolved in the oily core or adsorbed onto the shell. Additionally, based on the type of polymeric coating, the surface properties can be conveniently tailored to control their biodistribution and interaction with cells.

This review aims to provide the reader with an overview of the potential of nanoemulsions in biomedicine. It compiles information concerning manufacturing procedures, physicochemical characterisation, and summarises the main biomedical applications for this type of drug delivery systems.

6.2 O/W Nanoemulsions

Aqueous O/W nanoemulsions destined for pharmaceutical applications are typically made from materials listed as GRAS (Generally Recognised As Safe by United States' Food and Drug Administration agency). The selections of the oil, surfactants, and process of manufacturing are critical steps in obtaining nano-sized emulsions and ensuring colloidal stability. The different types of oils that have been reported, summarised in Table 6.1, have been selected from a wide range of materials. Non-ionic or amphoteric surfactants such as poloxamer, lecithin, and Tween® 80 are the most common surfactants used.

6.2.1 Emulsification Methods

Emulsification methods for manufacturing nanoemulsions can be divided into high- and low-energy processes (Tadros et al. 2004; Anton and Vandamme 2009) (for illustration see Fig. 6.1).

High-energy methods (Sect. 6.2.1.1) are based on the application of strong shear forces to break down the oil phase into small droplets (Schultz et al. 2004; Tadros et al. 2004; Delmas et al. 2011). Although highly effective in generating small droplets, the heat generated may be detrimental for labile drugs. In these cases, milder

Table 6.1 Components of O/W nanoemulsions

Oils	Common surfactants
Long chain triglycerides	Span® 85 (sorbitan trioleate)
Soybean oil	Labrafil M 1944 CS® (glycerides and PEG 300 ester mixture)
Carthame oil	Capmul® MCM (mono diglyceride of capric and caprylic acids)
Medium chain mono-, di-, or triglycerides	Vitamin E TPGS (α tocopheryl acid succinate ester/PEG 1000)
Miglyol® 812 N (capric and caprylic acid triglycerides)	Cremophor EL® (polyethoxylated ricin oil)
Captex® 355 (capric and caprylic acid triglycerides)	Myrj® 52 (polyoxyethylene glycol 2000 monostearate)
Labrafac Lipophile WL 1349® (capric and caprylic acid triglycerides)	Tween® 80 (polysorbate 80)
Imwitor® 742 (capric and caprylic mono-, di-, and triglycerides)	Labrasol® (caprylocaproyl polyoxyl-8 glycerides)
Short chain triglycerides	Natural lecithins
Triacetin (triester of glycerol and acetic acid)	Lipoid 80
Fatty acid esters	Phospholipids
Ethyl oleate	Poloxamer® F68
Capmul® PG8 (propylene glycol monocaprylate)	
Capric acid	
Other oils	
Castor oil	
Coconut oil	
Corn oil	
Cottonseed oil	
Bran rice oil	
Evening primrose oil	
Fish oil	
Jojoba oil	
Olive oil	
Linseed oil	
Peanut oil	
Pine nut oil	
Safflower oil	
Squalene	
Sunflower oil	
Sesame oil	
Wheatgerm oil	

low-energy emulsification methods (Sect. 6.2.1.2) may be more appropriate (Izquierdo et al. 2004; Tadros 2005; Anton and Vandamme 2009). Low-energy methods rely on adjusting the composition or environmental conditions to provoke emulsification, (Tadros et al. 2004; Anton and Vandamme 2009; McClements 2011; Gutierrez et al. 2008). Low-energy methods present the added benefit of potentially high yields and are easily scalable.

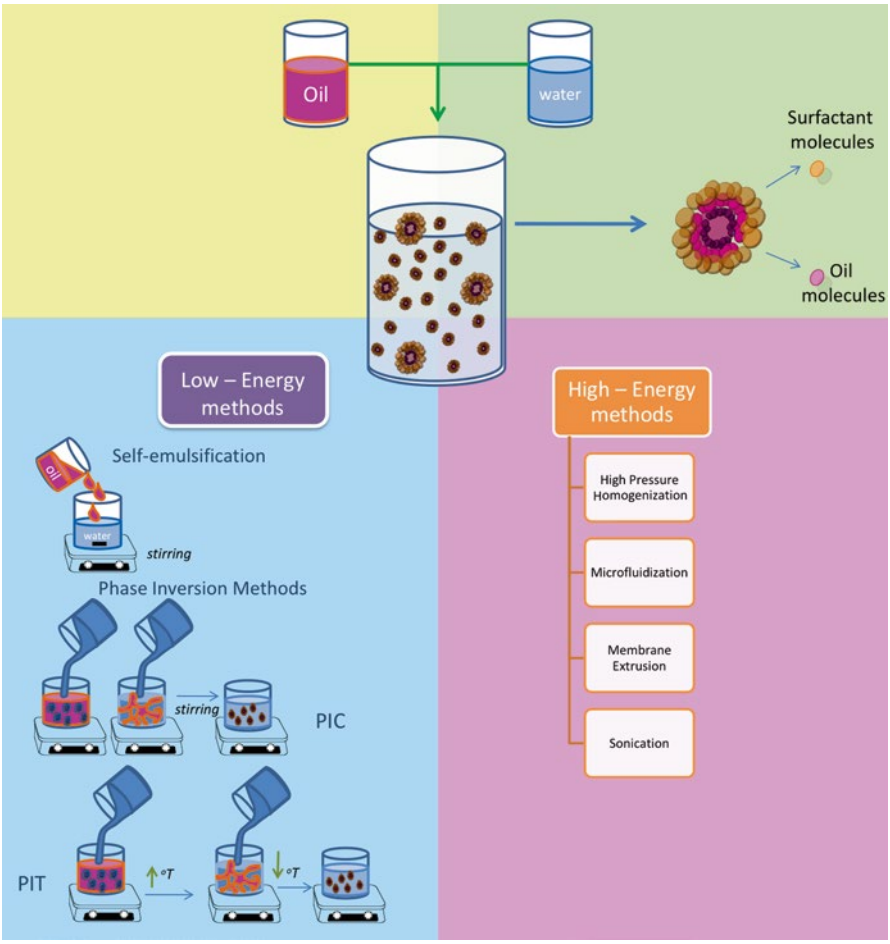

Fig. 6.1 Schematic representation of the most common high- and low-energy manufacturing methodologies applied to the preparation of nanoemulsions

6.2.1.1 High-Energy Emulsification Methods

Homogenization

This method consists of applying intense disruptive forces such as shear, turbulence, and cavitation, into a high-pressure valve homogenizer, to a coarse primary emulsion (Schultz et al. 2004; McClements 2011; Koroleva and Yurtov 2012). Surfactants with a high rate of absorption such as Tween® 20 are required to maintain stability (Taisne et al. 1996; Marie et al. 2002; Koroleva and Yurtov 2012).

Microfluidization

Microfluidization is based on the use of a network of microchannels allowing streams of both the oil and water phases to collide, resulting in the dispersion of the internal phase (Utada et al. 2007a, b). Here, droplet size is controlled not only by the nature and composition of each phase and the emulsifier concentration, but also by the geometry of the microchannels (e.g. co-directional, in opposite directions, T-shaped intersection), and the pressure applied to the liquids (the higher the pressure the smaller the size) (McClements 2010; Koroleva and Yurtov 2012).

Membrane Extrusion

In this method, the dispersion is obtained either by pumping an oil phase through the pores of an extrusion membrane (with the aqueous phase being on the other side of the extrusion phase), or by pumping a coarse emulsion in order to obtain smaller droplet sizes (van der Graaf et al. 2005; Koroleva and Yurtov 2012).

Ultrasonication

Probe sonication produces strong cavitation, a process generating growth, collapse, and oscillation of gas bubbles with release of energy, creating high local temperatures and high shear forces, allowing the preparation of fine nanoemulsions (Abismaïl et al. 1999; Gaikwad and Pandit 2008; Delmas et al. 2011).

6.2.1.2 Low-Energy Emulsification Methods

Self-emulsification

Self-emulsification is the simplest low-energy method. It consists in mixing a lipophilic phase (oil, surfactants, and a water-miscible solvent) with the aqueous phase, with or without additional stabilisers, under vigorous stirring at room temperature. This allows both phases, which were initially thermodynamically stable, to enter a metastable state in which spontaneous emulsification becomes possible. The turbulence created at the interface induces the displacement of the water-miscible solvent and the surfactant from the lipophilic phase to the aqueous phase. This transfer from the oil phase to the water phase results in an increase of the oil/water interfacial area, causing the spontaneous formation of oil droplets (Rahman et al. 2013; Anton and Vandamme 2011).

Emulsification Based on Phase Inversion

Phase inversion methods exploit the ability of an emulsion to switch from one type (W/O) to another (O/W) in response to changes in temperature volume ratio or

composition. Such alterations affect the surfactants' packing parameter (p), a property defined by (6.1):

$$p = v_o / a_e . l_o \tag{6.1}$$

where v_o is the surfactant tail volume, l_o is the tail length, and a_e is the equilibrium area per molecule at the aggregate surface (Nagarajan 2001; Yan et al. 2007).

In brief, surfactants with $p < 1$ are more soluble in water and favour the formation of O/W emulsions, while surfactants with $p > 1$ favour the formation of W/O emulsion as they are more oil-soluble (McClements 2011).

1. Phase inversion based on changes in temperature.

   This method uses temperature to control the affinity of surfactants for the different phases. The temperature at which the affinity of a given surfactant system changes from the dispersed to the external phase, is known as the *phase inversion temperature* (*PIT*) (Anton et al. 2007; McClements 2011).

   As shown in Fig. 6.2, at temperatures below the PIT the surfactant present a $p < 1$ (O/W emulsion). When the temperature increases, the affinity of the surfactant for the aqueous phase decreases, causing the droplets to coalesce leading to the formation of a bicontinuous microemulsion. When the temperature is raised well above the PIT the surfactant becomes more oil-soluble, $p > 1$, favouring the formation of a W/O emulsion. By cooling down to a temperature below the PIT the O/W system will form again, giving rise this way to an O/W nanoemulsion.
2. Methods based on changes in composition

   In the *Phase Inversion Composition* (*PIC*) *method*, the temperature is fixed and the relative affinity of the surfactant for the different phases (i.e. change in p) occurs by altering the composition (Solè et al. 2006; Maestro et al. 2008; McClements 2011).

   The *Catastrophic Phase inversion* (*CPI*) *method* also relies on changes to composition to induce emulsification. For example, phase inversion is achieved by slowly increasing the water volume fraction of a W/O emulsion. Here, however, the phase change is abrupt (catastrophic), without a transitional phase as occurs in the PIC. Droplet size and size distribution will vary with stirring speed and surfactant concentration (Fernandez et al. 2004; Bilbao-Sainz et al. 2010).

6.2.2 Physicochemical Characterization

As with many other colloidal systems, droplet size and surface charge are determined by dynamic light scattering and laser Doppler anemometry, respectively (McClements and Dungan 1995; Heurtault et al. 2003; Bohren and Huffman 2007). Additional information on size and shape can be obtained using Small Angle Neutron Scattering (SANS), Small Angle X-ray Scattering (SAXS), or electron microscopy. Furthermore, electron microscopy techniques, in particular sophisticated cryo-Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), can deliver high-quality images of nanoemulsions in their

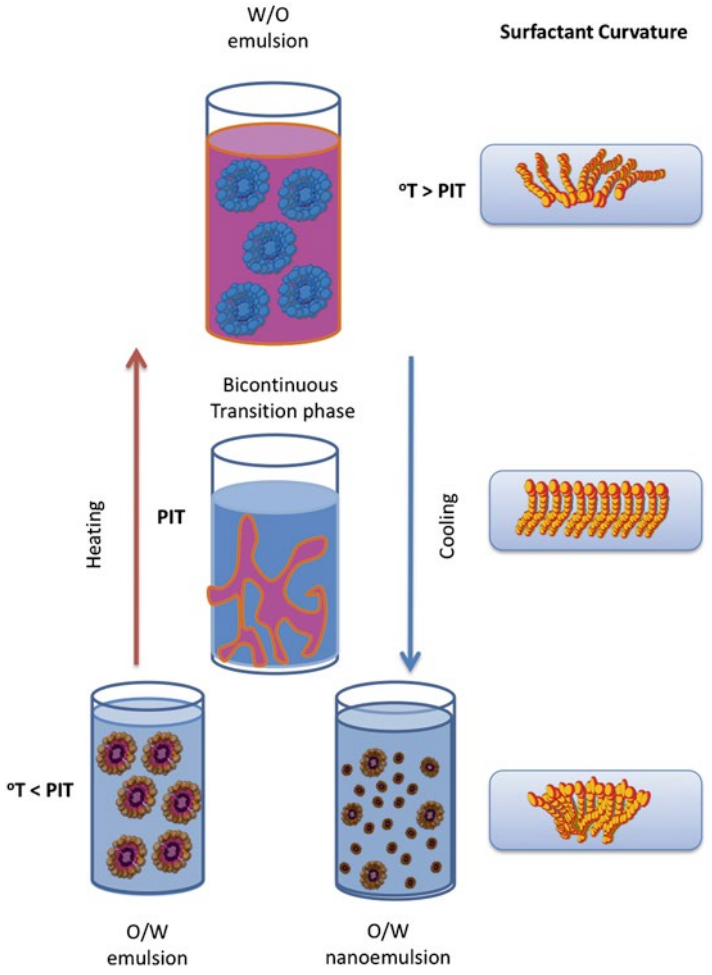

Fig. 6.2 Mechanism of the generation of nanoemulsions using the PIT method

natural state (Klang et al. 2012). In addition to droplet characterisation, the mechanical properties of nanoemulsions are often studied. Nanoemulsions are elastic systems and their elastic storage modulus G' can be measured by means of a rheometer (Howe and Pitt 2008; Pal 2011).

6.2.2.1 Modulating Droplet Size

Droplet size of nanoemulsions can be modulated through selection of proper manufacturing processes and composition. For example, small droplets are more easily obtained by high-energy methods which confer sufficient energy to the system to

breakdown even the smaller droplets (Tadros et al. 2004). Very fine nanoemulsions may also be prepared by low-energy methods, but require a careful selection of the materials, mostly with regard to the surfactants (Maestro et al. 2008). Indeed, surfactants lower the surface tension thus reducing the energy required to break a drop into small droplets. Several authors have reported that hydrophilic surfactants, such as Tween® 80 and Pluronic® 68, contribute towards reducing the oil droplet size compared to more hydrophobic ones (Seijo et al. 1990; Bouchemal et al. 2004). Surfactants with hydrophilic poly(ethylene glycol) (PEG) can also help to control droplet size (Wooster et al. 2008), although their ability to do so may decrease with the length of the PEG chain (Delmas et al. 2011).

The surfactant molecular geometry also influences the size of the dispersed phase and overall stability of the nanoemulsions. The length of the tail and the molecular conformation favour a more efficient packing of the surfactant molecules resulting in a reduction in size of the oil droplets. Most nanoemulsions are stabilised by synthetic surfactants, which tend to have long hydrophilic tails with a molecular size between 2 and 10 nm and consist of non-ionic and/or polymeric surfactants, as for example azo benzene-substituted poly(sodium acrylate) (HMPA-1) (Tadros 2005; Galindo-Alvarez et al. 2011).

6.2.2.2 Modulating Droplet Surface Charge

The surface charge of O/W nanoemulsions can be modulated through the addition of specific components. For example, by adding ionic surfactants, cationic or anionic nanoemulsions can be formed. Cationic nanoemulsions can complex negatively charged hydrophilic macromolecules, such as antisense oligonucleotides (Bruxel et al. 2011; Hagigit et al. 2012), and have increased cell adhesion/penetration properties as a result of interactions with the negatively charged cell membranes (Baspinar and Borchert 2012; Lallemand et al. 2012). Additionally, masking the charge of nanoemulsions by the addition of stealth polymers such as PEG is a strategy to avoid unspecific interactions with blood components after intravenous delivery, thus improving circulation times (Furtado Mosqueira et al. 2001; Huynh et al. 2009; Jokerst et al. 2011).

6.2.3 Kinetic Stability and Ostwald Ripening Effect

Emulsions, like most coarsely dispersed systems, are inherently unstable. As such, these systems will tend to revert to the state with the lowest free energy over time, under specified conditions of temperature, pressure, and composition (Atkins and De Paula 2010). There lies the main difference between microemulsions and nanoemulsions.

As shown in Fig. 6.3 microemulsion formation decreases the free energy of the systems rendering such formulations thermodynamically stable (Anton and Vandamme 2011; McClements 2012). In this respect, microemulsions are closer in

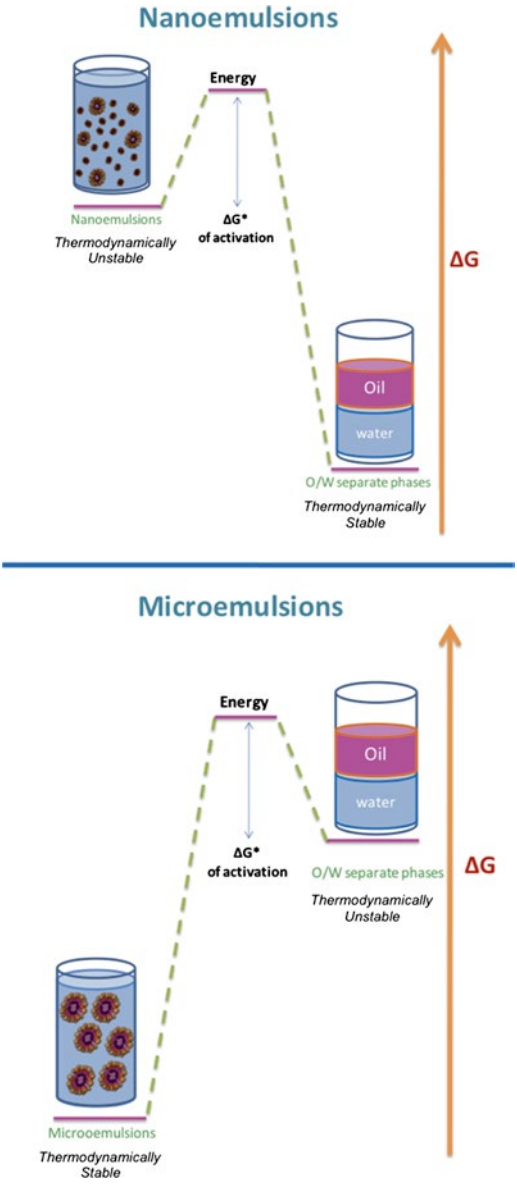

Fig. 6.3 Formation of nanoemulsions and microemulsions: free energy diagram of the two systems compared to the separated phase states

behaviour to micelles (Israelachvili 2011). Oppositely, nanoemulsions obey Gibbs' free energy law. These systems present higher free energy due to the increase in surface area produced by the dispersion of the oil phase. In time, the nanoemulsions will tend to revert back to two distinct phases: the system is said to be thermodynamically unstable. However, the rate of destabilisation is so slow that they are considered kinetically stable. In fact the height of the energy barrier that exists between the emulsified and non-emulsified states (Fig. 6.3) determines the stability

of the nanoemulsion: the higher the energy barrier to overcome, the longer is the stability of the nanoemulsion. In addition to this, the stability will also be influenced by other mechanisms responsible for the interaction between particles, such as Brownian motions, gravitational forces, and shear energy.

Physical instability of coarse emulsions usually manifest itself through flocculation (i.e. droplet aggregation), creaming (i.e. droplet rise through the medium), Ostwald ripening, or coalescence (i.e. when two droplets merge into a single larger droplet), with the latter potentially leading to the irreversible breaking of the formulation (Fredrick et al. 2010). As the very small droplet size of nanoemulsions reduces the incidence of coalescence, the Ostwald ripening effect is the main cause of instability (Anton and Vandamme 2011).

The Ostwald ripening effect is defined as the growth of the largest droplets at the expense of the smallest ones (Tadros et al. 2004). This phenomenon is due to differences in the chemical potential among oil droplets of different sizes. As the chemical potential increases for droplets of smaller size, due to a heightened Laplace pressure, the smallest droplets tend to transfer mass to the largest ones across the continuous phase (Delmas et al. 2011). The Ostwald ripening effect can be avoided by controlling the physical properties of the oil phase and the nature of the surfactant (Webster and Cates 1998). For example, Ostwald ripening was prevented by a large molar volume of long chain triglyceride (LCT) oils, which makes them insoluble in water thus providing a kinetic barrier to Ostwald ripening (Wooster et al. 2008). Another interesting strategy to increase the stability is the incorporation of well-known stabilising polymers, such as PEG (Wooster et al. 2008; Delmas et al. 2011).

As instability is associated with an increase in droplet size, measuring the change in size over time has been the basic method for studying nanoemulsion stability (Porras et al. 2004; Tadros et al. 2004; Wooster et al. 2008). When destabilisation is caused by coalescence, the dependence between size increase and time is given by the Deminière equation (6.2), where r is the average droplet radius after t, r_0 is the value at $t=0$, and ω is the frequency of rupture per unit of surface of the film (Deminière 1998; Peng et al. 2010).

$$\frac{1}{r^2} = \frac{1}{r_0^2} - \left(\frac{8\pi}{3}\right)\omega t \tag{6.2}$$

For Ostwald ripening, the rate is given by the Lifshitz-Slezov and Wagner theory (LSW) according to (6.3), where r_c is the critical radius of the system at any given time; c (∞), the bulk phase solubility; γ, the interfacial tension; V_m, the molar volume; ρ, the density of the water; R, the gas constant; and T, the absolute temperature (Lisfshitz and Slezov 1961; Taylor 1995).

$$\omega = \frac{dr_c^3}{dt} = \frac{8c_{(\infty)}\gamma DV_m}{\rho RT} \tag{6.3}$$

Plotting r^2 or r^3 against time can then identify the actual process driving instability. A linear relationship between the achieved former and time identifies coalescence as the main driving force while the plot between r^3 and time will be linear if Ostwald ripening is the main process.

6.2.4 O/W Nanoemulsions as Drug Carriers

Adequate selection of the manufacturing technique and materials allows tailoring of O/W nanoemulsions with specific physicochemical properties. This could be done to favour high entrapment efficiencies or favourable biodistribution of both lipophilic and hydrophilic drugs (Peltier et al. 2006; Makidon et al. 2008; Hamouda et al. 2010; Zhang et al. 2011b; Hagigit et al. 2012).

Nanoemulsions can host poorly soluble drugs in their hydrophobic core, providing additional protection from acid or enzymatic degradation (Kotta et al. 2012). Encapsulation may also facilitate membrane translocation and increase drug bioavailability (Beg et al. 2011; van Hoogevest et al. 2011). Interestingly, the use of O/W nanoemulsions is not limited to hydrophobic compounds. Although far less common, positively charged nanoemulsions can bind hydrophilic biomolecules through electrostatic interactions, thus enabling the delivery of new therapeutic entities such as oligonucleotides (Teixeira et al. 1999; Bruxel et al. 2011; Hagigit et al. 2012), plasmid DNA (Liu and Yu 2010) or antibodies (i.e. tyroglobulin) (Wang et al. 2012). More detailed information is provided in Sect. 6.5.

6.3 W/O Nanoemulsions

Water-in-oil (W/O) or oily nanoemulsions are systems where small water droplets are dispersed into an organic, water immiscible phase. The aqueous phase can consist of pure water, saline, a buffer, or a drug solution; while alkanes (Porras et al. 2008), carbon dioxide (Psathas et al. 2002), fluorocarbons (Krafft et al. 2003), isopropyl myristate (Sadtler et al. 1999; Uson et al. 2004), and squalene (Huang et al. 2009c) have been explored as the external phase.

6.3.1 Emulsification Methods

In order to achieve small droplet sizes, high-energy methods have been preferred for the preparation of W/O nanoemulsions (Sect. 6.2.1.1) (Sadtler et al. 1999; Landfester et al. 2000; Butz et al. 2002; Courrier et al. 2004b; Taden et al. 2004; Tang et al. 2012). Low-energy emulsifications methods have also been suggested, albeit with mixed results. On one hand, stable water-in-isopropylmyristate nanoemulsions (60–160 nm) were successfully obtained by phase inversion at constant temperature (Uson et al. 2004). Yet, this method failed when applied to water-in-decane systems (Porras et al. 2008). This was reportedly caused by the presence of a multiphase system including solid surfactant and liquid crystals (Porras et al. 2008). Equally, $NaCl_{aq}$-in- CO_2 miniemulsions prepared using the PIT method presented small droplet size (<300 vs >700 nm for formulations prepared using high-pressure

homogenisation), but were reported to be stable for only short periods of time (Psathas et al. 2002). Further evaluation of low-energy methods is therefore required to enable their widespread use for the preparation of oily nanoemulsions.

6.3.2 Pseudo-ternary Phase Diagrams

It has been suggested that phase diagrams alone may not allow for clear discrimination between O/W nanoemulsions and microemulsions (Anton and Vandamme 2011; Fryd and Mason 2012; McClements 2012). Despite these reservations, phase diagrams have repeatedly been reported in the study of W/O nanoemulsions (Porras et al. 2004, 2008; Uson et al. 2004), albeit often combined with light scattering analysis, as an additional tool for the identification of the type of emulsion formed (see below for details).

In order to determine the amount of oil, water, and surfactant required for the formation of nanoemulsions, phase diagrams are constructed at known surfactant: oil (S:O) ratios and varying water content (Porras et al. 2004). Combinations of surfactants are generally preferred to enable the dispersion of maximal amounts of water with one of the surfactants being hydrophobic (low hydrophilic–lipophilic balance, HLB) (Porras et al. 2004, 2008; Uson et al. 2004). The most advantageous mixing ratio (Smix) of each surfactant can be determined by assessing their ability to solubilise water at a constant S:O ratio, typically 15:85 or 5:95 for water in decane systems (Porras et al. 2004, 2008). Differences in the versatility of nanoemulsions stabilised with a single surfactant or a mix of surfactants are highlighted in a phase behaviour study of mixtures of isopropyl myristate, water, and hydrophobic Cremophor® WO7 alone or in combination with Cremophor® EL. In this case, the addition of Cremophor® EL allowed for the formation of more complex systems with a variety of phases that can accommodate larger amounts of water and are more amenable to the formation of W/O nanoemulsions (Uson et al. 2004). Alternatively, phase diagrams can be constructed for different Smix (Uson et al. 2004), which can be cumbersome when screening for a large number of different surfactants.

Once the surfactant combination has been decided, the phase diagram can be constructed (Fig. 6.4). The identification of the type emulsion, microemulsion, nanoemulsion, or coarse is usually performed by light scattering (Mengual et al. 1999). One implement calls for back-scattering measurements to be performed at different cuvette heights and time points (Porras et al. 2008). The formation of coarse emulsions will show changes in size depending on where the measurement is taken due to sedimentation of the dispersed phase. However, it is thought that neither microemulsions nor nanoemulsions will produce such changes and can only be differentiated by following size over time (Porras et al. 2008).

Using this method, the phase behaviour of water–decane mixtures revealed that microemulsions, nanoemulsions, and finally coarse emulsions were formed in sequence when the proportion of water was increased (Porras et al. 2004, 2008). For decane mixtures, stabilised with a combination of Span™ 20:Tween® 80 (62:38),

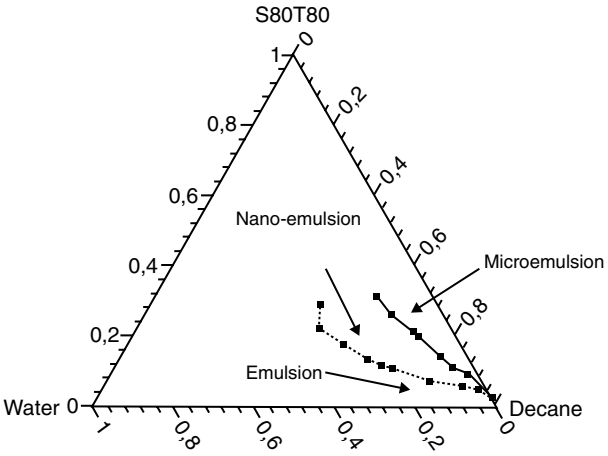

Fig. 6.4 Pseudo-ternary phase diagram showing regions of microemulsion, nanoemulsion, and emulsion for a decane in water nanoemulsions stabilised with Span™ 80 and Tween® 80 (51:49) at 25 °C. Surfactant:decane ratio 5:95. Reproduced from Porras et al. (2008) with permission

the nanoemulsion region was found at water contents between 3 and 20 %, for S:O ratios kept at 15:85 (ca. 68–82 % decane; 12–15 % surfactant) (Chiesa et al. 2008). In this region, emulsions were stable against phase separation, but prone to increases in droplet size, a distinctive feature confirming that nanoemulsions rather than microemulsions were obtained (Chiesa et al. 2008; Porras et al. 2008).

6.3.3 Particle Size, Surface Properties, and Stability

Similarly to aqueous emulsions, droplet size changes with the proportions of the different components and the nature of the surfactants (Uson et al. 2004; Porras et al. 2008). With other components kept at a constant ratio, increases in water and surfactant will produce larger (Porras et al. 2004; Uson et al. 2004; Peng et al. 2010) and smaller (Porras et al. 2004) droplets, respectively. Both occurrences can be explained by changes in interfacial area and interfacial tension (Friberg and Vesable 1985). Increasing the proportion of oil, at a constant water:surfactant ratio, will simply dilute the nanoemulsion and bears little impact on droplet size (Uson et al. 2004). Expectedly, the ratio of the different surfactants can also have an impact on droplet size. For example, in the case of water-in-isopropyl myristate nanoemulsions, droplet size initially decreases when the ratio of Cremophor® WO 7:Cremophor® EL, or hydrophobic to hydrophilic surfactant, was increased from 2:1 to 6:1 (Uson et al. 2004). Size then reached a plateau before increasing again slightly at a ratio of 9:1.

The stability of W/O nanoemulsions will be determined by the amount of surfactant, which allows uniform coating of the droplets and the formation of small and homogeneously dispersed droplets. The discrete size of the dispersed phase droplets

offers some protection from sedimentation (Uson et al. 2004). Yet, rapid phase separation may still occur when the surfactant mixture cannot adequately stabilise the water droplets. This has been observed in water-in-decane emulsions where formulations stabilised with mixtures of Span™ 20:Tween® 20 rapidly separated (Porras et al. 2008).

Ostwald ripening remains the most common cause of instability for W/O nanoemulsions (Courrier et al. 2004b; Porras et al. 2004, 2008; Uson et al. 2004). Yet, for given S:O ratios and surfactant mixtures, coalescence may become the prime destabilisation mechanism at higher water contents, as a result of the thinning of the protective surfactant layer (Porras et al. 2008). This was observed for water-in-decane nanoemulsions. At an S:O ratio of 15:85, emulsions were destabilised through Ostwald ripening for water contents of 11–14 wt% but mainly by coalescence for 16 wt% (Porras et al. 2008). In water-in-fluorocarbon nanoemulsions, destabilisation due to Ostwald ripening could be slowed down by decreasing the solubility of the droplets through the addition of NaCl to the dispersed phase (Sadtler et al. 1996).

6.3.4 W/O Nanoemulsions as Drug Carriers

W/O nanoemulsions have been studied for various applications including as a scaffold for the formation of nanoparticles (Landfester et al. 2000) or as coolants (Chiesa et al. 2008). In comparison to aqueous nanoemulsions, very few examples of pharmaceutical use of W/O formulations have been reported (see Sect. 6.5 for details). Indeed, many of the systems described as W/O nanoemulsions seem to better fit the definition of microemulsions as the formulations (1) were often described as being thermodynamically stable, (2) presented a high surfactant content (>40 %), and (3) could be prepared by simply mixing all the ingredients, although some have reported the use of probe sonication (Wu et al. 2001a, b; Wang et al. 2008a). Nevertheless, oily emulsions have shown potential for the delivery of water-soluble agents (Sadtler et al. 1998; Wu et al. 2001a, b; Courrier et al. 2004a; Wang et al. 2008a; Hwang et al. 2009; Fan et al. 2011; Zhang et al. 2011a; Tang et al. 2012) and to improve the oral bioavailability of Class III BCS (Biopharmaceuticals Classification System) drugs and transport of proteins through the skin (Zhi et al. 2005; Gundogdu et al. 2011; Russell-Jones and Himes 2011).

6.4 Polymer-Coated O/W Nanoemulsions: Nanocapsules

Nanocapsules, formed by an inner oil phase and coated by a hydrophilic or hydrophobic polymer, have been widely explored in the field of drug delivery over the last years (Damgé et al. 1988; Quintanar-Guerrero et al. 1998; Prego et al. 2005, 2006a, b; Peltier et al. 2006; Letchford and Burt 2007; Cattani et al. 2010;

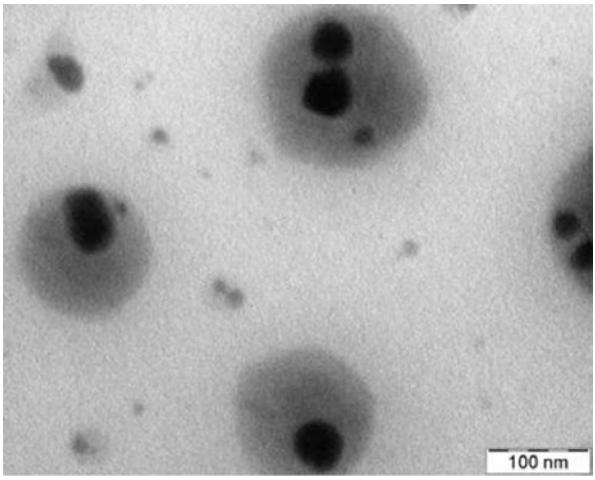

Fig. 6.5 TEM micrograph of poly(lactic acid) poly(ethylene oxide) nanocapsules prepared by interfacial deposition of preformed polymers

Ngwuluka 2010; Anton et al. 2012; Battaglia and Gallarate 2012). For illustration, Fig. 6.5. shows a TEM micrograph of poly(lactic acid)-poly(ethylene oxide) (PLA-PEG) nanocapsules.

6.4.1 Coating Materials

Shell-forming polymers may be selected from a wide range of biomaterials, including hydrophobic and hydrophilic materials, as compiled in Table 6.2. In turn, particle size, polydispersity index, and zeta potential of nanocapsules are clearly influenced by the molecular weight, charge, solubility, and chemical structure and architecture of the coating polymer (Prego et al. 2006a, b; Goldberg et al. 2007). The type of polymer, the nature of the drug, and the specific interactions between them and other components of the nanocapsules have a strong influence on drug loading and drug release properties (Cauchetier et al. 2003). For example, the use of polyarginine as a shell-polymer led to a significant reduction of the particle size, in comparison to the uncoated nanoemulsion, and to an inversion of the superficial charge from negative to positive (Lozano et al. 2013). In the case of chitosan nanocapsules, the amount of chitosan had to be carefully modulated to improve the association of salmon calcitonin as both drug and polymer competed for interaction with the negatively charged acid group of the stabiliser lecithin (Prego et al. 2006a, b).

Selection of the appropriate polymeric shell could also influence the behaviour of the systems in vivo (Damgé et al. 1988, 1990; Oyarzun-Ampuero et al. 2013). In this context, the mucoadhesive and permeation enhancer polysaccharide chitosan has been selected for the preparation of nanocapsules for transmucosal delivery of

Table 6.2 Polymers reported for the preparation of nanocapsules

Shell composition	Properties	Active agents	Pharmaceutical applications	Ref.
Poly(ε-caprolactone) (PCL)	Biodegradable	Indomethacin	Oral drug delivery	Cattani et al. (2010)
	Biocompatible		Ocular drug delivery	Cruz et al. (2006)
	Controlled Release			Poletto et al. (2008)
	Good mechanical properties			Calvo et al. (1997)
Poly(ε-caprolactone)-poly(ethylene oxide) (PCL-PEG)	Biodegradable	Carvacrol	Imaging and diagnostic	Ameller et al. (2003), Furtado Mosqueira et al. (2001)
	Biocompatible		Cancer therapy	
	Controlled release			
	Good mechanical properties			
	Long-circulating systems			
Poly(lactic-co-glycolic) acid (PLGA)	Biocompatible		Delivery of antimicrobial drugs	Abdel-Mottaleb et al. (2011)
	Biodegradable	Ibuprofen	Transdermal drug delivery	Iannitelli et al. (2011)
	Controlled release	Carvacrol		
	FDA approved			
Poly(lactic-co-glycolic)-poly(ethylene oxide) (PLGA-PEG)	Biocompatible	Carvacrol	Imaging and diagnostic	Ameller et al. (2003), Furtado Mosqueira et al. (2001)
	Biodegradable		Cancer therapy	
	Controlled release			
	Long-circulating systems			
Poly(lactic acid) (PLA)	Biocompatible	Fluconazol	Ocular drug delivery	de Assis et al. (2008)
	Biodegradable	Atovaquone	Cancer therapy	Cauchetier et al. (2003)
	Controlled release			
	Low toxicity			
Poly(lactic acid)-poly(ethylene oxide) (PLA-PEG)	Biocompatible	Fluconazol	Cancer therapy	Furtado Mosqueira et al. (2001), de Assis et al. (2008)
	Biodegradable	pDNA	Imaging and diagnostic	
	Controlled release			
	Long-circulating systems			

(continued)

Table 6.2 (continued)

Shell composition	Properties	Active agents	Pharmaceutical applications	Ref.
Chitosan	Mucoadhesive	Indomethacin	Oral drug delivery	Prego et al. (2005)
	Biocompatible	Salmon calcitonin	Ocular drug delivery	Calvo et al. (1997)
	Permeation enhancer			Prego et al. (2006a)
	Biodegradable			
	Transfection agent			
Chitosan-poly(ethylene oxide) (Chitosan-PEG)	Mucoadhesive	Salmon calcitonin	Oral drug delivery	Prego et al. (2006b)
	Biocompatible	Docetaxel	Cancer therapy	Torrecilla et al. (2013)
	Permeation enhancer		Nanovaccines	
	Biodegradable			
	Transfection agent			
	Long-circulating system			
Polyaminoacids	Biocompatible	Docetaxel	Cancer therapy	Lozano et al. (2013)
	Biodegradable	pDNA	Nanovaccines	González-Aramundiz et al. (2012)
	Permeation enhancer			
	Vaccination adjuvant			
	Transfection agent			
Hyaluronic acid	Biocompatible	Docetaxel	Cancer therapy	Oyarzun-Ampuero et al. (2013)
	Biodegradable			
	Transfection agent			
	Mucoadhesive			
	Active targeting			
	Long-circulating systems			

macromolecules such as pDNA, antigens, or peptides, as detailed in Sect. 6.5. Results demonstrated an improved effect of chitosan nanocapsules in drug retention, absorption, and therapeutic efficiency, in comparison with uncoated nanoemulsions, thus highlighting the positive role of the polymeric coating (Prego et al. 2005, 2006a, b, 2010; Shu et al. 2010; Vicente et al. 2010; Gaspar et al. 2011).

Further improvement of the properties of nanocapsules could be achieved through surface modification with PEG. PEGylation can be carried out by incorporation of PEG fatty acids esters, also reported as PEGylated nanoemulsions, or by using preformed PEGylated polymers such as poly(lactic acid)-PEG (PLA-PEG), poly(lactic-co-glycolic) acid-PEG (PLGA-PEG), poly(ε-caprolactone)-PEG (PCL-PEG), or chitosan-PEG to form the shell (Furtado Mosqueira et al. 2001; De Campos et al. 2003; Prego et al. 2006b). Apart from the well-known stealth behaviour that PEG confers on formulations (Gabizon 2001), PEGylation decreases the toxicity of the nanocarriers, improves nanocapsules stability, and has an influence on the physicochemical properties of the resultant nanosystems (de Assis et al. 2008; Vonarbourg et al. 2009; Battaglia and Gallarate 2012). Additional surface modification could lead to the design of stealth nanocapsules decorated with specific ligands, in order to actively target disease sites, improving drug accumulation (Béduneau et al. 2007; Morille et al. 2009; Laine et al. 2012; Torrecilla et al. 2013).

6.4.2 Formulation Technologies

Most of the methods described for the preparation of nanoemulsions can be adapted for nanocapsules manufacturing. High- and low-energy methods including emulsification-coacervation (Lertsutthiwong et al. 2008, 2009), simple or double emulsification (Moinard-Checot et al. 2006, 2008; Grigoriev and Miller 2009), phase-inversion temperature-based technology, (Heurtault et al. 2002), in situ polymerization (Ngwuluka 2010), and interfacial deposition of preformed polymers (Calvo et al. 1997; Prego et al. 2006a, b; Lozano et al. 2008, 2013) have all been described.

6.4.2.1 Simple or Double Emulsification

The selection of one or another for a pharmaceutical formulation will depend on the solubility of the drug to be entrapped into the oily droplets. In all cases hydrophobic polymers form the shell, and high-energy shear forces such as ultrasonication are required.

During *simple emulsification* (O/W), lipophilic drugs are incorporated into the organic inner phase, which is emulsified in an aqueous phase containing a stabilising agent such as cholic acid or poly(vinyl alcohol). The polymer that will form the capsule is typically added to the oil phase (Quintanar et al. 2005; Moinard-Checot, et al. 2008; Poletto et al. 2008).

The *double emulsion* method (W/O/W) combines two emulsification steps. In the first instance a W/O nanoemulsion is formed, which contains the drug, solubilised in the inner phase. This oily emulsion is then emulsified in a secondary aqueous phase (Quintanar-Guerrero et al. 1998; Poletto et al. 2008).

6.4.2.2 In Situ Interfacial Polymerization

This technique involves the formation of nanocapsules through the polymerization of monomers at the interface of two non-miscible liquids. Polymerization conditions differ depending on the selected polymer. For example, acrylic acid and acrylamide derivatives can be polymerized in the presence of persulfate salt (Scarioti et al. 2011; Zhao et al. 2011a, b). In the case of alkyl cyanoacrylate monomers, anionic polymerization can be initiated by the presence of nucleophilic groups (Aboubakar et al. 1999; Nicolas and Covreur 2009). Poly(alkyl cyanoacrylate) nanocapsules showed an exceptional ability to encapsulate insulin into the oily core and to improve its effectiveness in vivo by the oral route (Damgé et al. 1990, 1997). This method does not require the application of high energies. The main disadvantage is the toxicity that may be caused by free monomers. Toxicity of the monomers has been related with their local concentration, the length of the alkyl chain, and thus, cell membrane bioadhesion (Dillingham et al. 1983; Huang and Lee 2006).

6.4.2.3 Interfacial Deposition of Preformed Polymers

This is a mild technique to obtain O/W nanocapsules that involves the deposition of preformed polymers at the oil–water interface (Mora-Huertas et al. 2010). As it was described before for the self-emulsification method (Sect. 6.2.1), this procedure is based on the diffusion of the organic solvent to the aqueous phase in which the surfactant, if needed, is dissolved. Hydrophobic polymers are incorporated in the organic phase. As the organic solvent diffuses the polymer becomes insoluble, just allowing the deposition of the hydrophobic polymer at the oil nanodroplet interface (Cauchetier et al. 2003; Teixeira et al. 2005; Stella et al. 2007; de Assis et al. 2008; Cattani et al. 2010).

Hydrophilic polymers are incorporated in the aqueous external phase. In this case, additional electrostatic interactions are involved in their deposition at the oil nanodroplets interface (Lozano et al. 2008, 2013; Garcia-Fuentes and Alonso 2012). An alternative is to incubate the polymer with preformed anionic of cationic nanoemulsions.

6.4.3 Nanocapsules as Drug Carriers

O/W-polymer-coated nanoemulsions can host drugs both in the oil core and onto the polymeric shell. As a consequence, nanocapsules are seen as highly attractive

delivery systems and hold an enormous potential for a wide range of applications, being particular examples detailed in the next section (Calvo et al. 1997; Damgé et al. 2007; Huynh et al. 2009; Teixeira et al. 2010; Lozano et al. 2013; Torrecilla et al. 2013).

6.5 The Potential of Nanoemulsions in Therapeutics

In this section we aim to provide an overview of the potential of nanoemulsions in therapeutics, supported by specific examples that illustrate the most recent advances in the field.

6.5.1 Transmucosal Drug Delivery

The delivery of drugs and macromolecules by non-invasive transmucosal routes is a promising approach in therapeutics. Nanoemulsions can be specifically designed to overcome mucosal barriers and other limitations of every modality of administration.

6.5.1.1 Oral Delivery

Despite its advantages, i.e. easiest of administration and patient compliance, the oral route remains a challenge for formulation scientists. Drug nanocarriers are expected to (1) confront the gastrointestinal track and its harsh environment, preserving the integrity of the encapsulated drugs, (2) overcome the intestinal mucosa, and (3) promote drug absorption.

As mentioned in Sect. 6.2, O/W nanoemulsions are well-suited for the encapsulation of poorly soluble hydrophobic drugs (Shafiq et al. 2007; Chen et al. 2011; Parveen et al. 2011; van Hoogevest et al. 2011). An improvement in drug solubility is related to an increase in drug bioavailability as dissolution is often a rate-limiting step to absorption (van Hoogevest et al. 2011).

Examples of improved oral bioavailability upon encapsulation in nanoemulsions include the case of Sylimarin, a mixture of flavolignans rich in Silybin with hepatoprotectant properties, which saw its oral bioavailability in rats increased by six and fourfold compared to Sylimarin in suspension and the marketed formulation (SYLBON®) (Parveen et al. 2011), respectively. Similarly, upon oral administration of a nanoemulsion of the pro-drug ramipril, the bioavailability of the active metabolite ramiprilat was increased by 2.94 and 5.4-fold with respect to conventional capsules and a drug suspension (Shafiq et al. 2007).

Enhanced oral bioavailability can also be achieved by preventing the interaction of the encapsulated drug with efflux transporters such as the P-glycoprotein (P-gp). The essential oil eugenol is rich in terpenoids and is an inhibitor of P-gp-mediated transport (Yoshida et al. 2005). Eugenol-based nanoemulsions (isopropyl myristate,

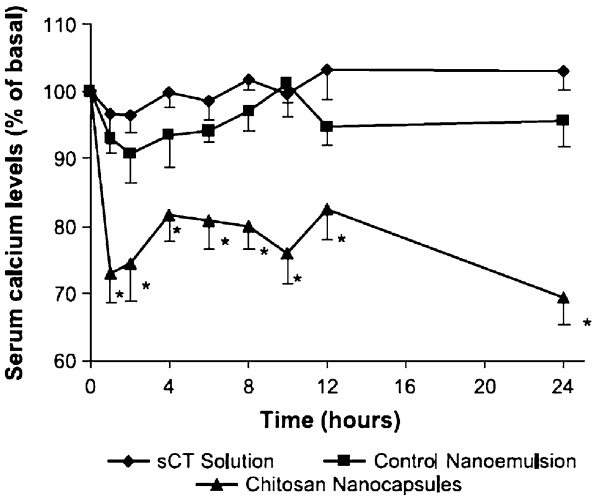

Fig. 6.6 Changes in serum calcium levels after oral administration to rats of salmon calcitonin associated to chitosan nanocapsules (*triangle*) or non-coated O/W nanoemulsions (*square*). As a control, a solution of the peptide in solution (*diamond*) was used. Reproduced from Prego et al. (2006a) with permission

eugenol, and Tween® 80) have then been proposed for the oral delivery of colchicine, a P-gp substrate (Shen et al. 2011). Results show a 2.1-fold increase in oral bioavailability of colchicine compared to the drug in solution (Shen et al. 2011). Nanoemulsions prepared without eugenol (isopropyl myristate and Tween® 80) also enhanced bioavailability but to a lesser extent (1.6-fold).

Finally, the inclusion of mucoadhesive polymers by increasing retention time through prolonged interaction with the intestinal mucosa can also improve the absorption of encapsulated drugs (Prego et al. 2005). Chitosan, a well-known mucoadhesive polymer, was used to coat medium chain triglyceride (Miglyol® 812) and lecithin nanoemulsions (chitosan nanocapsules) destined for oral peptide delivery. As it can be observed in Fig. 6.6, an improved efficacy (threefold) was observed when the peptide salmon calcitonin was associated with chitosan nanocapsules, compared to uncoated nanoemulsions (Prego et al. 2005, 2006a, b). Furthermore, polymer coating plays an important role in improving drug stability under physiological conditions (Fukui and Fujimoto 2009). In fact, insulin encapsulated in chitosan-glucomannan- (Wang et al. 2008b) and poly(isobutyl cyanoacrylate)-coated nanocapsules (Damgé et al. 1988, 1990; Watnasirichaikul et al. 2002) showed improved (1) stability and pH resistance of sensitive drugs in vitro, (2) drug absorption, and (3) a therapeutic effect in both rats and dogs, in comparison to the free insulin.

6.5.1.2 Pulmonary and Intranasal Delivery

Water-in-fluorocarbon nanoemulsions constitute an attractive carrier for the delivery of drugs to the lung (Sadtler et al. 1996; Krafft 2001; Krafft et al. 2003).

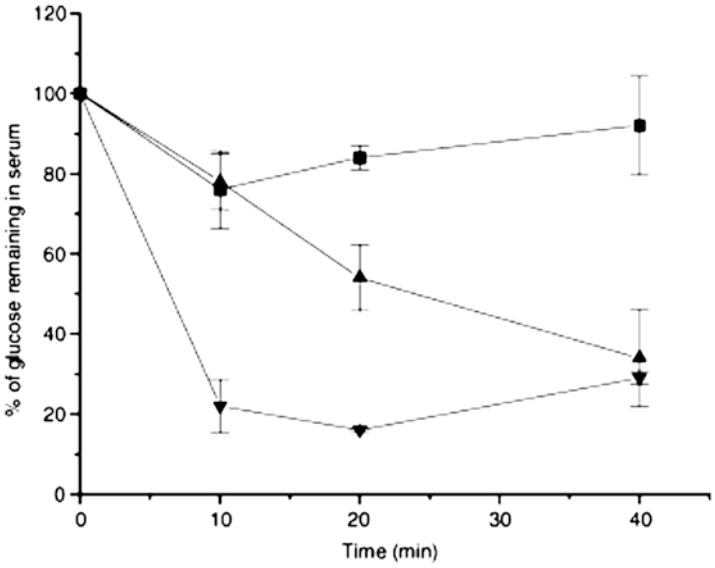

Fig. 6.7 Changes in blood glucose levels in mice following the intranasal instillation of a drug-free (*squares*) and insulin-loaded (*triangles*) water-in-perfluorooctyl bromide nanoemulsion or of an insulin aqueous solution (*inverted triangles*). Data is expressed relative to the blood glucose levels measured in control-untreated mice. Reproduced from Courrier et al. (2004a) with permission

Fluorocarbons are biologically inert materials, miscible with hydrofluoroalkanes (used as propellants) (Butz et al. 2002) and able to solubilise large amounts of oxygen (Riess 2005). One of the main challenges in the formulation of fluorocarbon is their high hydrophobicity and lipophobicity that limits the type of surfactants that can be used as stabilisers. To this end, several (*F*-alkyl)alkyl dimorpholinophosphate C_nF_{2n+1} $C_mH_{2m}OP(O)[N(CH_2CH_2)_2O]_2$ (F_nC_mDMP) surfactants were evaluated (Krafft et al. 1991; Sadtler et al. 1998). Out of the different candidates, F_8C_{11}DMP surfactant was able to produce stable fluorocarbon nanoemulsions with an average size around 100–120 nm (Sadtler et al. 1999; Courrier et al. 2004b), which were shown to be highly efficient in controlling the release of a model hydrophilic fluorescent probe. A later report verified that they could be used to homogenously and reproducibly deliver low-molecular weight caffeine from a CFC-free pressurised metered-dose inhaler, providing preliminary indications that water-in-fluorocarbon nanoemulsions could become successful inhaled drug delivery systems (Butz et al. 2002).

Water-in-fluorocarbon nanoemulsions were also evaluated for the delivery of insulin in a mouse model, following intranasal instillation (Courrier et al. 2004a). As depicted in Fig. 6.7, insulin loaded in the nanoemulsion produced a rapid hypoglycaemic effect (40 % of blood glucose levels after 20 min) though less pronounced than the insulin solution control (80 %). However, while the effect of the insulin solution seemed to cease after 40 min, the last available time point, glucose levels

continued to decrease when insulin was given as a nanoemulsion, suggesting a more sustained effect. Longer experimental times would thus be required in order to confirm the true potential of these formulations for insulin delivery.

6.5.1.3 Ocular Delivery

Eye diseases are commonly treated by topical administration of the selected drug. However, providing and maintaining the drug concentration at the administration site remains challenging. The design of drug carriers aimed for delivery onto the ocular mucosa is seen as an efficient strategy to prolong ocular residence time of drugs, limiting drainage, and increasing the pharmacological effect at the site of action. In addition nano-sized delivery systems have shown an improved interaction with the ocular mucosa and can even penetrate the corneal and conjunctival epithelia (Maincent et al. 1995; de la Fuente et al. 2008a, b, 2010; Contreras-Ruiz et al. 2011).

O/W nanoemulsions are considered an excellent option for topical delivery of lipophilic drugs to the eye. As a matter of fact, some formulations have already been commercialised (Klang et al. 2000; Lallemand et al. 2012). Restasis® (Allergan, Inc.) was the first O/W emulsion formulation to be approved for commercialization. It consists in an anionic emulsion of Cyclosporin A for the treatment of dry eye and is composed of glycerine, castor oil, polysorbate 80, carbomer copolymer type A, purified water, and sodium hydroxide to adjust pH. Nanoemulsion-based artificial tears such as Soothe® (Bausch & Lomb, Inc.) and Refresh Endura® (Allergan, Inc.) have also been marketed in the United States. Currently the pharmaceutical company Novigali Pharma is developing a technology marketed as Novasorb® for the treatment of dry eye disease with cyclosporine A. Novasorb® contains the cationic lipids benzalkonium chloride and cetylalkonium chloride and polyols such as glycerol, mannitol, or sorbitol. This formulation is now in its phase III Clinical Trial for the evaluation of efficacy, tolerance, and safety (Lallemand et al. 2012).

Since the corneal and conjunctival cell membranes are negatively charged at physiological pH, cationic nanoemulsions are currently explored for an improved interaction (Klang et al. 2000; Lallemand et al. 2012). Klang et al. (2000) compared positively and negatively charged nanoemulsions based on medium chain triglycerides (MCT) for ocular delivery of indomethacin. The positively charged nanoemulsions provided significantly higher drug levels than both the control solution and a negatively charged emulsion in the aqueous humour and sclera-retina. Furthermore, the spreading coefficient of the positively charged emulsion on the cornea was shown to be four times higher than that of the negatively charged emulsion (Klang et al. 2000). Promising results have been recently published by Hagigit et al. (2012) on the use of cationic nanoemulsions based on medium chain triglycerides and also a well-reported cationic lipid for transfection, DOTAP ((*N*-[1-(2,3-dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium methyl sulphate), for the complexation and delivery of antisense oligonucleotides directed at Vascular endothelial growth factor receptor (VEGF-R2) to reduce neovascularization. Results showed that, upon

topical administration to rats and mice (previously treated to develop corneal neovascularization), there was a substantial inhibition of the process.

Similarly, cationic polymer-coated O/W nanoemulsions have been shown to improve the ocular bioavailability of different drugs such as indomethacin, pilocarpine, metipronalol, or piroxicam (Jacob et al. 1990; Losa et al. 1993; Klang et al. 1996; Desai and Blanchard 1998; De Campos et al. 2003). The corneal penetration of indomethacin loaded into chitosan nanocapsules was improved by a factor of 2, according to drug concentrations measured in the aqueous humour, and with respect to uncoated cationic nanoemulsions upon instillation to rabbits (Calvo et al. 1997). A different composition, based on PECL forming the shell, has also shown potential for this particular application, providing a fivefold increase in cyclosporine concentrations in the cornea compared to the levels measured upon instillation of the free drug. Moreover, this particular nanocapsule's composition specifically target the cornea and decrease systemic absorption, which is usually related to undesirable side effects (Calvo et al. 1994, 1996).

6.5.2 Topical/Transdermal Drug Delivery

Transdermal delivery remains a highly desirable route of administration due to the large skin surface available for absorption as well as limited first-pass effect. However, those advantages are offset by the skin's well-organised structure and low permeability (Neubert 2011). Among the different carriers tested as transdermal delivery system, nanoemulsions seem particularly promising and hold several advantages for the treatment of skin conditions (Abdel-Mottaleb et al. 2011).

The efficacy of O/W nanoemulsions for topical/skin delivery depends heavily on the selection of oil, particularly with regard to its molecular weight: high molecular weight oils favour the formation of more stable nanoemulsions than low-molecular weight oils (Koroleva and Yurtov 2012). Yet, low-molecular weight oils increase the penetration rate and do not leave an oily and greasy skin (Sonneville-Aubrun et al. 2004). Therefore, oils should be selected based on the requirements of the final formulation. Oils containing high levels of antioxidants, such as rice brain oil (Oriza Sativa), a widely used component of sunscreen creams and anti-ageing creams, have also been formulated in anti-irritant nanoemulsions properties (Lerma-Garcia et al. 2009). Bernardi et al. have demonstrated that rice bran oil nanoemulsions have high hydration and moisturising properties when applied on the skin of patients affected by psoriasis or dermatitis while maintaining the normal skin pH (Bernardi et al. 2011). A large proportion of the literature on topical use of nanoemulsions comes from the cosmetic industry. Indeed, nanoemulsions have been included in various formulations, from hydrating creams to hair-colouring products. The L'Oreal group alone possesses dozens of proprietary technologies based on nanoemulsions.

Nanocapsules have also been proposed as potential carriers for transdermal delivery (Teixeira et al. 2010; Abdel-Mottaleb et al. 2011). More specifically, it is expected that an increased affinity of the nanocarriers for the stratum corneum due

to the polymeric coating (PCL nanocapsules) may promote a deeper penetration into human skin in comparison with non-coated nanoemulsions (Alves et al. 2007). This was demonstrated by Teixeira and co-workers for retinyl palmitate, a vitamin A derivative, loaded into PLA nanocapsules (Teixeira et al. 2010).

6.5.3 Nanoemulsions for Disease Management

6.5.3.1 Cancer

Anticancer drugs present biopharmaceutical issues that need to be resolved in order to achieve maximal therapeutic efficacy and low toxicity. Most anticancer drugs suffer from poor solubility or compromised stability in physiological fluids. Moreover, the mechanisms of action tend to be broad and non-specific to tumour cells, affecting the surrounding tissues. The design of suitable carriers intended to overcome these limitations is a key point in the development of novel anticancer therapies (Brigger et al. 2002).

Apart from the increasing solubility of poorly soluble or hydrophobic drugs, nanoemulsions can significantly improve anticancer drugs' therapeutic index. Current commercial taxane formulations include a high percentage of surfactants such as Cremophor® L for paclitaxel and Tween® 80 for docetaxel that are known to cause toxicity (Gelderblom et al. 2001; Engels et al. 2007). Docetaxel, re-formulated as a nanoemulsion manufactured by high-pressure homogenization and composed of egg lecithin, soybean oil, and co-surfactants, resulted in decreased toxicity in healthy mice when administered intravenously. The formulation was shown to be effective in a tumour model of glioma (Gaoe et al. 2012). Another example is provided by Zhang et al. (2011b), who reported the preparation of a lipophilic doxorubicin-oleic acid derivative which was subsequently emulsified in the presence of Lipoid® E80 (egg phospholipids), vitamin E, and soybean oil. This doxorubicin nanoemulsion was able to prevent drug accumulation in the heart, lung, and kidneys vs a doxorubicin solution, while still maintaining high blood levels after intravenous administration to healthy mice.

Another case where the drug therapeutic index was improved is that of melphalan, an anticancer molecule for the treatment of ovarian cancer, generally administered as a tablet or as an injection. After oral administration of the tablet the drug is subject to plasmatic metabolism resulting in: (1) its inactivation and (2) the reduction of bioavailability on repeating dosing; both problems have been addressed with a nanoemulsion formulation (Rajpoot et al. 2012). Capmul® MCM, Tween® 80, and Transcutol® P were used respectively as oil, surfactant, and co-surfactant. Oral administration in mice showed an almost fivefold increase in bioavailability compared to a suspension of the free drug.

Improved therapeutic index and drug pharmacokinetics can be achieved by increasing the drug circulation time through the incorporation of PEG (Beduneau et al. 2006; Allard et al. 2008; Bourseau-Guilmain et al. 2012; Laine et al. 2012).

Moreover, it has been suggested that certain PEGylated surfactants, as PEG-hydroxystearate, could have an inhibitory effect on P-glycoprotein (Huynh et al. 2009). An additional advantage of PEGylated nanoemulsions is that ligands for targeting concrete cell populations can be linked to the PEG moieties (Béduneau et al. 2007; Allard et al. 2008; Bourseau-Guilmain et al. 2012; Torrecilla et al. 2013). Ohguchi et al. have shown that the use of folate-PEG-linked nanoemulsions loaded with aclacinomycin inhibit the tumour growth of folate receptor-positive nasopharyngeal tumours after intravenous injection to mice (Ohguchi et al. 2008). Bourseau-Guilmain et al. reported the efficacy of functionalized PEG nanocapsules with a monoclonal antibody against AC133, a cancer stem cell marker (Bourseau-Guilmain et al. 2012). Furthermore, PEGylated chitosan nanocapsules conjugated to a monoclonal antibody anti-TMEFF-2 showed a significant decrease of the IC_{50} in A549 cells after 24 h of incubation, in comparison with free drug or non-conjugated nanocapsules (Torrecilla et al. 2013).

6.5.3.2 Infectious Diseases

Antimicrobial/Antiparasitic Therapies

Due to the intrinsic ability of O/W nanoemulsions to host drugs with poor aqueous solubility, many studies have focussed on finding new applications or extending the spectrum of application for drugs already known. For example, clortrimazole, a topical antimycotic (i.e. ear, skin), has been formulated in nanoemulsions composed of Capryol™ 90, Solutol® HS 15, and Gelucire® 44/14 as surfactants and tested for the treatment of malaria via oral administration. Borhade et al. (2012) demonstrated the ability of such formulations to inhibit the growth of *Plasmodium Berghei* in mice, showing higher suppression compared to the drug formulated as a suspension. Further, nanoemulsions protected the drug against degradation and remained stable over 6 months, which could turn out to be an economically attractive strategy for the development of anti-malaria therapeutics.

Nanocapsules have been proposed to overcome bacterial resistance to conventional treatments. Biofilms are an organised community of bacterial cells embedded within a hydrated matrix. In this ecosystem, microbial properties are altered and have an increased resistance to antibiotics. Nanocarriers have the potential to diffuse into the mucus environment surrounding the biofilm, deliver the active drug locally, and improve its residence time and effectiveness (Iannitelli et al. 2011; Peulen and Wilkinson 2011). Iannitelli et al. described the use of PLGA nanocapsules with carvacrol core (carvacrol acts as the oily core and is known to inhibit the growth of several bacteria strains) for the treatment of microbial biofilm and showed an improvement in the penetration of carvacrol deep into the core of the biofilm. An additional advantage is that other antimicrobial agents could be encapsulated in the oily carvacrol reservoir to increase the potency of this formulation.

Lastly, an interesting O/W nanoemulsion formulation has been commercialised under the name NanoProtect™. This preparation, made of GRAS materials, has

been tested and evaluated to act as decontamination agent of facilities and equipment contaminated with anthrax. Although not for use in humans, this application highlights the potential of O/W nanoemulsions as a preventive measure to limit transmission of anthrax (Bielinska et al. 2007).

Nanovaccines

Despite of the well-known efficacy of classical vaccines, attenuated, or killed pathogens, subunit vaccines have gained much attention over the last years due to their higher safety and purity. The main disadvantage of these subunit vaccines is their dependency on the use of adjuvants to produce an effective immune response (Correia-Pinto et al. 2013). Regarding this, drug delivery systems could improve the recognition of antigens by antigen-presenting cells, producing an effective immune response (Wadhwa et al. 2012).

The efficacy of W/O coarse emulsions (>1 μm) as adjuvants for vaccines is well-known. Freund's (Freund et al. 1937) and Montanide ISA (Aucouturier et al. 2002) adjuvants are both commonly used emulsion-based veterinary adjuvants. The high efficacy of W/O emulsions stem from their ability to control the release of the antigen and to enhance the immune reaction (Herbert 1968; Schijns 2000; Jansen et al. 2005). Consequently, nanoemulsions could be expected to be beneficial for the same purposes. To date, most of the examples on the use of nanoemulsions as adjuvants in vaccination relate to O/W nanoemulsions, which have served to improve the mucosal response to vaccines administered to the nasal mucosa (Myc et al. 2003; Bielinska et al. 2007; Hamouda et al. 2011; Stanberry et al. 2012).

O/W nanoemulsions have been formulated with the recombinant HIV viral protein gp120 and induced both systemic and mucosal antibody responses after intranasal administration to mice and guinea pigs (Bielinska et al. 2008). Makidon et al. have shown that intranasal needle-free immunisation against Hepatitis B can be achieved with an O/W nanoemulsion of cetyl pyridinium chloride, Tween® 80, and soybean oil as a particulate adjuvant for the recombinant Hepatitis B surface antigen (HBsAg) (Makidon et al. 2008). A nanoemulsion with similar composition formulated with outer membrane proteins of *B. multivorans* developed by NanoBio® Corporation was later shown to achieve in vivo immunisation and protection against pulmonary infection in mice after intranasal administration (Makidon et al. 2010). These formulations are currently being brought through Phase I clinical studies (Johnson et al. 2010; Makidon et al. 2010). Chitosan nanocapsules have similarly been assayed for intranasal needle-free immunisation against Hepatitis B (Vicente et al. 2009, 2010). HBsAg was adsorbed onto the chitosan shell, while immunostimulants were used in the core composition. Results have shown an enhanced effect in long-term immunisation in vivo, after both intramuscular and intranasal administration to mice (Vicente et al. 2010).

Still, the potential of W/O nanoemulsions has not gone unrecognised. Huang et al. have described an elegant multiple nanoemulsion, which draws on the

advantages of W/O nanoemulsions while countering syringeability problems by further dispersing the initial emulsion into an aqueous outer phase (Huang et al. 2009a, b, c, 2010). Their formulation, referred to as a PELC nanoemulsion, combines a polymeric surfactant (poly(ethylene oxide)-block-poly(lactide-co-ε-caprolactone) with a low-molecular weight sorbitan trioleate (Span® 85). The oil phase is composed of the GRAS-listed oil squalene. PELC nanoemulsions systematically produced stronger immune responses compared to vaccine administered without adjuvant, generating IgG titers 1.5-fold to sevenfold higher that were observable up until 26 weeks after intramuscular immunisation (Huang et al. 2009c, 2010); encapsulating the antigen in the internal water phase further improved the response to the antigen compared to formulations where it was contained in the external phase (Huang et al. 2010). However, the immunity bestowed by PELC vaccines was not significantly different from influenza vaccine administered with aluminium-salt adjuvants, an adjuvant commonly found in human vaccines (Gupta 1998).

6.5.4 Emerging Therapeutic Approaches

6.5.4.1 Gene Therapy

Gene therapy has been defined as an interesting therapeutic approach and is based on the administration of genetic material to alter protein expression. Vaccination, cancer therapy, and tissue regeneration are some of the fields in which gene therapy has been used as effective therapeutic tool (Schatzlein 2001; Rolland 2005).

Cationic nanoemulsions have successfully been used for the complexation and delivery of nucleic acids, for example to the ocular surface, (Hagigit et al. 2012). Cationic nanocapsules have also been proposed as gene carriers. In this regard, poly(arginine) (PARG) or PEGylated PARG were selected as cationic polymers to constitute the shell and allow the simple adsorption of nucleic acids (Lozano et al. 2013). Saulnier and co-workers have recently proposed a different approach for efficient association of nucleic acids into the oil core, which involves the encapsulation of preformed lipoplexes into PEGylated nanocapsules prepared by PIT (Huynh et al. 2009). The efficacy of this formulation has been demonstrated in NMRI nude mice (David et al. 2012). For that purpose, a gene encoding for an enzyme able to transform the pro-drug ganciclovir into a cytotoxic metabolite was selected as a new experimental anticancer treatment. The successful expression of the gene in the tumoural tissue (orthotopic SK-Mel28 luc and HTB-72 melanoma models in mice) after intravenous administration of DNA-loaded nanocapsules was evidenced by a significant reduction in the growth of the tumoural tissue after 4 days of treatment with the pro-drug Ganciclovir. Moreover, the specific targeting of these nanocapsules could also be achieved by modifying the PEG chains with galactose for hepatocyte targeting (Morille et al. 2009).

6.5.4.2 Tissue Engineering

Over the last decades, tissue engineering has emerged as a promising field in biomedical research. The goal is to improve cell reassembling into structures similar to the original tissue. A defined biomimetic environment is critical for cell differentiation and proliferation leading to functional tissues (Griffith and Naughton 2002; Ikada 2006). Growth factors play an essential function in this process as they interact with selected receptors. However, it is necessary to control their delivery and to protect them from proteolytic degradation (Malafaya et al. 2007; Chen et al. 2010).

Despite increasing interest in drug delivery systems for tissue engineering, the use of nanoemulsions in this field is still at an early stage. Encapsulation of growth factors allows the protection and controlled release critical to achieve an improved effect on both cell proliferation and differentiation (Shi et al. 2010; Dvir et al. 2011). PLGA and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules have been tested for the sequential delivery of bone morphogenic proteins (i.e. BMP-2 and BMP-7) to mesenchymal stem cells and led to a synergistic effect on osteogenic differentiation, due to the adjusted release of both components (Yilgor et al. 2010).

6.6 Imaging and Diagnosis

Nanoemulsions offer an interesting alternative to the use of radioisotopes for imaging purposes. For example, CS-1000 is a commercially available agent (Celsense Inc., USA) specifically formulated as an aqueous colloidal nanoemulsion of perfluorocarbon polymers. Perfluorocarbons are both hydrophobic and lipophobic and do not associate with cell membranes. They also provide a long-lasting intracellular labelling, as they are not subjected to enzymatic degradation. CS-1000 is undergoing studies as a novel Fluorine-19 delivery agent for detection and quantification of human dendritic cells using Magnetic Resonance Imaging (MRI) (Shan 2004; Bonetto et al. 2011).

Nanoemulsions can also act as platforms with the dual capacity to incorporate substances for drug therapy and detection (i.e. theranostics), in order to enable imaging-guided therapy, as illustrated in Fig. 6.8. This theranostic nanoemulsion is composed of soybean oil, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] ammonium salt (PEG-DSPE). The nanoemulsions incorporate the hydrophobic glucocorticoid prednisolone acetate valerate (PAV), as anticancer agent, iron oxide nanocrystals, for MRI imaging, and the fluorescent dye Cy7, for Near Infrared Fluorescence (NIRF) imaging. This nanotheranostic platform tested in a colon cancer murine model showed accumulation in the tumours, with both MRI and NIRF imaging, while the tumour growth was consistently inhibited due to the action of the encapsulated drug (in comparison with the free PAV, saline, or a drug-free control nanoemulsion) (Gianella et al. 2011).

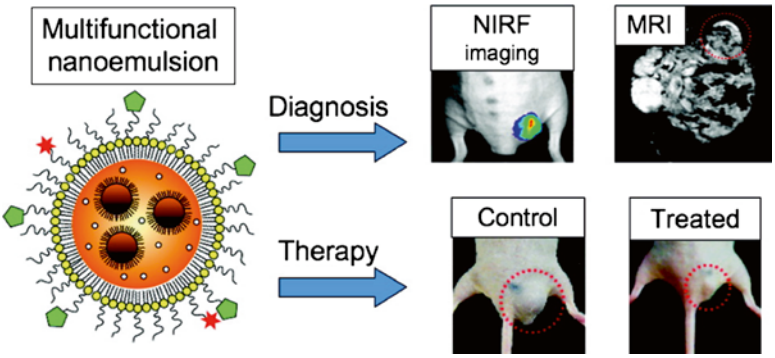

Fig. 6.8 Theranostic nanoemulsion with dual capacity to act as delivery system for (1) anticancer drugs, and (2) contrast agents and fluorescent dyes for MRI and NIRF imaging. Reproduced from Gianella et al. (2011) with permission

6.7 Conclusions

The knowledge accumulated thus far suggests nanoemulsions to be a promising strategy to confront current biomedical needs. Nanoemulsions can be made to contain well-defined, biocompatible, and biodegradable materials, all established and with a good safety record, and can be prepared by mild and scalable technologies. Additionally, nanoemulsion formulations can be tailored to the biopharmaceutical requirements and modality of administration. Importantly, nanoemulsions can be formulated in a case-by-case basis to encapsulate/associate a wide range of molecules, from poor-soluble drugs to highly hydrophilic complex macromolecules. Considering that the quantity of therapeutic molecules emerging from preclinical programmes has grown substantially over the last decade, advances in nanoemulsion development are expected to lead to a burst in the production of new therapeutic/diagnostic entities for biomedical applications.

Problem Box

Question 6.1

Which is the main mechanism related to nanoemulsions instability and how can it be prevented?

Answer 6.1

The Ostwald ripening effect, defined as the growth of the largest droplets at the expense of the smallest ones, is the main cause of instability of nanoemulsions. Controlling the physical properties of the oil phase and the nature of the surfactant can prevent destabilisation of O/W nanoemulsions, as well as coating

(continued)

Problem Box (continued)

the nanoemulsions with a polymeric shell. For W/O nanoemulsions, Ostwald ripening could be slowed down by increasing the ionic strength of the aqueous phase, therefore limiting the solubility of the droplets in the external phase.

Question 6.2

How nanoencapsulation can increase oral bioavailability of drugs/biomolecules? Name some mechaisms.

Answer 6.2

Nanoemulsions can promote drug absorption by (1) improving the solubility of hydrophobic drugs, (2) protecting labile molecules from degradation in the harsh environment of the gastrointestinal track, (3) increasing the interaction with the intestinal mucosa and the retention time, (4) preventing the interaction of the encapsulated drug with efflux transporters such as the P-glycoprotein (P-gp).

Glossary

Capmul® MCM-C8 Glyceryl monocaprylate
Capryol™ 90 Propylene glycol monocaprylate (type II)
Cremophor® WO7 (BASF Corp) Pegylated hydrogenated castor oil manufactured by reacting 1 mol of the oil with 7 mol of ethylene oxide
Cremophor® EL (BASF Corp) Pegylated hydrogenated castor oil manufactured by reacting 1 mol of the oil with 35 mol of ethylene oxide. Now known as Kolliphor EL®
Gelucire® Glycerides and esters of polyethylene glycol
Miglyol® 812 Caprylic/capric triglyceride
NIRF imaging Near-Infrared Fluorescent Imaging
Solutol® HS 15 2-Hydroxyethyl 12-hydroxyoctadecanoate. Now known as Kolliphor® HS 15
Span™ 20 Sorbitan laurate
Span™ 85 Sorbitan trioleate
Sylibin 2-[2R,3R-dihydro-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxin-6-yl]-2R,3R-dihydro-3,5,7-trihydroxy-4H-1-benzopyran-4-one
Sylimarin A mixture of flavolignans rich in Silybin with hepatoprotectant properties
Tween® 20 Polyoxyethylene(20) sorbitan monolaurate
Tween® 80 Polyoxyethylene(20) sorbitan monooleate

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Chapter 7
Polymer-Drug Conjugates

Cristina Fante and Francesca Greco

Abstract Polymer-drug conjugates are nanosized drug delivery systems, which comprise several drug molecules covalently attached to a polymeric carrier. This chapter provides an overview of this technology in the context of drug delivery. Particular emphasis is given to different approaches and techniques used to synthesise and characterise polymer-drug conjugates. In the final part of the chapter current applications of this technology are also discussed.

7.1 Materials Chemistry

7.1.1 Definition of Polymer-Drug Conjugates and General Background to This Technology

Polymer-drug conjugates are a drug delivery technology where a polymer carrier is used to improve the performance of a drug (e.g. improve drug selectivity towards the target site). Unlike other systems, such as liposomes or nanoparticles where the drug is physically entrapped within the carrier, in a polymer-drug conjugate the drug is *covalently* attached to the polymer via a biodegradable linker (Fig. 7.1).

The concept of conjugation of a drug to a polymeric carrier was first introduced in the 70s by Helmut Ringsdorf as a strategy to enhance the selectivity, cellular uptake and solubility of a drug (Ringsdorf 1975). Since then, extensive work has been carried out which has resulted in polymer-drug conjugates reaching clinical evaluation (Vasey et al. 1999; Seymour et al. 2009, also reviewed in Duncan 2006; Canal et al. 2011).

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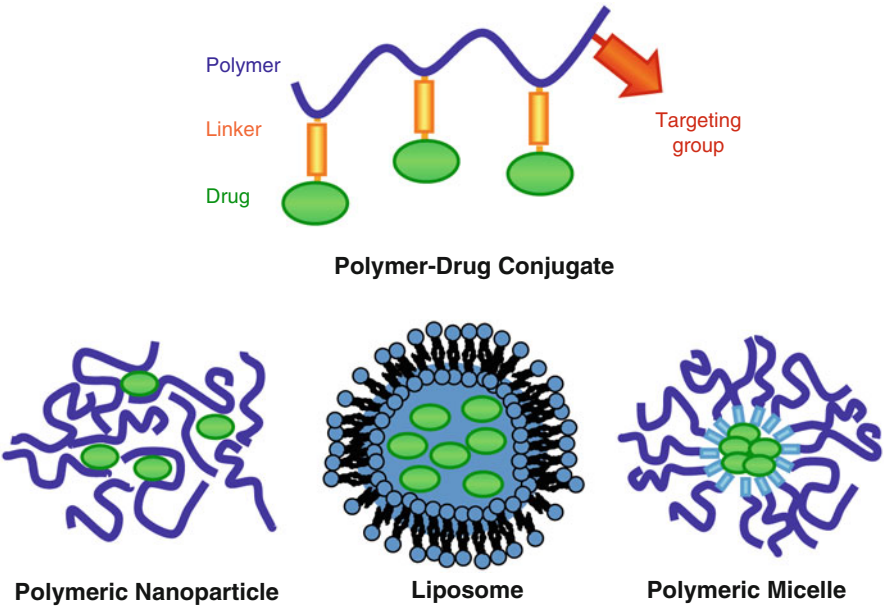


Fig. 7.1 Schematic representation of a polymer-drug conjugate and other drug delivery technologies. In polymer-drug conjugates the drug is *covalently* attached to the polymer via a biodegradable linker

The rationale for conjugating a drug to a polymer stems from the fact that the biological behaviour of the drug can be significantly altered by increasing its molecular weight (MW). Covalent conjugation to a polymer results in:

- *Prolonged circulation time of the drug*. The polymer can protect the conjugated drug from premature inactivation during its delivery to the site of action; in addition, the macromolecular size of the conjugate prevents the early elimination of the drug through renal filtration.
- *Restricted body distribution*. An intravenously administered drug is generally able to diffuse throughout the body, with no selectivity towards the target tissue. On the other hand, the macromolecular size of a conjugate prevents extravasation of the drugs in areas where the vascular endothelium is continuous. In fact, conjugation to a polymer restricts drug access to those tissues where the vasculature presents fenestrations and gaps of appropriate size (>20 nm). The tumour tissue, for instance, is characterised by a defective vasculature which is permeable to the conjugate. This and other features (discussed in Sect. 7.4.1) make the tumour tissue an ideal target for polymer-drug conjugates.
- *Selective drug release*. Polymer conjugation can also alter the cellular pharmacokinetics of the drug. Because of its size, the conjugate is taken up by cells exclusively by endocytosis, firstly into endosomes, then into lysosomes. The lysosomal compartment has two peculiar characteristics: an acidic pH (4–5) and a high concentration of proteolytic enzymes (e.g. cathepsin B). This unique environment

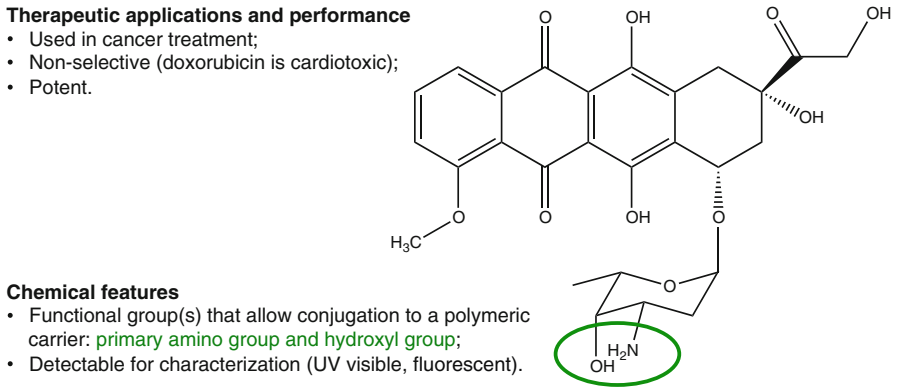


Fig. 7.2 Chemical structure of doxorubicin. Key characteristics that make this drug a suitable candidate for use in the context of polymer-drug conjugates are annotated

can be turned into a useful trigger for drug release from the conjugate. Indeed, polymer-drug conjugates have been designed with biodegradable linkers either sensitive to acidic pH or selectively degraded by proteolytic enzymes. This allows the drug to be released exclusively intracellularly and to effectively reach its biological target (generally the nucleus and the DNA in case of anticancer drugs).

The biological behaviour of a polymer-drug conjugate is strongly affected by each of its constituents and by the overall physico-chemical properties of the system (e.g. water solubility). The next section will look at each component of a polymer-drug conjugate.

7.1.2 Composition of a Polymer-Drug Conjugate.

As previously described, a polymer-drug conjugate is constituted by three (to four) components: a drug, a polymeric carrier, a linker and, optionally, a targeting group. In this section we will look at each component individually.

7.1.2.1 Drug

The vast majority of the polymer-drug conjugates that have been proposed to date have been designed for application in cancer therapy (see Sect. 7.4). Therefore, most of the drugs used in polymer-drug conjugates are anticancer agents; examples include: doxorubicin, paclitaxel and camptothecin.

With the help of an example (doxorubicin) we are now going to look at what characteristics make a drug suitable for conjugation to a polymeric system (also summarised in Fig. 7.2).

Physico-chemical characteristics
- Solubility in water;
- High molecular weight;

Biological behaviour
- Biodegradability: breaks down into smaller fragments in the body;
- Non toxic;
- Non immunogenic.

Chemical structure
Conjugation sites: one carboxyl group per monomer (high loading capacity).

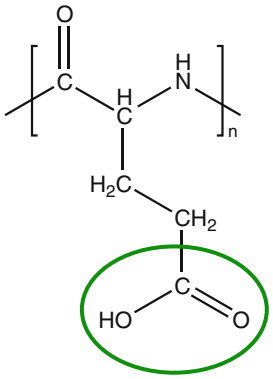


Fig. 7.3 Chemical structure of PGA. Key characteristics that make it a good polymer within the context of polymer-drug conjugates are highlighted

Doxorubicin is a potent anticancer agent, used for the treatment of metastatic breast cancer (Paridaens et al. 2000). The main issue with the therapeutic use of doxorubicin is its cardiotoxicity, which is a result of the lack of selectivity of this drug (Jensen 2006). Such lack of selectivity makes it an ideal candidate for polymer conjugation, as the polymer will promote drug accumulation in the tumour tissue. The chemical structure of doxorubicin also makes it a suitable candidate for conjugation. In particular, this molecule contains a primary amino group and a hydroxyl group. Both these functional groups can be exploited for linking the drug onto a polymer (e.g. via formation of an amide bond or an ester bond for the amino group or hydroxyl group, respectively). In addition, doxorubicin absorbs UV–vis light and is inherently fluorescent. These are two favourable properties as the presence of the drug can be detected using appropriate instruments (see Sect. 7.3).

7.1.2.2 Polymer

Many polymeric carriers have been suggested for use within the context of polymer-drug conjugates. In particular, four of them have been tested clinically: *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers, polyethylene glycol (PEG), polyglutamic acid (PGA) and oxidised dextran (Duncan 2006).

With the help of an example (PGA) we are now going to look at what characteristics make a polymer suitable for use in a polymer-drug conjugate system (also summarised in Fig. 7.3).

PGA is a polymer constituted by units of glutamic acid linked together via amide bonds. PGA is water-soluble and this is an important characteristic for intravenous drug delivery as biological fluids, such as the blood, are essentially water-based systems.

PGA is biodegradable, which means that in biological systems it is degraded to smaller fragments. This is an advantage for a polymeric carrier as it ensures that the

carrier is eliminated from the body after drug release. Non-biodegradable polymers, such as PEG, can also be used, but their size has to be such that allows excretion via the kidneys (typically lower than 40,000 Da).

Each monomer of PGA has a pendant side chain, which terminates with a carboxyl group. These carboxyl groups can be exploited for conjugation to a drug, for instance, to an amino group to produce an amide bond or to a hydroxyl group to produce an ester bond. All polymers suggested for use as a carrier need to have a functional group that allows conjugation to a drug. If a polymer has multiple conjugation sites (such as PGA), each polymer chain will be able to carry several drug molecules. In the case of PGA, each polymeric chain could carry one drug molecule per monomer (i.e. 200 drug molecules per chain, for 30,000 Da PGA). This property of a polymeric carrier (i.e. the ability to carry drug molecules) is called 'loading capacity'. In general, a higher loading capacity is to be preferred, as less of the carrier needs to be used in order to administer a therapeutic dose of the drug.

Finally, PGA is non-toxic and non-immunogenic (i.e. does not stimulate an immune response). These are key properties, as toxicity from a drug delivery carrier would not be considered acceptable.

7.1.2.3 Linker

In a polymer-drug conjugate the linker is the part that connects the drug to the polymeric carrier. Many types of linkers have been suggested, which includes peptidyl linkers and pH-labile linkers (Duncan 2006).

With the help of an example (the peptidyl linker Gly-Phe-Leu-Gly) we are now going to look at what characteristics make a linker suitable for use in a polymer-drug conjugate system (also summarised in Fig. 7.4).

Early studies on a series of HPMA copolymer-doxorubicin conjugates compared different peptidyl linkers, including Gly-Phe-Leu-Gly. The conjugate containing this linker was then progressed for evaluation into clinical trials (Vasey et al. 1999; Seymour et al. 2009). Stability tests carried out in plasma showed that the linker was stable (i.e. did not release the drug) in these conditions. This was a positive finding, as a good linker needs to be stable in the blood to avoid premature drug release. Other studies carried out on a mixture of lysosomal enzymes (mimicking the environment found by the conjugate in the lysosomes) showed drug release in these experimental settings. Biodegradability at the target site is also an important characteristic as drug release is necessary to drug activity.

7.1.2.4 Targeting Group

The targeting group is the fourth (but optional) structural component of a polymer-drug conjugate. Its role is to actively direct the conjugate towards the desired tissue. With the help of an example (galactosamine) we are now going to look at what characteristics make a targeting group suitable for use in a polymer-drug conjugate (also summarised in Fig. 7.5).

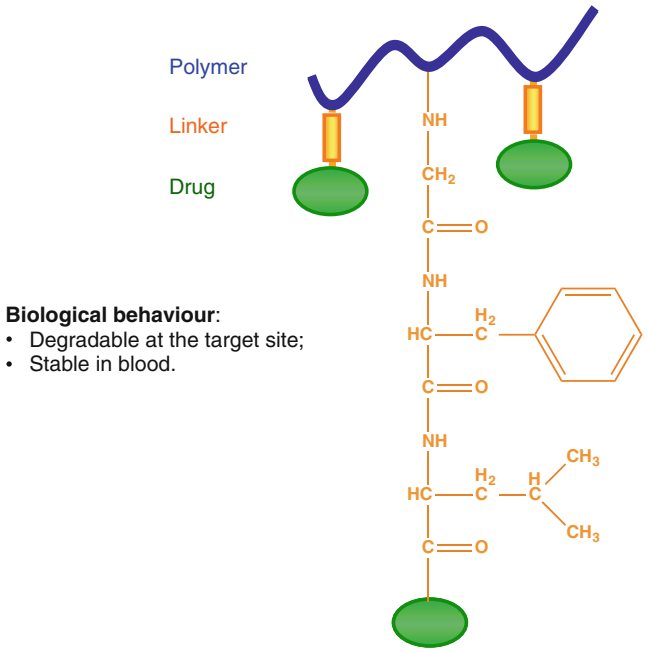


Fig. 7.4 Chemical structure of the peptidyl linker Gly-Phe-Leu-Gly. Key characteristics that make this linker a suitable candidate for use in the context of polymer-drug conjugates are highlighted

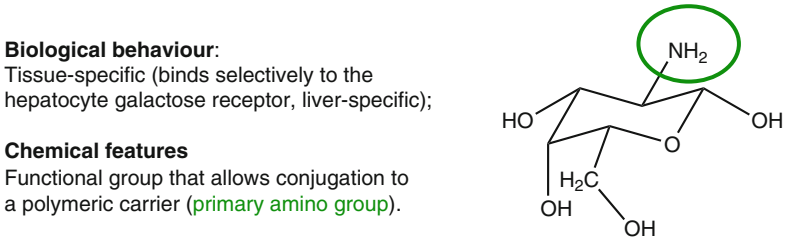


Fig. 7.5 Chemical structure of galactosamine. Key characteristics that make it a good targeting group within the context of polymer-drug conjugates are highlighted

Galactosamine is an amino sugar able to bind selectively to the hepatocyte galactose receptor, a liver-specific receptor (Ashwell and Harford 1982). The ability to bind to tissue-specific markers (which are generally proteins associated to a certain tissue and not expressed elsewhere in the body) is an essential requirement for a targeting group. In addition, galactosamine presents a primary amino group, which can be used for linking this molecule to the polymer (as for the conjugated drug). These features make galactosamine a suitable targeting group to be exploited in the context of drug delivery. Galactosamine was covalently bound to an HPMA copolymer-doxorubicin conjugate designed for the treatment of liver cancer (Seymour et al. 1991;

Pimm et al. 1993; Julyan et al. 1999). This conjugate was investigated clinically (Phase I and Phase II) and clinical imaging confirmed preferential accumulation in the liver (Seymour et al. 2002).

In this chapter we have already discussed the ability of a conjugate to target the tumour tissue with a passive mechanism based on its physico-chemical properties (i.e. its molecular weight, see Sect. 7.1.1). For this reason, the targeting group is an optional component in a polymer-drug conjugate designed for anticancer therapy.

7.2 Polymer-Drug Conjugates Synthesis

7.2.1 Synthetic Strategies

There are two main synthetic approaches that can be used to produce a polymer-drug conjugate: polymer-analogous reaction and copolymerisation of appropriate monomers (Fig. 7.6).

- *Polymer-analogous reaction*. This synthetic strategy starts with a polymeric precursor in which the sites of conjugation are chemically modified to increase their reactivity (e.g. a carboxylic acid converted into an ester containing an appropriate leaving group) and to produce a reaction with the drug (Fig. 7.6a).
- *Copolymerization of appropriate monomers*. This approach involves two steps: (1) coupling of the drug to the monomer; (2) polymerization of such drug-monomer derivatives with the monomers, to yield the conjugate (Fig. 7.6b).

The copolymerisation strategy presents the advantage that, at least theoretically, drug loading can be increased or decreased by adjusting the ratio between the monomers containing the drug and the other monomers. However, the polymer-analogous reaction is by far the most commonly used approach, as it normally requires milder reaction conditions compared to those used in polymerisation reactions. Hence, with this strategy there is generally less of a chance to degrade the drug during the conjugation process.

Conjugation of a drug to a polymeric carrier can also be defined in relation to the position of the conjugation site within the polymer chain. This is largely driven by the chemical structure of the polymeric carrier used. Two types of conjugation can be identified: terminal conjugation and pendant chain conjugation (Fig. 7.7).

- *Terminal conjugation*: this is typical of polymers with functional groups present only at the end termini (e.g. unmodified PEG). For unmodified linear polymers, this type of conjugation results in a conjugate with a low carrying capacity, as a maximum of two drug molecules can be carried by each polymeric chain. More recently, however, polymers with regular branching at the termini have been developed (dendronised systems) to accommodate additional drug molecules in each polymer chain (see Fig. 7.7a).

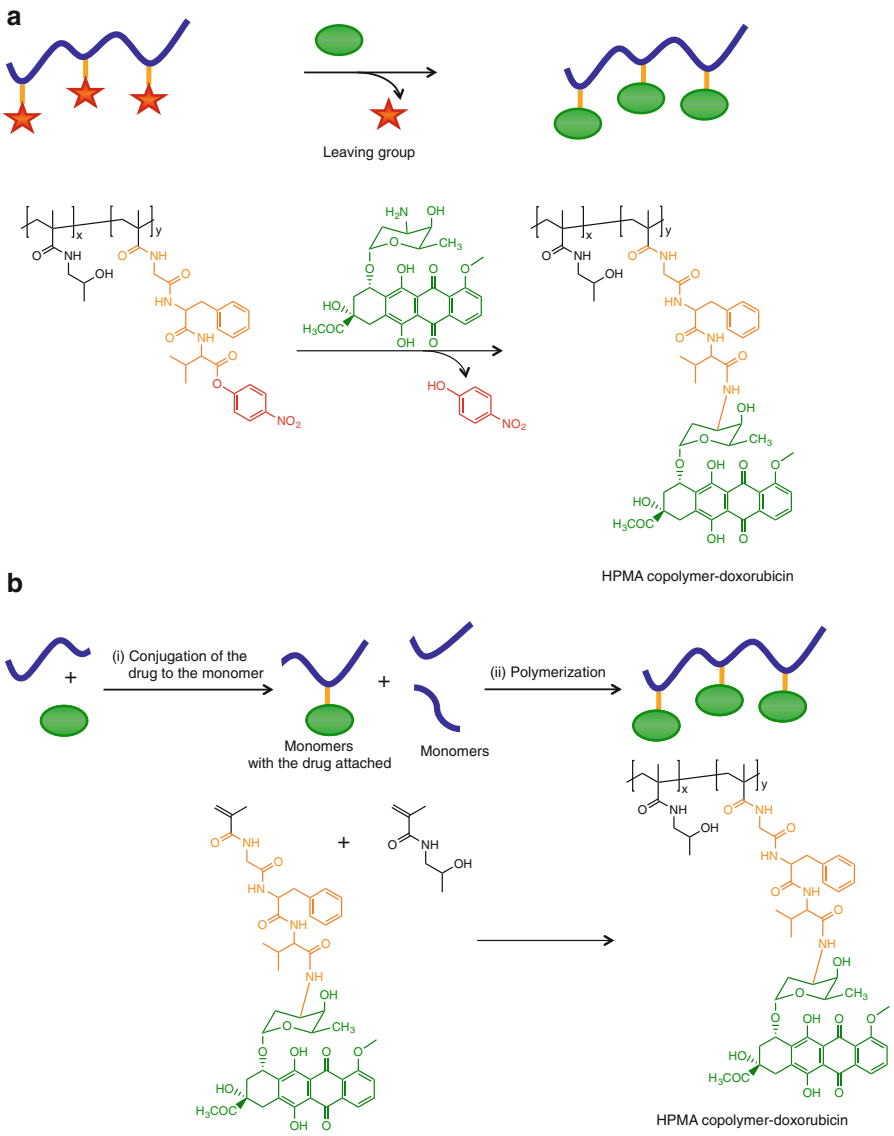


Fig. 7.6 General approaches for polymer-drug conjugation: (**a**) polymer-analogous reaction; (**b**) conjugation of the drug to a monomer and copolymerization of the polymeric precursors

- *Pendant chain conjugation*: this refers to the conjugation to polymers with suitable functional groups present along the chain (see Fig. 7.7b). This is the case with polymers such as the HPMA copolymer, where side chains are attached to the main polymeric backbone, or PGA that naturally contains a side carboxyl group per monomer and allows a theoretical maximum loading of one drug molecule

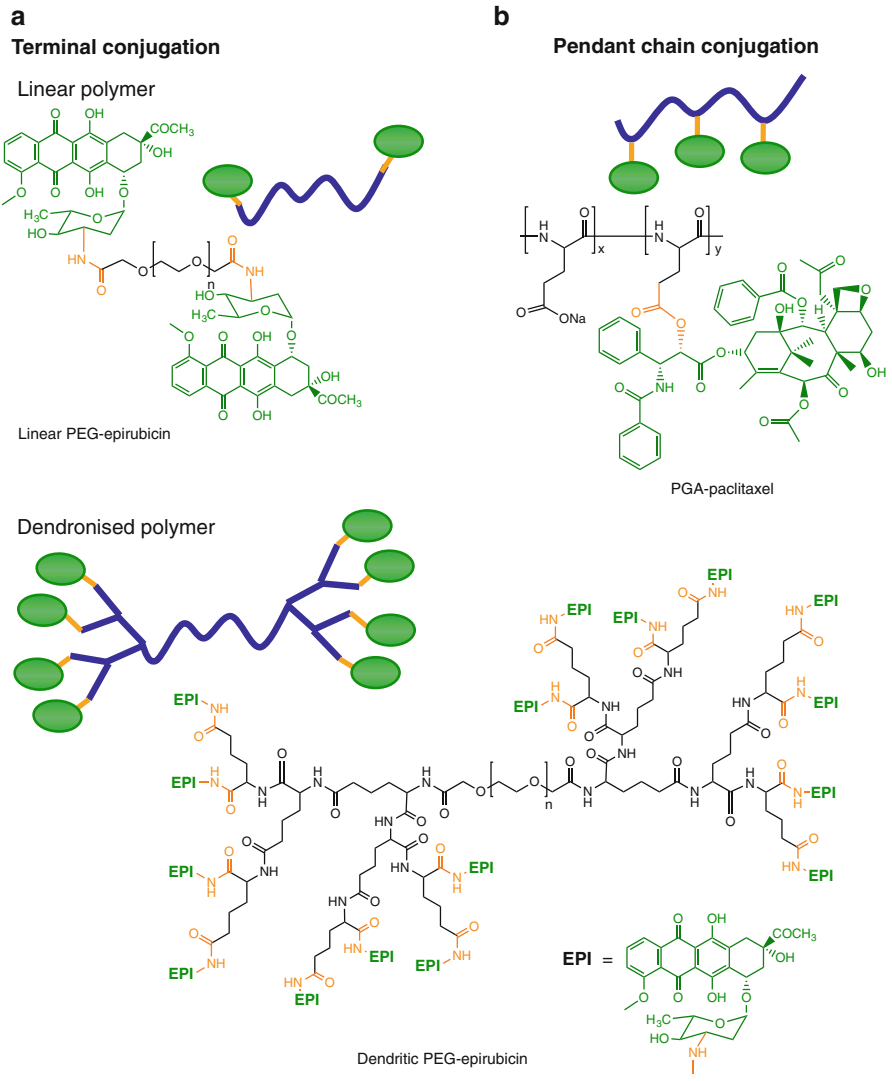


Fig. 7.7 Types of conjugation according to the position of the conjugation site within the polymer chain. The drug can be attached to the polymer through (**a**) its terminal groups; (**b**) its side chains

per monomer. Pendant chain conjugation has the advantage that the loading capacity is typically higher than that achievable with terminal conjugation.

With regard to the actual conjugation reaction, the choice is primarily driven by the functional groups present on the drug and on the polymeric carrier, but standard synthetic reactions can be employed. For instance, an amino group present in the drug can be linked to a carboxylic acid group on the polymer after appropriate

activation of the carboxylic acid group via standard coupling reactions (e.g. *N*,*N*′-*dicyclohexylcarbodiimide* (DCC) or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) coupling).

7.2.2 Purification

Once the conjugation reaction has been carried out, the conjugate needs to be purified from reaction by-products and from residual reagents, as these might present unwanted biological activity (e.g. they might be toxic). Standard purification techniques routinely used for purifying small molecules can be applied to the purification of the conjugates (e.g. precipitation, filtration). In addition, as the conjugate and the reagents differ significantly in size, size-exclusion chromatography is typically used in this context.

One type of impurity that needs particular attention is residual, unreacted free drug, as it will have a different biological activity and a different pharmacokinetic profile compared to the conjugated drug. This aspect is further discussed in Sect. 7.3.1.

7.3 Polymer-Drug Conjugates Characterisation

After preparation, polymer-drug conjugates must be carefully characterised. Thorough characterisation prior to any testing is essential to correctly interpret biological behaviours observed for the conjugate. In addition, as conjugates progress into clinical trials, it is important to guarantee the reproducibility of their preparation and the overall quality of the final products. Because the drug is covalently attached to the polymeric carrier, polymer-drug conjugates cannot be treated simply as a new formulation of the drug (even if they carry a well-known and clinically established drug). In fact, these technologies are considered by the regulatory authorities as ‘new chemical entities’ (i.e. as completely new drugs) and, as such, they need to undergo extensive testing to ensure their safety and efficacy. Both European (i.e. EMA) and American (i.e. FDA) regulatory authorities have set the standards for the quality of medicines in order to guarantee their efficacy and safety.

7.3.1 Characterisation Parameters

Proof of conjugation. The initial characterisation of a polymer-drug conjugate includes finding evidence that a covalent bond between the drug and the polymer

has been formed (i.e. that the drug is attached to the polymer and not physically 'entrapped' within the polymer). Several techniques have been used to achieve this purpose and, in many cases, different techniques have to be used in conjunction of one with the other (see Sect. 7.3.2).

Total drug content. When the formation of the conjugate is confirmed, it is essential to understand *how much* drug is bound to the polymer. The total drug content can be expressed in two different ways: (i) as the percentage in weight of the whole conjugate; (ii) as the percentage of functional groups contained in the polymer that have been conjugated to the drug. (i) and (ii) are strictly related but convey different information. In particular, (i) gives an immediate measure of total drug content. For example, a PGA-paclitaxel conjugate was synthesised that contained approximately 37 % w/w of paclitaxel (e.g. 100 mg of conjugate contain 37 mg of paclitaxel) (Li et al. 1998). Conversely, (ii) is often used to indicate how efficient a conjugation reaction was (e.g. 80 % of the functional groups available on a polymer reacted with the drug or, in other words, the drug loading was 80 % of the maximum theoretical loading). Different techniques can be used to assess the drug content of a conjugate, as described in more detail in Sect. 7.3.2.

Impurities and residual-free drug content. We have already discussed the importance of an accurate purification of the conjugate from excess reagents and reaction by-products (Sect. 7.2.2). One type of impurity that needs particular attention is residual, unreacted free drug. As the free drug will have biological activity and a different pharmacokinetic profile compared to the conjugated drug, removing it is particularly key. To this end, size-exclusion chromatography is the technique most frequently used, as the free and conjugated drug differ significantly in size. The content of residual-free drug is typically expressed as a percentage of the total drug content and, in general, the purification of the conjugate should aim to achieve levels below 1 %.

Size. Determining the size of the conjugate is very important, as this is arguably the key parameter that drives the distribution of the conjugate in the body and determines how quickly the conjugate (or the unloaded polymer) will be excreted from the body. However, it should be highlighted that most polymeric systems are polydisperse (i.e. polymers are constituted by a range of polymeric chains varying in length, depending on the number of monomers per chain). This means that most polymer-drug conjugates are polydispersed systems too. Therefore, the molecular weight stated is an average of the molecular weight of the various chains and should always be accompanied by the polydispersity value for that system.

7.3.2 Techniques Employed to Characterise Conjugates

To achieve the information described above, a variety of analytical techniques can be used.

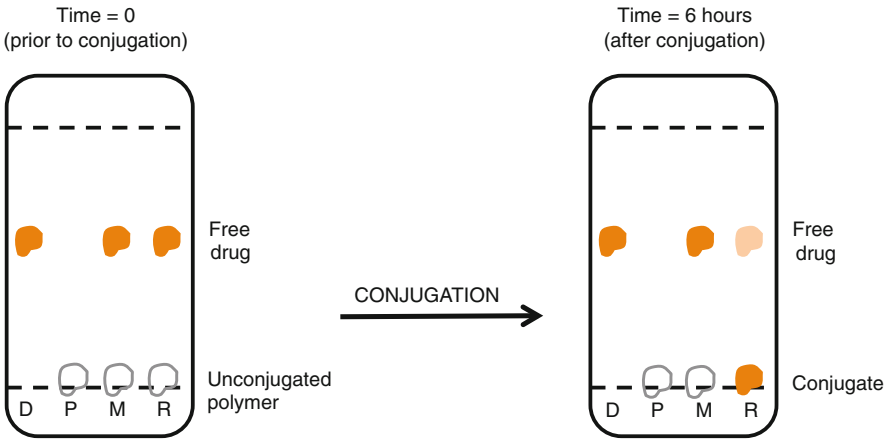


Fig. 7.8 Schematic representation of how to monitor a conjugation reaction by TLC. In this example, the TLC plate is placed under an UV lamp. The drug (in D) can be detected by UV, while the polymer (in P) does not absorb UV light, therefore is not visible; lane (R) contains the reaction mixture (the change in colour of the spot on the baseline refers to the drug being conjugated to the polymer; as a consequence the spot of the free drug tends to disappear); lane (M) contains the mixture of the polymer and the drug in absence of activators of the reaction

7.3.2.1 Thin Layer Chromatography (TLC)

TLC is a very simple technique that is routinely used during conjugation reactions to obtain an indication of how the reaction is progressing. A sample from the reaction mixture is spotted onto a TLC plate and run with an appropriate mobile phase. Typically, the TLC plate is then viewed under a UV lamp (if, the drug absorbs at UV). Alternatively, specific stains are used to detect functional groups present in the drug and make the TLC spot visible (e.g. ninhydrin stain, which turns the spot pink in presence of compounds containing primary amino groups). The mobile phase can be modified to ensure that the free drug and the conjugated drug have different retention times (Fig. 7.8). As the reaction progresses, the drug starts to get detected at the retention factor of the polymer (see Fig. 7.8).

7.3.2.2 UV–vis Spectroscopy

Many drugs have a chemical structure that absorbs light in the UV–vis region of the electromagnetic spectrum. As such, these drugs can be detected and quantified in solution by a UV–vis spectrophotometer. On the other hand, the most common polymers used in conjugation (PEG, PGA, HPMA copolymers) do not absorb UV–vis light. These represent very useful features when characterising a polymer-drug conjugate, as the UV–vis spectrum of the polymer is expected to change

dramatically after conjugation. However, for a correct interpretation of the conjugate's spectrum, it is important to remember the following considerations:

- UV–vis is a useful technique to identify and quantify the *total* drug content of the conjugate, but it cannot normally discriminate between *bound* and *free* drug as, typically, both absorb at the same wavelength; therefore UV–vis spectroscopy alone is not a valid proof of conjugation and it becomes meaningful only when supported by other techniques.
- Polymer conjugation often alters the extinction coefficient of a drug (i.e. the free drug and the conjugated drug might have different extinction coefficients; Vicent et al. 2005), which can lead to errors. In particular, if the drug content in a conjugate was estimated using a calibration curve of the free drug, this could result in an over- or under-estimation of the total drug content.

7.3.2.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is a technique commonly used to prove that conjugation has occurred. The presence of signals belonging to the drug in the spectrum of the conjugate is not an indication of conjugation per se, as they might belong to the unbound drug. However, a shift in the resonance of the protons and carbons adjacent to the conjugation site is an indication that their electronic environment has changed and this might be due to conjugation. In addition, the sharp peaks typical of the spectrum of small molecules become broad after conjugation to macromolecules, as a consequence of the slower molecular tumbling of the polymeric chains. A shift in resonance combined with the broadening of the peaks represents an indication of successful conjugation of the drug.

In some cases the overlapping of crucial peaks does not allow a complete interpretation of the spectrum. In these cases, 2D NMR spectroscopic analyses represent a valid and powerful alternative to the usual spectrum. For instance, the nuclear overhauser effect spectroscopy (NOESY) and the total correlation spectroscopy (TOCSY) have been used to prove the formation of the amide bond between HPMA copolymer and doxorubicin (Pinciroli et al. 1997).

NMR spectroscopy can also be used to determine drug content within the conjugate. This can be achieved by relative integration of the signals from the drug and those from the polymeric backbone. This allows the calculation of a molecular ratio between drug molecules and polymer monomers, which can be converted into drug content expressed as percentage in weight.

7.3.2.4 Infrared Spectroscopy (IR)

IR is a technique very useful to identify functional groups. Polymer conjugation often results in the formation of new bonds (e.g. ester or amide bonds) between the drug and the polymer, which can be detected in an IR spectrum. A comparison of

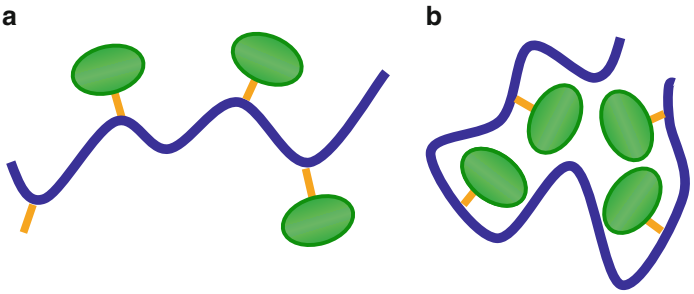


Fig. 7.9 Schematic representation of the different conformations that a conjugate may assume after conjugation to a drug: extended (**a**) or compact (**b**)

the spectrum of the conjugate with the spectra of the unbound drug and polymer can reveal the formation of the new bond. However, this might result in a challenging task when the newly formed bond is a functional group already present in the polymer. For example, PGA is constituted by monomers of glutamic acid joined together via amide bonds. If the drug is joined to the polymer via an amide bond, it might be difficult to discriminate which component of the IR signal is due to the amide bonds of the polymer and which is due to the conjugated drug. Conversely, formation of an amide bond in a PEG-based conjugate would be easier to detect as unmodified PEG does not contain this type of bond.

7.3.2.5 Matrix-Assisted Laser Desorption/Ionisation Time of Flight (MALDI-TOF)

MALDI-TOF analysis is a particular type of mass spectrometry that has been employed to characterise polymer-drug conjugates (Wu and Odom 1998). In particular, this technique can detect the shift in the mass of the conjugate due to conjugation with a drug. A mass increase in the polymer, which matches the added mass due to the presence of the drug, constitutes strong proof of conjugation. Therefore, MALDI-TOF is useful in the context of confirming covalent linkage and also in determining the molecular weight and the polydispersity of the conjugate.

7.3.2.6 Gel Permeation Chromatography (GPC)

GPC is a type of size-exclusion chromatography in which compounds are separated according to their size (or, more precisely, to the volume they occupy in solution, i.e. hydrodynamic volume): bigger molecules elute from the column faster (have shorter retention times) than smaller molecules. After conjugation to a drug, the hydrodynamic volume of a polymer may change significantly depending on the type of drug used (Fig. 7.9). The conjugate could adsorb molecules of water within its structure and adopt an extended conformation, which would result in a shorter

retention time compared to the free polymer. On the other hand, the conjugate may adopt a more compact conformation and display a smaller hydrodynamic volume than that of the corresponding free polymer, which would result in a prolonged retention time.

7.3.2.7 High Performance Liquid Chromatography (HPLC)

HPLC is a technique widely employed in the synthesis and characterisation of polymer-drug conjugates. The use of HPLC within the context of polymer-drug conjugates relies on the fact that the free drug and the conjugated drug are likely to interact differently with the stationary phase and the mobile phase, and therefore are likely to elute from an HPLC column at different times. Preparatory HPLC can be used to isolate and purify the conjugate from the free drug. Columns for preparative HPLC are relatively large (internal diameter: 5–20 mm) and allow loading of large amounts of a compound (up to milligrams of compounds). In a similar manner, analytical HPLC can be used to check that the conjugate is free from unconjugated drug or to quantify the total drug content and the content of residual-free drug. Columns for analytical HPLC are smaller (internal diameter typically 4.6 mm) and allow loading of small amounts of compound (up to few micrograms).

In addition, HPLC is often employed during stability studies, to verify that the conjugate is stable, and no drug is released from the conjugate during storage. Finally, HPLC can also be used to measure drug release under different physiological conditions. For example, samples from the blood or the urine of a patient can be analysed by HPLC to detect the stability of the conjugate in biological fluids and the rate of drug release.

7.3.2.8 Small Angle Neutron Scattering (SANS)

SANS is a technique that has been used to investigate the behaviour of the conjugates in solution (Paul et al. 2007). This type of analysis can provide key information about the conformation of the polymer (e.g. radius of gyration). A comparison between the scattering behaviour of different conjugates can also provide information about structure-activity relationships.

7.4 Application of Polymer-Drug Conjugates

This section describes the different therapeutic areas to which the concept of polymer-drug conjugates has been applied (Sects. 7.4.1 and 7.4.2). A recent development in the field (the use of polymer-drug conjugates to deliver drug combinations) is also reported (Sect. 7.4.3). The final section (Sect. 7.4.4) provides an update on the current status of polymer-drug conjugates.

7.4.1 Treatment of Cancer

The vast majority of polymer-drug conjugates that have been developed to date and that have undergone clinical evaluation are designed for application to cancer treatment. The tumour tissue represents an ideal target for polymer-drug conjugates. Firstly, it is characterised by a leaky vasculature, which allows extravasation of the conjugate from the capillaries feeding the tumour tissue to the tumour tissue itself. Conversely, the large size of the conjugate prevents extravasation of this system into normal tissues. In addition, the tumour tissue is also characterised by a poor lymphatic system, which is unable to effectively clear the extracellular fluid. As a consequence, the conjugate is retained in the tumour tissue for longer. The combination of these two factors (leaky vasculature and poor drainage) has been called 'enhanced permeability and retention (EPR) effect' and is a phenomenon typical of solid tumours (Matsumura and Maeda 1986; reviewed in Maeda et al. 2013).

The EPR effect results in the passive accumulation of macromolecules into the tumour tissue and makes polymer conjugation an ideal strategy to selectively target this disease. Unconjugated drugs are unable to take advantage of the EPR effect for the following reasons: (1) they are low MW molecules, able to diffuse throughout the body with no selectivity (i.e. size exclusion) for a specific tissue; (2) their clearance from the biological fluids does not rely on the lymphatic system, therefore they do not accumulate in the tumour tissue.

As the EPR effect promotes an improved selectivity towards the tumour tissue, the toxicity of polymer-drug conjugates compared to their parent-free drug is generally lower. For example, the maximum tolerated dose (a parameter indicating the toxicity of a therapeutic agent) of free doxorubicin is 60–80 mg/m^2 (Muzykantov and Torchilin 2003), while that of an HPMA copolymer-doxorubicin is 320 mg/m^2 (Vasey et al. 1999).

Within the context of cancer treatment, many established anticancer agents (e.g. paclitaxel and doxorubicin) have been proposed in the form of polymer-drug conjugates. More recently, the concept of polymer-drug conjugates has also been applied to experimental anticancer agents that had shown promising anticancer activity, but that had failed to progress into the market due to toxicity or other unfavourable properties. For instance, the antiangiogenic agent TNP-470 has been conjugated to an HPMA copolymer with a view to maintaining the anticancer activity while reducing its toxicity (TNP-470 is neurotoxic when administered as a free drug) (Satchi-Fainaro et al. 2004, 2005).

7.4.2 Polymer-Drug Conjugates in Diseases Other Than Cancer

Traditionally polymer-drug conjugates have been developed for the treatment of cancer, but recently the versatility of this approach has been exploited also in other

therapy areas. Representative examples of different therapeutic areas to which the concept of polymer-drug conjugates has been applied are described here.

Rheumatoid arthritis is a chronic inflammatory disease of the joints. Chronic inflammation is associated with leaky vasculature and enhanced vascular permeability. Therefore, the ability of macromolecules to selectively extravasate in correspondence of the inflamed tissue has supported the development of polymer-drug conjugates specific for this disease (e.g. PEG-dexamethasone, Liu et al. 2010; linear cyclodextrin-α-methylprednisolone, Hwang et al. 2008).

Leishmaniasis is a protozoal infection transmitted by the bite of a certain type of sand fly. The visceral form of the disease is caused by the migration of the parasite to vital organs and is often lethal. In order to improve the treatment of the disease, two HPMA copolymer conjugates of the anti-leishmanial drug 8-aminoquinoline have been developed (with and without the targeting moiety mannose) and have shown promising results in pre-clinical studies (Roy et al. 2012; Nan et al. 2001, 2004).

Polymer-drug conjugation is a relatively new concept and its development has focused on the use of well-known drugs, with the aim of proving the advantages of the new approach by comparison. With conjugation becoming a more established approach, innovative drugs targeting new molecular mechanisms have also been conjugated. For instance, it is now clear that cardiovascular and neurodegenerative disorders are often associated with increased apoptosis (i.e. the mechanism of programmed cell death) (Haunstetter and Izumo 1998; Mattson 2000). Consequently, anti-apoptotic agents have been investigated to limit inappropriate apoptosis and arrest the progression of the disease (Malet et al. 2006). One of these drugs (called ‘peptoid 1’) was shown to achieve only limited efficacy in cellular models because of its poor ability to permeate the cell membrane. A lysosomotropic delivery of the drug was then needed to improve its performance and polymer conjugation was the strategy selected. Indeed, conjugation to PGA resulted in enhanced uptake and intracellular trafficking of the anti-apoptotic drug (Vicent and Perez-Paya 2006).

7.4.3 Combination Therapy

Many diseases (e.g. cancer and HIV) are treated with cocktails of drugs rather than with a single therapeutic agent. The overall aim of this type of therapeutic regimen (combination therapy) is to maximise efficacy while decreasing toxicity. For instance, in the case of anticancer treatment, different chemotherapeutic agents are administered jointly over repetitive treatment cycles. The development of drug delivery platforms able to carry multiple types of drugs could allow the simultaneous administration of the drugs in combination, resulting in improved patient compliance and simplified therapies. To this purpose, in recent years, polymer-drug conjugates carrying two types of drugs have been developed and tested pre-clinically. Examples of this approach are reported in Table 7.1.

Table 7.1 Examples of polymer-drug conjugates containing combination therapy

Conjugate	Type of combination	Reference
HPMA copolymer-doxorubicin-aminoglutethimide	Chemotherapy; Endocrine therapy	Vicent et al. (2005), Greco et al. (2007)
PEG-NO-epirubicin	Chemotherapy; cardioprotective agent.	Pasut et al. (2009), Santucci et al. (2006)
HPMA copolymer-TNP470-alendronate	Antiangiogenic agent; bisphosphonate drug.	Segal et al. (2009)
HPMA copolymer-paclitaxel-alendronate	Chemotherapy; biphosphonate drug.	Miller et al. (2011)
HPMA copolymer-doxorubicin-dexamethasone	Chemotherapy; anti-inflammatory and anti-proliferative agent	Kostkova et al. (2011)
PEG-paclitaxel-alendronate	Chemotherapy; biphosphonate drug.	Clementi et al. (2011)

7.4.4 Current Status of Polymer-Drug Conjugates

Polymer-drug conjugates have shown promise for cancer therapy, but to date, no polymer-drug conjugate has reached the market. However, an increasing number of polymer-drug conjugates have entered and are progressing through clinical evaluation. Table 7.2 summarises the polymer-drug conjugates that have undergone clinical evaluation.

7.5 Conclusions

Polymer-drug conjugates are a drug delivery technology based on the covalent conjugation of drug molecules to a polymeric carrier. Conjugation to a polymer prolongs the circulation time of the drug, increases its selectivity for the target tissue (e.g. the tumour tissue) and allows selective drug release. The development of a polymer-drug conjugate requires a careful design and an accurate choice of its components (polymer, linker, drug and optional targeting moiety). Optimised synthetic conditions and extensive characterization are necessary to make a reproducible and 'high quality' polymer-drug conjugate. The application of this concept has mainly focused on the treatment of cancer, with encouraging clinical results. While the first marketed polymer-drug conjugate is still awaited, research is ongoing to further develop this concept. Progress in polymer chemistry is leading to innovative polymer architectures and new molecular targets are being investigated to extend this technology to new applications.

Table 7.2 Clinical status of polymer-drug conjugates that have entered clinical evaluation

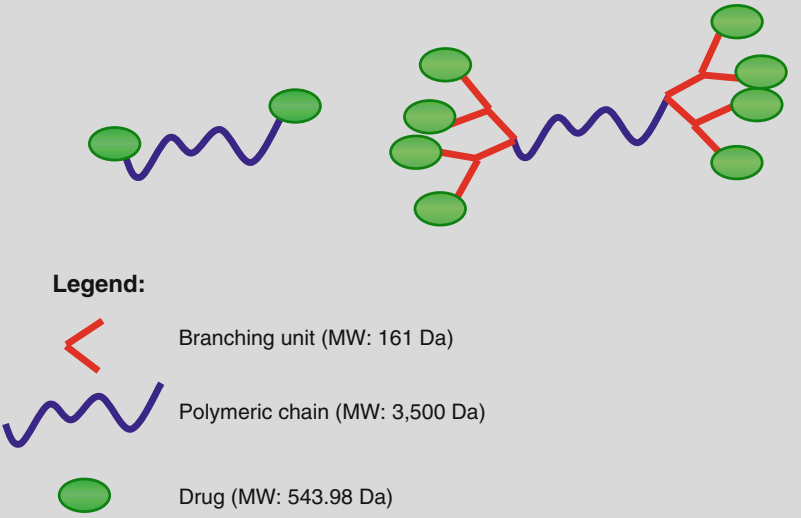
Status	Conjugate	Name	Reference
Phase III	PGA-paclitaxel	CT-2103; Opaxio®	O'Brien and Oldham (2005), Albain et al. (2006), Langer et al. (2008), O'Brien et al. (2008), Paz-Ares et al. (2008)
	PEG-naloxone	NKTR-118	Neumann et al. (2007), Webster and Dhar (2009)
Phase II	HPMA-DACH-platinate	AP5346; ProLindac®	Campone et al. (2007)
	HPMA copolymer-doxorubicin	PK1; FCE28068	Vasey et al. (1999), Seymour et al. (2009)
	PEG-Irinotecan	NKTR-102	Hamm et al. (2009), Borad et al (2008)
	HPMA copolymer-doxorubicin-galactosamine	PK2; FCE28069	Seymour et al. (2002)
	HPMA copolymer-carboplatinate	AP5280	Rademaker-Lakhai et al. (2004)
	PGA-camptothecin	CT-2106	Homsi et al. (2007), Daud et al. (2006)
	PEG-SN38	EZN-2208	Guo et al. (2008)
	Cyclodextrin polymer-camptothecin	IT-101	Oliver et al. (2008)
Phase I	Carboxymethyldextran-exatecan camptothecin	DE-310	Soepenberg et al. (2005)
	PEG-docetaxel	NKTR-105	Calvo et al. (2010)
	PHF-camptothecin	XMT-1001	Sausville et al. (2010)
Discontinued	PEG-camptothecin	Pegamotecan	Scott et al. (2009)
	PEG-paclitaxel	–	Beeram et al. (2002)
	HPMA copolymer-camptothecin	MAG-CPT	Schoemaker et al. (2002)
	HPMA copolymer-paclitaxel	PNU166945	Meerum Terwogt et al. (2001)
	Oxidised dextran-doxorubicin	DOX-OXD	Danhauser-Riedl et al. (1993)

Problem Box

Question 1.

Calculate the drug loading (expressed as % w/w) for a linear polymer-drug conjugate and for its dendronised derivative (schematic representation of the structures and the MWs of the various components are reported in figure below). Briefly discuss the importance of drug loading within the context of polymer-drug conjugates.

Important note. In your calculation assume that conjugation of the drug to the polymer or to the branching unit results in the loss of a molecule of water (loss of an H from the drug and loss of an OH from the polymer/branching unit). Addition of each branching unit also results in a loss of a molecule of water.



Answer 1.

Step 1. Calculate the % w/w of the linear polymer-drug conjugate.

(a) Calculate the drug content:
$(543.98 \times 2) - 2 = 1{,}085.96$
(b) Calculate the total weight of the conjugate
$(543.98 \times 2) + 3{,}500 - (18 \times 2) = 4{,}551.96$
(c) Calculate the % w/w:
(1085.96/4551.96) * 100 = 23.8%

Step 2. Calculate the % w/w of the dendronised polymer-drug conjugate.

(a) Calculate the drug content:
$(543.98 \times 8) - 8 = 4{,}343.84$

(continued)

Problem Box (continued)

(b) Calculate the total weight of the conjugate
$(543.98 \times 8) + 3{,}500 + (161 \times 6) - (18 \times 14) = 8{,}565.84$
(c) Calculate the % w/w:
$(4{,}343.84/8{,}565.84) \times 100 = 50.7\ \%$

Question 2.

The anticancer agent paclitaxel has been considered a promising candidate for delivery via polymer-drug conjugate technology. Indicate what characteristics of this drug molecule make it suitable for applications with this technology.

Answer 2.

(a) Paclitaxel is an anticancer drug with toxicity due to non-selective delivery (conjugation to a polymer can improve selectivity); (b) paclitaxel is poorly water-soluble and has to be administered in a oil-based formulation (Cremophor), which has a certain toxicity; conjugation to a polymer can improve solubility in biological fluids; (c) paclitaxel contains an hydroxyl group (this can be used for conjugation via, for instance, an ester linkage); (d) paclitaxel has a short plasma half-life (which can be prolonged by conjugation to a polymer).

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Chapter 8
Polymersomes and Filomicelles

Núria Sancho Oltra, Sharon M. Loverde, Takamasa Harada, Abdullah Mahmud, Karthikan Rajagopal, and Dennis E. Discher

Abstract Amphiphilic block copolymers represent a major field of research in the design and creation of innovative materials for biomedical applications. Self-directed assemblies of such copolymers have been of great value in the development of novel drug delivery systems. Polymeric vesicles (polymersomes) and worm-like micelles (or filomicelles) are particularly appealing due to their structure and composition that provides them with specific and tunable properties. The work reviewed at an introductory level in this chapter highlights some of the features of such aggregates and reviews the synthesis of their components, their assembly, and characterization. Degradation and drug release kinetics are described as well as their application in therapeutics.

8.1 Introduction

The more we learn about how materials behave in biological contexts, the greater is the demand for new materials with potential for biomedical applications. Biomaterials can help in the treatment of cancer and many other diseases. Over the years, the combined efforts of many different disciplines have enabled the design and creation of innovative materials with tailored or enhanced properties that have proved applicable to drug delivery (Lalatsa et al. 2012; Ma et al. 2012), gene therapy (Ledley 1995; Wagner 2012), or even tissue engineering (Ma 2008; Patterson et al. 2010). The development of new biomaterials with novel features is nevertheless challenging and requires precise design and development, sometimes through a combination of proteins, catalysts, quantum dots, and polymers (Adeli et al. 2011;

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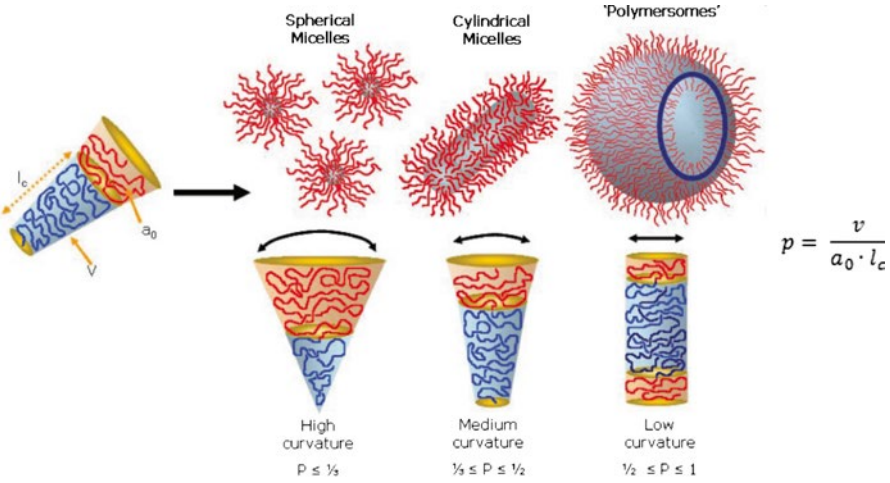


Fig. 8.1 Self-assembled structures from amphiphilic block copolymers in a block-selective solvent. p stands for packing parameter, v is the volume of the hydrophobic chains, a_0 is the optimal area of the head group, and l_c is the length of the hydrophobic tail (reprinted with permission from Blanazs et al. (2009). Copyright 2009 John Wiley and Sons)

Taylor-Pashow et al. 2010; van de Manakker et al. 2009; Zimmermann et al. 2011). Synthetic polymers represent a particularly important field because of the numerous possibilities for functionalization that allow for precise tuning of properties for specific applications. Block copolymers, which result from the combination of different polymeric blocks, have begun to be used in biomaterial designs as di-, tri-, etc. block copolymers (Zhao et al. 2012; Brinkhuis et al. 2011).

Copolymers with blocks of different polarity are particularly interesting due to their specific aggregation behavior in different solvents. Diblock copolymers composed of one block that is hydrophilic (from the Greek *hydros*, meaning water, and *philia*, meaning love) and another block that is hydrophobic (from the Greek *phobos*, meaning fear) are amphiphilic molecules that form aggregates of different morphologies in water (Fig. 8.1) (Blanazs et al. 2009). This assembly behavior results from the preference of each block to either contact water (hydrophilic) or avoid water (hydrophobic).

The ratio between the sizes of the two blocks determines the molecular curvature which influences the packing of the polymeric chains and therefore the resulting morphology of the aggregate. The packing parameter (p) allows for an estimation of the molecular curvature and hence for a prediction of the aggregate morphology. Block copolymers with large hydrophilic chains will generally yield molecular-scale spherical micelles, but increasing the size of the hydrophobic block progressively shifts the preferred assemblies to mesoscopic cylinder-shaped worm-like micelles (filomicelles), vesicles (polymersomes), and then lamellae, and inverted phases. Sonicating or shearing the latter, mesoscopic assemblies can disrupt and generate spherical micelles that are kinetically trapped, which means that post-synthesis processing is a critical aspect of working with these structures.

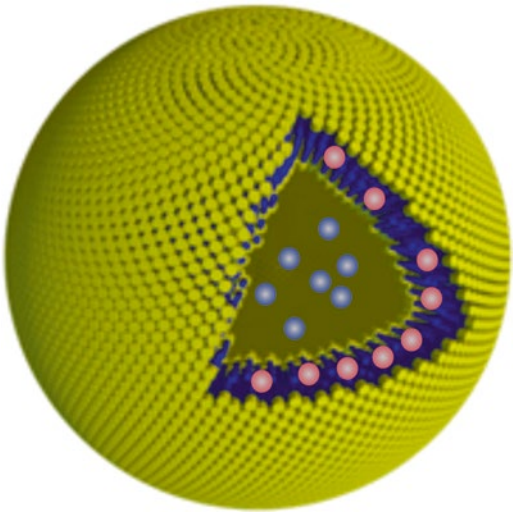


Fig. 8.2 Schematic representation of a polymersome containing a hydrophobic drug in the membrane and hydrophilic molecules in the water pool (figure adapted from http://www.ru.nl/bio-orgchem/research/polymers/)

The systematic structural diversity has allowed the design and study of numerous functional systems with block copolymers of very different nature and different properties (Gaucher et al. 2005). Biodegradability is a particularly attractive feature when designing materials for therapeutic applications. Many biodegradable synthetic polymers have been successfully applied in biomedicine, with key examples in drug delivery (Leong et al. 1985) as well as orthopedic devices (Middleton and Tipton 2000). Amphiphilic block copolymers made from biodegradable components are therefore promising for biomedical purposes.

8.1.1 Polymersomes

Polymer vesicles, also known as polymersomes (Discher et al. 1999; Discher and Eisenberg 2002), have been found to be particularly suitable for the construction of nanoreactors (Vriezema et al. 2007), medical imaging (Ghoroghchian et al. 2005), gene (Kim et al. 2009), and drug delivery (Lee and Feijen 2012a). While possessing many of the properties of natural liposomes, polymersomes have shown higher stability and lower permeability. Moreover, these and other characteristic features can be tuned on demand by manipulation of the synthesized polymers. These assemblies are especially interesting in the field of drug delivery since the hydrophobic membrane allows for the accommodation of hydrophobic drugs, while the confined aqueous lumen can encapsulate hydrophilic therapeutics, proteins, RNA, etc. (Fig. 8.2) (Christian et al. 2009a; Kim et al. 2009; Lee and Feijen 2012a).

Block copolymers used in drug delivery comprise biocompatible polymers and are often constituted by hydrophilic poly(ethylene oxide) (PEO) chains and

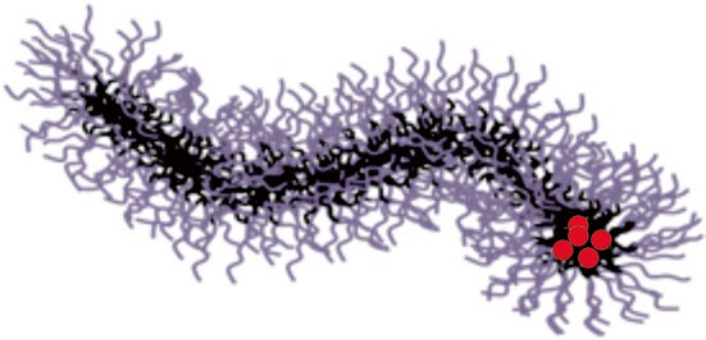


Fig. 8.3 Schematic representation of a worm-like micelle containing a hydrophobic drug in the core

hydrophobic polyesters like poly(lactic acid) (PLA) (Hagan et al. 1996) or poly(caprolactone) (PCL) (Soo et al. 2002). While PEO confers "stealthy" properties to the assemblies (Photos et al. 2003) avoiding clearance by phagocytosis and therefore resulting in longer blood circulation times, polyester cores are biodegradable and in time they hydrolyze into nontoxic products.

8.1.2 Worm-Like Micelles

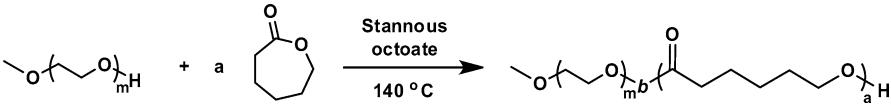
Worm-like micelles, also known as filomicelles, have been used in drug delivery as well. The bigger hydrophobic core of the assemblies compared to spherical micelles is able to encapsulate higher amounts of hydrophobic drugs (Fig. 8.3). Additionally, the elongated shape and flexibility of these micelles have proven to be of major importance for the avoidance of the phagocytes in the liver and the spleen (Geng et al. 2007) and therefore, for their passive accumulation into the tumors. The use of filomicelles from degradable copolymers poly(ethylene oxide)-*b*-poly(ε-caprolactone) (PEO-*b*-PCL or OCL) and poly(ethylene oxide)-*b*-poly(ε-caprolactone-*r*-L,D-lactide) (OCLA) have shown satisfactory results in the delivery of drugs to tumors (Geng et al. 2007; Kim et al. 2005).

8.2 Synthesis of Block Copolymers for Drug Delivery

The synthesis of block copolymers used in drug delivery is possible via numerous approaches that can usually be classified into two distinctive strategies: chain extension and conjugation. The chain extension strategy implies the controlled polymerization of one monomer followed by chain extension by polymerization of the other one. Either of the blocks can be polymerized following standard synthetic

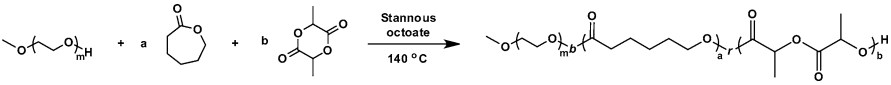
methods (free radical polymerization, ring-opening polymerization, atom transfer radical polymerization (ATRP), reversible addition—fragmentation chain transfer (RAFT), etc.) provided that the terminal functionality of the first synthesized block is or can be transformed into an initiating moiety for the polymerization of the second one. The conjugation strategy, however, involves the use of two blocks polymerized independently that posses end functional groups capable of reacting with each other to yield the resulting block copolymer.

Due to the extended number of synthetic strategies we will focus here on one broadly used route for the synthesis of block copolymers containing polyesters, the ring-opening polymerization. OCL block copolymers are generally synthesized by the ring-opening polymerization of ε-caprolactone using the required size of methoxy-terminated PEO as macro-initiator and stannous octoate as catalyst (Scheme 8.1).



Scheme 8.1 Synthesis of OCL block copolymer by reaction of poly(ethylene oxide) with ε-caprolactone

For the synthesis of OCLA copolymers, a mixture of ε-caprolactone and D,L-lactide is reacted with the required amount of PEO and stannous octoate (Scheme 8.2).



Scheme 8.2 Synthesis of OCLA block copolymer by reaction of poly(ethylene oxide) with ε-caprolactone and D,L-lactide

The obtained diblock copolymers can be characterized by ^{1}H-NMR and Gel Permeation Chromatography (GPC).

8.3 Aggregate Formation and Characterization

8.3.1 Preparation of Polymersomes

There are several techniques to prepare polymersomes from amphiphilic block copolymers. These techniques follow either of the following strategies: solvent exchange or thin film rehydration (Lee and Feijen 2012a; Kita-Tokarczyk et al. 2005).

Fig. 8.4 Fluorescence microscopy images of OCL aggregates in water using the hydrophobic dye PKH26 (reprinted with permission from Rajagopal et al. (2010a). Copyright 2010 American Chemical Society)

By the solvent exchange method organic solutions of the block copolymer are hydrated either by addition of the organic solution into water or by slowly adding water to the organic polymer solution. The traces of organic solvent can be then removed by dialysis or evaporation. For the thin film rehydration method, a thin film is prepared by the evaporation of a solution of the block copolymer in an organic solvent. The remaining film is then rehydrated by addition of water while stirring.

8.3.2 Preparation of Worm-Like Micelles

Worm-like micelles can be prepared using either of the two following methods: thin film rehydration, just explained, and solvent evaporation (Rajagopal et al. 2010a; Dalhaimer et al. 2004; Du et al. 2007; Geng et al. 2005). In the solvent evaporation method, a stock solution of the polymer in organic solvent is added to deionized water. The contents of the vial are stirred gently with the cap open at room temperature. Stirring is continued for 48 h or until no chloroform layer is observed.

8.3.3 Characterization of OCL Aggregates

Aggregates from OCL can be visualized by fluorescence microscopy using a hydrophobic fluorophore (PKH 26) (Rajagopal et al. 2010a). When the hydrophobic dye is added to an aggregate suspension, internalization of the dye into the hydrophobic core takes place due to the interaction between the dye and PCL. This encapsulation enables the visualization of the aggregates by fluorescence microscopy (Fig. 8.4). An alternative visualization technique involves the combination of OCL block copolymers with rhodamine-functionalized PCL (Fig. 8.5) (Rajagopal et al. 2010b). These dye-containing polymers integrate easily into the worm-like micelle assemblies without altering the micelle properties.

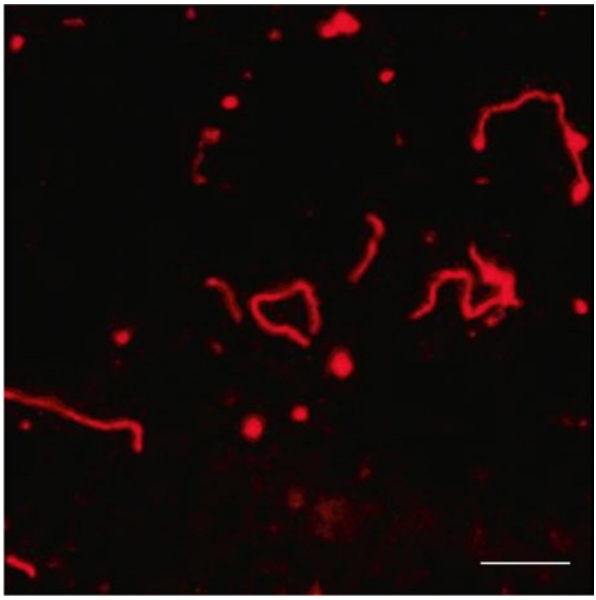


Fig. 8.5 Fluorescence microscopy images of worm-like micelles from OCL blended with 5 % rhodamine-PCL. Scale bar is 10 μm

8.4 Morphological Phase Behavior of OCL Assemblies

The preferred morphology of the assemblies from OCL has been studied for block copolymers containing different hydrophilic/hydrophobic ratios. The differences in morphology shown to be dependent on the lengths of the blocks, as previously observed (Du et al. 2007). Plotting the molecular weight of the hydrophobic tail (M_{CH_2}) versus the hydrophilic mass fraction, $f_{\text{hydrophilic}}$ (Fig. 8.6), the transitions between morphologies can be defined (Rajagopal et al. 2010a).

Aggregates were obtained for $f_{\text{hydrophilic}} > 0.36$. At lower values, precipitation of the polymer occurred due most probably to the inability of the short PEO chains to stabilize the colloidal assemblies. At low $f_{\text{hydrophilic}}$, the formation of vesicles is favored as a result of the parallel stacking of the polymer chains that contributes to the crystallite growth. The increase in $f_{\text{hydrophilic}}$ results in a higher curvature in the structure frustrating in that way the crystallization process and yielding flexible worm-like micelles (Rajagopal et al. 2010a). Interestingly, for OCL block copolymers the formation of worm-like micelles is only limited to a narrow morphological space that is buried within the vesicle region, while for other copolymers, such as poly(ethylene oxide)-*b*-poly(1,2-butadiene) (PEO-*b*-PBD or OB), worm-like micelles appear as a transition state between spherical micelles and vesicles (Jain and Bates 2003).

Aggregates from OCLA exhibit a similar worm-like micelle morphology, at the same hydrophilic fraction and molecular weight of the copolymer, that coincides with the worm-like micelle region of OCL (Fig. 8.6).

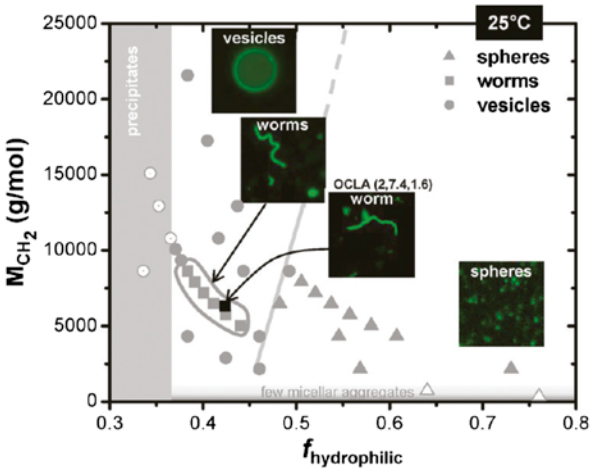


Fig. 8.6 Morphological phase behavior of OCL assemblies in dilute solution prepared at 25 °C by the solvent evaporation method. *Filled symbols in gray* represent each of the morphologies: *triangles* for spherical micelles, *squares* for worm-like micelles, and *circles* for vesicles. *Gray lines* indicate the approximate phase boundaries. Worm-like micelles formed from poly(ethylene oxide)-*b*-poly(ε-caprolactone-*r*-D,L-lactide) (OCLA) copolymer are indicated in the phase diagram with a *filled black square* (reprinted with permission from Rajagopal et al. (2010a). Copyright 2010 American Chemical Society)

8.5 Controlling the Rigidity of Worm-Like Micelles

Fluorescence microscopy images showed a persistent transversal fluctuation in worm-like micelles that suggests a soft, fluid core. This is, however, not observed for the whole molecular weight range of OCL that aggregates into worm-like micelles. There is a small population of OCL(2,9) (MW(PEO) = 2000 g/mol, MW(PCL) = 9,000 g/mol) that results in a fraction of rigid worm-like micelles (Fig. 8.7).

This rigidity is attributed to the semicrystalline nature of PCL and is dependent on the molecular weight of the hydrophobic block. For high molecular weight PCL a close packing of the polymer chains is possible and therefore crystallization of the core occurs. For lower molecular weights, however, the core curvature prevents this crystallization process resulting in flexible worm-like micelles. Crystallization of the hydrophobic core can also be prevented for higher molecular weights by the incorporation of defects in the structure. It has been found that by copolymerizing 10 % of D,L-lactide (LA) with PCL to obtain poly(ethylene oxide)-*b*-poly(ε-caprolactone-*r*-D,L-lactide) (OCLA), this rigidity is eliminated. The side methyl groups from D,L-lactide prevent the close packing of the chains suppressing in this way the crystallization of the core (Fig. 8.8). Few cases of half-rigid worm-like micelles have been also observed (Fig. 8.9), which is consistent with the nucleation and growth of the crystals along the PCL core.

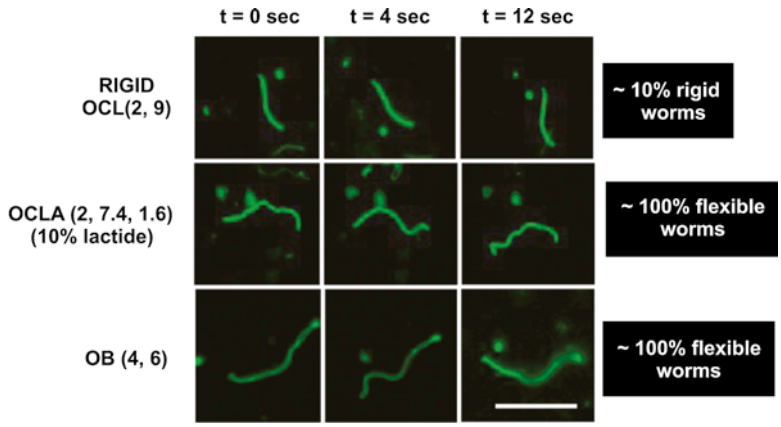


Fig. 8.7 Fluorescence microscopy images of worm-like micelles at different time intervals are shown for a rigid OCL(2,9) worm-like micelle (*top row*), a flexible OCLA(2,7.4,1.6) worm-like micelle (*middle row*) and a flexible OB(4,6) worm-like micelle (*bottom row*). Approximately 10 % of OCL(2,9) worm-like micelles showed rigid-body motion at room temperature, while all of OCLA and OB worm-like micelles are completely flexible. Scale bar is 10 μm (reprinted with permission from Rajagopal et al. (2010a). Copyright 2010 American Chemical Society)

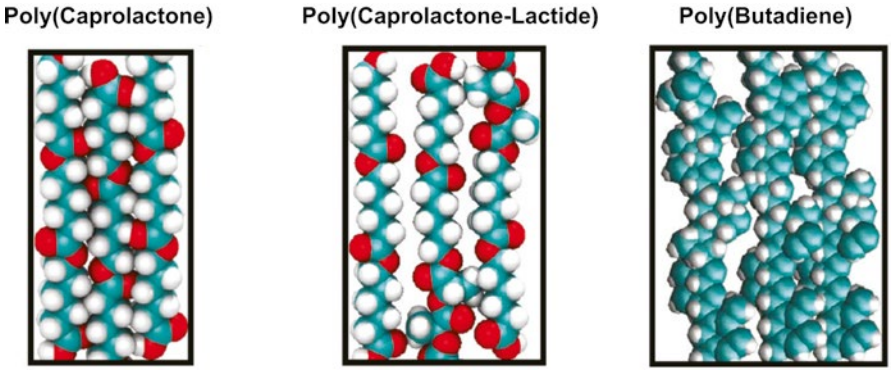


Fig. 8.8 Space filling models of poly(caprolactone), poly(caprolactone-*r*-D,L-lactide), and poly(butadiene) blocks (reprinted with permission from Rajagopal et al. (2010a). Copyright 2010 American Chemical Society)

8.6 Degradation of Polymersomes and Worm-Like Micelles

Polyesters have been used extensively in biomedical applications such as drug delivery and tissue regeneration (Grayson et al. 2005; Seyednejad et al. 2011). Many different intrinsic (i.e., molecular weight, chemical structure, degree of crystallinity, etc.) and external (i.e., temperature, pH, etc.) (Ivanova et al. 1997) factors

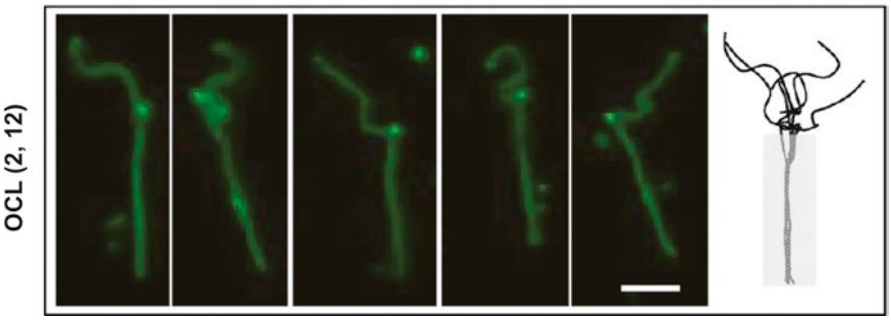


Fig. 8.9 Fluorescence microscopy images of partially rigid OCL worm-like micelles. Snapshots were taken every 30 s and the contour was traced, rotated, and overlaid to highlight the rigid segment. Scale bar is 5 μm (reprinted with permission from Rajagopal et al. (2010a). Copyright 2010 American Chemical Society)

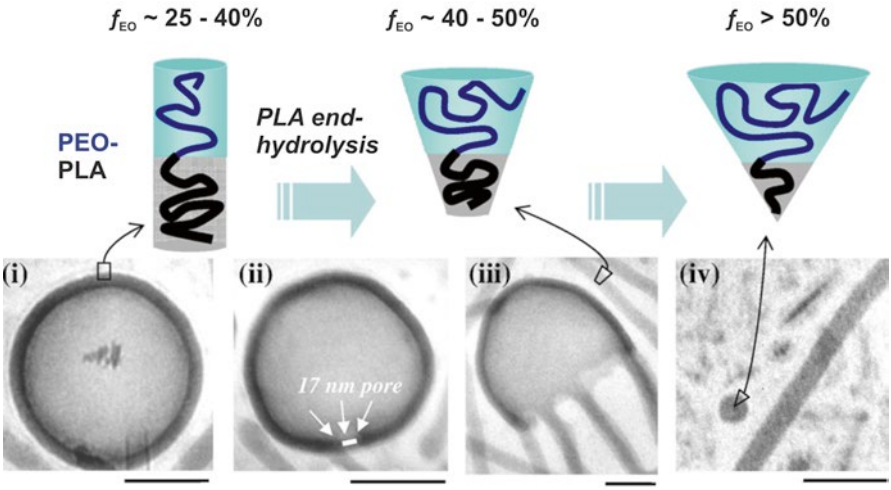


Fig. 8.10 Cryo-transmission electron microscopy (Cryo-TEM) images of PEO-PLA assemblies in water. Hydrolysis of PLA triggers the formation of pores and the transition from polymersomes to worm-like and spherical micelles. Scale bars are 100 nm (reprinted with permission from Ahmed et al. (2006a). Copyright 2006 Elsevier)

seem to be responsible for the degradation of polyesters. Additionally, the degradation kinetics of polyesters appears also influenced by the assembled macromolecular structures and therefore the susceptibility to degradation can be modulated (Höglund et al. 2007, 2010, 2012). The degradation of polyesters in polymersomes has been shown to have direct implications in the phase behavior of block copolymer amphiphiles and is expected to directly influence the drug release kinetics as observed for polymersomes of PEO-PLA (Ahmed et al. 2006a). The degradation of the hydrophobic core takes place over the period of hours to days and it results in porosity in the polymersome membrane (Fig. 8.10(ii)). As the propagation of the

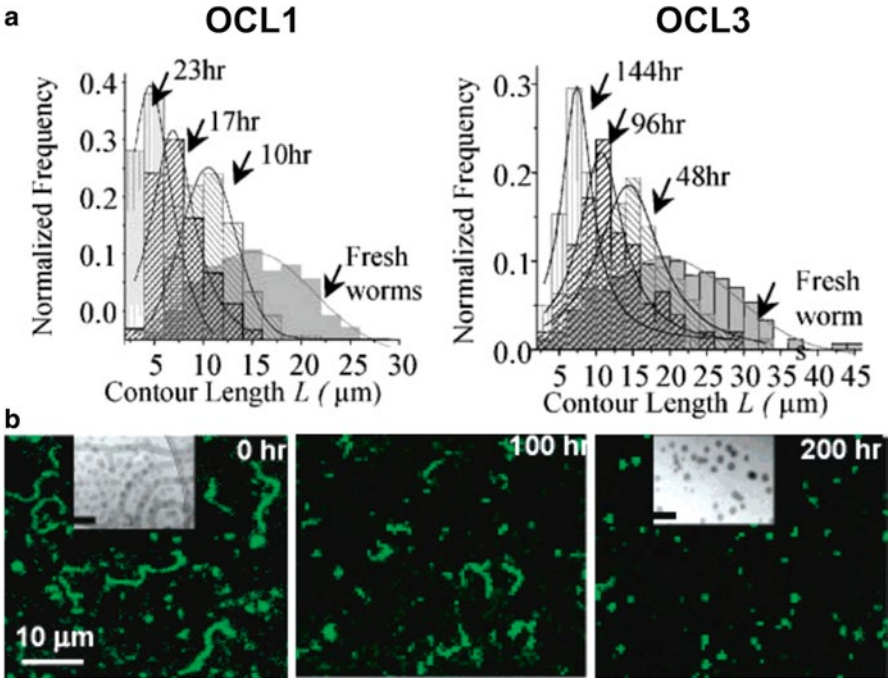


Fig. 8.11 (**a**) Contour length distributions of OCL1 (M_n = 2,000–2,770) (*left*) and OCL3 (M_n = 5,000–6,500) (*right*) worm-like micelles in time (reprinted with permission from Geng and Discher (2006). Copyright 2006 Elsevier). (**b**) Fluorescence microscopy and cryo-TEM (inset, bar = 100 nm) images of OCL worm-like micelles showing the transition to spherical micelles in time (reprinted with permission from Geng and Discher (2005). Copyright 2005 American Chemical Society)

pores increases, destabilization of the vesicular structure takes place leading to worm-like micellar aggregates (Fig. 8.10(iii)). This phase transition plays a major role in the delivery of encapsulants and can be correlated with the release of hydrophobic drugs like paclitaxel (vide infra). Further degradation of the hydrophobic chains present either in bilayers or worm-like micelles generates spherical micelles (Fig. 8.10(iv)).

Different approaches have been developed to control the degradation and subsequent drug release from polymersomes. Noteworthy approaches include cross-linkage (Ahmed et al. 2003; Discher et al. 2002), functionalization post-assembly (Egli et al. 2011), and blending with nondegradable polymers (Ahmed et al. 2002). The degradation mechanism and kinetics for OCL worm-like micelles have also been studied into detail (Geng and Discher 2005, 2006). From multiple frames of fluorescence microscopy images with well-resolved OCL worm-like micelles, a distribution of measurable contour lengths (>1 μm) was plotted from ~200 worm-like micelles and fit with a Gaussian curve (Fig. 8.11a). On time scales of days, these giant OCL worm-like micelles spontaneously shorten to spherical micelles as observed by fluorescence microscopy and Cryo-TEM (Fig. 8.11b). The contour length shortening of OCL worm-like micelles can be quantitatively analyzed by tracing contour

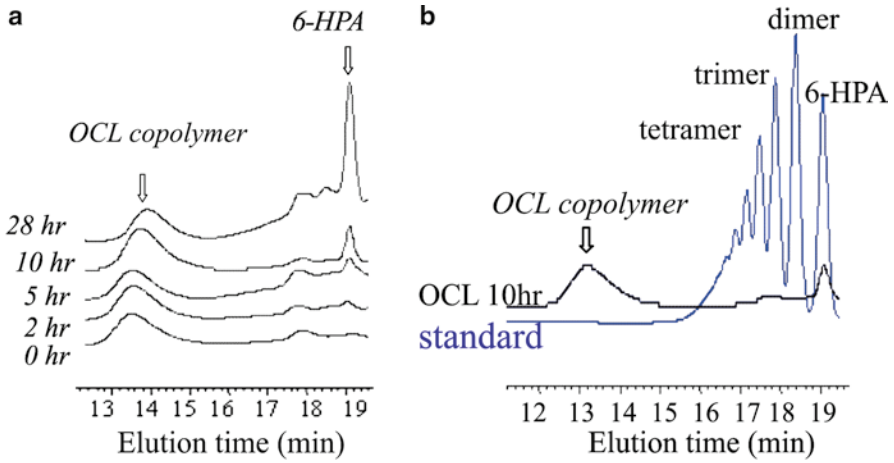


Fig. 8.12 (**a**) Gel permeation chromatography (GPC) chromatograms of OCL worm-like micelles at different degradation times, at 37 °C. (**b**) Overlaid GPC chromatograms of OCL and degradation product (monomer 6-HPA) with standards (reprinted with permission from Geng and Discher (2005). Copyright 2005 American Chemical Society)

length distribution curves changing with time (Fig. 8.11a). Results revealed that the mean contour length (L) clearly shrinks from the initial long worms towards spheres with time, while the distribution curve narrows as well. The total transition into spherical micelles takes ~28 and ~200 h for OCL1 and OCL3, respectively. It is estimated that chains have lost approximately 30 % of their molecular weight by hydrolysis by the time the worms transition into spheres.

For increasing molecular weights of the hydrophobic block, the transition time from worm-like to spherical micelles is considerably extended at 37 °C in water. The hydrolysis product was found by GPC and ^{1}H-NMR to be the monomer of the hydrophobic block 6-hydroxycaproic acid (6-HPA) and the polydispersity of OCL copolymer remained essentially the same (Fig. 8.12), demonstrating that PCL in these copolymers hydrolyzes from the end by "chain-end cleavage" rather than by "random-scission." "Random-scission" would yield various degradation products and broaden the polydispersity of the polymer.

End-hydrolysis of PCL increases the weight fraction of PEO (f_{EO}) and consequently shifts the preferred morphology towards a higher curvature structure (Jain and Bates 2003). This increase in f_{EO} favors the formation of spherical over worm-like micellar structures. The range of f_{EO} that favors worm-like micelle formation has been estimated to be very narrow when compared to spherical micelles explaining why worm-like micelles are so susceptible to morphological transformation.

Microscopy images illustrated that the worm-to-sphere transition occurs with bulb formation at the end of the worm, consistent with release of spherical micelles

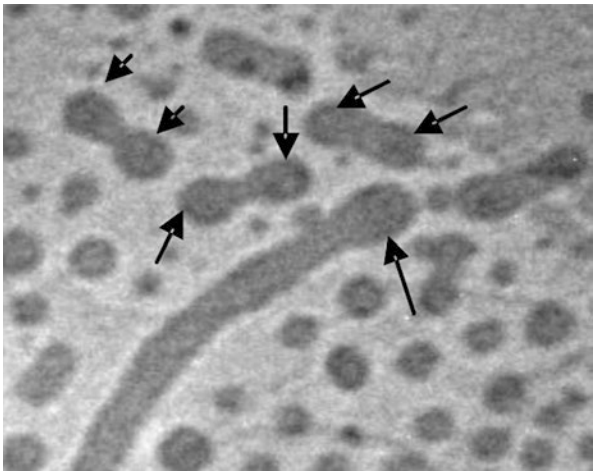


Fig. 8.13 Transmission electron microscopy (TEM) image of OCL worm-to-sphere transition. Bulb formations at the end of the worm-like micelles are indicated with *arrows*

from the end-cap of the worm micelle (Fig. 8.13) (Burke and Eisenberg 2001). This mechanism of micellar morphological change has been also confirmed by molecular simulations (Loverde et al. 2010). This morphological transition can be profitable if we consider a drug delivery system with the enhanced properties of the long worm-like micelles (i.e., higher drug loading capacity and longer circulation times in vivo) combined with the extremely useful features of spherical micelles (i.e., easier internalization) for therapeutic purposes.

The end-cleavage of PCL within the worm-like micelle structure has been found to be considerably faster than the hydrolysis of PCL homo/copolymers with different morphologies under the same conditions (Chen et al. 2000; Lee and Gardella 2000; Li et al. 1998). This enhanced hydrolysis takes place due to the specific effect of the micellar structure on the process. As speculated for spherical micelles (Nie et al. 2003), the terminal hydroxyl group on the PCL block is not completely embedded into the hydrophobic core of the micelle but it is drawn into the hydrophilic corona. This particular orientation allows for the interfacial water (Fendler and Fendler 1975), cooperatively with the terminal hydroxyl group (de Jong et al. 2001), to attack the ester group nearest to the terminal hydroxyl. The resulting product contains a new terminal hydroxyl group that can likewise restart the process of PCL end-cleavage. The essential role of the terminal hydroxyl in the degradation process was shown in experiments where the acetylated copolymer was used. In that case worm-like micelles were also obtained; however, no significant morphological change was observed after 24 h at 37 °C, by which time OCL worm-like micelles are completely degraded.

8.7 Biomedical Applications of Polymersomes and Filomicelles

8.7.1 Drug Loading into Polymersomes

Loading of drugs into polymersomes can be done following different methodologies. One common approach implies the injection of a solution of the block copolymer with the hydrophobic drug to be loaded in tetrahydrofuran into water with subsequent removal of the organic solvent by dialysis (Lee and Feijen 2012b). Alternatively, the loading of the hydrophobic drug paclitaxel (TAX) into the membrane of polymersomes of PEG-PLA/PEG-PBD obtained by the film rehydration method in citrate buffer pH 4 is done post-vesicle formation (Ahmed et al. 2006a, b). The drug solution in methanol is injected into the vesicle suspension followed by dialysis in phosphate buffer saline (PBS) pH 7.4. This dialysis step not only removes the nonencapsulated drug, but it also creates a pH gradient necessary for the encapsulation of doxorubicin (DOX) in the aqueous lumen. For the incorporation of the drug in the hydrophilic pool, a pH-gradient method established for liposomes is applied (Mayer et al. 1986). In this way, both hydrophobic and hydrophilic drugs are encapsulated in a single polymersome.

8.7.2 Drug Loading into Worm-Like Micelles

The hydrophobic core of the worm-like micelles, composed of PCL, is capable of encapsulating hydrophobic drugs like TAX. TAX is a hydrophobic drug with low solubility in water (~1 μg/mL). Due to the elongated shape of worm-like micelles, the hydrophobic core can accommodate higher amounts of hydrophobic drugs resulting in a considerable higher drug loading capacity compared to spherical micelles (Cai et al. 2007). Results showed that worm-like micelles of the same molecular weight of spherical micelles can load approximately twice as much hydrophobic drug (TAX) (Geng et al. 2007). Molecular dynamics simulations corroborate with these results, but also illustrate that the drug displays interfacial activity, particularly at experimentally relevant loading concentrations (Loverde et al. 2012). The high loading capacity of worm-like micelles increases in this way significantly the solubility of TAX in water; 10–15 wt% of OCL worm-like micelles can dissolve 3–7.5 mg of TAX per mL. This is comparable to Cremophor EL (6 mg/mL) which is a solubilizer for hydrophobic drugs widely used in the clinic. Moreover, the loading of TAX onto the worm-like micelles does not affect the flexibility of the assemblies as observed by fluorescence microscopy.

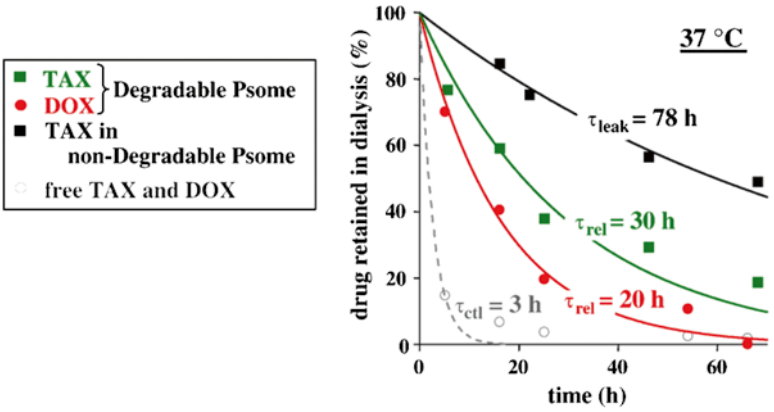


Fig. 8.14 In vitro release and leakage of DOX and TAX from degradable (PEO-PLA) and nondegradable (PEO-PBD) polymersomes (reprinted with permission from Ahmed et al. (2006a). Copyright 2006 Elsevier)

8.7.3 In Vitro Release of DOX and TAX from Polymersomes

The specific copolymer chemistry, and its respective interactions with the hydrophobic drug, will strongly influence both the loading and release behavior of the drug itself. Figure 8.14 shows the release kinetics of DOX and TAX using degradable polymersomes from loaded PEO-PLA and nondegradable polymersomes from PEO-PBD containing TAX at 37 °C (Ahmed et al. 2006a). The release of drugs from degradable polymersomes is clearly much faster than when nondegradable polymersomes, PBD-based copolymers, were used. Moreover, the release times in the tumor environment are expected to be shorter due to the acidic pH encountered that is known to accelerate degradation (Ahmed and Discher 2004).

8.7.4 In Vitro Release of TAX from OCL Worm-Like Micelles

Release kinetics of TAX from OCL worm-like micelles has been studied by the dialysis method under "sink condition" for different molecular weights (M_n (OCL1) ~ 4,700 and M_n (OCL3) ~ 11,000) and at different pH (pH 5 and 7) at 37 °C. Results indicated that the release was significantly slower for aggregates from OCL of higher molecular weight, but in either case a faster release was observed at acidic pH (Fig. 8.15). pH-dependent release kinetics has been observed previously in polymersomes of OCL in experiments using DOX (Ghoroghchian et al. 2006).

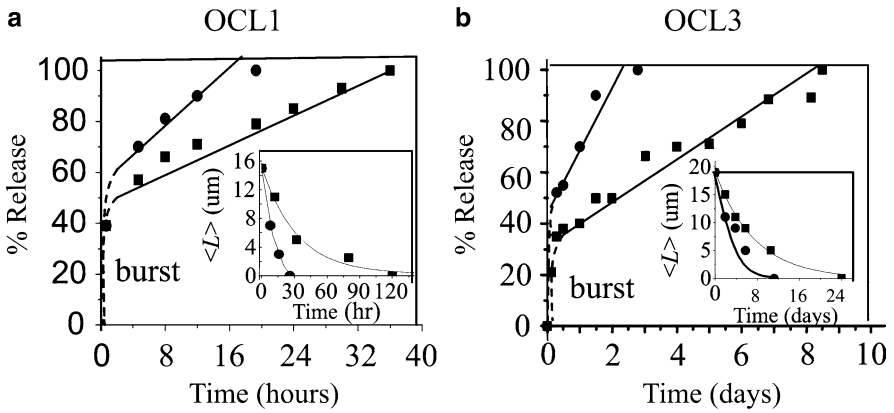


Fig. 8.15 Release profiles of TAX from (**a**) OCL1 and (**b**) OCL3 worm-like micelles in pH 5 Hepes (*filled circle*) and pH 7 PBS buffers (*filled square*) at 37 °C. Insets correspond to the degradation-induced OCL worm-like micelle contour length shortening with time (reprinted with permission from Geng and Discher (2006). Copyright 2006 Elsevier)

Control experiments of TAX release from OCL1 worm-like micelles at 4 °C, where degradation is negligible, showed that the diffusion-controlled release kinetics are similar under both buffers, demonstrating that the faster release in acidic buffer is not due to diffusion but rather degradation (Geng and Discher 2006). Further comparison of the TAX release rate with the corresponding OCL worm-like micelle shortening/degradation rate at 37 °C (inset) demonstrates that TAX release is indeed dominated by degradation. Shortening rates of worm-like micelles were shown to be correlated to the TAX release rate indicating a tight coupling between degradation and release. As alluded to previously, this pH-dependent release is particularly interesting for drug delivery purposes. The cell environment in cancerous cells is known to be acidic, therefore TAX would remain encapsulated in the aggregates when circulating in blood plasma (neutral pH) with relatively slow release. When cancerous tissues are reached, however, the release would be much faster due to the lower pH encountered (Shuai et al. 2004).

Cytotoxicity studies on A549 lung carcinoma cells carried out using unloaded spherical and worm-like micelles revealed that their toxicity was negligible when compared to Cremophor EL (Fig. 8.16a). Although Cremophor EL is extensively used in the clinic as a drug solubilizer, it presents side-effects due to its intrinsic toxicity (Onetto et al. 1993). The results indicate that the anticancer effects of TAX-loaded OCL3 micelles can be fully attributed to the drug instead to the toxicity of the carrier (Fig. 8.16b). Despite the fact that both OCL3 filomicelles and spherical micelles showed the same enhanced cytotoxicity, the use of filomicelles appears more attractive for drug delivery purposes since its loading capacity is considerably higher compared to spherical micelles (Cai et al. 2007).

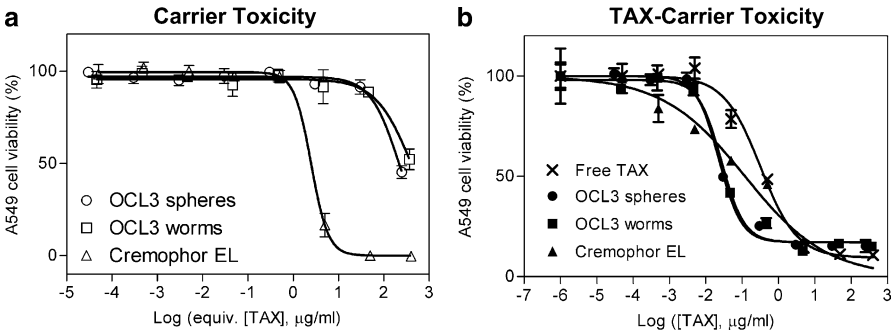


Fig. 8.16 (**a**) Cytotoxicity curves of excipients (OCL3 spheres, worms, and Cremophor EL) on A549 cells; (**b**) cytotoxicity curves of different TAX formulations on A549 cells (reprinted with permission from Cai et al. (2007). Copyright 2007 Springer)

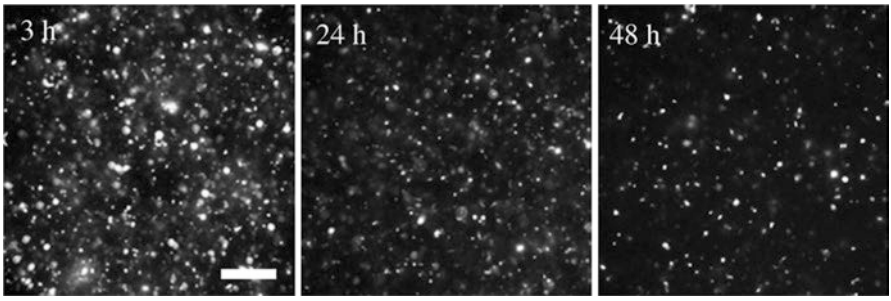


Fig. 8.17 Fluorescence microscopy images of blood samples from mice injected with fluorescently labeled polymersomes at different times. Scale bar is 5 μm (reprinted with permission from Ahmed et al. (2006a). Copyright 2006 Elsevier)

8.8 In Vivo Circulation of OCL Assemblies

Tumors are connected to the blood stream via blood vessels of several hundreds of nm in internal diameter. The ultimate goal of a drug-containing delivery system is to "leak" into the tumor vasculature to passively accumulate into the tumor via the EPR (enhanced permeation and retention) effect and eventually release the encapsulated drug. Long circulation times of the aggregates for drug delivery are thus desired when applied in vivo.

Nondegradable polymersomes have been found to persist in circulation longer with increasing PEO lengths (Photos et al. 2003). This is due to the steric stabilization that these PEO "brushes" confer to the aggregates and results in the avoidance, until certain extent, of the clearance from the circulation by phagocytosis. The influence of PEO chains on the circulation time has also been observed for polymersomes from PEO-PLA (Ahmed et al. 2006a). Such polymersomes were detected in the circulation of injected mice after 48 h (Fig. 8.17). That is a considerable longer time compared to ~200 nm polystyrene beads that did not contain PEO (<1 h).

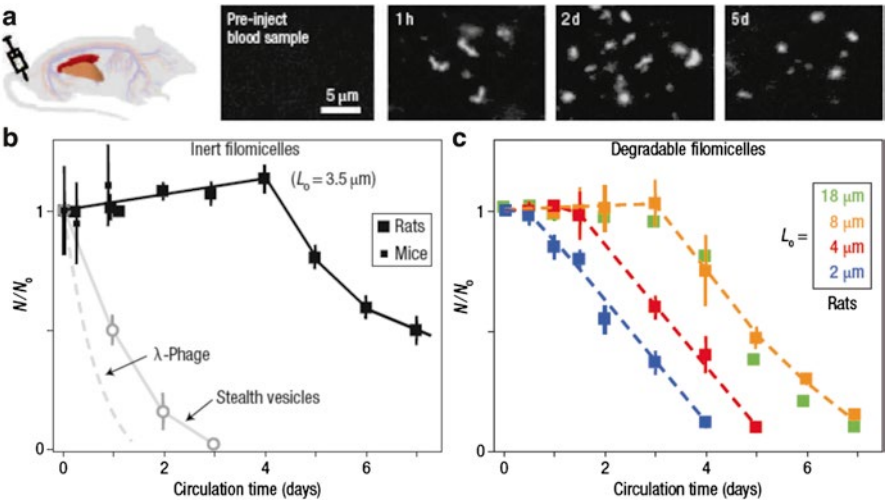


Fig. 8.18 Filomicelles and their persistent circulation. (**a**) Injection of fluorescent filomicelles into rodents, followed by fluorescent imaging of blood samples showed that filomicelles circulated in vivo for up to 1 week. (**b**) Relative numbers of inert filomicelles, stealth vesicles and λ-phages in the circulation over time. (**c**) Relative numbers of different sizes of degradable filomicelles from OCL in circulation. The error bars in (**b**) and (**c**) show the standard deviation for four or more animals (reproduced with permission from Geng et al. (2007). Copyright 2007 Nature Publishing Group)

Interestingly, worm-like micelles from OCL have shown even more prolonged circulation in vivo for up to a week (Fig. 8.18a) (Geng et al. 2007). These results show a clear difference between worm-like micelles, PEGylated "stealth" vesicles and quasi-linear λ-phages viruses, which are cleared within 2 and 1 days, respectively (Fig. 8.18b).

The circulation time of worm-like micelles was found to be dependent on their initial length. For worm-like micelles of L_0 up to ~8 μm, longer worm-like micelles persist longest in the circulation (Fig. 8.18c) (Geng et al. 2007). Interestingly, this length is similar to the diameter of blood cells which are likewise flexible and have a circulation time of several weeks (Pierigè et al. 2008). Longer worm-like micelles fragment rapidly with a decrease in length over time consistent with progressive shortening by mechanical forces.

To further understand the persistent circulation of worm-like micelles in circulation, the interaction of the aggregates with macrophages was investigated. Macrophages are phagocytic cells responsible for the removal of external particles from circulation found in the liver and the spleen. Results showed that contrary to the spherical assemblies, long worm-like micelles (≥3 μm) were able to avoid the phagocytosis resulting in an increase in the circulation time. Short worm-like micelles were internalized, however (Fig. 8.19a). The persistent circulation of the long worm-like micelles is attributed to the ability of the assemblies to align with the flow minimizing in that way interactions with the phagocytes. The images of the

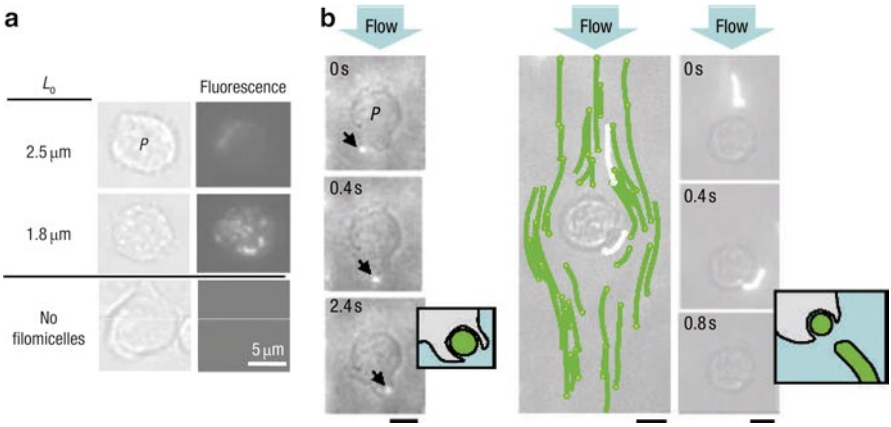


Fig. 8.19 (**a**) In vitro interactions between filomicelles and phagocytes (P). Fluorescent filomicelles of varying contour length were incubated with activated macrophages for 24 h in static culture. The fluorescence intensity of cells is proportional to the phagocytosis of filomicelles. (**b**) In a flow chamber with immobilized phagocytes, long filomicelles (*right*) flow past the cells, and occasionally leave a fragment, but smaller micelles and vesicles are captured (*left*, *arrows* point to small micelles and vesicles). Flow velocity is ~25 μm/s, which is similar to that in the spleen. The scale bars represent 5 μm (reproduced with permission from Geng et al. (2007). Copyright 2007 Nature Publishing Group)

assemblies in the presence of phagocytes showed that even if the worm-like micelles get in contact with the phagocytes, the dragging force that the flow exerts over the assemblies allows for the eventual avoidance of their internalization (Fig. 8.19b). This is not the case with spherical micelles that get much easier internalized. The in vitro results are in agreement with the in vivo studies in showing a clearance of submicron micelles and shorter worm-like micelles but not long worm-like micelles.

8.9 In Vivo Drug Release from OCL Assemblies

Studies with loaded OCL polymersomes on solid tumors derived from human breast cancer cells in mice showed that degradable polymersomes containing TAX and DOX had high antitumor activity (Ahmed et al. 2006a). The tumor size relative to controls was monitored over time after an injection of the loaded polymersomes (Fig. 8.20). The antitumor activity of the drug-loaded polymersomes was clear without any other toxic effect (i.e., no significant weight loss). The treated tumors shrank down to below 40 % relative to their initial area. Co-administration of free DOX and TAX did not result in such considerable tumor shrinkage; moreover, the tumor regrew rapidly by the third day after injection. By day 5, the tumor was 50 % larger with free drugs versus polymersome delivery.

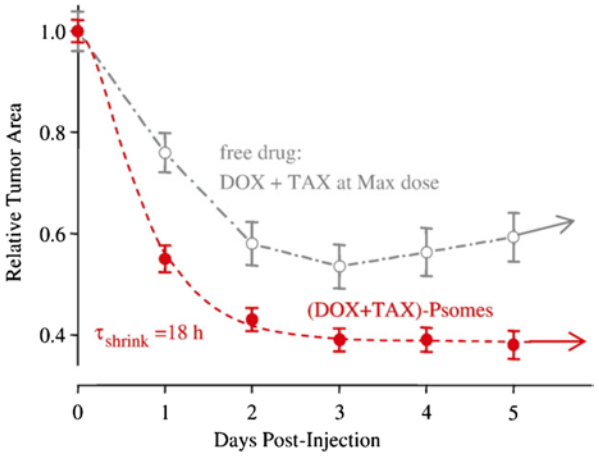


Fig. 8.20 Antitumor activity of TAX and DOX loaded OCL polymersomes compared to free drugs. The relative tumor areas (mean ± S.E.M.) are normalized by untreated control groups (reprinted with permission from Ahmed et al. (2006a). Copyright 2006 Elsevier)

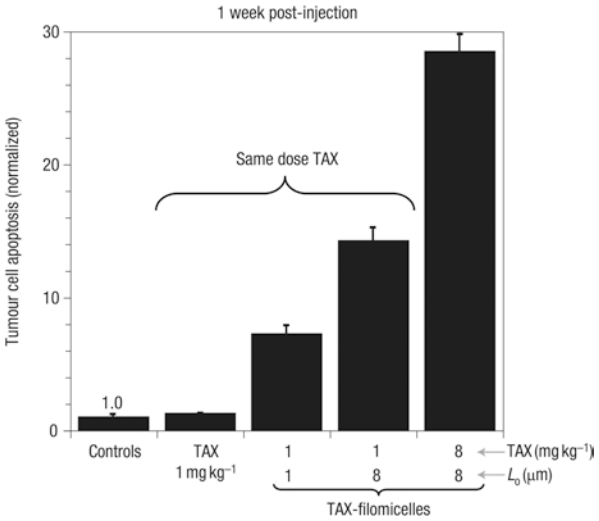


Fig. 8.21 Apoptosis measurements obtained by quantitative imaging of TUNEL-stained tumor sections after 1 week of injection. Results are obtained for free drug and OCL worm-like micelles loaded with different concentrations of TAX. All data shows the average from four mice. The error bars show the standard deviation (reproduced with permission from Geng et al. (2007). Copyright 2007 Nature Publishing Group)

The effectiveness of worm-like micelles in the in vivo delivery of drugs to tumors has also been investigated using worm-like micelles from OCL of different lengths loaded with TAX. For the same dose of drug, results showed that an eight-fold increase in worm-like micelle length resulted in a similar therapeutic effect as that of an eightfold increase in the TAX dosage (Fig. 8.21) (Geng et al. 2007).

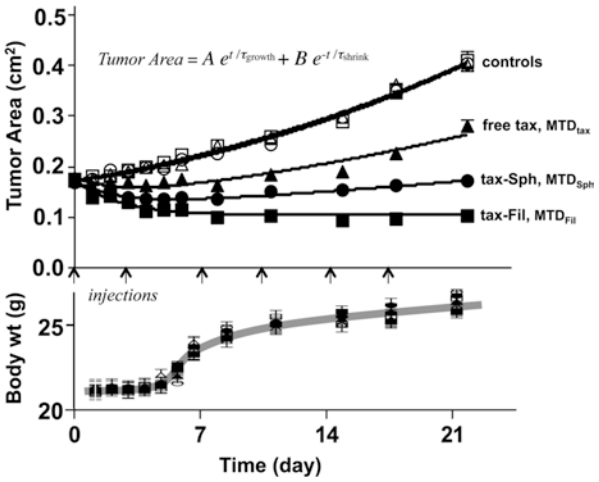


Fig. 8.22 Measurements of A549 tumor shrinkage after TAX administration. *Upper panel*: Tumor inhibition studies over a 3-week period with multiple injections of different treatments including controls (PBS alone, empty OCL3 spherical micelles or filomicelles), free TAX in PBS, OCL3 spherical micelles loaded with TAX at MTD (~8 mg/kg), and OCL3 filomicelles loaded with TAX at MTD (~16 mg/kg). The tumor growth profile was determined by tumor area monitored 24 h after each injection by measuring two orthogonal dimensions as $[(L_1 \times L_2)/2]$. The tumor growth-inhibition curves were fitted by applying the exponential modeling equation: $tumor\ size = Ae^{t/\tau_{growth}} + Be^{-t/\tau_{shrink}}$, where t is the time (week), A and τ_{growth} are constants of the tumor growth phase, and B and τ_{shrink} are constants of the TAX-inhibition phase. *Lower panel*: The body weight changes measured at 24 h after each injection during the 3-week experiment process show the mice gained similar weight for all experimental groups. All data points in this figure show (av ± SD) for four mice (reprinted with permission from Christian et al. (2009b). Copyright 2009 American Chemical Society)

In this way, the TAX dosage is significantly reduced compared to TAX-loaded spherical micelles of PEO-PLA (Kim et al. 2004). Once again, this illustrates the advantages of worm-like over spherical micelles, not only due to the higher loading capacity and longer circulation times, but also to their effectiveness in the in vivo drug delivery.

For comparison, worm-like and spherical micelles from OCL loaded with TAX were injected into mice. Mice injected with worm-like micelles showed a higher maximum-tolerated dose (MTD) ($MTD_{Fil} \approx 18$ mg/kg) compared to mice injected with OCL spherical micelles ($MTD_{Sph} \approx 10$ mg/kg). This shows once more that higher dosage is possible when using worm-like micelles injected into tumor-bearing mice.

The effect of the aggregate morphology on tumor shrinkage was studied by injecting loaded worm-like and spherical micelles into tumor-bearing mice. Although in all cases where TAX was administrated significant tumor shrinkage was observed, a clear difference in tumor size was observed between animals treated with free TAX, TAX-loaded spherical micelles, or TAX-loaded filomicelles decreasing accordingly (Fig. 8.22). Using worm-like micelles not only the tumor size of

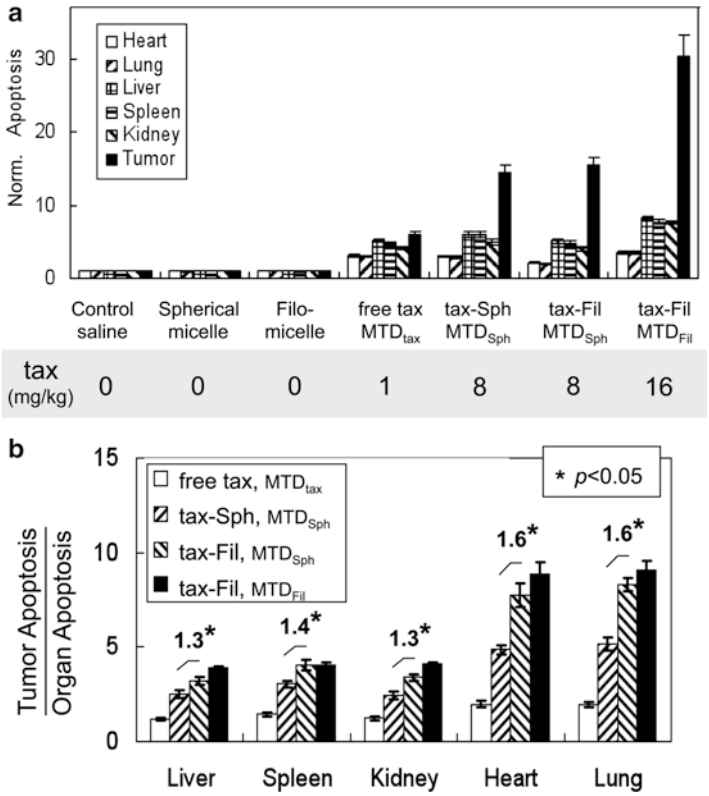


Fig. 8.23 Measurement of cell apoptosis in tumor and nontumor major organs. Measurements were made after a 22-day multiple injection experiment. (**a**) Cell apoptosis index measured by ELISA and calculated as: apoptosis index = enrichment factor of TAX treatment animal groups/enrichment factor of untreated animal groups. (**b**) Cell apoptosis index ratio between tumors and other nontumor organs (p < 0.05). All data points in this figure show (av ± SD) for four mice (reprinted with permission from Christian et al. (2009b). Copyright 2009 American Chemical Society)

treated mice was the smallest, but it also maintained its reduced size after 22 days of treatment.

Further studies showed higher specificity of tumor cytotoxicity when using worm-like micelles (Christian et al. 2009b). Comparing the apoptosis within tumor and nontumor organs, greater apoptosis was observed in the tumor without greatly increasing apoptosis in the nontumor organs when using TAX-loaded micelles compared to free drug (Fig. 8.23a). The apoptosis in nontumor organs was 15–30 % lower with filomicelles than with spherical micelles (Fig. 8.23b) and there was a clear increase in the tumor-selective cytotoxicity of TAX.

The biodistribution of TAX in vivo (as measured after extraction by HPLC) confirmed that TAX delivered by polymeric micelles accumulates in the tumors

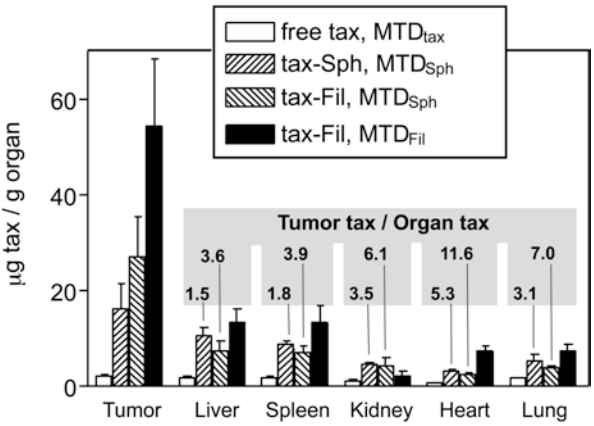


Fig. 8.24 Measurement of TAX biodistribution 24 h after single injections. All data points in this figure show (av ± SD) for four mice (reprinted with permission from Christian et al. (2009b). Copyright 2009 American Chemical Society)

more than TAX injected as free drug (Fig. 8.24). The higher tumor accumulation of aggregates with filamentous compared to spherical morphologies has also been reported for other block copolymers (Kim et al. 2012).

8.10 Summary and Conclusions

Self-assembled structures from amphiphilic block copolymers possess great potential for biomedical purposes such as drug delivery. As highlighted here, polymersomes and filomicelles made from biodegradable block copolymers possess specific features that seem suitable for such applications. Their specific chemical structure allows them to encapsulate drugs and therapeutics of different natures and their composition can be tuned for increasing their circulation time in blood. Likewise, the composition can be tuned to control their degradation and, thus, the release kinetics of the encapsulants.

Worm-like micelles have shown a particularly interesting behavior due to their flexibility and shape. On the one hand, the elongated structure allowed for higher loading capacity of hydrophobic drugs into the core and, on the other, their flexibility allowed them to escape from the phagocytes responsible for the clearance of external particles from the blood. Both structures have shown promising results in the delivery of drugs to tumors, as well as in the apoptosis of tumorous cells, when loaded with anticancer drugs. The shape as well as composition of the aggregates appears crucial to the efficacy of such drug delivery systems. Many additional possibilities remain to be explored with such nanocarriers, with many new and interesting materials for drug delivery likely to emerge in the near future.

Problem Box

Question 1:

List some of the key features in the design of a drug delivery system.

Answer

In the initial steps of the design of a drug delivery system several important questions to determine its specifications arise: what kind of tumor wants to be treated?, what drug is going to be delivered?, what delivery strategy is to be used?.

By answering these questions it is possible to decide on two things.

(a) The composition of the carrier.

The use of biodegradable materials which result into nontoxic subproducts would be desirable for in vivo applications. These materials will lead to a gradual release of the drug as a result of the degradation of the system.

Depending on the desired release mechanism different functionalities can be incorporated to trigger the degradation and therefore the release of the drug.

Incorporation of additional functionalities like targeting molecules would also influence the final performance of the nanocarrier.

(b) The morphology of the nanocarrier which can be controlled by the hydrophilic/hydrophobic ratios of the polymeric blocks. Factors like the nature of the drug to be delivered and the amount of drug to be loaded will favor the choice for spherical, worm-like micelles, or polymersomes among others.

Question 2:

What is one of the main disadvantages of intravenous administration of a free drug in solution in the treatment of cancer compared to the same loaded into a nanoparticle?

Answer

Intravenous administration of free drugs results in their fast renal clearance from the circulation. When encapsulated into nanoparticles, selective accumulation in tumor sites can be achieved by the EPR effect. This results in a higher concentration of drug and a greater influence on tumor proliferation. Other advantages of using nanoparticles include higher maximum-tolerated doses and higher specificity of tumor cytotoxicity reducing the damage to healthy tissue.

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Chapter 9
Polymeric Nanoparticles

Ijeoma F. Uchegbu, Aikaterini Lalatsa, and Dennis Wong

Abstract Self-assembling polymers, which are either amphiphilic block copolymers with hydrophobic and hydrophilic blocks, hydrophilic polymer backbones substituted with hydrophobic units or polymers with a low aqueous solubility, may all be used to prepare aqueous dispersions of polymeric nanoparticles. The amphiphilic variants form polymeric micelles and polymeric bilayer vesicles. The hydrophobic polymers form dense amorphous polymeric particles. Polymeric particles, of whichever nature, may be loaded with hydrophobic and hydrophilic drugs, and the bioavailability of the drug compound is altered by this encapsulation within a polymeric nanoparticle. This simple concept has been exploited heavily to yield enhancements in oral, tumour and brain bioavailability and some of these polymeric nanoparticle formulations have undergone clinical testing and even been commercialised, e.g. the nanoparticle paclitaxel formulation Abraxane.

9.1 Introduction

A book on pharmaceutical nanoscience would be incomplete without a chapter on polymer nanoparticles. Polymers are extensively used in pharmacy (Uchegbu and Schatzlein 2006) and form the backbone to a number of nanomedicines. Polymers

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are excellent materials to use in the fabrication of pharmaceutical nanoparticles as the size of the nanoparticle may be controlled via the polymer chemistry [i.e. polymer molecular weight (Wang et al. 2001a) and polymer hydrophobicity (Wang et al. 2004)] and polymer nanoparticles may be easily loaded with both hydrophobic (Qu et al. 2006; Siew et al. 2012) and hydrophilic (Dufes et al. 2000) drugs, with hydrophilic drugs being encapsulated within polymeric bilayer vesicles (Dufes et al. 2000), for example. The polymers that have been most commonly used to prepare polymeric nanoparticles include the polyesters [e.g. poly(D,L-lactide *co*-glycolide) (Seju et al. 2011; Yang et al. 2012), poly(D,L-lactide)-*co*-poly(ethylene glycol) (PLA-PEG) (Hrkach et al. 2012), poly(D,L-lactide *co*-glycolide)-*co*-poly(ethylene glycol) (Ensign et al. 2012; Hrkach et al. 2012)], chitosans [e.g. *N*-monomethyl, *N*,*N*-dimethyl, *N*,*N*,*N*-trimethyl, *N*-palmitoyl, 6-*O*-glycol chitosan—quaternary ammonium palmitoyl glycol chitosan (GCPQ) (Uchegbu et al. 2001; Qu et al. 2006; Lalatsa et al. 2012b), 5β-cholanic acid glycol chitosan (Min et al. 2008), cross-linked chitosan (Trapani et al. 2013), alginate–chitosan coacervates (Sarmento et al. 2007)], polyamino acids [e.g. amphiphilic poly(L-lysine) and amphiphilic poly(γ-glutamic acid) (Wang et al. 2000; Nakagawa 2008; Lalatsa et al. 2012c)], acrylates [e.g. poly(*n*-butyl cyanoacrylate)] (Wohlfart et al. 2011) and hyaluronic acid (Yoon et al. 2012). Polymer nanoparticles form either by polymer self-assembly in aqueous media due to their amphiphilic character (Qu et al. 2006), are formed by ionic gelation in the case of the aqueous-soluble forms of chitosan (Dyer et al. 2002; Trapani et al. 2013) or are precipitated in the presence of a suitable surface-active agent into nanoprecipitates in aqueous media due to their hydrophobicity (Win and Feng 2006). Polymer nanoparticles may take the form of polymeric micelles (Wang et al. 2004), polymeric bilayer vesicles from polymers bearing hydrophobic pendant groups (Brown et al. 1999; Wang et al. 2004), polymeric vesicles formed from block copolymers (the polymerosomes covered elsewhere in this volume) or indeed polymeric amorphous dense nanoparticles (Wang et al. 2004). This chapter will focus mainly on polymer nanoparticles (polymeric micelles, polymeric vesicles prepared from non-block copolymers and dense polymeric nanoparticles) that are prepared by the precipitation or self-assembly of polymers alone and will not deal with polymer coatings on liposomes or polymer–drug conjugates that form nanoparticles. Liposomes and polymer–drug conjugates are covered elsewhere in this volume. Dense polymer nanoparticles and polymeric micelles may be loaded with hydrophobic drug compounds such as propofol (Qu et al. 2006), cyclosporine A (Siew et al. 2012) or paclitaxel (Win and Feng 2006), while polymer bilayer vesicles may be loaded with hydrophilic compounds such as dextran (Dufes et al. 2000). The biodistribution of the encapsulated drug compounds is largely controlled thereafter by the properties of the nanoparticles: surface chemistry and particle size, with the core and surface characteristics determining to a large extent the residence time of the drug molecules within the particles.

9.2 Materials Chemistry

Polymer nanoparticles for biomedical use are invariably dispersed within an aqueous medium. These polymer nanoparticles may be formed in three broad ways and each method of fabrication calls for a particular polymer chemistry. Polymers which form nanoparticles may be hydrophobic, hydrophilic or amphiphilic (Fig. 9.1), and polymer nanoparticles may be formed from the precipitation of a hydrophobic polymer (Fig. 9.1a, b) from an organic solvent solution of the polymer with the polymer nanoparticle stabilised against aggregation by surfactant present in the aqueous medium (Ensign et al. 2012). Another method of preparing polymer nanoparticles from hydrophobic non-biodegradable polymers is to carry out an in situ polymerisation step using a monomer to produce a hydrophobic polymer (Fig. 9.1c), with the polymer nanoparticles once again stabilised by a hydrophilic polymer in the aqueous media (Wohlfart et al. 2011). Hydrophilic polymers (Fig. 9.1d, e) are used to prepare polymer nanoparticles by preventing their solubility in aqueous media using physical low molecular weight cross-linking agents such as tripolyphosphate (Shahnaz et al. 2012). Amphiphilic polymer (Fig. 9.1f–i) self-assembly to polymer nanoparticles takes place when the amphiphilic polymer is subjected to either simple shaking to produce polymeric micelles or probe sonication in the presence of a drug to produce polymeric nanoparticles (Qu et al. 2006; Lalatsa et al. 2012c).

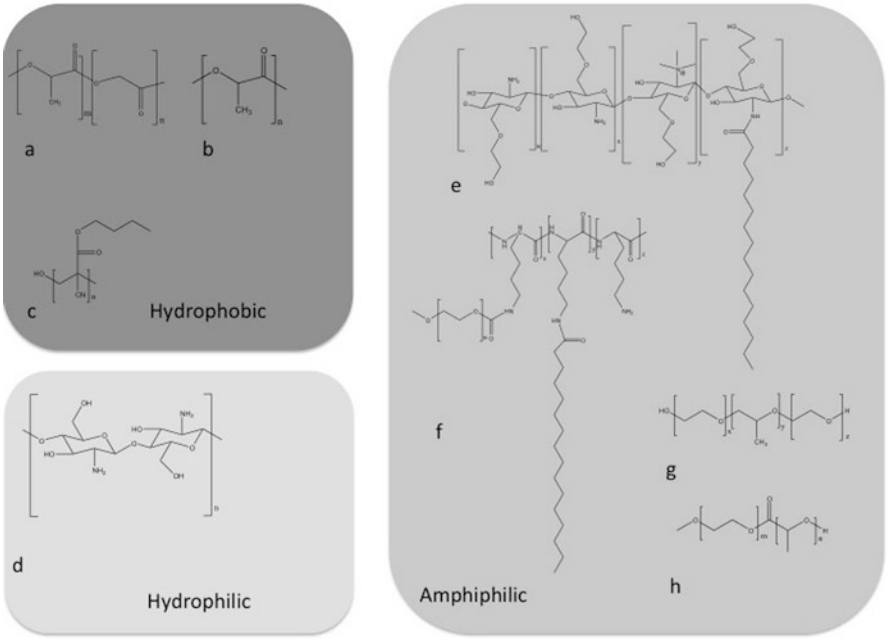


Fig. 9.1 Polymers used to prepare polymeric nanoparticles

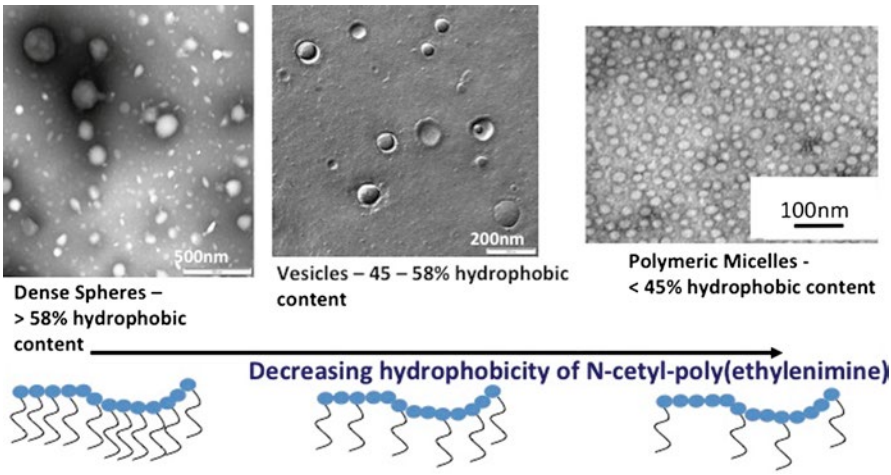


Fig. 9.2 Polymeric nanoparticles, polymeric vesicles and polymeric micelles all formed from various cetyl poly(ethylenimine) amphiphiles

The amphiphilic polymers have been widely studied and the resulting self-assembly found to be governed by the hydrophobicity of the polymer (Fig. 9.2) in the case of the amphiphilic polyelectrolyte *N*-cetyl-poly(ethylenimine) (Wang et al. 2004), with the more hydrophilic polymers forming polymeric micelles, the hydrophobic polymers forming dense amorphous polymeric particles and the polymers of intermediate hydrophobicity forming polymeric bilayer vesicles. Polymeric micelles arise from *N*-cetyl-poly(ethylenimine) (Wang et al. 2004) with a molar hydrophobic content of less than 45 % or a mol% cetyl substitution of less than 8 mol% (Wang et al. 2004). A similar situation has been observed with amphiphilic block copolymers comprising poly(ethylene glycol) and hydrophobic peptide blocks [poly(ethylene glycol)-*block*-poly(L-amino acids)], in which micelle-forming polymers are formed when 6.5–11.6 mol% of the monomers are hydrophobic (Van Domeselaar et al. 2003). The self-assembly of poly(*N*ε-palmitoyl-L-lysine)-graft-poly(ethylene oxide) amphiphiles into bilayer polymeric vesicles is also governed by the hydrophobicity of the polymer (i.e. the level of palmitoyl substitution) (Wang et al. 2001b). A vesicle formation index (*F*) has been derived for these self-assemblies (9.1).

$$F = \frac{H}{L\sqrt{DP}} \tag{9.1}$$

where H = mol% of underivatized polymer monomers, L = mol% of hydrophobic unit derivatized monomers and DP = degree of polymerisation of the polymer backbone. An F value in excess of 0.168 gives rise to vesicles in the case of poly(*Nε-palmitoyl-L*-lysine)-graft-poly(ethylene oxide) amphiphiles (Wang et al. 2001b), whereas an F in excess of 0.11 gives rise to vesicles in the case of *N-cetyl-poly*(ethylenimine)

amphiphiles (Wang et al. 2004; Lalatsa et al. 2012c). *F* levels below the values given above give rise to dense amorphous polymeric nanoparticles.

Unlike low molecular weight bilayer vesicle self-assemblies, the size of polymeric vesicles may be set by choosing a polymer of a particular molecular weight as there is a linear relationship between the square root of the molecular weight and the resulting vesicle size (9.2) for GCPQ amphiphiles (Wang et al. 2001a).

$$\sqrt{MW} = 0.78d_{\mathrm{v}} + 107 \tag{9.2}$$

where MW = polymer molecular weight, d_{v} = the *z*-average mean diameter of the resulting polymeric vesicles. There is also a linear relationship between the mol% polymer hydrophobic substitution (*Ct*) and the size of both polymeric vesicles (d_{v}—(9.3)) and polymer dense amorphous nanoparticles (d_{n}—(9.4)) for the *N*-cetyl poly(ethylenimine) polymers (Wang et al. 2004).

$$d_{\mathrm{v}} = 1.95Ct + 139 \tag{9.3}$$

$$d_{\mathrm{n}} = 2.31Ct + 5.55 \tag{9.4}$$

Just as the chemistry of the polymer may be used to predict the nature and size of the resulting polymeric nanoparticles, so also may the drug loading (Qu et al. 2006), in that more hydrophobic (>10 mol palmitoyl substitution) and higher molecular weight (15–20 kDa) GCPQ polymers encapsulate higher levels of the hydrophobic drug propofol. Additionally, the more hydrophobic *N*-cetyl, *N*-trimethyl, *N*-dimethyl, *N*-monomethyl-poly(ethylenimine) polymers encapsulate higher levels of cyclosporine A (Le et al. 2013). In essence for the delivery of hydrophobic drugs it is advisable to select the more hydrophobic and higher molecular weight variants.

9.3 Preparation and Characterisation of Polymeric Nanoparticles

9.3.1 Preparation of Polymeric Nanoparticles

Polymeric nanoparticles are generally formed from the precipitation of an organic solution of the polymer in aqueous media or by the dispersion of an amphiphilic polymer in aqueous media using probe sonication (Lalatsa et al. 2012c). The emulsion–solvent evaporation technique is a popular method of preparing polymeric nanoparticles from hydrophobic polymers (Hrkach et al. 2012; Hu et al. 2013). The drug and the polymer are dissolved in an organic solvent such as dichloromethane and then dispersed in an aqueous solution containing a hydrophilic surfactant and the emulsion produced by probe sonication (Hu et al. 2013) or microfluidisation (Hrkach et al. 2012) or a combination of probe sonication and homogenisation

(Kim et al. 2012). The resulting emulsion is then left stirring to evaporate the organic solvent, with the nanoparticles precipitating out of the oil phase of the oil in water emulsion and being stabilised by the surfactant in the aqueous phase. In some cases an emulsion is not formed and the nanoparticles are precipitated out of a water miscible organic phase (e.g. acetone) as the organic solvent is added drop wise to an aqueous phase (Ensign et al. 2012). In some instances the drug is added as an organic solution to an aqueous dispersion of the amphiphilic polymer and the organic solvent subsequently removed by dialysis (Lee et al. 2012). For amphiphilic polymers, the polymer and solid hydrophobic drug are added to aqueous media and probe sonicated to produce the drug-loaded polymeric nanoparticle dispersion (Uchegbu et al. 1998; Cheng et al. 2006; Qu et al. 2006; Lalatsa et al. 2012b; Chooi et al. 2013). This is the only method which does not use organic solvents. Water-soluble polymer-based nanoparticles formed by ionic gelation are prepared by the addition of a drug and a gelating agent, e.g. tripolyphosphate to an acid-soluble polymer such as chitosan (Shahnaz et al. 2012). Regardless of the method of nanoparticle formulation, unentrapped hydrophilic drugs are removed by ultracentrifugation (Uchegbu et al. 1998; Shahnaz et al. 2012), while unentrapped hydrophobic drug crystals are usually removed by filtration through 0.45 μM filters (Qu et al. 2006) or if destined for oral delivery not filtered at all (Siew et al. 2012).

9.3.2 Polymer and Nanoparticle Characterisation

Prior to the preparation of drug-loaded nanoparticles, the polymers themselves are structurally characterised using nuclear magnetic resonance spectrometry (Fig. 9.3) (Wang et al. 2000), a test for various chemical groups such as the primary amine function in poly(*N*ε-palmitoyl-L-lysine)-graft-poly(ethylene oxide) (Wang et al. 2000) is carried out and their molecular weight is measured using gel permeation chromatography-multi angle laser light scattering (GPC-MALLS) (Wang et al. 2001a) or matrix assisted laser desorption time of flight (MALDI-TOF) analyses (Chooi et al. 2010).

Amphiphilic polymers self-assemble in aqueous media at a critical concentration—the critical micellar concentration (CMC) (Alexandridis et al. 1994; Chooi et al. 2013). This self-assembly is normally entropy driven at ambient temperature. The water molecules adjacent to the hydrophobic groups in the molecule are at an entropy deficit and achieve an entropy gain, being free to hydrogen bond in all geometric dimensions on polymer aggregation (Chooi et al. 2010) in a similar manner to low molecular weight amphiphiles (Tanford 1980). The CMC (Table 9.1) is important as it is a measure of the stability of the amphiphilic aggregates and the CMC has an influence on drug loading, with lower CMCs leading to a higher % w/w drug loading on the nanoparticle (Table 9.2) (Chooi et al. 2013).

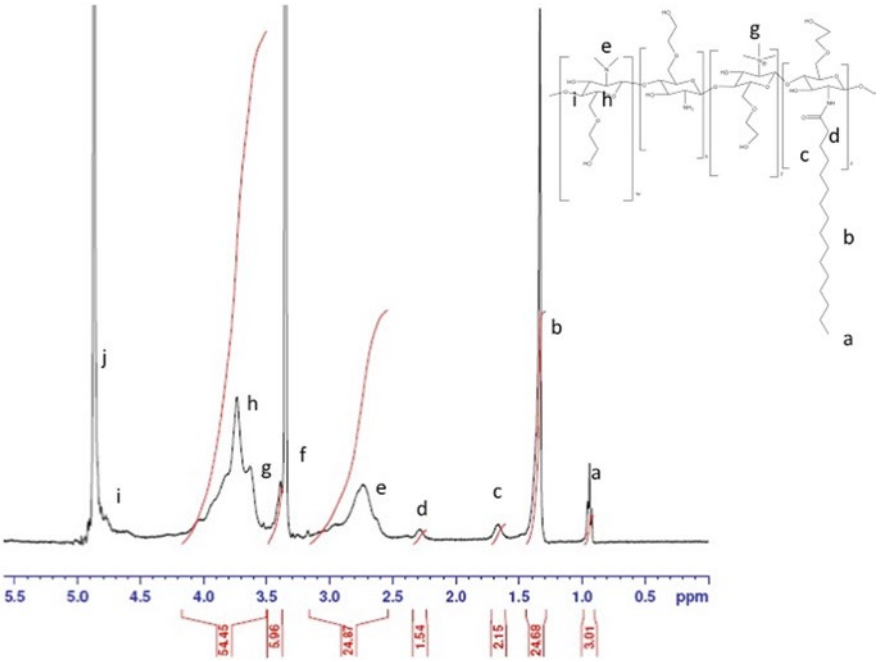


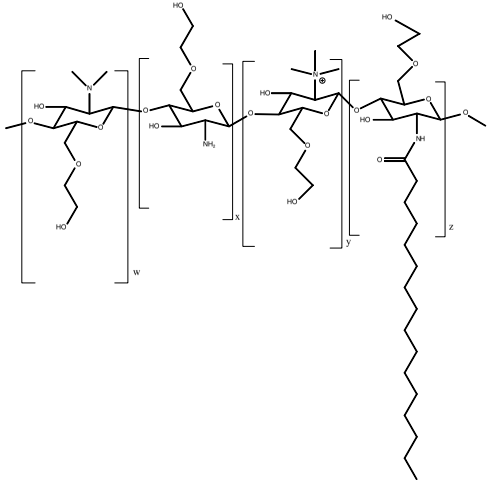
Fig. 9.3 GCPQ NMR and assignment. Peaks f and j originate from the methanol and water solvent respectively

CMC measurements are carried out as detailed in Chap. 2. The isothermal calorimetry method is the most sensitive method of determining an amphiphile's CMC and is considered the most accurate as it is probe free (Chooi et al. 2010, 2013; Siew et al. 2012). Once drug-loaded nanoparticles are formed, they are characterised for particle size, morphology, zeta potential and drug loading. The particle size distribution is measured using photon correlation spectroscopy and particle zeta potential (Fig. 9.4) using electrophoresis (Uchegbu et al. 2004) and reported as the *z*-average mean diameter and particle distribution polydispersity and surface charge (Chooi et al. 2013). Particle morphology is characterised using electron (Fig. 9.5) or atomic force microscopy. Electron microscopy is an excellent method to use to probe the shape of the particles (Fig. 9.5).

Drug loading is measured by removing unentrapped drug by filtration and centrifugation or dialysis, as outlined above, extracting the encapsulated drug (destroying the nanoparticles by the addition of organic solvent) and measuring the level of encapsulated drug using standard analytical methods such as high performance liquid chromatography (HPLC) (Qu et al. 2006; Shahnaz et al. 2012). Indirect methods of analysing drug content include analysing the level of unentrapped drug and working out the level of drug entrapped by difference (Sarmento et al. 2007).

Table 9.1 The critical micellar concentration of chitosan amphiphiles

Polymer	Polymer	Mw (kDa)	Mw/Mn	Mol % palmitoylation	Mol% quaternary ammonium units	Number of chitosan amphiphile arms	CMC (isothermal calorimetry, μM)
N-palmitoyl, *N*-dimethyl, *N*-monomethyl *N*-trimethylammonium, 6-*O*-glycol chitosan—GCPQ (Siew et al. 2012)	GCPQ24H	12.19	1.197	16.14 ± 1.0	8.30 ± 1.32	N/A	19
	GCPQQ24L	11.48	1.447	14.46 ± 2.29	8.07 ± 1.54	N/A	25
	GCPQ48H	8.70	1.025	15.48 ± 0.49	11.42 ± 0.64	N/A	20
	GCPQ48L	8.18	1.145	9.02 ± 1.27	14.50 ± 4.10	N/A	25



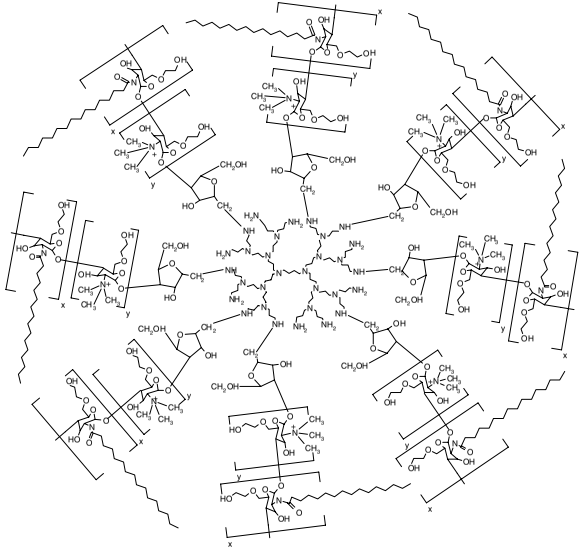
N-graft-(*N*,*N*,*N*-trimethyl, *N*,*N*-dimethyl, *N*-monomethyl, *N*-acetyl, *N*-palmitoyl, 6-*O*-glycol chitosan)-diaminobutane poly(propylenimine) dendrimer (Chooi et al. 2013) 	DAB-GCPQA30	33.0	1.093	4.10	7.80	6	0.013
	DAB-GCPQ70	103.3	1.486	2.5	7.8	14	0.009

Table 9.2 The in vitro cytotoxicity of hyaluronic amphiphile-based nanoparticles

Sample	IC_{50} (μg mL^{-1}) B16 F10 cell line (CD44 positive)	NIH 3T3 cell line (CD44 negative)
16A5	22.3	20.7
Etoposide alone	5.8	2.4
16A5 + etoposide (1:1.8 g g^{-1})	13.2	5.7
16A5 + etoposide (1:1.8 g g^{-1}) + hyaluronic acid (molecular weight >1,000 kDa, 100 μg mL^{-1})	>1,000	3.48

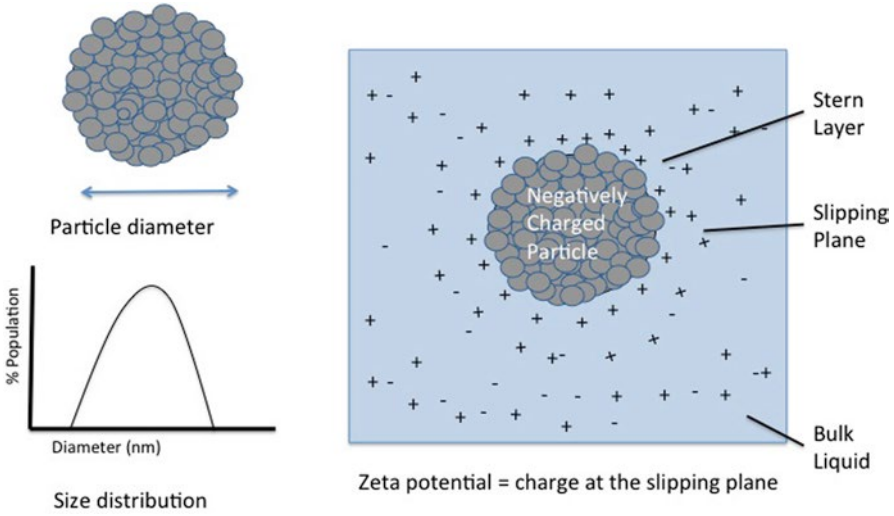


Fig. 9.4 Particle characterisation—measuring particle size and particle zeta potential

9.4 The Application of Polymer Nanoparticles to Pharmacy and Medicine

Drug-loaded polymer nanoparticles have been employed in pharmacy to achieve a change in drug biodistribution as once encapsulated, the drug biodistribution is governed by the characteristics of the particle, particularly the particle surface chemistry. The polymers most widely used in pharmaceutical formulations are shown in Fig. 9.1.

Polymeric nanoparticles have been administered by the intravenous, oral, topical ocular and nasal routes. These are the most common routes of administration that appear in the literature. They have been used to deliver drugs to tumour tissue,

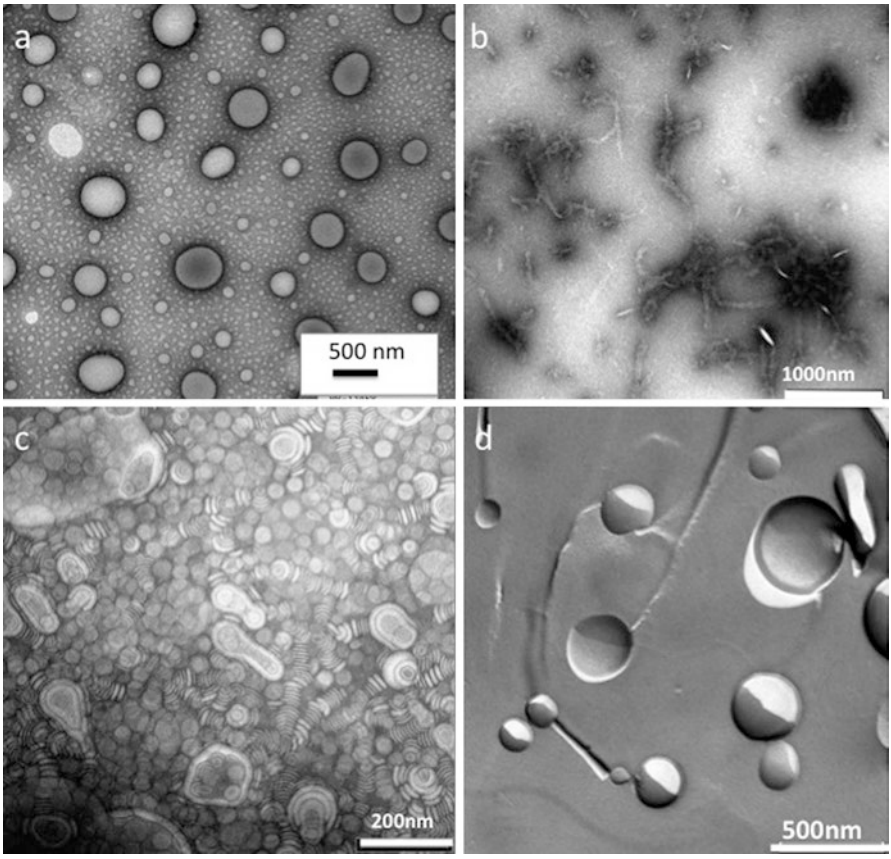


Fig. 9.5 (**a**) Negative-stained transmission electron micrograph of GCPQ nanoparticles (GCPQ, 10 mg mL^{-1}—cyclosporine A 2 mg mL^{-1}) nanoparticles in water; these GCPQ—cyclosporine A nanoparticles increase the bioavailability of cyclosporine by threefold and the C_{max} by fivefold, on oral administration to rats, when compared to cyclosporine A in water (Siew et al. 2012). (**b**) Negative-stained transmission electron micrograph of *N*-palmitoyl, poly(propylenimine) dendrimer (DPD5, 10 mg mL^{-1}) tubules in water; dendrimer amphiphiles bear an average of five palmitoyl groups per dendrimer molecule (Chooi et al. 2010). (**c**) Negative-stained transmission micrograph of *N*-cetyl poly(propylenimine) dendrimer (5 mg mL^{-1}) and cholesterol (2.5 mg mL^{-1}) disc-shaped nanoparticles dispersed in water; dendrimer amphiphiles bear approximately 1 cetyl unit per dendrimer molecule (Qu et al. 2008). (**d**) Freeze fracture electron micrograph of *N*-cetyl poly(ethylenimine) (4 mg mL^{-1}), cholesterol (2 mg mL^{-1}) bilayer vesicles in water; poly(ethylenimine) amphiphiles bear 37 cetyl groups per 100 ethylene amine monomers (Wang et al. 2004)

deliver drugs across the blood brain barrier via the intravenous and nasal routes, deliver peptides to the brain via the oral route, reduce side effects, prolong drug residence time within ocular tissue and deliver gut labile drugs such as peptides across the gut epithelium. Examples of all of these advances are presented in this chapter.

9.4.1 Intravenous

9.4.1.1 Anticancer Drugs

Beneficially altering drug biodistribution such that there is a higher level of drug at tumour sites and less drug accumulating in healthy tissue is one of the ways that polymer nanoparticles have been exploited. The most successful polymer nanoparticle therapeutic to date is Abraxane. Abraxane was licensed in 2005 for the treatment of refractory malignant breast cancer. There are currently 1,762 trials on the clinical trials database (clinicaltrials.gov), which were aimed at evaluating the new treatment regimens for Abraxane, either as part of a combination therapy or for new indications (Uchegbu and Siew 2013). Abraxane is composed of nanocrystalline paclitaxel stabilised by albumin (Fu et al. 2009; Cortes and Saura 2010). The albumin coating on the nanoparticles has been implicated as being responsible for the targeting of paclitaxel to metastatic tissue as albumin binds to secreted protein acidic and rich in cysteine (SPARC) (Cortes and Saura 2010), a protein which accumulates in the tumour microenvironment and a protein that is associated with a high metastatic potential in most human cancers (Podhajcer et al. 2008). The use of albumin-stabilised paclitaxel also solves a delivery problem associated with paclitaxel's poor water solubility (<2 μg mL^{-1}) as paclitaxel was erstwhile formulated with Cremophor EL, a surfactant associated with severe hypersensitivity reactions in patients (Gelderblom et al. 2001). The Abraxane formulation presents a solution to these anaphylactoid responses to the surfactant Cremophor EL. These hypersensitivity reactions have led others to seek alternative paclitaxel formulations, with many such formulations involving the use of polymers and as such there are clinical trials registered involving Samyang's Genexol PM, in which paclitaxel is solubilised in aqueous media within methoxy poly(ethylene glycol)-b-poly(lactide-*co*-glycolide) block copolymers (Uchegbu and Siew 2013). Genexol PM has been tested in metastatic breast cancer patients, for example. Other formulations of paclitaxel destined for the intravenous route include paclitaxel encapsulated within poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanoparticles stabilised with Vitamin E poly(ethylene glycol) succinate (Vitamine E TPGS) (Win and Feng 2006). This formulation gave a higher AUC when compared to Taxol (paclitaxel solubilised with Cremophor EL). Docetaxel, a paclitaxel analogue, has also recently been clinically evaluated in a poly(D,L-lactic acid)-*block*-methoxypoly(ethylene glycol) block copolymer nanoparticle decorated with a ligand specific for prostate-specific membrane antigen—*S*,*S*-2[3-[5-amino-1-carboxy pentyl]-ureido]-pentanedioc acid (ACUPA) and evaluated in prostate cancer patients (Hrkach et al. 2012). This new nanoenabled docetaxel formulation was found to be superior to docetaxel alone with respect to extending the plasma half-life of the drug (Hrkach et al. 2012).

Amphiphilic glycol chitosans, in which glycol chitosan is conjugated to palmitoyl units, were first described in 1998 (Uchegbu et al. 1998). GCPQ, a glycol chitosan amphiphile (Fig. 9.1e), forms drug-carrying nanoparticles and these particles do not accumulate in the liver on intravenous administration (Lalatsa et al. 2012b). Glycol chitosan amphiphiles prepared by derivatizing glycol chitosan with cholanic acid

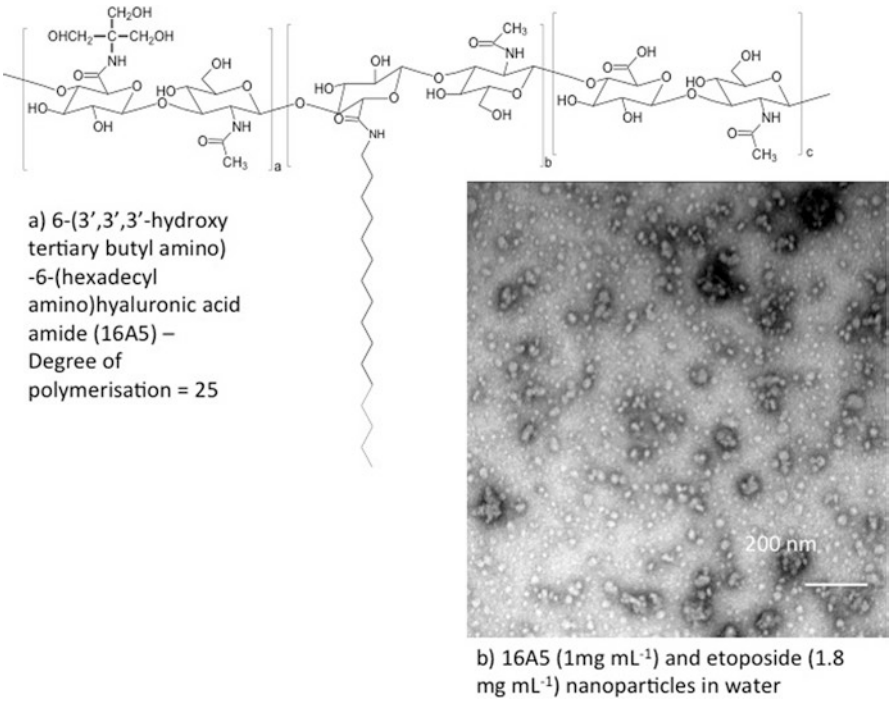


Fig. 9.6 (**a**) Amphiphilic hyaluronic acid (16A5) used to prepare etoposide-loaded hyaluronic acid-based nanoparticles, (**b**) transmission electron micrographs of hyaluronic acid-based nanoparticles. 16A5 was synthesised by the conjugation of hexadecylamine and trihydroxyltertiary butylamine to hyaluronic acid (degree of polymerisation = 25). The polymers form nanoparticles that are able to encapsulate hydrophobic drugs such as etoposide. The resulting nanoparticles show selectivity for cells possessing the CD44 receptor (Table 9.2)

have been found to localise within tumour tissue (Na et al. 2012), possibly as a result of the enhanced permeation and retention effect first described by Maeda (1992) and these glycol chitosan nanoparticles, when loaded with camptothecin, were superior in achieving tumouricidal activity when compared to the drug in solution (Min et al. 2008).

Chitosan amphiphile nanoparticles have also been targeted to tumour tissue using octreotide in the form of a stearylamine—poly(ethylene glycol)—octreotide to target *N*-octyl, 6-*O*-carboxymethyl chitosan nanoparticles to the somatostatin receptor on tumour cells (Zou et al. 2013). The use of the octreotide-targeting ligands improved tumouricidal activity in mice bearing the MCF-7 tumour cells.

An additional polymer that has been exploited for tumour targeting is hyaluronic acid. Hyaluronic acid nanoparticles have been prepared from dodecoyl hyaluronate (Fig. 9.6) and found to encapsulate drugs and show selectivity for the CD44 receptor (Table 9.2). Amphiphilic hyaluronic acid prepared by the reaction of hyaluronic acid with amino 5-beta-cholanic and amino poly(ethylene glycol)

also forms particles that are able to deliver the photosensitiser—chlorin e6—to tumour tissue bearing the CD44 receptor, generate singlet oxygen within HT-29 tumour tissue, on near-infrared irradiation and cause tumour regression (Yoon et al. 2012). The nanoparticles have also been used to image tumour tissue.

Finally, low molecular weight drugs have been employed as constituents of polyionic complexes (PICs), in which a nanoparticle is formed by electrostatic attractive forces. An example of this is the formulation of *cis-dichlorodiammineplatinum* II (CDDP) with poly(ethylene oxide)-*block*-poly(α,β-aspartic acid) to yield 20 nm PICs, which slowly release CDDP over time (Nishiyama et al. 1999, 2003) and significantly enhance tumour exposure to CDDP (Nishiyama et al. 1999; Uchino et al. 2005).

Polymeric micelle cancer drug formulations such as poly(ethylene oxide)-*block*-*poly*(benzyl-L-aspartate) doxorubicin (Kataoka et al. 2000) or poly(ethylene oxide)-*block*-poly(cetyl, benzyl-L-aspartate) formulations (Yokoyama et al. 1998b) increase the blood residence time of the encapsulated drug (Kataoka et al. 2000), enabling the micelles to accumulate in the tumour presumably by exploiting the enhanced permeation and retention effect (Maeda et al. 2000) in which particulate drugs escape the leaky tumour vasculature to become entrapped in the tumour tissue. Ultimately improved tumouricidal activity is observed with these polymeric micelles (Yokoyama et al. 1998a; Kataoka et al. 2000).

9.4.1.2 Anti-infectives

Specific drug delivery applications of polymeric micelles include the encapsulation of hydrophobic anti-infectives such as amphotericin B (Adams et al. 2003).

Amphotericin B-loaded poly(ethylene oxide)-*block*-poly(benzyl-L-aspartate) micelles decrease the haemolytic activity of amphotericin B and provide a means by which the aqueous incorporation of the hydrophobic amphotericin B may be increased by two orders of magnitude (Yu et al. 1998). Additionally, a derivatized aspartic acid-based poly(ethylene oxide) block copolymer poly(ethylene oxide)-*block*-poly[*N*-(6-hexylstearate)-L-aspartamide], when loaded with amphotericin B, combined a reduction in amphotericin B-mediated haemolytic activity with an equivalent level of efficacy with fungizone in a murine neutropenic model of disseminated candidiasis (Adams et al. 2003). The more hydrophobic poly(ethylene oxide)-*block*-poly[*N*-(6-hexylstearate)-L-aspartamide] amphiphiles (50–70 mol% stearic acid substitution) were more efficient modulators of haemolytic activity, as amphiphile hydrophobicity promoted drug retention within the polymeric particles (Lavasanifar et al. 2002).

9.4.1.3 Across BBB

Delivering drugs to the brain is severely limited by the blood–brain barrier, an anatomical and physiological barrier composed of the neurovascular unit, efflux

membrane transporters and degrading enzymes (Lalatsa et al. 2011). Various methods have been developed to overcome this barrier and polymers have figured largely as transport vectors. The exploitation of transport pathways on brain endothelial cells has resulted in polymer nanoparticles being transcytosed across brain endothelial cells (Zensi et al. 2009). The various transporters that have been used include the transporter specific to Apolipoprotein E (Zensi et al. 2009). When Apoliprotein E was conjugated to cross-linked albumin nanoparticles there was particle uptake across the brain endothelial cells. Exploitation of this transport of polymer particles across the blood–brain barrier for medicinal benefit was realised when doxorubicin was delivered to the brain following intravenous administration of doxorubicin poly(*n*-butyl) cyanoacrylate nanoparticles coated with polysorbate 80 (Wohlfart et al. 2011). Coated particles were superior to non-coated particles in delivering doxorubicin to the brain and this is thought to be due to the polysorbate 80-coated particle ability to recruit plasma apolipoprotein E to the particles' surface and use this ligand to access transport across the brain endothelial cells via the LRP1 receptor (Kreuter et al. 2002).

Other mechanisms by which polymer particle delivery across the blood–brain barrier may be achieved include the conjugation of F3 peptide (CKDEPQRRSARLAKPAPPKPEPKPKKAPAKK) to the surface of poly(ethylene glycol)-b-poly(lactic acid) nanoparticles (Hu et al. 2013). The F3 peptide targets nucleolin, a transport protein expressed on the surface of glioma cells and the tumour neovasculature brain endothelial cells. When these F3-decorated nanoparticles, loaded with paclitaxel, are administered in tandem with a cell-penetrating peptide (tLyp-1—CGNKRTR), which targets neurolipin-1, a transmembrane protein overexpressed on the tumour vasculature, there is improved survival in animals bearing intracranial C6 glioma cells (Hu et al. 2013).

The delivery of macromolecules to the brain has been achieved using receptor-mediated uptake technology as conjugating the rabies virus glycoprotein (RVG29) to the surface of a Pluronic–chitosan-based nanocarrier resulted in delivery of the protein beta-galactosidase to the brain after intravenous administration (Kim et al. 2013).

Although the uptake of polymer nanoparticles by brain endothelial cells is an attractive concept, the delivery of drugs to the brain may also be accompanied by no real particle transport across the blood–brain barrier. In the case of GCPQ particles loaded with leucine^5-enkephalin or a leucine^5-enkephalin lipidic prodrug tyrosine^1-palmitate, leucine^5-enkeplalin (TPLENK), the particles are not taken up by the blood–brain barrier (Lalatsa et al. 2012b; Moger et al. 2012) yet there is sufficient peptide drug delivery to the brain to elicit an antinociceptive response on intravenous administration of GCPQ nanoparticle-encapsulated leucine^5-enkephalin or TPLENK, whereas intravenous administration of leucine^5-enkephalin does not elicit an antinociceptive response. The positively charged GCPQ particles appear to adhere to the luminal side of the brain endothelial cells (Moger et al. 2012), enabling the particle cargo to achieve close contact to the blood–brain barrier, and the GCPQ particles also prevent peptide degradation within the plasma (Lalatsa et al. 2012b), both mechanisms resulting in the increased delivery of the peptide across the

blood–brain barrier. It is hypothesised that the lipidic prodrug—TPLENK is also able to diffuse more easily across the barrier when compared to the peptide alone due to a reduced level of hydrogen bonding of TPLENK with the water in the blood.

9.4.2 Oral

Polymer nanoparticles have been used experimentally to enable the delivery of gut labile materials and drugs with poor aqueous solubility. Most of these studies are experimental in nature and have not yet been commercialised.

9.4.2.1 Oral Insulin

The quest for a non-parenteral insulin formulation goes on and with over 100 million diabetic patients in Europe alone; the development of such a product offers rich rewards. The Exubra inhalation product proves that non-parenteral insulin is a technical possibility (Ghosh and Collier 2007) and hundreds of studies have experimented with the delivery of insulin by mouth, with some of these studies indeed demonstrating that the oral delivery of insulin is possible. It appears only a question of time before the insulin pill becomes available for human use. For example, Mukhpadhyay and others report on the formation of chitosan–insulin nanoparticles by coacervation at alkaline pH and the successful employment of these 100 nm particles to lower blood glucose diabetic rats (Mukhopadhyay et al. 2013), and as far back as 2007, Sarmento's and others reported the use of an alginate–chitosan coacervate nanoparticle formulation of insulin to lower blood glucose in diabetic rats (Sarmento et al. 2007), with the mucoadhesion offered by chitosan nanoparticles making the real difference to the efficacy of the system. Mucoadhesion promoted by cysteine residues on Eudragit nanoparticles also resulted in hypoglycaemia via the oral route in diabetic mice (Zhang et al. 2012), with this mucoadhesion promoting a longer gut residence time.

9.4.2.2 Hydrophobic Drugs

The delivery of hydrophobic drug compounds is becoming a real problem for the pharmaceutical industry (Kirkpatrick 2003) as the pharmacophore trends towards a higher Log *P* and molecular weight phenotype. There are a number of ways that oral absorption may be enhanced (Strickley 2004) and various solubilising excipients abound, most of them micelle forming and macromolecular in nature. These micelle-forming macromolecules include the poly(ethylenimine) amphiphiles (Cheng et al. 2006; Le et al. 2013) and the glycol chitosan-based amphiphiles (Siew et al. 2012). Such amphiphiles result in polymer nanoparticles once they have been loaded with hydrophobic drugs such as Cyclosporine A (Figs. 9.5a and 9.7)

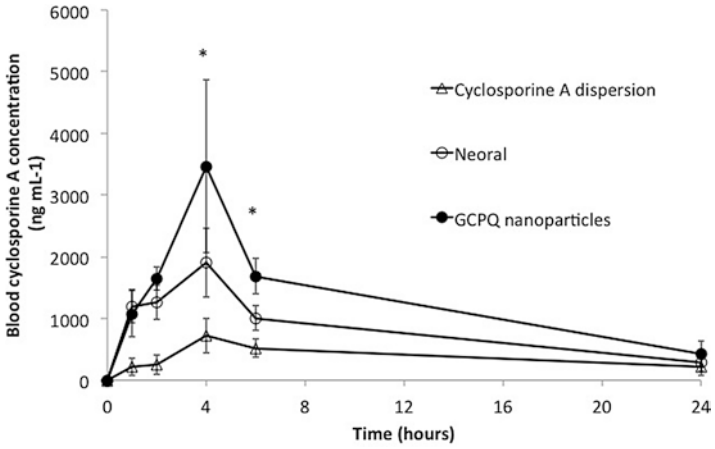


Fig. 9.7 GCPQ oral delivery of Cyclosporine A: AUC (ng mL^{-1} h) = 9,065 (Cyclosporine A dispersion), 18,997 (Neoral), 30,703 (GCPQ nanoparticles). C_{max} (ng mL^{-1}) = 721 (Cyclosporine A dispersion), 1,903 (Neoral), 3,465 (GCPQ nanoparticles) (Siew et al. 2012)

(Siew et al. 2012). Drug-loaded nanoparticles arising from such polymeric amphiphiles increase drug bioavailability by at least threefold when compared to the drug alone and increase the C_{max} by as much as sixfold (Siew et al. 2012). The glycol chitosan-based nanoparticles act by increasing dissolution within the gastrointestinal tract due to the high surface area of the nanoparticle dispersion and the amorphous nature of the drug (Siew et al. 2012), increase drug absorption by prolonging residence time at the functional aspects of the gastrointestinal tract due to mucoadhesion (Siew et al. 2012) and promote drug absorption by the particles being taken up by the enterocytes (Garrett et al. 2012; Lalatsa et al. 2012a) (Fig. 9.8). Furthermore, the more hydrophobic the polymer, the higher the level of hydrophobic drug loading and the more the drug is absorbed (Le et al. 2013). Paclitaxel was loaded into *N*-((2-hydroxy-3-trimethylammonium) propyl) chitosan nanoparticles made via an oil in water in oil emulsification followed by glutaraldehyde cross-linking and these nanoparticles improved survival in non-small cell lung tumour-bearing mice when compared to Taxol (Lv et al. 2011), although it is not clear if the particles were actually taken up.

9.4.2.3 Oral to BBB

Undoubtedly, the oral delivery of gut labile molecules to the brain remains a formidable challenge and there are no oral neuropeptides on the market as a result. However, recently it has been shown that the oral delivery of leucine^5-enkephalin to the brain is indeed possible, providing the dose was high enough and the peptide was encapsulated within GCPQ nanoparticles, either as the peptide alone or as the

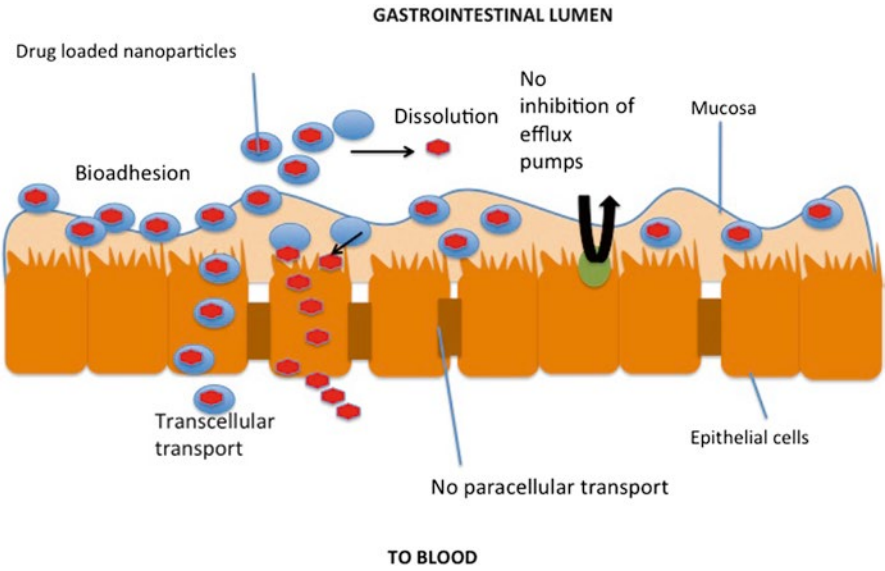


Fig. 9.8 The uptake of GCPQ particles by gut enterocytes

peptide lipidic prodrug TPLENK (Lalatsa et al. 2012a, b). The positively charged chitosan-based particle protects the peptide or the peptide prodrug from degradation within the gastrointestinal tract and enable the peptide or peptide prodrug to be taken up by enterocytes. Once within the plasma the particle-encapsulated peptide is protected from degradation by plasma enzymes and uptake by the brain capillaries is enabled.

9.4.3 Ocular

The delivery of drugs to the ocular surface requires that drugs enjoy a long enough residence time to reduce the need for short dose intervals. Transport of drugs through the cornea to the aqueous humour and into the conjunctiva and connective tissue of the eye may also be influenced by the nature of the delivery system. Prednisolone transport to the aqueous humour, on topical application, is significantly enhanced with the use of GCPQ nanoparticles (Qu et al. 2006). The improved pharmacodynamic activity of polymer nanoparticle formulations has been demonstrated with chitosan–hyaluronic acid nanoparticles when applied topically to the eye and containing dorzolamide hydrochloride or timolol maleate as this formulation reduced intraocular pressure to a greater extent than the marketed formulation of the drugs (Wadhwa et al. 2010). Additionally, dexamethasone loaded into cross-linked poly(*N*-isopropylacrylamide)-*co*-poly(vinyl pyrrolidone)-*co*-poly(methacrylic acid)

micelles to yield dexamethasone polymer nanoparticles showed better pharmacodynamic activity against uveitis when compared to dexamethasone solution and this was attributed to the mucoadhesion of the particles (Rafie et al. 2010).

9.4.4 Nasal

The nasal route of administration is favoured because of the highly vascularised nature of the nasal mucosa and the possibility of delivering drug to the brain via the neuronal routes. As such nasal formulations of insulin (Dyer et al. 2002) and leuprolide (Shahnaz et al. 2012) in chitosan nanoparticles have been used as a non-parenteral route for the systemic delivery of such peptides. Additionally, a nasal formulation of Olanzapine PLGA nanoparticles delivers more drug to the brain than the drug in solution (Seju et al. 2011).

9.5 Conclusion

Polymer nanoparticles are prepared from amphiphilic polymers, water-insoluble polymers and even from hydrophilic polymers. They have been demonstrated to improve the bioavailability of drugs via the oral, intravenous, intranasal and topical ocular routes. They act by protecting the drug from degradation, reducing drug clearance and even by transporting drugs across biological barriers while still encapsulated in the nanoparticles.

Problem Box

Q1: Describe how nanoparticles may be formed from polymers in aqueous media.

Answer

Amphiphilic polymers when dispersed in water or other aqueous medium spontaneously self-assemble to form nanoparticles. This self-assembly may be promoted by probe sonication, high pressure homogenisation or by microfluidisation, for example. Probe sonication is the method used to make polymers from the chitosan amphiphile: *N*-monomethyl, *N*,*N*-dimethyl, *N*-trimethyl, *N*-acetyl, *N*-palmitoyl, 6-*O*-glycol chitosan (GCPQ). Hydrophobic polymers such as poly(lactide-*co*-glycolide) (PLGA) may be precipitated from an organic solvent emulsified into aqueous media, with the formed nanoparticles

(continued)

Problem Box (continued)

stabilised by surfactants present in the aqueous media, e.g. PLGA nanoparticles formed in the presence of poly(oxyethylene) 20 sorbitan monooleate. Hydrophilic polymers such as chitosan may be prepared by using ionic gelation in which a counter anion such as tripolyphosphate is used to form nanogels with the positively charged chitosan.

Q2: Give examples of how polymer nanoparticles have been used to provide a therapeutic benefit either in clinical or preclinical settings.

Answer

Most polymer nanoparticles have demonstrated their benefits in cancer chemotherapy. Docetaxel poly(DL-lactic acid)-*block*-poly(ethylene glycol) nanoparticles have been evaluated in prostate cancer patients and found to extend plasma half-life of the drug, an observation which could lead to better tumour targeting with the drug. Furthermore, *N*-monomethyl, *N*,*N*-dimethyl, *N*-trimethyl, *N*-acetyl, *N*-palmitoyl, 6-*O*-glycol chitosan (GCPQ) nanoparticles have been demonstrated to facilitate the oral delivery of peptides to the brain, a first for any type of nanoparticle.

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Chapter 10
Porous Silicon Nanoparticles

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Abstract Micro- and nano-based technologies are presently recognized as promising potential tools for drug delivery applications in almost every field of health sciences, aiming to overcome the adverse physicochemical properties of conventional drug molecules, which often lead to poor drug bioavailability. A large amount of the new chemical entities developed by the pharmaceutical industry are poorly water-soluble compounds, which in order to subsist as efficient drugs with improved and controllable in vivo behaviour require the aid of more advanced technologies. In this context, porous silicon (PSi) nanocarriers have received considerable attention for the delivery of a wide range of therapeutics, particularly due to their excellent in vivo biocompatibility, easy surface chemical modification and easy control over their porous network structure. The literature has extensively demonstrated the successful use of PSi for controlling the loading and release of poorly water-soluble drugs; however, in this chapter we will mainly focus on the applications of the PSi-based nanoparticles for biomedical applications. In this chapter, we start by addressing the issues of poorly water-soluble drugs and then introduce PSi-based materials as potential drug carriers for such drugs. We then highlight the fabrication methodology of PSi materials, the drug loading and release, and present several examples of the significant potential of PSi in biomedical imaging and in drug delivery applications. These applications exploit these promising features of PSi for

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future translation to the clinic. We will conclude the chapter with a brief overview of our visions of the future of the PSi nanomaterials and their implications in the pharmaceutical and biomedical field.

10.1 Introduction

Over the past years great advances in nanotechnology-based platforms have shown remarkable improvements towards more advanced delivery systems in order to efficiently direct the drug molecules to unhealthy tissues or cells. Nowadays, many nanoparticles are used in the clinic and the development of nanodelivery systems may accommodate single- or multifunctionalities on the same entity. However, the biological barriers are very heterogeneous, which may prevent the therapeutic and imaging agents from reaching their intended targets in sufficient amounts. Therefore, sophisticated delivery systems are emerging in order to develop multimodular nanoassemblies, in which different components with specific functions may act in a synergistic manner, as discussed below.

The unique physicochemical properties, as well as potential biomedical applications of nanomaterials have long been a subject of intense research worldwide. Nanomaterials are engineered structures with at least one dimension below 100 nm, encompassing highly advantageous features in terms of mechanical, electrical, chemical and optical features unlike their bulk materials. According to the size, morphology/shape, chemical composition and surface chemistry (Mitragotri and Lahann 2009), nanoparticulate systems may have different stability and behaviour in the biological microenvironment and cellular distribution (Moghimi et al. 2005), as well as elicit undesired biological or toxicological effects (Oberdorster 2010). Therefore, safety and biocompatibility of such nanomaterials are crucial requirements both in the manufacture of the dosage forms and in the treatment of patients. Consequently, a full characterization of the in vitro and in vivo behaviour of such nanomaterials is of utmost importance in the preclinical assessments.

Two attractive and emerging areas of research are pharmaceutical nanotechnology and nanomedicine development, where nanocarriers are used to mediate drug delivery. The advantageous characteristics of the nanocarriers in drug delivery applications can be summarized as follows (Farokhzad and Langer 2009):

1. Improved delivery of poorly water-soluble drugs
2. Cell-specific or tissue-specific targeted delivery of compounds
3. Transcytosis of drugs across tight epithelial and endothelial barriers
4. Delivery of large macromolecules to intracellular sites of action without disturbing any other cellular function, even after degradation
5. Co-delivery of two or more drugs or therapeutic modality for combination therapy
6. Visualization of drug delivery sites by combining therapeutic agents with imaging modalities
7. Real time read of the in vivo efficacy of a therapeutic agent
8. Non-toxic, non-immunogenic and preferably biodegradable nature

When administered intravenously, nanocarriers generally face the opsonization phenomenon, which may to the entrapment of the carrier by the mononuclear phagocytic system (MPS) and fast removal from the bloodstream to the liver and spleen (Moghimi et al. 2001). The blood opsonization, fast clearance and cell uptake are governed by, among other factors, the size and surface properties of the nanosystems. Adequate size design of the nanocarriers is therefore crucial, not only to accommodate high amounts of therapeutic agents, but also to avoid to some extent, premature capture by the MPS. Although important, the discussion related to the opsonization and the nanocarrier's parameters affecting the opsonization is vast and out of the scope of this chapter.

10.1.1 Delivery of Poorly Water-Soluble Drugs

Micro- and nanotechnologies have also offered great perspectives for the development of drug delivery systems in order to overcome the most unfavourable physicochemical and biopharmaceutical properties of many drug molecules. This is particularly important considering that many drug molecules suffer from very poor bioavailability, i.e. solubility in aqueous solutions is less than 100 μg/mL, with slow dissolution rates, as well as poor permeation characteristics across biological barriers and very high first-pass metabolism. When considering the oral dosing of such drugs, this typically leads to defective biological performance.

It is well acknowledged that 40–60 % of the new currently available active pharmaceutical ingredients (APIs) and ca. 70–90 % of the new drugs under development are poorly water-soluble drugs. These drugs are therefore not well suited for the preferred oral delivery using conventional tablet formulations. Even today, the delivery of poorly water-soluble drugs is still considered a bottleneck in drug development in pharmaceutical sciences, which has led to an extensive amount of research to develop delivery technologies that could enhance drug dissolution and increase drug absorption. In 2010, the pharmaceutical industry investments in nanocarriers to improve drug solubility and bioavailability accounted for ca. US$139 million of the total costs (Cientifica 2012). There is a clear trend for increasing investment in this area in the following years to come.

In pharmacological therapy, a response from an administrated drug is elicited in order to reverse a pathological condition. This response depends on the plasma drug concentrations and on the availability of the active drug at the receptor site. Therefore, the oral bioavailability of a drug is mainly determined by its solubility and permeability. In order to achieve effective oral medical treatment in patients, the drug molecules need both to dissolve in the gastrointestinal (GI) tract and to permeate across the intestinal wall. Although many drugs have reasonable membrane permeation properties, the rate limiting process of absorption is still the drug dissolution step (Singh et al. 2011).

The pharmaceutical means to improve oral drug bioavailability focuses mainly on improving the drug solubility and dissolution rate (Fahr and Liu 2007), for

example, by altering the solid state properties of a drug, which include different crystalline forms and amorphous disordered structures of the molecules. The dissolution of the amorphous form of a drug can be markedly better than its crystalline counterparts (Hancock and Parks 2000). The amorphous state of a compound is thermodynamically less stable and has a tendency to convert into the more stable one, crystalline form—thus affecting the drug dissolution rate. Moreover, in order to be effectively absorbed in the body, orally administered drugs also need to be dissolved at the site of absorption in the GI tract (Avdeef 2001), and then cross the intestinal barrier into the systemic circulation.

According to the biopharmaceutical classification system (BCS), four different categories of orally dosed drugs can be found (Amidon et al. 1995): BCS class I—highly water-soluble and intestinally permeable; BCS class II—poorly soluble and highly permeable; BCS class III—soluble and non-permeable; BCS class IV—non-soluble and non-permeable. Because for the BCS class II drugs the dissolution is the predominant rate-limiting factor in controlling absorption, a large number of formulation strategies have been investigated for improving the solubility and dissolution rate of such drugs, for example, by means of porous silicon (PSi) nanomaterials. Poorly water-soluble drugs can be loaded into the pores of PSi, with rather high loading levels, and upon oral dosing the drug can be released at a faster rate than from a conventional tablet, hence increasing the drug absorption in the GI tract.

10.1.2 Nanostructured PSi-Based Materials as a Carrier for Water-Insoluble Drugs

Among many nanosized materials, PSi has made an impact in biomedical applications, particularly in the last decade. PSi materials were initially studied in non-pharmaceutical applications, but after evidence of their outstanding properties in drug delivery (Canham 1997), PSi has also been widely studied in pharmaceutical and medical applications, including for example, delivery of drugs, proteins/peptides and bone tissue engineering.

PSi is a material traversed by a network of empty pores in a crystalline Si structure. These materials have large surface-to-volume ratio properties and are classified according to their pore diameter. The most common PSi nanostructures are referred as mesoporous (pores with diameters between 2 and 50 nm). The internal surface area of PSi per volume unit can be as large as hundreds of m^2/cm^3, and can act as reservoirs for storing therapeutic compounds for drug delivery applications. PSi materials have several unique physicochemical properties for biomedical applications such as (a) tuneable nanoscale sizes, (b) tailored shapes and pore sizes, high surface areas and pore volumes, (c) easy functionalizable surfaces and (d) the capability of hosting the required amount of compounds with a reproducible loading and release.

Besides the abovementioned remarkable structural features, PSi materials also present suitable biocompatibility, degrade into non-toxic products and have a large specific

capacity for drug loading. The use of PSi, as an effective delivery vehicle for pharmaceuticals and bioactive molecules to desired intracellular sites or as a host material for controlled drug delivery, bio-imaging and the treatment of diseases including cancer has been widely recognized and these applications are further explored in this chapter (Salonen et al. 2005, 2008; Heikkila et al. 2007; Kaukonen et al. 2007; Laaksonen et al. 2007; Godin et al. 2008; Salonen and Lehto 2008; Tasciotti et al. 2008; Kilpelainen et al. 2009, 2010, 2011; Bimbo et al. 2010, 2011b, 2012a, b; Santos et al. 2010, 2011a, b, 2013a, b; Kinnari et al. 2011; Sarparanta et al. 2011, 2012a, b; Serda et al. 2011; Kaasalainen et al. 2012; Kovalainen et al. 2012; Lehto et al. 2012; Liu et al. 2013; Mäkilä et al. 2012; Santos and Hirvonen 2012; Shahbazi et al. 2012; Tahvanainen et al. 2012; Vale et al. 2012; Shahbazi and Santos 2013).

When the drug molecules are loaded inside the nanostructured pores, the dissolution-related problems can be partly surmounted. By confining poorly water-soluble compounds into the PSi materials, the bioavailability of many drugs can be improved as well as their pharmacological and therapeutic properties with reduced collateral effects. In this case, the crystallization of the loaded material is avoided by the restricted pore size of the materials and the interactions between the drug and the pore surfaces. The physicochemical properties of the drug are dramatically changed to a non-crystalline (amorphous-like) disordered state (Salonen et al. 2005). Consequently, the drug can be delivered at the site of absorption and also targeted to a specific site and, at the same time, be sheltered from any unwarranted degradation. Furthermore, the PSi materials can be designed to be suitable for any type of payload molecules without compromising the payload capacity and the stability of the molecules. The PSi-based product Brachysil™ (pSivida Ltd.) is already in clinical trials phase III for inoperable primary liver and pancreatic cancer therapy (Zhang et al. 2005; Goh et al. 2007).

In summary, the properties that make PSi an attractive material for drug delivery applications are presented in Fig. 10.1 and include (Salonen et al. 2008; Salonen and Lehto 2008; Santos et al. 2011a, b, 2013b):

- Electrochemical etching production (top-down approach) of PSi enables pore sizes to be controllably tailored from microns to nanometers.
- Engineering of a wide range of porosities (up to 90 %) to enable high drug loading levels, typically in the range 5–40 wt.%.
- According to its porosity and surface chemistry, PSi is highly biodegradable and biocompatible, i.e. its dissolution is catalyzed by alkaline conditions and forms silicic acid, a non-toxic form of Si found widely in the body.
- A number of well-established chemistries exist for the modification of PSi surfaces that can be used to control the drug loading, drug interactions and drug release, and the readsorption rate of the PSi host matrix. These include thermal carbonization (TCPSi), thermal oxidation (TOPSi) and thermal hydrocarbonization (THCPSi) (Salonen et al. 2008; Salonen and Lehto 2008).

This chapter highlights the most common methods of preparation and characterization of PSi-based materials, stresses their potential in pharmaceutical and biomedical applications in preclinical imaging and enhancing drug dissolution and permeation of poorly water-soluble drug compounds.

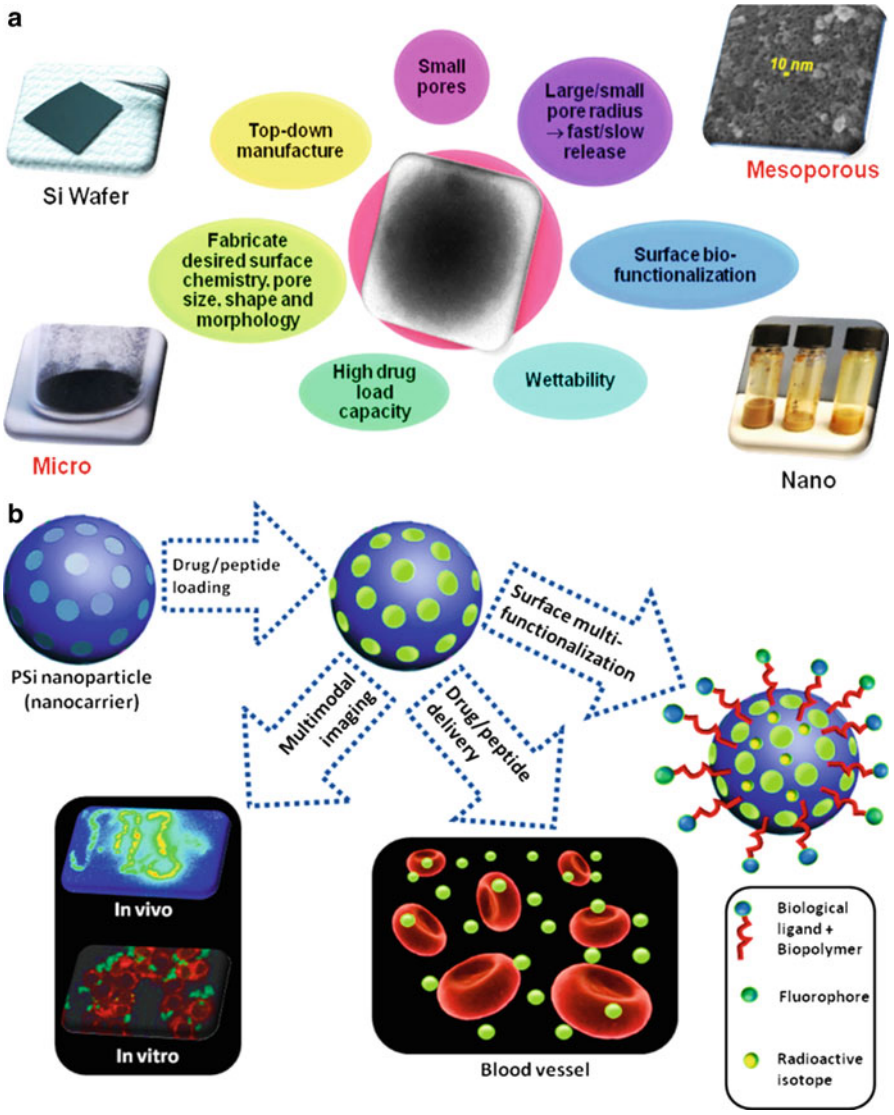

Fig. 10.1 (**a**) Most relevant properties of the PSi materials, showing the Si wafer and the PSi powder-based microparticles (*left*), the mesopores (~10 nm) and the PSi nanoparticle suspension (*right*). Other properties of PSi materials are also described in the figure. (**b**) Schematics of the different applications of the PSi nanocarrier in drug delivery applications, including (from *right* to *left*) the loading/release of drugs/peptides, and transport and release in the bloodstream of therapeutic compounds to unhealthy cells or tissues, as well as surface functionalization with different biological ligands and polymers, and bio-imaging using fluorophores and/or radioactive isotopes. Reprinted with permission from Santos et al. (2013a); copyright © 2013, Materials Research Society

10.2 Fabrication of PSi Nanoparticles

10.2.1 Overview of the Common Fabrication Methods

Several methods have been reported in the literature for the fabrication of PSi materials. The most frequent method for the production of PSi is the electrochemical anodization of monocrystalline Si wafers in aqueous hydrofluoric acid (HF)-ethanolic electrolytes, with the ethanol acting as a surfactant (Korotcenkov and Cho 2010). In its simpler set-up, the Si wafer acts as anode while a platinum (Pt) plate serves as cathode, when both are immersed in the electrolyte solution under an applied potentiostatic (voltage-controlled) or galvanostatic (current-controlled) mode (Salonen et al. 2008). The almost exclusive use of Pt as a cathode material is due to the fact that Pt is practically the only conductive material which can repeatedly withstand the stringent cathodic conditions and the constant exposure to HF. The cell in which the anodization takes place must also be made of HF-resistant material, such as Teflon or polypropylene, in order to endure the whole etching process (Fig. 10.2).

High anodic currents produce smooth Si surfaces in a process called electropolishing. With lower anodic current pores are etched on the surface of the Si producing a porous layer. The core properties of the obtained PSi when operating below the electropolishing current threshold, such as porosity, porous layer thickness, pore size and shape are all determined by the fabrication conditions (Salonen et al. 2000). These conditions include current density, wafer type and resistivity, HF concentration, chemical composition of the electrolyte, crystallographic orientation, temperature, duration of anodization and illumination conditions. Despite the overwhelming number of affecting parameters, much of these are related to each other enabling effective reproducibility of the material in addition to an unprecedented control over structure and morphology of the obtained PSi. With small changes in the fabrication

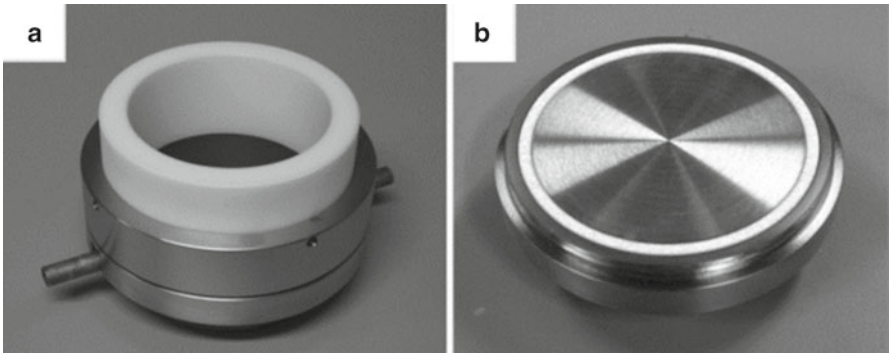

Fig. 10.2 (**a**) A single tank PSi etching cell for 100 mm Si wafers, fabricated from Teflon with a stainless steel frame. (**b**) Lower part of the etching cell, showing the metallic back contact for the Si wafer

parameters, PSi can contain, e.g. micro- or mesopores of sponge or fir tree-like pore morphology with interconnected or branched pores (Hamilton 1995; Foll et al. 2002). The nature of the anodization process enables the fabrication of stacked PSi layers with different porosities, as the already formed layers are not affected by the electrochemical reactions. This property allows also the lift-off of the porous layer from the bulk Si as a free-standing thin film by applying an electropolishing current when the layer detachment is wanted.

10.2.2 Production of PSi Nanoparticles

As PSi is essentially a top-down-type nanomaterial in contrast to ordered mesoporous silicas, the fabrication of size-controlled nanoparticles is usually done by mechanical size reduction using ultrasonication or milling. Also, photolithographic methods commonly used in semiconductor technology have been employed effectively for PSi nanoparticle fabrication.

Production of the nanoparticles through mechanical size reduction starts with the fabrication of PSi thin film. The characteristics of the material, such as porosity and pore diameter are selected at this stage. The porous thin film may be constituted from a single uniform layer etched with a constant current, or from a multilayer stack, where periodically repeating high porosity layers function as fracture planes (Park et al. 2009; Bimbo et al. 2010). As the size reduction by both milling and sonication is a relatively high-energy process and a considerable amount of surface is formed, the dispersion medium may react with the newly formed external surface of nanoparticles. While the initial thin films may be chemically modified prior to the size reduction, further reactions with the dispersion medium can be used to control the surface chemistry of the PSi nanoparticles. Final size control and selection can be carried out using both membrane filters and centrifugation (Fig. 10.3).

The utilization of photolithographic methods in the nanoparticle fabrication provides considerably more control over the final particle size and shape (Serda et al. 2011; Godin et al. 2012), but requires more advanced production facilities. Briefly, a masking layer is initially deposited on the Si wafer, after which a photoresist is spun and patterned with the selected photomask. After transferring the pattern on the wafer, electrochemical anodization selectively etches the patterned areas. The masking layer is then removed, and the pre-patterned PSi particles can be released by ultrasonication.

10.2.3 Surface Stabilization and Modification of PSi for Biomedical Applications

Following anodization, the native surface of PSi is hydrogen-terminated ($Si–H_x$). These bonds can be hybrids Si–H, $Si–H_2$ and $Si–H_3$ and provide a passivating layer on the otherwise highly reactive Si. However, the hydrogen passivation is unstable,

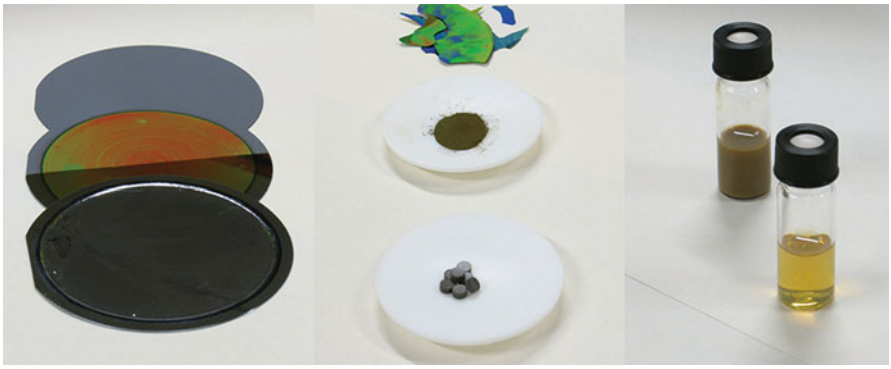

Fig. 10.3 From Si wafers to pharmaceutical use. Fabrication of PSi step by step, starting from a clean Si-wafer, etched with a PSi layer. The detached films can be milled down to fine particles and, finally, compressed into tablets as a part of formulation or processed even further into stable PSi nanoparticle dispersions

causing PSi to undergo a slow, spontaneous oxidation with oxygen or water molecules even in dry ambient air. The transformation from hydrophobic hydrogen terminated to hydrophilic oxidized surface takes several months at room temperature. Complete native oxidation takes much longer time, depending on the storage conditions. In addition, there are also some impurities present in the PSi, which are commonly detected after the fabrication procedure, such as fluorine, oxygen and trace amounts of hydrocarbons (Grosman and Ortega 1997).

For most applications, a non-reactive and stable surface is crucial, and the unstable hydrogen termination of the freshly etched PSi has to be replaced. This inherent property of fresh PSi has prompted the development of various surface stabilization methods. By replacing the reactive hydrogen groups with a more stable oxidized, silylated or carbonized layer, the PSi surface can withstand harsh physiological conditions for extended durations. Several surface stabilizations also retain enough reactivity for even further chemical functionalization.

10.2.3.1 Thermal Oxidation

The stabilization of the anodized Si-surfaces by thermal oxidation is one of the more straightforward ways to oxidize Si-surfaces; the product of this treatment is called thermally oxidized PSi or TOPSi (Salonen et al. 2008; Salonen and Lehto 2008). Thermal oxidation starts to occur at a threshold temperature near 210 °C, where the reaction proceeds selectively oxidizing Si–Si backbonds, producing O_xSiH_y species. Increasing the temperature near 250 °C, where there is the first evidence for loss of hydrogen from the Si surface, and where the Si dangling bond sites produced are able to chemisorb oxygen dissociatively, both the Si–Si backbonds and the surface hydrides are oxidized simultaneously. Oxidation also leads to the formation of surface Si–OH species as a result of the oxygen atom insertion into Si–H bonds. The subsequent formation of Si–O–Si modes is originated from

oxidation of the PSi surfaces. At low temperatures (below 450 °C), the surface oxidation saturates after a few hours, forming a thin SiO_2 layer. Consequently, this treatment changes the PSi surface from hydrophobic to hydrophilic (Salonen et al. 2008; Salonen and Lehto 2008). Increasing the oxidation temperature even further, oxygen diffusion through the thin SiO_2 layer may proceed, and the PSi will eventually oxidize completely to silica (Pap et al. 2004).

10.2.3.2 Thermal Carbonization

Another method for stabilizing fresh PSi surfaces is the thermal carbonization of PSi. The thermal carbonization process for PSi, introduced by Salonen et al. utilizes acetylene (C_2H_2) as a carbon source for an SiC-based stabilizing layer formation (Salonen et al. 2000). The method is based on the adsorption and subsequent absorption of C_2H_2 in the PSi crystalline structure under thermal treatment. Utilizing the dissociation temperature of C_2H_2 and the hydrogen desorption from PSi at elevated temperatures, the thermal carbonization process can be divided into two distinct surface modifications (Salonen et al. 2004).

When the process is conducted below the C_2H_2 dissociation temperature (450–650 °C), a continuous C_2H_2 feed can be maintained. This enables high surface coverage, as much of the desorbing hydrogen can be replaced with C_2H_2 molecules. The hydrogen termination is thus replaced by a hydrocarbon termination ($-CH_x$), yielding a stable, hydrophobic surface chemistry called thermally hydrocarbonized PSi or THCPSi.

At higher temperatures (above 650 °C), the adsorbed C_2H_2 molecules will be completely dissociated. The surface of the PSi starts to reorder at this temperature range, causing the C_2H_2 fragments to absorb into the PSi structure, forming a non-stoichiometric SiC-layer below the pore walls with a thin oxide layer covering the outer surface. The result is called thermally carbonized PSi or TCPSi, and the surface chemistry is hydrophilic and extremely stable (Salonen et al. 2008; Sarparanta et al. 2011). The thermal carbonization can also be done subsequently to an initial THCPSi layer, resulting in a more stable surface; the high temperature annealing will not cause significant alterations to the porous structure. Both THCPSi and TCPSi process pathways are shown in Fig. 10.4. The high stability and relatively low cytotoxicity of these PSi treatments has made the C_2H_2-based approach a highly viable solution for drug delivery purposes (Salonen et al. 2005; Bimbo et al. 2010, 2011b; Santos et al. 2010; Kilpelainen et al. 2011; Liu et al. 2013; Sarparanta et al. 2012a, b). Besides surface stabilization, thermal carbonization has also been used as a post-fabrication pore size control method (Fang et al. 2010).

10.2.3.3 Hydrosilylation, Silanization and Multifunctionalization

Hydrosilylation methods are used especially when the PSi surface is directly functionalized in order to obtain a specific surface termination, such as a carboxylic acid

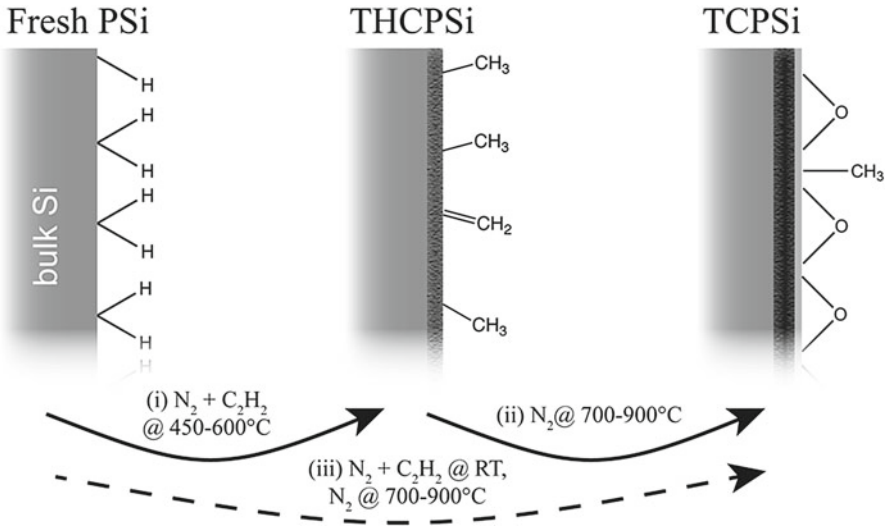

Fig. 10.4 C_2H_2-based thermal hydrocarbonization and carbonization for fresh PSi. The hydrocarbonization process (i) is done by exposing the PSi to C_2H_2 during a moderate temperature (450–600 °C) heat treatment to form THCPSi. The carbonization can be done immediately after the THCPSi treatment (ii) to improve surface stability or directly for fresh PSi (iii) at high temperature (700–900 °C) to produce TCPSi

group for biomolecule conjugation (Boukherroub et al. 2002; Sciacca et al. 2011). However, the wet chemistry processes with long-chained hydrocarbons suffer from limited surface coverage, leaving the parts of the PSi surface still hydrogen terminated, susceptible for spontaneous oxidation, and hydrolysis in aqueous conditions (Lees et al. 2003).

Methods for alleviating the limitations in hydrosilylation coverage have been recently reported. An electrochemical end-capping of the remaining hydrides with an organohalide, such as iodomethane has been shown to improve the stability directly by modified PSi against oxidation (Lees et al. 2003). A more recent approach to an enhanced surface stabilization is based on combining the strengths of a gas phase treatment, such as thermal hydrocarbonization with a liquid phase hydrosilylation into a two-step treatment where the bulk structure of the PSi is protected with a highly stable hydrocarbon termination (THCPSi) and a specific functionality is provided by a secondary treatment. Recently, a radical coupling of a dicarboxylic acid with a peroxide initiator was demonstrated with THCPSi, yielding a partial carboxylic acid (–COOH)-terminated surface (Sciacca et al. 2010). A similar result was obtained also by adapting the thermal hydrosilylation method for undecylenic acid with THCPSi, for providing a –COOH terminated stable surface for controlled delivery of peptides (Kovalainen et al. 2012). This method has been shown to be far superior in regard of the aqueous stability compared to the direct hydrosilylation of hydrogen-terminated PSi (Sciacca et al. 2010; Jalkanen et al. 2012).

Table 10.1 Outline of surface modification routes currently used in stabilization of PSi

Modification	Surface termination	Stability	References
Native PSi	$Si–H_x$	Low	Canham (1995)
Oxidation	Si–O–Si, OSi–H, –OH	Moderate/high	Park et al. (2009) and Godin et al. (2012)
Hydrocarbonization	$–CH_x$	High	Bimbo et al. (2010), Liu et al. (2013), and Sarparanta et al. (2012a)
Carbonization	Si–O–Si, –CSi–O, –OH	High	Salonen et al. (2005)
Silylation	e.g. $–CH_x$ or –COOH	Moderate/high	Sciacca et al. (2010)
Silanization	e.g. Si–O–Si, $–NH_2$	Moderate	Park et al. (2009)
Multifunctionalized	$–CH_x$ + –COOH	High	Sciacca et al. (2010) and Kovalainen et al. (2012)

Methods utilizing silane coupling chemistry more commonly used with silica-based materials have also been studied with PSi. The attachment of organofunctional molecules through siloxane (Si–O–Si) bridges on silanol-terminated silica or oxidized PSi is a fairly straightforward process that can be done either in liquid or by vapour deposition (Zhang et al. 2010; Sweetman et al. 2011). Silanization with a common organosilane APTES (3-aminopropyltriethoxysilane) has been reported on oxidized PSi (Arroyo-Hernández et al. 2006; Serda et al. 2010) as well as with HF-activated TCPSi (Mäkilä et al. 2012). It is often used to produce PSi with a positive surface charge due to the abundance of terminal amine groups, which in turn can be used for further bioconjugation processes (Serda et al. 2011; Mäkilä et al. 2012).

The versatility of PSi is emphasized by the broad selection of available surface modification routes. These enable the selection or even development of a specific functionalization for the desired application. A summary of the different surface modification of PSi routes is presented in Table 10.1, together with suitable references on the modification procedure and its applications.

10.2.4 Drug Loading in PSi

The loading of a drug within the porous structure of Si is generally achieved through capillary action, either by dropping the drug solution onto the PSi surface or by immersing the PSi into a concentrated drug solution usually followed by sonication (Foraker et al. 2003; Bimbo et al. 2011a). The mechanisms by which drugs can be retained within the PSi matrix are covalent attachment, physical trapping and spontaneous adsorption. Covalent attachment is the most robust approach for loading drugs into the porous matrix and typically uses organic molecules that contain carboxyl species on the distal end of an aliphatic chain for the grafting. The hydrosilylation reaction between the end of alkenes and Si surface leaves the carboxyl terminal free (Boukherroub et al. 2002), where a drug payload can be attached by activating the acid group with, e.g. *N*-hydroxysuccinimide in the presence of a

carbodiimide, leading to the generation of succinimidyl ester functional groups to the PSi surface (Sam et al. 2010).

The PSi surface modified by thermal hydrosilylation can promote the attachment of different-size molecules such as amino acids and enzymes to the surface (Hart et al. 2003; Sam et al. 2010). Anticancer drugs such as doxorubicin have also been covalently attached onto the PSi matrix and investigated in vitro (Wu et al. 2008). Covalent attachment represents the strongest drug-matrix attachment. However, the release of the drug requires breaking the covalent bonds or degrading the porous matrix. Physical trapping of drugs by oxidation can also occur by a process that causes PSi to expand to accommodate the extra volume of oxygen atoms from a deliberated oxidation leading the pores to shrink, trapping the drug molecules inside (Mattei et al. 2002).

Spontaneous adsorption of drug molecules into the porous matrix, on the other hand, results from the simple immersion of the PSi structure in a drug solution. Loading of drug molecules such as furosemide and bovine serum albumin onto PSi has already been reported (Tay et al. 2004; Kaukonen et al. 2007). As the isoelectric point of Si is found at pH of 2, it is generally negatively charged in most aqueous solutions, and therefore, at the appropriate pH, PSi spontaneously adsorbs positively charged molecules, such as protein A (Schwartz et al. 2007).

PSi can also be extremely advantageous for the adsorption and delivery of small hydrophobic molecules such as doxorubicin (Vaccari et al. 2006), dexamethasone (Anglin et al. 2004) and porphyrins (Chirvony et al. 2006). The surface chemistry controls the affinity of PSi particles for a particular molecule, and thus, the amount of drug loaded and the rate of release. Adsorption-based loading procedures can be optimal for protein formulation since the process can be performed at room temperature without exposing the protein to harsh chemical conditions, therefore preserving the delicate tertiary structure of these molecules (Pastor et al. 2011). However, it is not feasible for long release periods, since the attachment is weaker than covalent or physical trapping. The fast dissolution of the PSi matrix, which increases over time at alkaline pH and temperatures of 37 °C, can also be used as an advantage for releasing the loaded drug (Anderson et al. 2003).

10.2.5 Physical Characterization of PSi: A Small Toolbox

In drug delivery applications the physical properties of the PSi have to be well known. As the fabrication process parameters control several key features, such as porosity, pore diameter and morphology, these have to be determined carefully. An unsuitable pore size may prevent the adsorption of the drug molecules inside the pores due to steric hindrance, or cause the adsorbed drug to release at a wrong rate. Below, a short overview of a few commonly used analytical methods for PSi characterization is provided. The cursory overview is by no means exhaustive, as various other methods are in equally common use.

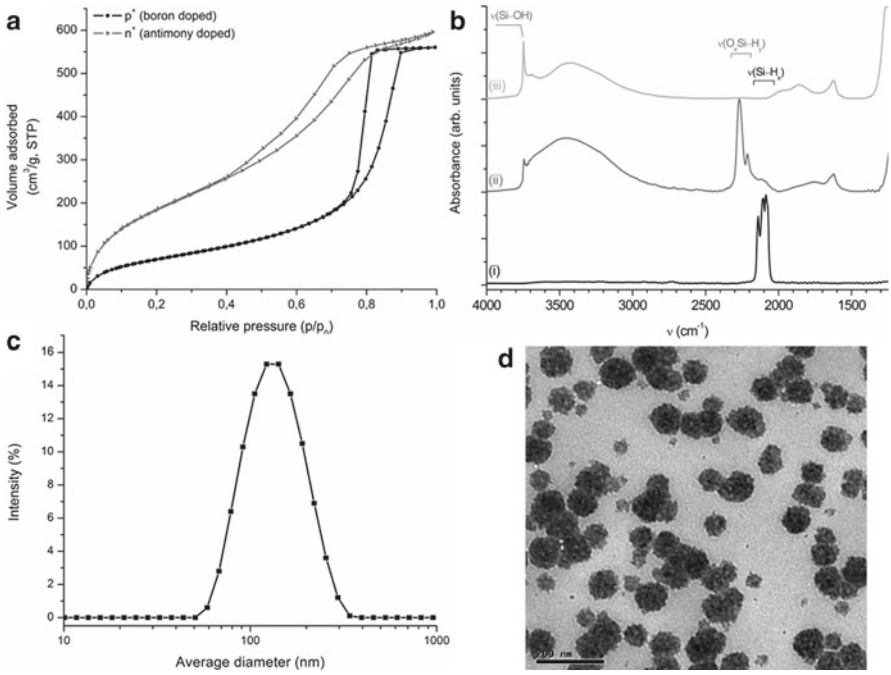

Fig. 10.5 (**a**) Nitrogen isotherms of PSi films electrochemically etched from p^+ and n^+-type Si wafers. In both cases the electrolyte and the etching current density have been identical. Both films are mesoporous; both indicate significantly different pore morphologies. The specific surface areas of the films are 265 and 730 m^2/g for the p^+ and n^+ films, respectively. The average pore diameters are 12 and 5 nm, respectively. (**b**) FTIR absorbance spectra from, a free-standing fresh PSi film (*bottom*), TOPSi films oxidized at 300 °C (*middle*) and TOPSi film oxidized at 750 °C (*top*). The spectra show the generation of backbond-oxidized surface layer and the removal of the surface hydrides during the transformation of the film to $PSiO_2$. (**c**) DLS results of THCPSi-nanoparticles with an average size of 145 nm. (**d**) TEM picture of TOPSi nanoparticles providing more information on the shape of the PSi nanoparticles

Gas sorption, such as nitrogen sorption at 77 K, is a straightforward tool for determining the porous characteristics of PSi. The obtainable adsorption/desorption isotherm provides a considerable amount of information on the physical properties of PSi. Figure 10.5a shows the nitrogen sorption isotherms of two different, fresh PSi samples. While both are mesoporous, the shape of the isotherm differs considerably between the two. The specific surface area of material can be calculated from the initial portion of the isotherm ($p/p_0 = 0.05–0.3$) using Brunauer–Emmett–Teller (BET) theory: At high relative pressures ($p/p_0 = 0.9–0.99$), both isotherms show a plateau indicating the filling of the pores. Taking this value as the volume of liquid nitrogen adsorbed, the total pore volume can be estimated. Utilizing the obtained values for specific surface area and total pore volume, while assuming the pore shape to be, e.g. cylindrical, the average pore diameter can be evaluated. Further information from the isotherm can be gained by studying the

shape of the adsorption/desorption hysteresis loop. For more detailed analysis of the pore morphology, there are various computational methods available such as the widely used Barrett–Joyner–Halenda (BJH) method and several models based on density functional theory (Thommes 2010).

While the morphology of the PSi has a considerable effect on its properties regarding adsorption and release of drug molecules, their possible interactions with the surface of the PSi has to be taken into account. There are multiple methods that enable efficient characterization of the surface chemistry of PSi. These can be used for verification of the success of the surface stabilization, but also to study the drug–pore wall interactions.

A common tool for routine PSi surface modification analysis is Fourier transform infrared spectroscopy (FTIR), which currently implies the study of fundamental vibrations of molecules in the mid-infrared region (approximately from 4,000 to 400 cm^{-1}). Figure 10.5b presents the FTIR spectra obtained from a fresh PSi free-standing film and from two TOPSi films. The spectra provide detailed information on the changes occurring on the PSi surface during the thermal oxidation process. As discussed above, the Si–H_x groups undergo at low temperatures a backbond oxidation. This can be observed from the shift of the characteristic Si–H_x bands at 2,050–2,150 cm^{-1} to higher wave numbers (2,200–2,300 cm^{-1}), due to the formation of O_x–SiH. Also, the material becomes hydrophilic, as a broad moisture-related band appears between 3,200 and 3,600 cm^{-1}. The strong Si–O-related features would appear at lower wave numbers (1,100 cm^{-1}). If the oxidation temperature is increased enough, the backbond-oxidized Si hydrides are removed, and as can be observed from the spectra, the features around 2,250 cm^{-1} have disappeared. The strong band appearing at 3,750 cm^{-1} is related to Si–OH terminal groups. Analyses of the FTIR spectra obtained from different surface chemistries of PSi are broadly reviewed in the literature. Some examples for FTIR band interpretation can be found elsewhere (Sam et al. 2009; Sciacca et al. 2010; Sarparanta et al. 2011; Mäkilä et al. 2012).

Aside from the porous morphology and surface chemistry, information about the final nanoparticle size and shape cannot be emphasized enough with the techniques described above. Dynamic laser scattering (DLS) and transmission electron microscopic (TEM) methods are commonly employed to provide details on the external properties of PSi nanoparticles. DLS (Fig. 10.5c) is a fast method that provides information about the average particle size in the sub-micron range (Sarparanta et al. 2012a). In DLS, the Brownian motion of the nanoparticle in suspension is measured and correlated with its size, giving the hydrodynamic diameter of the particles. As DLS provides the diameter of an equivalent sphere that provides a similar result, further information on the shape of the nanoparticles has to be obtained with other methods, such as TEM (Fig. 10.5d). Many modern commercial DLS instruments include the option to perform electrophoretic mobility measurements in addition to the size determination. With nanoparticles intended for biomedical use, this enables calculation of the zeta–potential of the nanoparticles in the selected suspension. By titrating over a wide pH range, the isoelectric point of the particular surface chemistry of the PSi materials can be determined (Kaasalainen et al. 2012).

10.3 Biomedical Applications of PSi Nanoparticles

There are a vast number of studies described in the literature regarding the potential applications of PSi in biomedical applications, particularly dealing with PSi microparticles (Salonen et al. 2008; Santos et al. 2011a, b, 2013a, b; Lehto et al. 2012; Liu et al. 2013; Santos and Hirvonen 2012; Shahbazi et al. 2012), and thus, in this chapter we will mainly focus on the most relevant in vitro and in vivo aspects involving PSi nanoparticles. Several examples on the biocompatibility issues of PSi nanomaterials, as well as on PSi acting as drug nanodelivery system and imaging platform are presented and discussed, while pointing out to the most relevant references.

The evaluation of the biocompatibility and/or cytotoxicity of a nanomaterial involves an extensive screening of in vitro assays and animal testing under certain, controlled conditions. In order to be translated into the clinic, PSi should be regarded as a safe and biocompatible material, as well as allowing a safe drug distribution in the body after release. This is because there are many factors that can trigger any reaction in the cells/body, e.g. particle concentration, surface area, size distribution, chemical composition, surface structure, solubility, shape and particle aggregation. In this context, besides the functionality of the system, a series of preclinical tests including biodistribution, immune response, toxicity, reactive oxygen species (ROS) and clearance, together with the effects of the interaction between any residual solvents, degradation products, the nanomaterials themselves and the cells and their surrounding environment must be evaluated (Bimbo et al. 2010, 2012a; Santos et al. 2010). Any absence of toxicity by a material is not necessarily an indication of biocompatibility of the material, because several inflammatory reactions that can be harmful in the body can still take place after long exposure times.

The biodegradation of the PSi nanomaterials into non-toxic products also ensures the biocompatibility of PSi, where the former can relatively be controlled by the overall porosity and pore size of the material (Canham 1997); PSi with a porosity <70 % is bioactive and slowly biodegradable, whereas PSi with a porosity >70 % dissolves in all simulated body fluids, except gastric fluids (Salonen et al. 2008). Monomeric silicic acid [$Si(OH)_4$] is the main degradation product of PSi, which is the most natural form of Si in the environment and very important in human physiology in protecting against the poisonous effects of aluminium (Popplewell et al. 1998), and, most importantly, all [$Si(OH)_4$] can be excreted in urine (Reffitt et al. 1999).

10.3.1 Biological Safety of PSi

The element Si is one of the most abundant chemical elements on the Earth's crust, commonly found as silicon dioxide, SiO_2. In the human biology the role of Si is still matter of debate, although it is known to be absorbed daily from food in the form of [$Si(OH)_4$], and also having a positive role in the health of connective tissues and bone (Jugdaohsingh et al. 2002; Sripanyakorn et al. 2004).

Silicon dioxides (SiO_2) are generally recognized as safe food substances and listed in the U.S. Food and Drug Administration (FDA) inactive ingredients database as used in, e.g. oral and topical drug products (FDA 1979). The emergence of PSi-based materials in drug delivery applications has boosted extensive safety studies on these materials in the field, although our knowledge of the toxicological effects in vitro and in vivo of these materials is still very limited and far of being systematic (Bimbo et al. 2012a).

Some of the major hurdles when fabricating PSi materials is to ensure a successful controlled and targeted release for a diversity of therapeutics in the body with minimal side effects and, simultaneously, ensuring drug efficacy. Examples of the in vitro and in vivo studies employing PSi nanoparticles are discussed in the following sections. For the direct assessment of the response of the various cancer cell lines when exposed to PSi materials, different parameters and end points have to be considered such as cell viability, cell apoptosis, DNA synthesis and fragmentation, cell proliferation, cell membrane integrity tests, cell cycle, cellular stress and inflammation, and cell–biomaterial association/uptake studies. The assays used should not interfere with the PSi materials in order to avoid erroneous results (Laaksonen et al. 2007). Indirect assessments should also be considered and these include exposure to drugs, residual solvents, degradation products, etc. The in vitro screening is generally faster, less expensive, less time-consuming and ethically more appropriate than the animal tests.

Following the promising drug delivery applications of PSi materials, particularly in oral drug delivery (Foraker et al. 2003; Salonen et al. 2008), there have been several studies on the in vitro interactions of PSi-derived materials in the GI-derived cancer cell lines, as well as evaluating the inflammatory and immune responses and cellular uptake in macrophage cells (Laaksonen et al. 2007; Bimbo et al. 2010, 2011a, b, 2012b; Santos et al. 2010; Liu et al. 2013; Mäkilä et al. 2012; Sarparanta et al. 2012a, b; Tahvanainen et al. 2012; Vale et al. 2012). Following the in vitro studies, animal tests provide information on the biodistribution of the PSi nanomaterials after administration via different routes (e.g. oral, subcutaneous and intravenous), providing also additional information on the particle accumulation, local inflammation and clearance. Unlike the in vitro models, the in vivo set-up also takes into account the biological environment.

10.3.1.1 In Vitro Biocompatibility

PSi nanocarries administrated in the body have to overcome several physiological barriers before reaching their sites of action, regardless of the administration route. During their translocation, the particles also interact extensively with the cellular environment and will interact with the body's natural defence mechanisms, which can limit, sequester or dissolve the particles before they reach their targets. For example, nanoparticles of different concentrations, sizes and surface chemistry can elicit different types of responses at different levels in the cells and tissues which will activate their effective antioxidant defences that deal with ROS generation (Ainslie et al. 2008; Low et al. 2010).

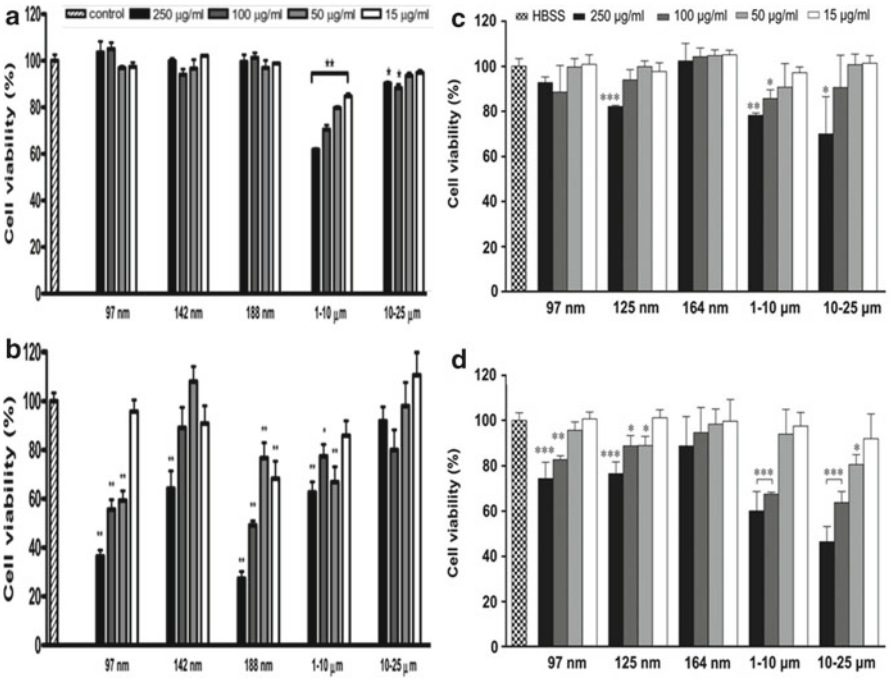

Fig. 10.6 Cell viability of Caco-2 intestinal (**a**, **c**) and RAW 264.7 macrophage (**b**, **d**) cells assessed by a CellTiter-Glo® luminescent assay after 24 h incubation with different concentrations (µg/mL) of THCPSi (**a**, **b**) and TOPSi (**c**, **d**) particles. Error bars represent SEM (**a**, **b**) or s.d. (**c**, **d**) ($n \geq 3$); the statistical analysis by ANOVA shows the level of significance at probabilities of $^{*}p < 0.05$ $^{**}p < 0.01$ and $^{***}p < 0.001$. Modified with permission from Bimbo et al. (2010, 2011a); copyright © 2010 and 2011, American Chemical Society and Elsevier B.V., respectively

In order to evaluate the possible harmful effects of nanostructured PSi nanoparticles, Bimbo et al. assessed the cellular toxicity and oxidative response elicited by different concentrations, sizes and PSi particle types (Bimbo et al. 2010, 2011a). In the first set of experiments, the toxic response of the THCPSi and TOPSi nanomaterials were incubated with intestinal and macrophage cell lines for 24 h and the cell viability was assessed by two different methods (Santos et al. 2010): (1) a luminescent method based on the quantification of the metabolically active in terms of ATP-content cells; and (2) a fluorescent method based on the detection of a conserved and constitutive protease activity associated with intact viable cells. Figure 10.6 shows a size and concentration-dependent toxicity of the PSi nanoparticles when assessed with the luminescence-based assay. It can be see that in general the PSi microparticle elicited greater cytotoxic response than the PSi nanoparticles of sizes of 142 and 164 nm for THCPSi (Fig. 10.6a, b) and TOPSi (Fig. 10.6c, d), respectively.

One strategy to make the THCPSi surfaces even more biocompatible and chemically stable is by self-assembling a biofilm of protein hydrophobin class II (HFBII) onto its surface (De Stefano et al. 2007, 2009). Hydrophobins are a family of

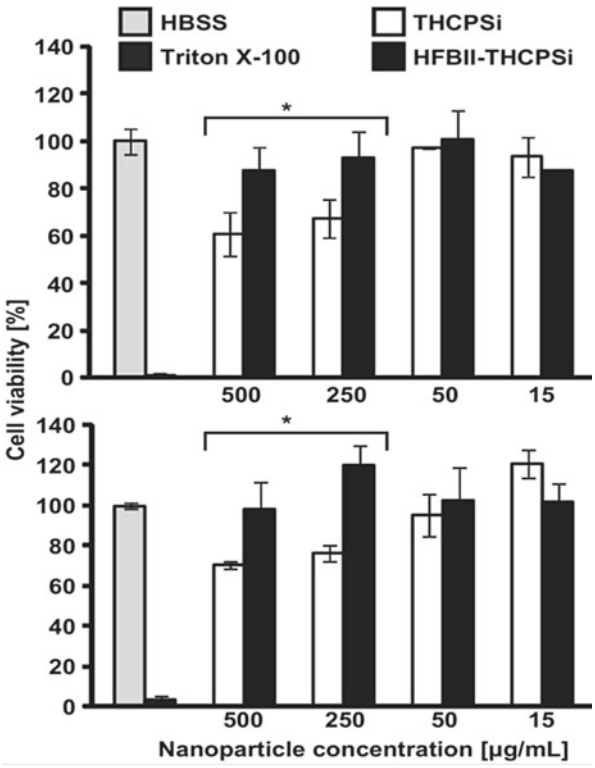

Fig. 10.7 Viability of stomach AGS cells incubated with HFBII-THCPSi nanoparticles for 6 h (*top*) and 24 h (*bottom*). Values represent mean ± s.d. ($n \geq 3$) and statistical analysis by Student's *t*-test show the level of significance at the probability of $*p < 0.05$. Reprinted with permission from Sarparanta et al. (2012b); copyright © 2012, American Chemical Society

surface active proteins of fungal origin that have the ability to form self-assembled layers on hydrophobic materials and modify the surface binding properties of the materials (Linder 2009). Hence, the wettability properties of hydrophobic THCPSi can be enhanced, leading to the production of a chemically and mechanically stable homogenous monolayer of self-assembled proteins with improved characteristics in terms of surface interaction.

In this context, we developed a protocol in order to reach the maximum coating efficiency onto THCPSi materials with minimum loss of protein (Bimbo et al. 2011b, 2012b; Sarparanta et al. 2012a, b). The HFBII solution is prepared using McIlvaine's buffer pH 4.0 as a solvent and, after the THCPSi particles are added to the HFBII solution at a mass ratio of 4:1 (particles: HFBII), the mixture is incubated at 80 °C for 30 min. Using this simple physical HFBII adsorption onto the surface of THCPSi nanoparticles (Bimbo et al. 2011b, 2012b; Sarparanta et al. 2012a, b), the THCPSi surface turns from hydrophobic to hydrophilic and, consequently, the biocompatibility of the particles is significantly improved (Fig. 10.7).

Besides the toxicity, nanomaterials may also induce oxidative stress to the cells which can elicit different types of responses at different levels, even if they are not cytotoxic (Nel et al. 2006). We have demonstrated that no significant increase in ROS is observed for both TOPSi (Bimbo et al. 2011a) and THCPSi (Bimbo et al. 2010) nanoparticles for the range of concentrations and sizes tested. In addition, inflammatory responses measured in terms of TNF-α production (tumor necrosis factor a; polypeptide cytokine produced by macrophages that stimulates acute phase reaction and used as an inflammatory response marker) have not shown a clear concentration dependence in the cells when treated with THCPSi nanoparticles (Bimbo et al. 2010) and also triggered in general a less pronounced inflammatory reaction than THCPSi microparticles.

The studies described above evidenced that particle size and concentration dependencies are important parameters in safety issues of PSi nanoparticles. From the range of particle sizes tested so far by our research group, TOPSi and THCPSi nanoparticles of 164 and 142 nm appeared to be the safest particles in terms of cytotoxicity, oxidative stress and inflammatory response.

10.3.1.2 Cellular Interactions

When considering the use of nanoparticles for oral and intravenous drug delivery, it is important to consider a delivery system where the particles do not cross the intestinal wall for safety reasons and are minimal recognized by the macrophages, respectively. For example, it is known that the particle's surface charge and size plays a crucial role in cell association or internalization (Gratton et al. 2008; Jiang et al. 2008), with positively charged particles below 100 nm being internalized/associated to a greater extent than negatively charged and larger ones, which also induce reduced cell viability.

In this context, we have demonstrated that THCPSi nanoparticles strongly associated in a concentration-dependent manner with both intestinal and macrophage cells (Fig. 10.8a) and that the majority of the particles remained attached to the cell surface when compared to controls (Bimbo et al. 2010). It was hypothesized that the negative zeta-potential of THCPSi nanoparticles together with their hydrophobic nature favoured a strong particle–cell membrane interaction, but prevented extensive internalization.

The interaction of uncoated and HFBII-coated fluorescent (FITC)-labelled THCPSi nanoparticles with HT-29 and Caco-2 intestinal cells showed that HFBII-coated THCPSi nanoparticles interacted more with the cells compared to uncoated ones (Bimbo et al. 2012b), but did not evidence a significant cellular internalization for either uncoated or HFBII-coated THCPSi nanoparticles (Fig. 10.8b). Both uncoated and HFBII-coated THCPSi nanoparticles are negatively charged, but HFBII coating provides a slightly higher zeta-potential than in the absence of coating, which seemed to played an important role in modulating the cellular association and, therefore, in augmenting the number of particles that associated with the

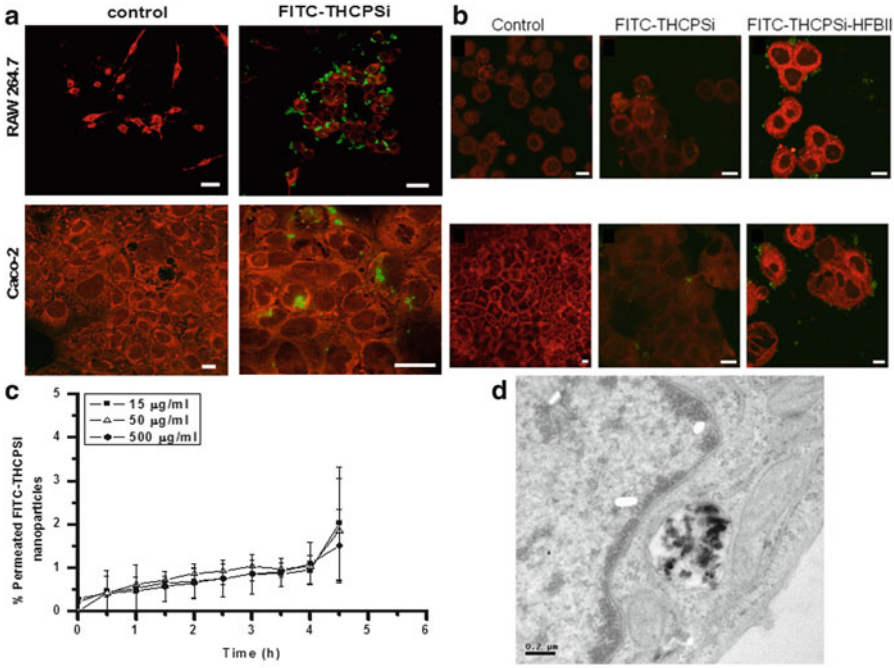

Fig. 10.8 (**a**) Confocal microscopy images with *z*-stack planes of the particle association of FITC-labelled THCPSi nanoparticles (155 nm; 15 and 50 µg/mL) incubated for 3 h with Caco-2 and RAW 264.7 macrophage cells ($n \geq 3$; mean ± SEM; scale bars = 20 µm). (**b**) Confocal microscopy images (scale bars = 10 µm) of HT-29 (*upper*) and Caco-2 (*lower*) incubated for 2 h with THCPSi nanoparticles: control cells (*left*), cells incubated with FITC-labelled THCPSi nanoparticles (*centre*) and cells treated with HFBII-coated FITC-labelled THCPSi nanoparticles (*right*). (**c**) Permeation of FITC-labelled THCPSi nanoparticles (155 nm) across Caco-2 cell monolayers as a function of time ($n \geq 3$; mean ± SEM). (**d**) TEM pictures of flat embedded ultrathin sections of TOPSi nanoparticles (164 nm; 50 mg/mL) inside the lysosomal compartments of RAW 264.7 macrophage. Modified with permission from Bimbo et al. (2010, 2012b); copyright © 2010 and 2012, American Chemical Society and the Royal Society of Chemistry

two different cell lines. The association was more pronounced in HT-29 cells than in Caco-2 cells due to its mucus-secreting nature, indicating that HFBII can possibly act as a mucoadhesive agent in the intestine.

The permeability of THCPSi nanoparticles across Caco-2 monolayers showed that less than 2 % of the particles permeated across the monolayers in 4.5 h regardless of the particle concentration (Fig. 10.8c), demonstrating their potential for oral drug delivery applications (Bimbo et al. 2010). The TOPSi nanoparticles internalized by macrophages did not penetrate the nucleus of the cells and were confined within the cytoplasmic vesicles of the cells (Fig. 10.8d).

10.3.2 Biodistribution, Accumulation and Clearance of PSi

Following in vitro testing, the in vivo assessment of a potential drug nanocarrier is crucial in order to understand the pharmacokinetics, pharmacodynamics and biodistribution of such a carrier in the body, and thus, anticipate its harmful reactions. The accumulation and clearance of the nanoparticulate systems are also critical parameters to address when such nanocarriers are to be translated to the clinic. The biodistribution studies concerning PSi nanoparticles are still in their infancy. Therefore, a thorough understanding of particle–cell interactions should be developed before a drug carrier can come into the clinical setting. Safety and biocompatibility of PSi engineered structures are important requirements for nanocarriers, particularly when in vivo exposure or administration is concerned. In this context, there are several different responses that the nanomaterials may induce when in contact with cells/tissues. Consequently, a full characterization of the toxicological issues regarding these structures is crucial in the preclinical stage.

One of the pioneering works reported in 2009 by Park et al. demonstrated that luminescent PSi nanoparticles of 126 nm in size and loaded with doxorubicin, slowly released the drug under physiological conditions reaching a maximum concentration within 8 h (Park et al. 2009). No significant toxicity could be related to the degradation product of the material in the animal tissues, which after intravenous injection were able to clear from the body within 4 weeks. Interestingly, by using the intrinsic properties of the PSi materials such systems were also shown to be suitable for in vivo imaging after intramuscular and subcutaneous injections in nude mice. The biodistribution and histological studies in mice 24 h post-injections showed that the PSi nanoparticles accumulated preferentially in the spleen rather than in the liver, whereas dextran-coated PSi accumulated more in the liver than in the spleen. The injection of the dextran-coated PSi nanoparticles into tumor bearing nude mice led to an accumulation of the nanoparticles in the tumor, aiding its visualization.

Another strategy to follow the biodistribution of PSi nanoparticles is to use radiometric methods (Bimbo et al. 2010; Huhtala et al. 2012; Sarparanta et al. 2012a, b). For example, Huhtala et al. investigated the release of insulin-like growth factor 1 (IGF-1) in infantile neuronal ceroid lipofuscinosis mouse model by using ^{125}I-labelled IGF-1 and ex vivo tissue radioactivity counting (Huhtala et al. 2012). It was shown that the THCPSi nanoparticles complexed ^{125}I-IGF-1 accumulated and remained in the liver, and from there IGF-1 was steadily released into the blood circulation.

10.3.2.1 ^{18}F-Radiolabelled PSi

As discussed above, radiometric methods such as autoradiography and ex vivo tissue radioactivity can provide detailed knowledge on drug carrier distribution, carrier behaviour in the body and binding of drug into its target. In this context, positron emission tomography (PET) and single photon emission computed tomography

(SPECT) are highly sensitive and non-invasive imaging modalities, which have the ability to accurately localize and quantify tracer biodistribution and accumulation, and only tracer amounts (pg–ng) of radiolabelled compound are needed for the detection.

Recently, the suitability of PSi nanocarriers for drug delivery applications was evaluated by studying the biodistribution of PSi-based materials in vivo using radiometric methods (Bimbo et al. 2010; Sarparanta et al. 2012a, b). Fluorine has a high affinity for Si, which has been exploited in labelling of PSi materials with a PET compliant radioisotope—^{18}F. Sarparanta et al. have successfully ^{18}F-fluorinated TOPSi, THCPSi and TCPSi materials (Sarparanta et al. 2011). Fluorine-18 is produced in a ^{18}O(p,n)^{18}F reaction on a cyclotron and the ^{18}F-fluorination with ^{18}F-nucleophiles proceeds likely via direct substitution of ^{18}F–Si for residual Si–H groups or for oxygen in Si–O–Si structures on the PSi surface (Fig. 10.9). The hydrolytic stability of the Si–F bond is strongly influenced by the nature of the substituents on the Si atom, and thus, the surface chemistry of the Si material mainly determines the stability of the ^{18}F–Si bonds in physiological conditions. TOPSi has an oxidized layer on its surface and it exhibits slightly lower hydrolytic stability of the ^{18}F–Si bond in alkaline conditions and in plasma (Sarparanta et al. 2011), when compared to the THCPSi and TCPSi materials.

Recently, we have also proved that ^{18}F-labelled THCPSi nanoparticles can be used as a reliable tool in studying biological properties of PSi-based nanocarriers in vivo (Bimbo et al. 2010). The great advantages are that fluorine is small in size and the nucleophilic ^{18}F-fluoride used for labelling the material can be produced with high specific activity. Consequently, ^{18}F-fluorination of the THCPSi's surface can be attained with minimal influence on the surface chemistry of the material, which in turn also has negligible influence on the particles behaviour in vivo. Furthermore, the fact that free ^{18}F-fluoride is efficiently taken up from the blood circulation by bone osteoblasts, makes it an excellent internal control for the stability of the radiolabel on the surface of the particles in vivo (Fig. 10.10). Also, ^{18}F has a half-life of ca. 109.8 min which is long enough to allow the follow-up of slower physiological processes, such as the passage in the GI tract (Bimbo et al. 2010). Because it is a short-lived positron emitter, ^{18}F can be used for non-invasive visualization of the particle biodistribution using, e.g. PET.

Following the approach described above, first we used ^{18}F-THCPSi nanoparticles as tracers to study the biodistribution of nanoparticles in rats (Bimbo et al. 2010). The biodistribution studies were performed after administration of THCPSi nanoparticles orally (dose ~0.214 mg; 18.95 MBq), subcutaneously (dose ~0.075 mg; 12.44 MBq) and intravenously (dose ~0.030 mg; 9.18 MBq) and by analysing the ex vivo tissue radioactivity counting. The activity concentration of the labelled nanoparticles in these experiments was 207 ± 139 MBq/mg, corresponding to ca. 5–100 ng of ^{18}F per 1 mg of the THCPSi nanoparticles.

Macroautoradiographical analysis of the lower GI tract provided a quantitative analysis of the biodistribution of THCPSi nanoparticles administered orally, as shown in Fig. 10.10a. The particles given orally did not cross the intestinal wall, as proved by the negligible amount of radioactivity detected in the systemic circulation

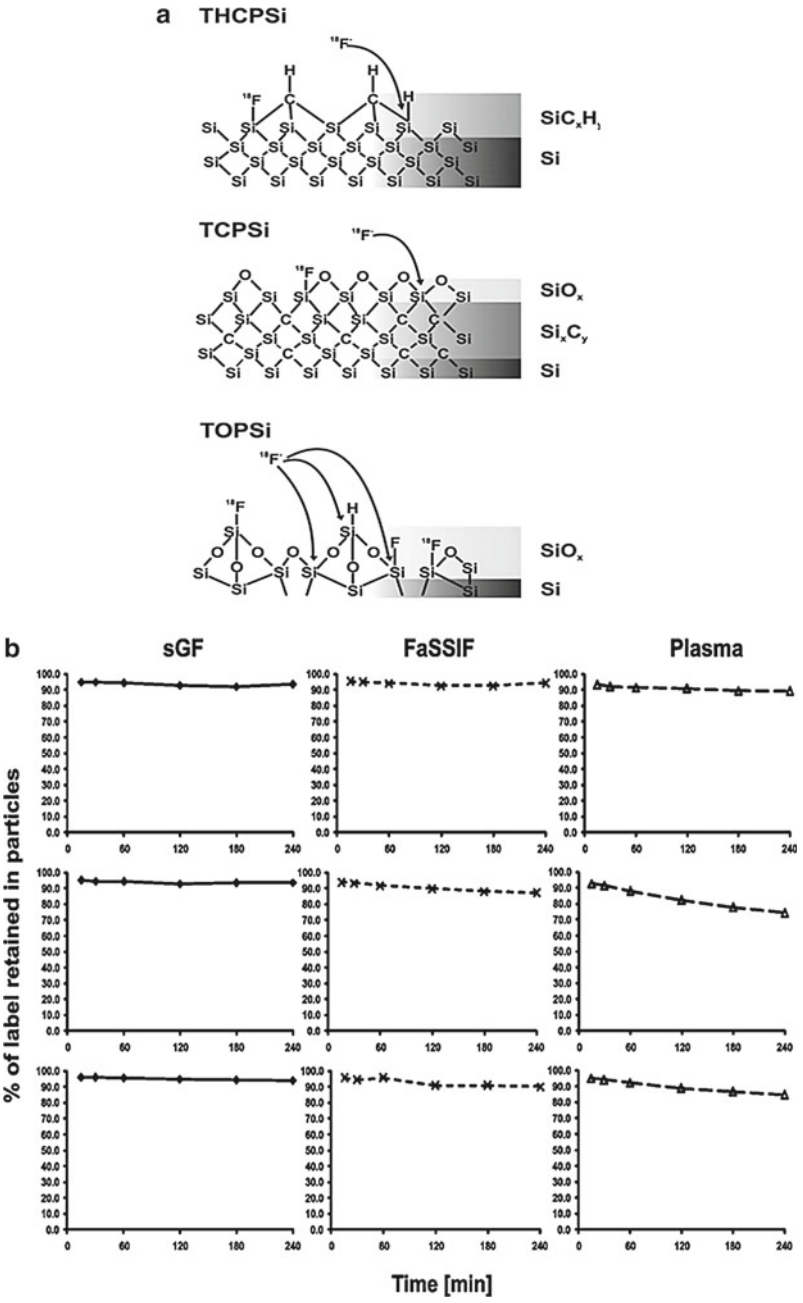

Fig. 10.9 Possible mechanisms taking place for the ^{18}F incorporation on PSi surfaces (**a**). ^{18}F-label stability (**b**) in simulated gastric (sGF), fasted-state intestinal fluids (F) and in human plasma at 37 °C: ^{18}F-THCPSi (*top*), ^{18}F-TOPSi (*middle*) and ^{18}F-TCPSi (*bottom*). Modified with permission from Sarparanta et al. (2011); copyright © 2011, American Chemical Society

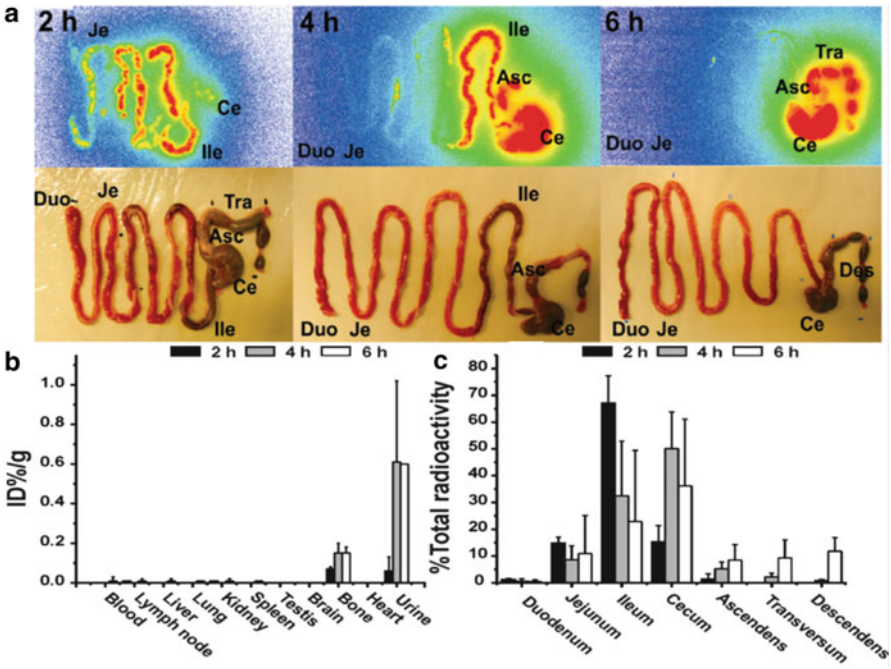

Fig. 10.10 (**a**) Macroautoradiographs and respective photographs of the GI tracts of rats after oral administration of ^{18}F-THCPSi nanoparticles 2, 4 and 6 h (from *left* to *right*). *Duo* duodenum, *Je* jejunum, *Ile* ileum, *Ce* cecum, *Asc* ascendens, *Tra* transversum, *Des* descendens. Biodistribution of ^{18}F-THCPSi nanoparticles after oral administration (**b**) and their radioactive distribution in the lower GI tract (**c**), as quantified from autoradiography ($n = 3–4$; mean ± s.d.). Modified with permission from Bimbo et al. (2010); copyright © 2010, American Chemical Society

or organs outside the GI tract (Fig. 10.10b). Remarkably, only a very minor fraction of radioactivity was detected in bone and urine (<0.6 ID%/g), indicating minor detachment of the ^{18}F-label from the surface of the THCPSi nanoparticles. This detachment of the label was probably due to the in vivo defluorination or disintegration of the particles themselves in the harsh conditions of the GI tract. The results show that the majority of the administered nanoparticles reached the distal parts of colon in 6 h, demonstrating that the half-life of ^{18}F is sufficient to follow the passage of the particles through the GI tract (Fig. 10.10c).

The ^{18}F-THCPSi nanoparticles administered subcutaneously under the loose skin at the back of the animal resided in the subcutaneous space for at least up to 4 h post-injection. In this case, only minimal leakage (<1 ID%/g) of dissociated ^{18}F-label into the circulation and subsequent accumulation in bone and urine was observed. After intravenous administration, the ^{18}F-THCPSi nanoparticles were found in the liver and spleen, indicating fast elimination of the particles from the circulation. In this case, the ^{18}F–Si bond also exhibited excellent hydrolytic and enzymatic stability with less than 0.3 %ID/g detected in bone at later time points (Bimbo et al. 2010).

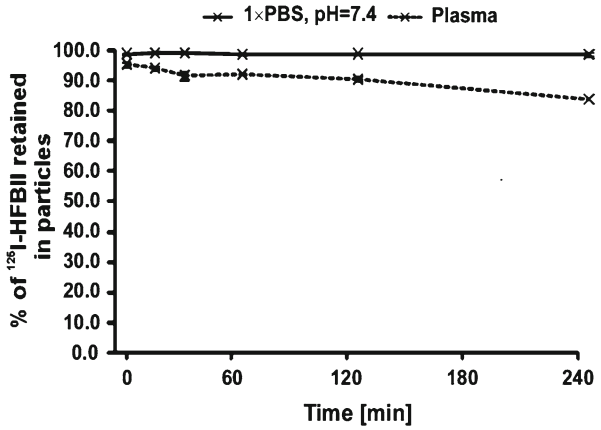

Fig. 10.11 Stability of the HFBII coating on THCPSi nanoparticles in PBS (pH = 7.4) and in human plasma at 37 °C ($n = 2$; values represent the average ± s.d.). Reprinted with permission from Sarparanta et al. (2012a); copyright © 2012, American Chemical Society

10.3.2.2 Stability, Plasma Protein Adsorption and Biodistribution of Protein-Functionalized PSi

Following the previous successful works, we next employed radiometric methods to assess the influence of HFBII-coated THCPSi nanoparticles in vivo (Sarparanta et al. 2012a, b). In this approach, the efficiency of the HFBII self-assembling on the THCPSi surface was monitored after radiolabelling the protein with ^{125}I using the Bolton–Hunter method for labelling proteins with radioisotopes of iodine (Sarparanta et al. 2012a); ^{125}I-HFBII was then used to coat the THCPSi nanoparticles. Figure 10.11 shows the stability of the coating of the ^{125}I-HFBII-THCPSi nanoparticles in different physiologically relevant conditions. The results indicated that the coating was stable in phosphate buffered saline (PBS), with over 98 % of the ^{125}I-HFBII still bound to the particles after 240 min. In plasma, the HFBII coating detached gradually, but slowly, with still 83 % of the coating intact after 240 min.

In order to avoid possible protein detachment from the surface of the particles and to follow the radioactivity signal of the particles (not from the detached or decomposed protein), the in vivo biodistribution studies were performed with the ^{18}F-labelled THCPSi nanoparticles, which were subsequently coated with the protein. After intravenous administration in rats, we assessed the biodistribution of the HFBII-^{18}F-THCPSi nanoparticles by ex vivo tissue radioactivity counting (Sarparanta et al. 2012a). The results showed no significant improvement in the blood circulation times of the particles. Instead, a high uptake of the nanoparticles was observed in the major MPS organs, liver and spleen, already after 15 min. Despite of this, it was found that the HFBII coating significantly altered the liver-to-spleen ratio of ^{18}F-THCPSi uptake, with roughly equal amounts of the HFBII-^{18}F-THCPSi nanoparticle-dosed animals distributed to the spleen and liver, whereas in

Table 10.2 Proteins adsorbed onto THCPSi and HFBII-THCPSi nanoparticles after 120 min of incubation in 100 % human plasma

Protein	THCPSi	HFBII-THCPSi
Apolipoprotein B-100	–	–
Complement C3	–	+
α-2-Macroglobulin	–	–
Inter-α-trypsin inhibitor heavy chain H4	+	+
Fibrinogen α chain	+	–
Serotransferrin	+	–
Serum albumin	+	–
Fibrinogen β chain	+	–
Clusterin (apolipoprotein J)	–	+
Hemopexin	–	–
Fibrinogen γ chain	+	–
IgM μ chain C region	–	+
Apolipoprotein A-IV	–	+
Apolipoprotein E	–	+
IgG γ-1 chain C region	+	+
IgG γ-2 chain C region	+	+
Apolipoprotein A-I	+	+

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the case of ^{18}F-THCPSi-treated animals the spleen uptake remained twofold to the liver uptake.

Based on the biodistribution results, we further investigated the composition of the plasma protein corona on the two nanoparticle types. The surface biofunctionalization of THCPSi nanoparticles with HFBII clearly modified the plasma protein adsorption, resulting in significant alteration in their accumulation to the liver and spleen. Table 10.2 shows the remarkable differences in protein adsorption between HFBII-coated and uncoated THCPSi nanoparticles (Sarparanta et al. 2012a).

Only inter-α-trypsin inhibitor heavy chain H4, immunoglobulin G (IgG, γ-1 and γ-2 chain C regions) and apolipoprotein A-I were found in both THCPSi and HFBII-THCPSi nanoparticles. Interestingly, abundant plasma components fibrinogen, serotransferrin and serum albumin were found only in THCPSi nanoparticles, whereas complement C3, IgM, apolipoproteins E and A-IV, and clusterin (apolipoprotein J) were found only in HFBII-THCPSi nanoparticles. The proteins α-2-macroglobulin, hemopexin and apolipoprotein B-100 were not detected in either of the nanoparticles tested. Since IgG was found on both THCPSi and HFBII-THCPSi nanoparticles, the differences in their liver and spleen uptake cannot be solely accounted for by immunoglobulin-mediated uptake. In addition, the increase in size of ^{18}F-THCPSi as a result of plasma protein adsorption could have contributed to the increased splenic filtration and concentration of the particles in the spleen. Furthermore, proteins associated with immune recognition and phagocytosis dominated on the HFBII-THCPSi surface, whereas the known dysopsonin protein serum

albumin was not detected. The removal of HFBII-^{18}F-THCPSi from the circulation and the increase in the liver uptake versus accumulation in the spleen was hypothesized to be due to IgM (a promoter of the complement activation and opsonization), the complement factor C3 (induces phagocytosis into Kupffer cells) and apolipoproteins E and J (promote recruitment of "non-professional" phagocytes, e.g. hepatocytes and fibroblast cells).

10.3.2.3 Mucoadhesive and Gastroretentive Properties of Protein-Coated PSi Nanoparticles

In order to assess the passage of HFBII-coated THCPSi nanoparticles in the GI-tract after oral administration, we used the same radiolabelling and coating procedure as described before (Sarparanta et al. 2012b). The biodistribution of the nanoparticles in the GI tract was again monitored with macroautoradiography and compared to the previously analysed distribution of the uncoated ^{18}F-THCPSi nanoparticles. During the first 2 h after administration, HFBII-^{18}F-THCPSi nanoparticles were retained longer time in the stomach than the uncoated ^{18}F-THCPSi nanoparticles (Fig. 10.12a).

The coated particles persisted in the stomach for up to 3 h after administration and, after entering the small intestine, behaved similarly to the uncoated ones. This was explained by the pH-response of the HFBII coating to the environment conditions of the GI tract. In sGF the coating exhibited good stability, but in FaSSIF the stability of the coating was dramatically decreased with almost 45 % of the coating detached immediately. Similarly as described above, the apparent adhesion to the mucosa was monitored by autoradiography of cryo-sections from the stomach and ileum (Sarparanta et al. 2012b). In order to increase the resolution of digital autoradiography, the sections were subsequently stained with hematoxylin–eosin (H&E) and examined with microscopy along the mucosa (Fig. 10.12b). The results confirmed two important phenomena: (1) the gastric retention was a result of the mucoadhesion of the HFBII-THCPSi nanoparticles, leading to an increase in the transit time of the drug carrier in the GI tract of the rat; and (2) the particles lost their mucoadhesive properties after entering the small intestine, as can be seen from the macroautoradiographs in Fig. 10.12c.

10.3.3 Drug Delivery of PSi-Based Nanomaterials

In order to enhance the dissolution properties of poorly water-soluble drugs, as well as to facilitate the delivery of larger molecules, drug molecules are confined inside the PSi nanopores. The improvement in drug dissolution behaviour is due to the fact that the adsorbed molecules do not retain the original crystalline structure but exist (at least partly) in an amorphous state (Salonen et al. 2005). A decrease in the Gibbs free energy makes the amorphous system physically stable. Drug molecules

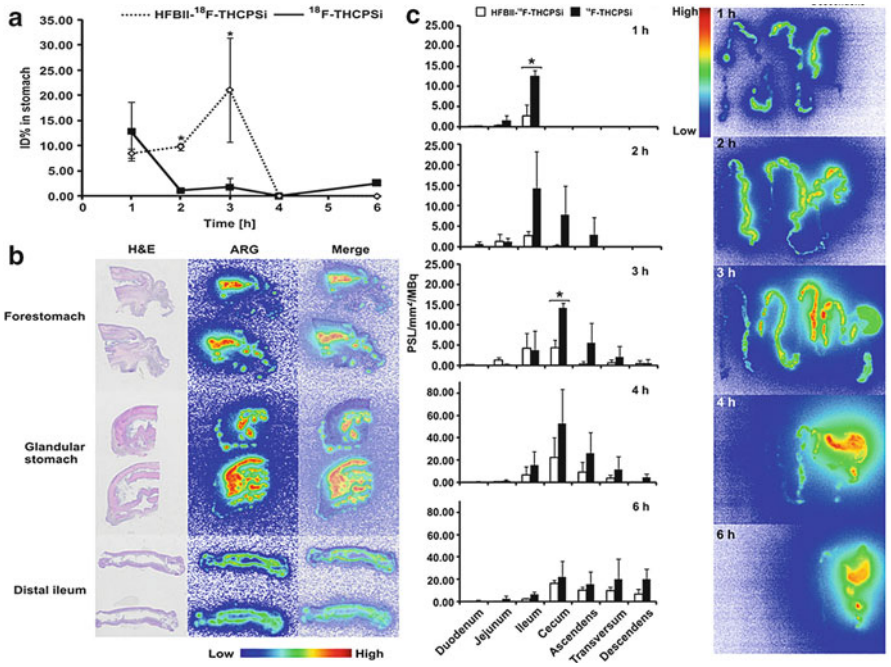

Fig. 10.12 (**a**) Gastric clearance of HFBII-^{18}F-THCPSi nanoparticles versus uncoated ^{18}F-THCPSi nanoparticles. Values represented as mean ± s.d. ($n = 3$; $*p < 0.05$). (**b**) Autoradiography and H&E stain of rat stomach and ileum. (**c**) Distribution of HFBII-^{18}F-THCPSi and ^{18}F-THCPSi nanoparticles in the rat lower GI tract (*left*) as a function time quantified from macroautoradiography (*right*) at 1–6 h after oral administration and expressed as units of photostimulable luminescence (PSL) per mm^2 (values represented as mean ± s.d. ($n = 3$; $*p < 0.05$). Modified with permission from Sarparanta et al. (2012b); copyright © 2012, Elsevier B.V.

confined in the PSi pores behave very differently to those in bulk. This ensures that the therapeutic compounds carried by the PSi materials will be efficiently released from the pores and dissolved in external medium. The release of the drug from the pores is usually associated with simple diffusion or triggered (controlled) release processes, as well as with combined effects of leaching and PSi matrix dissolution, which can be slower or faster depending on the PSi surface treatment and surrounding conditions. The drug release usually follows first-order kinetics with respect to the drug concentration and is affected by the surface chemistry, morphology and pore size of the PSi particles.

PSi-based materials can be prepared to meet the needs of oral drug delivery, including fast drug dissolution, enhanced drug permeation across the intestinal barriers and reduction in pH dependency of the oral drug absorption (Salonen et al. 2005; Heikkila et al. 2007; Kaukonen et al. 2007), thus improving drug bioavailability (Wang et al. 2010). Currently, major challenges faced in the formulation of poorly water-soluble drugs are the solid state stability and the establishment of a reliable in vitro–in vivo correlation. High local concentrations and possible

supersaturation provided by the enhanced drug dissolution properties can be highly beneficial for drug absorption in the GI tract.

Several studies have shown that different biologically active agents can be loaded and, subsequently, released from PSi-based materials (Salonen et al. 2005; Kaukonen et al. 2007; Bimbo et al. 2011a, b). The dissolution of poorly water-soluble drugs from the particles is usually faster than that of bulk APIs. However, when the solubility of the API is high, a delayed release can be achieved from the particles (Salonen et al. 2005). The fast dissolution from the particles is related to the confined space inside the pores that prevents long range ordering, thus preventing the crystallization of the loaded substances. The pore diameter of the particle and the surface chemistry are important factors affecting the release rate of the loaded drugs (Limnell et al. 2007; Kinnari et al. 2011), and in turn the release rate is always a combination of the properties of the loaded substance and the particle.

10.3.3.1 Enhanced Drug Solubility and Permeability

In order to evaluate the potential of PSi-based materials as drug carriers, Caco-2 (a human colon epithelial cancer cell line) has been widely used as a model for human intestinal absorption (Salonen et al. 2005; Kaukonen et al. 2007; Artursson et al. 2001). To validate the efficacy of TOPSi nanoparticles (170 nm) as a drug delivery system, the permeation properties of the nanoparticles loaded (17.3 wt.%) with griseofulvin (an antifungal drug belonging to BCS class II) were assessed (Bimbo et al. 2011a). The results showed a significant increase in drug permeation across the Caco-2 monolayers compared with the bulk drug at pH 5.5 (Fig. 10.13a) and 7.4. This was attributed to the enhanced drug solubility obtained as a result of the drug loading into the TOPSi nanoparticles, leading to high local concentrations and even drug supersaturation, which could increase the net drug absorption and respective drug permeation in the intestine (Kaukonen et al. 2007).

We also evaluated the effect of HFBII-coating onto PSi materials on the drug release and permeation across Caco-2 cell monolayers (Bimbo et al. 2012b). Due to the size and porosity of the THCPSi nanoparticles, the self-assembled protein layer could block the pores and prevent or, to some extent, hinder the drug release from the pores. Therefore, to assess if the HFBII could permeate across the Caco-2 monolayer, ^{125}I-HFBII-THCPSi nanoparticles were tested and the radioactivity of the apical and basolateral compartments of the Transwells® was measured. Negligible radioactivity was measured in the basolateral compartment over a period of 240 min, with less than 1 % of the administered dose permeated. The permeation of indomethacin (IMC; a non-steroidal anti-inflammatory drug belonging to BSC class II) across a differentiated Caco-2 cell monolayer from the THCPSi nanoparticles showed that the permeation rate of the loaded IMC (15.2 wt.%) from the particles was similar to the drug solution (Fig. 10.13b). The IMC amount permeated across the differentiated Caco-2 monolayer was ca. 22 % for both HFBII-coated THCPSi and the drug solution after 240 min. Therefore, HFBII coating of the nanoparticles did not hamper in any way the normal release of IMC from the THCPSi nanoparticles.

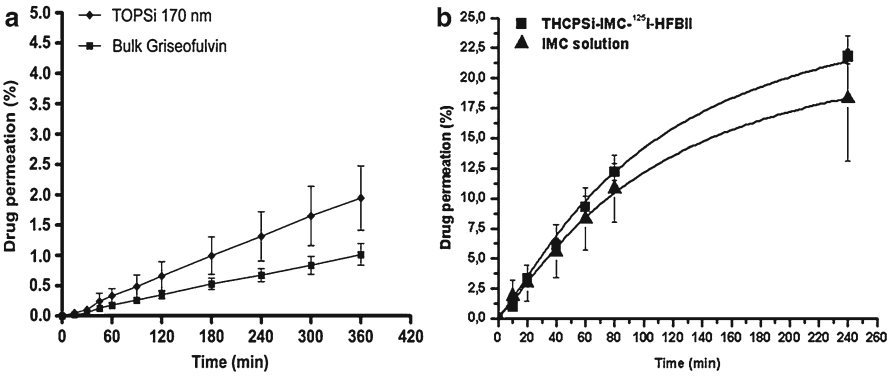

Fig. 10.13 Permeation of bulk (**a**) griseofulvin-loaded TOPSi nanoparticles (170 nm) and (**b**) IMC-loaded THCPSi nanoparticles across a differentiated Caco-2 cell monolayer at apical pH of 5.5 (**a**) and 7.4 (**b**). The basolateral solution in the permeation experiments was kept at pH 7.4. Error bars represent s.d. ($n \geq 3$). Modified with permission from Bimbo et al. (2011a, 2012b); copyright © 2011 and 2012, Elsevier B.V. and the Royal Society of Chemistry

The drug release and permeation of the drugs from the TOPSi and THCPSi nanoparticles suggests that PSi can simultaneously enhance the solubility and, consequently, the drug permeation of poorly water-soluble drugs across biological barriers, regardless of particles' surface chemistry.

10.3.3.2 Advanced PSi-Based Nanocarriers

One of the major obstacles in drug delivery is the fabrication of nanocarriers with excellent stability under physiological conditions, which can both efficiently encapsulate therapeutic agents and control the release of their payloads. Obtaining high loading degrees in nanostructured PSi materials is only one requirement for efficient drug delivery (Salonen et al. 2008; Santos et al. 2011a, b). In addition, the PSi nanomaterials are required to retain and protect the loaded therapeutic molecules within the mesopores before reaching the targeted sites, without major leaks to maximize their treatment and/or imaging efficacy, and minimize their toxicity (Santos et al. 2011a, 2013a; Bimbo et al. 2012a; Santos and Hirvonen 2012). This is also important when drug nanocarriers administered intravenously have to be transported in the bloodstream to the tumor cells, avoiding the MPS screening and capture by the liver and spleen, and thus, achieving prolonged circulation times.

Several strategies have been developed to incorporate payloads within the PSi nanostructure, e.g. by using covalent attachment and trapping the cargos by oxidation (Wu and Sailor 2009). The majority of these strategies involve chemical reactions which may lead to degradation and inactivation of the payloads. In order to tackle this problem, Liu et al. have recently developed a new approach to control the release of drugs from the PSi nanoparticles presenting the following important

features for targeted/controlled drug delivery (Liu et al. 2013): (a) superior suspensibility and better stability against aggregation in aqueous solutions; (b) increased particle smoothness; (c) improved cytocompatibility, in particular with rather sensitive cell lines, such as macrophages; (d) reduced cellular association; (e) improved stability in human plasma in vitro; (f) prolonged drug delivery over a longer period of time, preventing the initial drug release burst.

In the aforementioned work, a novel nanocomposite based on the encapsulation of THCPSi nanoparticles with solid lipid nanoparticles (SLNs) on a 1:1 ratio to form a THCPSi-SLN nanocomposite (...) THCPSi-SLNC (...) was prepared using a solid-in-oil-in-water (S/O/W) emulsion solvent evaporation method. In this method, glycerol monostearate, phosphatidylcholine and THCPSi nanoparticles are dispersed in ethanol at 70 °C forming the oil phase, whereas the aqueous phase is composed of 1 % polyvinyl alcohol (PVA, Mw 31–50 kDa), 1 % polyethylene glycol 6000 (PEG 6000) and heated to the same temperature. The oil phase is then added into the aqueous phase drop-by-drop under vigorous stirring, and after solvent evaporation the hot emulsion is gradually dropped into 1 % PVA containing 1 % PEG 6000 under vigorous stirring at 2 °C to allow the solidification of the SLN matrix.

This approach allowed the successfully encapsulation of spherical-like THCPSi nanoparticles (Fig. 10.14a) in the SLN matrix, to form a nanocomposite with rather different surface smoothness and hydrophobicity compared to the bare THCPSi nanoparticles (Fig. 10.14b, c). In addition, the stability of the nanocomposite in human plasma was enhanced drastically (Fig. 10.14d), which is an important parameter regarding the intravenous administration of this nanocomposite drug formulations. Another remarkable feature of this drug delivery system is that after encapsulation, the cytocompatibility of the THCPSi nanoparticles in macrophage (Fig. 10.14e), intestinal and liver cancer cells was also greatly improved. This was explained as a result of the reduced cellular association between the nanocomposite and the cells compared to the bare nanoparticles, particularly in the case of macrophages, which can also be regarded as advantageous for parenteral drug delivery as it may provide better protection against opsonization. Loading of a model drug (furosemide; FUR; BCS class IV) into the nanocomposite further demonstrated its capability to sustain the release of the drug, indicating that the SLN matrix successfully seals the pores of the THCPSi nanoparticles, avoiding the common initial burst drug release and acting as a promising nanovector system for drug delivery applications.

10.4 Conclusions and Future Perspectives

In this chapter we presented a brief overview on the techniques used to fabricate and characterize nanostructured PSi-based materials, as well as the commonly used methodologies for drug loading and studying drug release from PSi nanomaterials. We further highlighted some of the promising features of PSi nanoparticles in drug delivery and biomedical applications with several in vitro and in vitro examples.

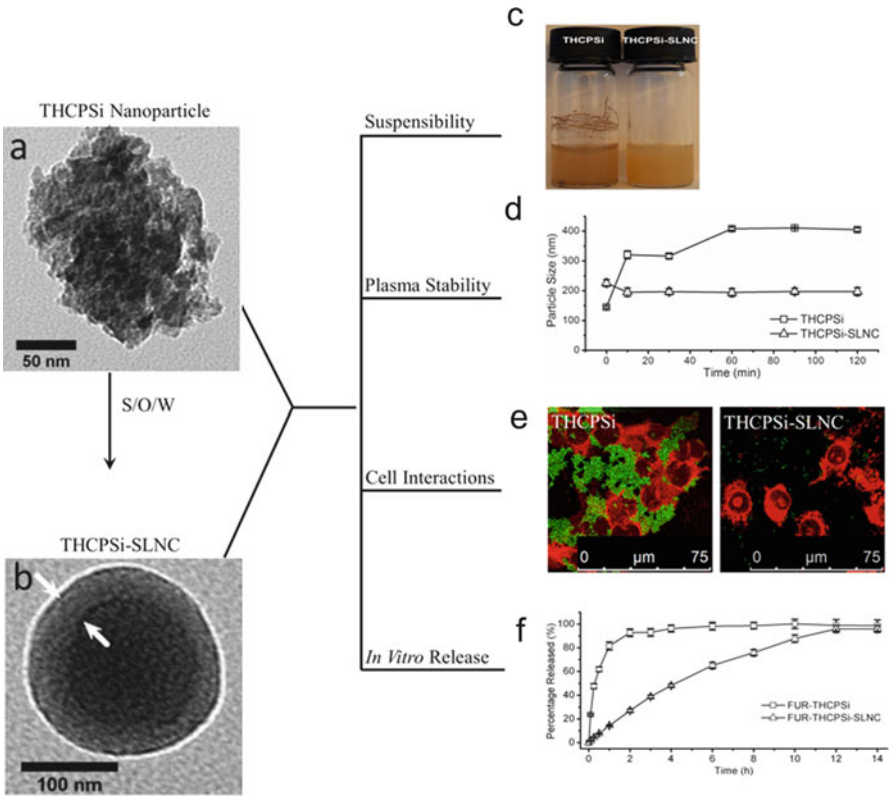

Fig. 10.14 TEM images of (**a**) THCPSi nanoparticles and (**b**) THCPSi-SLNCs prepared using an S/O/W emulsion evaporation method. (**c**) Dispersions of the THCPSi nanoparticles and THCPSi-SLNCs in aqueous solution, demonstrating the higher stability of the particles. (**d**) Impact of the human plasma on the particle size for both the THCPSi nanoparticles and the THCPSi-SLNCs within 2 h incubation at 37 °C (values denote the mean ± s.d.; $n=3$). (**e**) Confocal fluorescence microscopy images of RAW 264.7 macrophages cells (stained with *orange* CellMask©) after 3 h incubation with FITC-labelled THCPSi nanoparticles (stained in *green*) and FITC-labelled THCPSi-SLNCs at 37 °C (scale bars = 75 µm), showing a reduction in cellular association to the later particles. (**f**) Controlled FUR release from THCPSi nanoparticles and THCPSi-SLNCs at pH 5.5 and 37 °C (error bars represent the mean ± s.d.; $n=3$). Modified with permission from Liu et al. (2013); copyright © 2013, WILEY-VCH Verlag GmbH & Co. KGaA

The discovery of PSi over 50 years ago has led to vast applications of this material in several areas, with particular emphasis in the health sciences in the last decade. Nowadays, PSi is an acceptable biomaterial with a number of attractive features as a solid carrier for poorly water-soluble drugs, rendering it an efficient drug carrier. These outstanding features include:

(a) The fabrication parameters of PSi such as the surface chemistry, particle and pore sizes, and shape and morphology can easily be controlled to attain good biocompatibility and biodegradability of the materials.

(b) High porosities (>70 %) of the PSi particles offer the possibility to reach high drug loading degrees into PSi.
(c) Restricted confinement of drug molecules into the mesopores in an amorphous- or non-crystalline-like state, preventing crystal growth and consequent stabilization of the disordered structure of the drug.
(d) The mesopores are able to protect the payload molecules against the harsh conditions of the body environment and from enzymatic degradation, particularly with very sensitive compounds (e.g. proteins and peptides).
(e) The precise control of the surface properties and the tunability of the pore size of PSi allow suitable controllable release kinetics of the drug molecules.
(f) The production of hydrophilic PSi particles, such as TOPSi nanoparticles, can be used to improve particle/drug wettability.
(g) Easy surface chemistry modification via oxidation, carbonization and hydrosilylation for further surface functionalization of the PSi materials provides an alternative for more advanced controlled drug delivery or targeting.
(h) The chemistry of ^{18}F–Si is simple and very stable, enabling the modification of the PSi's surface for biomedical imaging applications.
(i) Finally, because the PSi production is based on a top-down approach, easy scaling-up for industrial use can be attained.

As described in this chapter, PSi has been shown to be a promising nanocarrier for drug compounds due to its numerous attractive properties that can also be synergistically exploited in the development of advanced drug delivery systems. Proof of concept for the use of PSi as a solid dispersion carrier for the oral delivery of drugs has been established (i.e. to overcome the challenge of low solubility, low permeability across the GI tract and variable drug bioavailability) without any particular requirements for the chemistry modification of the drug molecules. After the loaded drug is released, all that is left in the body is pure Si, which dissolves into non-toxic silicic acid and is safely excreted from the body. In addition, a ^{18}F-radiolabelled PSi surface can act as an imaging agent, which can be used with highly sensitive and non-invasive imaging modalities to accurately localize and quantify tracer biodistribution and accumulation in vivo.

Although a vast literature can be found on drug delivery using PSi particles, the clinical potentialities of these materials remain to be shown in the future. Furthermore, the production of PSi materials at an industrial level is still limited. Another important aspect of PSi is its safety, particular when dealing with nanoparticles. The biodistribution of the PSi nanoparticles has been followed and the biocompatibility has been demonstrated in vitro and in vivo, but the effect of the accumulation of such materials in the long term is not yet fully understood. More in vivo and clinical-oriented studies are urged to confirm the potential of PSi as a drug nanocarrier.

The external surface of the PSi nanoparticles can be modified to be, e.g. gastroretentive and mucoadhesive, and thus, the drug bioavailability can still be further increased. In order to avoid a burst release of the drugs, the PSi nanoparticles can also be encapsulated in more complex delivery systems such as SLNs, in order to sustain the drug delivery. It is now well-acknowledged that the high specific area, high pore volume, tuneable pore structures and the physicochemical stability render

the PSi materials excellent multifunctionalities. Furthermore, these materials can be strictly designed for triggering a proper response and in the future deliver the payloads according to the clinical needs of the patient and pathology. Interfacing these nanostructures with biological entities is a significant advance to resolve many key challenges in the pharmaceutical and medical fields, which include the development of novel drug delivery vehicles for early diagnosis, prognosis and treatment of complicated human diseases, such as cancer diseases. Regardless of the aim, nanostructured PSi is therefore a versatility platform and its emerging properties will certainly enable the creation of personalized solutions with potential broad clinical implications in the future.

Problem Box

1. Which kind of parameters and material properties determine the versatile utilization of the mesoporous silicon (PSi) materials as excipients in drug delivery applications?

Answer:

PSi materials have the advantage of delivering large dosages of poorly water-soluble drugs without premature releasing the drugs from the pore channels. This is because of their large surface area (>700 m^2/g) and large pore volume (>0.9 cm^3/g). PSi-based materials have well-defined structures and surfaces, they are relatively inexpensive, chemically inert, thermally stable, biocompatible and biodegradable, which makes them promising candidates as immediate release or controlled drug delivery systems. The stable and rigid Si frame makes them resistant to mechanical stress, pH and fast degradation. Their pore diameters can also be tuned (2–50 nm) allowing for different drug loadings and drug release properties. The PSi can act as a reservoir for storing hydrophobic drug molecules, and can be easily tailored—via the size and surface chemistries of the pores—for selective storage of different molecules of interest, including bioactive peptides. These materials can be fabricated with various particle sizes, ranging from nano- to microsizes enabling the potential utilization in various drug delivery routes, including the oral and intravenous routes. The cellular uptake can also be maximized by tuning the shape, size, pore or surface functionalizations of the PSi materials. Moreover, the absorption and distribution properties of drugs from the PSi particles may be modified and functionalized with different chemical species.

2. True (T) or false (F) questions.

   (a) PSi materials have relevant characteristics for drug delivery applications. Some of these characteristics are adequate pore size, large surface

(continued)

Problem Box (continued)

area and pore volume, top-down manufacture, biodegradability, easy surface functionalization, easy to scale-up, high drug load capacity and biocompatibility.

(b) The physicochemical properties (e.g. dissolution, solubility) of drug molecules can be improved by using PSi materials because the confinement of the drug molecules inside the pores stabilizes the crystal structure of the drugs.
(c) Thermal oxidation and thermal carbonization result in hydrophilic surface chemistries of the PSi.
(d) Dissolution and diffusion are the main drug release mechanisms from the PSi-based materials.
(e) The enhanced drug permeation across intestinal cell monolayers of drugs loaded into PSi particles has been attributed to the fast drug release (dissolution) from the particles and also to the high local drug concentrations in the vicinity of the cell monolayer.
(f) PSi nanoparticles with a diameter of less than 200 nm permeate easily across epithelial barriers like Caco-2 cell monolayers.
(g) Regarding drug delivery applications, PSi nanoparticles cannot be modified to enhance particles' mucoadhesion and gastroretention properties.
(h) The surface biofunctionalization of PSi particles with hydrophobin (HFBII) protein can affect the biodistribution of the PSi particles as well as to improve the cellular interactions between the cell surface and the particles.
(i) PSi nanoparticles provide a versatile platform as a multifunctional drug carrier for drug targeting purposes.

Answers:

(a) (T).
(b) (F).
(c) (T).
(d) (T).
(e) (T).
(f) (F).
(g) (F).
(h) (T).
(i) (T).

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Chapter 11
Drug Nanocrystals

Leena Peltonen

Abstract Drug nanocrystals are nanosized particles, where the solid drug is covered with a stabilizer layer. Their main benefit is faster dissolution, but they are also used for increasing the bioavailability of drugs, which have narrow absorption window or suffer large differences in oral absorption between the fed and fasted states. Drug nanocrystals can be produced either by top-down (e.g., wet milling or high-pressure homogenization) or bottom-up (e.g., antisolvent precipitation) techniques. Compared to other nanotechnological approaches in medicine, drug nanocrystals are considerably simple to produce. Accordingly, pharmaceutical nanocrystals were first time presented in the beginning of the 1990s and already after 10 years the first commercial product was on market. First products were oral tablets, but nanocrystals have been utilized also for ocular, parenteral, dermal, pulmonary, and buccal drug delivery. The main problem with drug nanocrystals is their inherent instability, which can lead to unwanted aggregation, and the selection of a proper stabilizer is crucial for successful nanocrystal formulation.

11.1 Nanocrystals Structure

Drug nanocrystals are crystals in the nanometer size range (1–1,000 nm). They contain 100 % drug without any matrix material. Stabilizing agents, such as surfactants or polymers, are located on the surface of the nanocrystals (Fig. 11.1). In theory nanocrystals are 100 % crystalline material. However, during the processing of nanocrystals, changes from the crystalline to the amorphous form may occur and often also these partly amorphous nanostructures are called nanocrystals (or sometimes "nanocrystals in the amorphous state") (Ali et al. 2011; Wang et al. 2012a).

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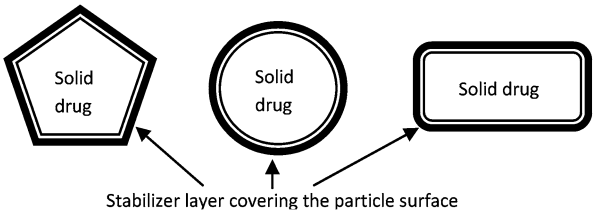


Fig. 11.1 Nanocrystals are formed from a solid drug core, which is covered by a stabilizer layer. Depending on the production process and material properties of the drug, nanocrystals may have different shapes, e.g., spherical, cylindrical, discoidal, tabular, ellipsoidal, cubic, or polygonal

Typically, drug nanocrystals are sized between 200 and 500 nm, depending on the purpose and drug delivery route, but also smaller and larger nanocrystals can be utilized. Nanocrystals have been studied for pharmaceutical use since the beginning of the 1990s, and first commercial product was approved by the FDA (U.S. Food and Drug Administration) in the year 2000 (Keck and Müller 2006; Merisko-Liversidge and Liversidge 2008; Liversidge et al. 1992; Müller et al. 2011; Bansal et al. 2012; Junghanns and Müller 2008; Chingunpituk 2007).

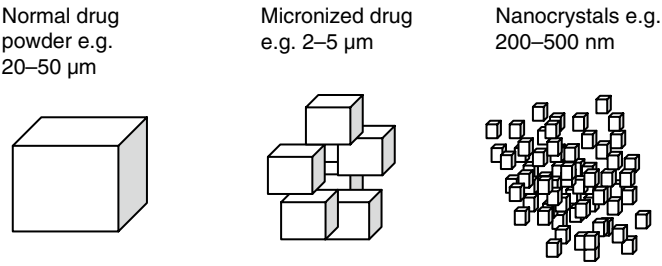
11.2 Benefits of Drug Nanocrystals

Nanocrystals are utilized for increasing the solubility and dissolution rate of poorly soluble drugs (Müller et al. 2011). Today in pharmaceutical research 70–90 % of new chemical entities are poorly soluble, e.g., BCS (Biopharmaceutics Classification System) class II or class IV drugs. The class II drugs are best candidates for nanocrystals, because they have poor solubility but the permeation properties are good, meaning that the low bioavailability is mostly caused by poor solubility. Today approximately 70 % of new chemical entities belong to class II. In class IV, the problem is also poor permeability, and, hence, nanocrystals are not that beneficial for those drugs.

If a constant volume of material is taken and the particle size is decreased, the surface area of the particles is increased (Fig. 11.2). Furthermore, according to the Noyes–Whitney equation, which is a simplified expression for drug dissolution, surface area is directly related to the dissolution rate (11.1):

$$\frac{\mathrm{d}\,m}{\mathrm{d}\,t} = kA\left(C_\mathrm{s} - C\right) \tag{11.1}$$

where dm/dt is the dissolved amount of drug (mass) as a function of time t, k is the dissolution rate constant, A is the surface area of dissolving solid, C_s is the saturated concentration of the dissolving solid, and C is the concentration of the dissolved solid in the dissolution medium at time t. Accordingly, when the surface area of the particles is increased (for example, by decreasing the particle size), the dissolution rate is also increased.



Volume ratio	**1**	**:**	**1**	**:**	**1**
Number of particles -ratio	**1**	**:**	**1 000**	**:**	**1 000 000**
Surface area of particles -ratio	**1**	**:**	**10**	**:**	**100**

Fig. 11.2 The effect of particle size on the number of individual particles and the surface area, when the total volume of the drug material is kept constant. For example, the surface area is 100 times higher, when the particle size is decreased from 20 µm to 200 nm, and at the same time number of particles is increased by a factor of 1,000,000

Besides the faster dissolution rate due to the larger surface area, finely divided solids have higher solubilities than coarse-grained materials. This can be expressed by the modified Kelvin equation:

$$\frac{S}{S_0} = \exp\left(\frac{\gamma V}{RTd}\right) \tag{11.2}$$

where S is the solubility of particles with size d, S_0 is the solubility of the bulk material, γ is the surface free energy, V is the molar volume, R is the gas constant, and T is the temperature (Kaptay 2012). However, this increased solubility has a marked effect only with very small particles, e.g., particle sizes below approximately 100 nm (Anhalt et al. 2012; Kesisoglou and Wu 2008; van Eerdenbrugh et al. 2010). Accordingly, the most crucial benefits with drug nanocrystals, when the typical size is from 200 to 500 nm, are a faster dissolution rate.

Increased dissolution rates also decrease the effect of food on drug absorption, meaning that the absorption in fed and fasted states is very similar (Gao et al. 2013). Accordingly, drugs with high lipophilicity, which require food to be solubilized, are possible candidates for nanocrystallization, because the technique can make them bioequivalent both in the fed and fasted states. Another potential group of drugs, which benefit from nanocrystallization, is drugs with a narrow absorption window. With these drugs the faster dissolution rate of nanocrystals in the absorption area can increase the bioavailability to acceptable levels. All materials behave differently in nanocrystallization, but the best candidates for nanocrystallization are materials with a high crystal lattice energy (high melting point), high molecular weight, and a solubility of less than 200 µg/mL, because the benefits gained by a smaller particle size are the highest with these types of compounds (Rabinow 2004; Lee et al. 2008; Merisko-Liversidge et al. 1996).

11.3 Stabilization of Drug Nanocrystals

The most crucial problem with nanosized particles is the low stability of the particles; particles tend to aggregate back to larger structures. Therefore, they need to be stabilized by a stabilizer. Typically stabilizers are either surfactants (surface-active agents, for example, polysorbates) or polymers (for example, ethylene oxide/propylene oxide block copolymers) (Van Eerdenbrugh et al. 2007, 2008a, 2009; Verma et al. 2009a; Lee 2003; Sharma et al. 2009; Grau et al. 2000; Hecq et al. 2005, 2006; Hernández-Trejo et al. 2005; Chen et al. 2009). Stabilizers are adsorbed on the surface of the drug particle and this hinders particle aggregation. Stabilization can be based on two different mechanisms: either steric stabilization or electrostatic stabilization (charge stabilization). Also a combination, both steric and electrostatic stabilization, is possible; this is known as electrosteric stabilization. Electrosteric stabilization can be achieved, for example, by combining chemical functionalities on the stabilizer or utilizing a combination of stabilizers. Sometimes this combination of more than one stabilizer has been linked to enhance long-term stability (Chaubal and Popescu 2008; Niwa et al. 2011; Rosen 2004).

If pure electrostatic stabilization is utilized, the zeta potential (ζ) of the nanocrystals should be either less than −30 mV or more than +30 mV. If electrosteric stabilization is in question, lower values may be enough depending on the efficiency of steric stabilization. Typical electrostatic stabilizers are ionic surfactants and polymers, and depending on the molecular weight (chain length) the stabilization may be electrosteric (Hecq et al. 2006). Steric stabilizers are typically nonionic surfactants and polymers (Grau et al. 2000; Hecq et al. 2006; Hernández-Trejo et al. 2005).

Surface charge is dependent on the environmental factors and, hence, electrostatic stabilization may be decreased, if other charged materials are present (Sun et al. 2012), e.g., when milling in aqueous buffer solution. Nonionic stabilizers have no charged groups and according to the DLVO theory (Derjaguin, Landau, Verwey, and Overbeek theory), the repulsive barrier against agglomeration is not sufficient (Derjaguin and Landau 1941; Verwey and Overbeek 1946). Their efficacy as a stabilizer is based on steric action, i.e., when the long molecular chains form physical barriers on the particle surfaces, which prevent the close contact between particles and hinder the van der Waals' attractive forces between them (Fig. 11.3). Typically with equal chain lengths nonionic surfactants have a higher adsorption potential as compared to polymers (Palla and Shah 2002). On the other hand, small molecular surfactants, like sodium dodecyl sulfate (SDS), may solubilize drugs by micelle formation and hence destroy the crystal structure of drug particles (Lee et al. 2008; Choi et al. 2005). Steric stabilization is more sensitive to temperature changes. Electrostatic stabilization is effective in aqueous environments, but the ionized state is not the same in dry material, and this may cause stability problems (Farrokhpay 2009). Also changes in pH or ionic strength may affect the efficiency of ionic stabilization. These factors are important to take into account if nanosuspensions are dried or sterilized for further formulation purposes. Nanocrystal suspensions can be sterilized successfully, for example, by gamma irradiation, sterile filtration, and steam sterilization (Van Eerdenbrugh et al. 2007; Zheng and Bosch 1997; Na et al. 1999).

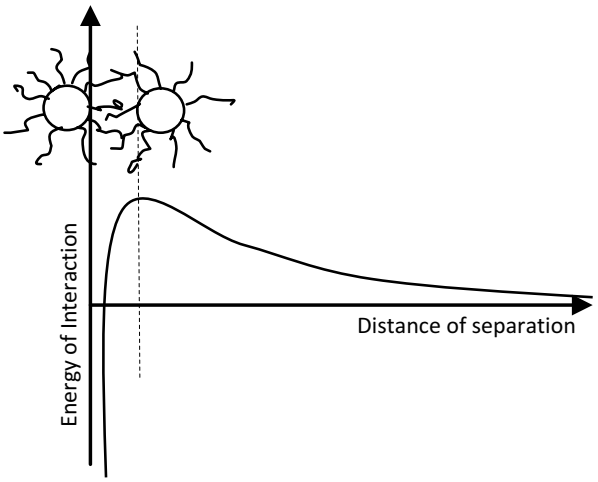


Fig. 11.3 The energy of interaction of colloidal particles presented as a function of distance of separation between individual particles; the combined curve for attraction and repulsion forces. Particles are sterically stabilized, when the long molecular chains of the stabilizer hinder the close contact between individual particles. The *vertical dashed line* in the figure shows the minimum distance, below which the attractive van der Waals forces start to dominate and cause particle aggregation

11.4 Nanocrystal Synthesis

Different techniques for the manufacturing of nanocrystals exist, and they can be divided roughly into two classes, namely bottom-up and top-down techniques (Fig. 11.4). In bottom-up techniques the nanocrystals are formulated by building larger structures from smaller ones, e.g., precipitation from a solution (Verma et al. 2009a). Though, with bottom-up techniques it is possible to formulate very small structures, the most common problem with these techniques is how to end the process and achieve one particle size fraction, and not to end up microparticles (Müller and Moschwitzer 2007). In top-down methods the starting point is with larger entities and during the process their particle size is diminished, for example, by milling or by high-pressure homogenization (Keck and Müller 2006; Merisko-Liversidge and Liversidge 2008).

With top-down techniques, the main problem is the difficulty in producing the smallest particle size fractions, namely particles under 100 nm. Also the size deviation in top-down methods is often high when compared to bottom-up techniques. The heterogeneity of the final product can be lowered by increasing the processing time, meaning that longer milling times or an increased number of homogenization cycles are required (Keck and Müller 2006; Liu et al. 2012; Sulaiman 2007; Hao et al. 2012).

The product formed by the different techniques is nanosized drug particles, which are covered with a stabilizer layer; the efficiency of stabilization of a particle

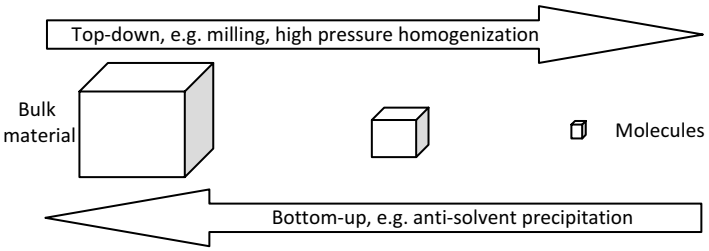


Fig. 11.4 Nanocrystals may be produced by top-down or bottom-up techniques. In top-down methods particle size is decreased, for example, by milling or by high-pressure homogenization. In bottom-up techniques, nanocrystals are structured molecule by molecule, for example, by precipitating drug from a solvent with an antisolvent addition

system differs even when the same material is used in different processes (Verma et al. 2009a; Kocbek et al. 2006; Mishra et al. 2009). Accordingly, the most important aspect of the particle formation step is the attachment ability of the stabilizer to the newly formed particle surfaces (Verma et al. 2009b). The particle shape of nanocrystals depends on the processing technique and the properties of the drug material. Particles can, for example, have shapes like spherical, cylindrical, discoidal, tabular, ellipsoidal, cubic, or polygonal shapes (Liu et al. 2011; Valo et al. 2011).

Though, the product formed by the different techniques consists of nanosized particles, which are covered by a stabilizer layer, the amount of stabilizer needed and the stabilization efficiency are very different with each of the techniques (Verma et al. 2009a; Kocbek et al. 2006; Mishra et al. 2009). This indicates that the method for stabilization and the attachment of the stabilizing material on the particle surfaces are process-related phenomena. In milling techniques, a stabilizer is needed during the whole process to protect the newly formed surfaces against aggregation, and the particle size achieved is related to both the process parameters and the amount of stabilizer (Liu et al. 2011). In high-pressure homogenization small particles are formed during the process independently with no correlation to the amount of the stabilizer, but stabilizer is needed after the process and during storage in order to stabilize the end product (Mishra et al. 2009). In precipitation techniques the role of the stabilizer is to control the particle formation and to stop the growth process to a certain particle size fraction and the exact amount of stabilizer is very crucial for successful nanoparticle formation (Valo et al. 2010; Matteucci et al. 2006).

11.4.1 Bottom-up Techniques: Antisolvent Precipitation

The most studied bottom-up technique for nanocrystallization is the antisolvent precipitation technique (Ali et al. 2011; Valo et al. 2010, 2011; Matteucci et al. 2006; D'Addio and Prud'homme 2011). The basic idea behind the technique is that first drug material and stabilizer (e.g., surfactant or polymer) is dissolved in a solvent,

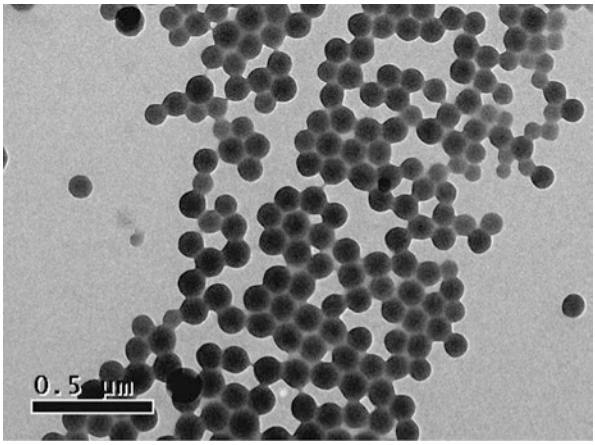


Fig. 11.5 Transmission electron micrograph (TEM) of itraconazole nanocrystals prepared by antisolvent technology with hydrophobic HFBII as a stabilizer. Particle size is below 100 nm and the size distribution is very narrow, which is typical for bottom-up techniques

after which an antisolvent (for the drug) is added, causing precipitation of the drug. The most important step for nanocrystal formation by antisolvent precipitation is efficient stabilization. Finding the right composition for successful nanocrystal formation is often demanding but when optimal process conditions are found, very small particles with a narrow size distribution can be produced (Fig. 11.5). The poor solubility of the drug may limit the utilization of the technique, because many poorly soluble materials are poorly soluble not only in aqueous but also in organic solvents, and difficulties can be faced in order to find proper solvents for performing the precipitation process. Solvent removal after the process can be difficult, especially when organic solvents are used, and solvent residues may cause problems. Often the control over particle size is difficult and micron-sized particles may be formed instead of nanocrystals. Other rarely used bottom-up techniques are liquid atomization-based spray drying, electrospraying, and aerosol flow reactor methods (Eerikäinen et al. 2003; Lee et al. 2011; Peltonen et al. 2010).

11.4.2 Top-down Techniques: Wet Milling and High-Pressure Homogenization

The most utilized top-down techniques to produce drug nanocrystals are media milling and high-pressure homogenization. Milling is performed in a dispersion, which contains solid drug material and a stabilizer (Liversidge and Cundy 1995; Merisko-Liversidge et al. 2003). Water or suitable aqueous buffer solution is used as a dispersion medium. The dispersion is loaded onto a milling vessel together with milling pearls. The efficiency of nanomilling to produce nanosized particles is

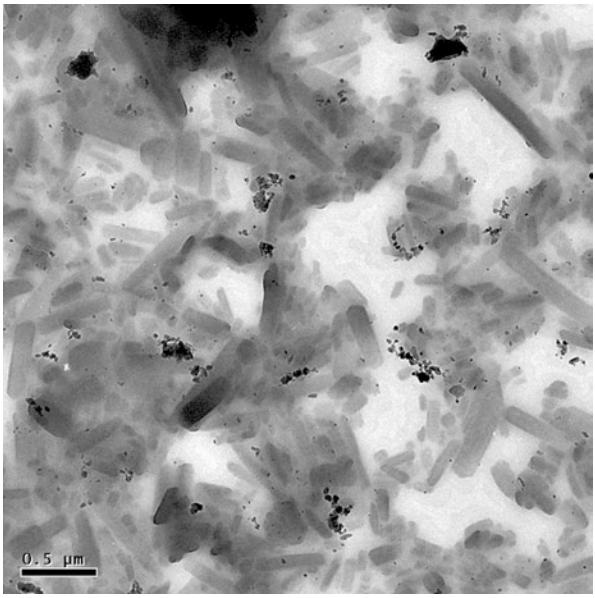


Fig. 11.6 TEM figure of itraconazole nanocrystals prepared by nanomilling. Ethylene oxide/propylene oxide block copolymer (Pluronic F127) was used as a stabilizer and the total milling time was 30 min. In order to avoid warming up of the milling vessel, milling was performed by keeping a 15 min break after each of the 3 min milling period. The drug:stabilizer ratio was 5:3, and water was used as dispersion medium. Milling was run with 1,100 rpm and the size of the milling pearls was 1 mm. The particle shape seen in the figure is typical for milled itraconazole nanocrystals

mostly related to the process parameters (Liu et al. 2013). The most important process parameters in nanomilling are the amount of drug, the selection and the amount of stabilizer, the amount and size of the milling pearls, the milling speed, the milling time, and the temperature. For example, with high rotating speeds (over 1,000 rpm) and small milling pearls (0.5–1 mm) nanocrystals with the particle size of around 300 nm can be produced in a few minutes (Fig. 11.6) (Liu et al. 2011). Also scaling up of the milling process is possible (Van Eerdenbrugh et al. 2008b; Kesisoglou et al. 2007). After optimization of the milling parameters, process is very reproducible (Merisko-Liversidge et al. 2004). Nanosized drug particles, which are covered by a stabilizer layer, are formed by the shear forces induced by impaction of the milling pearls with the drug. Earlier lower milling speeds (80–90 rpm) together with long milling times (1–5 days) were common (Merisko-Liversidge et al. 1996; Van Eerdenbrugh et al. 2009; Lee 2003; Liversidge and Cundy 1995), but nowadays high milling speeds (1,000–4,000 rpm) and short milling times (30–60 min) are utilized more often (Lee et al. 2008; Choi et al. 2005; Liu et al. 2011, 2013).

The most important concerns related to nanomilling are contamination due to wear of the milling vessel or milling pearls. This wear is exacerbated with increased temperature during milling and by the high shear forces involved in the milling process. In order to avoid extra wear, ceramics like zirconium oxide have been used as a

material for the vessel and the pearls, and pearls and the vessel may also be coated (Liu et al. 2011; Merisko-Liversidge et al. 2003; Merisko-Liversidge and Liversidge 2011). During the milling process drug material is under increased mechanical stress and this may induce chemical degradation, polymorphic changes, or the drug material may turn into amorphous forms. The formation of amorphous material is closely related to the properties of the milled drug, the stabilizer used, possible interactions between the drug and stabilizer, and the process parameters used for the milling. Often the build up of amorphous regions is unwanted, because their formation may not be controllable and they are unstable. If the amorphous areas formed during milling can be stabilized, then formation of amorphous material may even be one goal.

In milling two opposite processes are competing simultaneously, namely fragmentation of materials into smaller particles and particle growth due to collisions (Annapragada and Adjei 1996; Gubskaya et al. 1995). Process parameters and material properties of the milled material are crucial to the occurrence of these phenomena, and also to the properties of the end product. Normally with certain process parameters, a constant particle size is reached after a certain time point (Merisko-Liversidge and Liversidge 2011; Knieke et al. 2009, 2010). Sometimes particle size or heterogeneity of the product may even increase, if the milling is continued further (Lee et al. 2008; Brodka-Pfeiffer et al. 2003). Accordingly, a very important factor for stable nanocrystal formation is that particle surfaces are efficiently stabilized by an appropriate stabilizer; the smaller the particles the higher the surface energy and tendency to aggregate.

High-pressure homogenization techniques for the production of nanocrystals are piston gap (both in water and in nonaqueous environment) and microfluidizer technologies (Keck and Müller 2006; Liu et al. 2012; Hao et al. 2012; Kakran et al. 2012). In piston gap homogenization, drug is dispersed in the stabilizer solution and forced by a piston through the narrow homogenization gap (Fig. 11.7). Pressure may be up to 4,000 bars, but normally values from 1,500 to 2,000 bars are used. The diameter of the homogenization gap is typically from 5 to 20 μm, depending on the pressure and viscosity of the suspension. When the material is forced through the homogenization gap the dynamic pressure is increased due to the increased flow speed and this causes lowering of the static pressure below the vapor pressure of water. Accordingly, water starts to boil causing gas bubble formation; the bubbles will collapse immediately when the suspension arrives at the normal pressure area after the homogenization gap. Formation and implosion of gas bubbles and cavitation induce shock waves. Nanocrystals are formed in high-pressure homogenization equipment due to the shock waves, high shear forces, particle collisions, and turbulent flow.

If water cannot be used as a medium, high-pressure homogenization is possible also to use the piston gap technique in oils or in polyethylene glycols. In this case, gas bubbles and cavitation are not reached due to the lower vapor pressure of the oils or polyethylene glycols, but particle fracturing is caused by particle collision, shear forces, and turbulent flow. In microfluidizer technology, nanocrystals are formed by a frontal collision of two fluid streams and the pressure can be up to 1,700 bar. Particle fracturing is caused by particle collisions, shear forces, and cavitation forces (Bruno and McIlwrick 1999).

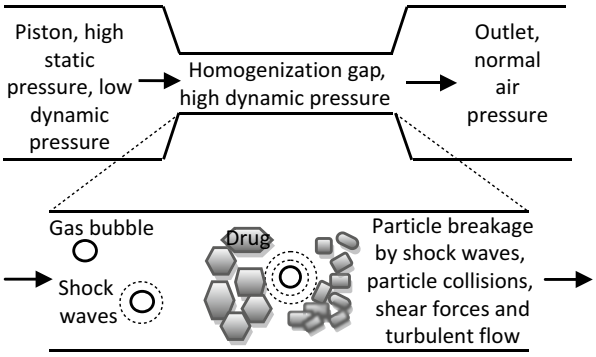


Fig. 11.7 Schematic presentation of high-pressure homogenization by a piston gap technique in an aqueous medium. Drug suspension is forced through the homogenization gap by a piston. In homogenization gap (*lower figure*), particle breakage is caused by shock waves induced by air bubbles, turbulent flow, particle collisions, and shear forces

In high-pressure homogenization, the particle size depends on the homogenization pressure, number of homogenization cycles, and hardness of the drug. In microfluidizer techniques, a considerably high number of homogenization cycles are required (from 50 to 100), while in the piston gap technique, typically 10–20 cycles are enough. The stabilizer affects the particles only after the process by stabilizing the formed particles, but it does not affect the achieved particle size of the process. Contamination due to wear of the process equipment can cause problems in high-pressure homogenization. Processes also require a high energy input.

When processing nanocrystals, the most critical question is how to compensate for the high free energy of the newly formed surfaces. For stabilization to be effective, strong and fast adsorption of the stabilizer on the surfaces is needed. Typically, the weight ratio of drug to stabilizer is approximately from 20:1 to 1:1, depending on the stabilizer and drug properties, production method and process parameters, and the aimed final particle size (Merisko-Liversidge and Liversidge 2011).

The factors affecting the efficiency of stabilization of a particular kind of drug material are not so clear, but different parameters have been tried in order to understand the stabilization effect (Peltonen and Hirvonen 2010). Surface energy values may be calculated from the static contact angle measurements. In some cases the stabilization has been found to be more efficient, if the surface energy values of the drug and stabilizer are close to each other, but the total role of the surface energy is not yet clear (Lee et al. 2008; Choi et al. 2005; Parsons et al. 1992). The molecular weight of the stabilizer affects the physical barrier and the viscosity of the system (Badia et al. 1997; Peltonen and Yliruusi 2000). A higher viscosity normally increases the process times, but in the final product it stabilizes the particles against aggregation (Van Eerdenbrugh et al. 2009; Matijasic et al. 2008; Fuerstenau and Abouzeid 2002; Lee et al. 2005). The interactions between the functional groups in the drug and stabilizer structures should also be taken into account (Choi et al. 2005; Lee et al. 2005; Tian et al. 2007; Sabnis et al. 1997). For example, hydrogen

bonding between the stabilizer and drug may hinder the efficient stabilization. Drugs have also functional groups like phenols, amines, hydroxyls, ethers, or carboxylic acids, which may interact with the stabilizer. Particle formation may be enhanced or hindered by strong ionic interactions, hydrogen bonding or dipole-induced, van der Waals' or London forces. The hydrophobicity of the stabilizer is important for efficient adsorption on the particles' surfaces, because the formed surfaces are often hydrophobic (Van Eerdenbrugh et al. 2009; Lee et al. 2005; Law and Kayes 1983). The driving force for surface adsorption to hydrophobic particle surfaces increases, when the hydrophobicity of the surfactant increases, and it seems that the hydrophobicity of the stabilizer is one of the main factors for successful stabilization.

11.5 Physical Characterization

The characterization of nanocrystals is very demanding due to their small size. The most important measured parameters in order to characterize drug nanocrystals are particle size and size distribution, particle shape and morphology, zeta potential, dissolution behavior, and crystallinity and crystal form determinations. Surface properties like zeta potential affect the stability of the particle suspension. Particle size has an effect on the solubility and dissolution properties, e.g., the efficiency of the formulation. Colloidal stability is assessed using size measurements, also. A visual inspection of the formulation is not reliable due to the small particle size.

The surface characteristics of the nanoparticles also govern the interactions with biological materials. Accordingly, a thorough investigation of the particle properties is essential for proper control of the physicochemical as well as biopharmaceutical behavior of the formulations in vivo.

11.5.1 Particle Size, Shape, and Morphology

Particle size measurements can be performed with various techniques depending on the mean particle size and the homogeneity of the sample. Also the purpose of the measurement is important; for example, for process control purposes a fast measurement is needed. Often more than one technique is more reliable than a single technique and a combination of techniques, where also particle shape information is available, is recommended (Yegin and Lamprecht 2006). The most commonly used techniques are light scattering (Donini et al. 2002; Bohren and Huffman 1983) and electron microscopic techniques (e.g., SEM—scanning electron microscopy, TEM—transmission electron microscopy, and ESEM—environmental scanning electron microscopy) (Liu et al. 2011; Abdelwahed et al. 2006; Swarbrick and Boylan 2001). AFM (atomic force microscopy) and capillary hydrodynamic fractionation can also

be used but so far these techniques have been used in very limited extent (Shahgaldian et al. 2003; van Zyl et al. 2004).

The benefits of using electron microscopy techniques are that at the same time also the particle shape and morphology information can be achieved. The limitation of these techniques is that sometimes a coating of the sample may be needed, which may alter the properties of the sample. For example, some surface properties may be hidden or masked by the coating. Also finding a proper coating material may be difficult. Most often platinum or gold sputtering is used but carbon may also be used. In ESEM the coating is not needed, which makes the analysis easier, but so far ESEM is used less frequently. The high vacuum in SEM may also be problematic especially with aqueous samples, which do not survive in the vacuum. Losing the water during the SEM analysis may cause changes in the material, and, hence, freeze fracturing can be utilized. The sample may also be dried before the SEM analysis, but then it must be taken into account that this may also alter the properties of the sample when compared to original nanosuspension. Electron microscopy analysis is also very time-consuming and the interpretation of the results needs extra input. A rough idea about the particle size and shape may be reached considerably easily, but reliable particle size and size distribution analyses take time. Often the problem may also arise that due to the small sample size analyzed, the sample may not be representative of the whole batch.

As mentioned earlier, one benefit of SEM and AFM studies is that besides the particle size and shape, the morphology of the particles can be studied simultaneously. Morphology is a very important property, which may impact on further formulation ability (e.g., flowability). AFM is more demanding and it is so far more rarely utilized for nanoparticle studies as compared to electron microscopy. Furthermore determining the real internal structure/ultrastructure is very difficult with nanoparticles due to the small particle size.

Compared to electron microscopy techniques, light scattering measurements for particle sizing are fast (a typical measurement takes a few minutes), and in that sense the techniques are especially suitable for process control purposes. Light scattering measurements are also precise and sensitive. However, heterogeneous particle samples (a wide size distribution) are problematic in light scattering techniques, because the larger particles are overstressed in the measurements (Yegin and Lamprecht 2006). For these samples with a wide size deviation (polydisperse samples) pre-handling, like size fractioning, of the sample before the measurement may be used. If pre-handling is used, careful interpretation of the results is needed, especially for quantitative analysis.

In light scattering techniques the scattering intensity is related to the scattering angle, the absorption and the size of the particles, and the refractive indices of the particles and the medium. Often approximations of these values are used for determinations due to the difficulty in precisely measuring them. These approximations may cause some unreliability to the results. Dynamic processes like Brownian motion may have an impact on the results, especially with time-sensitive measurements. Problems may arise also due to particle shape. Light scattering information is transformed to size information by using special algorithms.

These algorithms are, for example, based on the assumption that the particles are spherical, present in sufficient dilution, and that there are large difference between the refractive indices of the particles and medium. Often nanocrystals are spherical or near spherical, but care needs to be taken about the validity of the information obtained using these techniques and it is highly recommended that results and their validity are confirmed with other techniques like SEM (Yegin and Lamprecht 2006; Bohren and Huffman 1983).

The selection of medium in light scattering measurements is also very important, because depending on the medium, different layers of solvent/solute may be attached to the particle surfaces or bulky surface groups may interact differently with the medium; both these phenomena may affect the results. After processing, the nanocrystal sample needs normally to be diluted before the light scattering measurements. This is also a very important step, due to the high solubility of the nanocrystals. Unwanted dissolution of the smallest nanocrystals during the dilution and measurement, and, hence, misleading results, is avoided by using a saturated drug solution as a dilution medium while measuring the particle size of the drug nanocrystals (Liu et al. 2011).

11.5.2 Chemical and Physical Stability

Thermal and X-ray diffraction (XRD) analysis can be used for determinations of chemical and physical stability of the material. From DSC (differential scanning calorimetry) thermal behavior, like melting point, glass transition temperature (T_g), solvates/hydrates, changes in crystalline form, or formation of amorphous material may be detected (Liu et al. 2011; Wang et al. 2012b). From DSC quantitative data of the energy, which is adsorbed (endothermic reaction) or evolved (exothermic reaction) by the material during the analysis, are determined. If the thermal reactions are very mild (small amount of material), very close to each other, or they are overlapping, the interpretations of the results are difficult, and normally DSC analysis is supported by, for example, XRD measurements (Liu et al. 2011; Peltonen et al. 2002). Infrared spectroscopy (IR, FT-IR) can be used for interaction studies between the materials (Moon et al. 2009; Gupta et al. 2010).

11.5.3 Zeta Potential

The zeta potential can be determined by measuring electrophoretic mobility with light scattering techniques (Baba et al. 2007; Fan et al. 2006; Hinrichs et al. 2006). The zeta potential can be calculated from electrophoretic mobility of the particles with Smoluchowski's equation, which is based on the assumption of rigid spherical particles, which means that the radius of the particles is considerably larger than Debye screening length (double layer thickness). In Hückel's equation with hard

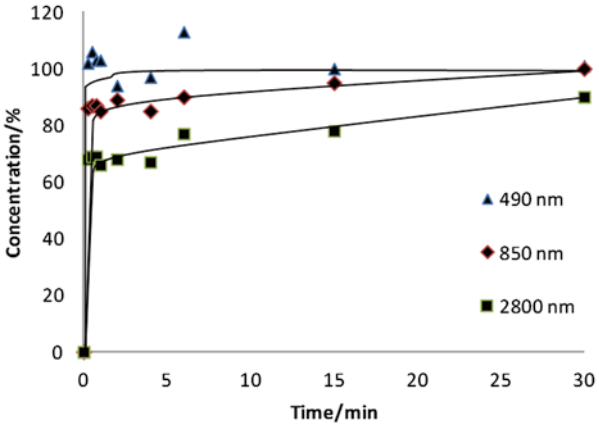


Fig. 11.8 Dissolution profiles of indomethacin nano- and microcrystals. Nanocrystals were made by media milling technique with ethylene oxide/propylene oxide block copolymer (Pluronic F68) as a stabilizer. The drug:stabilizer ratio was 5:3, and water was used as dispersion medium. Different particle size fractions were produced by milling with different sized milling pearls: 1 mm pearls for nanocrystals sized 490 nm, 5 mm pearls for nanocrystals sized 850 nm, and 10 mm pearls for microcrystals sized 2,800 nm. Dissolution is very fast with smallest particles (490 nm), while with larger particle sizes complete dissolution takes more time

spheres, the particle radius is smaller than the double layer thickness. Also other equations for calculation of zeta potential are used, but Smoluchowski's equation is most often used with pharmaceutical applications and it gives reasonable accuracy for nanocrystal zeta potential measurements. A more rarely used method for zeta potential measurements is high performance capillary electrophoresis, a technique that can differentiate charged materials based on their electricity differences in electrolyte solutions. In zeta potential measurements besides the particle characteristics extra emphasis should be put on the measurement environment. Variables such as viscosity, ion concentration, and pH may alter dramatically the measurement output (Hirsjärvi et al. 2006).

11.5.4 Dissolution

Dissolution testing of nanocrystals is often complicated, because of the very fast dissolution rate of small particles (Fig. 11.8) (Liu et al. 2011). Traditional pharmacopeia tests (e.g., the paddle method) can be utilized, but finding differences between different particle size fractions with these techniques is very difficult. More discriminating dissolution results can be achieved, for example, by testing batches under non-sink conditions (Liu et al. 2013). Special care needs to be taken against sampling in order to avoid removing undissolved solid nanoparticles from the dissolution vessel.

With nanosized particles even small changes in properties, for example, due to scaling up, may drastically affect the formulation properties and performance in vivo. Thus, the importance of thorough and reliable in vitro characterization cannot be overstated. Many analysis methods are also complementary, meaning that correctness of interpretations is always good to check with utilization of more than one technique.

11.6 Application of Nanocrystals

First-generation nanocrystallization technologies, like media milling or high-pressure homogenization, are relatively simple and fast processes. Accordingly, when compared to other nanotechnological approaches under current interest in drug delivery, the benefit of nanocrystals is that their scale up is comparably easy. Processes to produce nanocrystals are also quite cost-effective when compared to many other much more complicated and multistage systems. In the pharmaceutical industry oil- or microemulsion-filled capsules for the oral drug delivery of poorly soluble drug materials are still the first choice for formulation development, but nanocrystal formulations are very possibly the next option. Newer techniques, i.e., the so-called second-generation smarter nanocrystals, are more complex and they are utilized for solving more demanding delivery problems and they include nanocrystals smaller than 100 nm, coated or functionalized nanocrystals for targeting purposes, and more efficient production techniques by combining more than one process, e.g., pretreatment before milling or high-pressure homogenization (Keck and Müller 2006; Junghanns and Müller 2008; Chingunpituk 2007).

Nanocrystals have been studied for pharmaceutical use since the beginning of the 1990s, and first commercial product, Rapamune by Wyeth, was approved by the FDA in 2000 (Liversidge et al. 1992). Today, after over 20 years of intensive research numerous products are undergoing clinical trials or have been approved worldwide by regulatory agencies in, e.g., the USA, Canada, the European Union, and Japan (Keck and Müller 2006; Junghanns and Müller 2008; Chingunpituk 2007). In the beginning, nanocrystals were utilized as novel formulations for replacing already marketed products, but later also new chemical entities were widely studied. Below, the first commercial nanocrystalline-based products and their benefits, when compared to the traditional formulations, are presented.

Sirolimus (Rapamune®) was originally marketed as an oral suspension. It required cold storage and before administration it required mixing with water or orange juice. In the new formulation drug was nanocrystallized by a media milling technique so that the particle size of the drug was below 200 nm. Due to the higher solubility of nanocrystals, a tablet formulation was possible; tablet formulations are much more patient-friendly when compared to the original oral suspension. Also the oral bioavailability of nanocrystals in tablets is 21 % higher compared to the original product. The drug amount in tablets is 1 and 2 mg, and the corresponding tablet weights 365 and 370 mg, respectively, meaning that the relative amount of the nanocrystals in the tablet composition is very low.

An example of drug material with a narrow absorption window is aprepitant (Emend®). It is used for the treatment of emesis and it is absorbed only in the upper gastrointestinal tract. The original product on market was made from micronized drug powder and high doses were needed, which caused increased side effects. In the nanocrystalline product pelletized nanocrystals are packed inside of hard gelatin capsules. The faster dissolution in vivo with nanocrystals was followed by a higher absorption rate and improved bioavailability.

Fenofibrate (Tricor®) is typical lipophilic drug, which suffers high deviation in bioavailability between fed and fasted states. Fenofibrate is practically insoluble in water and the higher absorption of fenofibrate in fed state is due to the presence of lipids and other surfactants in the food, which are able to solubilize the drug. With the new nanocrystal formulation, the bioavailability of the drug is similar in the fed and fasted states. Also the dose of the drug is smaller in the new formulation due to higher bioavailability of nanocrystals.

Abraxane® is a Cremophor EL free nanocrystal formulation of albumin-bounded paclitaxel. Drug nanocrystals are produced by high-pressure homogenization, where albumin is functioning as a stabilizer and the particle size of the product is approximately 130 nm. The administration route is by intravenous infusion. The benefits of nanocrystalline formulation as compared to Cremophor EL containing formulation are a lowered hypersensitivity, shorter infusion time, and smaller infusion volume due to the higher drug concentration in the product. Additionally the formulation avoids the problematic leaching of plasticizers from infusion bags or infusion tubings.

The first commercial nanocrystal products were for the oral drug delivery route, but other routes (e.g., intravenous, intrathecal, pulmonary, topical) have also been studied (Merisko-Liversidge et al. 2004; Kassem et al. 2007; Bhol and Schechter 2005; Zhang et al. 2011; Rao et al. 2011). However, intravenous products have turned out to be more complex, because dissolution of nanocrystals is slower when compared to the corresponding marketed solutions. One problem is also that nanocrystals may be phagocytosed by liver and spleen macrophages. The current research in intravenous products is aiming to formulate smaller nanocrystals, which mimic the pharmacokinetic profiles of solutions.

The size of the formed nanocrystals is important from an in vivo efficiency and formulation point of view. An appropriate particle size needs to be decided with reference to the dosage form design and in vivo performance. For example, a very small particle size makes powders too cohesive for tabletting. Also, if the total nanocrystal content is very high, the possibility of nanocrystals touching each other and fusing into larger entities is increased, and as such the benefits to be gained from nanocrystal formation will be lost. Depending on the drug nanocrystal's properties, the rough limit for the maximum amount for nanocrystals in tabletting mixture is approximately 30 %. Many studies today are also examining the production of microparticles from nanocrystals, e.g., nanocrystals are formulated into micron-sized particles so that they still retain the nanocrystal properties (Laaksonen et al. 2011). These nanos-in-micros structures will ease further formulation of products with an even higher drug loading. As a conclusion, the final particle size has to be decided according to the wanted end product properties, and often even a small particle size decrease may be enough for successful in vivo performance.

Problem Box

Question 1. What are the most important benefits of nanocrystals as compared to bulk drug materials (e.g., particle size typically 20–50 μm)?

Answer 1. Due to larger surface area of nanocrystals the dissolution rate is faster. With very small particles (less than 100 nm) the saturated solubility is also higher as compared to bulk drug materials.

Question 2. What kinds of drug materials do benefit most from nanocrystallization?

Answer 2. Materials, whose bioavailability is limited by the low solubility (typically BCS class II drugs), materials which suffer from large fluctuation in bioavailability between fasted and fed states, and drugs with narrow absorption window.

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Part II
Concepts Underpinning the Application of Biomedical Nanomaterials

Chapter 12
Biological Barriers: Transdermal, Oral, Mucosal, Blood Brain Barrier, and the Blood Eye Barrier

Preethi Marimuthu and Andreas G. Schätzlein

Abstract Compartmentalisation is a precondition for the development of life, allowing concentration gradients to be maintained, facilitating selective transport of molecules, functional polarisation, protection of cells and tissues. Consequently, organisms have evolved highly sophisticated structures and mechanisms that allow compartmentalisation to be maintained and controlled in a highly regulated fashion.

Under normal conditions these compartmentalising structures are essential building blocks of life, their smooth functioning being central to our health. However, the same effectiveness that is a bonus under physiological conditions means the same structures may become considerable barriers to the pharmacotherapy of diseases, as access of drugs to the sites of disease may be severely restricted. This chapter describes the architecture, organisation, and function of key barriers that therapeutic nanoparticles may encounter for the most important routes of drug administration. The epithelial barriers (skin, mucosa of the airways, and gastrointestinal tract) and endothelial barriers share many commonalities as they all share key design elements that have evolved to support compartmentalisation.

12.1 Introduction

A key biological principle is organisation by compartmentalisation. This approach is key to creating defined environments within which conditions can be finely controlled and tuned. This is a common principle shared by all living organisms from

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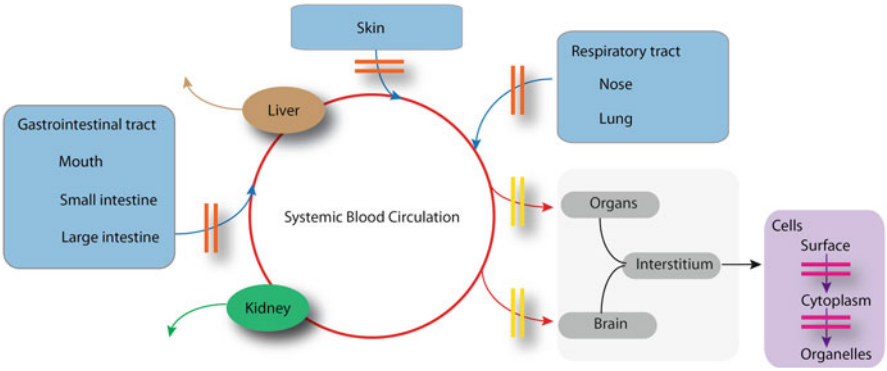

Fig. 12.1 The organisation of barriers in the body. The compartmental organisation of the human body defines barriers for drug delivery at the body, organ, and cellular level. Epithelial tissues control access from the environment to the systemic blood circulation from where distribution occurs throughout the body (*orange bars*). The vessel walls and specifically the endothelial cells control access from the systemic circulation to the organs and their interstitial tissue (*yellow bars*). At the cellular level uptake is controlled at the surface and by surface membrane-derived organelles (endocytosis) as well as between the cytoplasm and various intracellular organelles (*magenta bars*)

microorganisms to plants and animals. All organisms that are metabolically active and have the ability to grow and multiply exist in a dynamic equilibrium with their environment to allow exchange of nutrients, waste products, and information. These requirements have led to the evolution of a sophisticated system of barriers and exchange mechanisms on all organisational levels of the organism, i.e. from cell compartments to cells, and for multicellular organisms also at the level of tissues, organs, and the individual. This principle of compartmentalisation allows the maintenance of homeostasis, nutrient exchange, and protects the organism. Damage or malfunction of these compartmental barriers posses a severe risk to physiological function of the organism.

From a pharmacological point of view the compartmentalisation also defines the barriers that need to be overcome in order to allow therapeutic molecules to reach their target. Understanding of the underlying biology of the barrier and the physiological mechanisms for exchange with the outside environment thus provide the rationale for the strategies employed by drug delivery scientists in an effort to overcome the barriers in a controlled and reversible fashion.

This review will be focusing on those barriers currently most relevant to drug delivery; the barriers will be considered in the sequence in which they typically will be encountered in situations in which a drug needs to be delivered to a remote, not directly accessible site in the body (Fig. 12.1).

All defined biological barriers in the body are created by epithelial or endothelial tissues. The epithelial tissues form the barriers between the body and the environment, but also create the boundaries between the organs and tissues within the body. Endothelial tissues form the lining of the circulatory system, i.e. the lymphatic and

blood vessel. They take on different organisation and functionality depending on their location but share some common design principles.

These tissues have evolved a specific type of architecture to allow control of the interface between the body and its environment as well as between different organs and tissues. In their simplest form they consist of one or multiple layers of endothelial or epithelial cells anchored to a dense layer of extracellular matrix material (basal membrane). These tissues are avascular—thus creating a spatial separation between the barrier and the systemic circulation. As a consequence nutrient supply of the cells in these tissues occurs by diffusion, typically from the underlying tissues. The specific architecture of the barrier tissues can differ considerably but in addition to the epithelial cells the barrier will normally also involve layers of connective and muscle tissue. In the case of epithelial tissues, the layer underneath the basal membrane is made up from connective tissue (including blood and lymphatic vessels, and nerves etc.). In the skin this layer is the dermis, whereas for the gut mucosa it is known as the lamina propria mucosa. In the case of endothelial tissues of larger vessels the underlying layer typically consists of muscle cells.

The epithelial cell layer can consist of only a single cell layer, e.g. the alveoli of the lungs, but more frequently consist of multiple cell layers, often organised in a stratified fashion with different cell layers (strata) having more specialised function. This is accomplished by adult epithelial stem cells in close proximity to a basal membrane layer dividing asymmetrically, i.e. into a daughter cell that remains in place and retains stem cell functionality and another daughter cell, which will then start to undergo differentiation. With each cell division near the basal layer the differentiation daughter cells are displaced further towards the surface where they will eventually be shed.

In terms of their barrier function, the way individual cells are linked to each other is of major importance. These links provide mechanical cohesion and strength to the epithelial and endothelial tissue but can also effectively seal the intracellular space between these cells. On of the key building blocks to achieve this seal is formed by tight junctions. Tight junctions are specialised parts of the cell membrane designed as a continuous barrier. In addition to mechanical stability, they provide a highly effective barrier to transport. Proteins in both adjoining membranes, predominantly occludins and claudins, interlink directly to form continuous sealing strands. In tight junctions, a network of these sealing strands connects larger areas of neighbouring cells. The quality of the barrier created is proportional to the extent of the network. The seal created is sufficient to completely abrogate any transport between cells (paracellular), thus allowing transport to be controlled entirely by the transport functions provided by the barrier cells, e.g. molecule specific pumps and pores or endo- and transcytotic processes. By contrast, gap junctions, i.e. specialised patches where the cell membranes of adjoining cells come together very closely (4 nm), can connect cells. Here the space between the neighbouring cells is bridged by specialised proteins, which form a connecting cylinder with a hydrophilic channel that connects the cytoplasm of both cells. The narrowing of the intracellular space can hamper transport of larger molecules and particles, but does not create a continuous barrier.

12.2 Epithelial Barriers

In multilayered epithelia cells that are moved towards the barrier surface undergo a process of terminal differentiation in which they take on the specific characteristics of the tissue. Once the cells reach the surface layer of the epithelium they die, are shed, and then replaced. This process means that epithelial barriers tend to be highly dynamic and are continuously regenerated.

In epithelia subjected to mechanical stress the differentiation process leads to the formation of keratinocytes, i.e., the cytoplasmic cell surface is strengthened with a proteinaceous layer of keratin. In very exposed surfaces the cells differentiate further and stop being metabolically active, loose their nucleus, to become 'dead' corneocytes which act as building blocks for the stratum corneum. Here the keratin creates the cornified cell envelope, while lamellar lipid granules secreted into the intracellular space fuse into continuous sheets of crystalline, multi-layered interlocked lipids. Together the corneocytes and intracellular lipids create a brick and mortar architecture that gives mechanical and chemical protection to underlying tissues and acts as a tight renewable barrier (Bragulla and Homberger 2009).

12.2.1 The Skin

12.2.1.1 Architecture

The skin forms an effective barrier between the organism and the environment, preventing invasion of pathogens and fending off chemical and physical assaults, as well as the unregulated loss of water and solutes. The physical barrier is mainly localised in the stratum corneum (SC), which prevents permeation of all molecules larger than around MW 500 into the skin, but also avoids loss of water by diffusion through and evaporation from the skin. The layers of keratinocytes just below the stratum corneum (stratum granulosum) also contribute to the barrier through the special ways in which the cells are connected, i.e. tight, gap, and adherens junctions, as well as through desmosomes and cytoskeletal elements (Fig. 12.2).

The space between the corneocytes is filled by multiple layers of lipid lamellae, each with a thickness of around 10 nm (Madison et al. 1987). The intracellular lipid lamellae contain ceramides, free fatty acids, and cholesterol, ceramide A and B. These form layers of highly crystalline lipids, covalently bound to neighbouring cells and interlocked by long lipid chains. Between the lamellar layers only a minimal amount of water is present which is required for the hydration of the polar elements.

On top of its outermost cellular layer, the SC, the skin is covered by a thin (0.4–10 μm), irregular, and discontinuous layer consisting of sebum secreted by the sebaceous glands, along with sweat, bacteria, and dead skin cells. At the microscopic level a direct path into the depth of the epidermis presents itself as a series of hydrophilic (cell) and hydrophobic (intracellular lipids) domains.

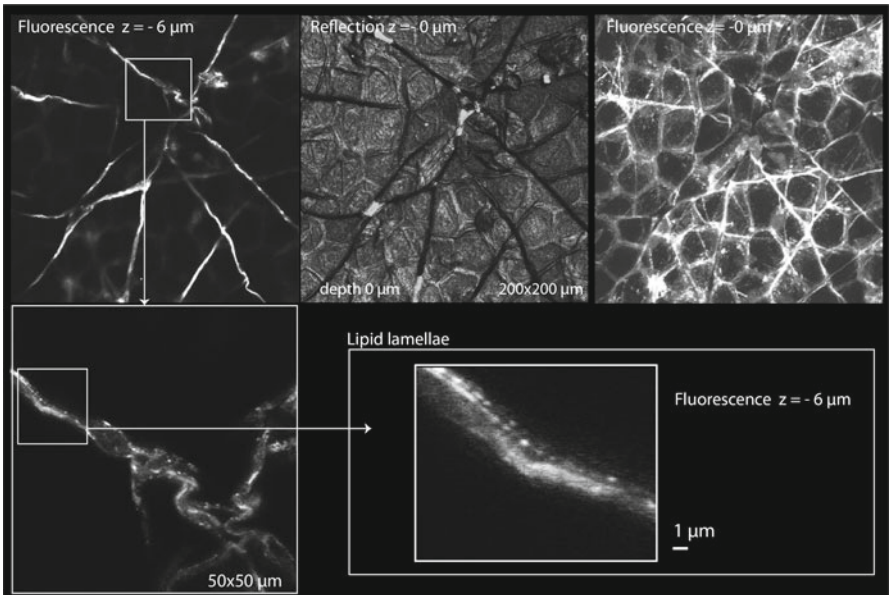

Fig. 12.2 The microscopic structure of the stratum corneum barrier illustrated using reflected and fluorescent confocal signals. The stratum corneum relief can be highlighted when looking at the surface (*XY* plane) using reflected light (*top middle*) and shows corneocytes organised in clusters separated by deeper clefts. The signal from fluorescently labelled hyperelastic vesicles predominantly stains the intracellular lipids between corneocytes (*top left* and *right* and *bottom*). Optical sectioning at a depth of 6 µm shows that the lipid filled clefts are the major pathways for hyperelastic vesicle penetration (*top left*). A detail zoom of these lipid pathways at higher magnification reveals that these vesicles follow along a pathway with lamellar structure (*bottom middle*) reminiscent of the lipid lamellae observed in the intracellular space by electron microscopy (*bottom right*)

12.2.1.2 Passive Barrier Function

The skin predominantly acts as a barrier and from a physiological perspective no exchange with the environment is necessary. In addition, the skin needs to provide a flexible and resilient mechanical barrier against external insults. Any exchange with the environment is provided strictly by the specialised appendages such as eccrine and apocrine sweat glands or hair follicles which are interspersed throughout the epidermis. The stratum corneum provides the main barrier functionality of the skin. The layer secreted by the glands is considered to have a negligible effect as an additional barrier, whereas permeation through the skin's appendages can actually be enhanced compared to the SC proper. Few compounds have physical chemical properties that would be compatible with an efficient diffusion along the direct path into the skin, as this presents itself as a 'patchwork' of hydrophilic and hydrophobic domains (cutting across 'bricks' and 'mortar'). It is thus much more likely for molecules to follow the much more tortuous pathway along the intracellular lipids domains (between the 'bricks'). Despite the optimised architecture, a small amount of water exists between these crystalline lipid layers and at

the junctions between adjacent layers. This creates a tortuous hydrophilic pathway for minimal diffusion of small molecules. It is along this path that the physiological loss of water through the skin occurs (transepidermal water loss, TEWL, insensible water loss) that, in contrast to sweating, cannot be controlled by the body. Changes in epidermal differentiation and lipid composition can lead to a disturbed skin barrier architecture, which may allow the entry of environmental allergens, the development of immunological reactions, and inflammation in atopic dermatitis (Proksch et al. 2008). For example, in atopic dermatitis a malfunction in the secretion of lipid granules is thought to lead to a disturbed formation of the lipid layers ('mortar') and downstream barrier malfunction (Janssens et al. 2011).

12.2.1.3 Active Barrier Function

The skin is the largest organ and constantly exposed to potential threats from the environment. Consequently, it has important functionality as a staging post for the body's immune defences, but also plays a role (similar to the mucosa) as a potential target for immunisation. The skin also functions as an active chemical and metabolic barrier with enzymes in the viable epidermis ready to break down a broad range of xenobiotic molecules and drugs that have entered the skin; this can create a first-pass effect similar to that of the liver after oral drug absorption.

12.2.1.4 Nanoparticle Delivery to the Skin

The skin barrier is highly efficient for all molecules with a molecular weight greater than 500 Da and transdermal drug delivery is limited to a few small molecules with suitable physicochemical properties such as nicotine or morphine (Schneider et al. 2009).

Consequently, a multitude of strategies to increase drug transport through the skin have been developed. As the skin offers little by the way of dedicated transport functionality that could be exploited, most approaches have been aiming to disrupt the barrier in a reversible fashion. A number of technologies use minimal mechanical disruption of the stratum corneum, e.g., using micro needles or ballistic nanoparticles, to bypass the skin barrier (Prausnitz and Langer 2008).

Another approach aims to reduce the barrier properties of the intercellular space filled with the lipid lamellae domains and the associated minimal hydration water. These intracellular structures form a tortuous but potentially continuous pathway from the surface into the deeper layers of the epidermis. Permeability can be enhanced by extraction of lamellar lipids or disruption of their lamellar structure, e.g. by using solvents and non-crystalline lipids. Alternatively, enhanced penetration along the aqueous 'pathway' of interlamellar water can be achieved by increasing skin hydration. This could be a consequence of either prolonged exposure to external moisture or enhanced accumulation of water normally lost by evaporation from the skin (TEWL), e.g. after occlusive application of patches or humectants such as the lipid films formed by, e.g. moisturisers.

The use of such permeation enhancing approaches can increase transport of suitable small molecules temporarily. However, based on the structure of the SC, i.e. solid keratinocyte blocks embedded in a crystalline matrix of lipid layers, the skin resembles a microporous barrier, with the typical size of aqueous pseudo pores available being considerably smaller than the diameter of even nanoparticles. Not surprisingly, there is therefore little evidence for the transport of significant amounts of nanoparticles into and through the skin.

However, we have previously shown that specially engineered vesicles possessing a hyperelastic membrane (also known as Transfersomes) can adopt their shape to allow penetration through pores significantly smaller than their diameter. Such vesicles have been shown to be able to penetrate the skin along the intercellular pathway when applied under suitable conditions (Schatzlein and Cevc 1998; Cevc et al. 2002; Honeywell-Nguyen et al. 2004).

12.2.2 The Mucosa

The mucosa is an epithelial layer lining the body cavities, which not only provides protection but is also involved in secretion and absorption. The mucosa has a similar histological architecture to the skin, but lacks a typical stratum corneum, i.e. corneocytes. Instead, multiple layers of keratinocytes, i.e. keratinised cells retaining a nucleus and some metabolic activity, can provide mechanical strength, where needed. The process of differentiation proceeds analogous to the epidermis with lipid granules being excreted to form intercellular lipid lamellae. The mucosa has a higher permeability for water and typically mucosal tissues are found in wet or moist environments, e.g. mouth or nose. Mechanical stress influences the structure of the mucosa. For example, in the mouth cavity tissues with varying degrees of keratinisation exist, e.g. the gingiva and palate which are exposed to stronger mechanical forces consist of keratinised mucosa while the sublingual and buccal mucosa tends to be non-keratinised. The level of keratinisation is also linked to the type of lipids involved in creating the intracellular lipids, with the less keratinised sites containing a composition of lipids that are more polar and fluid. These types of lipids lead to a less well-organised lipid structure in the intracellular space and increased permeability to drugs.

12.2.2.1 Mucus

Mucus, predominantly formed by a family of secreted specialised mucin proteins, acts as sticky semi-permeable barrier that covers and protects most epithelial surfaces in the body. It has a number of physical-chemical properties that make it uniquely suited for this task.

Mucin monomers are 0.2–0.6 μm long protein fibres containing long flexible stretches (PTS domains rich in proline, threonine, and serine) covered by covalently linked negatively charged glycan (25–200 nm) and separated by ‘naked’ hydrophobic regions which aggregate into cysteine cross-linked globular domains (5 nm).

Disulfide-rich domains at either end serve to link the monomers end to end with branches allowing for covalent and non-covalent cross-linking. The linked mucin monomer chains can extend over several micrometer. Mucins can be secreted or can remain linked to the cell surface by SEA (sea urchin sperm protein, enterokinase, and agrin) domains. Under mechanical stress the covalent links in the SEA domains of the cell bound mucins are transformed into non-covalent bonds that allow shedding of mucus without damage to the underlying epithelia.

Mucus is a highly dynamic material, typically undergoing a process of continuous secretion at the epithelial base of the mucus blanket and a corresponding amount of shedding near the surface. For example, in the human GI tract around 10 L of mucus containing between 2 and 5 % of mucins are produced daily. Thus, any ingress of material into the mucus towards the epithelia is counterbalanced by transport of the mucus towards the surface. In the respiratory tract ciliated epithelial cells affect a similar transport from the lower parts of the lung towards the trachea. The speed of this counterflow can be rapidly adjusted, e.g. in response to noxious stimuli, by increasing the amount of secreted mucus. In the stomach the rate of secretion is such that it counteracts the speed of diffusion of pepsin.

In the gastrointestinal tract (GIT) the thickness of the mucus is strongly dependent on site and digestive activity, with stomach and colon having mucus blankets of a couple to several hundreds of nanometres thickness. The viscoelastic properties of mucus have to be tightly regulated to allow it to perform its protective function, i.e. exclusion of molecules and particulates, but also to ensure that the physiological transport mechanisms can function. In the respiratory tract that means that the mucus needs to be sufficiently ‘thin’ to allow the ciliated epithelia to move the mucus towards the trachea. On the other hand, if the mucus loses too much of its viscoelastic properties it will simply flow with gravity to accumulate in the lungs. Another example of this regulation is provided by cervico-vaginal mucus which shows cyclical changes that affect its barrier properties. During most of the cycle the cervico-vaginal mucus has a high level of viscoelasticity making it near impossible for sperm to penetrate. During ovulation the mucin concentration decreases by 2–4-fold to around 1 % and the viscoelastic properties are dramatically reduced.

The viscoelastic properties of mucus are such that an unstirred non-mixing layer is maintained near the cell surface even under conditions of extensive mixing and in the presence of other strong shear forces. This is possible because mucus reacts to mechanical stress dynamically, i.e. the response to stress changes gradually. In response to small forces with little displacement, the mucin chains extend in an elastic fashion like a spring. When exposed to larger forces however, the chains gradually untangle leading to a dramatic fall in viscosity at the interface. This reduction in viscosity creates a layer of highly lubricating mucus which effectively dissipates the mechanical stress and allows underlying layers of mucus to maintain their viscous and elastic entangled state and avoid mixing, thus limiting transport in this region to diffusion only.

Diffusion of molecules into the mucus layer depend on the concentration gradient and also on the size of the molecule. This is not only because of the slower diffusion observed with larger molecular weight compounds but also because of the retardation of movement experienced as a consequence of the mesh formed by the cross-linked mucin network.

Numerous attempts have been made to determine the effective 'pore' size in native mucus, however, imaging of mucus without preparation artefacts has proven challenging. Current data suggest a random mesh with fibres of 30–100 nm diameter—this is in contradiction to the size one would expect based on the chemical structure (3–10 nm)—suggesting ready aggregation of individual fibres into larger strands. Comparisons of diffusion constants of molecules in mucus vs. water give some indication of mesh size and suggest a fibre spacing in the order of 400 nm. Spacing does, however, also depend on the relative concentration of mucus, as at higher concentrations viscosity increases sufficiently to trap material effectively. Similarly acidic conditions appear to lead to a denser structure compared to more alkaline conditions.

Some agents can effectively increase the mesh size of mucus by aggregation of the individual fibres.

Diffusion of molecules and particles in mucus is also affected by their non-covalent interactions. Mucus is known for its 'stickiness', i.e. the ability to adhere to a broad range of materials—this is achieved by the ability of mucins to create multiple low affinity interactions with a broad range of materials. This includes hydrophobic interactions with the hydrophobic, lipid-coated 'naked' domains as well as ionic interactions with the mostly anionic groups in the glycan-covered domains. Although the individual interactions are of low affinity and can be easily disrupted, mucus very efficiently binds to larger molecules where these interactions are of a polyvalent nature, as for any bond broken others have already formed readily. These interactions can thus retain particles and molecules with multiple interaction sites very effectively. Consequently, the speed with which materials can move in mucus depends not only on their size, but also on the surface properties and the availability of polyvalent interaction sites. Water-soluble small molecules and most globular proteins are typically not significantly retarded in mucus. Such molecules will only have one or few hydrophobic or ionic interaction sites and thus will be able to move once those individual non-covalent bonds are broken. Furthermore, their hydrodynamic size falls well below the mucus mesh exclusion size. In contrast, polyvalent small cationic molecules can experience significant retardation. Lipophilic molecules can experience retardation even when they are small presumably because of interaction with the hydrophobic mucus domains. Other molecules will experience strong retardation either because of the polyvalent non-covalent interactions or like bacteria or some viruses because of their size (Cone 2009).

The barrier properties of mucus with respect to nanoparticles thus depend strongly on the specific nanoparticle size and surface characteristics. In general smaller uncharged particles will be less retarded than large charged particles. In particular positive charges lead to strong interactions with the mucus. Interestingly particles interacting extensively with the mucus could also change the ultrastructure of the mucus mesh and thus reduce its barrier properties (Wang et al. 2011).

Hydrophobic particles including those made from polystyrene, poly(lactic acid), and poly(cyanoacrylates) do not only show evidence of retardation by their interaction with mucus, but also show an extensive interaction with mucus through their sometimes irregular distribution (Ensign et al. 2012). Coating of such surfaces with hydrophilic polymers such as PEG creates a steric barrier which reduces some

non-specific mucus interactions and thus can minimise retardation of such particles in the mucus (Lai et al. 2007). Nanoparticle interaction with mucus has recently been reviewed (Ensign et al. 2012).

12.2.2.2 Gastrointestinal Tract

The mucosa of the GIT follows the organisational and functional principles outlined for mucosa in general. However, in particular where secretion and absorption occur, GIT mucosa has developed a number of highly specialist tissues.

12.2.2.2.1 Oral Mucosa

The oral mucosa is made up of keratinised and non-keratinised stratified epithelia with a thickness of around 500 μm. The buccal mucosa is exposed to an environment containing a changeable array of fluids, types of foods, microbes, etc. Furthermore, as part of the function of the oral cavity it is exposed to mucus and saliva containing a range of proteolytic enzymes, esterases, oxidases, and reductases originating from buccal epithelial cells, as well as phosphatases and carbohydrases present in saliva.

As a site for drug administration the mucosa of the oral cavity has the advantage of ease of access, potentially allowing facile dosing and removal, if required. In addition, the buccal epithelium blood supply is linked to the internal jugular vein, thus allowing drugs to potentially bypass the first-pass metabolism in the liver that can occur after drug absorption from the intestine. On the other hand, prolonged drug exposure of any particular area of oral mucosa is not straightforward to achieve as saliva and mucus facilitate a constant movement and exchange. An additional challenge comes from the exposure to the microbial, digestive, and metabolic enzymes, potentially leading to rapid drug degradation.

12.2.2.2.2 Gastrointestinal Mucosa

The GIT mucosa has dual functionality. In addition to providing the typical barrier functionality, the mucosa lining the various parts of the intestine forms part of a well-orchestrated absorption machinery that has evolved to process food and allow uptake of key nutrients and water. With the exception of a few enzymes which are already active in the oral cavity, the upper GI tract (mouth, oesophagus, and stomach) breaks down food stuffs using mechanical and non-specific acid degradation to create chime, a semisolid food pulp that can then be fed into the absorptive sections of the GIT, i.e. the upper and lower intestine as and when required. Depending on the amount of food this process can take from 40 min to several hours. The stomach volume typically ranges from around 200–300 mL empty, to up to 1,400 mL after a meal. The specific volume as well as the process of emptying is regulated by hormones, e.g. GLP-1 (Delgado-Aros et al. 2002).

The Stomach

The stomach can be divided into a number of anatomical and histological regions, i.e. the entrance or cardiac region, the fundus and the body, and the exit, i.e. pyloric region. Its body contains multiple muscle layers and folds (rugae) which vigorously mix the stomach content to facilitate the mechanical breakdown of the food. The chemical food degradation here is based on enzymes and, most importantly, the high level of hydrochloric acidic (HCl) in the stomach (pH 2). HCl is secreted in response to various stimuli by specialised glands which are localised in the lamina propria of the gastric mucosa. Specifically, the mucosa forms invaginations, gastric pits (foveoli), through which the glands open into the stomach. The gastric glands are simple tubular glands with multiple tubular lobes merging in the isthmus region to then open via a neck into an individual pit in the stomach. The cells producing stomach hormones and proteases (pepsin, rennin) can be found towards the base of the glands, followed by the HCl producing (parietal) cells, and finally towards the 'neck', the mucus cells. The mucus cells produce a continuous layer of mucus that lines the stomach and is interrupted only by the opening of the gastric glands. The secretion of the mucus layer separates the cells from the corrosive stomach content. The stomach secretions (HCl, enzymes) are powerful enough to destroy most swallowed microorganisms and break down all types of food. However, the protection provided by mucus is so effective that the stomach mucosa only consists of a single layer of columnar mucus secreting cells.

The barrier function of the gastric mucus depends on a number of properties. The gastric mucus consists of an outer, loosely adherent layer and an inner layer that is firmly attached to the underlying mucosa (Phillipson et al. 2008). Both layers consist of mucins, but their thickness is differentially regulated. The mucins form a gel-like, fluid-retaining protein mesh, which prevents ingress of any particle larger than the mesh size and slows down diffusional transport of smaller molecules and even hydrogen ions. The mucus also limits the access of acid to the cell surface by trapping bicarbonate ions secreted by the parietal cells to neutralise any back diffusion of hydrogen ions from the lumen. As the cells secret equivalent amounts of bicarbonate and HCl, the process is self-regulating. The thickness of the mucus in the stomach ranges from around 80 to 100 μm for the firmly adherent layer and around 100–120 μm for the looser layer facing the gastric lumen. The thickness of the mucus barrier in the GIT depends on the exact location within the GIT and changes in response to physiological regulation. It also differs for different species and is considered in more detail below (Ensign et al. 2012; Varum et al. 2012).

The Small Intestine

The small intestine, consisting of the duodenum, jejunum, and ileum, follows on from the pylorus of the stomach. The strong ring muscles at the exit of the stomach play an important role in regulating gastric emptying, i.e. the transition of chyme into the small intestine. The duodenum, linking stomach and jejunum, is only around 30 cm long but is central to the enzymatic breakdown of food. In its central section it contains the major duodenal papilla with the opening of the pancreatic duct and the bile duct. In response to chyme (partially digested stomach content) entering the

duodenum, the release of cholecystokinin and secretin hormone triggers the release of bile from the liver and gall bladder. The same hormones, as well as gastrin, are responsible for triggering the secretion of pancreatic juice.

Although the underlying architecture remains constant, the mucosa of each of the different sections of the small intestine has a typical phenotype. One the most striking features of the mucosa is the dramatic increase of surface area, a feature that helps to maximise absorption. Specifically, the mucosa possesses a large number of folds that, in turn, are covered with numerous finger-like 1–1.5 mm long villi. A single layer of specialised epithelial cells, the enterocytes, covers these villi. Interspersed between these cells are some mucus-producing goblet cells and the follicle-associated epithelium (FAE). The individual enterocytes again have thousands of microvilli at their apical end, creating a brush border membrane. The individual microvillus is a finger-like membrane protrusion with a diameter of 100 nm and a length of 1–2 μm. The microvilli are linked to the underlying cytoskeleton network and are supported by a polarised bundle of actin filaments oriented with the plus ends extending into the tips. This brush border membrane architecture leads to a 100-fold increase of membrane area per cell. The brush border membrane is also packed with enzymes, creating a zone of very high digestive and metabolic activity. Taken together these surface features lead to an enormous increase of the mucosal surface with a total absorptive area in the order of 200–400 m^2 (Hunter et al. 2012).

But not only the lining of the gut is optimised for digestion: Together with the highly acidic environment of the stomach, the digestive juices present a significant barrier to any form of therapeutic drug delivery, as they are able to break down most biological materials and molecules very rapidly. The most important constituents of the bile are the bile acids (cholic acid, chenodeoxycholic acid) and their conjugates taurocholic acid and glycocholic acid, which account for around 80 % of the bile salts. Bile acids and derivatives are surface-active compounds which can help to solubilise lipids by stabilising the lipid water interface. Once secreted, these compounds can bind to alimentary lipid materials which they solubilise through the formation of mixed micelles. Around 95 % of the bile acids are reabsorbed in the ileum and recycled in the liver. A further 2–5 % are reabsorbed after metabolic transformation by gut bacteria in the large intestine (Keating and Keely 2009). The total bile pool of 2–4 g recirculates up to 10 times a day to provide the 20–30 g bile acid required for fat digestion (Silbernagl and Despopoulos 2009).

In addition to the bile secretion there are also 1-2 L of pancreatic juice secreted by the exocrine pancreas. The juice contains high levels of bicarbonate which neutralises the hydrochloric acid in the chyme to create the pH (pH 7–8) at which the pancreatic enzymes are optimally effective.

In response to food stimuli, the intestinal glands in the duodenum (crypts of Lieberkühn) secrete enteropeptidase. Enteropeptidase, in turn, is able to activate the proteolytic enzymes secreted as pro-enzymes (trypsino*gen*, chymotrypsino*gen*) by cleavage (trypsin, chymotrypsin) which then go on to activate other enzymes. Carbohydrate digestion depends on α-amylase, which is secreted as an active enzyme in the pancreatic juice. It is responsible for the initial breakdown of starch and glycogen—further breakdown happens with the help of other enzymes at the brush border membrane (see below).

The most important enzyme for lipid digestion is pancreatic lipase, which breaks down the triacylglycerol with the help of co-lipases and bile salts. One of the key elements of lipid digestions is the emulsification of the lipids with the help of oral and stomach enzymes and the mechanics of stomach movement. The resulting 1–2 μm lipid droplets are then further reduced in size in the small intestine (enzymes and bile) to create nanometre-sized mixed micelles (20–50 nm) with a 50× increased surface area, which can be absorbed by the brush border membrane of the jejunum mucosa. Other important enzymes include phospholipase A_2, ribonuclease, deoxyribonuclease, and carboxylesterase.

The breakdown of food into nutrients starts in the mouth and stomach and is then most active when the chyme reaches the small intestine and its enzymes. However, the brush border membrane on the enterocytes is emerging as another highly metabolic region. The membrane composition of these enterocytes is optimised to withstand high detergent concentrations and many membrane-bound enzymes and transport proteins are concentrated in detergent-resistant lipid rafts (Danielsen and Hansen 2008). Recent reports suggest that the microvilli may also be actively secreting enzyme-loaded vesicles into the gut lumen (McConnell et al. 2009a). The vast majority of nutrients are thus taken up in this zone as small molecules or after digestion of larger biopolymers (carbohydrate, peptide/protein, or nucleic acids) into oligo- or monomers by passive diffusion or via specific active transport mechanisms.

In addition to the digestive and absorptive function, the intestinal tract is also a key site for the body's interaction with potentially dangerous microorganisms. In order to control these the GIT has an extensive system of cells and tissues involved in immune regulation. For example, the FAE in the intestinal mucosa provides access to the gut-associated lymphatic tissue (GALT), an extensive system of gut-associated immune tissues, including the Peyer's patches. The number, size, and density of Peyer's patches vary between species but also stage of development. For example, in humans the patches have a diameter of around 1 cm and reach their highest density in the ileum (Brayden et al. 2005). The Peyer's patches have a physiological role in the mucosal immune system. As part of the surveillance system they are involved in constant uptake and sampling of gut content. Specifically, microfold cells (M cells) provide the interface of gut and GALT with the ability to take up larger particles and bacteria, in order to present them to immune cells, i.e. dendritic cells. In order to enable efficient uptake of larger particles these areas have only a sparse covering of mucus, no active P-glycoprotein pumps, and the ability to transcytose material to underlying layers.

The Large Intestine

The large intestine comprises the caecum (poorly defined in humans), the colon (ascendens, transversum, descendens, sigmoid), the rectum, and anal canal. Its diameter is much wider than that of the small intestine and the mucosa takes the form of a simple columnar epithelium. In humans the colon has a length of around 1.5 m, but a much smaller surface area (in the order of 1/50–100th) than the small intestine because of the mucosa's lack of villi and microvilli/brush border membrane. Its main function is the continued reabsorption of water from the undigested waste, the

formation of the faeces, and defecation. This leads to a volume reduction of chyme and waste from 1.5–2 L to 100–200 mL and dramatic changes in viscosity and solubility of dissolved materials. The mucosa has little by the way of GALT, but possesses a high number of goblet cells responsible for the production of a 800–900 μm thick, mostly loose, mucus cover (Ensign et al. 2012). The mucus is thought to facilitate the movement of the more viscous and solid waste. The transit time in the colon can vary widely from around 1 h to several days (Wilson 2010), but may also depend on size (pellets move slower than tablets) and local factors, as content can come to rest unstirred in pockets for prolonged periods (McConnell et al. 2009b).

The bacterial flora of the gut in general, but in particular of the large intestine, consist of an estimated 10^{10}–10^{12} colony-forming units/g compared to around 10^{6} found in the ileum. The population comprises many hundreds to thousand of different species. Complex interactions of this distinct ecosystem and the body's metabolism and immune response are beginning to emerge (Gravitz 2012). The ability of the bacteria to thrive in the colon and produce nutrients depends on their ability to digest the waste products left behind after digestion by human enzymes. This means the content of the colon is exposed to a whole host of additional enzymes, many of which are tailored to the breakdown of complex carbohydrates, which also could contribute to degradation of drugs or drug carriers. The bacteria are also responsible for the production of some nutrients/vitamins which are then absorbed in the colon.

Although the absorptive activity of the colon is much lower than that of the small intestine, it does contain a range of transporters, some of which are used to facilitate uptake of drugs in the colon, e.g. after administration of suppositories (McConnell et al. 2009b). One clear advantage in this context stems from the fact that material absorbed in the colon avoids the first-pass effect of the liver. In addition, the activity of most endogenous metabolic enzymes and P-gp type efflux pumps is also lower in the colon compared to small intestine, potentially resulting in an increased bioavailability of some drugs delivered to the lower GIT with the help of delayed release formulations. While the colon is a potentially promising site for drug delivery, there is currently relatively little known about the direct uptake of nanoparticles in this gut region under physiological conditions. Florence and colleagues have also found evidence of uptake of polystyrene particles in the colon (Florence et al. 1995). In general uptake was much reduced compared to the small intestine, although there was evidence that in some cases the upper GIT may act as 'sink' preventing materials actually reaching the lower GI. Delivery with nanoparticles after delayed release in the colon may help to increase any innate uptake in this gut region. Additional opportunities may exist for the treatment of the inflamed colon, e.g. colitis/Crohn's disease, but the importance of transcytosis in this context remains unclear.

12.2.2.2.3 Nanoparticle Delivery

The general design principle of biological barriers is to minimise any exchange of materials and molecules across the barrier (lockdown) and then to utilise specific and selective transport processes to allow controlled exchange of required molecules (Fig. 12.3).

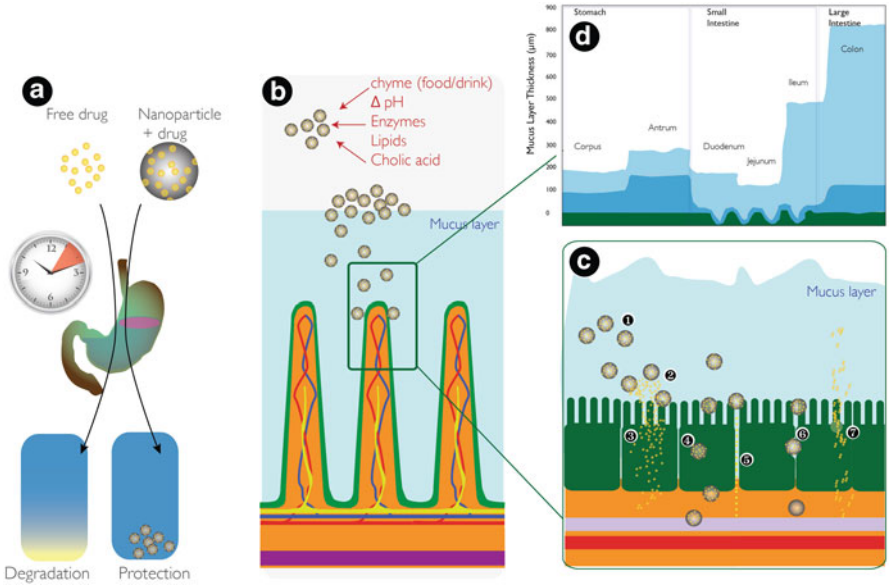

Fig. 12.3 The biological barriers to nanoparticle delivery in the gut: (**a**) Nanoparticles can increase the amount of drug that reaches the absorbing regions of the intestine by protecting encapsulated content from degradation in the stomach. (**b**) The particles reach the small intestine where they are exposed to a range of materials, degrading enzymes and lipids and bile salt-derived surface-active compounds. Nanoparticles need to adhere to the mucus blanket (bioadhesion) to be retained and/or penetrate the mucus (mucoinvasion) to get between the villi and close to the cells of the gut epithelium. (**c**) The surface area of the small intestine is increased by villi and in addition by microvilli on the individual enterocytes. Nanoparticles can increase the bioavailability of encapsulated material by increasing solubility (1) and dissolution (2) to increase the local drug concentration. The transcellular pathway allows uptake and transcellular transport of suitable drugs (3) and nanoparticles (4). Alternatively, paracellular pathways could allow passage of small water-soluble molecules (5). Paracellular transport of larger molecules or particles would normally require a temporary disruption of the tight junctions (6). In addition, efflux pumps such as P-gp pumps are expressed in the gut epithelium which rapidly pump substrate drugs and xenobiotics back out (7). (**d**) The mucus blanket of the gut typically possesses a denser and more tightly bound layer adjacent to the cell surface and an additional looser layer facing the gut lumen (mucus thickness redrawn from Atuma et al. 2001)

A large body of literature demonstrates that micro- and nanoparticulate materials can increase the oral bioavailability of drugs. The mechanisms by which this can be achieved include increased drug solubility, increased dissolution, protection from GIT degradation, and an increase in GIT residence time. Ultimately these mechanisms utilise physiological uptake processes that apply to small molecules or oligomers as part of normal digestion.

An area of continued interest and research is focused on opportunities for delivery linked to the uptake of drugs in nanoparticles rather than of free drug molecules released from particles. Four potential routes for NP uptake/transcytosis commonly considered include endocytotic uptake by either M cells or enterocytes, paracellular transport between the tight junctions, and persorption, i.e. passage of particles through temporary gaps created by shedding of cells at the tip of the villi.

The paracellular route is thought to be of less practical importance because the total surface area of the intercellular space is comparatively small (~1 %) and transport along intact tight junctions is limited to very small molecules (~1 nm). Some materials such as soluble chitosan have been reported to be able to open tight junctions. The mechanism involved has been shown to be protein kinase C-dependent (Smith et al. 2005), but does not seem to be active when nanoparticles are used (Prego et al. 2005). However, a recent report suggests that chitosan nanoparticles can be transported across the gut epithelium by opening of tight junctions, while the mucus barrier is able to prevent simultaneous transport of the potentially toxic lipopolysaccharide (Sonaje et al. 2011). Overall, the long-term implications of the temporary breakdown of this physiological barrier remain still unclear. In our research we have been able to show that tight junction opening is not important for the transport of modified glycol chitosan particles (Siew et al. 2012).

Persorption of particles through the gaps left after shedding of cells from the tip of a villus has previously been shown to allow passage of colloidal gold particles with smaller particles being taken up in a more efficient way (Hillyer and Albrecht 2001). However, this mechanism is again unlikely to be of practical importance for conventional pharmacotherapy because of the very small relative surface area affected under physiological conditions (Prego et al. 2005).

Uptake of particles by the M-cells is a very well-understood process with a clearly defined physiological role in the interplay between GALT and the gut environment. M-cells associated with the FAE covering Peyer's patches have the capacity to effectively transcytose a range of materials including nanoparticles using a number of endocytotic mechanisms (Buda et al. 2005). The limited mucus coverage and spare glycocalix of the M-cells are thought to facilitate the process (des Rieux et al. 2006). Specific interactions and binding to M-cells membrane structures and receptors plays an important role in some infectious diseases, but, conversely, can also be exploited for the targeting of particulates to M-cells (Devriendt et al. 2012). In in vitro models the transport of nanoparticles across Caco2 monolayers increases up to 1,000-fold with M-cells (des Rieux et al. 2005). On the other hand, this route is again limited to a relatively small area of only 1 % of the total gut area (des Rieux et al. 2006). Nevertheless, with respect to immune therapies these sites represent a potentially promising route and their targeting by particulate-based therapies remains an important area of interest.

In principle all cell membranes are able to undergo endocytosis. Therapeutic delivery with nanoparticles across the epithelium via enterocytes would have to be based on an innate ability for endocytotic uptake and transport.

It is currently not entirely clear what role these processes play in the physiology of enterocytes and as part of the physiological digestion process. Endocytotic mechanisms are responsible for the ability of neonatal animals to take up immunoglobulins. However, the ability is rapidly lost after birth and little is known about physiological endocytosis by enterocytes in later life.

There is some evidence that endocytosis forms part of the physiological digestive process. For example, the absorption of ferritin by endocytotic processes has recently been demonstrated in Caco2 monolayers (Kalgaonkar and Lönnerdal 2009)

and may play a physiological role, as at least a proportion of ferritin is thought to remain intact in vivo to allow binding to the receptor. Interestingly, pathogens such as *Vibrio cholera*, the gram-negative bacterium that can give rise to cholera, use endocytotic mechanisms. The cholera toxin secreted by *V. cholera,* for example, uses binding of its B subunit to GM1 gangliosides in the glycocalix to induce endocytotic uptake of the protein toxin.

These findings of pathophysiological endocytosis processes in the enterocytes provide plausible mechanisms that may be exploited by therapeutic nanoparticles. Over the recent number of years an increasing amount of evidence has emerged that demonstrates endocytotic uptake and transcytosis of nanoparticles. Florence and co-workers have used stable polystyrene model particles to explore particle uptake in animal models (Hillery et al. 1994). Recent data demonstrates that not only model particles but also nanoparticulate drug delivery systems can be taken up by endocytosis including lipid nanocapsules (Roger et al. 2009; Beloqui et al. 2013), chitosan nanoparticles (Behrens et al. 2002), and micelles (Mathot et al. 2007; Lalatsa et al. 2012). Interestingly, polystyrene particles cannot only be shown to be transcytosed across the intestinal epithelium, but can in fact undergo a process similar to enterohepatic circulation (Jani et al. 1996). Novel imaging techniques such as label-free Raman spectroscopic microscopy have been used to demonstrate that nanoparticles can be taken up by enterocytes and can undergo recirculation (Garrett et al. 2012). The bioavailability of such glycol chitosan-based micellar clusters is around 24 % and they are sufficiently stable to allow deliver of small peptides to the brain (Lalatsa et al. 2012).

The uptake of some nanoparticles can be increased by using ligands such as lectins which are able to bind to enterocytes (Gabor et al. 2004) as has been shown with tomato lectin covalently linked to polystyrene nanoparticles (Hussain et al. 1997). Another example is utilising the built-in ability for endocytosis of transferrin (Lim et al. 2007); this endocytotic activity may potentially exploited to allow specific uptake of targeted particles (Lim et al. 2007). Given the range of physiological and pathological uptake mechanisms potentially available, e.g., viruses (Smith and Helenius 2004), it is likely that these particle-cell interactions will offer some possibility for enhanced enterocyte-targeted nanoparticle uptake.

12.2.2.3 Airways and Lungs

The most important function of the lung is external respiration, i.e. the exchange of gas with the environment. This exchange involves the transport of gases by convection along the *conducting* airways (mouth, nose, trachea, bronchi, and larger bronchiole) to the *respiratory* airways (alveoli) where the gas exchange occurs with the blood via diffusion. Together the airways and the lung resemble an inverted tree with trachea and bronchi, etc. as stem and branches and the alveoli as leaves. There are around 300–500 million alveoli each with a diameter of around 0.3 mm. The airways have a surface area of around 2–3 m^2 and divide around 16 times into smaller branches until reaching the alveolar ducts and sacs with their large surface

area of around 100–150 m^2. In addition to gas exchange the lungs also have some metabolic function (e.g. angiotensin I → II), buffer the blood volume, and trap small blood clots.

The airways are lined by a pseudo-stratified epithelium, comprising basal (stem) cells, mucus-producing goblet cells, brush cells, and ciliated cells covered by a layer of mucus. In the larger bronchi the epithelium and liquid/mucus have a thickness of 58 and 8 μm, respectively. They become thinner in the smaller branches proportionally towards the bronchioles where the epithelium (10 μm) is covered by a much thinner (2 μm) mucus layer. The mucus layer consists of a low viscosity sol layer close to the cell surface in which the ciliae can move with ease and a higher viscosity gel layer on top which allows the mucus to be moved by the beating ciliae. This 'mucociliary escalator' mechanism allows particles and debris that have entered the lungs to be moved rapidly (~5 mm/min) towards the trachea and mouth. In this fashion the respiratory mucus blanket is replaced every 20 min (Beck-Broichsitter et al. 2012), although removal of particles from deeper regions of the lung can take up to 24 h (Patton and Byron 2007).

Gas exchange occurs in the respiratory epithelium of the lungs, i.e. the alveoli. The distinct architecture of this epithelium has evolved to maximise gas exchange, i.e. to provide a large surface area with minimal diffusion distances. Specifically, more than 95 % of the surface of the hundreds of millions of alveoli is occupied by type I cells which cover a large surface area, but are extremely thin (<0.1–0.2 μm). They are covered by an equally thin (70 nm) fluid/mucus layer. The remaining area at the edges of the individual sacs is made up by Type I progenitor cells and the lung surfactant producing Type II cells. The surfactants produced by the Type II cells are critical in reducing the surface tension at the air–liquid interface to minimise the forces required to fully ventilate the lungs and balance the forces exerted between individual large and small alveoli to avoid collapse.

In addition, for each of the alveoli around 13 alveolar macrophages are actively clearing insoluble material from the air-side surface. Their concentration at the edges means that sometimes particles that are deposited in the alveoli can remain there for prolonged periods of time.

12.2.2.3.1 Nanoparticle Delivery to the Airways

Drug delivery to the lung faces two distinct challenges. The first challenge stems from the problems associated with delivering the drug or particle to the correct part of the lung, the second barrier is then to achieve uptake to gain access to the systemic circulation.

Under ideal conditions deposition, predominantly in the alveolar regions of the lungs, of up to 90 % of aerosol particles of a size of 1–2 μm can be achieved when particles of the correct properties are inhaled deeply and slowly. However, it is technically quite complex to create cheap, portable, and safe devices that can deliver fixed dose aerosols with the correct properties reproducibly to a wide range of patients and with sufficient shelf life. A considerable amount of effort has therefore gone

into the development of pressurised metre-dosed inhalers, dry powder inhalers and nebulisers, and the respective formulations. Today such devices routinely deposit in the order of 50 % of the nominal dose in the lung (Dolovich and Dhand 2011).

The interaction between airflow, particle size, particle density, and anatomy of the lung is complex and achieving deposition in the desired lung region is not trivial. Large, dense particles are normally just carried along with the airflow. When the flow changes its direction such particles, due to their inertia, will continue to travel along a straight line, separate from the flow and impact on airway surfaces in the mouth, trachea, or large bronchi. Somewhat smaller particles will initially follow the airflow but, as the flow velocity reduces in the lower parts of the lungs, will tend to be deposited in bronchi due to gravity. Only the smallest particles of around 1–5 μm in diameter will remain suspended in the air, able to reach the alveoli. Particles that are still smaller, i.e. in the nanometre range, would again be deposited with lower efficiency because they are likely to stay suspended in the air over the duration of the typical 5-s breath hold and therefore would be exhaled again. For example, 50 % of 20 nm particles, but only 30 % of 5 nm particles are deposited in the alveolar region; practically no deposition in this region would occur for 1 nm particles (Oberdörster et al. 2005; Bur et al. 2009).

Once the drug or nanoparticle has been deposited in the lungs, further processing will depend on the interactions with the mucus and epithelium and may therefore be different depending on the lung region (e.g. alveoli vs. trachea). To avoid some of the challenges related to the presence of particulates in the lungs (see below), many inhaled formulations contain drug-containing particles that rapidly dissolve to release the drug cargo for absorption.

For low molecular weight drugs or drugs that separate rapidly from the particles, the considerations regarding absorptions are similar to any other epithelial barrier. In the absence of specific transporters, uptake will be best for small molecules with moderate hydrophobicity, less favourable for highly hydrophobic drugs, or positively charged hydrophilic drugs. For some small drugs bioavailability of material deposited in the lungs can approach 100 %, helped by a rapid absorption, low levels of metabolic enzymes, and absence of first-pass metabolism. For this mode of administration, the rapid absorption kinetics, in the order of 1 min to 1 h, are matched by equally short plasma half-lives (Patton and Byron 2007). The lung mucosal barrier is more penetrable than that of the gut allowing the passage of molecules with a molecular weight of up to 160 kDa. However, absorption of macromolecules such as peptides and proteins decreases with increasing molecular weight. On the other hand, larger peptides and proteins have a tertiary and quaternary structure, which can help to reduce the level of degradation by peptidases, which for smaller peptides can be considerable.

After deposition in the lungs nanoparticles need to overcome the mucus barrier, as particles trapped in the mucus are cleared rapidly, independent of size (50 nm to 6 μm) (Bur et al. 2009). (Nanoparticle interactions with mucus are discussed in more detail below.) Once nanoparticles reach the epithelium, it is highly likely that they will be phagocytosed by the multitude of macrophages, estimated to be 1.2 (mouse) to 12 (human) per alveolus (Geiser 2010). After particle uptake the

macrophages gradually (up to 70 days in rats) migrate so that disposition can occur via the mucociliary escalator mechanism. Similar to the initial particle deposition, this interaction process is size-dependent. For example, particles of a size of 1–2 μm show efficient deposition in the lungs but are also rapidly taken up by macrophages; smaller and significantly larger particles are being phagocytised to a lesser extent (Geiser 2010). With respect to potential toxicity, the persistent presence of particles can cause granuloma formation and inflammation. The form factor of particles appears to also be important, with long axial ratio particles being more problematic (e.g. asbestos fibres and various nanotubes) (Oberdörster et al. 2005). It is clear that surface charge and chemistry will affect particle properties, but it is unclear whether there is a difference between hard (inorganic) and soft (organic) particles in terms of processing and response. For ultrafine (nano) inorganic particles the inflammatory response induced appears to be proportional to the total particle surface area rather than size alone (Oberdörster et al. 2005).

Whereas around 80 % of μm-sized particles (0.5–10 μm) can be recovered from alveolar macrophages, this is the case for only 20 % of the particles in the 15–20 nm size range, suggesting that those particles interact with epithelial cells. Indeed, a number of studies show that such particles, depending on size and surface chemistry, can gain access to epithelial cells and lung interstitial sites within 15 min. The propensity for this translocation appears to be greater in larger animal species (Oberdörster et al. 2005). These cells share the basic ability to take up many types of materials by endocytosis with most cells, although the extent, specific receptors involved, and relative importance of specific mechanisms differ (Conner and Schmid 2003).

Uptake of nanoparticles can occur in a non-specific way simply based on adsorption, e.g. as a consequence of electrostatic interactions. This approach is frequently used in the context of delivery of genetic therapies with the help of polymers where the resulting particles typically are positively charged (Beck-Broichsitter et al. 2012). In this context it is also important to remember that viruses have the ability to subvert endogenous endocytotic mechanisms when infecting cells (Smith and Helenius 2004). For example, up to 80 % of the millions of cases of common cold every year are caused by rhinovirus which use the ICAM-1 receptor in upper respiratory tract epithelial cells for infection (Dreschers et al. 2007). Nanoparticles have been targeted in this fashion using a number of receptors such as galactose, lactose, lactoferrin, or PECAM-1 antibodies and Fab fragments (Beck-Broichsitter et al. 2012).

There is evidence that nanoparticles made from, e.g. gold, iridium, carbon, and polystyrene, in the size range from $5 \leq 240$ nm after reaching epithelium or interstitium can translocate further to reach the systemic circulation. Similar to uptake and translocation into epithelial cells and interstitium, the translocation into the circulation does not require specific receptor interactions, e.g., 6 nm ZnO particles (Gilbert et al. 2012), but the coating of the particles with, e.g. albumin or lecithin, potentially facilitates the transcytosis.

12.2.2.4 Nasal Mucosa

The nasal cavity is divided into two halves, which in humans have a combined volume of around 13 mL and a surface area of 150 cm^2. This large surface area is achieved through the presence of three mucosa-covered seashell-shaped bone protrusions, the conchae (inferior/low, middle, superior/high), which divide the airflow coming from the nostrils along four groove-like channels to flow to the rhinopharynx at the back of the mouth and then the trachea and lungs. This respiratory part of the nose is covered in the same pseudo-stratified epithelium as the other conducting airways, containing ciliated and non-ciliated columnar cells, goblet cells, and basal cells. The mucosa is covered by the same dual viscosity mucus which allows the ciliae to easily move in the low viscosity sol (3–5 μm), but also to move the high viscosity upper mucus layer (2–4 μm) from front to back, towards the pharynx. Movement (~5 mm/min) is achieved by the coordinated beating of the ciliae which hold the mucus like a 'hook' (Mistry et al. 2009). The blood supply, lymphatics as well as sensory neurons are found in the underlying *lamina propria* of the nasal mucosa. These neurons, in particular the ophthalmic and maxillary branches of the trigeminal nerve, ultimately link directly to the brain stem. With respect to lymphatics the nasal cavity contains the nasal-associated lymphatic tissue (NALT) which in humans is concentrated in the Waldeyer's tonsillar ring surrounding the pharynx. Here the mucosa contains M-cells, similar to those found in the gut, which are involved in the processing of particulates. In addition, a more diffuse layer of lymphoid tissue can be found directly under the mucosa (Illum 2007).

At the roof of the nasal cavity lies the olfactory part of the nose, responsible for our sense of smell. This region is not part of the normal airflow but transport of molecules relies on diffusion or turbulent airflow after 'sniffing'. This part of the nose is covered by olfactory mucosa and in humans accounts only for 5 % of the surface area of the nose (50 % in rats) (Oberdörster et al. 2005). In addition to the olfactory neurons the olfactory mucosa also contains supporting or sustentacular cells and basal or progenitor cells. The olfactory neurons produce non-motile cilia of up to 200 μm length which entangle with the microvilli on the surface of the sustentacular cells (Mistry et al. 2009). The *lamina propria* underneath the epithelial layer contains the blood vessels, lymphatics etc. as well as the Bowman glands which open onto the surface of the olfactory epithelium. Within the *lamina propria* multiple axons coming from the neuronal cells in the olfactory epithelium are bundled together into *fila olfactoria*, which then are collected into fascicles. A few of these are again bundled together and ensheathed by an individual Schwann cell to pass through the porous bone of the cribiforme plate to reach the olfactory bulb inside the skull. The neuronal cells of the olfactory epithelium belong to the peripheral nervous system; however, their axons terminate in the olfactory bulb, which is an extension of the forebrain and part of the CNS. Thus, olfactory neurons connect the nasal cavity directly to the brain. Therefore, the nose and the nasal mucosa are an interesting route for nanomedicines-mediated delivery of drugs as they potentially provide access to the systemic circulation but also directly to the brain.

12.2.2.4.1 Nasal Delivery of Nanoparticles

The respiratory mucosa of the nose has the same potential advantages for delivery (e.g. no first-pass metabolism), but also the same barriers as the other conducting airways (trachea, bronchi). However, one key difference stems from the difference in deposition related to the size and density of nanoparticles. The oral and nasal cavity are the sites in which either very large or very small particles are preferentially deposited, whereas intermediate-sized particles are required to achieve deposition in the upper airways and the alveolar region of the lungs (Oberdörster et al. 2005). The respiratory mucosa is rich in lymphatic cells and relatively little is known about the role of the NALT, nasal M-cells, and lymphatic cells in particle transport. However, it has been suggested that some observations showing a lack of nanoparticle translocation in in vitro experiments but transport in the in vivo situation could be explained by the absence of the lymphatic cells in the in vitro setting (Illum 2007). The amount of particles reporting to have translocated from the respiratory mucosa into the blood stream are quite low, e.g. 1–3 % for 500 nm polystyrene particles in rabbit and rat.

The brain represents a part of the body that is particularly well protected by barriers, namely the blood brain barrier (BBB) which is practically impenetrable to most molecules with a MW >500 (see below). However, historical evidence suggests a direct route from the nose to the brain exists, which circumvents this barrier. This is thought to predominately occur via the olfactory nerves. For example, long-term exposure to dust particles in mine workers has been linked to the presence of particles in the brain. Furthermore, the infection of the brain by neurotropic viruses, i.e. intranasal instillation of 30 nm polio virus in monkeys (but also meningitis and herpes infections in humans) also supports the notion of a direct route for nanoparticles from the nose to the brain (Oberdörster et al. 2005). The speed of this process estimated to be 2.4 mm/h is also in agreement with the velocity observed for axonal transport of other particles. Transport along this pathway has also been demonstrated for colloidal gold (50 nm) and MnO_2 (30 nm). Other observations showing a lack of transport for much larger (>1 μm) particles can be explained by the fact that the individual axons and interaxonal spaces are less than 200 nm in diameter. In addition, it has been shown that 20–200 nm particles can reach the brain via the sensory nerves feeding into the trigeminus nerve (Oberdörster et al. 2005).

While there is evidence of nose-to-brain direct nanoparticle transport, it is important to note that this route is severely restricted in terms of capacity due to the small volume/surface area of the nasal cavity, the small area ratio of olfactory mucosa to nasal mucosa, and the limitations of low dose and short retention time.

Access to the neuronal network can only occur after the mucus barrier has been overcome. Similar to other epithelia there is evidence suggesting that chitosan-based polymers may increase interaction with the mucus, whereas PEG modifications may facilitate passage through the mucus. Furthermore, ligands such as lectins or wheat germ agglutinin may increase the ability of nanoparticles to trigger endocytotic uptake (and potential transcytosis) similar to other epithelia (Mistry et al. 2009).

In summary, there is a significant body of work demonstrating an increase of drug bioavailability, either to the systemic circulation or to the brain via the nose. Similarly, there is evidence of nose-to-brain transport of mostly inorganic and solid nanoparticles. However, to date there is relatively little evidence clearly linking these observations to the mechanism of transport of nanoparticles across the barrier rather than a supportive role of the nanoparticles, e.g. in increasing drug transport by protection from degradation, retention in close proximity to barrier, etc.

12.2.2.5 The Vagina

The vagina is a fibromuscular tube, 7–10 cm in length, leading from the vulva to the cervix. At the top of the vagina the external part of the cervix (ectocervix) protrudes into the vaginal cavity. The inside of the vagina has a number of longitudinal folds (rugae). Its wall is formed by the epithelial lining, a smooth muscle layer, and the connective tissue layer of the tunica adventitia. From the opening towards the cervix the epithelial lining changes from a keratinised to a non-keratinised stratified epithelium. The epithelium of the ectocervix is similar to that of the vagina; in the cervix canal (endocervix), a simple columnar cell layer containing numerous glands forms the epithelium. The epithelium at the transition between endo- and ectocervix can undergo physiological changes between stratified and columnar type of epithelium (metaplasia) to adapt to the environment. The vaginal mucosa is an important site for the interaction of the immune system and the environment and is rich in immune cells that carry out defence and surveillance functions. Interestingly, recent research has revealed that the top layers of the stratified squamous epithelium of the ectocervix and vagina are unique. While the lower layers of this epithelium have very well-developed ‘seals’, the upper layers lack tight cell-cell connections, i.e. tight junctions (Blaskewicz et al. 2011). This allows penetration of IgG and potentially of other large molecules/particles into the top epithelial layers and could affect immune modulation, infection (e.g. HIV), or particle/drug delivery. However, the epithelial cells are rich in peptidase activity which may facilitate rapid degradation of material which has reached deeper layers.

The vaginal epithelium does not contain mucus-producing cell or glands. Instead, it is covered by vaginal fluid, a combination predominantly containing cervical mucus secreted from the glands in the endocervix and transudate (clear extracellular fluid low in protein and cells) produced by the vaginal epithelium. It can also contain, to a varying degree, sloughed off cells and cell debris, and mucus produced by glands at the entrance to the vagina (Bartholdi and Skene’s). The vaginal fluid is rich in enzymes (lysozyme, defensine, and other antimicrobial peptides), but also contains some plasma proteins and nutrients, e.g. lipids and glycogen as a consequence of breakdown of cells. Together these support the growth of a specific vaginal bacterial flora which is particularly rich in lactobacilli that help to maintain the acidic milieu of the vagina (pH 3.5–5) by metabolising glycogen to lactic acid.

One of the most remarkable properties of the vaginal environment is its adaptation in response to the hormonal changes of the menstrual cycle or menarche/menopause.

For example, the thickness of the vaginal epithelium changes between 200 and 500 μm, depending on the hormonal status, i.e. being thicker in response to high levels of oestrogen and showing involution/atrophy, e.g. in the menopause. Similarly, the amounts and consistency of mucus/vaginal fluid change, e.g. leading to a reduced viscosity of the cervical mucus to allow passage of sperm during ovulation. Other factors that can dramatically change the environment of the vagina include menstruation, intercourse (increased lubrication from transudate, increased pH from sperm), or disease (e.g. *T. vaginalis*).

12.2.2.5.1 Vaginal Delivery of Nanoparticles

The vagina is an important site for the local delivery of drugs, e.g. antibiotics, and can also allow systemic delivery of suitable low MW drugs. As a site for nanoparticle delivery the vagina thus has potential advantages in terms of avoidance of first-pass effects, good blood supply, and richness in immune cells. However, there are also considerable challenges, most of which are shared with other surfaces of the body. In addition, there are specific conditions that may require consideration, e.g. the relative ease of access is balanced by the challenges related to achieving long-term retention. The vaginal mucus is relatively porous (Lai et al. 2010), but the environment on the other hand has a low pH and is rich in enzymes.

While most applications of nanoparticles serve to enhance the delivery of free drug, a number of studies now suggest that delivery of particles may be possible. For example, a derivative of the relatively small RANTES/CCL5 protein able to interfere with HIV infection was delivered to the basal layers of the exocervical epithelia ex vivo using PLGA particles (Ham et al. 2009). Fluorescent 200 nm PLGA particles were observed up to 75 μm below the surface of the epithelium after 24 h (Woodrow et al. 2009). These particles, when loaded with siRNA and administered in mice, were able to induce gene silencing throughout the reproductive tract for a duration of 14 days. PLGA particles surface-modified with PEG to minimise mucus interactions were found to be superior in their ability to reach epithelial cells and penetrate to the submucosa 6 h after administration into the vagina in mice (Cu et al. 2011).

12.2.3 The Eye

The eye is a complex organ covered by the eyelids and sitting in the bony eye socket. It is surrounded by its appendages such as tear glands as well as connective tissue. Inside the eyeball or globe (diameter ~24 mm), the lens separates the front of the eye (anterior chamber) containing the aqueous humour from the back (posterior chamber), which is mostly filled with the vitreous humour (Tasman 2012).

The eyelids and the eyeball are connected by the conjunctiva, a non-keratinised stratified squamous epithelium. Starting at the rim of the eyelids, it covers the inside of the eyelids (palpebral conjunctiva), then forms deep pockets (fornix) at the top

and bottom of the eyeball, and continues to attach to the sclera ('white of the eye') through the bulbar conjunctiva, ending in a rim (limbus). The conjunctiva contains high numbers of mucus-producing goblet cells around the fornix and, to a lesser extent, at its periphery (limbus, lid margin).

The eyeball itself is made of three concentric layers, the sclera, the uveal layer, and the retina. Each of these layers takes a somewhat different form at the front and back of the eye. For example, the outermost layer of the eyeball is formed by the sclera or 'white of the eye'. This tough, fibrous membrane contains collagen and elastic connective tissue. However, at the centre of the front of the eye the sclera transitions into the transparent cornea. The second, or uveal layer of the eye is a pigmented vascular layer. At the front of the eye it forms the iris and ciliary body and at the back of the eye the choroid membrane. The innermost layer of the eye is formed by the complex-layered light-sensitive retina which together with the optical nerve is actually a part of the CNS and only covers the back of the eye.

The cornea forms a dome-shaped cover over the anterior chamber, iris and pupil. The cornea is rich in sensory nerve fibres, but lacks blood vessels and immune cells. It has a five-layered architecture: a thin non-keratinized stratified squamous epithelium with cells connected by tight junctions sits on the collagen-rich Bowman's layer/membrane (Urtti 2006). Underneath, the corneal stroma is formed from around 200 layers of collagen interspersed with a few keratocytes. With a thickness of around 500 μm it accounts for around 90 % of the cornea. The transparency is achieved through the highly regular arrangement of the very thin (<200 nm) collagen fibrils (Knupp et al. 2009). On the inside, the cornea is lined by a non-regenerating endothelium (different from vascular endothelium below) formed by simple squamous to cuboid cells with a hexagonal shape on a modified basement membrane ('Descemet's membrane'). These mitochondria-rich cells have microvilli and regulate the transport of nutrients into the avascular stroma. They are also pumping back out excess water from the stroma which would otherwise interfere with the cornea's transparency. The cornea also is an immune privileged tissue, separated form the normal immune system.

The iris forms a mobile diaphragm that separates the front and back of the eye. In the centre the iris leaves a hole, the pupil, whose diameter can be adjusted from 1.5 to 8 mm by the dilator and sphincter muscles that together with nerves, blood vessels, and connective tissue are contained in the body of the iris. The level of pigmentation of the anterior surface determines the apparent colour of the eyes. At the periphery the iris transitions into the ciliary body.

The ciliary body is of a triangular shape and contains the muscles used during the adjustment of focal length of the eye, accommodation. The muscles are connected to the lens via fibres that run from small ciliary processes to the equator of the lens. The surface layers of the ciliary body play a central role in managing the production and resorption of the aqueous humour and the formation of the vitreous mucopolysaccharide. The fluid produced by the cells on the posterior side of the ciliary body flows via the smaller posterior chamber (volume 60 μL) through the pupil into the anterior chamber (volume 250 μL) where it will be absorbed into the Schlemm's canal. This canal can be found at the periphery of the anterior chamber where the

ciliary body forms a triangular junction with the sclera/cornea. Here a trabecular meshwork stretches between these surfaces acting like a sieve to limit access of large particulate matter to the collector channels that flow into the Schlemm's canal. The canal has a flattened elliptical cross section (15 × 300 μm) and channels the aqueous humour via collector veins into the venous network of the sclera.

Directly behind the iris sits the elliptical lens (~10 × 4 mm, depending on accommodation and age) held in place by the ciliary fibres inserting at its equator. At its back the lens rests in an indentation in the vitreous to which it is lightly connected. The lens is enclosed into an outer elastic capsule formed by a thick basement membrane. The lens body is made from the lens fibre cells that are long and transparent (12 × 4 μm) and organised in a complex pattern of concentric layers. With ions and nutrients entering the lens from the aqueous humour to supply the lens fibres, the simple epithelial cells directly underneath the capsule actively maintain the osmolarity of the fluid in the lens body.

The back of the eye, between lens and retina, is filled with the vitreous, a clear gel-like material consisting of 99 % of water in a matrix of fibres and hyaluronic acid. This watery vitreous is surrounded by a 100 μm-thick layer of denser *cortical* vitreous which also contains collagen fibrils and cells. The vitreous tightly fills the back of the eye pressing against the choroid membrane and thus the underlying retina which it helps to hold in place. A small channel, the hyaloid or Coquet's canal, exists which connects the anterior part of the vitreous with the posterior part and which is thought to allow some fluid movement to allow the shape changes of the lens during accommodation.

Finally, the back of the eye directly adjacent to the vitreous consists of the choroid layer and the retina. The choroid, a thin (~0.2 mm) pigmented and highly vascular tissue layer, is a continuation of the iris and ciliary body. The retina forms the innermost layer, lightly attached to the choroid via the retinal pigment epithelium. This layer is also responsible for the regulation of transport of nutrients and metabolites through the blood-retinal barrier to the rod and cone layer in which the light is detected.

Tears and the tear film are one of the unique features of the eye (Paulsen 2006; Tiffany 2003). As the cornea is an avascular tissue, one of the key functions of tears is to carry nutrients to the cornea and carry away metabolic waste products. The tear film has three functional layers that are produced separately: The conjunctival goblet cells produce the mucins that are in direct contact with the epithelial cells and cornea. Although mucus is only a minor component (<100 μg/mL) of the tear film and the viscosity is markedly lower than that of other mucus, it does have the typical shear-thinning properties that facilitate excellent lubrication (Lai et al. 2009). The tear or lacrimal gland that produces the majority of the tear fluid by volume is situated towards the outside margin of the upper eyelid. The gland contains many lobes that are connected by ducts that join to form a number of excretory ducts from which their secretion are released into the fornix (upper recess) of the conjunctiva. This aqueous tear fluid is variable in volume and consistency, and production can be adjusted rapidly, e.g. when crying or to wash away foreign material. In addition to water, the fluid contains electrolytes, small molecules such as glucose, and a number of proteins, the most abundant ones include lysozyme, lactoferrin, lacritin,

lipocalin, and IgAs. Finally, the tear film is covered by a lipid layer produced by the 25–50 Meibomian or tarsal glands in the lower and upper eye lids, respectively. The secretions are unique, containing mainly apolar lipids (nonpolar lipids of wax ester, cholesteryl ester, and triacylglycerides) but also proteins.

The presence of the lipid film is considered important in order to reduce evaporation of the tear film, as well as to regulate tear film surface tension to avoid it flowing off. The blink rate and the pressure applied to the eye/eye lids, e.g. by squinting, can vary tear fluid volume as well as composition. The basal tear production is sufficient to create a constant flow (~1 μL/min) of tears which flows from the outside of the eye. There it forms the lacrimal lake, a fluid pool (~10–20 μL) formed in the corner of the eye from where it is then drained by capillary action through the opening of the lacrimal duct into the nose. The tear fluid once produced is spread over the conjunctiva and cornea by blinking. The tear film improves the optical properties of the cornea by smoothing out irregularities; it also provides a constant stream of nutrients and functional, e.g. anti-microbiotic, proteins to the surface of the eye. The tear flow and blinking action constantly wash any debris and particulate matter off the surface of the eye.

12.2.3.1 Nanoparticle Delivery to the Eye

While the eye is connected to the systemic circulation and, via the optical nerve, to the brain, the complex nature of its barriers and relatively small surface areas involved mean that in practice delivery to the eye is focused on treating local conditions. Due to the nature of barriers and fluid flow, in the eye delivery is often considered separately for the front segment (conjunctiva, sclera, cornea, anterior chamber, front of iris/ciliary body) and back segment (back of iris/ciliary body, lens, vitreous, choroid, retina) of the eye (Urtti 2006). For suitable drugs the front of the eye is in principle accessible via topical administration. By contrast, the back of the eye is thought to require invasive access, e.g. via intravitreal injection, or subconjunctival or peri-ocular depot. Alternatively some drugs may be able to reach the site after high-dose systemic administration.

One of the first challenges to topical delivery to the eye is the difficulty of retaining material on the surface of the eye in the presence of the tear film. In addition, any excess non-viscous fluids administered are drained via the lacrimal duct within a few minutes. Furthermore, rapid absorption into the conjunctival capillary bed or nasal cavity after drainage can reduce the amount of drug in contact with the eye surface rapidly.

The cornea is the main route of drug transport to the aqueous humour and thus front of the eye. As the cells of the cornea are connected by tight junctions, paracellular transport is dramatically reduced in favour of transport of lipidic compounds. Typically suitable lipidic drugs will reach the inside of the eye around 20–30 min after applications with peak concentrations of around $1/10^{th}$ to $1/100^{th}$ of what was originally applied. More than 95 % of the material will instead be drained away to the nose with the tear fluid.

The conjunctiva has a substantially higher surface area (20×) than the cornea and is also much more permeable, in particular for larger and more hydrophilic molecules. Permeation of the conjunctiva and scleral layers would in principle also provide access to the eye. However, the good vascularisation of the conjunctiva (and uvea/choroid towards the back of the eye) makes it likely that it would in fact act as a significant absorptive sink and would reduce the amount of material that could be transported beyond this layer. For this route the sclera provides an additional challenging barrier due to its dense fibrous nature. Once the drug has crossed the barrier to reach the anterior chamber, access to and uptake by iris and ciliary body are relatively easy. In contrast, access to the back of the eye is hampered by the direction of fluid flow (back to front), high viscosity vitreous and the denser tissues involved (e.g. lens).

These barriers also need to be considered with invasive administration routes such as peri-ocular depots. Treatment of conditions at the back of the eye thus will typically require intraocular injections. Alternatively, systemic drug administration at high doses can reach the blood vessels at the back of the eye. Transport from there to the retina is, however, a specialised endothelial barrier, the blood-retina barrier (see below).

Nanoparticles do play a role in the development of formulations for topical administration or depot injections. However, typically the rationale here tends to focus on drug solubility and enhanced local drug concentration to increase transport by passive diffusion. Ensuring extended residence times/minimal washout, e.g. by exploiting bioadhesion, is also well-understood strategy for nano-enhanced delivery to the eye. To date, there is little evidence of active transport mechanisms being involved in uptake process or for nanoparticle being able to overcome the barriers of the intact corneal epithelium.

12.3 Endothelial Barriers

The blood circulation serves as a central transport system that connects all organs to allow exchange of materials and information through molecules and cells. Therapeutic molecules and particles that are administered or taken up at one site (e.g. the gut), but need to act on a remote site, need therefore also to be compatible with this specific environment and its potential barrier properties.

The cardiovascular system is a complex organ consisting of the blood with a volume of more than 5 L in humans, the heart and the blood vessels. In the blood circulation nanoparticles are exposed to a large number of circulating cells, proteins, macromolecules, etc. with which they are likely to interact in some form. Nanoparticles are also likely to interact with the blood vessels while being carried in the blood, but may also extravasate (leave the blood vessels) to gain access to the interstitium and cells of a target organ.

Depending on their specific function, blood vessels differ in the level of anatomical complexity; typically large blood vessels and arterial vessels carrying blood under

pressure from the heart have multiple layers of muscle and tissue to provide stability and withstand pressure. These larger conduit vessels (0.02–12.5 mm in diameter) carry the blood to (arteries) and from (veins) the peripheral tissue with high speeds of ~40 cm/s. While these vessels are the ones most visible, they only account for a minute fraction of the total blood vessel cross section. A much larger cross section (around 4,000× more) is represented by the capillaries which actually distribute the blood in the various tissues. The length of this meshwork of tiny vessels has been estimated to be more than 60,000 miles covered by 60 trillion individual endothelial cells (Aird 2005).

The interactions of the nanoparticles with this barrier, i.e. their relative stability against modification, e.g. by adsorption of blood components or adsorption to the vessel wall, will directly affect the chances of carriers reaching their target site (Aggarwal et al. 2009). One significant barrier here is represented by the highly efficient uptake of circulating nanoparticles such as liposomes by macrophages, e.g. in the liver and spleen, which severely limits the ability of such particles to reach other sites in significant amounts. The extent of this effect will depend on the size and surface characteristics of the particles. Strategies such as surface modification with hydrophilic polymers have been developed to minimise these effect (Aggarwal et al. 2009). Binding of a large variety of proteins leads to rapid uptake by macrophages, which can be minimised by modification of particles with hydrophilic polymer surfaces (e.g. poly(ethylene glycol)—PEG). Other particle surface modifications may facilitate binding of particular proteins that can in fact re-target the particles. This effect has, for example, been described for polysorbate-coated particles which enhance transport across the BBB (below).

Typically only small molecules and electrolytes will be able to freely move from the capillaries into the interstitium of the adjacent tissue. About 90 % of the volume that has reached the interstitium will be absorbed back into the circulation (venules), while 10 % of the volume will eventually reach the lymphatics. The retention of larger molecules such as proteins within the blood vessels is in fact critical for the hydrodynamic balance as these molecules exert oncotic pressure which helps to retain fluid in the blood vessels and thus helps to maintain the correct intravascular pressure. Enhanced vascular permeability will cause fluid imbalances and can, for example, lead to oedemas.

Nanoparticles are not normally able to leave the vascular bed of the conducting vessels or smaller vessels in most organs as the endothelial cells provide a continuous barrier (Debbage and Thurner 2010). Exemptions to this rule include the observation of an enhanced permeation and retention (EPR) effect in solid tumours (Maeda et al. 2013). The effect is based on the fact that newly formed tumour blood vessels do have various defects and therefore become ‘leaky’, allowing extravasation and subsequent accumulation of macromolecules and nanoparticles in the tumour.

In some areas of the body the normal barrier function of the endothelial cells is enhanced further, effectively creating compartments which are particularly well protected, e.g. from xenobiotics, and immunologically isolated from the rest of the body and the systemic circulation.

Examples of these privileged sites include the brain with the blood brain barrier and the blood–cerebrospinal fluid barrier, the eye with a barrier between the blood and the aqueous humour and one from the blood to the retina, and also the blood–epididymis barrier in the testes. The BBB is an excellent example of the underlying mechanisms and because of its therapeutic importance will be discussed in more detail.

12.3.1 The Blood Brain Barrier (BBB)

At 1.4 kg the brain makes up only 2 % of our body by weight. Nevertheless it consumes around 20 % off the total energy our bodies utilise at rest. Consequently, with a vascular surface area of more than 10 m^2, the brain is very well vascularised and its blood flow accounts for around 15 % of the heart output (Abbott et al. 2010). Anatomically the brain and CNS are well protected and isolated by layers of bone and tissue to avoid injury. However, the brain is also separated from the rest of the body, in particular the systemic blood circulation, by the BBB. The BBB acts as a selective barrier that protects the brain from exogenous compounds and pathogens, facilitates transport of nutrients and selected endogenous molecules, and regulates the composition of the extracellular fluid in the brain. The BBB acts on the principle of minimising all forms of transport across the barrier, while at the same time providing regulated transport mechanisms for selected compounds that do need to be shuttled across. In practical terms this means that transport of all compounds apart from low molecular weight (MW $\leq$500 Da) hydrophobic compounds is effectively blocked—unless they can utilise some of the selective transport mechanisms active at the BBB, e.g. for nutrients or specific proteins.

Specifically, the BBB thus involves an extremely tight initial barrier in the form of the blood vessel endothelial cells, followed by a second barrier consisting of multiple highly efficient efflux transporters in place to rapidly pump back out any substrate compounds that have crossed the barrier.

The endothelial cells forming the BBB sit on a 30–40 nm thick dense basal lamina. On their abluminal side they are surrounded by pericytes and the astrocyte end foot processes which together sheath the cerebral capillaries.

These endothelial cells differ from typical endothelial cells because they lack any pores/fenestrations and are linked to each other through extensive and well-developed tight junctions connecting the adjacent cells. This creates a continuous membrane barrier that effectively stops any paracellular transport. The cells involved also have a very low basal pinocytotic activity and high levels of mitochondrial activity.

Where the blood vessels are adjacent to the brain's internal fluid spaces (circumventricular organs), the BBB is replaced by a different but similarly structure, the blood–cerebrospinal fluid barrier (BCSFB) which separates the blood and the cells of the choroid plexus that produce the cerebrospinal fluid. The CSF also surrounds the outside of the brain which is covered by the arachnoid membrane and separated

from the blood by the arachnoid barrier. In practical terms the quality of this barriers is comparatively less crucial as it only represents 0.5 % of the area of the BBB.

12.3.1.1 Nanoparticle Delivery Across the Blood Brain Barrier

While the excellent barrier properties of the BBB effectively keep the brain separate from the systemic circulation, an extensive level of exchange across the BBB is required to maintain brain homeostasis. Nutrients and metabolites need to be exchanged across the BBB and a number of mechanisms are in place to allow this to happen for specific types of molecules. The transport processes that do exist at the BBB can be categorised into the following types:

(a) Transcellular transport for lipophilic, low molecular weight compounds
(b) Carrier-mediated processes for sugars, organic ions, nucleosides, and amino acids
(c) Receptor-mediated endocytosis for insulin, iron, and lipids
(d) Absorptive endocytosis suitable for some cationic molecules

While some strategies for overcoming the BBB involve a temporary disruption of the tight junction, most nanoparticle-based strategies typically aim to exploit one of the physiological transport mechanisms to enhance uptake of the nanoparticle and/or its associated cargo.

The rate of transport of any drug carried across the BBB not associated with a nanoparticle will be enhanced by measures that increase the local drug concentration. Mechanistically some approaches may therefore not even require direct interaction with the BBB: although the BBB is a highly efficient barrier, some drugs, in particular low MW lipophilic compounds, show a limited intrinsic permeability. The concentration of such drugs found in the brain therefore corresponds to the level of drug found in the blood. Any nanoparticle formulation that increases the drug concentration at the BBB and increases the duration of the exposure is likely to enhance transport across the BBB. This could be the effect of increases to the drug half-life, e.g. by protection from degradation, reduction of protein binding, and any favourable effects on pharmacokinetics which sometimes are reported to improve BBB transport despite any lack of direct interaction of a carrier with the BBB.

This effect of increased local concentrations can be enhanced further by binding to the brain endothelial cells as has been shown for some types of nanoparticles. This binding can be based on non-specific interactions (Lalatsa et al. 2012) or could be due to targeting of carriers with ligands for receptors found at the BBB. Some non-targeting particles have also been shown to acquire targeting proteins by adsorption from the plasma (Kreuter et al. 2003). Some of these receptors are involved in endocytotic uptake of materials and transcytosis and have been shown to be able to carry associated nanoparticles across the BBB. The most frequently studied receptors in this context include the transferrin receptors (TfR), insulin receptors (INSR), leptin receptors, insulin-like growth factor receptors, the

low-density lipoprotein receptor-related protein (LRP) family [including low-density lipoprotein—LDL, very LDL, LRP1, megalin, and apoE-receptor 2 (LRP8)]. A recent study demonstrated the principle that effective endocytotic transport across the BBB not only requires efficient binding to the target receptor, but also relies on subsequent transcytosis (Yu et al. 2011). The high affinity binding to the receptor observed with some antibodies can effectively abrogate downstream transcytosis. Antibodies with an attenuated affinity may in fact lead to more effective transport across such barriers. Overall, the transport capacity of these receptors is determined by number of factors such as the receptor number, activity and turnover, as well as ligand affinity, and the concentration of potentially competing endogenous ligand in the blood.

12.4 Conclusions

This article has summarised the most important biological barriers to drug and, in particular, nanoparticle uptake and transport. The key anatomical and histological features specific to each of these barriers have been illustrated. While the barriers differ in many of their specific features, they also share some underlying principles and mechanisms. Animals have evolved complex bodies which are organised into organs and are compartmentalised in a number of ways. In order to allow these compartments to function independent of each other and also independent from the environment, barriers have evolved that limit exchange of molecules and particulate materials. This is achieved through an optimised histological architecture that has evolved to maximise the barrier properties, i.e. by linking adjacent cells together through a network of tight junctions. In addition, cells can express various pumps to pump out any molecules that may have entered cells, e.g. after initial diffusion into the cell membrane. However, as ultimately the organs also have to fulfil the requirements of the whole organism, and do so appropriately and in response to the environmental conditions, a range of mechanisms exist to allow regulated exchange of selected types of molecules.

The mechanisms that allow this to happen typically involve a specific interaction and recognition between a membrane carrier and its substrate molecules which will allow direct cytoplasmic access for smaller molecules. Alternatively, non-specific or receptor-specific membrane interactions, e.g. of macromolecules or particles, can trigger endocytotic uptake and potentially transcytosis using a number of different mechanisms (Conner and Schmid 2003).

These physiological barrier mechanisms also apply to therapeutic drugs, macromolecules, and nanoparticles. Understanding the biological basis of these biological barriers underpins the rational design of nanomedicines, which may then, in turn, be engineered and optimised to overcome the barriers.

Problem Box

Question 1:

What are the key organising principles of biological barriers?

Answer 1:

Biological barriers tend to achieve their key functions of compartmentalisation and controlled exchange using some common design features.

The barrier-forming structures, e.g. cells and their connections with neighbouring cells, are organised so as to produce a mechanical/steric exclusion barrier for all but the smallest molecules. The bulk of the barriers are based on structures that alternate hydrophilic and hydrophobic elements such as cell walls, cytoplasm, and extracellular space to prevent direct diffusion of all hydrophilic or lipophilic molecules; cellular pumps pump out any drug material that does diffuse across. Such effective barriers are then opened up for desirable molecules in a targeted and specific fashion using pumps and receptors.

Can you recognise these design principles in the epithelial and endothelial barriers described in the chapter?

Question 2:

The epithelial barriers are dynamic structures. Why is replacement of the barrier an important aspect of their function?

Answer 2:

Replacement as a strategy for maintenance of barrier integrity can be seen at the level of epithelial cells as well as with respect to the mucus barrier that covers most internal epithelia.

The cells making up the immediate barrier layer are created by the division of stem cells typically located near or on a membrane matrix layer. With each new generation the daughter cells produced by previous divisions are shifted towards the luminal side of the tissue. Having multiple layers of cells allows cells to specialise, e.g. cells near the surface develop characteristics such as keratinisation which are useful for the mechanical stability of the barrier, but which would be incompatible with rapid proliferation required at the base of the tissue. Replacement of the surface cells provides protection against gradual destruction of the barrier.

An additional less effective but much more dynamic barrier is provided by the mucus. The production of mucus creates a mechanical and a diffusion barrier which limits access of particles and molecules to the epithelial barrier itself. Superficial mucus layers are routinely replaced on an on-going basis, e.g. in the gut by sloughing off. Mucus consists predominately of water (>95 %) and only contains relatively small amounts of mucin which in the gut can also be reabsorbed. Furthermore, mucus production (and similarly the mucin containing tear film) can be rapidly increased in response to stimuli or irritants.

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Chapter 13
Active Targeting

Dolores Remedios Serrano Lopez and Aikaterini Lalatsa

Abstract Actively targeted nanomedicines are drug delivery systems based on nanocarriers loaded with a therapeutic and/or imaging agent in which a targeting moiety has been attached onto their surface, with the aim of targeting and interacting with a specific receptor, in order to elicit their effect. Three major components should be carefully considered in order to design an optimal nanomedicine including: the nanocarrier (e.g. liposomes, particles, dendrimers, micelles), the targeting moiety (e.g. proteins, peptides, oligonucleotides, carbohydrates), and the therapeutic and/or imaging agent. Nowadays, promising approaches have been developed, especially in the field of cancer and central nervous system diseases. However, very few active targeted nanomedicines have progressed from the proof-of-concept to clinical trials.

13.1 Active Targeting of Nanomedicines

13.1.1 Principles of Active Targeting of Nanomedicines

Actively targeted nanomedicines are drug delivery systems engineered using nanocarriers loaded with therapeutic and/or imaging agents possessing on their surface a targeting moiety. This targeting moiety can be an antibody or other protein, a carbohydrate, or a nucleic acid sequence in order to allow the nanoparticles to interact specifically with cells possessing the respective receptor. It is important to

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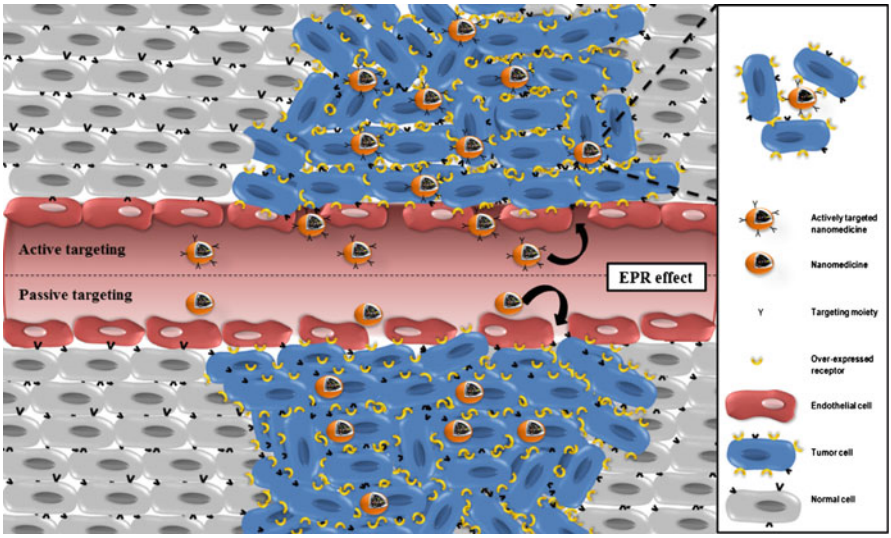

Fig. 13.1 Active and passive targeting of nanomedicines. Nanomedicines can reach tumours selectively using biological mechanisms such as the EPR effect which results from the disorganised pathology of angiogenic tumour vasculature with its discontinuous endothelium, leading to hypermeability to circulating nanoparticles, and the lack of effective tumour lymphatic drainage, which leads to subsequent nanoparticle accumulation. On the other hand, active targeted nanomedicines accumulate in cells (in this case cancer cells) based on the interaction between the targeting moieties grafted at the surface of the nanoparticle that can bind specifically to receptors over-expressed by the cells

distinguish the term "targeted drug delivery" from "targeted therapy" which is commonly used in drug discovery referring to specific interactions between a drug and its receptor at the molecular level (Gerber 2008; Bae and Park 2011). Four key requirements are usually necessary for an effective actively targeted nanomedicine: (a) evasion from opsonisation and clearance by the liver and kidneys allowing sufficient circulation in the organism; (b) retention within intended sites; (c) specific interaction between the targeting moiety of the nanomedicine and its respective receptor, and (d) therapeutic and/or imaging agent release at the targeted site (Mills and Needham 1999; Bae and Park 2011). The major difference with passively targeted nanomedicines is the fact that they make use of biological mechanisms to reach specific organs or disease sites such as phagocytosis by the cells of the reticuloendothelial system (RES) or the enhanced permeation and retention (EPR) effect observed for example in tumours with leaky vasculature (Duncan 2006). Figure 13.1 illustrates the differences between active and passive targeting of nanomedicines.

13.1.2 Targeted Drug Delivery Processes

Apart from paracellular and transcellular transport across epithelial cell membranes, carrier-mediated transport (CMT), receptor-mediated transcytosis (RMT), and

adsorptive-mediated transcytosis (AMT) are important processes upon which the endothelial cells rely to facilitate the entry of essential polar nutrients (such as glucose and amino acids) and other molecules selectively into the cell (Xu et al. 2013).

Cells exhibit a polarised expression of transport (carrier) proteins in the luminal and the abluminal membranes with some transporters expressed exclusively in one of these interfacial membranes and some in the other, whereas some are inserted into both membranes (Di et al. 2012). As some transporters are unidirectional and some bi-directional in their transport of solutes across the cell membrane, this polarisation means that some solutes can be preferentially transported into the cell and some out of the cell in an energy-driven process (Pardridge 2012; Pardridge and Boado 2012). Thus, the transport of some solutes can be facilitated in either direction depending on whether the concentration gradient favours entry into or out of the cell. These transporters (mostly glucose and various amino acid or nucleoside transporters) can be targeted for transport of nanoparticles in order for them to transport the loaded cargo within the cell of interest.

On the other hand, endocytosis is the main route of entry for intact large molecular weight biopharmaceuticals via specific and non-specific mechanisms. Binding of the ligand, which could be attached to nanoparticles, to its specific membrane receptor on the cell surface induces a modification of the receptor protein and triggers an endocytotic event in the luminal membrane most likely involving the formation of a caveolus (clathrin-coated pits visualised with electron microscopy) that triggers the formation of endocytotic vesicles (Brasnjevic et al. 2009; Xu et al. 2013). These endocytotic vesicles fuse with an endosome (pre-lysosomal compartment with acidic pH) and dissociation of the ligand from the receptor takes place, allowing the free receptor to be recycled to the cell surface (Xu et al. 2013). Transferrin receptors are diffusely distributed over the entire plasma membrane and migrate to coated pits only upon binding to their ligand. The low-density lipoprotein (LDL) receptors are predominantly localised at the membrane surface area where coated pits are found (Xu et al. 2013). The ligand containing vesicles can be either exocytosed leading to transport across the cell membrane or fused with a lysosome leading to intracellular degradation. Another intracellular pathway may involve trafficking of endosomes, containing intact receptor-ligand, to their inner saccule of the Golgi complex, where the enzymes can cause dissociation of the ligand from the receptor, and the separated ligand may then be exported in vesicles destined for lysosomal degradation (Xu et al. 2013). Receptor-mediated endocytosis (RME) has been shown for a few peptides and proteins like insulin, transferrin, certain cytokines, and leptin.

Contrary to RMT that involves specific plasma membrane receptors, cationic large molecular weight biomacromolecules or particles can be taken up by the brain via adsorptive-mediated transcytosis (Lalatsa et al. 2012a). AMT requires an excess positive charge on the molecule, which renders it cationic to electrostatically interact with the anionic sites of acid residues of acidic glycoproteins of the cell surface (comprised of type IV collagen, laminin, fibronectin, and heparin sulphate) triggering endocytosis and subsequent transcytosis (Csaba et al. 2006; Lalatsa et al. 2012a). The processes following endocytosis are similar to RMT. However, AMT has a higher capacity for transport compared to RMT, as transport becomes saturated at higher concentrations. Cationised albumin is known to utilise this pathway to gain

entry to the brain. Other molecules involve avidin, histone, cationised polyclonal bovine immunoglobulin, E-2078 (small dynorphin-like basic peptide) and the cell-penetrating peptides HIV transactivator of transcription (TAT) protein (Frankel and Pabo 1988) or other arginine-rich peptides such as SynB5 (RGGRLAYLRRRWAVLGR) and pAnt-(43–58) (RQIKIWFQNRRMKWKK) (Drin et al. 2003; Lalatsa et al. 2012a). The arginine content of these oligomers is a critical factor (Schmidt et al. 2010) and the important structural features of guadinium-rich cell-penetrating vectors (Wender et al. 2008) are now better understood. However, the concentration of cationic peptides in tissues may be limited by the fact that cationic agents are more readily taken up by the liver and kidney, so that the actual mass taken into the tissue is unchanged and is only a fraction of the intravenously injected dose (Lee and Pardridge 2001). Masking the cell-penetrating vector by another oligopeptide, which is designed to be cleaved off at the target tissue by specific extracellular proteases allowing exposure of the cationic vector to promote absorption, is one way to achieve better drug targeting (Jiang et al. 2004). Lipidisation of the cationic polypeptide has also been used as a strategy to enhance transcytosis of a myristoylated polyarginine vector (Pham et al. 2005).

Thus, attaching ligands to nanoparticles that are able to act as substrates for carrier-facilitated diffusion or receptor-mediated transcytosis can allow the transport of the nanoparticles within the cell and can be exploited for specific transport in tissues over-expressing these carrier or receptor-mediated proteins.

13.1.3 Components of Actively Targeted Nanomedicines

Actively targeted nanomedicines are composed of three major components: (a) the targeting ligand/moiety, (b) the nanocarrier system, and (c) the therapeutic (such as gene, radiopharmaceutical, or chemotherapeutics) and/or imaging (optical, fluorophore, radiopharmaceutical, and magnetic) agent (Veiseh et al. 2010) (Fig. 13.2).

Targeting ligands are conjugated either directly to the surface of the nanocarriers or via a linker molecule [such as poly(ethylene glycol)] in order to avoid steric hindrance that can limit the interaction of the ligand to the specific receptor and ensure successful delivery of the therapeutic and/or imaging agent to the target site (Byrne et al. 2008; Danhier et al. 2010). However, in some circumstances, targeting ligands can be conjugated directly to the therapeutics and/or imaging agent too (Allen 2002; Daniels et al. 2012). Carbodiimide-mediated conjugation (based on the formation of amide bonds between carboxylic acid groups of the nanocarrier and primary amine groups of the ligand) is commonly non site-specific (Chapman 2002). However, a site-specific binding can be performed through maleimide-based conjugation chemistries which use native or engineered thiol-containing cysteine residues localised on known positions far from the antigen-binding sites (Chapman et al. 1999).

It is important to consider the characteristics of the targeting ligand per se; the targeting ligand plays a key role in the binding affinity, cell specificity, circulation time, extravasation, and cellular uptake of the nanocarrier systems (Byrne et al. 2008).

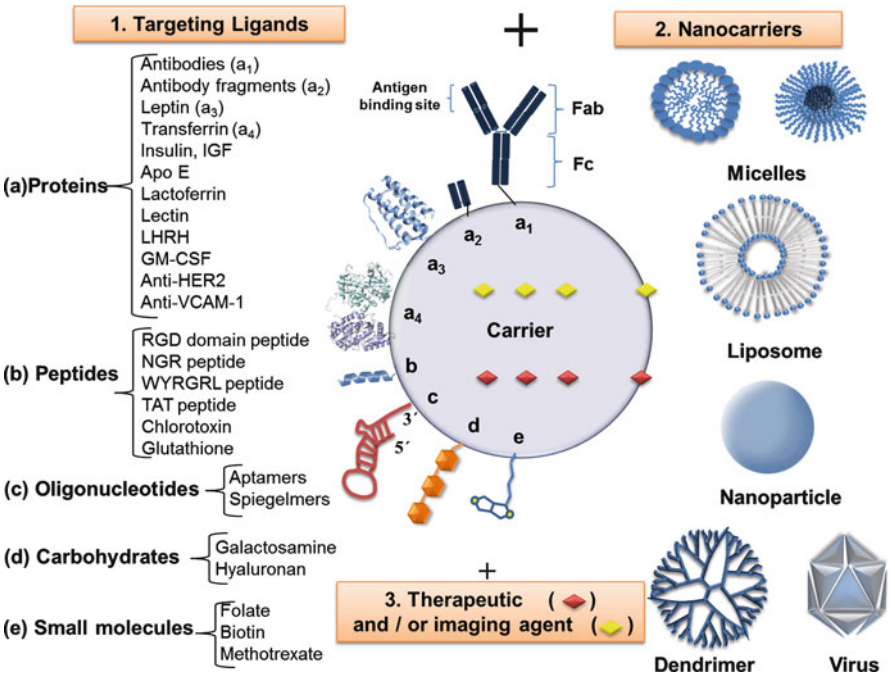

Fig. 13.2 Components of actively targeted-based nanomedicines: (1) Targeting ligands (2) nanocarrier systems, and (3) therapeutic (gene, radiopharmaceutical, or chemotherapeutic) and/or imaging (optical, fluorophore, radiopharmaceutical, and magnetic) agent. The targeting ligands are directly conjugated to the surface of the nanocarrier or through a linker molecule in order to enhance the site-specific delivery of the therapeutic and/or imaging agent. *LHRH* luteinizing hormone-releasing hormone, *IGF* insulin-like growth factor, *GM CSF* granulocyte macrophage colony-stimulating factor, *HER* human epidermal receptor, *VCAM-1* vascular cell adhesion molecule-1, *RGD domain* Arginine-Glycine-Aspartic acid, *NGR* Asparagine-Glycine-Arginine, *WYRGRL* Tryptophan-Tyrosine-Arginine-Glycine-Arginine-Leucine, *TAT* polycationic peptide (Glycine-Arginine-Lysine-Lysine-Arginine-Arginine-Glutamine-Arginie-Arginine-Arginine-Proline-Glutamine)

Other factors pertaining to the successful development of actively targeted nanocarriers involve the purity and biocompatibility of the ligand and the ease of synthesis (conjugation with the drug delivery system) and industrial production (e.g. cost, stability, and scalability) (Swami et al. 2012).

13.1.3.1 Nanocarriers

An increasingly large number of novel therapeutics are poorly soluble drugs; nanocarriers able to encapsulate these therapies are useful delivery technologies to enable the in vivo administration of these therapies via parenteral and non-invasive routes (Danhier et al. 2010). To date most of the actively targeted nanomedicines

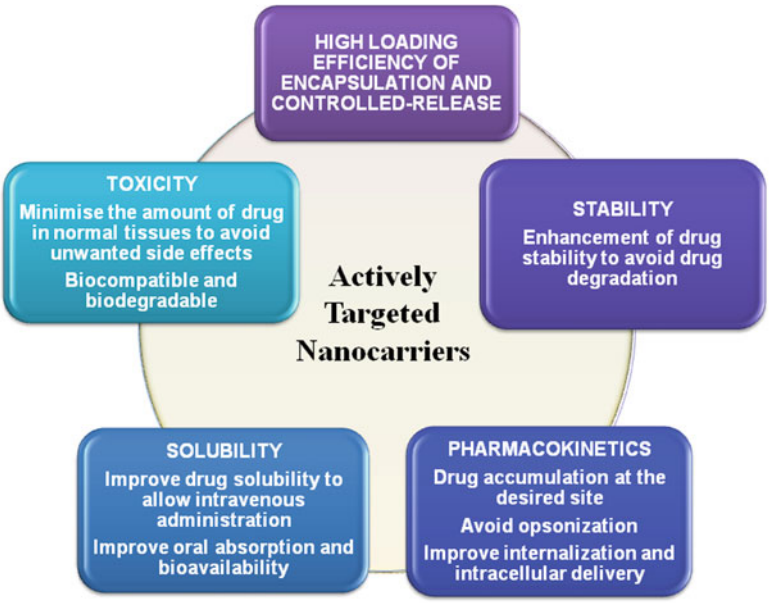

Fig. 13.3 Advantages of actively targeted nanocarriers

are intravenously administered and their advantages are summarised in Fig. 13.3. The primary goal of any actively targeted nanomedicine should be to deliver the majority of therapeutic and/or imaging molecules load at the targeted active site avoiding drug degradation and opsonisation during their circulation in the body. Opsonins are blood proteins that can quickly bind to the surface of the nanocarriers, render them easily recognisable by macrophages of the reticulo-endothelial system, and lead to the nanoparticle being removed from the circulation by phagocytosis before they can elicit their function (Owens and Peppas 2006). To protect the nanocarriers from opsonisation and then phagocytosis by macrophages, the most common method employed relies on coating the nanocarriers with a hydrophilic polymer such as polyethylene glycol (PEG), which would limit the absorption of opsonin proteins on the surface of the nanocarrier by steric repulsion (Owens and Peppas 2006). However, it should be born in mind that the active region of the targeting moiety (especially of small molecules) could be blocked by the coating in which case a linker allowing exposure of the ligand on the surface of the coated particle is essential for a successful strategy. The second advantage of actively targeted nanotechnologies is that they are able to minimise the amount of drug delivered to non-targeted tissues or tissues lacking the specific receptor, while ideally being prepared from biocompatible and biodegradable carriers to avoid undesirable toxicities (Danhier et al. 2010). Additionally, as with other nanoparticulate technologies, the nanocarrier needs to be able to encapsulate the therapeutic and/or imaging agent with a high loading efficiency and to temporally and spatially control the release of the therapeutic (Swami et al. 2012).

Various types of nanocarriers have been used for active targeting, including nanoparticles, micelles, liposomes, dendrimers, and viruses (Fig. 13.2). Nanoparticles are solid and spherical structures where therapeutic and/or imaging agents are encapsulated within the polymeric matrix, ranging in size from 10 nm to 1 μm (colloidal range). However, to minimise uptake by the RES, sizes are preferentially between 20 and 200 nm (Jain 2008; Bader 2012). Liposomes are closed spherical vesicles formed by one or multiple phospholipid bilayers which are surrounding an aqueous core (Danhier et al. 2010) and can present as small unilamellar vesicles (SUVs) of less than 100 nm in size, large unilamellar vesicles (LUVs), or multilamellar vesicles (MLVs) that are larger than 100 nm (Jain 2008). Based on the physicochemical characteristics of the therapeutic and/or imaging molecule, these will be either entrapped in the aqueous core if they are hydrophilic or intercalated into the phospholipid bilayer if they are amphiphilic or hydrophobic molecules. Polymeric micelles are formed through the self-assembly of amphiphilic block co-polymers resulting in core-shell structures able to entrap drugs with low aqueous solubility (Kwon 2003; Lalatsa et al. 2012b). Micelles are characterised either by a hydrophobic core and hydrophilic shell or by a polar core and hydrophobic shell (reverse micelles) (Jones et al. 2008) and are usually smaller than liposomes, ranging around 5–50 nm in size (Reddy and Swarnalatha 2010). Dendrimers are synthetic, branched macromolecules with a controlled three-dimensional architecture based on an initiator core of either an ethylene diamine (EDA) core or ammonia core and multiple layers with active terminal groups (amino groups) on the surface, ranging between 1 and 20 nm in size (Dufes et al. 2005; Paleos et al. 2010). Drug or imaging molecules can be either covalently conjugated to the surface or encapsulated in the core through chemical linkage, hydrophobic interactions, or hydrogen bonds (Lee et al. 2005; Swami et al. 2012). Finally, virus capsid proteins can be potentially useful in tumour targeting gene therapy because of their dual behaviour as carriers and targeting species (Xu et al. 2008; Manosroi et al. 2012). For example, parvovirus particles loaded with different genes (26 nm in size) were shown to be able to bind to receptors over-expressed in a variety of tumour cells, such as the transferrin receptor (Jain 2008).

13.1.3.2 Targeting Moieties

There is a great variety of targeting ligands that can be employed with nanoparticulate actively targeted delivery strategies (Fig. 13.2) and can be divided into five major groups, involving: proteins, peptides, oligonucleotides, carbohydrates, and small molecules.

Proteins

Today, antibodies are one of the most widely used classes of targeting moieties characterised by a broad range of binding affinities and a high degree of specificity for cellular receptors due to the presence of two epitope-binding sites in a single

molecule (Torchilin 2008). However, there are two major challenges regarding the use of antibodies as targeting ligands. First of all, they are complex and large molecules (~150 kDa) which results in necessitating expensive and time-consuming manufacture with some degree of variation from batch to batch and stability problems (Weinberg et al. 2005; Yu et al. 2012). Secondly, the administration of antibodies can cause immunogenicity (Yu et al. 2012). The antibodies are composed of two domains: Fc (fragment crystallisable) and Fab (fragment antigen binding). The Fc domain binds to Fc receptors localised particularly on macrophages, leading to liver and spleen uptake and resulting in an increase in the immunogenicity of the attached particle (Allen 2002). Advances in the field of antibody engineering partly mitigated this problem by the use of humanised or fully human antibody fragments that are less immunogenic (Allen 2002). Antibody fragments such as scFv (single-chain variable fragments) and Fab are smaller in size and lack the Fc domain and the complement system-activating region while keeping their antigen-binding affinity (Carter 2001; Swami et al. 2012) making them attractive alternatives to targeting ligands due to their decreased immunogenicity, ease of identification, and production compared to whole antibodies (Allen 2002). Even though antibodies and antibody fragments exhibit very effective and site-specific binding, new targeting moieties are being developed as antibodies can have lower receptor affinity as a result of conjugation, bind to non-specific Fc receptors, cannot account for possible changes in the antigen or circulating competing free antigen, and have scarce tumour penetration due to their large size (Byrne et al. 2008).

Apart from antibodies, many other proteins have been used as active targeting ligands such as hormones (e.g. luteinizing hormone-releasing hormone (LHRH), insulin, insulin-like growth factor, leptin), glycoproteins (e.g. transferrin, lactoferrin), and lipoproteins (Apo E) (Beduneau et al. 2007; Singh et al. 2011; Taheri et al. 2011; Daniels et al. 2012; Kanwar et al. 2012; Tosi et al. 2012; Wagner et al. 2012). Protein conjugation onto the surface of the nanocarrier can result in loss of the binding affinity as more than one functional groups from the protein can react with the nanocarrier making the conjugation not site-specific (Veiseh et al. 2010). Additionally, non-specific physical interactions (electrostatic, hydrophobic) between the nanoparticles and the protein can result to aggregation and loss of binding affinity (Veiseh et al. 2010). Proteins, as antibodies, can cause reactions similar to those of antibodies.

Peptides

Peptides have shown an increasing targeting potential because of their smaller size, higher stability, lower immunogenicity, and relative ease of large-scale synthesis, which has allowed a decrease in the production cost (Swami et al. 2012; Yu et al. 2012). Moreover, engineered peptides can be synthesised in order to have a single active functional group allowing for site-specific anchoring to the nanocarrier surface and avoiding loss of binding affinity (Hong et al. 2007). New peptide-targeting domains have been discovered that possess high binding activity against different targets such as integrins (RGD domain peptide), collagen (WYRGRL peptide),

or matrix metalloproteinases (MMPs) (NRG peptide) (Danhier et al. 2010; Swami et al. 2012). Glutathione is a tripeptide used as a targeting ligand to enhance drug delivery to the brain (Kannan et al. 1990). Fusion of chlorotoxin, a potent toxin isolated from scorpion venom, to IgG-Fc results in an immunotoxin targeted to glioblastoma cells (Kasai et al. 2012).

Oligonucleotides

Selected nucleic acids known as aptamers are composed of single-stranded DNA or RNA oligonucleotides between 15 and 40 bases which fold into unique 3D conformations exhibiting high affinity and specificity for protein targets and have been used as targeting ligands (Lee et al. 2006; Swami et al. 2012; Yu et al. 2012). Similar to peptides, aptamers have several advantages over antibodies such as better tissue penetration due to their smaller size (15 kDa), lower immunogenicity, and the ability to be chemically synthesised with minimal variations between batch to batch and a faster and easier industrial production through systematic evolution of ligands by exponential enrichment process (SELEX) (Fang and Tan 2010; Swami et al. 2012). Nevertheless, one of the major disadvantages of the aptamers is their rapid blood clearance mainly as a consequence of nuclease degradation (Lee et al. 2006). To overcome their low serum stability, aptamers can be chemically modified with PEG or by incorporation of 2′-fluoro in their backbone (Potti et al. 2004; Nimjee et al. 2005), but alternatively they can be chemically synthesised as spiegelmers (mirror image of natural oligonucleotides) composed of L-oligonucleotides instead of the natural D-oligonucleotides (Eulberg and Klussmann 2003). Spiegelmers can be considered as biostable aptamers (up to 60 h stable in biological fluids) due to L-OLIGONUCLEOTIDES not being good substrates for nucleases (Eulberg and Klussmann 2003).

Pegaptanib (brand name Macugen®) is the first aptamer therapeutic approved for use in humans. It is a pegylated RNA aptamer that binds with high specificity and affinity to extracellular vascular endothelial growth factor ($VEGF_{165}$) which is a protein involved in angiogenesis, vascular permeability, and inflammation processes involved in the progression of age-related macular degeneration disease (EMEA 2006). In particular, $VEGF_{165}$ is the isoform preferentially responsible for the pathological ocular neovascularisation, which leads to vision loss (Ng et al. 2006). Pegaptinib can be directly injected into the vitreous cavity where it will bind to $VEFG_{165}$ inhibiting its function and delaying the progression of the disease (Lee et al. 2006; Ng et al. 2006).

Carbohydrates

Carbohydrates such as lactose, galactose, mannose, and galactosamine interact with lectins (specific carbohydrate-binding proteins) on the surface of cells and thus can be exploited as targeting ligands (Berg et al. 2002). Lectins have been found to

be over-expressed on numerous cancer cells (Minko 2004; Swami et al. 2012). For example, the asialoglycoprotein receptor is only expressed in high density (500,000 receptors/cell) on hepatocytes and can specifically bind to ligands such as galactosamine, which can be used as targeting ligand to target nanocarriers encapsulating chemotherapeutics to liver tumour (Shen et al. 2011; Yu et al. 2012). Hyaluronan (hyaluronic acid) is a biocompatible and biodegradable polysaccharide composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine (Toole 2004) is known to bind specifically to cell-surface receptors (such as CD 44) which are over-expressed on various cancer cells (Choi et al. 2010). Carbohydrates as targeting ligands suffer from low binding affinity to the target receptors necessitating multivalent interactions for efficacy that can be achieved by increasing the targeting ligand density on the surface of the particles (Managit et al. 2003; Swami et al. 2012).

Small Molecules

Apart from proteins, peptides, oligonucleotides, and carbohydrates, many other small molecules with various structures and properties have potential as targeting ligands. Using a small molecule as a targeting ligand possesses advantages such as low molecular weight, low synthetic cost, ease of conjugation to the nanocarriers, and ability to attach multiple ligands to the particle surface easily (Swami et al. 2012). One of the most studied molecules is folic acid (also called folate or vitamin B_6), which is vital in many metabolic processes for cell survival. Folate has a high specificity and binding affinity for the folate receptor (dissociation constant, $K_d = 10^{-10}$ M), which is over-expressed in many types of cancer cells (Ross et al. 1994; Low et al. 2008; Yu et al. 2012). Folate is a versatile ligand and has been successfully conjugated with a variety of nanocarriers (such as liposomes, nanoparticles, dendrimers) in order to develop targeted theranostic agents (therapeutic and diagnostic capabilities in the same system) (Low et al. 2008). Nevertheless, one of the major concerns regarding the use of folates as targeting moieties is the over-expression of folate receptors not only in cancer cells but also in normal tissues such as the placenta and kidneys (Swami et al. 2012). Similarly, methotrexate which is a potent cytotoxic and anti-inflammatory agent closely resembling folate (Phillips et al. 2003) has been used as a ligand with a binding affinity a thousand times higher than folate and is an ideal ligand for targeting the folate receptor and active chemotherapeutic strategies (Goodsell 1999; Thomas et al. 2012). Whereas folic acid has been widely studied, other therapeutic compounds are being developed as potential ligands against over-expressed receptors in targeted cells such as biotin (against breast carcinoma MCF7 and murine lung cancer M109) (Taheri et al. 2011), vitamin B_{12} (Russell-Jones et al. 2004), and riboflavin (Karande et al. 2001). Additionally, high-throughput screening libraries and phage display can yield high affinity ligands for targeting nanocarriers to specific receptors or sites (Weissleder et al. 2005; Kuohung et al. 2010).

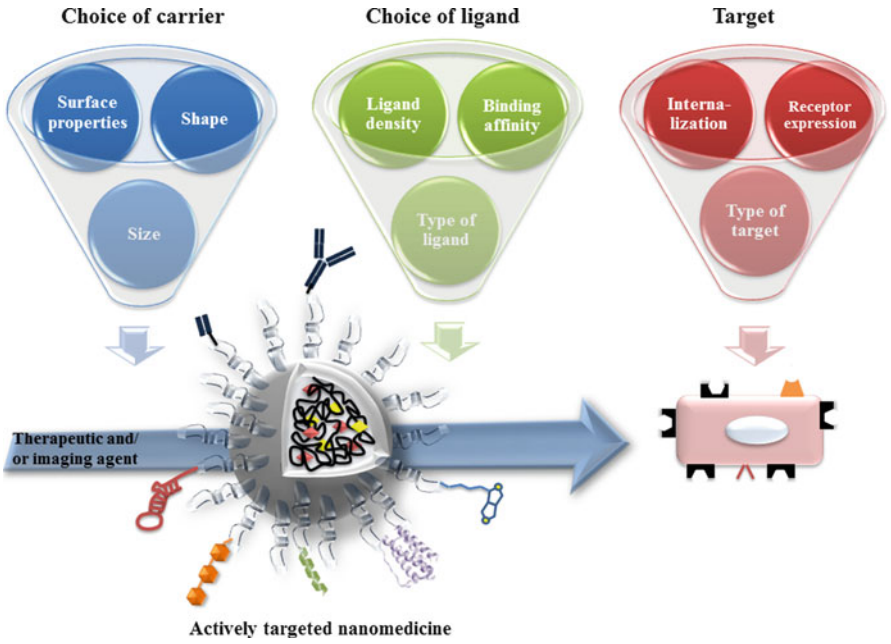

Fig. 13.4 Optimal design of actively targeted nanomedicines. Three important parameters have to be considered in designing actively targeted nanomedicines: (1) the target, (2) the nanocarrier, and (3) the targeting ligand. Based on the characteristics of the target cell (over-expressed receptors, heterogeneity of cells, internalisation of the ligands), the carrier and the ligand will be chosen. The surface properties, the shape, and the hydrodynamic size of the carrier should be considered in order to achieve a suitable pharmaco- and toxicokinetic profiles of the nanomedicine. The specificity of the ligand and the ligand density per particle need to be considered for a suitable binding affinity to the receptor for stability of the system

13.1.4 Optimal Design of Actively Targeted Nanomedicines

Engineering actively targeted nanomedicines requires consideration of the nature of the targeted receptor, the particle characteristics (particle size, zeta potential, surface functional groups, drug loading ability), and the ligand's specificity and affinity (Fig. 13.4).

13.1.4.1 The Target

Target cells should possess a high density of the targeted receptor on their surface ideally, at least several orders of magnitude in more abundance compared to non-targeted cells. Similarly the antigen needs to be over-expressed at the tissue of interest (Allen 2002). The larger the difference in receptor density between targeted and

normal cells is, the higher the specificity and the lower the toxicity of the nanomedicine will be. The target cells should not have a high degree of heterogeneity in relation to their receptor or antigen expression as this can result in the growth of drug-resistance cells (Allen 2002; Wu et al. 2006). Also, the targeted antigen or receptor should not be down-regulated or shed because any circulating shed antigen could bind to the targeted nanomedicine resulting in complexes that would be quickly cleared from the bloodstream (Allen 2002).

An important aspect in relation to the selection of the target involves receptor-mediated internalisation as a result of ligand binding (Allen 2002), especially if drug internalisation is necessary for efficacy. Internalisation of the receptor can be detrimental for certain active strategies such as ADEPT (antibody-directed enzyme prodrug therapy). ADEPT is a strategy designed to overcome the problems of lack of tumour selectivity and involves an antibody designed/developed against a tumour antigen that is linked to an enzyme and injected to the blood, resulting in selective binding of the enzyme in the tumour. When the discrimination between tumour and normal tissue enzyme levels is sufficient, a prodrug is administrated into the blood circulation, which is converted to an active cytotoxic drug by the enzyme, only within the tumour. Selectivity is achieved by the tumour specificity of the antibody and by delaying prodrug administration until there is a large differential between tumour and normal tissue enzyme levels. However, if the antibody-enzyme complex is internalised, no enzyme will be available at the target tumour site to activate the chemotherapeutic prodrug necessary for antitumour efficacy (Senter and Springer 2001).

The type of target cell dictates the pharmacokinetic properties of the actively targeted nanomedicine. Targeting vascular tumour cells (haematological cancers or metastatic cells distributed via the blood circulation) does not require nanosystems with a long circulation half-life as nanoparticles can quickly bind to the target cells after intravenous administration (Allen 2002). However, when solid tumours are the target, it is important that the nanomedicine is able to circulate in the bloodstream long enough to be taken up by the tumour, i.e. long enough to extravasate from the blood vessels and penetrate the solid tumour (Allen 2002); this phenomenon is illustrated by reports of large targeted particles (100–150 nm in size) taking 48 h or longer to reach peak levels in solid tumours (Allen et al. 1998).

13.1.4.2 Choice of Nanocarrier

The hydrodynamic size, shape, and surface properties such as charge and hydrophobicity and the ligand density play a critical role in cellular uptake, clearance, and the biodistribution of the targeted nanomedicine (Chouly et al. 1996).

Nanomedicine particle size has a great influence in distribution of the nanoparticles in the body. Nanomedicines with a particle size smaller than 10 nm can be quickly cleared by the kidneys (Petros and DeSimone 2010), while particles below 100 nm usually experience reduced hepatic filtration and can circulate in the bloodstream for a longer time (Swami et al. 2012). Particles larger than 400 nm have a higher tendency to be cleared by the macrophages of the RES and to be

accumulated in the liver and spleen (Litzinger et al. 1994; Yokoyama 2005). Spherical and small particles have a higher diffusion rate and circulate at the centre of the blood vessel resulting in reduced interactions with the endothelial cells (Decuzzi et al. 2006). Blood vessels have a pore cut-off size between 4 and 25 nm, whereas the pore cut-off size of tumour blood vessel allows extravasation of particles between 380 and 780 nm, which explains the higher accumulation of the nanomedicines within tumour cells than normal tissues (Hobbs et al. 1998; Rippe et al. 2002). Particle size and surface coating is significant for permeation across the blood–brain barrier (BBB) with reports of particles between 40 and 100 nm over-coated with PEG being able to preferentially permeate across the BBB (Nance et al. 2012). Smaller size nanoparticles are able to penetrate easier into solid tumour tissues as long as their size is big enough to escape kidney clearance (Allen 2002), and a size ranging between 10 and 250 nm (Alexis et al. 2008) is deemed optimal compared to smaller (<5–10 nm) and bigger particle sizes (>400 nm), particles that are useful to target the kidney or liver and spleen, respectively.

Surface hydrophobicity and charge are important parameters for nanocarriers as both properties result in shorter blood circulation times due to either particle agglomeration resulting in opsonisation or complement activation and non-specific binding to untargeted cells (for highly cationic particles) (Chouly et al. 1996; Owens and Peppas 2006; Salvador-Morales et al. 2009). Therefore, for a long circulation half-life, actively targeted nanomedicines with minimal non-specific interactions, a hydrophilic surface resulting from surface engineering with a hydrophilic coating such as PEG, chitosan, dextran, and with a zeta potential between −10 and +10 mV, is required (Davis 2009; Swami et al. 2012).

Regarding the shape, its effect on nanomedicine biodistribution has not been completely elucidated yet. To date, it has been suggested that anisotropically (irregular) shaped nanocarriers are able to avoid bioelimination better than spherical systems (Liu et al. 2007). Other studies have shown that the increase in the length-to-width aspect ratio of nanosystems is correlated with an increase in their blood circulation time (Geng et al. 2007; Veiseh et al. 2010).

13.1.4.3 Choice of Targeting Moiety

When selecting the targeting ligand for an actively targeted nanoparticle to a specific receptor, it is important to identify whether a high specificity of an antibody or antibody fragment is necessitated or whether a non-antibody ligand can be used (Table 13.1).

The major advantages of antibodies or antibody fragments are the high degree of specificity for the target cell, the wide range of binding affinities that can be achieved, and the possibility of synergism between the signalling antibodies and the therapeutic agents resulting in a dual and more efficient targeting (Maynard and Georgiou 2000; Baselga 2001; Allen 2002). Non-antibody ligands such as transferrin and folic acid have relatively non-selective expression, thus they can bind to a greater extent to non-target tissues (Allen 2002). The non-antibody ligand used

Table 13.1 Selection of targeting ligands and advantages and disadvantages of using whole antibody, antibody fragment, or non-antibody ligands as targeting moieties

	Whole antibody	Antibody fragment	Non-antibody ligand
Advantages	High degree of specificity	High degree of specificity	Readily available
	Broad range of binding affinities	Broad range of binding affinities	Low-cost manufacture
	Signalling synergism	Signalling synergism	Ease of scale-up synthesis
	Higher binding avidity	Lower immunogenicity than whole antibodies	Minimal variations from batch-to-batch
			Ease of handle
	Antibody and complement-dependent cytotoxicity	Easier identification and production than whole antibodies	Easier conjugation
			Higher stability
			Better tissue penetration
			Low immunogenicity
Disadvantages	High cost manufacture	Lower stability than whole antibodies	Limited selective expression
	Batch-to batch variation	Lower binding avidity	Weak binding affinity
	Time-consuming production		
	Immunogenicity		
	Binding to non-specific Fc receptors		
	Low tumour penetrability		
	Complexity of the conjugation process		

should have sufficient high affinity for the receptor, but be able to allow the cargo to be released into the cell. The endogenous ligand should not compete with the delivery vector for receptor occupancy, thus careful consideration of the relative binding affinities and the physiological levels of the endogenous ligand need to be considered. Transferrin is thought not to be a suitable vector as its plasma concentration is >1,000-fold higher than the K_d of 5.6 nM, although this is not an absolute principle. Ideally the vector should not be pharmacologically active (insulin). The vector conjugate should have a high receptor affinity that can be affected by the linker/spacer strategy used. If the binding affinity is very high, the nanomedicine will bind strongly to the first target molecule encountered and dissociation and further diffusion would not be possible. This might be useful for readily available targets such as in the case of vasculature tumours, but could be detrimental in the therapy of solid tumour that necessitates penetration deeper in the tumour (Adams et al. 2001). Whole antibodies exhibit the highest affinity because they have two binding sites per molecules unlike certain antibody fragments (scFv and Fab), which have only one binding domain. Some non-antibody ligands (such as carbohydrates) show a weaker binding affinity counteracted by several ligands (multivalency) attached on the surface of the carriers (Allen 2002). High ligand density on the surface can increase the binding to target cells at the expense of a higher manufacturing cost and complexity for the proposed targeted nanomedicines due to enhanced possibility of clearance and instability (Lopes de Menezes et al. 1998; Allen 2002).

In spite of the use of humanised or fully human antibodies, immunogenicity remains a problem to be addressed and presents in the form of anti-idiotypic responses, especially with the use of whole antibodies (Dillman 2001). The presence of the Fc domain in the whole antibodies can lead to binding to Fc receptors of normal tissues (particularly macrophages which might increase their immunogenicity), and also to enhancement of their activity because of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (Allen 2002).

One of the major advantages of the non-antibody ligands is the ease of scale-up of their synthesis resulting in low-cost and time-efficient manufacture with minimal variation from batch to batch, and reduction of overall cost and complexity of engineered nanomedicines. Compared to antibodies, the smaller number of active sites for modification present in protein and peptide ligands allows for uncomplicated site-specific conjugation to the nanoparticles (Swami et al. 2012). Other advantages of these targeting moieties are their higher stability upon storage and better tissue penetration, which can be potentially useful in the treatment of solid tumours.

13.1.4.4 The Therapeutic Agent

The last point to consider is the potency of the encapsulated or loaded drug. When the ratio between the ligand to drug is too high, more potent drugs are needed, especially when the drug is conjugated directly to the targeting ligand (e.g. antibody) without a carrier. In this case, each ligand molecule can deliver only a few drug necessitating large amounts of ligand which increases the cost as well as the immunogenicity of the system. However, when the drug is encapsulated in nanocarriers, the number of drug molecules delivered per carrier molecule is exponentially higher, which is important for low potency drugs (Swami et al. 2012).

13.1.5 Examples of Actively Targeting Nanomedicines

13.1.5.1 Focus on Cancer Active Targeting

EPR Effect

Despite the fact that active targeting of nanomedicines results in specific binding to receptors localised on cancer cells, the EPR effect plays a critical role too as it allows accumulation of nanomedicines in solid tumours than in normal tissues (Allen 2002). With the exception of prostate and pancreatic cancers, which are hypovascular tumours, the EPR effect can influence targeting to almost all human cancers (Maeda et al. 2001). Permeation and then extravasation of nanomedicines from the blood vessel to the tumour interstitial space are enhanced because of the unique properties of tumour vasculature such as the lack of tight junctions between adjacent vasculature and endothelial cells (Ruoslahti 2002) and the presence of porous blood vessels with a pore cut off size of 380–780 nm (Hobbs et al. 1998).

At the same time, retention of nanomedicines within solid tumours is enhanced due to the poor lymphatic drainage and slow venous return from the interstitium of the tumour (Maeda et al. 2001; Danhier et al. 2010). However, poor lymphatic drainage can lead to higher osmotic pressures within the tumours resulting in an outflow of fluids which might severely restrict the nanomedicine distribution to some areas of the tumour (Stohrerm et al. 2000).

Cell Proliferation Targeting

One of the advantages of actively targeted nanomedicines is their potential to interact specifically with endocytosis-prone surface receptors (over-expressed by cancer cells) leading to cellular internalisation of the nanomedicine resulting in direct cell death (Kirpotin et al. 2006; Danhier et al. 2010). Table 13.2 summarises several approaches of actively targeted nanomedicines towards (a) membrane bound internalisation-prone receptors and (b) intracellular receptors:

Membrane bound internalisation-prone receptors

1. *Folate receptor*. Folic acid is crucial for the synthesis of purines and pyrimidines and binds to folate receptors being internalised via RME. Because of the high replication rate of cancer cells, the folate receptor is up-regulated compared to normal tissues by up to two orders of magnitude (Low and Antony 2004). The alpha isoform of the folate receptor is over-expressed on ovarian, renal, and pancreatic carcinomas and the beta isoform is over-expressed on malignant haematopoietic cells (Kelemen 2006).
2. *Transferrin receptor*. Transferrin is a glycoprotein that transports iron through the blood into the cells by which it is internalised via RME after binding to transferrin receptors and is expressed in many organs (liver, spleen, lung, and brain). These receptors are over-expressed (up to 100-fold higher) in cancer cells, which makes them an attractive target for cancer therapy (Danhier et al. 2010; Daniels et al. 2012). However, competition of the transferrin-conjugate with high levels of endogenous transferrin limits its use as a vector. The anti-transferrin monoclonal antibody (mAb) (OX26) has also been used as a ligand for nanoparticulate drug delivery (Ulbrich et al. 2009; Zhang and Pardridge 2009).
3. *Epidermal growth factor receptor* (*EGFR*). EGFR belongs to the tyrosine kinase receptor family. It is expressed in epithelial, mesenchymal, and neuronal tissues playing a crucial role in proliferation, differentiation, and development. It is over-expressed in a variety of solid tumours (such as colorectal, lung, ovarian, kidney, pancreatic, brain, and prostate cancer) and its activation is correlated with tumour progression and poor prognosis (Yano et al. 2003).
4. *Lectins and glycoproteins expressed on cell surfaces*. Lectins are glycan-binding proteins which selectively recognise and bind to carbohydrate moieties expressed on cell surfaces and can be differently expressed in cancer cells compared to normal tissues (Clark and Mao 2012). Thus, two strategies can be used, the first involving direct lectin targeting where lectins attached onto the surface of the

Table 13.2 Examples of actively targeted nanomedicines to tumors

Target	Targeting ligand	Nanocarrier (particle size)	Therapeutic agent	Indication (outcome)	Last known status	References
Membrane bound internalisation-prone receptor targeted nanomedicines						
Folate receptor	Folic acid	Dendrimer (<5 nm)	Methotrexate	Human epithelial cancer (tenfold higher efficacy)	Preclinical	Kukowska-Latallo et al. (2005)
		Chitosan-based polymeric micelles (100–200 nm)	10-Hydroxy-camptothecin	Gastric carcinoma, hepatoma, leukaemia, tumour of head and neck (62 % reduction in tumour growth rate)	Preclinical	Zhu et al. (2013)
		PEGylated PLGA-core nanoparticles (~194 nm)	Paclitaxel	Human cervix carcinoma (more than threefold reduction tumour growth and threefold drug accumulation in tumour)	Preclinical	Zhao et al. (2012)
		PEGylated β-cyclodextrin	Rhodamine-B	19 % higher accumulation in KB cells	In vitro	Salmaso et al. (2004)
Transferrin receptor	Transferrin	PEGylated liposomes—*MBP-426* (180 nm)	Oxaliplatin	Metastatic solid tumour, gastric, and gastroesophageal carcinoma (sixfold reduction in tumour growth ratio)	Phase Ib/II	Suzuki et al. (2008) and Mebiopharm Co. (2013)
	Single-chain antibody fragment	Liposome—*SGT 53* (<400 nm)	Plasmid DNA coding for p53 gene for tumour suppression	Solid tumours (increase of transfection efficiency in 70–80 % of the gene encoding)	Phase Ib/II	Camp et al. (2013) and SynerGene Therapeutics (2013)
	Transferrin	Polymeric nanoparticle with cyclodextrins on their surface and adamantane-PEG conjugate—CALAA-01 (~70 nm)	siRNA against ribonucleotide reductase subunit 2	Solid tumours (inhibition of tumour engraftment with antitumour efficacy at the dose of 0.6–1.2 mg siRNA/kg and safer profile without liver toxicity and complement activation)	Phase Ib	Davis (2009) and Calando Pharmaceuticals (2013)

(continued)

Table 13.2 (continued)

Target	Targeting ligand	Nanocarrier (particle size)	Therapeutic agent	Indication (outcome)	Last known status	References
EGFR	GE11 peptide	PLGA-PEG-GE11 construct conjugated to PCL-nanoparticles (120–160 nm)	Paclitaxel/ Lonidamine	Multi-drug resistance cancer (maximal tumour accumulation occurs at 3 h after intravenous administration; fivefold decrease in tumour weight is achieved at 28 days post-treatment)	Preclinical	Milane et al. (2011a, b)
	Anti HER-2 MAb fragment (scFv)	PEGylated liposomes (~100 nm)	Doxorubicin	BT-474/MCF-7 breast cancer (prolonged circulation as stable constructs without drug leakage or MAb dissociation resulting in superior antitumour efficacy with 50 % cure rates)	Preclinical	Kirpotin et al. (1997) and Park et al. (2002)
	Aptamer J18	Gold nanoparticles (~20 nm)	—	A431 epidermoid carcinoma (specific internalisation of the nanoparticles conjugated to EGFR-targeting aptamer via receptor-mediated endocytosis)	In vitro	Li et al. (2010) and Master and Sen Gupta (2012)
Hepatic asialoglycoprotein receptor	Galactosamine	HPMA polymer-drug conjugate—PK2	Doxorubicin	Hepatocellular carcinoma (24 h after intravenous administration, 16.9 % of the administered dose of doxorubicin was targeted to the liver)	Phase I/II	Seymour et al. (2002)
CD44 receptor	Hyaluronan	PEGylated hyaluronic acid nanoparticles with cholanic acid core (~320 nm)	Camptothecin	SCC7 and MDAMB-231 cancer cells (specific internalisation into cancer cells via receptor-mediated endocytosis and low uptake by normal fibroblast resulting in higher accumulation in tumour tissue compared to bare nanoparticles)	Preclinical	Choi et al. (2011)
Intracellular receptor targeting						
Oestrogen receptor	Estrone	Liposome (~200 nm)	Doxorubicin	Breast cancer (13.9-fold higher accumulation in cancer cells)	Preclinical	Rai et al. (2008)

LHRH receptor	Gonadorelin	PEGylated liposome (120–150 nm)	Mitoxantrone	MCF-7 breast cancer (higher affinity and better antitumour efficiency)	In vitro	He et al. (2010)
Angiogenesis targeting						
VEGF	Anti-VEGF MAb (Sc-7269)	Dextran magnetic nanoparticles	^{131}I	Hepatic carcinoma cells (high efficacy in tumour growth delay and safer treatment than non-targeted nanomedicines)	Preclinical	Chen et al. (2006)
VEGFR-2	Anti-VEGFR-2 MAb	Dextran-coated polymerised nanoparticles	^{90}Y	K1735-M2 melanoma cells (significant tumour growth delay and marked decrease in vessel density in tumours)	Preclinical	Li et al. (2004)
	Anti-VEGFR-2 MAb	Gold nanoparticles (~10 nm)	Doxorubicin	NIH-3T3 and BAEC cells (specific intracellular uptake via endocytic mechanism)	In vitro	Das et al. (2011)
Integrins ($\alpha_v\beta_3$)	RGD peptide	PEGylated stearic acid-grafted chitosan micelles (~25 nm)	Doxorubicin	BEL-7402 hepatocellular carcinoma cells (7.2-fold higher cytotoxicity in cancer cells compared to free drug)	In vitro	Cai et al. (2011)
	RGD peptide	Poly(D,L-lactic acid) nanoparticles (~105 nm)	Oridonin	Hepatocarcinoma 22 (fourfold longer retention time and fourfold decrease for tumour volume)	Preclinical	Xu et al. (2012)
	Cyclic RGD peptide	PEGylated liposomes (<100 nm)	Paclitaxel	A549 lung adenocarcinoma (lower tumour microvessel density compared to taxol-treated group)	Preclinical	Meng et al. (2011)
VCAM-1	Anti-VCAM MAb	PEGylated liposomes (~83 nm)	–	Human Colo 677 tumour (effectively targeted to endothelial cells in vivo; 91.7 % and 73.1 % liposomes were co-localised in endothelial cells 30 min and 24 h post-administration, respectively)	Preclinical	Gosk et al. (2008)
MMP-2, MMP-9	Activatable low molecular weight protamine	PEGylated PCL-nanoparticles (~121 nm)	Paclitaxel	Glioblastoma (specific accumulation in tumour cells via lipid raft-mediated endocytosis and energy-dependent macropinocytosis)	Preclinical	Gu et al. (2013)

(continued)

Table 13.2 (continued)

Target	Targeting ligand	Nanocarrier (particle size)	Therapeutic agent	Indication (outcome)	Last known status	References
MT1-MMP	GPLPLR peptide	Liposomes (~191 nm)	Hydrophobised derivative of CNDAC	Colon 26 NL-17 carcinoma (fourfold higher accumulation in tumour than unmodified liposomes and significant tumour growth suppression)	Preclinical	Kondo et al. (2004)
Aminopeptidase N/CD13	NGR peptide	Liposome	Doxorubicin	Neuroblastoma (10 times higher uptake than non-targeted liposomes 24 h post-administration; complete tumour eradication is induced in all animals after metronomic administration)	Preclinical	Pastorino et al. (2003)

Aptamer J18 80-residue aptamer that binds specifically to EGFR, *CNDAC* (1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl) cytosine), *EGFR* epidermal growth factor receptor, *GE11* 12-residue peptide (YHWYGYTPQNVI) with affinity for EGFR, *GPLPLR peptide* stearoyl-Gly-Pro-Leu-Pro-Leu-Arg, *HPMA N*-(2-hydroxypropyl) methacrylamide, *LHRH* luteinizing hormone-releasing hormone, *MAb* monoclonal antibody, *MMPs* matrix metalloproteinases, *NGR peptide* Asn–Gly–Arg peptide, *PEG* polyethylene glycol, *PLGA* poly(D,L-lactide-*co*-glycolide), *PCL* poly(epsilon-caprolactone), *RGD peptide* Arg-Gly-Asp peptide, *scFv* single-chain variable fragments, *siRNA* small interfering RNA, *VCAM-1* vascular cell adhesion molecule-1, *VEGF* vascular endothelial growth factor, *VEGFR* vascular endothelial growth factor receptor, antitumour nucleoside, *Y* yttrium

nanocarriers loaded with the therapeutic agent will target cell-surface carbohydrates or reverse lectin targeting based on carbohydrates moieties grafted at the nanocarriers directed to lectins on the surface of the cancer cells (Minko 2004).

Intracellular receptors:

1. *Oestrogen receptor*. Oestrogen receptors belong to the nuclear hormone family of intracellular receptors. Once receptors are activated by oestrogens, they are translocated into the nucleus and bind to DNA being able to regulate diverse genes (Osborne 1998). Oestrogen receptors are over-expressed up to 60–80 % in breast cancers (oestrogen-positive breast cancers) (Paliwal et al. 2011).
2. *LHRH receptor* (*luteinizing hormone-releasing hormone*). LHRH is a hypothalamic decapeptide known as gonadotropin-releasing hormone (Kakar et al. 2008). The LHRH receptor is an intracellular G-protein-coupled receptor expressed on the nuclear membrane (Re et al. 2010). LHRH receptors are barely expressed in healthy visceral organs, but are over-expressed in many tumours like breast, ovarian, endometrial, prostate, pancreatic, hepatic and colorectal cancers, melanomas, oral, laryngeal and renal cell carcinomas, and nervous system tumours (He et al. 2010).

Angiogenesis-Related Targeting

Solid tumours rely on an adequate blood supply to grow. Tumour and host cells secret different pro-angiogenic factors to develop new blood vessels including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), interleukin-8, platelet-derived growth factor, platelet-derived endothelial cell growth factor, tumour necrosis factor-α, basic fibroblast growth factor, angiogenin, angiotropin, and transforming growth factor-α (Baker and Fidler 2007). Inhibiting the angiogenesis process may regulate the size and the metastatic capabilities of the tumours (Folkman 1996). Moreover, there are other advantages of actively targeting the tumour vasculature, such as: its broad application spectrum because the tumour vasculature is not specific for the type of cancer, lower risk of drug resistance due to the fact that endothelial cells are more genetically stable than cancer cells, and the fact that the nanomedicines could bind directly to their receptors without being required to extravasate and penetrate within the solid tumour (Kumar and Li 2001). Table 13.2 summarises actively targeted nanomedicines developed against the major angiogenic targets.

– *VEGF and their receptors*, *VEGFR-1 and VEFR-2*. VEGF levels are up-regulated by tumour hypoxia and oncogenes which leads to up regulation of VEGFR on tumour endothelial cells, with VEGFR-2 being highly expressed in tumour neovasculature (Veikkola et al. 2000). Thus, the two main approaches are to target: (a) VEGF to inhibit its binging toVEGFR-2 receptor or (b) to target the VEGFR-2 receptor to decrease the VEGF binding (Carmeliet 2005).

- *$\alpha_v\beta_3$ integrin*. This is an endothelial cell receptor (which contains Arg-Gly-Aps or a RGD sequence) for extracellular matrix (ECM) proteins such as fibrinogen, fibronectin, vibronectin, von Willebrand factor, osteopontin, and thrombospondin (Desgrosellier and Cheresh 2010). $\alpha_v\beta_3$ integrins are highly expressed in tumour and angiogenic endothelial cells having a key role in the calcium-dependent signalling pathway responsible for endothelial cell migration (Nisato et al. 2003; Desgrosellier and Cheresh 2010). Thus, RGD sequence peptides or non-peptide mimetics are potentially useful as targeting ligands in cancer therapy.
- *Vascular cell adhesion molecule-1* (*VCAM-1*). VCAM-1 is an immunoglobulin-like transmembrane glycoprotein expressed on the surface of endothelial cancer cells being able to promote cell-to-cell adhesion (Osborn et al. 1989). During angiogenesis and metastasis, integrins bind to ECM proteins or cell surface immunoglobulins like VCAM-1 (Chen and Massague 2012). On normal vasculature, VCAM-1 is present and is inducible by angiogenesis and over-expressed in lymphomas, leukemias, melanoma, lung, breast and gastric cancer, and renal cell carcinoma (Byrne et al. 2008).
- *MMPs*. MMPs belong to the zinc-dependent endopeptidases family which is able to degrade all ECM proteins (Vihinen et al. 2010). MMPs have been implicated in many pathological processes such as carcinogenesis. Their role in angiogenesis and metastasis is crucial as they are involved in forming the capillary tubes and recruiting accessory cells (Danhier et al. 2010). In particular, the membrane type 1 matrix metalloproteinase (MT1-MMP) activates MMP-2 (Gelatinase-A, 72 kDa gelatinase) which hydrolyses one of the major components of the basement membrane, the Type IV collagen, but also MT1-MMP cleaves the $\alpha_v\beta_3$ integrin which enhances its binding activity (Sato et al. 2005). Thus, additive anticancer effects can be obtained by targeting the MT1-MMP. MT1-MMP is expressed on endothelial tumour cells and on a variety of cancer cells such as gliomas, melanomas, malignancies of lung, gastric, breast, cervical, and colon carcinomas (Genis et al. 2006). Another targeted metalloproteinase is the aminopeptidase N/CD13, which is an endothelial cell-surface receptor, involved in tumour-cell invasion, tumour metastasis, and ECM degradation by tumour cells. NGR (Asn–Gly–Arg) peptides are used as targeting ligands because of their ability to bind this aminopeptidase (Pasqualini et al. 2000; Danhier et al. 2010).

13.1.5.2 Actively Targeted Nanomedicines for Transport Across the Blood-Brain Barrier

Currently, more than 1.5 billion individuals are affected by CNS diseases such as cancer, psychiatric disorders, neurodegenerative, inflammatory, and infective diseases (McGonigle 2012). There is an unmet clinical need and then the development of actively targeted nanomedicines can be a potential useful non-invasive approach to treat brain diseases. The major obstacle to deliver any therapeutic and/or imaging

agent to the brain is the blood-brain barrier (BBB) which is an unique membranous barrier composed of tightly bound endothelial cells and perivascular astrocytes whose function is to segregate the brain from the systemic blood circulation to ensure CNS homeostasis in order for neuronal functions to optimally take place (Lalatsa et al. 2012a; Serrano-Lopez and Lalatsa 2013). The influx transport systems expressed within the cerebral endothelial have been exploited with the aim of designing BBB active targeting nanomedicines. These transport systems are CMTs, RME, and adsorptive-mediated endocytosis (AME) which play a key role in the delivery of essential substance to the CNS (Beduneau et al. 2007). One of the major approaches is based on the use of targeting moieties able to mimic endogenous substances, which exhibit specific affinities with CMT, RME, or AME systems. Table 13.3 summarises several examples of CNS active targeted nanomedicines.

The passage of nutrients of low molecular weight is mediated by CMT which are highly expressed in the cerebral vessels of the BBB, including carriers for glucose (GLUT), monocarboxylic acids (MCT1), large neutral amino acids (LAT1), cationic amino acids, excitatory amino acids (EAAT), organic cations, choline, adenine, nucleoside, etc. (Begley 2004; Tsuji 2005; Beduneau et al. 2007) Among all the transporters, GLUT and choline transporters have exhibited the greatest potential as transporters in order to cross the BBB. The GLUT1 transporter facilitates the transport from the blood to the brain not only of the D-glucose, but also of other molecules with similar structure such as galactose, mannose, 2-deoxyglucose, and glucose analogues (Pardridge 1995). One advantage of the GLUT1 transporter is its high capacity to transport glucose (1,420 nmol/min/g tissue) compared to other transporters like MCT1 (91 nmol/min/g tissue) and LAT1 (28 nmol/min/g tissue) (Tsuji 2005). The choline transporter exhibits an anionic-binding area that interacts with cationic molecules such as choline (precursor for the neurotransmitter acetylcholine), but also with other molecules like carnitine and thiamine with positively charged quaternary ammonium groups (Kang et al. 1990; Lockman and Allen 2002). The major advantage of this transporter is the fact that there is no saturation under physiological concentration, thus other components could be transported without altering the choline transport to the CNS (Allen and Smith 2001).

On the contrary, in order to gain access to the CNS, large molecules required for the normal function of the brain are bound to specific receptors, which are highly expressed and localised on the endothelial cells of the BBB (Gabathuler 2010). The binding of the ligand to a specific receptor results in the internalisation of the complex (receptor-ligand) within an endocytic vesicle (Beduneau et al. 2007). The main receptors used in the design of active targeted nanomedicines to deliver different compounds across the BBB are the insulin receptor, folate receptor, transferrin receptor and low-density lipoproteins receptor (LDLR), and other protein related (Gabathuler 2010).

LDLR binds lipoprotein particles carrying ApoE and ApoB100 and targeting this receptor is a very promising strategy for endocytosis of nanoparticles across the BBB (Lalatsa et al. 2012a). Lipoprotein receptor protein (LRP) is a multifunctional

Table 13.3 Examples of CNS active targeted nanomedicines

Target	Targeting ligand	Nanocarrier-name (particle size)	Therapeutic agent	Outcome	Last known status	References
Carrier-mediated transport						
GLUT1	Glucose	PEGylated liposomes (<100 nm)	Coumarin	Higher accumulation in brain 6 h after intravenous administration	Preclinical	Xie et al. (2012)
	Glucose	Gold nanoparticles (~4 nm)	–	More efficiently transport across primary human brain endothelium than non-brain endothelium	In vitro	Gromnicova et al. (2012)
Choline transporter	Choline	Dendrigraft[a] poly-L-lysines PEGylated nanoparticles	Plasmid DNA	Higher uptake efficiency in vitro and higher gene expression in vivo	Preclinical	Li et al. (2011)
Receptor-mediated endocytosis						
Insulin receptor	Insulin or anti-insulin receptor MAb (29B4)	Albumin nanoparticles (~150 nm)	Loperamide	Significant increase of antinociceptive effect prolonged over 2 h after intravenous administration	Preclinical	Ulbrich et al. (2011)
Transferrin receptor	OX26 MAb or R17217—PEG 5 kDa—MAL linker	Albumin nanoparticles (150 nm)	Loperamide	Significant increase of antinociceptive effect prolonged over 2 h after intravenous administration in rats	Preclinical	Ulbrich et al. (2009)
	Transferrin	Nanoparticles of PLA-TPGS diblock co-polymer (~137 nm)	Docetaxel	Higher efficient (229 %) in terms of IC_{50} than Taxotere® 24 h post-treatment; higher accumulation in brain	Preclinical	Gan and Feng (2010)
	OX26 MAb conjugated to streptavidin[a]	PEGylated chitosan nanoparticles conjugated to biotin (~590 nm)	Peptide Z-DEVDFMK	Enhanced brain translocation after intravenous administration	Preclinical	Aktas et al. (2005)

	OX26 MAb—PEG 2 kDa—MAL linker	Liposomes (~100 nm)	Digoxin	Inhibited p-gp efflux and increased uptake across the BBB	Preclinical	Huwyler et al. (2002)
	OX26 MAb	PEGylated Liposomes (~100 nm)	GDNF plasmid DNA	Three weeks treatment of intravenous therapy with these immunoliposomes loaded with a glial-derived neurotrophic factor led to near complete recovery of rats with neurotoxin-induced Parkinson's disease	Preclinical	Zhang and Pardridge (2009)
	Anti-transferrin—MAb	PEGylated liposomes (~100 nm, PEG 2 kDa)	shRNA, siRNA	Weekly, intravenous RNAi with pegylated liposomes enables a 90 % knockdown of the human epidermal growth factor receptor, which results in a 90 % increase in survival time in mice with intra-cranial brain cancer.	Preclinical	Pardridge (2007)
Folate receptor	Folate	Solid lipid nanoparticles (~80 nm)	Docetaxel ketaconazole[a]	20-fold increase uptake in brain endothelial cells and 44-fold enhancement in brain permeation coefficient value both compared to Taxotere®	Preclinical	Venishetty et al. (2013)
LDL receptor	ApoE	PEGylated albumin nanoparticles (~186 nm)	–	Enhance brain uptake of the nanoparticles through the LDL receptor-related protein without opening tight junctions	In vitro	Wagner et al. (2012)
	Angiopep-2	PEGylated PAMAM dendrimers	DNA	Higher brain uptake and enhanced gene expression in vivo	Preclinical	Ke et al. (2009)
	Lactoferrin	PEGylated PAMAM dendrimers	Neurotrophic factor gene	Neuroprotective effects in a rotenone-induced rat model of Parkinson's disease	Preclinical	Huang et al. (2010)

(continued)

Table 13.3 (continued)

Target	Targeting ligand	Nanocarrier-name (particle size)	Therapeutic agent	Outcome	Last known status	References
Leptin	$Leptin_{61-90}$	PEGylated Poly-lysine dendrimers	DNA	Poly(L-lysine) dendrimer: PEG: DNA (1:5:2 w/w/w) resulted in higher uptake in the brain and enhanced gene expression	Preclinical	Barrett et al. (2009)
Adsorptive-mediated endocytosis						
Anionic sites in the BBB	Cationic albumin	PEGylated nanoparticles (~109 nm)	Plasmid DNA	Accumulation in brain upon intravenous administration; significantly delay in brain tumour growth	Preclinical	Lu et al. (2006)
	TAT peptide	Nanoparticles formed by self-assembly of an amphiphilic peptide with a hydrophobic cholesterol core (<150 nm)	TAT peptide	Suppression of bacterial growth in *Staphylococcus aureus*-infected meningitis rabbits	Preclinical	Liu et al. (2009)

GLUT D-glucose transporter, *dendrigraft* polymer that has the form of a dendrimer, *GDNF* glial-derived neurotrophic factor, IC_{50} drug concentration required to induce the death of 50 % cell incubated in a designated period, *MAb* monoclonal antibody, *MAL* maleimide, *OX26 MAb* monoclonal antibody with high affinity for transferring receptor and was conjugated to the surface of the PEGylated chitosan nanoparticles through the specific interaction streptavidin-biotin, *PAMAM* poly(amidoamine) dendrimers, *PEG* polyethylene glycol, *peptide Z-DEVDFMK*-specific caspase inhibitor which significantly reduces vulnerability to the neuronal cell death being potentially useful after cerebral ischemia, *PLA-TPGS* poly(lactide)-D-α-tocopheryl polyethylene glycol succinate, *TAT peptide sequence* Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg

[a]Ketaconazole was used to inhibit the p-gp efflux of docetaxel at the BBB

lipoprotein receptor which interacts with a great variety of ligands such as ApoE, tissue plasminogen activator, amyloid precursor protein (APP), lactoferrin, and others mediating their endocytosis and is expressed in many tissues and the CNS (Rebeck et al. 1993). LRP is expressed in the cerebellum, on neuronal cells, and in astrocytes. LRP is over-expressed in malignant astrocytomas, especially glioblastomas (Yamamoto et al. 1997). Polymeric nanoparticles over-coated with polysorbate 80 have been shown to adsorb ApoE and ApoB from the blood and it is postulated that they are taken up via the LDLR. Rather than coating particles with surfactants and relying on spontaneous adsorption of ApoE from plasma, covalent attachment of ApoE on the particles has been engineered and elicited particles able to be taken up both into BCECs (Bovine caruncular endothelial cells) and brain parenchyma without any effect on the tight junctions (Zensi et al. 2009). Lactoferrin, on the other hand, is normally present in very low physiological levels unlikely to compete with a vector conjugate making it a good target as it is transcytosed across the BCECs and is not intracellularly degraded. Lactoferrin conjugated via a PEG spacer to poly(amidoamine) dendrimers was targeted to the brain and used to deliver a neurotrophic factor gene to a rotenone-induced rat model of Parkinson's disease with some neuroprotective effects being observed (Huang et al. 2010). BBB permeable peptides based on the structure of aprotinin called Angiopeps is a family of 19 amino acids peptides derived from the kunitz domain that have a high transcytosis rate using LRP-1 and LRP-2 (Demeule et al. 2008). Angiopep-2 has also been studied for gene delivery when covalently linked to a PEG spacer linked to a PAMAM dendrimer (Ke et al. 2009). An increase loading of Angiopep-2 was required to achieve a 0.25 % of injected dose to the brain with majority of the dose detected in the kidneys at 2 h post-administration. This technology is applicable to both small and larger molecules. The advantages of the lipoprotein receptor system over other receptor systems such as the hexose receptor systems include more efficient cellular delivery and the potential for transcytosis of ligands across tight endothelia, including the blood-brain barrier. Several examples of nanomedicines targeting RME are included in Table 13.3.

Regarding AME system, the transport across the BBB takes place through a less specific mechanism based on an electrostatic interaction between the negatively charged membrane of the BBB and a positively charged ligand (Beduneau et al. 2007). Thus, cationic proteins because of their basic isoelectric point are positively charged at physiological blood pH; thus makes them able to bind to the luminal plasma membrane triggering adsorptive endocytosis (Gabathuler 2010). In fact, it has been reported that the transport of cationised albumins or immunoglobulins into the CNS is significantly enhanced compared to native proteins (Triguero et al. 1989; Vorbrodt et al. 1996). Related to positively charged ligands that can be conjugated to nanomedicines to enhance their passage across the BBB, cell penetrating peptides have become a very effective class of transporters composed of short cationic sequences with a remarkable capacity for membrane translocation with minimal toxicity (Fonseca et al. 2009). To date, TAT (RKKRRQRRR), polyarginine (RRRRRRRRR), penetratin (RQIKIWFQNRRMKWKK), and transportan (GWTLNSAGYLLGKINLKALAA LAKKIL) are some of the most used cell-penetrating peptides that have successfully

delivered small molecule therapeutics, proteins, nucleic acids, contrast agents, etc. (Fonseca et al. 2009). Table 13.3 summarises several examples of CNS active targeting nanomedicines.

13.2 Conclusion and Future Perspectives

To date, a few passive targeted nanomedicines have been commercialised (Lammers et al. 2012). However, despite the fact that significant progress has been made in the field of active targeted nanomedicines, translation of promising approaches from bench to bedside has been poor at best (Bae and Park 2011). Many times the potential usefulness of active targeting has been exaggerated and misunderstood, especially in cancer therapy where there are several important pitfalls that hamper the clinical success of these nanomedicines. These are highlighted below.

(a) Over-interpretation of the EPR effect. Although the EPR effect enhances the accumulation of nanomedicines at the intended site, this does not mean that 100 % of the dose administered will be accumulated at the target site. Actually, in the majority of the intravenous administered formulations, above 95 % of the dose ends up at unintended sites (Bae and Park 2011).
(b) Poor tumour penetration. Considering that the nanomedicine had the appropriated size, shape, and surface charge to be extravasated through the opening between endothelial vascular cells within the tumour tissue, it should not be forgotten that higher interstitial fluid pressure (ranging from 5 to 40 mmHg) is developed inside most solid tumours compared to normal tissues (typically <3 mmHg) which makes difficult the accumulation and diffusion of the nanomedicine within the tumour tissue (Milosevic et al. 2004).
(c) Over-expression of the target and tumour heterogenicity. There are two major problems regarding these concepts: first it is very difficult to find a target that is exclusively expressed by cancer cells (or the blood-brain barrier), and secondly, the target is not usually expressed by all the cancer cells. Despite the fact that certain receptors could be over-expressed on cancer cells, it should be taken into account how many times the mass of non-target cells is larger than the target cell mass (Bae and Park 2011).

Overcoming the drawbacks regarding the formulation design such as solubility, aggregation, instability, suitable particle size, shape, and surface can result in too complex nanomedicines and low-cost effectiveness which make them not widely applicable. Besides the difficulties in the formulation design and manufacture, once the nanomedicine has been administered, it will face both anatomical and physical barriers that have to be overcome in order to elicit its pharmacological effect (Fig. 13.5) (Lammers et al. 2012). For these reasons, it is essential to develop appropriate animal models able to reproduce all the hurdles mentioned above and predict more accurately the clinical situation.

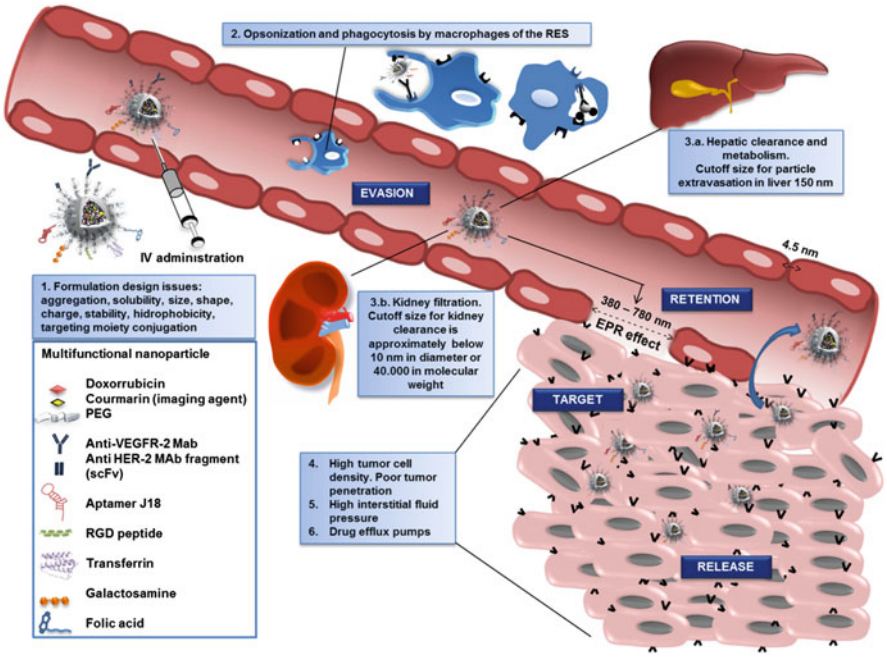

Fig. 13.5 Requirements for optimal active targeted nanomedicines and problems to be addressed. There are four essential requirements in order to ensure optimal active targeting: evasion, retention, target, and release. Thus, multifunctional nanomedicines possessing appropriate ligands conjugated on their surface are necessary. Additionally, the nanocarriers have to exhibit certain characteristics in order to overcome all the hurdles before reaching their target. These include addressing issues such as: hydrophilicity, charge, particle size, shape, solubility, etc. Upon intravenous administration, nanomedicines have to avoid opsonisation by plasma proteins in order to evade phagocytosis by the macrophages of the RES. Also, nanomedicines should exhibit sufficient residence in the bloodstream which may be enhanced by manipulating the particle size. Because of the larger blood vessel fenestrations in the liver (100–175 nm), particles can easily be extravasated and be at risk of hepatic clearance and the drug payload metabolised (Ballet 1990). Alternatively, very small nanomedicines (diameter <10 nm or molecular weight <40,000) will be cleared by kidneys (Yokoyama 2005). In the case of tumor active targeting, the EPR effect plays a key role in enhancing the retention of the nanomedicine within the tumour tissue. The pore cut-off size of the blood vessels in the majority of the tumours ranges from 380 to 780 nm unlike the small pores (around 4.5 nm) present in normal tissue endothelium (Ballet 1990; Rippe et al. 2002). Once nanomedicines are accumulated in the tumour, specific interactions between the targeting moieties and the receptors of the intended tissue take place where the therapeutic and imaging agent will be released. However, other problems have to be faced in order to ensure the diffusion and accumulation of the nanomedicines within the intended site such as the high interstitial fluid pressure, poor penetrability in the tumour tissue and active drug efflux

In conclusion, future active targeting nanomedicines are pointing to multifunctional personalised theranostic therapies which will combine a dual role between therapeutic and imaging nanomedicines adjusted to the clinical need of every patient.

Problem Box

Question 1

Apart from the hydrodynamic size and the shape, which other characteristic is important to bear in mind in the choice of nanocarrier?

Surface properties such as charge and hydrophobicity play a key role in cellular uptake, clearance, and biodistribution of the nanomedicine. For example, charged nanocarriers and nanocarriers not coated with hydrophilic polymers have shorter blood circulation times because of the adsorption of opsonin proteins onto their surface that render them easily recognisable by macrophages of the reticulo-endothelial system and thus they will not arrive to the desired target to elicit their effect.

Question 2

Which are the major advantages of using antibodies fragments as targeting moieties compared to use whole antibody?

Antibody fragments are smaller in size and lack the Fc domain and the complement-activating region (which might reduce the immune response) while keeping their antigen binding affinity. In addition, their identification and manufacture is easier compared to whole antibodies.

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Chapter 14
Hydrophobic Drug Solubilisation

C. Hoskins and W.P. Cheng

Abstract Poorly soluble drugs have always been a challenge to the pharmaceutical industry. Today, it has been estimated that 40 % of new chemical entities fail in development because of adverse physical properties such as poor aqueous solubility. In this chapter, the authors look at the principals of drug solubility and dissolution and evaluate the use of drug nano-crystals and nano-size delivery systems such as liposomes, polymeric micelles and solid lipid nanoparticles in enhancing drug solubility. Additional benefits such as achieving site-specific delivery and facilitating cellular uptake are also discussed. The key advantages that nanotechnologies are able to offer and the challenges in the exploitation of these technologies are outlined with appropriate examples. Clinical experience is included to demonstrate the successful approach in using nanotechnologies for hydrophobic drug solubilisation.

14.1 Clinical Need

14.1.1 Introduction

Drug efficacy and bioavailability are largely dependent on drug physico-chemical properties such as water solubility, pKa and Log *P*. It has been reported that as much as 40 % of all new drugs in the developmental stage have physical and chemical properties that impact negatively on bioavailability. This issue possesses a major problem for drugs given via most of the routes of administration. For intravenous

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Table 14.1 British pharmacopoeia categories of descriptive solubility

Descriptive term	Approximate volume of solvent in millilitres per gram of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1,000
Very slightly soluble	From 1,000 to 10,000
Practically insoluble	More than 10,000

administration it is desirable for drugs to be formulated as aqueous-based injections. Thus, drug aqueous solubility is a prerequisite (Yeh et al. 2009). If the drug is not water-soluble, one of the methods to achieve an aqueous-based formulation is to formulate the drug in an emulsion. One example is propofol, a general anaesthetic drug available in a soya bean-based formulation. However, the viscous solution can cause patient discomfort and usually a prerequisite painkiller needs to be administered beforehand (Ravenelle et al. 2008). For oral drug delivery, drugs must be released from the formulation and dissolved in the aqueous gastrointestinal fluid before absorption can take place in the gastrointestinal tract (GIT) (Aulton 2007). The bioavailability of an oral drug is also complicated by the fact that the drug must also be able to diffuse across biological membranes, i.e. the GIT mucosa. Therefore, for drugs which have high permeability but with poor solubility (Class II according to Biopharmaceutics Classification System (BCS)), the rate of dissolution is a major rate-limiting step for the absorption of these drugs on oral administration. As a result, this undesirable property often leads to poor oral bioavailability and erratic GIT absorption.

14.1.2 Drug Solubility

The solubility of a drug (solute) is defined as the maximum amount of drug that will dissolve in a fixed volume of solvent at any certain temperature and form a solution (Florence and Atwood 2006). Here the solvent can be composed of one or more miscible phases. Drug solubility is classified in seven categories in the British Pharmacopoeia (Table 14.1).

14.1.3 Dissolution

The transfer of drug molecules from a solid state into solution is known as dissolution. The process of dissolution occurs in consecutive stages for any solid (Fig. 14.1). Firstly, individual drug molecules must break away from their bulk crystal structure.

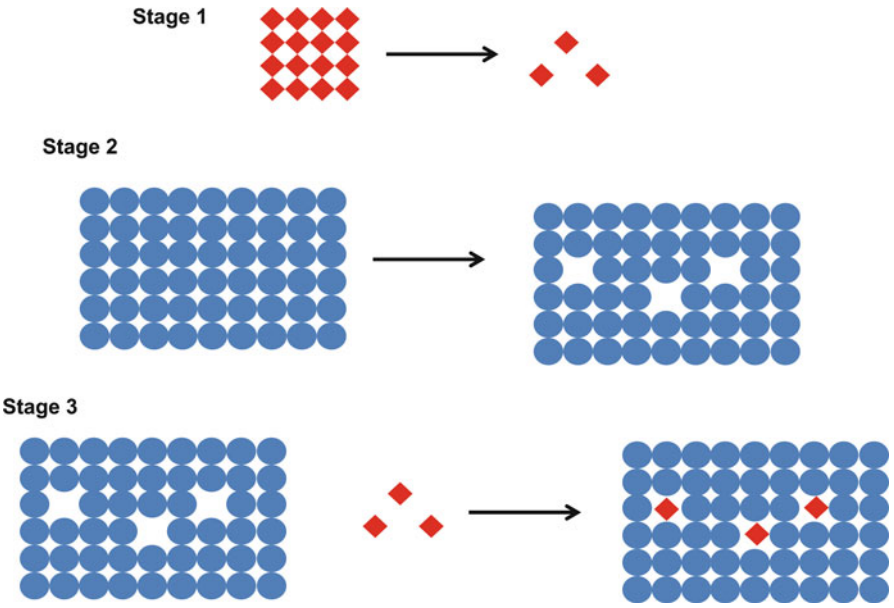


Fig. 14.1 Stages in drug solubility. Stage 1 Drug molecules move away from their bulk form, Stage 2 Water molecules form tiny cavities and Stage 3 Drug molecules are accommodated within water cavities resulting in solubilisation

As a result tiny (molecular scale) cavities form between the solvent molecules allowing the free drug molecules to be accommodated. Once the molecules have been inserted into these cavities, the drug is said to be solubilised. Once all of the cavities have been occupied by drug molecules and no more drugs can be dissolved, the solution is said to be supersaturated.

The rate of dissolution of a solid into a liquid phase is illustrated using the Noyes-Whitney equation (14.1). Here $\frac{dw}{dt}$ is the rate of dissolution of a solid, C_s is the saturation solubility of the drug in the dissolution layer, D is the diffusion coefficient of the dissolved solute, σ is the thickness of the diffusion layer and A is the surface area of the solid (Florence and Atwood 2006).

$$\frac{dw}{dt} = \frac{DAC_s}{\sigma} \tag{14.1}$$

Therefore, if the surface area of the drug particle is increased via particle size reduction then the rate of dissolution will increase. Also saturation solubility directly impacts on the rate of dissolution of solids. Other factors also influence the rate of dissolution of solids such as temperature (due to increasing C_s), introduction of stirring/agitation and the amount of solute already in solution (where small amounts of drug molecule are in solution, dissolution occurs at an increased rate). Environmental temperature and pressure also directly impact the degree of

solubilisation of drug molecules. In general, an increase in the temperature will lead to increased solubility. However, there are a few exceptions to this rule where lower temperature solvents exhibit higher drug solubility.

14.1.4 Log P

Once in solution drug molecules can partition (P) between different phases. When a drug is added to a solvent system containing two immiscible solvents, the drug will partition into each solvent relative to their concentration and its affinity for each phase. The extent of partitioning into each phase can be quantified as the partition coefficient (14.2). The partition coefficient is defined as the degree of hydrophobicity of a molecule, whereby C_0 is the concentration of a drug in the hydrophobic phase and C_w is the concentration of drug in the aqueous phase.

$$P = \frac{C_0}{C_w} \tag{14.2}$$

Drugs possessing high P values have higher affinities for the lipophilic phase, while drugs with low P values prefer the hydrophilic (aqueous) phase. However, the partition coefficient is usually quoted in log form (Log P). Log P helps to estimate in vivo bioavailability of drug molecules. Those with high Log P values theoretically should achieve higher bioavailability due to their greater ability to partition across the intestinal epithelium. However, this is not always true, as illustrated by oral drugs whereby the prerequisite for oral absorption to occur is that drugs must exhibit sufficient water solubility to dissolve in the aqueous GIT fluid before passively diffusing across GI tract membrane.

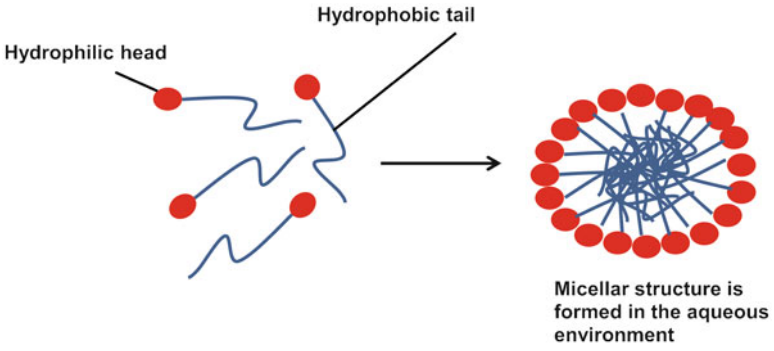


Fig. 14.2 Schematic diagram of a surfactant molecule and spontaneously formed micelle structure in aqueous environments

Table 14.2 BCS classification for an oral drug

Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

14.1.5 Biopharmaceutics Classification System

The BCS is a guide for predicting oral drug absorption (Table 14.1). This system is used to predict the oral bioavailability of drugs using the parameters of water solubility and intestinal permeability. For example Class I and III both have high solubility; therefore, the drug is readily released from the formulation and dissolved in an aqueous environment. However, for Class III drugs, the absorption is limited by the permeation rate (Dressman et al. 1997). In contrast, Class II drugs possess low solubility and high permeability. Therefore, the rate-limiting step is the rate of dissolution. The use of excipients is crucially important to increase the dissolution of these drugs in the aqueous GI fluid and subsequently the bioavailability of the drugs (WHO 2006). Conventional excipients are commonly used to improve drug solubilisation for Class II, but in later examples, we will demonstrate that nanotechnologies can be used for Class II as well as Class IV drugs which have both low permeability and solubility (Table 14.2).

14.2 Technologies to Improve Drug Solubilisation

Delivery systems are employed to increase the solubility and bioavailability of hydrophobic drug compounds. These include traditional formulation technologies utilising surfactants (Malmstein 2002; Farías et al. 2009), co-solvents (Sinko 2005; Millard et al. 2002), micronization (Han et al. 2011) and salt formation and nanotechnologies such as liposomes (Nii and Ishii 2005; El-Ridy et al. 2007), solid lipid nanoparticles (Subedi et al. 2009), amphiphilic polymers (Nishiyama and Kataoka 2006; Kwon and Okano 1996), polymer nanoparticles (Shaikh et al. 2009) and nano-crystals (Chen et al. 2011).

14.2.1 Conventional Excipients

Surfactants are low molecular weight amphiphilic compounds which possess both hydrophobic and hydrophilic moieties (Fig. 14.2). The hydrophobic portion of a surfactant is usually composed of saturated/unsaturated hydrocarbon chains, aromatic or heterocyclic ring systems.

Due to the amphiphilic nature of surfactants, in aqueous media they spontaneously aggregate to form micelles (Fig. 14.2). Micelles are core-shell structures where the hydrophobic 'tails' of the surfactants aggregate surrounded by the hydrophilic 'head groups'. Micelles exist in dynamic equilibrium possessing stability ranging from milliseconds to seconds. Spontaneous aggregation only occurs in surfactants above a critical solution concentration known as the critical micellar concentration (CMC). Surfactants possessing a lower CMC value will exhibit increased stability upon dilution. In the micellar form, the hydrophobic core of the surfactant serves as a lipophilic reservoir for hydrophobic drugs. The exterior hydrophilic shell reacts with the biological milieu allowing absorption into the body and enabling transport to the target areas. To date the commercial surfactants available for human use include sodium dodecyl sulphate (SDS) (Zhou and Rhue 2000), the Tween surfactants (Lawrence 1994) and Cremophor EL (BASF 2001). However, micelles made of surfactants possess a relatively high CMC making them relatively unstable. After in vivo administration, they become highly diluted resulting in disruption of their structural integrity leading to premature drug release. Secondly, extremely high quantities of excipient are required to solubilise relatively small amounts of drugs (excipient/drug ratios, 15:1–1000:1); this can be costly or cause undesirable side effects.

The use of co-solvents is one of the conventional techniques to improve drug solubility. Different drug molecules experience different affinities for liquids according to the liquid phase, as evident from the Log *P* value. For instance, a drug molecule may possess a high drug solubilisation in ethanol, however be sparingly soluble in toluene. Some solvent systems comprise of two or more miscible phases in order to enhance drug solubilisation. Commonly glycerol, ethyl alcohol and propylene glycol are used in aqueous formulations.

The formation of salt crystals to increase aqueous solubility is often employed in liquid formulations for injectables (Serajuddin 2007). Salts of weak acidic and basic drug molecules possess differing physical properties and often have higher aqueous solubility. For solid dosage forms, the dissolution rates experienced from using salts of weakly acidic compounds under physiological GIT conditions are much higher compared to the free acid form. The increase in dissolution rate of a salt can be attributed to an increase in its solubility. With advances in the chemical sciences, salt formation has become the most commonly applied technique of increasing solubility and dissolution rate in drug product development (Serajuddin 2007).

Micronization is a technique which increases the total surface area of the drug resulting in faster dissolution rates. The dissolution of drugs is often directly related to the particle size (14.1). Particle size reduction (or increasing of surface area) can lead to an increase in dissolution rates. Micronization of drugs is carried out by milling of the drug into uniform micron-sized particles, which usually enhances aqueous dissolution.

14.2.2 Nanotechnology

The use of high-throughput screening in drug discovery has resulted in 70–90 % of new chemical entities being poorly soluble, e.g. BCS Class II and IV compounds.

Depending on the type of nano-systems, use of nanotechnology can overcome a number of issues that conventional technologies are unable to achieve: (1) higher encapsulation with less excipient, (2) multiple drug encapsulation, (3) improvement of drug permeability, useful for class IV drugs and (4) versatility with the capacity to be modified to achieve targeting and other desirable properties. The growing need to design delivery systems which are capable of encapsulating high levels of active ingredient while exhibiting excellent safety profiles has resulted in a plethora of technologies utilising the unique physical properties arising from the use of nano-sized particulate systems. Nano-crystals, liposomes, polymeric micelles and solid lipid nanoparticles have been extensively explored to improve the efficacy of hydrophobic drugs (Table 14.3). Table 14.4 lists the key advantages and limitations of the systems. All these systems are discussed in detail in various chapters of this book and readers are encouraged to refer to these respective chapters.

14.3 Preclinical Proof of Concept

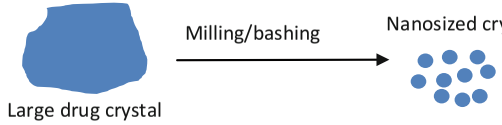
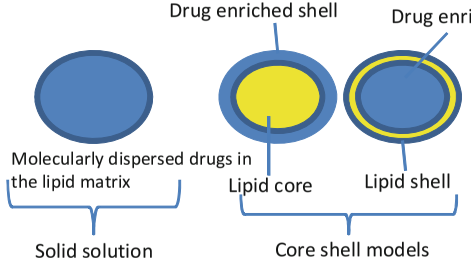
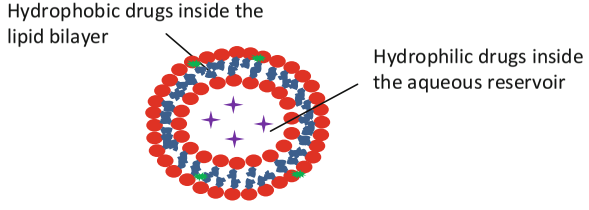
14.3.1 In Vitro Assays

14.3.1.1 In Vitro Drug Dissolution Test

As demonstrated by the Noyes Whitney equation (14.1), in oral drug delivery, the greater surface area of a drug particle results in faster dissolution and greater levels of absorption across the GIT. This leads to an increase in drug bioavailability. The drug nano-crystals result in an increased surface area to volume ratio. The enhanced dissolution of nano-crystals compared to larger drug crystals has enabled them to be used in oral dosage forms and more recently drug nano-crystals have been used in intravenous preparations. As bioavailability is enhanced via the oral route, lower doses can be administered and this results in reduced side effects and ultimately a more efficient and cost effective system. For instance, the in vitro dissolution profile of the hydrophobic drug megestrol acetate (MA) used in hormone therapy was investigated in both nano-crystals and the coarse crystal forms (Sylvestre et al. 2011). The data showed that after only 10 min 95 % of the total drug content of the nano-crystals had dissolved (Fig. 14.3). This was significantly more rapid than for the coarse crystals which took 3 h for full dissolution.

Dissolution is often the rate-limiting step in oral bioavailability. Encapsulation of lipophilic drug molecules into various nano-systems eliminates this step as the drug molecules are dispersed within the core/matrix of the aggregate (Hoskins et al. 2010). In this case, drug release from the nano-system is the rate-limiting step. Hoskins et al. reported in vitro drug release of model drugs propofol, griseofulvin and prednisolone from polymeric micelles formed by poly(allylamine) (PAA)-based amphiphiles. The data indicated (Fig. 14.4) that the drug release from the cholesterol grafted PAA (CH5) resulted in an increased drug release compared

Table 14.3 Types of nanotechnologies used to improve drug solubility

Nano-systems	Schematic diagram	Examples of the composition	Method to increase the drug solubility	Drugs that have been attempted
Nano-crystals		Drug crystals only	Crystal volume milled into nano-sized spherical particles	Megestrol acetate Sylvestre et al. (2011) Baicalein Zhang et al. (2011a)
Solid lipid nanoparticles (SLN)		Lipid constituents (e.g.) • Labrafec • Lipoid • Solutol • CP-Togo Care 450 • Goat fat	Hydrophobic drugs are molecularly dispersed in the lipid matrix or incorporated into the drug enriched shell/ core	Dicolfenac sodium Attama et al. (2008) Tocophenyl acetate Moddaresi et al. (2010) Ketoprofen Kheradmandnia et al. (2010) Bufalin Liu et al. (2011) Psoralen Fang et al. (2008)
Liposomes		Phospholipids (e.g.) • Phosphatidylcholines • Choline glycerolphospholipid • Ethanolamine glycerolphospholipid • Phosphatidylglycerol • Phosphatidylinositol • Phosphatidylserine	Hydrophobic drugs are physically entrapped in the bilayer	Paclitaxel Yang et al. (2007) Clofazamine Mehta (1996) Cytochalasin D Huang et al. (2012) Ibuprofen Mohammed et al. (2004) Amphotericin B Gulati et al. (1998)

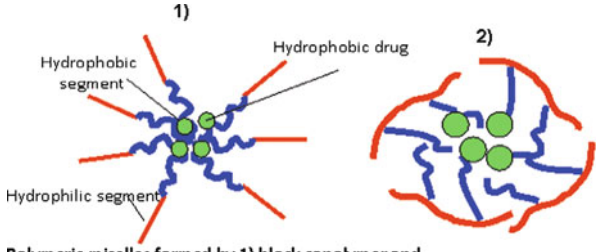
Polymeric micelles	 **Polymeric micelles formed by 1) block copolymer and 2) hydrophobically modified polymer**	**Block copolymers** • PEG-*b*-poly(aspartic acid) • PEG-*b*-poly(D,L-lactide) • PEG-*b*-polyglutamic acid • PEO-PPO-PEO (Pluronics) **Hydrophobically modified polymers** • Poly(allylamine) modified with cholesteryl, dansyl or palmitoyl groups • Chitosan modified with a range of hydrophobic pendant groups	Hydrophobic drugs are encapsulated within the hydrophobic core of the polymeric self-assemblies, mainly via non-covalent interactions	Cyclosporine A Cheng et al. (2006) Paclitaxel Iqbal et al. (2011) Propofol Hoskins et al. (2012a) Etoposide Mo et al. (2011) Griseofulvin Hoskins et al. (2012a) Prednisolone Hoskins et al. (2012a)

Table 14.4 Key advantages and limitations of the nanotechnologies used to improve drug solubility

Nano-systems	Key advantages	Limitations
Nano-drug crystals	• Increase drug solubility • Increase dissolution rate • Decreased absorption variation in fed/fasted state	• Low physical stability • Additional stabilisers required such as surfactants, liposomes, polymeric self-assemblies
Solid lipid nanoparticles	• Ability to optimise loading and release characteristics • Low chronic toxicity • Protection of incorporated drugs • Physical stability • Long shelf life • Site-specific targeting • Change biodistribution of drug	• Low drug loading levels • Risk of drug expulsion on storage • High water content in structure
Liposomes	• Ability to optimise loading and release characteristics • Low chronic toxicity • Protection of incorporated drugs • Site-specific targeting • Ease of fabrication/functionalization • Change biodistribution of drug	• Instability on drug entrapment • Instability on storage • Relatively high excipient:drug ratio • Relatively expensive
Polymeric micelles	• Ability to optimise loading and release characteristics • Low chronic toxicity • Protection of incorporated drugs • Site-specific targeting • Ease of fabrication/functionalization • Change biodistribution of drug • Low excipient:drug ratio	• Instability on dilution • Instability on storage

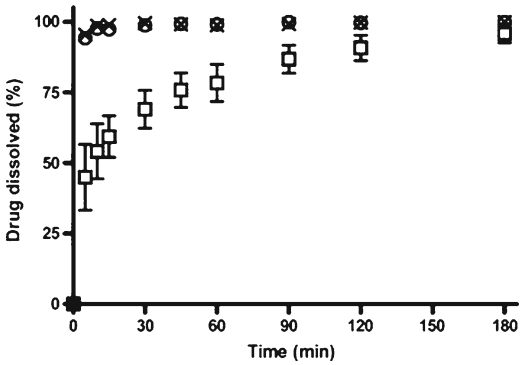


Fig. 14.3 Dissolution profiles in 900 mL of SDS (0.5 % w/v) of 2.5 mg MA from suspensions prepared from untreated MA powder (*squares*), medial milled MA (*crosses*) and fs laser (250 mW, 30 min, 2 mL) fragmented MA (*circles*) in a solution of poloxomer 188 (0.8 % w/v)/Mean ± SD ($n = 3$) (Sylvestre et al. 2011)

with the Dansyl derivative (DANSYL10). These results show the importance of understanding the drug release and tailoring it to specific applications in order for appropriate therapeutic effects to be achieved in the desired time frame (Hoskins et al. 2010).

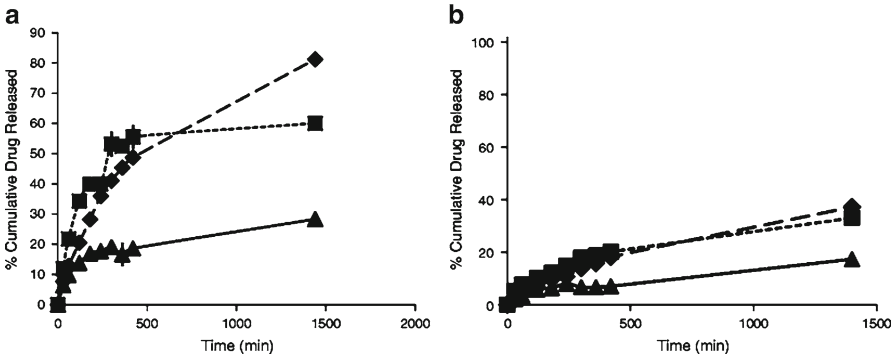


Fig. 14.4 In vitro drug release of hydrophobic drugs from (**a**) CH5 and (**b**) Dansyl10 formulations carried out in sink conditions (*diamonds*) propofol; (*squares*) prednisolone and (*triangles*) griseofulvion. Data presented as $n = 3$, ave. ± SD (Hoskins et al. 2012a)

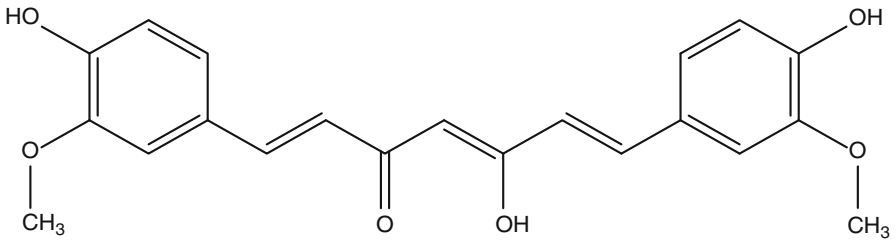


Fig. 14.5 Chemical structure of curcumin

Historically, most of the nano-systems were developed initially for drug solubilisation in intravenous preparations. However, increasing studies are now focussing on other drug delivery routes such as the oral and topical routes. Solid polymer nanoparticles composed of poly(lactide-co glycolic acid) (PLGA) using a poly(vinyl alcohol) (PVA) stabiliser have been fabricated to solubilise the hydrophobic drug curcumin (Fig. 14.5) (Shaikh et al. 2009). The drug was encapsulated inside the PLGA-PVA (5 % PVA) nanoparticles. These particles were stable over the 3-month test period. In vitro release studies were carried out and the data showed a biphasic profile with a rapid release of drug in the first 24 h (24 %) followed by a period of sustained release where after 20 days only 43 % of total drug content had been released. These nanoparticles reportedly enhanced oral absorption of curcumin by up to ninefold compared to free drug administered with an absorption enhancer in male Sprague Dawley rats dosed at 250 mg/kg. These findings indicate that while the long-term release data is of no relevance to the oral dosage form, the use of these nanoparticles did increase systemic exposure to the drug (Shaikh et al. 2009).

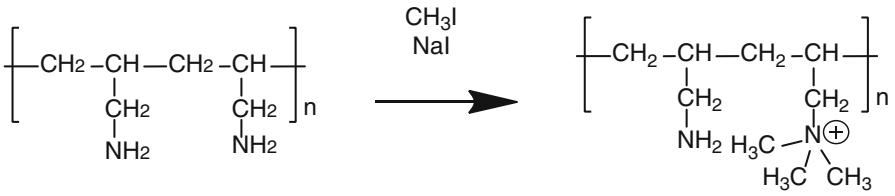


Fig. 14.6 Addition of a quaternary ammonium group to poly(allylamine)

14.3.1.2 In Vitro Nanotoxicity

In order for a drug delivery system to be used clinically, it must be biocompatible. in vitro toxicity information is gathered by incubation of nano-delivery systems with immortalised cell lines followed by investigation into cellular viability, metabolism and cellular responses. Common cytotoxicity tests include the MTT assay, measurement of lactate dehydrogenase (LDH) which is indicative of cell membrane disruption, reactive oxygen species (ROS) and lipid peroxidation (LPO) assays which measure free radical levels inside the cells which can cause internal stresses and ultimately lead to cell mortality. For nano-systems intended for intravenous administration, it is common that haemolysis assays are conducted to ensure that these nano-systems do not lyse red blood cells. In vitro cytotoxicity assay of various types of unloaded nano-systems has shown that the constituent components have a direct impact on the toxicity of the delivery system. Cortesi et al. reported the cytotoxicity on human erythroleukemic (K562) cells in the presence of three cationic liposomes (Cortesi et al. 1996). Here cetyl-trimethyl-ammonium bromide (CTAB), didodecyl-dimethyl-ammonium bromide ($DDAB_{12}$) and dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$) were studied. The data showed that the CTAB- and $DDAB_{12}$- 1iposomes showed a much larger toxic effect (IC50 = 0.88 μM and 0.85 μM, respectively) compared with the $DDAB_{18}$ liposome (IC50 >25 mM) (Cortesi et al. 1996).

Polymer architecture has been shown to influence the cytotoxicity of amphiphilic block copolymers. Rouxhet et al. reported on the cytotoxicity of monoglyceride-based copolymers with varying poly(ethylene glycol) chain lengths attached (Rouxhet et al. 2009). The cytotoxicity studies (MTT assay) were carried out on human colorectal adenocarcinoma cells (Caco-2). They reported that higher polymer concentrations resulted in decreased cell viability as expected. However, by varying the PEG chain length the safety profile of the polymer also varied. They concluded that using longer PEG chains resulted in an increase in cell viability compared with their short chain counterparts (Rouxhet et al. 2009). Similarly, Thompson et al. reported that polymer architecture of PAA amphiphiles had an impact on cytotoxicity. Addition of hydrophilic quaternary ammonium ions onto the polymer backbone of modified poly(allylamine) (PAA) polymers (Fig. 14.6) not only increased the aqueous solubility of the polymers, but also made the polymers less cytotoxic against human epithelial colorectal adenocarcinoma (Caco-2) cells (Thompson et al. 2008; Thompson et al. 2010).

In vitro cellular assays have also shown that some nano-sized systems are capable of reducing toxic side effects exhibited by drug molecules. Liposomes composed of Dimyristoylphosphatidyl choline (DMPC)-dimyristoylphosphatidyl glycerol (DMPG) (7:3 M ratio) reduce the toxicity clofazimine (Log $P = 7.3$) (Mehta 1996). Clofazimine is an antimycobacterial drug clinically used in the treatment of AIDS. However, use of the drug has been limited due to its toxic side effects and poor solubility. The toxic side effects arise due to drug degradation into toxic metabolites. Once incorporated into the DMPC-DMPG liposomes, the liposome acts as a protection barrier from this degradation. Investigation has shown that a liposomal formulation of clofazimine is far less toxic in vitro than the free drug in macrophages and erythrocytes (Mehta 1996). Interestingly when nano-systems are used to increase the solubility of an anticancer drug, they often also exhibit the capacity to increase the cytotoxicity of the drug in vitro (in line with the drug action) (Celano et al. 2004). Other reports have shown amphiphilic polymers (Matsumura et al. 1999), star-shaped polymers (Rezaei et al. 2012) and dendrimers (Bielawski et al. 2011) act in a similar manner. This is probably due to the efficient solubilising capacity of these nano-systems resulting in less drug being required to achieve a similar effect and their ability to promote cellular uptake.

14.3.1.3 Cellular Uptake and Trafficking

Nano-delivery systems have been shown to increase in vitro cellular uptake of cytotoxic agents such as paclitaxel (Miglietta et al. 2000), doxorubicin (Miglietta et al. 2000), etoposide (Mo et al. 2011), cisplatin (Xiao et al. 2011) and camptothecin (Cho et al. 2012). The increase in cellular uptake results in a greater cytotoxic effect in vitro and hence the efficiency of the drug is increased. Cellular uptake can be monitored using fluorescent tags attached to the nano-delivery system (Morgen et al. 2011) or via incorporation of fluorescent moieties into the constituent components of the system (Hoskins et al. 2011). Recently, interesting work has been published on the delivery of poorly water-soluble potassium channel opening drug UK-157,147. Here the drug was encapsulated inside ethyl cellulose: sodium glycocholate/poly[methyl methacrylate-co-(fluorescein-co-methacrylate)] nanoparticles (Morgen et al. 2011). Encapsulation efficiencies of up to 90 % were achieved. In vitro drug release studies showed that drug was rapidly released and the formulation was stable over a 3-month period. In vitro studies were carried out in order to determine the targeting effect of the nanoparticulate formulation after dermal application. In vitro imaging was carried out on rabbit ear tissue treated with fluorescent nanoparticles (with Nile red tag) and the images showed that the nanoparticles achieved delivery to the hair follicles (Fig. 14.7). A subsequent study was carried out in vivo and the study showed that delivery was achieved to the sebaceous glands after topical administration of the formulation. This work demonstrates nanoparticle-based drug delivery to the hair follicles and sebaceous glands on topical administration (Morgen et al. 2011).

The absorption of nano-sized delivery systems across membrane barriers has not yet been fully understood. There are many proposed mechanisms of cellular uptake

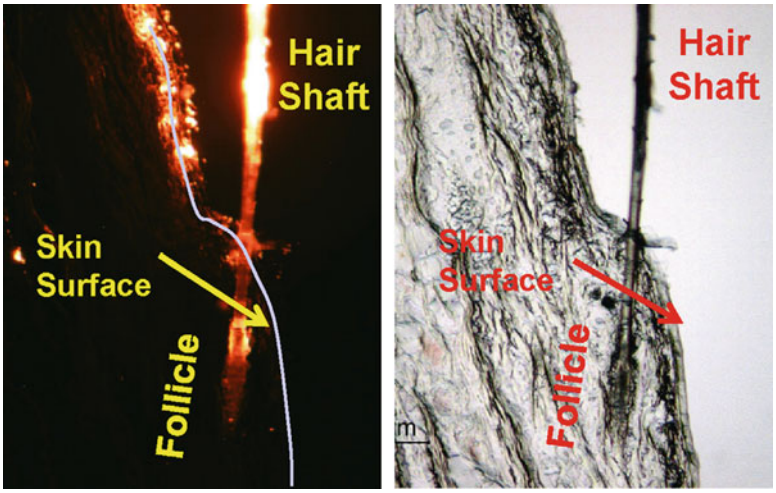


Fig. 14.7 Fluorescent (*left*) and bright-field (*right*) microscopy images of rabbit ear tissue incubated for 2 h with 19:1:0.6 ethyl cellulose: MEH-PPV: NaGC fluorescent nanoparticles (Morgen et al. 2011)

of these systems such as via non-specific endocytosis, ligand-mediated endocytosis or paracellular transport. A few key assays have been developed in order to determine the in vitro fate of nano-sized particles (Li 2005). In vitro intracellular trafficking is used to determine the mechanism of cellular uptake in the cell to visualise exactly where the drug and vehicle reside (Savic et al. 2003). This can be carried out by staining the organelles in the cell, e.g. the nucleus, cell membrane and mitochondria. Savic et al. have performed an extensive investigation of cellular uptake of polymeric micelles incorporated with a dye. They incorporated the hydrophobic membrane-selective dye—diaminofluorescein (DAF), into fluorescent tetramethylrhodamine-5-carbonyl azide-poly(caprolactone)-block-poly(ethylene oxide) (TMRCA-PCL-b-PEO) polymeric micelles and tracked their cellular internalisation in PC12 cells (Savic et al. 2003). Figure 14.8 shows that the cellular distribution of the micelles (Fig. 14.8b) and the incorporated agent (Fig. 14.8c) were different. The green colour in Fig. 14.8d shows the localization of DAF and the yellow shows the colocalization of the DAF with the TMRCA-PCL-b-PEO micelles. These images suggest that the PCL-b-PEO micelles were able to passively target the PC-12 cells as well as alter the subcellular distribution of the incorporated agent (Fig. 14.8d, h). The authors proposed that once internalised the dye was released from the micelle carrier; additionally some of the block copolymer micelles broke down into unimers and acted locally to permeate the membrane of cellular organelles. Overall an increase in incorporated agent efficacy was observed (Savic et al. 2003).

The use of a range of inhibitors such as sodium azide (metabolic inhibitor) or cytochalasin D (endocytosis inhibitor) is important to determine the mechanism of cellular uptake, e.g. endocytosis or active transport or a different type of transport (Maysinger et al. 2007; Mathot et al. 2007). The Caco-2 cell monolayer is an

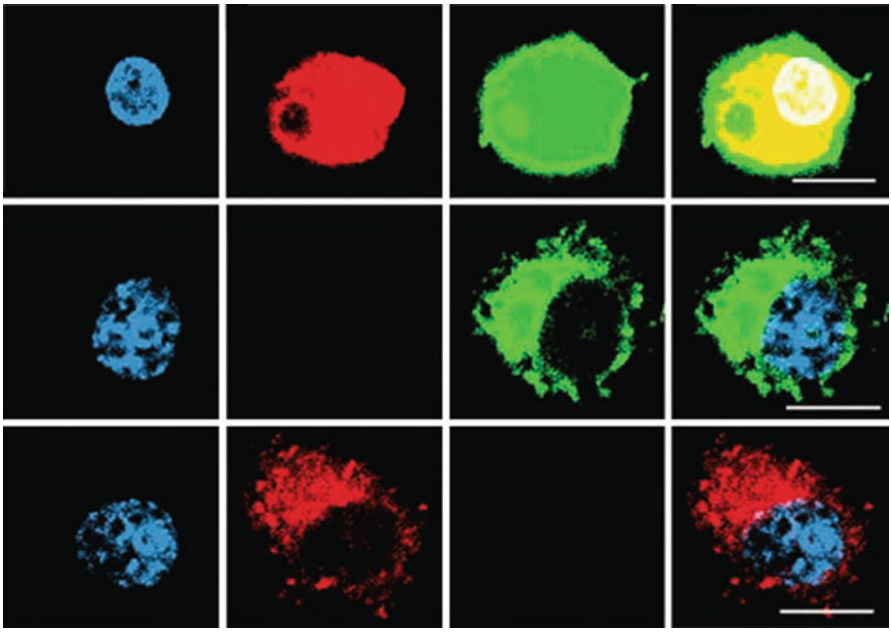


Fig. 14.8 Increased internalisation of DAF in micellar form. PC12 cells were incubated for 24 h in the presence of either (**a**–**d**) TMRCAPCL-b-PEO micelles that contained DAF, (**e**–**h**) free DAF or (**i**–**l**) TMRCAPCL-b-PEO micelles with no DAF. Scale bars = 10 μm (**a**, **e** and **i**). Nuclei stained with Hoechst 33342. (**b**) TMRCAPCL-b-PEO micelles with DAF. (**c**) DAF incorporated in TMRCA-PCL-b-PEO micelles. (**d**) An overlay of (**a**–**c**). (**f**) Control for bleed-through of *green* fluorophore into the *red* channel. No DAF was detected under settings identical to those used in (**b**) and (**j**) for imaging of the TMRCA-PCL-b-PEO micelles. (**g**) Free DAF. (**h**) An overlay of (**e**–**g**). (**j**) TMRCA-PCL-b-PEO micelles with no DAF. (**k**) Control for bleed-through of *red* fluorophore into the *green* channel. No TMRCA was detected under settings identical to those used in (**c**) and (**g**) for imaging of the free DAF. (**l**) An overlay of (**i**–**k**) (Savic et al. 2003)

in vitro cell culture model commonly used to study the transport of nano-systems across gut enterocytes. This method commonly evaluates if paracellular transport is involved in the absorption pathway. Additionally, canine kidney cells (MDCK) also develop tight junctions and can be used to study paracellular absorption routes (Li 2005). MDCK cells may also be transfected to express transporters to incorporate the efflux mechanism (Li 2005). Mathot studied the absorption pathway of monomethylether poly(ethyleneglycol)750-poly(caprolactone-co-trimethylene carbonate) (mmePEG750P(CL-co-TMC)) across the intestinal barrier (Mathot et al. 2007). The polymer was radiolabelled before carrying out a Caco-2 mono layer study by disrupting tight junctions and by inhibiting endocytosis. The unloaded polymer and polymers loaded with model drugs such as carbamazepine and chlorothiazide formed micelles which independently crossed the Caco-2 cell monolayer. Neither the paracellular route nor M-cells were used as a mechanism. The polymer was also shown to have no effect on the P-gp pump. Mathot suggested that the

drug-loaded micelles were absorbed by fluid-phase endocytosis, whereas the polymeric unimers diffused passively across the membrane concomitantly with micellar endocytosis (Mathot et al. 2007).

14.3.1.4 Initial In Vivo Evaluations

Initial in vitro evaluations are carried out in order to gain an understanding of the pharmacokinetic and pharmacodynamic properties and toxicity of the drugs and their delivery systems. These are often first carried out using small groups on small animals (typically rodents) and the data provides essential information before the formulations can be used in more comprehensive preclinical studies and human studies. Liposomes have been around longer than most of the other nano-systems for drug solubilisation. As such initial in vitro studies have been carried out on a number of liposome–drug combinations. Recently, pegylated liposomes prepared from cholesterol, lethicin and PEG reportedly increased therapeutic efficacy of cytochalasin D (CytD) (Huang et al. 2012). CytD is a cytotoxic agent used in chemotherapy. Its low aqueous solubility and high occurrence of severe side effects result in a drug which requires careful formulation before use. Huang and colleagues administered the drug-liposome formulation (CytD-PEGL) intravenously to tumour bearing mice [C57BL/6 N inbred mice injected with melanoma (B16) and BALB/c albino mice injected with colorectal carcinoma (CT26) and hepatoma (H22) cells]. They reported that CytD-PEGL was capable of increased accumulation in tumour cells when compared with the free drug. The liposomal formulation also experienced increased circulation times and significantly increased antitumour activity which was comparable with clinically used cisplatin (Huang et al. 2012).

Polymeric micelles formed by block copolymers have been widely studied for their in vitro potential in drug delivery via both the oral and parenteral administration routes. Iqbal et al. fabricated a poly(acrylic) acid-block-cysteine amphiphile to encapsulate the hydrophobic paclitaxel (Iqbal et al. 2011). After oral administration into Sprague Dawley male rats, the formulation was capable of increasing blood plasma concentration up to 7.3-fold in comparison to the free drug (Iqbal et al. 2011). Other studies involving the formulation of paclitaxel in polymeric micelles formed by triblock copolymers (poly(ethylene oxide)-b-poly(propylene oxide)—PEO-PPO-PEO) have also shown exciting in vitro activity (Zhang et al. 2011b). Here the PEO-PPO-PEO amphiphiles were loaded with paclitaxel (10 mg/Kg) and administered intravenously to C57BL/6 xenograft mice bearing subcutaneous tumours. The total plasma drug concentration was found to be up to 2.48 times higher than those administered with clinically used Taxol® (10 mg/Kg) ($p < 0.05$). The authors speculated that this increase in drug concentration could be due to the prolonged circulation times and the enhanced permeability and retention (EPR) effect. Consequently, a significant delay in tumour growth was observed with attendant increased survival rates (Zhang et al. 2011b).

Limited in vitro studies have been carried out using polymeric micelles formed by hydrophobically modified polymers, compared to amphiphilic block copolymers. Recently, poly(allylamine) (PAA) modified with cholesterol (CH5) and dansyl

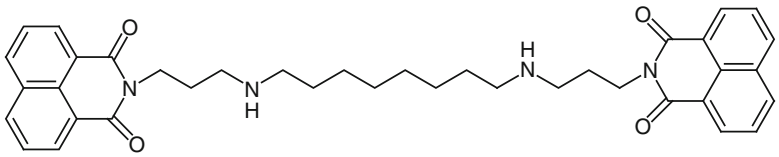


Fig. 14.9 Chemical structure of BNIPDaoct

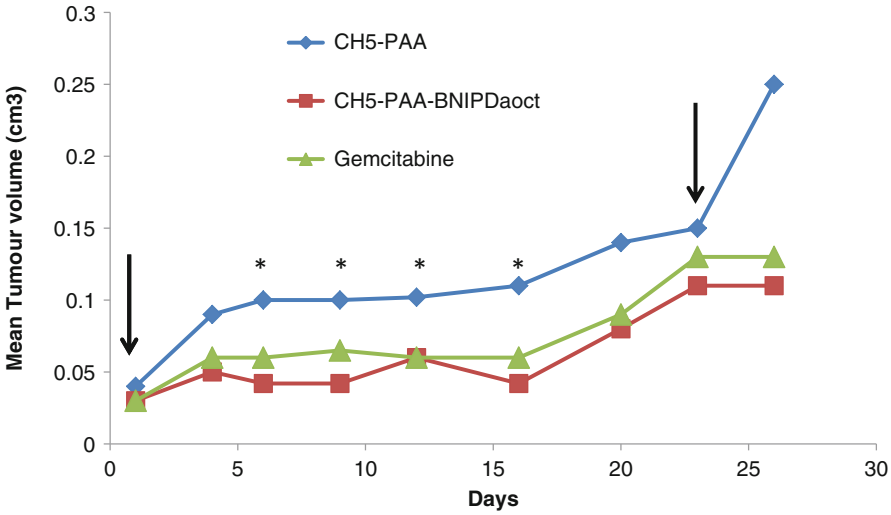


Fig. 14.10 Scatter plot of mean tumour volume in mice implanted with BxPC-3 cell line and treated with CH5-PAA ($n=6$), CH5-PAA-BNIPDaoct formulation ($n=13$) or gemcitabine ($n=11$). Treatment was started 2 weeks after implantation of the xenograft, when tumour was palpable. Error bars = s.d. and if not visible are obscured by the data point. *$p<0.05$, CH5-PAA-BNIPDaoct formulation vs. CH5-PAA; **$p<0.05$, CH5-PAA-BNIPDaoct formulation vs. gemcitabine. *Arrows* indicate first and last injection (Hoskins et al. 2010)

(DANSYL10) pendant groups have shown the ability to increase griseofulvin uptake in vivo into blood plasma after oral administration (Hoskins et al. 2010). Both the CH5 and DANSYL10 formulations of griseofulvin significantly enhanced the drug absorption in rats compared with an aqueous formulation ($p<0.001$) without any adverse side effects (Hoskins et al. 2010). Additionally, CH5 was also investigated for the delivery of the novel anticancer agent bisnaphthalamido propyl-diaminooctane (BNIPDaoct, Fig. 14.9) via peritoneal injections in xenograft-bearing mice.

The nude xenograft-bearing mice were treated over a 4-week period in a pancreatic cancer model. The novel formulation showed a similar response to clinically used gemcitabine in that there was a retardation of tumour growth using an eightfold less dose (Fig. 14.10) (Hoskins et al. 2010). This study demonstrated the success of using a nano-system to improve the solubility of a novel, practically insoluble drug which otherwise would be unable to be tested in vitro.

Table 14.5 Importance of various evaluations in each developmental stage of a nano-system for cancer therapy (Yokoyama 2010)

Stages/relative importance	Initial in vitro study	Later in vitro study	Clinical trials
In vivo anticancer activity	Very important	Very important	Critical
Pharmacokinetic profile	Very important	Critical	Moderately important
Toxicity	Moderately important	Very important	Critical

A nano-crystal suspension of MA in poloxamer was administered orally to male Sprague Dawley rats (5 mg/kg). The data showed that the bioavailability of the nano-crystal suspension was significantly improved compared to the coarse MA suspension with the AUC0-24 h more than doubling. This work highlights the impact of nano-crystals on oral delivery (Sylvestre et al. 2011). In pulmonary drug delivery, smaller particles are able to be delivered more effectively deep into the tissues of the lungs; once at the target site their increased dissolution aids in their absorption across mucosal membranes. Baicalein is a bioactive flavonoid naturally occurring in the roots of *Scutelleria baicalensis* (Zhang et al. 2011a). It possesses anti-inflammatory and anti-proliferative activity; however, it is poorly absorbed. Nano-crystals of baicalein have been reported for the pulmonary delivery of the drug. Here the data showed that after pulmonary delivery of baicalein nano-crystals dispersed in water (4.4 mg/mL) to male Sprague Dawley rats (10 mg/kg), the drug nano-crystal exhibited rapid absorption and was comparable to an intravenous formulation. These results show the potential of using nano-crystal suspensions for pulmonary drug delivery (Zhang et al. 2011a).

As mentioned in the earlier sections, in contrast to conventional excipients which generally only enhance the solubilisation of hydrophobic drugs, the use of nano-systems (apart from nano-crystals) has additional benefits of achieving site-specific delivery especially for cancer therapy. As a result, the vast literature on nano-systems for hydrophobic drug delivery typically focuses on cancer therapy. For the subsequent sections in this book chapter, we will mainly look at the use of these nano-systems in cancer therapy.

Once activity has been established with the cancer drug nanoformulations, for example, further studies typically examine the anticancer activity in a greater variety of cancer models. Additionally, extensive pharmacokinetic evaluation and toxicity studies are conducted. The toxicity studies involve various animal models and are aimed at gathering acute toxicity and chronic (repeat dose) toxicity on the experimental medicine. Yokoyama illustrates the importance of evaluating the pharmacodynamic activity, pharmacokinetic activity and toxicological evaluations during the stages of polymeric micelle formulation development for example (Table 14.5) (Yokoyama 2010). He reveals the efficacy, i.e. anticancer activity is more important in the initial in vivo studies than in the later in vivo studies, while toxicity tests are significant during the later preclinical and early clinical phases of development. However, Yokoyama emphasises the importance of the toxicity evaluations even in the initial in vivo studies, if one is to ensure a successful clinical outcome. For example, initial in vitro studies often use a maximum tolerated dose that is not lethal to experimental animals. However, unlike the homogeneity of experimental

animals, human patients have varied genetic characteristics and are also physiologically different from the animal models. Therefore, a mild toxic effect observed in an initial in vivo study might be manifested as a severe toxicity in some patients or a toxicological effect may not be observed in preclinical studies, but could be observed in early clinical studies. Although not an example of a hydrophobic drug, this phenomenon is illustrated by the severe side effect: palmar-plantar syndrome (skin eruptions on the hands and the feet), observed in patients when using doxorubicin liposomes for example. However, this side effect was not a dose-limiting toxic effect in any of the preclinical mouse models.

14.4 Clinical Experience

To date, there are a number of nanotechnologies for hydrophobic drug solubilisation which have been approved for clinical use while a few are in various clinical trial stages (Table 14.6). For cancer therapy, polymeric micelles are more advanced for the delivery of hydrophobic drugs than other types of nano-systems and we will look at these systems in this section.

The clinical trial Phase I data to date has demonstrated that most of the polymeric micelles formulations (those listed in Table 14.6) rapidly release the drugs upon intravenous administration where the concentrations of the drug in the blood and pharmacokinetic behaviour are similar to the free drug (Yokoyama 2010). However, these micellar formulations exhibit a number of benefits compared to conventional formulations. For example, Genoxol-PM containing Taxotere is superior to Taxol®. The reasons being that Taxol consists of Cremophor EL surfactants which cause toxic side effects in susceptible patients, while the poly(ethylene glycol)-b-poly(DL lactide) block copolymer is an effective solubiliser with a non-toxic profile. These Phase I clinical studies demonstrated the toxicity profile of these polymeric micellar formulations is similar to the free drugs, which is possibility due to the result of drug release from the carriers into the bloodstream. As Yokoyama 2010 pointed out that we need to wait for the final results of Phase II and Phase III clinical trials to know if polymeric micelles are going to be valuable in chemotherapy; however, based on the Phase I results, it is clear that these solubilisers are non-toxic and effective in enhancing the solubility of the hydrophobic anticancer drugs.

14.5 Conclusions and Future Outlook

As shown in Table 14.5, liposomes were the earliest nanotechnology products approved in late 1990s after their discovery in 1965, while the history of other nanotechnologies is relatively shorter than liposomes. Within the next 10 years it is expected that many clinical trials using nano-sized formulations will be completed and their use in the clinics should improve treatments and the patient's quality of life. The development of biocompatible polymers/materials for the production of

Table 14.6 Types of nanotechnology products approved on the market/in clinical trials

Type of nano-systems	Product	Hydrophobic drug	Indication	Route of delivery	Technology by/licenced to	Year of approval/progress in the clinical trials
Nano-crystals	Rapamune	Sirolimus	Immunosuppressant	Oral	Elan/Wyeth	2000
Nano-crystals	Emend	Aprepitant	Antiemetic	Oral	Elan/Merck	2003
Nano-crystals	Tricor	Fenofibrate	Treatment of high cholesterol and high triglycerides	Oral	Elan/Abbott	2004
Nano-crystals	Megace ES	Megestrol acetate	Palliative treatment	Oral	Elan/Par Pharm	2005
Nano-crystals	Triglide	Fenofibrate	Treatment of high cholesterol and high triglycerides	Oral	SkyePharma/First Horizon Pharmaceuticals	2005
Nano-crystals	Invega sustena	Paliperidone palmitate	Treatment of schizophrenia	IV	Elan/Johnson and Johnson	2009
Liposomes	Ambisome	Amphotericin B	Severe fungal infections	IV	Gilead	1990
Liposomes	Abelcet	Amphotericin B	Severe fungal infections	IV	Cephalon	1995
Liposomes	Vidusyne	Verteporfin	Age-related molecular degeneration	IV	Novartis AG/QLT	2000
Liposomes	Depocyt	Cytarabine	Neoplastic meningitis and lymphomatous meningitis	Epidural	Napp	2002
Polymeric micelles	NK-10S	Paclitaxel	Cancer targeting	IV	Nippon Kayaku Co, Japan	Phase I
Polymeric micelles	NK-012	SN-38	Cancer targeting	IV	Nippon Kayaku Co, Japan	Phase II
Polymeric micelles	Genexol-PM	Paclitaxel	Solubilisation	IV	Samyang Corp, Korea	Phase II

This list is not exhaustive Hoskins and Cheng (2012b)

nano-carriers, accompanied by advanced techniques to study the cellular uptake mechanisms and the fate of nano-carriers, will fuel greater exploitation of nanotechnology in drug delivery. Additionally, newer generations of these nano-systems, in particular polymeric micelles and liposomes, have been designed to precisely control the release of the drugs based on stimuli release mechanisms. In the future, these new excipients could be part of the decision tree in the development and formulation of new or existing hydrophobic chemical entities.

Problem Box

1. What are the benefits of using nanotechnologies for hydrophobic drug solubilisation?

   Answer: The benefits are (1) higher encapsulation with less excipient, (2) multiple drug encapsulation, (3) improvement of drug permeability, useful for class IV drugs, (4) versatility with the capacity to be modified to achieve targeting and other desirable properties.

2. A pharmaceutical company has a novel BCS class II drug. As a formulation scientist, you are asked to formulate this drug in polymeric micelles. Describe the delivery system and evaluate the use of this system to improve the solubility of the drug.

   Answer: Polymeric micelles are formed spontaneously by amphiphilic polymers which consist of hydrophobic and hydrophilic segments, for example amphiphilic block copolymers or hydrophobically modified water-soluble polymers. In aqueous environments, a hydrophobic core is formed spontaneously upon aggregation of the hydrophobic moieties, while the hydrophilic segments will remain in contact with the aqueous environment. This BCS class II drug will be encapsulated within the hydrophobic core of the polymeric self-assemblies, mainly via non-covalent interactions. There are a number of advantages using this delivery system including (1) ability to optimise loading and release characteristics; (2) low chronic toxicity; (3) protection of incorporated drugs; (4) ease of fabrication/functionalization and (5) low excipient:drug ratio. However, compared to other nano-size delivery systems, polymeric micelles might be unstable upon dilution or on storage.

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Part III
Therapeutic and Diagnostic Applications

Chapter 15
Cancer Chemotherapy

Hideaki Nakamura and Hiroshi Maeda

Abstract The enhanced permeability and retention (EPR) effect is the first essential step for selective delivery of macromolecular drugs to tumor tissues. The EPR effect is based on the aberrant architecture of tumor blood vessels and the impaired lymphatic drainage system in tumor tissue. This effect is facilitated by overproduction of multiple vascular mediators such as bradykinin, nitric oxide, prostaglandins, vascular endothelial growth factor (VEGF), and other cytokines in tumor tissue, which may also affect surrounding normal tissues. The biocompatibility, molecular size, and surface charge of macromolecular drugs, i.e., nanomedicines, are critical determinants of tumor-targeted drug delivery based on the EPR effect. However, ineffective treatment can result from the heterogeneity of the EPR effect in tumor tissues, which impedes drug delivery to some tumors. In this chapter, we also discuss how to overcome this problem by using specific therapeutic methods, such as angiotensin (AT) II-induced high blood pressure, angiotensin-converting enzyme inhibitors, nitric oxide-releasing agents, tumor necrosis factor-α, transforming growth factor-β, and heme oxygenase-1 inducer, some of which were demonstrated to be effective in clinical settings.

Abbreviations

ACE	Angiotensin-converting enzyme
AT-II	Angiotensin II
BSA	Bovine serum albumin
CO	Carbon monoxide

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COX	Cyclooxygenase
CT	Computer topography
EPR	Enhanced permeability and retention
HIF-1α	Hypoxia-inducible factor 1 alpha
HO-1	Heme oxygenase-1
HPMA	Hydroxypropyl methacrylamide
ISDN	Isosorbide dinitrate
IgG	Immunoglobulin G
NADPH	Nicotinamide adenine dinucleotide phosphate
NCS	Neocarzinostatin
NG	Nitroglycerin
NO	Nitric oxide
NOS	Nitric oxide synthase
PEG	Polyethylene glycol
SCID	Severe combined immune deficiency
SEM	Scanning electron microscopy
SERCA	Sarcoplasmic/endoplasmic reticulum ATPase
SMA	Styrene maleic acid
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

15.1 Introduction

One primary goal of cancer drug development is to produce safe and effective drugs. At the end of the nineteenth century, Paul Ehrlich proposed the concept of a "*magic bullet*," that is, a drug should selectively target pathogens and not harm normal cells of a host. The discovery of antibiotics successfully advanced this concept, because most antibiotics target specific molecules that are unique to bacteria and do not occur in mammalian cells or tissues. Most antibiotics are quite safe and have a large therapeutic safety window. Extension of Ehrlich's concept led to the development of molecular-targeted anticancer drugs. Since the 1990s, many attempts were made to develop such drugs, to affect only tumor-specific kinases or receptors. As part of this strategy, focusing on monoclonal antibodies that target such epitope molecules became a mainstream trend. However, recent clinical data related to these molecular drugs revealed that this approach, despite a significant prolongation of survival in a few patients in clinical settings using such agents as imatinib, has limitations and is far from a panacea for cancer treatment (Fojo and Grady 2009; Editorial 2008; Mayer 2009; Sinha 2008; Tol et al. 2009). In a similar vein, cancer treatment by means of vaccines has been extensively investigated by using multiple cancer antigen-specific peptidyl epitopes based on certain multiple motifs. However, recent multi-institutional phase II/III studies of 153 patients with pancreatic cancer that were carried out in Japan yielded no significant benefits of cancer vaccine treatment (Sankei Shinbun 2012).

Most solid tumors diagnosed in patients are essentially polyclonal in origin. In the last 10–20 years, these tumors have evolved extensive genetic diversification, as demonstrated by recent results for technological advances in cancer genomics. For example, Maeda and Akaike et al. showed excessive generation of reactive oxygen species and reactive nitrogen species during infection and inflammation (Oda et al. 1989; Akaike et al. 1990, 1996, 2003; Yoshitake et al. 2004; Kuwahara et al. 2009), and this excessive generation resulted in the formation of mutants or drug-resistant bacteria accompanying damage to the RNA or DNA, or DNA repair systems. Consequently, Sjöblom et al., Wood et al., and others reported highly diversified genetic polymorphism in the human cancer patients (Sjöblom et al. 2006; Wood et al. 2007).

These data are in clear contrast to those for the experimental severe combined immunodeficiency (SCID) mouse tumor model, which utilizes a human tumor xenograft that does not elicit a host reaction (immunological or inflammatory reaction) and produces no reactive oxygen species, and thus no mutation such as genetically altered epitopes or genetic polyclonality is expected. In this content, strategies using molecular-targeted drugs such as monoclonal antibodies, however, would not be as effective as hoped because human cancers do indeed produce such genetically altered epitopes or genetic polyclonality. In fact, the therapeutic benefit for patients treated with these molecular-targeted drugs is usually an extension of a survival time to at most 1–2 months on the 3–5 years of expected overall survival of untreated controls or those given conventional anticancer agents (Mayer 2009; Sinha 2008; Tol et al. 2009), in addition to the high cost of such drugs (Editorial 2008; Mayer 2009).

In view of these data, anticancer drugs with greatly improved efficacy and affecting more broad cancer types are clearly needed. Development of macromolecular or nanoparticle-based drugs, which utilize the enhanced permeability and retention (EPR) effect for selective drug delivery to tumors, will meet such requirements.

15.2 Background and Characteristics of the EPR Effect

In 1986, Matsumura and Maeda (1986) originally discovered the EPR effect, in which plasma proteins with large molecular weight, such as albumin (65 kDa), transferrin (75 kDa), and IgG (160 kDa), exhibited tumor-tropic accumulation (Fig. 15.1), whereas small proteins such as ovomucoid (29 kDa) and neocarzinostatin (NCS, 12 kDa) did not exhibit this phenomenon. Later, Maeda et al. clearly demonstrated the EPR effect by using a synthetic biocompatible polymer of N-(2-hydroxypropyl) methacrylamide (HPMA) larger than 40–800 kDa (Noguchi et al. 1998; Seymour et al. 1995; Maeda 2001a; Fang et al. 2011) (Fig. 15.2a). This unique feature of the EPR effect in tumor tissue is based on the defective nature of tumor blood vessels, which have an aberrant architecture and are highly permeable, and on the impaired lymphatic drainage system of tumor tissue (see discussion below). To manifest the EPR effect, anticancer drugs must satisfy specific criteria such as biocompatibility, molecular size range, and surface charge, as described below.

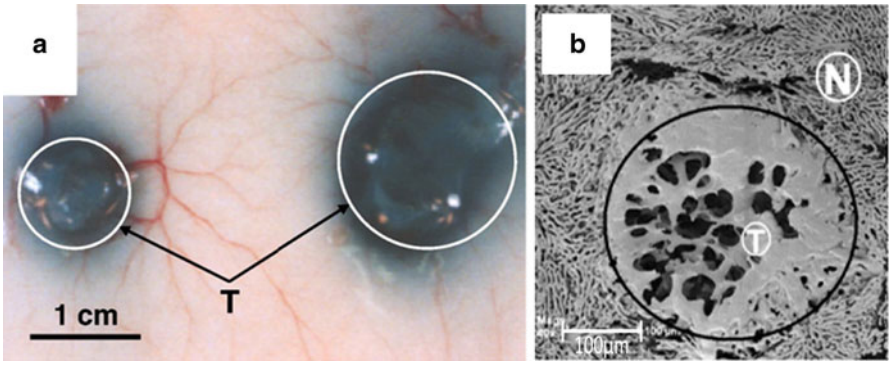

Fig. 15.1 Illustration of the EPR effect. (**a**) Tumor-selective accumulation of the putative macromolecular drug Evans blue-albumin complex (MW 67 kDa). The *blue color* in the macroscopic image indicates macromolecular drug delivery to S-180 tumor implanted in the skin of mice at 24 h after i.v. injection of Evans blue (10 mg/kg). The tumor sites (T, *circles*, and *arrows*) show progressive accumulation of Evans blue-albumin, in both small and large tumors. (**b**) Scanning electron microscopic image of metastatic liver cancer. The tumor (T, *circle*) is a micrometastatic tumor nodule; even this small nodule shows leakage of a polymer (polyarylate), which is not seen in the surrounding normal tissue (N, in the liver) (adapted from Maeda 2010a). Dewhirst et al. showed that tumor angiogenesis becomes visible when 100–300 tumor cells present (Li et al. 2000)

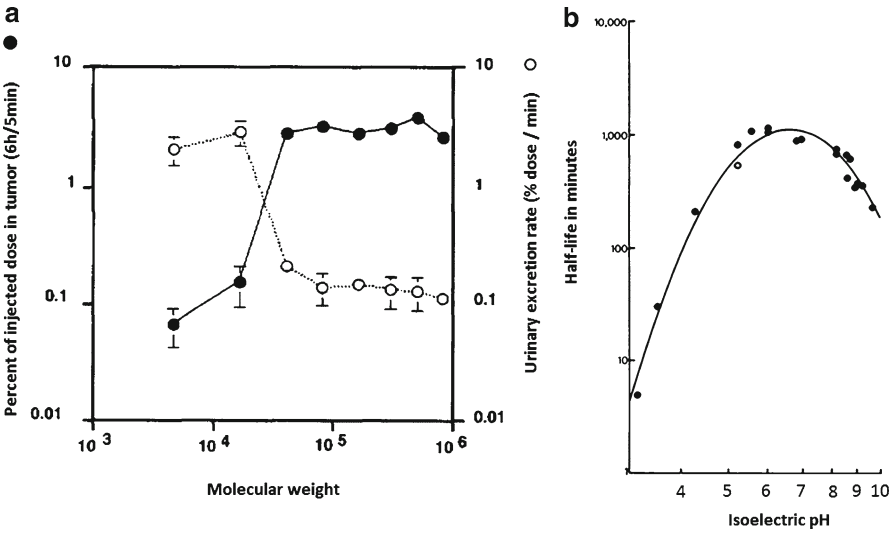

Fig. 15.2 Influence of the size and charge of macromolecules on their distribution in tumors and plasma concentration. (**a**) HPMA copolymers, labeled with ^{125}I and of different sizes, were injected i.v. into tumor-bearing mice. The percentage of the injected dose of HPMA in tumor and in urine was calculated (adapted from Noguchi et al. 1998; Maeda 2010a, 2012a). (b) L-Asparaginase derivatives (MW 120 kDa) with different isoelectric points (pI) after chemical modification were injected i.v. into rabbits (2,500 IU/kg), after which the remaining activity of each L-asparaginase derivative was measured and their half-life values in systemic circulation were calculated (adapted from Rutter and Wade 1971)

Table 15.1 Characteristics of the EPR effect of nanomedicine or macromolecular drugs

Biocompatibility	No interaction with blood components or blood vessels, no antigenicity, no clearance by the reticuloendothelial system, no cell lysis
Molecular size	Greater than 40 kDa (larger than the renal clearance threshold)
Surface charge	Weakly negative to near neutral
Time required to achieve	Longer than several hours in systemic circulation in mice
Drug retention time	Mostly days to weeks, in great contrast to passive targeting (in which low-MW molecules are rapidly cleared into the systemic circulation in a few min cf. low molecular weight contrast agent (see text)

That the EPR effect does not occur in a few minutes, as does passive targeting, should be emphasized; macromolecules must remain in circulation for several hours, and drug retention by tumor tissue occurs in days to weeks. Passive targeting, however, can last a few minutes, as seen by means of tumor angiography with low molecular weight (MW) contrast reagents.

This unique, universal phenomenon—the EPR effect—which occurs in most solid tumors is believed to be the first requirement before the uptake of drugs by cancer cells, in the development of tumor-targeted nanomedicines. Table 15.1 presents the molecular criteria required for the EPR effect to occur in solid tumors.

15.2.1 Factors Involved in Enhanced Vascular Permeability and the Reason for Retention of Macromolecules in Tumor Tissue

Enhanced vascular permeability in tumor tissue is a key factor contributing to the accumulation of macromolecules in the tumor. Elevated vascular permeability was originally discovered in inflamed tissue: extravasation of plasma protein in inflamed tissue is now known as edema. We also know that tumor tissue and inflamed tissue share many common parameters, which lead to the elevated vascular permeability (Maeda 2001a, 2010a, 2012a; Fang et al. 2011; Maeda et al. 2003, 2009; Wu et al. 1998). Enhancement of vascular permeability in both tumor and normal tissue types is mediated by various factors including bradykinin, nitric oxide (NO), prostaglandins, vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), heme oxygenase-1 (HO-1), which generates carbon monoxide (CO), and others (Fang et al. 2011, 2012; Maeda et al. 2003, 2009; Wu et al. 1998; Maeda 2010a, 2012a). These factors open the intercellular gap in blood vessels and facilitate extravasation of plasma components and other macromolecules into the tissue interstitium. Leaky blood vessels in tumors can be readily seen with the use of a scanning electron microscope, which can show vascular casts of extravasated polymer resin in microtumor nodules less than 0.2 mm in diameter (Fig. 15.1b) (Maeda 2010a, 2012a; Konerding et al. 1995). Leaky tumor vessels are observed even in

tumors larger than 10 mm in diameter (Fig. 15.1a) (Maeda 2012a). The EPR effect is thus seen in most solid tumors, and it ensures a sufficient supply of nutrients for rapidly growing tumors.

Aberrant vascular architecture including unusually large pores in the vessel walls of many tumor blood vessels, which range from 200 nm to 2 μm or larger (Konerding et al. 1995), contributes to extravasation of large nanoparticles. Hashizume et al. reported similar findings (Hashizume et al. 2000). Bacteria such as *Lactobacillus* and *Salmonella* spp. also reportedly accumulated preferentially in tumor nodules in mice (Kimura et al. 1980; Zhao et al. 2005; Hoffman 2009).

Another key factor contributing to the EPR effect is impairment of the lymphatic drainage system in tumor tissue, which is the primary reason for prolonged retention of macromolecules in tumor tissue. Lymphatic drainage is the main route of clearance of macromolecules or lipid particles from the tissue matrix or the interstitial space of normal tissues (Papillon et al. 1963). For instance, Lipiodol® (iodinated poppy seed oil) is used as a contrast agent for lymphangiography (imaging of lymphatic ducts and networks), because Lipiodol is largely recovered via the lymphatic system in normal tissues, and its presence is evidence of lymphatic vessels, which can be visualized with X-ray imaging (Papillon et al. 1963; Konno et al. 1983, 1984; Maki et al. 1985; Iwai et al. 1984). Once Lipiodol is delivered to tumor tissues, it remains there for a prolonged time (Konno et al. 1984; Maki et al. 1985; Iwai et al. 1984).

15.2.2 Biocompatibility, Molecular Size, and Surface Charge of Macromolecules and the EPR Effect

15.2.2.1 Biocompatibility

(1) *Plasma proteins and plasma residence time*. One of the most important factors that govern the EPR effect is the biocompatibility of macromolecules. We demonstrated earlier that even the most biocompatible plasma proteins, which have very long plasma circulation times (more than weeks), would lose this characteristic after chemical modification. Also, in vivo examination of the circulation time of proteins that had originated in different species (xenogenous proteins) showed that macrophages or scavenger receptors identify these proteins as nonself proteins, capture them, and clear them rapidly. Interaction of a protease and an inhibitor, such as plasmin and α_2-macroglobulin, respectively, causes a great reduction in the plasma residence time ($t_{1/2}$), from about 140 h to a few minutes (Tables 15.2 and 15.3). These findings mean that not only the size of the macromolecules but also their affinity to the host, or biocompatibility, controls the residence time in plasma during circulation.

(2) *Conjugation with polyethylene glycol (PEG) and other polymers*. Conjugation of proteins with PEG, or pegylation of proteins, is one way to overcome the disadvantage of rapid clearance of macromolecules or foreign proteins. PEG is nonadhesive, nonimmunogenic, and usually nonantigenic, and it confers high solubility

Table 15.2 Plasma clearance times of selected modified and native proteins in vivo

Protein	Species difference, original/test animal	Probe modification	pI[a]	MW (kDa)	$t_{1/2}$[b]	Note	Ref.
• Albumin	Mouse/mouse	None	4.8	68	72–96 h	Native, syngeneic	Maeda et al. (1986, 2003)
Albumin	Mouse/mouse	DTPA (^{51}Cr)[d]	≦4.8	–	6 h	Slightly surface-modified, loss of amino group, syngeneic	
Albumin	Cow/mouse	DTPA (^{51}Cr)[d]	≦4.8	–	1 h	Slightly surface-modified, loss of amino group, xenogeneic	
Formaldehyde-modified albumin	Human/rat	Formaldehyde ^{125}I	≦4.8	–	25 min	Denatured, loss of amino group, xenogeneic	
• α_2-Macroglobulin	Human/mouse	^{125}I	5.3	180×4	140 h	Native, xenogeneic	Maeda et al. (1986, 2003)
α_2-Macroglobulin-plasmin complex	Human/mouse	^{125}I	–	180×4	5 min	Inhibitor-protein complex, xenogeneic	
• Immunoglobulin (IgG)	Mouse/mouse	DTPA[d]	≦6.8	159	60 h	Slightly surface-modified, loss of amino group, syngeneic	Maeda et al. (1986, 2003)
• Interferon α	Human/human	None		18	8 h (sc)[e]	$t_{1/2}$ 4 min	Maeda et al. (2003)
Pegylated interferon α2a	Human/human	PEG		52	80 h (sc)[e]		
• Adenosine deaminase (ADA)	Cow/mouse	None	4.9	38	<0.5 h	Native, xenogeneic	Davis et al. (1981)
Pegylated ADA	Bovine/mouse	PEG[d]	–	>38	28 h, 3–6 days in humans	60 % of primary amine conjugated with PEG (5,000 Da), xenogeneic	
• Arginine deiminase (ADI)	*Mycoplasma hominis*/mouse	Native	5.5	46	<5 h	Native, xenogeneic	Ensor et al. (2002)
Pegylated ADI	*M. hominis*/mouse	PEG[d]	–	>46	~7 days	10–12 PEG (20,000 Da) attached to each ADI, xenogeneic	
• Bilirubin oxidase, native[c]	Microbial/mouse			52	0.25 h		Maeda et al. (1992), Kimura et al. (1988)
Bilirubin oxidase PEG conjugate	Microbial/mouse			110	5 h		

(continued)

Table 15.2 (continued)

Protein	Species difference, original/test animal	Probe modification	pI[a]	MW (kDa)	$t_{1/2}$[b]	Note	Ref.
• D-Amino acid oxidase native	Pig/mouse			39	14 h		Fang et al. (2002)
D-Amino acid oxidase PEG conjugate	Pig/mouse			63	36 h		
• Neocarzinostatin (NCS)	*Streptomyces*/ mouse	DTPA (^{51}Cr)[d]	3.4	12	1.8 min	Slightly surface-modified, loss of amino group, xenogeneic	Maeda et al. (1986, 2003)
SMA-conjugated NCS (SMANCS)	*Streptomyces*/ mouse	DTPA(^{51}Cr)[d], SMA	>3.0	17	19 min	Two chains of SMA (1,200 Da) attached to each amino group of NCS; SMA is polyanionic, xenogeneic	

[a]Isoelectric point
[b]Half-life in systemic circulation (minutes, hours, or days), given i.v. unless otherwise stated
[c]From microbe, *Myrothecium verrucaria*
[d]DTPA or PEG was reacted with the primary amino group of a lysine residue or N-terminal residue, which made the group much less cationic
[e]Subcutaneous

Table 15.3 Selected parameters affecting plasma residence times of different nanoparticles

Type of nanoparticle	Test animal	ζ potential (mV)	Mean particle size (nm)	Plasma residence time		Remarks	Ref.
				$T_{1/2}$	$T_{1/10}$		
• Liposome (nonpegylated)	Mouse	−7.31	124	9.08 h	>24 h	Doxorubicin loaded, DPPC:Chol = 1:1	Zhao et al. (2011)
Liposome, weakly cationic	Mouse	+5.58	131	4.51 h	15 h (mean)	Doxorubicin loaded, DPPC:Chol:DC-Chol = 5:4:1, slightly positive	
Liposome, strongly cationic	Mouse	+24.25	95	<30 min	<1 h	Doxorubicin loaded, DPPC:DC-Chol = 5:5, strongly positive	
• pLL-DNA complex	Mouse	Positive	–	<5 min	30 min	^{32}P-labeled 8-kbp DNA	Dash et al. (1999)
• Chitosan nanoparticle, weakly anionic	Mouse	−13.2	149.2	–	12 h (mean)	CMC/MMA = 1:2, slightly negative	He et al. (2010)
Chitosan nanoparticle, strongly anionic	Mouse	−38.4	156.0	–	3 h (mean)	CMC/MMA = 2:1, strongly negative	
Chitosan nanoparticle, weakly cationic	Mouse	+14.8	150.1	–	<1 h	CH/MMA = 1:1, slightly positive	
Chitosan nanoparticle, strongly cationic	Mouse	+34.6	152.7	–	<1 h	CH/MMA = 2:1, strongly positive	

DPPC L-α-dipalmitoylphosphatidylcholine, *Chol* cholesterol, *DC-Chol* 3β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]cholesterol, *pLL* poly(L-lysine), *CMC* carboxymethyl chitosan, *MMA* methyl methacrylate, *CH* chitosan hydrochloride

in water. Thus, pegylation, although attachment of the PEG chains increases the size of target molecules, results in a longer plasma circulation time (Tables 15.2 and 15.3) (Veronese and Pasut 2005; Sahoo et al. 2002; Ogino et al. 1988; Rutter and Wade 1971; Kimura et al. 1990; Fang et al. 2002). PEG-modified proteins also have increased resistance to proteolytic degradation (Veronese and Pasut 2005; Ogino et al. 1988; Kimura et al. 1990). Maeda et al. reported that conjugation of poly(styrene-co-maleic acid) (SMA) to NCS (MW 12 kDa), to produce an agent named SMANCS (Maeda et al. 1979), had an effect similar to that of pegylation. We also showed that conjugation with divinyl ether-maleic acid (pyran) copolymer (DIVEMA), or polyvinylpyrrolidone, as well as gelatin, yielded the same advantage (Akaike et al. 1996; Kojima et al. 1993; Hirano et al. 1994; Maeda 2001b; Maeda and Matsumura 1989).

15.2.2.2 Molecular Size

We stated earlier that the EPR effect is characteristic of macromolecules (>40 kDa). Low-MW drugs (<40 kDa) diffuse more freely through blood vessels, so they distribute in tumor as well as normal tissues throughout the body. Low-MW drugs given by infusion via a tumor-feeding artery first reach the tumor tissue because of the "first-path" effect and will distribute throughout the tumor tissue in only a few minutes, but they then quickly diffuse out from the interstitial space into the bloodstream and are excreted via the kidney into the urine. Therefore, in most cases, low-MW drugs are not retained in tumor tissue for long periods (Noguchi et al. 1998; Maeda 2010a, 2012a; Greish et al. 2003; Zhang et al. 2005). However, because of the large endothelial gap openings (0.2–2 μm) in the tumor vasculature, macromolecules extravasate specifically into tumor tissue and do not enter normal tissue because of tight endothelial intercellular gaps (<5 nm) (Maeda 2012a; Konerding et al. 1995). This finding indicates that macromolecules cannot leak out of vasculature in normal tissue. Furthermore, extravasated macromolecules are not cleared from the tumor tissue as described above. These two advantages—tumor-selective leakage and retention of macromolecules—result in tumor-selective drug delivery (Maeda et al. 2009; Maeda 2010a, 2012a; Maeda and Matsumura 1989; Greish et al. 2003). It should be also emphasized that molecular size (>40 kDa) is not the main criteria for the EPR effect, but biocompatibility governs the prolonged circulation that results in sustained extravasation for the EPR effect as discussed above, and shown in Tables 15.2 and 15.3.

15.2.2.3 Surface Charge

With regard to the surface charge of macromolecules, polycations such as a cationic liposome-DNA complex, poly(L-lysine)/DNA, at 50–100 nm in diameter, are rapidly cleared from systemic circulation with a half-life in blood of less than 3 min (Zhang et al. 2005; Dash et al. 1999; Kwoh et al. 1999). This rapid clearance is

similar to that of small proteins such as superoxide dismutase (Akaike et al. 1996, 2003; Hirano et al. 1994) and NCS (Maeda et al. 1979; Kojima et al. 1993; Maeda 2001b), but these are excreted via urine. Rutter and Wade reported that macromolecular proteins with different isoelectric points, and thus different surface charges in physiological solution, clearly affected the blood half-life of proteins in circulation (Rutter and Wade 1971) (Fig. 15.1). Chunbai et al. also reported that chitosan nanoparticles with both negative and neutral charges (ζ potential = −10 to −40 mV) have a longer blood half-life and accumulate more in tumors than those with a positive charge (ζ potential = +10 to +35 mV) (He et al. 2010). These results suggest that a slightly negative to a neutral charge is preferable for prolonged systemic circulation and facilitation of the EPR effect (Tables 15.2 and 15.3).

15.3 Heterogeneity of the EPR Effect

Although the EPR effect is a common phenomenon in most solid tumors, the effect varies from one tumor type to another. Various tumors have different histopathological characteristics, vascularity, genetic diversity, implanted or spontaneous origins of primary or metastatic, hypoxic or normoxic features in the microenvironment, and so on. For instance, a tumor exhibiting the EPR effect could be as small as <1 mm in diameter (Maeda 2010a, 2012a; Daruwalla et al. 2010). However, clinical and experimental tumors can be as large as 50–100 mm in diameter, which means that some part of a large tumor may be necrotic or rich in fibrotic tissue or clotted vessels. When we studied a large S-180 tumor (>3 cm), accumulation of blue albumin (or a putative macromolecular drug) was extensive primarily in the peripheral tumor tissue; the inner core of the tumor held much less dye (Maeda 2010a, 2012a; Maki et al. 1985; Daruwalla et al. 2010; Li et al. 1993). However, after intravenous (i.v.) injection of the dye-albumin complex into a small S-180 tumor (4–6 mm), the tumor tissue had more uniform uptake throughout the whole tumor.

Such heterogeneity occurs in human cases as well. For example, when SMANCS/Lipiodol was injected into patients with metastatic liver cancer, the staining, visualized by computed tomography (CT), was seen predominantly at the tumor periphery (Maeda 2010a, 2012a; Daruwalla et al. 2010; Li et al. 1993). The reasons for this may be sparse, compressed, or occluded blood vessels, or an extremely limited blood supply in the inner core of the tumor. These tumors were identified as hypoxic or avascular, or they frequently became necrotic, and thus drug delivery to the inner core of the tumor would be limited. However, tumor periphery showed the most active angiogenesis and rich blood flow. These features are accompanied by the generation of vascular mediators, as discussed in Sect. 15.2.1, which would result in active tumor growth and extravasation of plasma components and other macromolecules. This heterogeneous vascular environment would lead to heterogeneous drug delivery to tumor tissues. Pancreatic and prostatic cancers, both of which are hypovascular tumors, exhibit a poor EPR effect (Maeda 2012a; Iwai et al. 1984; Nagamitsu et al. 2009). Such a poor EPR effect would result in ineffective treatment

with conventional as well as macromolecular anticancer drugs. Therefore, overcoming the problem of heterogeneity of the EPR effect and augmenting drug delivery are of great importance. Accordingly, we developed methods to augment the EPR effect, as described below.

15.4 Augmentation of the Accumulation of Macromolecular Drugs in Tumors

15.4.1 Arterial Infusion of Lipiodol and Augmentation of Its Accumulation Under Angiotensin II (AT-II)-Induced High Blood Pressure

We developed several techniques to overcome the EPR-related heterogeneous accumulation of macromolecular drugs in tumors. The most direct delivery of anticancer agents to tumors involves the infusion of a drug into the tumor-feeding artery, which utilizes the first-path effect. However, only marginal therapeutic benefit occurs with conventional low-MW anticancer agents because these drugs diffuse rapidly and return to the circulation, an effect that would be similar to that after i.v. infusion (Li et al. 1993). When a lipid-formulated drug such as SMANCS/Lipiodol is given via this arterial route, however, its delivery to the tumor is more efficient. The high affinity of SMANCS to Lipiodol, because of its highly lipophilic nature, means that it mixes well in Lipiodol. This type of drug and the method of administration offer many advantages, as follows. First, SMANCS/Lipiodol can most effectively target tumors. The tumor/blood ratio of the drug concentration favored the tumor more than 2,000-fold. Second, SMANCS/Lipiodol stays in the tumor interstitium for several weeks because of the sticky nature of the lipid and the macromolecular characteristics of the agent (Maeda 2012a; Konno et al. 1983, 1984; Maki et al. 1985; Iwai et al. 1984; Nagamitsu et al. 2009), and it thus has a markedly prolonged antitumor effect. This arterial infusion was applied to primary hepatoma and renal cancer, which are hypervascular. Other tumors such as metastatic liver cancer and cancers of the gallbladder and pancreas do not demonstrate such a remarkable effect, so forced intraarterial infusion is preferred. The third advantage is the clear CT imaging of the target tumor; for example, the image of CT scan reveals the tumor size and the spread in the liver as a bright area, which indicates Lipiodol deposition. The last advantage is semiquantitation of the drug delivered to the tumor, which informs clinicians about any needed additional administration.

In contrast to primary hepatoma and renal cancer, certain tumors such as prostatic, pancreatic, and metastatic liver cancers are hypovascular, as revealed by angiography, and thus demonstrate a poor EPR effect, as mentioned earlier. To address the poor drug delivery to these hypovascular cancers, or difficult-to-treat tumors, the method of arterial infusion under AT-II-induced hypertension was developed (Nagamitsu et al. 2009). Tumor blood vessels lack a vascular smooth muscle layer

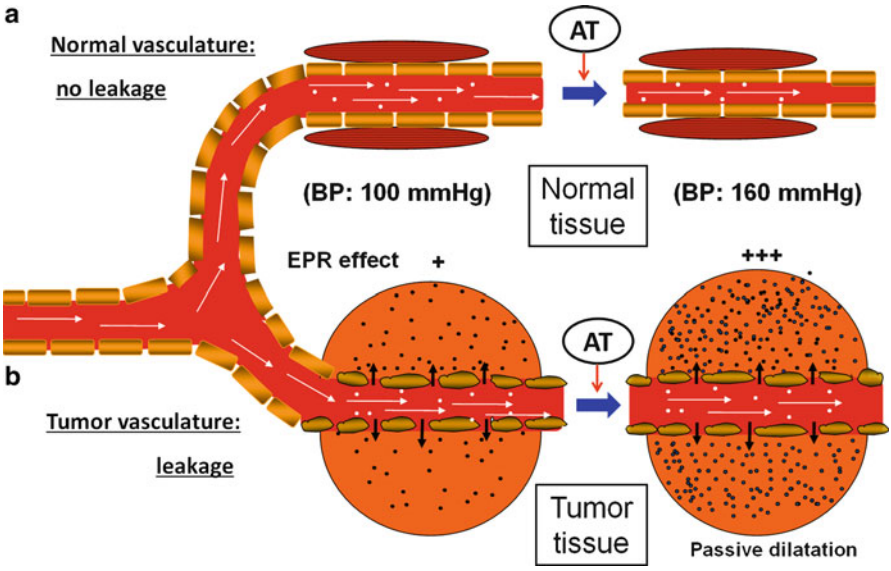

Fig. 15.3 Diagrammatic representation of the EPR effect and the effect of AT-II-induced enhancement of macromolecular drug delivery to normal and tumor tissues. In the *lower part* (tumor tissue), angiotensin II (AT-II) infusion induced high blood pressure (e.g., 100 mmHg → 160 mmHg), which caused endothelial cell–cell junctions in the tumor to open and blood flow to increase, with leakage of the macromolecular drug (*dark dots*). In contrast, normal blood vessels (*upper part*) constrict in response to AT-II, and tighten the endothelial cell–cell junctions that cause high blood pressure, with no leakage of the drug. AT-II-induced hypertension thus resulted in greater (two- to threefold) leakage of drug into the tumor without increased drug accumulation into normal tissue (adapted from Maeda 2012a)

or pericytes surrounding the vessels that mediate vasoconstriction. Tumor blood vessels therefore exhibit an extremely small constrictive response to AT-II, whereas normal blood vessels constrict in response to i.v. AT-II, which leads to systemic hypertension. Under such hypertensive conditions, as Fig. 15.3 shows, tumor vessels open by means of hydrodynamic pressure, and thereby the blood flow volume in tumor vessels increases (Suzuki et al. 1981), accompanied by polymeric drugs being forced out, into the tumor tissue. This tumor-specific event leads to more effective drug delivery to tumor tissue than to normal tissue. Under AT-II-induced hypertension (e.g., 100 mmHg → 160 mmHg), therefore, nanomedicines (including albumin, SMANCS, IgG, HPMA-drug conjugates, and SMA micelles, as well as liposomes, and lipid particles) leak out more into the interstitial space of tumor tissue than into the interstitial space of normal tissue, which results in an augmented therapeutic effect (Greish et al. 2003; Li et al. 1993; Nagamitsu et al. 2009) (Fig. 15.3). A two- to threefold increase in drug accumulation in tumor tissue occurred, and drugs remained there for more than 6 h. This situation is a great contrast to the one in normal blood vessels.

Also important is a significantly reduced adverse effect of these drugs on bone marrow and intestines (Li et al. 1993; Nagamitsu et al. 2009). Further, under AT-II-induced hypertension, delivery of low-MW drugs to tumors had increased only transiently (20–25 %) at 15 min after injection, so no significant therapeutic advantage occurred, in contrast to the situation with high-MW agents (Li et al. 1993). AT-II-induced hypertension during normal clinical procedures is usually maintained for 10–20 min, when AT-II is given by arterial infusion by an experienced angiographer (Nagamitsu et al. 2009); it will return to normotensive state within 3–5 min of the AT-II infusion ending.

Also, under AT-II-induced hypertension, delivery of low-MW drugs to tumors had increased only transiently (20–25 %) at 15 min after injection, so no significant therapeutic advantage occurred, in contrast to the situation with high-MW agents.

Although delivery of macromolecules to solid tumors was reportedly difficult because of an elevated interstitial fluid pressure in tumors (Jain 1990), we achieved progressively increased drug delivery by using various plasma proteins, Lipiodol, and a monoclonal antibody against a tumor (Konno et al. 1983, 1984; Maki et al. 1985; Iwai et al. 1984; Nagamitsu et al. 2009; Noguchi et al. 1992). Therefore, AT-II-induced hypertension, which led to significantly increased blood flow in tumor tissue, is an effective way to overcome obstacles such as interstitial fluid pressure in tumors (Nagamitsu et al. 2009; Suzuki et al. 1984; Noguchi et al. 1992; Hori et al. 1991). Also important is a significantly reduced adverse effect of these drugs on bone marrow and intestines (Li et al. 1993; Nagamitsu et al. 2009).

In the future, after many polymeric drugs and nanomedicines have become available, AT-II-induced hypertension may be recommended as a therapeutic modality that not only enhances bioavailability of the drug to tumor but also reduces drug delivery to normal tissues and hence produces fewer adverse effects.

15.4.2 Bradykinin (Kinin) and ACE Inhibitors

Kinin (a nonapeptide) is a major mediator of inflammation that is also induced in infection and cancer. Kinin dilates blood vessels (vasodilation) and enhances vascular permeability and thus causes extravasation of body fluids in inflamed (edema) and cancerous tissues (Maeda et al. 1988, 2003; Wu et al. 1998; Maeda 2012a; Matsumoto et al. 1984; Kamata et al. 1985; Matsumura et al. 1988, 1991). Maeda et al. reported that kinin and [hydroxyprolyl$_3$] bradykinin were found in the plasma and peritoneal fluid in advanced cancer patients (Maeda et al. 1988, 2003; Matsumura et al. 1988, 1991). During activation of the protease cascade of kinin generation, called the kallikrein-kinin cascade, serine protease kallikrein and cancer cell-derived plasminogen activator are responsible (Maeda et al. 1988, 2003; Matsumura et al. 1991). Administration of nanomolar amounts of kinin into guinea pig skin significantly increased vascular permeability, and extravasation of Evans blue-bound albumin at the injection site was observed (Maeda et al. 2003; Wu et al. 1998; Maeda 2001b; Matsumoto et al. 1984; Matsumura et al. 1988, 1991). In the

peritoneal fluid of patients with ascites tumor and pleural effusion of lung cancer patients, the kinin level was significantly elevated (1–40 ng/mL) and was sufficient to result in an enhanced permeability effect (Maeda et al. 1988).

Administration of a kinin receptor antagonist (HOE 140) suppressed vascular permeability and inhibited extravasation of body fluid in a subcutaneous tumor and ascites in ascites tumor-bearing mice (Maeda et al. 2003; Wu et al. 1998). Administration of soybean trypsin inhibitor, a known inhibitor of plasma kallikrein, also suppressed kinin generation and thus reduced ascites formation (Matsumura et al. 1988).

With regard to the effect of kinin, NO and prostaglandins interacted during mediation of the EPR effect in a tumor model. Extravasation of the Evans blue-albumin complex was suppressed about 46 % after administration of HOE 140, 39 % after administration of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), and 49 % after administration of a cyclooxygenase inhibitor (indomethacin) (Maeda et al. 2003; Wu et al. 1998; Matsumura et al. 1991). These studies thus showed that kinin is one of the most potent mediators of vascular permeability in tumors, and an increased kinin level in a tumor would lead to augmented vascular permeability in the tumor. One method of increasing the kinin level in the tumor is direct local infusion. However, the half-life of kinin in plasma is only a few seconds, and the infusion of intact kinin does little to enhance the EPR effect while possibly inducing pain.

Another way to increase the kinin level in the tumor is to inhibit the degradation pathway of kinin. Kinin is known to be degraded by kininase 2, which is also called angiotensin-converting enzyme (ACE). ACE inhibitors such as enalapril and captopril are widely used as standard antihypertensive drugs that can inhibit both conversions of angiotensin I (AT-I) to AT-II as well as degradation of kinin. Because of the similar amino acid sequences of AT-I and kinin at the C-terminal end, ACE inhibitors can inhibit the degradation of kinin and the conversion of AT-I to AT-II, so administering ACE inhibitors will increase the kinin level at the site of its generation (tumor). This working hypothesis was confirmed by using ^{51}Cr-labeled bovine serum albumin and ^{125}I-labeled monoclonal antibody in tumor-bearing mice (Noguchi et al. 1992; Matsumura et al. 1988, 1991) (Fig. 15.4). Dr. Felix Kratz, University of Freiburg, also confirmed this effect of ACE inhibitors in different tumor models; in tumor targeting of albumin doxorubicin conjugate (personal communication; unpublished data).

15.4.3 NO and Other NO-Releasing Agents

NO is an endogenous free radical molecule that plays important roles as a signaling messenger in cells. NO is naturally synthesized from L-arginine by nitric oxide synthase (NOS) in the presence of O_2, NADPH, and calmodulin. With respect to blood vessels, NO enhances the activity of soluble guanyl cyclase (200-fold), which leads to dephosphorylation of myosin light chain followed by smooth muscle

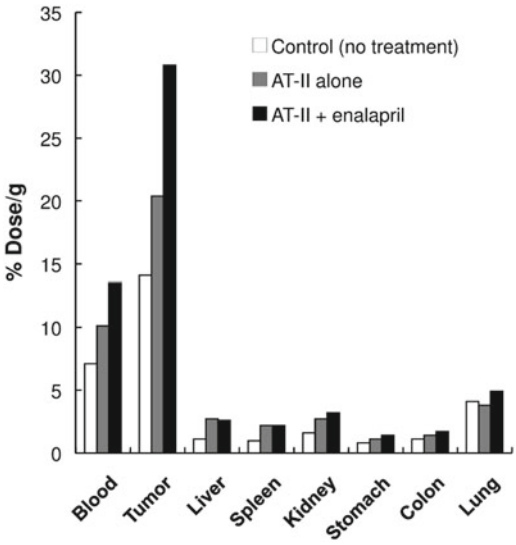

Fig. 15.4 Augmentation of the EPR effect and delivery of monoclonal antibody to the tumor by using AT-II and the ACE inhibitor enalapril. Human SW11116 colon cancer-bearing nude mice were injected i.v. with ^{125}I-labeled monoclonal antibody A7 with or without AT-II and enalapril (adapted from Noguchi et al. 1992)

relaxation and vasodilation (Cohen and Adachi 2006; Lincoln 1989). NO also induces calcium uptake by the sarcoplasmic reticulum by activating calcium ATPase in the sarcoplasmic/endoplasmic reticulum, which results in a reduced intracellular calcium level and smooth muscle cell relaxation (Cohen and Adachi 2006). NO also serves as a growth promoter in solid tumors and can enhance vascular permeability and increase blood flow (Wu et al. 1998; Doi et al. 1996).

In clinical settings, nitroglycerin, isosorbide dinitrate (ISDN), amyl nitrite, and other NO-releasing agents are frequently used to treat angina pectoris and myocardial infarction. These NO-releasing drugs are administered orally or applied transdermally and are converted to nitrite NO_2^- in hypoxic conditions such as those found in infarcted or tumor tissues. In such tissues, nitrite is further reduced to NO by nitrite reductase (Maeda 2010b; Seki et al. 2009). The tissue environment in many tumors is hypoxic and acidic, similar to the environment in infarcted heart tissue. We would therefore expect NO production to occur preferentially in tumor tissue after application of an NO-releasing agent such as nitroglycerin. In fact, Seki et al. in our laboratory reported that transdermal application of nitroglycerin (0.01–2.0 mg/site over the skin) increased blood flow in the tumor but not in the normal tissue (muscle), which led to enhanced accumulation of macromolecular drugs in the tumor (Seki et al. 2009) (Fig. 15.5). The application site of nitroglycerin ointment can be anywhere, even at a site distal from the tumor, because nitroglycerin reaches the blood circulation readily.

Jordan et al. also reported that ISDN treatment of patients increased the blood flow in prostate tumors, which led to increased tissue pO_2 in this hypoxic tumor (Jordan et al. 2000). Use of ISDN therefore improved the therapeutic response (Jordan et al. 2000). They suggested that the increased blood flow and accompanying higher pO_2 would result in decreased VEGF, with tumor growth suppression and

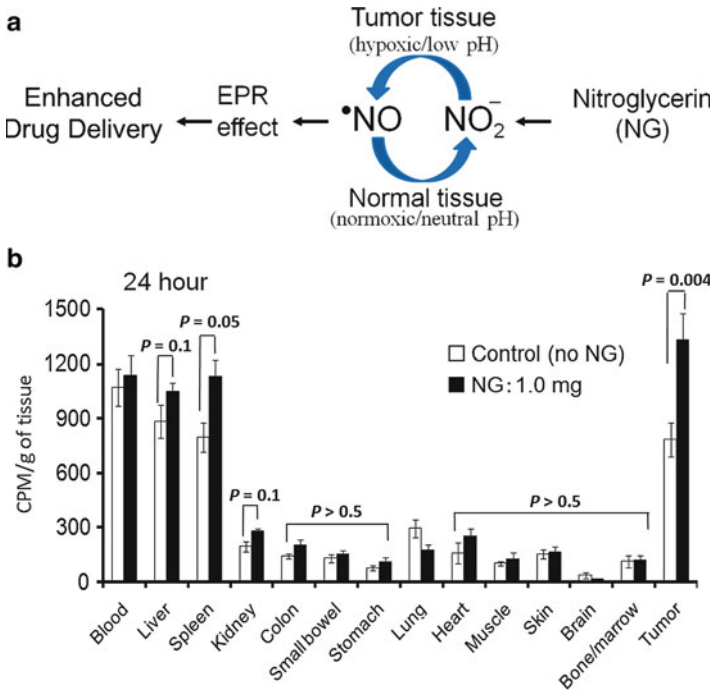

Fig. 15.5 Nitroglycerin (NG)-induced increase in accumulation of polymer-conjugated drug in tumors. (**a**) Mechanism of selective NO generation in tumor. NO was generated from nitrite, predominantly in hypoxic tumor tissue, not in normal tissue. (**b**) In vivo evaluation of the potentiation of drug delivery to tumor by nitroglycerin that was applied as an ointment to anywhere on the skin of S-180 tumor-bearing mice at a dose of 1.0 mg/mouse. Pegylated-^{65}Zn-labeled Zn-protoporphyrin was then injected i.v. into the tumor-bearing mice. After 24 h, anesthetized mice were dissected and radioactivity of each tissue was counted (adapted from Maeda 2010a; Seki et al. 2009)

reduced generation of P-glycoprotein in the tumor, via rapid decreases in the expression of hypoxia-inducible factor-1α (Yasuda et al. 2006a). Similarly, NO donors such as *S*-nitroso-*N*-acetyl-D-penicillamine increased the intracellular accumulation of doxorubicin in a doxorubicin-resistant cancer cell line via reduction of doxorubicin efflux by tyrosine nitration in multidrug resistance-associated protein 3 (Riganti et al. 2005). In a different context, radiosensitization by inducing iNOS using γ-interferon was also reported in hypoxic EMT-6 mammary adenocarcinoma, in which NO is the key player (Janssens et al. 1998).

In addition, Yasuda et al. observed an improved clinical response with vinorelbine and cisplatin in non-small cell lung carcinoma (Yasuda et al. 2006b), and with docetaxel and carboplatin in lung adenocarcinoma (Yasuda et al. 2006a), both studies demonstrating a significant benefit of using nitroglycerin. Our interpretation of the beneficial effect of nitroglycerin is that the benefit is primarily due to enhanced drug delivery (via the EPR effect). In addition, findings of both Yasuda et al. (2006a) and Seki et al. (2009) showed that nitroglycerin alone significantly suppressed tumor growth.

15.4.4 TNF-α and Rho Kinase

TNF-α is a pleiotropic proinflammatory cytokine that has permeability-enhancing and direct toxic effects on tumor-associated vasculature in vivo (Hagen and Eggermont 2004). TNF-α substantially alters the integrity of endothelial monolayer cells and remodels the cytoskeleton (Blum et al. 1997) as well as redistributes cell–cell adhesion molecules such as PECAM-1 (Romer et al. 1995) and vascular endothelial cadherin (Lampugnani et al. 1992). All these events lead to enhanced vascular permeability in vivo. In subcutaneous tumor models, systemic administration of TNF-α increased the vascular permeability of only the tumor, which resulted in higher tumor uptake of monoclonal antibodies (Folli et al. 1993) and viral particles as well as the Evans blue-albumin complex (Seki et al. 2011), without any change in the tumor blood flow volume. The TNF-α-induced increase in vascular permeability can be reversed by treatment with Rho kinase inhibitor (Y-27632), which suggests the important role of Rho signaling in TNF-α-induced enhancement of vascular permeability. van Nieuw Amerongen et al. reported that a Rho signaling inhibitor (simvastatin) suppressed vascular leakage of the Evans blue-albumin complex into the thoracic and abdominal aorta in the atherosclerosis-induced Watanabe heritable hyperlipidemic rabbit model (van Nieuw Amerongen et al. 2000). In a rat sarcoma model, isolated limb perfusion with TNF-α increased the concentrations of melphalan and doxorubicin in the tumor, whereas no increased uptake occurred in the normal muscle and skin (de Wilt et al. 2000; van der Veen et al. 2000).

Notwithstanding above effects of TNF-α, it was used as anticancer biologic agent in cancer patients. The major effect was severe fever and therapeutic effect was marginal or not beneficial.

15.4.5 Transforming Growth Factor-β (TGF-β) Type 1 Receptor Inhibitor

TGF-β is a multifunctional cytokine, similar to TNF-β, that plays a pivotal role in the suppression of growth and the differentiation of tumor cells and the tumor environment: TGF-β signaling suppresses the growth of both epithelial and endothelial cells and induces the production of extracellular matrix (Roberts and Wakefield 2003). Hypovascularity and extensive fibrosis found in pancreatic adenocarcinoma and diffuse-type gastric carcinoma are thought to be major obstacles to achieving efficient delivery of drugs to tumors in conventional chemotherapy as well as EPR effect-based therapy (Sofuni et al. 2005; Takahashi et al. 1996). Inhibition of the TGF-β receptor in tumor tissue should produce an effect similar to inhibition of VEGF: growth progression of endothelial cells and decreased production of extracellular matrix and fibrosis, so that delivery of anticancer drugs to tumors should be facilitated.

Treatment with low doses of TGF-β type 1 receptor inhibitor (LY364947; 1 mg/kg) inhibited TGF-α signaling in the vascular endothelium but not in cancer cells and most interstitial cells (Kano et al. 2007). Abnormal blood vessels with aberrant and

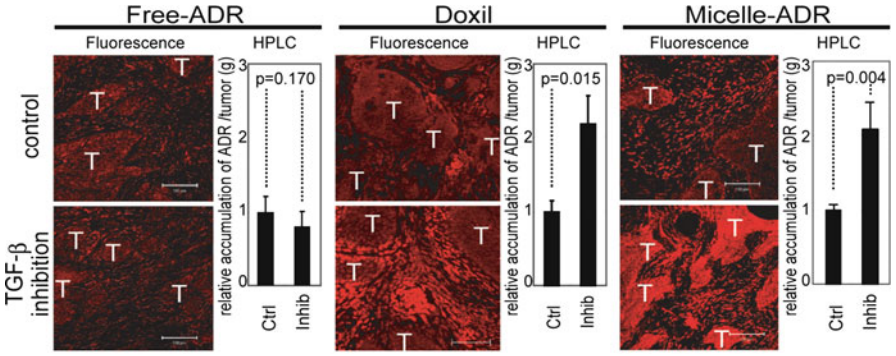

Fig. 15.6 Biodistribution of free ADR, Doxil, and ADR micelles in the pancreatic cancer BxPC3 model in mice. Distributions of free ADR, Doxil, and ADR micelles (each at 8 mg/kg) with or without TGF-β receptor inhibitor (LY364947) (1 mg/kg) were evaluated via fluorescent microscopy at 24 h after drug administration. Bar graphs at the right show relative quantitative results for the accumulation of drugs in tumors obtained by high-performance liquid chromatography (HPLC). Treatment with TGF-β receptor inhibitor resulted in about a twofold enhancement of accumulation of Doxil and ADR micelles. Error bars in the graphs represent standard errors; *P* values were calculated by using Student's *t* test. *T* nests of tumor cells in tumor tissues, *Doxil* pegylated liposome, *Ctrl* control without the inhibitor, *Inhib* inhibitor. See text for detail (adapted from Kano et al. 2007)

irregular dilation were found in typical untreated tumors in mice, whereas the vasculature of mice treated with TGF-β type 1 receptor inhibitor was narrower and rounder than untreated controls, which suggests vascular normalization (Kano et al. 2007). It is interesting that TGF-β type 1 receptor inhibitor treatment normalized blood flow in the tumor, i.e., unidirectional constant flow (as in normal blood flow) was observed (Minowa et al. 2009). Minowa et al. (2009) reported that inhibiting TGF-β receptor (by using A-83-01) increased the number of pericytes surrounding the tumor vasculature. Kano et al. (2007), however, reported a reduced number of pericytes surrounding the tumor vasculature. This controversial issue may be due to the different tumor types and/or different types of TGF-β receptor inhibitor used.

Both reports did show, however, the positive effect of TGF-β receptor inhibitor for enhanced drug delivery to tumors in a molecular size-dependent manner, as seen with the EPR effect. At 24 h after injection of macromolecular drugs, i.e., 2×10^6 Da dextran with a 50-nm hydrodynamic diameter, Doxil® with a 108-nm diameter, and adriamycin (ADR) micelles with a 65-nm diameter, and treatment with TGF-β type 1 receptor inhibitor, all drugs effectively accumulated in the tumors (Kano et al. 2007) (Fig. 15.6). However, treatment with TGF-β type 1 receptor inhibitor did not significantly enhance accumulation of the low-MW free ADR (MW 543) and bromodeoxyuridine (MW 307) in the tumors (Fig. 15.6). This augmented accumulation of particulate therapeutic agents (Doxil and ADR micelles) resulted in a marked suppression of tumor growth, even in the hypovascular solid tumor, which is difficult to treat. Thus, augmenting the EPR effect by using TGF-β type 1 receptor inhibitor warrants further exploitation.

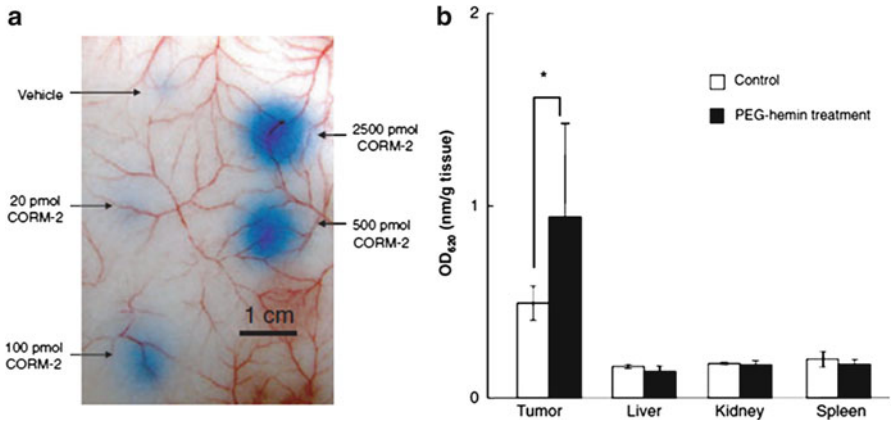

Fig. 15.7 CO-enhanced accumulation of Evans blue-albumin complex in tumors. (**a**) Different concentrations of the CO-releasing agent CORM-2 were administered subcutaneously, followed by i.v. injection of Evans blue (10 mg/kg). The dye-albumin complex was allowed to extravasate for 2 h. (**b**) At 24 h after the i.v. injection of an HO-1 inducer, pegylated hemin (10 mg/kg hemin equivalent), Evans blue was injected as in (**a**). After another 24 h, mice were killed and dissected to obtain the tissues. Control mice were not treated with pegylated hemin. The blue dye complexed with albumin in each tissue was extracted with formamide, and the degree of extravasation was quantified by means of absorbance at 620 nm (adapted from Fang et al. 2012)

15.4.6 HO-1 Induction and the CO-Based Enhanced EPR Effect

Similar to NO, CO, a gaseous molecule that has a critical role in vascular physiology, has recently been receiving considerable attention. An excessive dose of CO may be hazardous, but CO given as a microdose is now considered to be an important vascular modulator, particularly in transplantation surgery (Kohmoto et al. 2006; Nakao et al. 2005). CO causes the vascular tonus to dilate and facilitates erythrocyte trafficking, which transports molecular oxygen to peripheral tissues. CO is naturally generated in the body by HO-1 together with biliverdin during heme catabolism. Heme oxygenase-1 (HO-1) is responsible for 80 % of CO production in the body (Abraham and Kappas 2008) and it is highly upregulated in most cancers in vivo (Doi et al. 1999). Recently, Fang et al. in our laboratory reported that a subcutaneous injection of recombinant HO-1 facilitated extravasation of Evans blue-albumin complex as a result of the EPR effect (Fang et al. 2012). More direct evidence of involvement of CO in the EPR effect was demonstrated by subcutaneous administration of a CO-releasing agent, tricarbonyldichlororuthenium (II) dimer (CORM-2) (Fang et al. 2012) (Fig. 15.7). Furthermore, the EPR effect-based macromolecular drug delivery was enhanced by inducing HO-1 in cancer tissue, which was accomplished by administering pegylated hemin as a polymeric drug, which, because of the EPR effect, is selectively delivered to tumor tissue rather

than to normal tissue. Hemin is a typical inducer of HO-1, and we developed a water-soluble micellar form of hemin for tumor targeting by means of the EPR effect (Fang et al. 2012) (Fig. 15.7).

15.5 Concluding Remarks

Nanomedicine, i.e., macromolecular drug, development for cancer chemotherapy is thought to represent a new paradigm in cancer treatment and has been the focus of considerable attention. The major advantage of nanomedicine in cancer chemotherapy is the ability of drugs to target tumor tissue on the basis of the EPR effect, to improve therapeutic efficacy and reduce the adverse effects. Tumor tissue usually has aberrant blood vasculature: irregular networks of vessels, lack of surrounding smooth muscle layer cells, irregular blood flow, and impaired lymphatic clearance. Vascular permeability factors such as kinin, prostaglandins, NO, and inflammatory cytokines are overexpressed in both cancer and inflamed tissues. Clearly, these factors also affect normal vessels near the tumor tissue. These upregulated vascular factors and the architectural defects of tumor blood vessels are the major contributors to the EPR effect. Once macromolecules extravasate into the interstitial space of tumor tissue, they are retained in the tumor for long periods, more than a week; they are not cleared because of the impaired lymphatic recovery system in the tumor, and thus they have a sustained pharmacological effect.

Tumor-specific accumulation and retention of drugs that function based on the EPR effect pertain to biocompatible macromolecules with MW of >40 kDa. Nevertheless, the EPR effect is frequently heterogeneous, especially in large tumors. Also, tumors such as pancreatic, metastatic liver, and prostatic cancer have hypovascular features and thus demonstrate less of an EPR effect. These obstacles to drug delivery must be overcome to achieve more efficient and more uniform delivery of drugs to tumors.

In this chapter, we discussed different methods of augmenting the EPR effect and thereby providing more homogeneous drug delivery to tumors. AT-II-induced hypertension increases leakage of drugs, such as SMANCS/Lipiodol given by intra-arterial infusion, into the tumor interstitium. Similarly, ACE inhibitors, NO-releasing agents such as nitroglycerin, TGF-β receptor inhibitors, and HO-1 inducers may be useful for an augmentation of the EPR effect. Early clinical evaluation of drug administration based on the EPR effect did improve drug delivery, as demonstrated with SMANCS/Lipiodol administered by the intraarterial route under induced hypertension, the result with this specific formulation being improved therapeutic efficacy without serious adverse effects (Nagamitsu et al. 2009). Also, use of nitroglycerin or other NO-releasing agents in combination with conventional cancer chemotherapeutic agents was reportedly beneficial in clinical settings (Jordan et al. 2000; Yasuda et al. 2006a, b; Maeda 2012b). In conclusion, under these circumstances as described herein, EPR-based drug design is becoming an important issue for the development of macromolecular drugs used to treat solid tumors.

Problem Box

Macromolecular drugs or nanomedicines are known to exhibit the EPR effect in solid tumor in vivo.

(1) In relation to inflammation, discuss what factors facilitate the EPR effect.
(2) What makes the EPR effect different to the passive targeting?

[Relevant answers]

A to (1) Vascular permeability factors common to inflamed tissue and solid tumors are nitric oxide, bradykinin, prostaglandins, free radicals (ROS), inflammatory cytokines, and others. In cancer, structural integrity of the blood vessels is also defective; lack of pericytes, hyperdilated feature, large gap between endothelial cell–cell junction, etc. These facts also lead to higher vascular permeability in cancer tissue.

A to (2) Passive targeting can be seen not only for macromolecules but also for low molecular weight X-ray contrast agent (i.e., in angiography), showing a stained image of the tumor. However, they are rapidly washed out within a few minutes; namely there is no retention of the drug in tumor tissue by passive targeting. What makes the EPR effect different from passive targeting is the long period of retention of drugs in the tumor tissue, that is seen for days to weeks, and this is only seen for nanomedicines. This is particularly so with the lipid contrast agent Lipiodol® when it is injected into the tumor-feeding artery.

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Chapter 16
Anti-infectives

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Abstract Infectious diseases caused by viruses, bacteria, fungi and parasites are becoming a major health concern worldwide. Several serious diseases such as leishmaniasis, malaria, tuberculosis, hepatitis C and human immunodeficiency virus are caused by intracellular pathogens. Fatal systemic infection, e.g. invasive candidiasis, is caused by extracellular fungi. Delivery systems that can target these intracellular or extracellular pathogens can be effective in curing these diseases. Over the last 20 years, several nano-sized delivery systems have shown to be a potential tool for targeting drugs to the site of infection. There are many clinically used nanomedicines for the treatment of infectious diseases such as liposomes (e.g. AmBisome®) and protein-polymer conjugates (e.g. Intron® A). In addition numerous preclinical nano-delivery systems, e.g. polymeric nanoparticles, drug–polymer conjugates and complexes, dendrimers, lipid nanoparticles, cochleates and niosomes have been investigated for delivery of anti-infective agents. In this chapter, a description of these delivery systems, examples of infectious diseases and the rationale of using these delivery systems to treat certain infections will be discussed.

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16.1 Introduction

Infectious diseases are a major health problem worldwide. The cause of these infections can be: (a) viral, e.g. hepatitis, (b) parasitic, e.g. leishmaniasis, (c) fungal, e.g. candidiasis and (d) bacterial, e.g. tuberculosis. Most of these diseases are caused by intracellular pathogens. The great challenge in these types of infections is to deliver an intracellular dose of the drug to kill the pathogen within the cell (Armstead and Li 2011). The host cell membrane can act as barrier for drugs to reach the pathogen. Many antibiotics and antivirals have a short half-life, which requires frequent administration and high doses of the drug which might result in toxic side effects and low patient compliance. Poor patient adherence can result in the development of resistance due to exposure of pathogens to sub-therapeutic doses of drugs (Goossens 2009). Key properties of drugs to treat intracellular pathogens include: (a) an ability to cross the host cell membrane and reach the pathogen, (b) be non-toxic to host cells and (c) sustained and site-specific release at a therapeutic level (Briones et al. 2008). Many strategies have been examined to address the challenges to deliver a drug intracellularly to selective tissues and cells. Particulate-associated medicines derived from colloid forming excipients to encapsulate a drug or the direct covalent conjugation of a drug to a polymer have been extensively examined during the last few decades.

Particulate-associated medicines and polymer conjugates are often nanometre sized and include vesicular or carrier systems such as liposomes, polymeric nanoparticles, cochleates and polymeric micelles, and polymeric complexes and conjugates. These main classes of nano-sized carriers are used for the delivery of drugs, imaging agents and proteins (Duncan and Gaspar 2011). Liposomes are uni- or multilamellar bilayer vesicles composed of phospholipids and cholesterol encompassing an aqueous core (80–200 nm). There are examples of liposome-based medicines which have been translated to clinical use (Duncan and Gaspar 2011). Niosomes are also lipid-based vesicles composed of cholesterol and a non-ionic surfactant (Uchegbu and Florence 1995). Lipid cochleates are composed mainly from phosphatidylserine and calcium. The lipid bilayer is formed by electrostatic interactions of the phosphatidylcholine and calcium to form cylinders of rolled up bilayers that are suitable for entrapment of hydrophobic or amphiphilic molecules such as amphotericin B (AmB) (Perlin 2004; Zarif 2005).

A polymer drug–protein conjugate is defined as the covalent conjugation of a drug or protein to a polymer via a covalent bond (~5–25 nm). There are several examples of polymer–protein conjugates in the clinic, all derived from poly(ethylene glycol) (PEG), this is often referred to as ‘protein PEGylation’, or simply PEGylation (Veronose 2009).

Many different types of polymers have been examined for both (a) non-covalent association and (b) covalent conjugation of drugs. The vast majority of drugs used have been poorly soluble and/or toxic molecules. Water-soluble polymers can be used to aid the solubilisation of poorly soluble drugs. Dendrimers are hyperbranched polymers. In contrast to linear polymers, they display low viscosity and a large number of chain end groups. Block co-polymers comprise

hydrophilic–hydrophobic parts (amphiphilic) that can self-assemble to form polymeric vesicles. In water, a hydrophilic shell and hydrophobic core can be formed. This is often referred to as a polymer micelle (50–200 nm).

These systems are referred to as 'nanomedicines' (Duncan 2011). They are also complex formulations; the vast majority are designed to be parenterally administered. Challenges in terms of scalability, ease of sterilisation and cost often exist; however, these systems also provide the hope to selectively deliver drugs to specific tissues and to deliver drugs intracellularly. While much preclinical research has been published, there are also many examples of nanomedicines that have been translated to the clinic (Duncan and Gaspar 2011). However much remains to be accomplished to translate nanomedicines effectively into the clinic (Juliano 2013; Venditto 2013).

Overall these delivery systems are being developed to: (a) increase drug solubility, (b) reduce systemic drug toxicity, (c) enhance drug pharmacokinetics, (d) increase drug stability and (e) target the drug to specific sites of action. These efforts are designed to maximise the efficacy of the drug against the pathogen (Seale-Goldsmith and Leary 2009; Uchegbu 1999a, b). The rationale behind using nano-sized particulates to deliver anti-infective agents are: (a) nanoparticles can be taken up by cells via endocytosis to enable cellular internalisation of the drug (Vasir and Labhasetwar 2007), (b) a targeting ligand can be associated to the particle that can, in principle, facilitate delivery of the drug to specific tissues and cells (Seleem et al. 2009), (c) modulate the release of the drug from the carrier particle to reach the pathogen where it resides in the intracellular compartment and this can be achieved, for example, by using pH-responsive polymers (Murthy et al. 2003) and (d) optimise whole body pharmacokinetics to achieve sustained therapeutic levels resulting in reduced cumulative doses and reduced frequency of administration, e.g. covalent conjugation of PEG to drugs (Chaudhari et al. 2012) and therapeutic proteins (Jevsevar et al. 2010). In the case of polymeric conjugates, due to their large size, they circulate longer in the blood without being systemically absorbed. The vasculature at diseased and malignant tissue sites that are inflamed (i.e. due to infection) is often more permeable than healthy tissue. This allows for the preferential uptake of polymeric and other nano-sized particulates.

There is much unmet medical need for the treatment of infectious diseases. The use of nanomedicine-based systems may offer some advantages for the treatment of infectious diseases. Four example diseases are described here where the development of nanomedicines have had a positive impact.

16.2 Viral Infections: Hepatitis C

16.2.1 Pathology

Hepatitis C affects around 170 million people, making up around 3 % of the world's population (Ray 2002). Chronic infection was reported to progress in about 85 % of patients to liver cirrhosis, end stage liver disease and hepatocellular carcinoma (Luxon et al. 2002). Hepatitis C is the main cause of liver transplantation in Europe

and the United States. Approximately 20–30 % of those chronically infected will still die within 20–25 years (Strader and Seeff 1996).

The causative agent of this infection is the Hepatitis C virus (HCV) which is an enveloped single-stranded positive sense RNA virus belonging to the Flaviviridae family. Diagnosis of hepatitis C infection is made by measuring the amount of viral RNA present in the blood by one of the two methods (a) quantitative polymerase chain reaction (Q-PCR) or (b) signal amplification technologies (Rosen and Gretch 1999). Contraction of the disease is principally a result of contact with contaminated material either blood or bodily fluids by blood transfusion or as a result of intravenous drug use. Upon contact the virus travels to the hepatocytes of the liver and enters these cells via endocytosis where it replicates (Firpi and Nelson 2007). Elucidating the exact mechanisms of viral pathogenesis, however, has so far been hampered by the lack of an efficient cell culture system and animal models. So far six major genotypes of this virus and more than 50 different subtypes have been identified. Each genotype differs by around 30 % in its nucleotide sequence while subtypes reportedly differ by around 20 % (Ramadori and Meier 2001). Virus genotypes also differ in their geographical distribution and have been found to have a substantial effect on the effectiveness of any therapy.

16.2.2 Conventional Therapy

The objective of any treatment is to rid the body of detectable levels of HCV and for the virus to remain at undetectable levels for a period of 6 months after the termination of treatment. This is known as a sustained viral response (SVR) and correlates with a favourable prognosis. A list of all the currently available treatments for Hepatitis C is shown in Table 16.1. Interferon alpha 2 (IFNα-2) is a cytokine with both immune modifying and antiviral properties. The binding of IFN to its receptor (IFNR) activates Janus kinase (JAK) and induces phosphorylation of STAT1 and STAT2. The phosphorylated STATs translocate from the cytoplasm to the nucleus and complex with p48 which activates transcription of IFN-stimulated genes (ISGs) (Taylor et al. 2000). Interferon assists the immune response by enhancing memory T cell proliferation, natural killer cell activation and inhibiting T cell apoptosis (Feld and Hoofnagle 2005).

Ribavirin a guanosine analogue with broad spectrum activity against both DNA and RNA viruses is often used in combination with IFNα-2 and offers several advantages over IFN monotherapies. Ribavirin is thought to inhibit inosine monophosphate dehydrogenase, an enzyme that catalyzes a rate-limiting step in guanosine triphosphate (GTP) biosynthesis. This then leads to a decrease in intracellular GTP levels, which indirectly inhibits viral RNA synthesis (Tan et al. 2004). Ribavirin, like IFNα-2 also has significant side effects (e.g. anaemia).

Since a significant number of patients fail to exhibit an SVR, there was a need for new and improved therapies. New drugs for the treatment of Hep C currently fall into one of the following categories: (a) modified IFNs, (b) alternatives to ribavirin,

Table 16.1 Drugs for the treatment of Hep C on the market and in clinical trial (Firpi and Nelson 2007)

Drug trade name	Company	FDA approval	Details
Interferon-based therapies			
Intron® A	Schering-Plough	1991	Native IFNα-2b
Roferon® A	Hoffman-La Roche	1996	Native IFNα-2a
Intergen® A	Amgen	1997	Concensus IFN (c-IFN)
Beleroferan®	Nautilus Biotech	Phase I	IFNα with a single amino acid mutation
Ribavirin and associate analogues			
Ribivarin®	Valeant Pharmaceuticals International	1980	Prodrug interferes with RNA metabolism
Viramidine®	Valeant Pharmaceutical International	Phase III	Prodrug of ribavirin
Alternative drugs			
Telaprevir®	Vertex, Johnson & Johnson	2011	Protease inhibitor
Victrelis®	Merck	2011	Protease inhibitor
Civacir®	Nabi Pharmaceutics	Phase II	Polyclonal antibody against HCV
Isatoribine®	Anadys Pharmaceuticals	Phase I/II	TLR7 agonist
Zadaxin®	SciClone	Phase III	Synthetic version of thymosin alpha 1

(c) immune stimulating drugs and (d) HCV inhibitors. Current first-line treatment is weekly injections of a modified IFNα-2 known as PEGylated interferon (PEG-Intron and PEGasys; Table 16.2) in combination with ribavirin. This treatment has been refined so that many patients can be cured of HCV (i.e. genotype 2 and 3) (Thitinan and McConville 2009) which was not possible with native IFN-α2. A number of drugs are being evaluated in clinical trials (Table 16.1).

The use of PEG-interferon (PEGasys and PEG-Intron) will continue as new chemical entities are introduced since the trials are generally conducted with PEG-interferon and to decrease the risk of resistance by using these medicines alone. Combination regimens will also be expected to require the use of PEG-IFN. Many of the new medicines require stringent dosing regimens and also display side effects that may impact compliance.

16.2.3 Rationale of Using Nano-Delivery Systems for Anti-Hep C

Protein-based therapeutics such as IFNα-2 show great potential as medicines but their use in a clinical setting is severely limited by their short circulation half-life as a result of their small size and potential for immunogenicity (Harris and Chess 2003). In reality this often translates to frequent dosing regimens, low patient compliance

Table 16.2 Examples of nano-delivery systems for IFNα on the market and at the preclinical stage

Delivery system	Formulation name	Composition	Route	Efficacy (in vivo)	Status	References
Albumin fusion	Albuferon	IFNα-2b genetically fused to human serum albumin (HSA)	SC	69 % of patients achieved a 2 log reduction in HCV, mean elimination half-life of 159 h supporting 2–4 week dosing intervals	Development terminated	Bain et al. (2006) and Balan et al. (2006)
Polymer conjugation	PAS-IFNα	IFNα-2b conjugated to biodegradable α(2 → 8) linked polysialic acid (PSA)	SC	Exhibited 2.5–4.5 lower activity than native IFNα-2b	Preclinical	Hirst et al. (2002)
	Pullulan-IFNα	IFNα conjugated to the polysaccharide pullulan	IV	122-fold increased accumulation in the liver after 24 h following IV administration in mice	Preclinical	Tabata et al. (1999)
	FMS_7-IFNα-2	IFNα-2 with seven molecules of 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) attached to it its amino groups	SC	Maintained 4 % of its biological potency, ninefold improved circulation half-life compared to native IFNα-2a	Preclinical	Shechter et al. (2001)
	PEGylated IFNα-2b (PEG-Intron®)	IFNα-2a conjugated to a 12 kDa linear PEG	SC	A sevenfold lower clearance rate and a fivefold greater in vivo half-life than Intron A. 33, 41 and 49 % of the Peg-Intron treatment groups showed a detectable loss of viral RNA, compared with 24 % of the Intron A recipients	FDA approved	Glue et al. (2000) and Lindsay et al. (2001)
	PEGylated IFNα-2a (PEGasys®)	IFNα-2b conjugated to a 40 kDa branched PEG	SC	Increased serum half-life from 9 to 77 h, in a 48-week trial SVR observed in 68 % of patients compared to 28 % receiving the unPEGylated protein	FDA approved	Zeuzem et al. (2000)
Hydrogels	Medusa	IFNα-2b incorporated into a polymer composed of the hydrophilic chain of poly-L-glutamate and hydrophobic molecules of α-tocopherol	SC	Extended release over 7 days, ninefold reduction in C_{max} compared to the native protein	Phase II	Trepo et al. (2006)

and a poor clinical outcome, especially in the case of treating an infectious disease. Native unmodified forms of IFNα-2 are rarely used now because of the rapid clearance of the protein. Rapid clearance leads to dose dumping with the need for frequent administration of the protein. Sub-therapeutic, non-efficacious concentrations result between each administered dose and the frequent dosing leads to the loss of compliance because of the severe side effects of interferon treatment. Risk of immunogenicity also increases with frequent dosing of native IFNα-2.

PEGylation, the covalent attachment of PEG to protein molecules is a clinically proven strategy that continues to be developed to improve the efficacy of protein-based medicines and has already resulted in a large number of marketed products including that of IFNα (Alconcel and Maynard 2011). When bound to a protein, PEG increases the protein's molecular weight, decreasing its rate of glomerular filtration and sterically shields the attached molecule from exposure to antigenic determinants and proteolytic enzymes (Harris and Chess 2003). Although other half-life extension technologies for proteins exist (Kontermann 2011), PEGylation is by far the most widely utilised in the clinic. The first of these conjugates was obtained by conjugating an amino-reactive 12 kDa linear PEG with interferon-α2b (PEG-Intron®, Schering-Plough) and the second by coupling an amino-reactive branched 40 kDa PEG with interferon-α2a (PEGasys®, Roche). Following PEGylation PEGasys® maintains only 7 % of its activity but shows a tenfold increase in circulation half-life. PEG-Intron®, on the other hand, retains 28 % of its activity but has a lower blood residence time (50-80 h) due to the slow release of polymer from the primary attachment site (His34) (Grace et al. 2001). However despite their differences both of these conjugates have been found to show similar efficacy and have found extensive use in a clinical setting.

Several new forms of IFN have been or are currently in clinical trials (Table 16.2), which use half-life extension techniques other than PEGylation, such as (a) protein fusion, (b) encapsulation of the drug into nanoparticles or hydrogels and (c) through the conjugation of other polymers. A significant amount of effort is focused on decreasing the frequency of administration and the extent of side-effects felt by the patient; however, none of these new forms of IFN are expected to be as efficacious as PEGasys or PEG-Intron. Included in these new approaches are a number of other polymer conjugation strategies including the linkage of biodegradable polysialic acid. Following conjugation IFN-α exhibited activity 2.5–4.5 % lower than that of the native enzyme suggesting further optimisation of the conjugation conditions is required (Gregoriadis et al. 2005). Attempts have also been made to conjugate the linear non-ionic polysaccharide pullulan to IFN-α. The resulting conjugate was found to retain 60 % of the antiviral activity of the native protein and an increased liver accumulation (122-fold after 24 h) further evaluation of conjugate toxicity is now required (Tabata et al. 1999). Another conjugate prepared by the conjugation of molecules of 2-sulfo-9-fluorenylmethoxycarbonyl (FMS) to the amino groups of human IFN-α also showed promising results. While showing lower biological activity than the native protein (~4 %) and a reduced receptor binding (33 %) this conjugate exhibited a ninefold improved circulation half-life and was completely resistant to the activity of proteases (Shechter et al. 2001).

Other potential delivery systems include Albuferon® (Novartis) made by the genetic fusion of human serum albumin (HSA) and IFN-α with a molecular weight of 85.7 kDa which showed an improved circulation half-life and similar tolerability to that of PEGasys® following phase III clinical trials. Despite this, development by Novartis was recently terminated before gaining regulatory approval for unknown reasons (Kratz and Elsadek 2012). A hydrogel formulation of IFN-α (Flamel technologies) involving a polymer matrix composed of poly-L-glutamate and hydrophobic molecules of α-tocopherol grafted to glutamate units through hydrolysable ester bonds is also under development. As IFN-α non-covalently and reversibly binds to hydrophobic domains this system allows the slow and sustained released of native fully active IFN-α. Formulation was found not to affect the biological potency of the protein and give an extended release over 7 days with fewer adverse events. It is hoped the improved release should support a weekly dosing regimen although clinical trials are still ongoing (Trepo et al. 2006).

16.3 Viral Infections: HIV

16.3.1 Pathology

Human immunodeficiency virus (HIV) is the retrovirus responsible for acquired immunodeficiency syndrome (AIDS). A condition that causes progressive failure of the immune system making those infected at risk of opportunistic infections and cancers. The virus resides mainly in the CD4+ lymphocytes and to a lesser extent macrophage and dendritic cells. This RNA virus codes for the enzyme reverse transcriptase, which transcribes the RNA genome into a DNA version and then integrates it into the host cell genome (Fauci 1988). Immune dysfunction occurs as a result of low levels of CD4+ T-cells due to the following mechanisms: (a) direct viral cell killing, (b) apoptosis of infected T cells and (c) indirect killing by CD8 cytotoxic T-lymphocytes (Warr and Sljivic 1974). Without effective treatment, death from AIDS occurs rapidly (2–4 years from the initial time of diagnosis) (Mirchandani and Chien 1993).

16.3.2 Conventional Therapy

Treatment involves the use of antiretroviral drugs which act by inhibiting different stages in the virus life cycle. These tend to fall into one of the following categories: (a) reverse transcriptase inhibitors which inhibit viral DNA replication either by incorporation and chain termination or by blocking enzyme binding (e.g. zidovudine, etravirine, delaviridine, stavudine, lamivudine), (b) protease inhibitors (PIs) that prevent maturation of newly formed virions by preventing the cleavage of HIV's polyproteins

(e.g. ritonavir, saquinavir, indinavir), (c) fusion inhibitors that act to block the fusion of the virus with the cell membrane and its subsequent entry into host cells (e.g. enfuvirtide), (d) integrase inhibitors that block the incorporation of viral DNA into the host cells (e.g. raltegravir) and finally (e) entry inhibitors that prevent virus entry into the host cells (e.g. maraviroc) (Mallipeddi and Rohan 2010). Treatment regimens using three or more of these drugs at the same time are described as highly effective antiretroviral therapies (HAART) and have proved more effective.

16.3.3 Preclinical Proof of Concept

Various types of nano-carrier-based drug delivery systems have already been investigated for the treatment of HIV including liposomes, nanoparticles, dendrimers and bioconjugates (Gunaseelan et al. 2010; Gupta and Jain 2010). Nano-carrier-based drug delivery approaches offer the following benefits in the treatment of HIV: (a) the specific recognition of HIV infected cells, (b) the ability to deliver drug payload to viral reservoirs and cross physiological barriers (e.g. BBB, blood cerebrospinal fluid barrier), (c) as well as the capacity to deliver multiple drugs simultaneously. The maintenance of higher effective drug concentrations at the sites of viral replication should result in enhanced viral killing and minimise the production of resistant strains.

16.4 Parasitic Infections: Visceral Leishmaniasis

16.4.1 Pathology

Leishmaniasis is a parasitic disease complex with various manifestations. The causative parasites are kinetoplastid flagellates (Family Trypanosomatidae) (Stuart et al. 2008). It is transmitted by female sandflies (Phlebotomine species). There are two main clinical forms of leishmaniasis, visceral (kalazar) and cutaneous (ulcerative skin lesions), as well as less common manifestations, for example, mucocutaneous leishmaniasis (destructive mucosal inflammation) (Croft and Coombs 2003). Leishmaniasis is prevalent in 47 countries in tropical and subtropical regions across the world. It is estimated that 500,000 cases of visceral leishmaniasis are reported every year and more than 50,000 deaths (Desjeux 2004).

Visceral leishmaniasis (VL) is caused primarily by *Leishmania donovani* in India and East Africa and *Leishmania infantum* and *Leishmania chagasi* mainly in the Mediterranean basin, the Middle East, Central Asia and South America (Chappuis et al. 2007; Guerin et al. 2002). It can lead to death if untreated resulting from opportunistic infections which cause pneumonia and diarrhoea (Croft and Coombs 2003). Clinical symptoms of VL are fever, weight loss, anaemia, abdominal

pain, depression of the immune system and enlargement of liver and spleen. The clinical manifestation of VL can take up to 4–6 months to appear. The disease is localised mainly in the spleen and liver macrophages, in addition to macrophages of the bone marrow and lymph nodes (Chappuis et al. 2007).

The life cycle of the parasite occurs in two hosts, insect (sandfly) and the mammalian host (Bates 1994, 2007). In the insect, the parasite is found as a uni-flagellated form referred to as the promastigote stage. A rounded non-motile form of the parasite is present in the mammalian host as the amastigote stage (Alexander et al. 1999). The parasite targeted by chemotherapy when it exists as the intracellular amastigotes which is the clinically relevant stage. These amastigotes are present inside the macrophage in a specific cell compartment called the phagolysosome which has an internal pH of 4.5–5.0 (Zilberstein 1993). The acidic and hydrolytic environment of the lysosome leads to degradation of macromolecules such as proteins and RNA to a lower molecular weight derivatives (amino acids, nucleoside and phosphate) which are important for parasite nutrition (Burchmore and Barrett 2001). The properties of chemotherapeutic agents which determine their uptake by phagolysosome are molecular weight, pKa, lipophilicity and transporters. Efficient drugs should be able to cross the phagosomal membrane and be taken up by the amastigotes (Croft and Yardley 2002).

16.4.2 Conventional Therapy

The standard treatments for leishmaniasis are pentavalent antimonials (sodium stibugluconate, Sb^V) and Amphotericin B (AmB). These drugs are toxic, costly and require repeated administration. The recommended dose of Sb^V for VL is 20 mg/kg/day for 28–30 days (Chappuis et al. 2007). It can be administered intramuscularly (which is painful due to irritation and administration of a large volume) or intravenously which is less painful but impractical when treating large numbers of patients.

AmB is a polyene antibiotic that was first isolated from *Streptomyces nodosus* in 1955 (Gold et al. 1956). It is the gold standard for the treatment of disseminated life-threatening systemic fungal infections such as *Candida albicans*, *Histoplasma capsulatum* and *Aspergillus niger* (Michael 2006; Moen et al. 2009). The antileishmanial activity of AmB was discovered in the 1960s (McMill 1960). AmB is currently recommended as a first-line for the treatment of VL (especially in immune-competent and immunodepressed patients) in Europe and the United States (Sundar and Chakravarty 2010). It has recently being considered a first-line therapy in India, Nepal, Africa and Brazil for the treatment of VL due to the incidence of resistance to antimonials (Sundar and Chakravarty 2010; WHO 2005). Resistance to AmB in eukaryotic microorganisms has been rarely reported (Espuelas et al. 2000; Ghannoum and Rice 1999). A list of drugs used for the treatment of leishmaniasis is shown in Table 16.3. All the mentioned drugs are administered parenterally except miltefosine (oral administration).

Table 16.3 Chemotherapeutic agents for the treatment of visceral leishmaniasis

Drug	Chemical nature	Mode of action	Drawbacks
Pentavalent antimony compounds (sodium stiboglucanate SbV, SSG, meglumine antimonite)	Derivatives of stibonic acid (Alvar et al. 2006)	1. Affects the metabolism of glutathione and trypanothione (Wyllie et al. 2004) 2. Killing the parasite by the process of apoptosis through DNA fragmentation (Sereno et al. 2001; Sudhandiran and Shaha 2003)	Incidences of resistance to SSG have emerged in Bihar state (India) and toxicity (Sundar et al. 2000)
Miltefosine	Alkylphosphocholine (Croft et al. 1987)	1. Affects ether lipid metabolism, cellular signal transduction (Lux et al. 1996) 2. Induction of apoptosis (Navin and Chinmoy 2004)	Teratogenicity and the risk of emergence of resistance (Bhattacharya et al. 2007; Bryceson 2001; Sundar and Olliaro 2007)
Paromomycin (aminosidine)	Aminoglycoside antibiotic (Chunge et al. 1990)	1. Interaction with *Leishmania* ribosome 2. Induction of respiratory dysfunction by affecting cell metabolism (Davidson et al. 2009)	Aminoglycoside toxicity (ototoxicity)
Pentamidine	Aromatic diamidine (Croft and Yardley 2002)	Inhibition of arginine transport and its effect on polyamine biosynthesis (Bray et al. 2003)	1. Toxicity, diabetes, nephrotoxicity, and tachycardia (Soto et al. 1994) 2. Emergence of resistant strains in India
Amphotericin B	Polyene antibiotic (Gold et al. 1956)	Alteration of cell membrane integrity. It interacts with ergosterol in the fungal cell membrane and ergosterol precursor in *Leishmania parasite* cell membrane. This interaction increases the permeability of the fungal cell or *Leishmania* parasite cells and results in leakage of its content causing its death (Brajtburg and Bolard 1996)	Infusion-related toxicity and nephrotoxicity

16.4.3 Preclinical Proof of Concept: Strategy of Drug Delivery to the Macrophages

The location of the parasite inside the phagolysosome of the resident macrophages in different anatomical sites in the body presents a critical barrier for antileishmanial drugs to overcome (Romero and Morilla 2008). There is an urgent need to develop selective drug formulations for macrophage uptake that will minimise undesirable systemic side effects. Upon systemic administration of the drug, only a small amount of the drug typically reaches the macrophages (Ahsan et al. 2002). The main challenge in drug delivery of antileishmanial drugs is to control the distribution of the drug to the target macrophages and to minimise the interaction of the drug with non-target tissues after systemic administration (Gupta and Vyas 2007).

Colloidal drug carriers such as vesicular systems (liposomes and niosomes) and particulate carriers (microparticles, nanoparticles, nanospheres and microspheres) have a crucial effect on the biodistribution of the drug (Gupta et al. 2010). These colloidal carriers can be used for passive targeting to the macrophages because they can be taken up by the mononuclear phagocyte system (MPS) in the liver and spleen via phagocytosis (or endocytosis) (Ahsan et al. 2002). This process involves cellular uptake by invagination of the outside layer of the cell membrane to 'capture' a small volume of extracellular material. Vesicular formation occurs with invagination to allow impermeable extracellular material to be taken up by the cell. Entry into the cytoplasm then proceeds with extracellular material entrapped within vesicles (e.g. endosome or phagosome). Cell trafficking events including fusion with other cytoplasmic vesicles result in a change of the environment (i.e. decreased pH, increased proteolytic enzymes) within the vesicle containing the extracellular material resulting in pathogen death and protein degradation.

Particulate drug carriers are recognised by phagocytic cells of the MPS as an exogenous particle in intact form or opsonised form. Both colloidal carriers and the *Leishmania* parasite are taken up by the macrophages. This makes colloidal carriers potentially ideal delivery systems for antileishmanial agents. Examples of nanodrug delivery systems used for passive targeting of macrophages are shown in Table 16.4.

Another strategy for delivering a drug to the macrophages is by active targeting of the delivery system by inclusion of specific macrophage receptor ligands. Macrophages have various surface receptors such as Fc, complement, fibronectin lipoprotein, and mannosyl and galactosyl receptors. These receptors are responsible for controlling macrophage activities such as activation, recognition, endocytosis and secretion (Ahsan et al. 2002). Many attempts have been made to target macrophages using liposomes grafted with targeting ligands such as mannose, tuftsin and IgG antibodies (Basu and Lala 2004; Dasgupta et al. 2000; Guru et al. 1989; Irache et al. 2008; Veerareddy et al. 2009) (Table 16.4). All these techniques have not reached the clinic because of difficulties in scaling up and high cost (Wagner et al. 2002).

Table 16.4 Examples of delivery systems for anti-leishmanial agents

Delivery system	Drug	Composition	Route	Efficacy	Status
Colloidal dispersion	AmB (Fungizone®)	AmB:deoxycholate (2:1)	IV	Cure rate of 93 % in patients (*L. donovani*) at a dose of 1 mg/kg on alternate days for 30 days (total dose of 15 mg/kg) in India (Sundar et al. 2011) and 95 % cure rate in patients with antimony-resistant VL (Das et al. 2009)	Clinic
Liposome	AmB AmBisome®	Hydrogenated soy phosphatidyl-choline:cholesterol:distearoyl-phosphatidylglycerol:AmB (2:1:0.8:0.4)	IV	Cure rate of 97.5 % in patient with VL (*L. donovani*) at a single dose of 5 mg/kg in India (Sundar et al. 2011) It is effective in the treatment of visceral leishmaniasis in humans caused by *L. infantum* or *L. donovani* (Bern et al. 2006)	Clinic
	Sodium stibugluco-nate (Sb^V)	Dimyristoylphosphatidylcholine: cholesterol:dicetyl phosphate (5:1.5:0.22)	IV	Higher activity (700 times) against *L. donovani* in hamsters than the free drug (Alving et al. 1978). The formulation development was halted by its toxicity in monkeys in 1981 (New and Chance 1980)	Preclinical
Mannose-grafted liposome	Pentamidine	Phosphatidylethanolamine (PE):cholesterol:dicetyl phosphate (7:2:1). Mannose is covalently bound to PE	SC	The reduction of parasite burden in spleen was 46.6 and 85.1 % for drug encapsulated in non-mannose and mannose-grafted liposome compared with 18.5 % for free drug *in hamsters* (*L. donovani*) at a dose of 80 mg/kg (Banerjee et al. 1996)	Preclinical
Tuftsin-bearing liposome	Sb^V	Tuftsin in lipid bilayer (egg phosphatidylcholine and cholesterol)	IV/SC	High activity against experimental visceral leishmaniasis in hamsters (SC) and mice (IV) infected with *L. donovani* at doses of 60 and 75 μg/animal for 3 days (Guru et al. 1989)	Preclinical
Cochleate	AmB	Phosphatidylserine and calcium	Oral	99.0 % inhibition of the hepatic parasite burden in BALB/c mice (*L. donovani*) (10.0 mg/kg twice a day for 5 days) (Wasan et al. 2010)	Preclinical

(continued)

Table 16.4 (continued)

Delivery system	Drug	Composition	Route	Efficacy	Status
Polymer conjugate	AmB	AmB covalently bound to *N*-(2-hydroxypropyl)methacrylamide) (HPMA) via a biodegradable linker (GlyPheLeuGly)	IV	The inhibition of parasite burden was 50 % at a dose of 1 mg/kg body weight in BALB/c mice compared to AmBisome® (98 %) at the same dose (Nicoletti et al. 2009)	Preclinical
Polymer complex	AmB	AmB non-covalently attached to poly(α-glutamic acid)	IV	The complex (29 % AmB loading) showed a similar activity (ED_{50} of 0.24±0.03 mg/kg) to AmBisome (0.24±0.06 mg/kg) in vivo in BALB/c mice against *L. donovani* (Mohamed-Ahmed et al. 2013)	Preclinical
Polymeric nanoparticles	Pentamidine	Poly(L,D-lactide)	IV	It is 3.3 times more active than free pentamidine against VL caused by *Leishmania infantum* in a murine model (ED_{50} value = 0.32 mg/kg versus 1.05 mg/kg for free drug) (Durand et al. 1997a)	Preclinical
	Pentamidine	Polymethacrylates	IV	It is six times more active than free pentamidine in murine model of VL induced by *Leishmania infantum* ED_{50} of 0.17 mg/kg versus 1.06 mg/kg for free drug (Durand et al. 1997b)	Preclinical
Niosome	Sodium stibogluconate (Sb^v)	Mono-*n*-hexadecylether tetraethylene glycol:cholesterol:dicetylphosphate (3:3:1)	IV	It clears MPs organs from parasite (98 % suppression of infection) at a single dose of 296 mg Sb^V/kg as AmBisome® (dose of 8 mg/kg) (Mullen et al. 1998) but did not inhibit reinfection (Carter et al. 1999) Dextran (10 kDa) coated niosomes showed a difference in pharmacokinetics compared to free Sb with higher antileishmanial activity (*L. dononvani*) but there was dosing-related toxicity when tested in dogs (Buates and Matlashewski 1999)	Preclinical
	AmB	Tetraethylene glycol mono-*n*-hexadecylether:cholesterol:dicetylphosphate (3:3:1)	IV	Effective suppression of parasite burden (79 and 89 % in spleen and liver) in *L. donovani* infected BALB/c mice at a dose of 2.5 mg AmB/kg on days 7–11 post-infection (Mullen et al. 1997)	Preclinical

Polymers can be used for lysosomotropic drug delivery and can passively deliver drug into the macrophages. They can also be modified by attachment of a ligand for specific targeting of the macrophages, for example, grafting the polymer with a mannose moiety has been shown to target drug-polymer conjugates to the macrophages via their mannose receptor (Nan et al. 2004). The linker can be specifically cleaved by lysosomal enzymes or by hydrolysis. Polymers grafted with mannose moieties, for example, *N*-(2-hydroxypropyl) methacrylamide (HPMA) were used in the form of conjugates to deliver AmB (Nicoletti et al. 2009). HPMA was grafted with mannose to facilitate its interaction with the mannose receptor in the macrophages in a similar way as *Leishmania* parasite interacts with the mannose receptor to get internalised inside the macrophages (Green et al. 1994). Therefore, these conjugates become internalised via the mannose-dependent receptor-mediated endocytosis and can completely destroy the parasite inside the macrophages (Nan et al. 2004). There was no significant difference between the non-mannosylated and mannosylated conjugate in their observed in vivo antileishmanial activity in BALB/c mice infected with *L. donovani*. This was partially explained by the presence of conformational hindrance that prevents interaction of the polymer-bound mannose with the mannose receptor (Nicoletti et al. 2009). Another type of polymer-based delivery system was also reported, in which AmB was non-covalently associated with poly(α-glutamic acid) (PGA) in the form of an AmB-PGA complex (20–50 % AmB loading) (Mohamed-Ahmed et al. 2012, 2013). This complex was non-toxic to mammalian cells and highly active against active against *L. donovani* in vitro and in vivo (Table 16.4).

16.5 Parasitic Infection: Malaria

16.5.1 Pathology

Malaria is a parasitic disease caused by protozoa of the *Plasmodium* genus (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*). It is transmitted to humans by the bites of the mosquito (female, *Anopheles* genus). It is endemic in 109 countries and causes 1.5 million deaths (Gardella et al. 2008). The life cycle of the parasite is in two hosts: human and mosquito. In humans, the parasite is found in the blood and liver. The parasite infects hepatocytes and at this stage are both exo-erythrocytic schizonts and hypnozoites. The hypnozoites reside in the liver and can cause relapse. The most important stage of the parasite is the blood stage (late trophozoites and schizonts) and this causes the of the disease after 2 weeks from inoculation of the mosquito-vectored trophozoites. At the invasive stage, the parasites are called merozoites and they infect red blood cells (RBCs). The parasites multiply inside the RBCs causing lysis of these cells and this causes symptoms of the diseases (headache, recurrent high fever, myalgia, anaemia, hepatitis and splenomegaly, (Greenwood et al. 2008).

A more dangerous form of the disease is cerebral malaria which is caused by *P. falciparum*, where sequestration of the parasite in the blood vessels leads to blockage of vessels in the brain, cerebral oedema and intracranial hypertension (Coltel et al. 2004). Cerebral malaria can lead to death if untreated (Coltel et al. 2004).

16.5.2 Conventional Therapy

The anti-malaria agents can be classified according to their selective action at different phase of the parasite life cycle into: (1) drugs that eliminate tissue parasites (liver schizonts) are called tissue schizonticides, e.g. primaquine and (2) drugs acting on blood schizonts are called blood schizonticides or suppressive agents, e.g. chloroquine, amodiaquine, pyrimethamine and quinine. Most of these anti-malaria agents target the intra-erythrocytic stage. Those drugs that act on the tissue parasite prevent relapse of the infection. The problems of the conventional chemotherapy are: (1) development of resistant strains (Greenwood et al. 2008), (2) complexity of dose regimen and use of combinations of drugs can cause poor patient compliance and (3) undesirable toxic side effects of the drugs (Winstanley and Ward 2006).

16.5.3 Rationale of Using Nano-Delivery System for Anti malaria Agents

The rationales for using a nano-carrier for delivering antimalarial agents are: (1) protections of the drug from extracellular degradation, (2) increased selectivity of the drug to the parasite, (3) reduced frequency of administration and duration of the treatment and (4) improved pharmacokinetics of the drug (Vauthier and Couvreur 2007). The nano-carrier should be designed to maintain the level of the drug in the therapeutic range in the blood and minimise drug toxicity against human cells. The most important criteria of nano-carriers for delivery of antimalarial agents is the ability to remain in the blood circulation for a long time allowing interaction of the drug with infected RBCs and parasites (Mosqueira et al. 2004). Furthermore nano-carriers can be targeted to specific sites by attaching certain ligands to them (Date et al. 2007). In cerebral malaria, nanoparticulate carriers play an important role as the therapy is administered intravenously. In uncomplicated malaria, the oral route of administration is preferred which reduces the need for using a nano-carrier.

Targeting of anti-malaria drugs to the infected RBCs and hepatocytes is via intravenous administration using passive and active targeting. Passive targeting involves the accumulation of the drug carrier at a specific body site due to physicochemical or pharmacological factors (Barratt 2003). It can be achieved by using conventional nano-carriers such as liposomes and hydrophobic polymeric nanoparticles (Barratt 2003), surface-modified long circulating nano-carriers, e.g. PEG-conjugated carriers (Vauthier and Couvreur 2007). RBCs have no phagocytic activity; this might hinder

the use of particulate carriers to deliver antimalarial drugs. The particulate carriers are cleared from the blood quickly by the mononuclear phagocytic cells and the drug is delivered inside the macrophages (Gupta et al. 2010). This might reduce the rapid action of anti-malaria drugs although the macrophages can act as a depot for slow release of the drug and alter the pharmacokinetics of the drug. Furthermore, using particulate carriers can play an important role in the treatment of *P. vivax* where the parasite resides in the hepatocytes and the drug carrier will be taken by the liver macrophages (Santos-Magalhaes and Mosqueira 2010). Surface modification with PEG reduces the uptake of the particulate carrier by the MPS and prolongs the half-life of the drug carrier (Torchilin 2006), e.g. using halofantrine-poly(lactic acid)-PEG nanocapsules (Mosqueira et al. 2006).

Erythrocytes in the blood and hepatocytes in the liver are the main target for anti-malaria agents. Active targeting can in principle be obtained by attaching a targeting moiety such as carbohydrates, proteins and peptides, e.g. plasmodium amino acid sequences incorporated into PEG liposomes (Longmuir et al. 2006) or antibodies, e.g. delivery of chloroquine in antibody-bearing liposomes (Agrawal et al. 1987). There are two challenges to using specific targeting strategies: (1) some ligands can induce an immune response and (2) the need for optimisation of ligand numbers on the surface of the carrier for cell recognition (Torchilin 2006).

16.6 Fungal Infections: Candidiasis

16.6.1 Pathology

Candidiasis is the most common systemic fungal infection and is associated with 1.5 % of mortality (Badiee and Alborzi 2011). There is a high increase in the incidences of candida infection in hospitalised patients ranging from 0.20 to 0.38 per 1,000 hospital admissions (Kullberg et al. 2011). It occurs in 45 % of renal transplant patients. Most intensive care infections are caused by *Candida* species (*albicans*, *parapsilosis*, *tropicalis*, *glabrata* and *krusei*). The most common causative organism of candidiasis is *Candida albicans* which accounts for 40–50 % of the infections. *C. albicans* is a common organism in the oral, vaginal and gastrointestinal tracts. Under certain circumstance, this organism can cause systemic infection which is considered to be very serious and can be fatal (33–54 % mortality rate).

Candidiasis can be categorised into superficial and invasive depending on the nature of the pathology and immune response. Superficial candidiasis is related to skin and mucosal surfaces including thrush, chronic atrophic stomatitis, chronic mucocutaneous candidiasis and vulvovaginitis (Eggimann et al. 2003b). This type of infection is self-limiting in non-immunocompromised persons and requires local treatment and basic hygiene measures (Eggimann et al. 2003b). Invasive candidiasis involves infections of the bloodstream and subsequent spread of the organisms to organs. It includes candidemia, disseminated candidiasis, endocarditis, meningitis,

endophthalmitis and other deep organ infection (Pfaller and Diekema 2007). In systemic candidiasis, there is hematogenous spread of *C. albicans* to the brain, kidneys, lungs, liver and heart. The heart is most often colonised while spleen and liver are less frequently infected. This pathogen cause necrotic nodules or abscesses resulting in severe damage and failure of the infected organs. Invasive candidiasis is a life-threatening infection; it starts with penetration of pathogen through the mucosal barrier to the blood stream and subsequent spread throughout the body (Vincent et al. 1998). External introduction of the pathogen through surgical implants and catheters have been reported (Romano et al. 1994; Voss et al. 1994). There are several risk factors for systemic candidiasis such as intravascular catheters, burns, abdominal surgery, solid organ transplants, autoimmune diseases, HIV/AIDS infection, malnutrition and liver disease (Hajjeh et al. 2004; Husain et al. 2003; Pfaller 1996). The innate immune system especially neutrophils and macrophages are responsible for eliminating systemic fungal infection. Consequently, most of the systemic fungal infections occur in patients with neutropenia or defects in neutrophils or macrophage function (Grubb et al. 2008; Tuite et al. 2004).

16.6.2 Conventional Therapy

The most common treatment for hematogenous and organ candidiasis is intravenous polyene and azole antifungal agents. Polyene antifungal agents (amphotericin B and nystatin) have been used to treat fungal infection since 1957 (Gallis et al. 1990). AmB has been a first-line treatment for fungal infections since the 1960s because of its broad spectrum activity and with little documented incidence of mycological resistance (Rex et al. 2001; Tumbarello et al. 1996). Intravenously administered AmB either in the form of deoxycholate or lipid-based formulations have been used in the treatment of severe life-threatening fungal infections (Michael 2006; Ostrosky-Zeichner et al. 2003; Pappas et al. 2009). AmB is used for the treatment of invasive fungal infections in immunodeficient patients (HIV and immune-suppressive therapy recipients) (Gallis et al. 1990). It has been widely used to treat candidiasis (Bohme et al. 2009); however, AmB use is limited by its toxicity (Laniado-Laborin and Cabrales-Vargas 2009). Nystatin was discovered in 1949 and isolated from *Streptomyces noursei* (Brown and Hazen 1957). It has good activity against *Candida* species. It has been used for the treatment of cutaneous, vaginal and oral fungal infection since the 1950s (Pace and Schantz 1956; Stark 1967). The poor water solubility and toxicity of nystatin hindered its intravenous use in the treatment of systemic fungal infection (Ng et al. 2003). The toxicity of both AmB and nystatin is due to their ability to self-associate and form toxic aggregates.

Azole antifungal agents such as fluconazole have been used to treat fungal infections since the 1970s and are active against most of *Candida* species (Pfaller et al. 2007). Fluconazole is considered to be safe, however, sometimes there might be risk of alopecia (prolonged high dose) (Pappas et al. 1995) and more serious side effects such as the development of Stevens–Johnson syndrome (Thiyanaratnam et al.

Table 16.5 Conventional therapy for the treatment of invasive candidiasis

Drug	Route	Mode of action
Fluconazole	IV	Inhibition of the cytochrome P450-dependent enzyme lanosterol 14α-demethylase causing damage to cell membrane and death of fungal cells
Voriconazole	IV/oral	Same as fluconazole
Amphotericin B	IV	Interaction with cell membrane and formation of pores that lead to cells death
Nystatin	IV	Same as amphotericin B

2010). The main concern about fluconazole is the presence of some resistant *Candida* strains (Anaissie et al. 1996). Voriconazole is a second generation synthetic azole and has fungistatic activity against *Candida* species similar to fluconazole (Pfaller et al. 2007). It has broad spectrum antifungal activity and retains activity against fluconazole-resistant *Candida* strains (Ruhnke et al. 1997). Side effects associated with voriconazole are ocular toxicity (abnormal vision) related to high serum level (Tan et al. 2006) and rash. A list of conventional drugs used in the treatment of invasive candidiasis is shown in Table 16.5.

16.6.3 Rationale of Using Nano-Delivery System for Antifungal Agents

The rationale of using a nano-carrier for delivery of antifungal agents is to: (a) increase solubility, (b) reduce toxicity and (c) improve pharmacokinetic and distribution to infected tissues. The problem of poor water solubility is common with antifungal agents, e.g. nystatin, voriconazole (0.61 mg/mL) and AmB (<1 μg/mL). Intravenous delivery of antifungal agents is important for the treatment of critically ill patients (Eggimann et al. 2003b). The development of injectable formulations of azoles and polyene antifungal agents is hindered by their hydrophobicity. Among these delivery systems, the lipid-based formulation of AmB is the most successful in improving the therapeutic index of AmB (Adler-Moore and Proffitt 2008). There are several hypotheses by which AmB in a lipid-based formulation can reach the fungal cells (Brajtburg and Bolard 1996). Upon endocytosis, AmB can be released from the lipid vesicle inside the cell. It might also be possible that the AmB-lipid vesicle interacts directly with the fungal cell. Furthermore, the lipid formulation might be phagocytosed by the macrophages which function as reservoir of AmB for intracellular and extracellular antifungal activity of AmB (Brajtburg and Bolard 1996). AmB might dissociate from the lipid and the released AmB will kill intracellular fungi or exit the macrophages as a free AmB to kill extracellular fungi. Examples of delivery systems of antifungal agents in the clinic and preclinical stage are shown in Table 16.6.

Table 16.6 Examples of nano-delivery system of antifungal agents in the treatment of invasive candidiasis

Delivery system	Drug	Composition	Route	Efficacy (in vivo)	Status
Colloidal dispersion	Amphotericin B (Fungizone)	AmB:deoxycholate (1:1)	IV	79 % of patients with candidemia were treated by a dose of 0.5–0.6 mg/kg daily for 7 days and then three times a week (duration of treatment was 14 days) (Rex et al. 1994). It is used for the treatment of invasive candidiasis in critically ill patients (Eggimann et al. 2003a)	Clinic
Liposome	Amphotericin B (AmBisome®)	Hydrogenated soy phosphatidyl choline:cholesterol:distearoy lphosphatidylglycerol:AmB (2:1:0.8:0.4)	IV	Highly effective in the clearance of *C. albicans* from kidneys and other organs in both immunocompetent and leucopenic mice at a dose of 7 mg/kg/day for 5 days (Van Etten et al. 1993). LD_{50} in mice is >175 mg/kg. Reduction of *C. albicans* colony forming units/mg in the kidney (mice) by 99 % and improved survival by 40 % relative to untreated control mice at single and multiple doses of AmBisome® (Adler-Moore et al. 1991). AmBisome® is used in the treatment of invasive candidiasis in humans (Bassetti et al. 2011; Juster-Reicher et al. 2003; Miceli and Chandrasekar 2012; Ruhnke et al. 2008; Zaoutis 2010)	Clinic
	Nystatin (Nytoran®)	Dimyristoylphosphatidylcholine :dimyristoyl phosphatidylg-lycerol (7:3)	IV	Significant clearance of *C. albicans* from blood and tissues in neutropenic rabbits with disseminated candidiasis after a dose of 2 mg/kg/day for 10 days (Groll et al. 1999) It is used to treat invasive fungal infections in humans (Arikan and Rex 2001; Gotzsche and Johansen 2002)	Development hindered by unacceptable toxicity (Larson et al. 2000)
Lipid complex	Amphotericin B (Amphocil®)	Cholesteryl sulphate:AmB (1:1) (lipid complex, colloidal dispersion)	IV	Highly effective in mice infected with *C. albicans* at a dose of 1 mg/kg (Mitsutake et al. 1994). LD_{50} in mice is >75 mg/kg. ED_{50} of 0.05–0.3 mg/kg/day in mice having systemic candidiasis (Clark et al. 1991). Rapid treatment of the heart and kidney was achieved with a dose of 10 mg/kg/day for 1 week in rabbits having disseminated candidiasis (Perfect and Wright 1994)	Clinic

Cochleate	Amphotericin B	Phosphatidylserine and calcium	IP	Highly effective in the treatment of candidiasis in mice at a dose as low as 1 mg/kg/day given for 10 days (Zarif et al. 2000)	Preclinical
			Oral	100 % treatment of mice with systemic candidiasis at dose of 0.5 mg/kg/day for 15 days (Santangelo et al. 2000)	Preclinical
PEGylated liposome	Amphotericin B	PEG-distearoylphosphatidyl-ethanolamine, hydrogenated soy phosphatidylcholine: cholesterol	IV	Complete clearance of *C. albicans* in leukopenic mice at a dose of 5 mg/kg given as single dose 20 h after infection (Van Etten et al. 1995)	Preclinical
Polymeric nanoparticles	Amphotericin B	Poly(ε-caprolactone)	IV	Equivalent activity to Fungizone® (0.5 mg/kg/day) against systemic candidiasis in neutropenic mice at a dose of 2 mg/kg/day for 3 days (Espuelas et al. 2003). LD_{50} is 18 mg/kg in mice	Preclinical
Lipid nanosphere	Amphotericin B	Soybean oil, lecithin and maltose	IV	Highly effective at a dose of 1 mg/kg given 4 h after infection in immunosuppressed mice (Fukui et al. 2003)	Preclinical
Polymeric conjugates	Amphotericin B	AmB covalently bound to arabinogalactan	IV	Complete treatment of systemic candidiasis in mice (100 %) at a dose of 1 mg/kg/day for 5 consecutive days (Falk et al. 1999)	Preclinical
	Amphotericin B	AmB covalently bound to PEG	IV	80 % of mice infected with *C. albicans* survived after 10 days post-treatment with a single dose of 1 mg/kg given 1 h post-infection (Conover et al. 2003)	Preclinical
Polymeric micelle	Amphotericin B	Polymersomes of copolymer of PEG and poly (lactic acid)	IV	Complete clearance of *C. albicans* from the lungs, kidneys and spleen in immunosuppressed mice at a single dose of 5 mg/kg given 6 h post-infection (Jain et al. 2011)	Preclinical
	Amphotericin B	Methoxypoly(ethylene oxide)-*block*-poly (L-aspartate)	IV	Dose response effect similar to Fungizone® in neutropenic mice having disseminated candidiasis (Adams et al. 2003)	Preclinical
Polymer complex	Voriconazole	Sulfobutyl ether β-cyclodextrin	IV/oral	It is used in the treatment of invasive candidiasis in human (Von Mach et al. 2006; Walsh et al. 2000)	Clinic
Emulsion	Nystatin	Nystatin dispersed in intralipid® (oil/water emulsion stabilised with lecithin)	IV	Reduced toxicity of nystatin and improved activity against *C. albicans* in mice after a dose of 8 mg/kg/day for 5 days (Semis et al. 2011)	Preclinical

16.7 Bacterial Infection: Tuberculosis

16.7.1 Pathology

Tuberculosis is (TB) is the second most common infectious disease that causes death after HIV/AIDS. The danger of tuberculosis increases in areas where co-infection with HIV exists. TB is spread by airborne droplets (1–5 μm) containing *Mycobacterium tuberculosis* which remain suspended in the air for minutes to hours after their expectoration by infected people (Loudon and Roberts 1967). The droplet can be inhaled by people and then enter the alveoli. In the lung, alveolar macrophages take up the mycobacterium and either eliminate the bacteria or continue to develop the infection (Brighenti and Andersson 2012). The development of the disease depends mainly on the host's immunity and age. The mycobacterium slowly and continuously replicates inside the alveolar macrophages and spreads to the lymphatic nodes. The development of the disease depends mainly in the cell-mediated immune response that develops 2–8 weeks after infection. Activated T-lymphocytes and macrophages form granulomas that limits the spread of the disease (Gonzalez-Juarrero 2012). The infection can be contained unless there is a defect in the cell-mediated immunity.

16.7.2 Conventional Therapy

The goal of the treatment is to stop progression of the disease with complete cure and no relapse and to prevent emergence of drug resistance and death (WHO 2010). Drug susceptible TB is cured by a combination of rifampicin, isoniazid, pyrazinamide and ethambutol given daily for 6–8 months orally (du Toit et al. 2006). The long period of the treatment and serious side effects reduce patient compliance, but the period of treatment is necessary to kill all pathogens and to avoid resistance. The standard treatments for TB are summarised in Table 16.7.

Table 16.7 Conventional therapy for the treatment of TB

Drug	Route	Mode of action
Isoniazid	Oral or IM	Inhibition of synthesis of mycolic acids (essential component of mycobacterial cell wall)
Rifampicin	Oral or IV	Inhibit bacterial RNA synthesis (Nuermberger et al. 2010)
Pyrazinamide	Oral	Inhibit fatty biosynthesis and protein translation
Ethambutol	Oral	Inhibit mycobacterial arabinosyltransferases involved in formation of arabinoglycan which is an essential component of the MB cell wall
Streptomycin	IM, IV	Inhibit ribosomal protein synthesis

16.7.3 Rationale of Using Nano-Delivery Systems for Anti-TB

The concept of using nanoparticles technology for the delivery of anti-tubercular drugs is that a protective coat is provided for the drug. These drug–polymer nanoparticles after oral or aerosol administration bind to epithelial cells and transport actively across the epithelial layer before being taken up by the phagocytic cells (macrophages). The alveolar macrophages can take up particles in the range of 200 nm and up to 10 μm through non-specific surface receptor (Desjardins and Griffiths 2003). The effective particle size for delivering anti-tubercular agents in the form of polymeric nanoparticles is between 200 and 400 nm (Pandey and Khuller 2004a). The uptake of nanoparticles and *M. bacterium* by the macrophages is via endocytosis (Lawlor et al. 2011). Ligand-targeted nanoparticles are taken up by receptor-mediated endocytosis (Lawlor et al. 2011). Once the polymeric particles are inside the lysosome the polymer degrades releasing the drug locally to kill intramacrophage pathogens and then releases the drug systemically into the blood (Pandey and Khuller 2004a). Several biodegradable polymers have been investigated for the delivery of anti-tubercular drugs such as synthetic polymers, e.g. poly(lactide-*co*-glycolide) (PLGA) or natural polymers, e.g. alginate and chitosan. Examples of nano-delivery systems that have been investigated to deliver anti-tubercular agents are given in Table 16.8.

16.8 Conclusion

Worldwide there are a large number of people suffering from infectious diseases such as hepatitis-C, leishmaniasis, candidiasis and tuberculosis. The overall disease burden continues to increase and often those people most at risk and the most difficult to treat are in resource limited regions of the world. While key drivers for the development of medicines are cost, ease of use and the stability of the final dosage form, another major challenge in treating these diseases is to be able to deliver an effective dose specifically to the site of the target pathogen with minimal effect to non-target tissues and cells. This has led to intense research efforts during the last few decades, with the development of more complex formulations, specifically with particulate-associated drug delivery systems and polymer conjugates. In recent years both of these strategies have shown increasing utility and have become important enabling technologies in efforts to develop more effective medicines to treat infectious diseases. Although significant challenges remain with ensuring clinical efficacy, scaling up production and containing costs, and with many strategies presented still in preclinical stages of development, there are notable nano-medicines that have made the full journey through development and into clinical use.

Table 16.8 Examples of preclinical nano-therapy for the treatment of TB

Delivery system	Drug	Composition	Route	Efficacy (in vivo)	References
Polymeric nanoparticles	Rifampicin Isoniazid Pyrazinamide	PLGA	Aerosol	Complete clearance of *M. bacterium* bacilli from the lung after five doses given every 10 days in guinea pigs (aerosol)	Pandey et al. (2003)
	Streptomycin	PLGA	Oral	Eight oral doses of the nanoparticles can replace 24 IM injections of free streptomycin in mice infected with *M. bacterium*	Pandey and Khuller (2007)
	Rifampicin, Isoniazid and Pyrazinamide	PLGA	SC	Complete clearance of *M. bacterium* bacilli from the lung and spleen after single SC injection in mice	Pandey and Khuller (2004b)
		PLGA coated with lectin	Oral aerosol	Complete clearance of *M. bacterium* bacilli from the lung in guinea pigs after administration of nanoparticles fortnightly (three doses) for 45 days and it can replace free drug given orally daily for 45 days	Sharma et al. (2004)
Liposomes	Pyrazinamide	Dipalmitoylphosphatidylcholine and cholesterol	SC	Significant reduction in bacterial load in the lungs in mice infected by *M. bacterium* after 10, 20 and 30 days after last dose treatment (25 mg/kg) given twice weekly (7 doses)	El-Ridy et al. (2007)
	Rifampicin Isoniazid	Stealth liposome composed of cholesterol, phosphatidylcholine, distearoyl-phosphatidyl-ethanolamine-PEG and amylopectin	IV	The stealth liposome above or below therapeutic concentration was more active than free drug in clearance of *M. bacterium* bacilli from the lung in mice following 12 injections given twice a week for 6 weeks	Deol et al. (1997)

Solid lipid nanoparticles	Rifampicin, isoniazid and pyrazinamide		Oral	Complete suppression of bacterial load in lungs and spleen after 5 doses at every tenth day in mice infected by *M. bacterium*, whereas 46 daily doses of the free drugs is required to reach a similar therapeutic effect	Pandey et al. (2005)
Dendrimers	Rifampicin	PEGylated poly(propylene imine)	IV	High accumulation at the lung compared with non-PEG dendrimers	Kumar et al. (2006)
Niosomes	Rifampicin	Span 85 and cholesterol	Oral/IV	Selective accumulation in lungs compared to free drug following intravenous administration in rats	Jain and Vyas (1995) and Mullaicharam and Murthy (2004)

Problem Box

Q 1. Amphotericin B (AmB) is an example of an active drug substance used to treat fungal and parasitic diseases that has shown little documented resistance. Unfortunately AmB has a narrow therapeutic window and is poorly soluble. Briefly describe how it has been possible to improve the properties of AmB for use as a medicine.

A 1. AmB was initially discovered in the 1950s and shortly afterwards registered for use as a micellar deoxycholic acid formulation which solubilised AmB but did not improve its narrow therapeutic window. Several new classes of antifungal agents have continued to be developed, but AmB continued to be used in spite of its toxicity. Then in the 1990s several new lipid-based formulations of AmB were registered for use, particularly liposomal AmB. Liposomal AmB is a vesicular formulation that (a) helps to mask the AmB form to minimise toxicity and (b) alters the biodistribution of AmB so that it is not systemically distributed.

Q 2. Achieving optimal pharmacokinetics for medicines is important to treat infectious disease. How was interferon α-2 (IFN) improved so that some forms of hepatitis C could be essentially cured?

A 2. Recombinant IFN is a cytokine protein with a molecular weight of almost 20 kDa. Upon administration, it rapidly clears which means for most of the time between doses there is a sub-therapeutic concentration in the body. IFN also has very serious side-effects so daily administration is not possible and as an endogenous protein, such frequent dosing would increase the chance for secondary antibodies against endogenous to form. IFN was improved by PEG conjugation which slowed renal clearance rates to prolong circulation times of IFN and to maintain therapeutic levels between each time the dose was administered. In this way IFN could be dosed once weekly. When used weekly and in combination with ribavirin, this has allowed some forms of hepatitis C to be cured.

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Chapter 17
Vaccines

Yvonne Perrie, Randip Kaur, and Malou Henriksen-Lacey

Abstract Vaccines continue to offer the key line of protection against a range of infectious diseases; however, the range of vaccines currently available is limited. One key consideration in the development of a vaccine is risk-versus-benefit, and in an environment of perceived low risk, the benefit of vaccination may not be recognised. To address this, there has been a move towards the use of subunit-based vaccines, which offer low side-effect profiles but are generally weakly immunogenic. This can be compensated for by the development of effective adjuvants. Nanotechnology offers key attributes in this field through the ability of nanoparticulates to incorporate and protect antigens from rapid degradation, combined with their potential to effectively deliver the antigens to appropriate cells within the immune system. These characteristics can be exploited in the development of new adjuvants. This chapter will outline the applications of nanosystems in vaccine formulations and consider the mechanisms of action behind a range of formulations.

17.1 Exploiting Nanotechnology in Vaccine Formulation

Infectious diseases remain among the leading causes of death worldwide. Vaccination offers one of the most effective strategies in global healthcare to address this. However, there is an on-going need to develop new and more effective vaccine formulations so as to offer protection against new emerging diseases [e.g. severe acuter respiratory syndrome (SARS) virus coronavirus, and human immunodeficiency

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virus (HIV)] and re-emerging old and/or persistent infectious diseases (e.g. Tuberculosis, malaria, and foodborne infections). As we have already seen within this book, nanotechnology can play a key role in many pharmaceutical applications, and vaccines are no exception. The application of nanoscience to vaccine formulation offers the potential to enhance the efficacy of vaccination by promoting enhanced protection and effective delivery of antigens. To effectively exploit nanotechnology in vaccine development, we must first consider the possible mechanisms which support effective immunisation.

17.1.1 Vaccines—an Introduction

The immune system comprises of many cellular and humoural components which protect the host from disease, a term which includes infection, autoimmune syndromes, injury, and mutations. To do so, the host has acquired the ability to evolve to its environment, a classic example being the gut whereby commensal microbes live in harmony with the host. The immune system has two main functions; to recognise invading pathogens and to activate mechanisms that will destroy them. Such pathogens are controlled and terminated by the innate immune response which is ready to react quickly (Fig. 17.1a). Most components of innate immunity are present before the onset of infection and constitute a set of disease-resistance mechanisms that are not specific to a particular pathogen. These mechanisms include cellular and molecular components that recognise classes of molecules as different to the frequently encountered pathogens (Goldsby et al. 2003). Phagocytic cells such as neutrophils, macrophages, in addition to pattern recognition receptors, NK cells, complement, and variety of antimicrobial compounds synthesised by the host all play important roles in innate immunity (Goldsby et al. 2003).

The adaptive immune response (Fig. 17.1b) is made up of B and T lymphocytes that have unique receptors specific to various microbial antigens (Sudhakar and Subramani 2005), in contrast to the receptors of the innate immune system which are of many different types but not specific to a particular pathogen (Parham 2009). These antigen-specific receptors are encoded by genes generated during a complex process of gene rearrangement that occurs during the course of lymphocyte development. As each B and T lymphocyte contains a unique antigenic receptor, it allows for large and diverse population of cells capable of recognising a wide spectrum of pathogens. This is termed the lymphocyte repertoire (Sudhakar and Subramani 2005). In response to an infection, lymphocytes-bearing receptors specific for the pathogen are then selected to participate in the immune response. The proliferation and differentiation of these cells, termed clonal selection and expansion, generates a large population of specific effector cells. To assist in future invasion by the same pathogen, some of the lymphocytes persist in the body and provide long-term immunological memory, thus resulting in a faster and stronger response (Parham 2009).

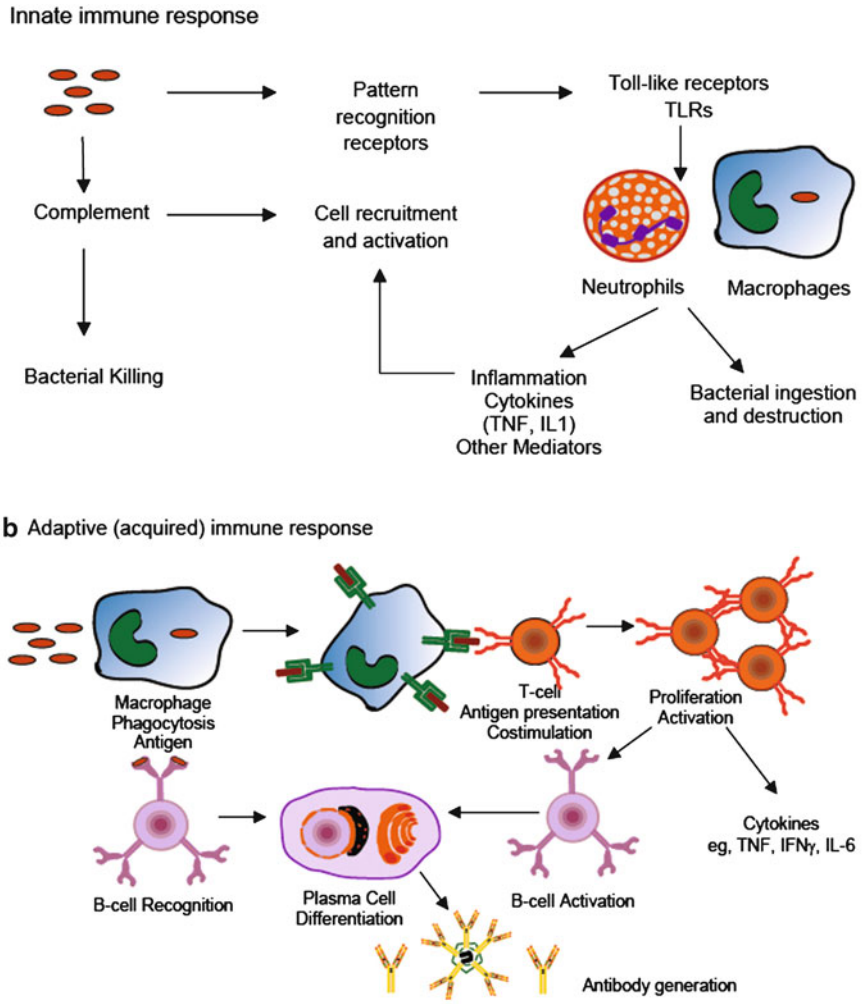

Fig. 17.1 Schematic representation of the immune response. (**a**) Innate immune response. Non-self-cells are rapidly attacked in the innate immune system. Key players in the innate system are neutrophils, macrophages, pattern recognition receptors, NK cells, and complement. The desired end result is the destruction of the foreign substance, the non-self-cell. (**b**) Adaptive immune response. The major players in this response are the B lymphocytes, T lymphocytes, and NK cells. The desired end result is destruction of the non-self-cell but through a more complex and tightly orchestrated series of events (based on Bingham 2008)

Therefore, the immune system is developed to offer a wide range of protection—however, one issue is that during an infection the body must be able to respond quickly and vigorously enough to provide the appropriate protection without the individual suffering the potentially lethal consequences of the infection. To address this vaccines have been developed; vaccines have been defined as ‘any preparation

made from a pathogen that is used for vaccination and provides protective immunity against infection with the pathogen' (Parham 2009). The ultimate goal of a vaccine is to develop long-lived immunological protection, whereby the first encounter with a pathogen is remembered and recognised by the immune system and therefore the immune system can generate a rapid, protective response against the infection.

17.1.2 The History of Vaccination

The origins of vaccination lie with smallpox, a disease which once ravaged most, if not all parts of the world. Initial descriptions of smallpox stem from as early as 1122 BC from texts originating in China. Smallpox was known to spread rapidly and resulted in disfigurement, blindness, and death. It was also known that smallpox was infectious, and early reports dating from 430 BC describe survivors of smallpox being used to treat those infected in a process known as inoculation (Gross and Sepkowitz 1998). Over many hundreds of years the concept of inoculation began to take form and involved a small swab of infectious material (otherwise known as pus) being placed on the skin of non-infected persons, and in 1,722, members of the English Royal Family were successfully immunised against smallpox using this method (Riedel 2005). However, it was the work of Edward Jenner, born in 1749 in Gloucestershire, UK, that supported the development of vaccination. Jenner, after overhearing a young dairymaid claim that she may never have smallpox as she had had cowpox, decided to further investigate this. In 1796 Jenner successfully inoculated a young boy with infectious material from a dairymaid who had cowpox lesions; 2 months later he exposed the boy to smallpox and no disease resulted. This was the beginning of what we now term vaccination.

17.2 Current Conventional Vaccines

In their traditional organisation, vaccines are grouped into four categories: killed, attenuated, toxoids, or subunit vaccines.

- *Live attenuated vaccines*
  Live attenuated vaccines consist of a live microbial agent that has mutated so that it has a reduced ability to grow in human cells and is no longer pathogenic to humans (Parham 2009). These microorganisms are still able to infect their target cells. However, infection is inefficient (mild) and there are limitations in the replication of the microorganisms. Vaccines produced in this manner include the bacillus Calmette-Guérin (BCG) and the measles, mumps, yellow fever vaccines, and rubella combination vaccine (MMR), and such vaccines are generally capable of stimulating both a humoural and cell-mediated immune response. However, there is a risk of reversion to virulence, and this type of vaccine is not considered safe for use in immunocompromised individuals.

Table 17.1 European licensed adjuvants for inclusion in vaccines

Adjuvant	Produced by	Disease
Aluminium salts	Various	Various
MF59® (squalene)	Novartis	Influenza
AS03 (squalene + tocopherol)	GSK biologicals	Influenza
AS04 (MPL + aluminium hydroxide)	GSK biologicals	HPV, HBV
Virosome	Crucell	Influenza, HAV

GSK GlaxoSmithKline, *MPL* monophosphoryl lipid A, *HPV* human papillomavirus, *HBV* hepatitis B virus, *HAV* hepatitis A virus
Adapted from (Friede 2009; Mbow et al. 2010)

- *Inactivated vaccines*
  Inactivated vaccines consist of microorganisms or viruses that have been treated with, e.g. heat or chemicals such as formaldehyde, thereby removing their ability to be infectious while retaining immunogenicity. While offering advantages in terms of safety, such vaccines are generally less effective than live attenuated vaccines, usually only stimulating humoural immunity and often requiring booster doses. Examples of these vaccines include: trivalent inactivated influenza vaccines, cholera, and hepatitis A vaccines.
- *Toxoids*
  Some microorganisms produce toxic compounds that are the responsible for causing the disease (i.e. tetanus toxin and diphtheria toxin). Toxoids are inactivated forms of these toxic compounds. In addition to being successful vaccines in their own right, toxoids may also be used to increase the immunogenicity of some other vaccines, such as the *Haemophilus influenzae* type B (Hib), which contains a polysaccharide unit from the virus conjugated to diphtheria or tetanus toxins.
- *Subunit vaccines*
  Subunit vaccines initiate strong immune responses using a small part of the organism that could include a gene from the genome. Recombinant DNA technology has greatly facilitated the development of such vaccines, a process where foreign genes are introduced into yeast or bacteria expression systems. This allows the production of large quantities of antigen that is purified and used as a vaccine (Arvin and Greenberg 2006). The principle benefits of subunit vaccines are their inability to revert to a pathogenic form, decreased toxicity, reproducible production, and improved antigen specificity; however, the immune response induced by such vaccines is short-lived and thus several boosts are required to achieve protection. For Hepatitis B virus for example, only the surface protein of the virus is used to generate the subunit vaccine.

As is highlighted in Table 17.1, there is no clear rule as to which type of vaccine (recombinant/killed/attenuated, etc.) may be superior against a certain disease. Certainly the present aim of vaccinologists is to focus on safety (including low toxicity and prevention of reversion to virulence); however, immunogenicity obviously plays a major role. With regard to smallpox, while the live attenuated composition of the vaccine led to the successful eradication of the disease, there were numerous

other factors which certainly contributed including the lack of an animal reservoir, non-zoonotic disease, stability of the vaccine formulation, and clear disease symptoms. However, it is interesting to note that the side effects from this vaccine were also sufficiently bad for the vaccination campaign in the United States to be halted in 1972, 8 years prior to the declaration by the WHO that the world was free from smallpox (Kennedy et al. 2009). Therefore, even the only vaccine to have ever led to the eradication of a disease had its faults and it is most probable that if the smallpox vaccine was still required today, it would not be used as it would fail clinical trials.

17.3 Improving Subunit Vaccine Efficiency with Adjuvants

The first marketed subunit vaccine, available in the United States in 1981, was for protection against Hepatitis B (Hilleman 2000). Therefore, in terms of arrivals onto the vaccine market, the subunit vaccines are latecomers with development rather than discovery being hindered by their poor immunogenic profile. However, they have the very important advantage over attenuated or killed vaccines in that there is no chance of reversion to a virulent form. Subunit vaccines are based on solely the antigenic epitopes originally derived from a virulent organism; there is a total loss of the molecules which would have typically alerted the host to the dangerous nature of the pathogen, in addition to the loss of particulate nature. This appears to be the downfall of subunit vaccines—they lack sufficient resemblance to pathogens. Therefore in an effort to improve the immune responses to subunit antigens, adjuvants are included in the formulation.

Adjuvants, whose name stems from the Latin ‘adjuvare’ meaning to aid, are defined as substances used in combination with a specific antigen that produce a robust immune response when compared to the antigen alone (Gupta et al. 2005; Vogel 1995). Adjuvants come in such a wide range of shapes and sizes that even though documentation on adjuvants has existed for nearly 100 years, there is still no universally approved grouping system. Figure 17.2 summarises some of the current suggestions for mechanisms of adjuvant action.

One of the first papers published by Ramon described the adjuvant effect of a range of compounds including tapioca, agar, and starch oil (Ramon 1925). Following this, the use of inorganic compounds including aluminium phosphate and aluminium hydroxide was documented (Glenny et al. 1926) and these aluminium-based compounds became the first licensed adjuvants in commercial vaccines. Until late 2009, aluminium-based compounds remained the only US-licensed adjuvants, while in Europe a wider scope of adjuvants had been recognised (e.g. Table 17.1). However, adjuvant development from bench to marketed vaccines has been slow with the first non-aluminium salt adjuvant being licensed less than 20 years ago (Mbow et al. 2010; O’Hagan and Gregorio 2009). The slow output and lack of successful licensing is due to numerous reasons including poor scale-up and inflexibility with regard to the scope of antigens with which they can be administered (O’Hagan and Gregorio 2009). However, when the varied immunising abilities of different vaccine adjuvants bearing

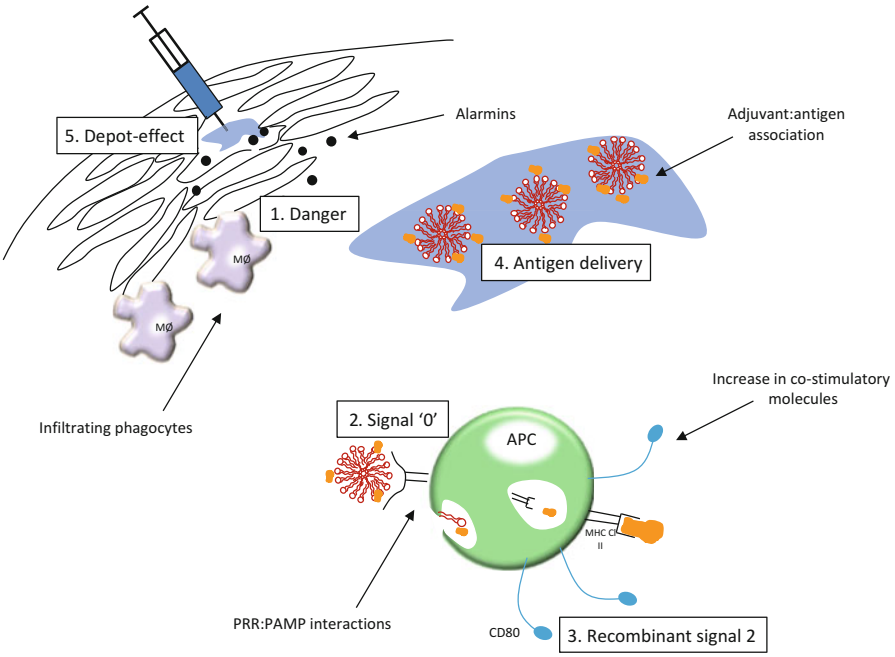

Fig. 17.2 Classification of adjuvants: the five steps of adjuvant action are described in the text. *APC* antigen-presenting cells, *MØ* macrophage, *PRR* pathogen recognition receptors, *PAMP* pathogen-associated molecular patterns

remarkably similar structure and composition are considered, these requirements are hardly surprising. For these reasons adjuvants are licensed in individual vaccines as opposed to being considered a separate entity (Fig. 17.3).

17.3.1 'Alum'—The Original Particulate Adjuvant

'Alum' is a collective term often used to refer to a group of aluminium salts including aluminium hydroxide, aluminium phosphate, and aluminium potassium sulphate. The correct term for this group of adjuvants is aluminium salts and not Alum, which correctly refers to aluminium potassium sulphate (Marrack et al. 2009). The widespread use of aluminium salts as vaccine adjuvants is due to a combined ability to (generally speaking) improve vaccine immune responses as well as provide an excellent safety profile. While occasional local reactions including inflammation, erythroma, subcutaneous nodules, and allergic reactions are reported, when the numbers of people who have been vaccinated are considered, aluminium salts are exemplary adjuvants (Clements and Griffiths 2002). The major downfall of aluminium salts is the polarised immune response which they activate being predominate activators of Th2-biased immunity, i.e. aluminium salts are ideal adjuvants in vaccines requiring

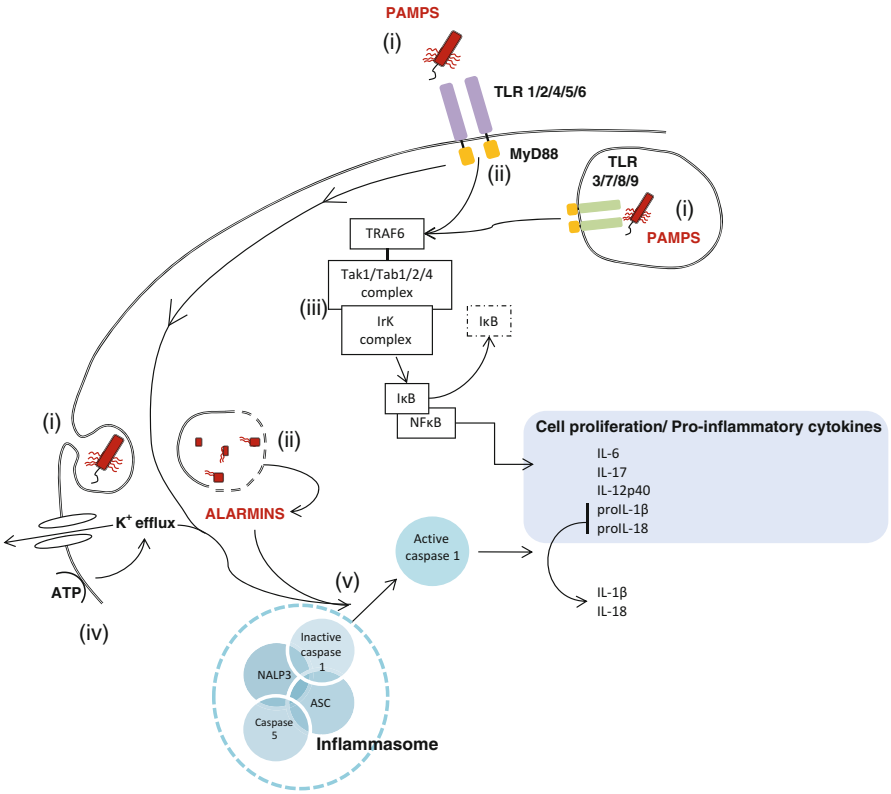

Fig. 17.3 Mechanisms of immunological action of aluminium salts: downstream signalling events after activation of Toll-like receptors (TLR) and the inflammasome. (i) Pathogens are endocytosed and activate TLRs via their pathogen-associated molecular patterns (PAMPs) resulting in the initiation of intracellular events including release of alarmins and activation of MyD88 adapter proteins respectively (ii). The TRAF6 signalling pathway becomes activated leading to cleavage of IκB and activation of the transcription factor NFκB with production of pro-inflammatory cytokines such as IL-1β and IL-18, both of which are produced with 'pro' inhibitory domains (iii). Endogenous ATP can act as an alarmin to activate the P2X7 receptor leading to potassium efflux from the cell (iv). The combination of potassium efflux and TLR activation lead to cleavage of the active caspase component of inflammasomes which is then able to cleave pro-domains of the cytokines IL-1β and IL-18 (v)

strong humoral immunity with high levels of IgG1 antibodies and cytokines such as IL-4. Examples of this include vaccines against extracellular pathogens such as parasitic diseases (e.g. leishmaniasis) or toxoid-producing pathogens (e.g. diphtheria, tetanus). Among the methods used to mediate the Th1/Th2 balance, one has involved combining aluminium salts with adjuvants known as strong stimulators of Th2 responses. Examples include aluminium hydroxide combined with cationic liposomes (Agger et al. 2008) or monophosphoryl lipid A (MPL), the latter of which is now the licensed adjuvant AS04 (GlaxoSmithKline (GSK) Biologicals).

It is only recently that the mechanisms by which aluminium salt adjuvants act have started to be uncovered, challenging the previous dogma that adjuvanticity was

simply due to longer retention of antigen at the injection site, also known as the depot-effect (Marrack et al. 2009). The importance and mechanisms of antigen association to aluminium adjuvants remains debated (e.g. Morefield et al. 2005); however, it is clear that aluminium salts prevent the rapid removal and degradation of antigen normally seen upon injection of free antigen. There is now a wide range of literature on the mechanisms of aluminium action (for in-depth reviews see (Brewer 2006; Marrack et al. 2009)) which strongly suggest a role for the inflammasome and uric acid release upon local tissue damage (Marrack et al. 2009; O'Hagan and Gregorio 2009). Briefly, injection of aluminium salts is known to cause tissue damage and cell death with release of alarmins such as endogenous uric acid, infiltration of neutrophils, and inflammatory mediators. Uric acid is capable of activating caspase 1, a component of the inflammasome, which can subsequently cleave pro-units of IL-1β and IL-18 to their active forms (Kool et al. 2008, see reference within Kennedy et al. 2009; Mariathasan 2007; Martinon et al. 2002; Monie et al. 2009) (Fig. 1.5).

17.3.2 Improving our Understanding of How Adjuvants Work

Generally speaking, adjuvants can be divided into groups depending on their physical properties, such as inorganic salts, liposomes, oil in water (o/w) emulsions, surfactants, etc. This classification poses the problem that the method in which the adjuvant acts is unknown and therefore does not help with eliminating or identifying potential nanosystems as future adjuvant candidates. Another classification which will become more complex with time is proposed by O'Hagen and De Gregorio (2009) whereby adjuvants are classed as first- or second-generation adjuvants. In this system a first-generation adjuvant refers to one of the more traditional substances normally composed of one immunostimulatory compound. These include aluminium salts, liposomes, and MF59 among others. Addition of further immunostimulatory compounds to these existing first-generation adjuvants results in a second-generation adjuvant such as the aforementioned 'Adjuvant Systems' (GSK Biologicals), IC-31® (Intercell, Austria), and ISCOMS. While the possibility exists to extend the system to third-generation adjuvants (and fourth and fifth, etc.), the system becomes increasingly awkward and does not give any indication as to how the adjuvants may work. In 2000 Virgil Schijns proposed a system whereby adjuvants can be divided into groups depending on their method of immunostimulatory action (Schijns 2000). While five groups were suggested, it is possible that further mechanisms may be deduced in the future, in addition to the difficulty in classification when one adjuvant has more than one mode of action. Finally and possibly the simplest involves a classification system based on whether the adjuvant works via TLRs or not (Mosca et al. 2008). In this instance only two groups exist (TLR-dependent and TLR-independent); however, for adjuvants containing two or more immunogenic components (such as GSK's AS04 adjuvant containing aluminium hydroxide and MPL), they may act via both TLR-dependent and TLR-independent mechanisms

further complicating matters. For the purposes of identifying the mechanisms of action of present and future adjuvants, the classification system by Schijns is the most appropriate and the basis of how these adjuvants work will be described herein.

17.3.3 Concepts of Adjuvant-Mediated Immunogenicity

According to Schijns classification, there are five methods in which adjuvants may be immunostimulatory. Highlighted in Fig. 17.1, these methods provide an ideal way to explain the immune system with relevance to systemic delivery of vaccines. Upon parental vaccine delivery there is localised tissue damage which results from the physical insertion of the needle and vaccine components into the tissue milieu. Cells will inevitably be ruptured releasing their intracellular contents including mitochondria, uric acid, and heat shock proteins (HSPs), all of which are termed 'alarmins' (Bianchi 2007). Alarmins are host-derived substances released upon non-intentional cell death (in contrast to apoptosis) and, unsurprisingly signal to the host that there is a problem such as tissue destruction or stress. This can be considered the first mechanism of adjuvant action (Shi et al. 2000) and is termed the danger signal, in reference to the 'danger model' originally described by Matzinger (1994). The danger model assumes that the host can differentiate between harmless and harmful as opposed to self and non-self [the later model originally described by Metchnikoff and thoroughly reviewed by Janeway (1992)]. Overlapping with the danger model is the concept of signal 0, the second class by which adjuvants act. Signal 0 can include alarmins but with respect to adjuvant composition and formulation the signal 0 group principally includes exogenous pathogen-associated molecular patterns (PAMPs). Importantly the signal 0 concept is the physical binding of alarmins or PAMPS [collectively termed danger-associated molecular patterns, or DAMPS (Bianchi 2007)] to pathogen recognition receptors (PRR's) and the resulting intracellular signalling cascade that results in activation of the cell (see Fig. 17.4 for an schematic of DAMPS and their TLRs). PRRs are constitutively expressed on or within cells of the innate immune system (Schijns 2000) and include, among others, TLRs, inflammasomes, integrins, c-type lectins, and antibody Fc receptors. The specific signalling cascades initiated by these DAMP-PRR interactions will be discussed later but some of the resulting actions include the production of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules required for the productive activation of the T cell upon antigen presentation. In fact, antigen presentation without simultaneous co-stimulatory molecule interactions has been shown to lead to tolerance. Co-stimulatory molecules such as CD80 and CD86 are consequently very important for successful T cell activation and while being expressed constitutively on DCs, they are only expressed on other APCs including macrophages and B cells upon activation. Adjuvants which are able to activate macrophages and B cells to induce expression of CD80 or CD86, and those able to upregulate expression of the said markers on DCs can be grouped under the third adjuvantal category, 'recombinant signal 2'. The principle adjuvants in this group are cytokines which can be considered as endogenous adjuvants and

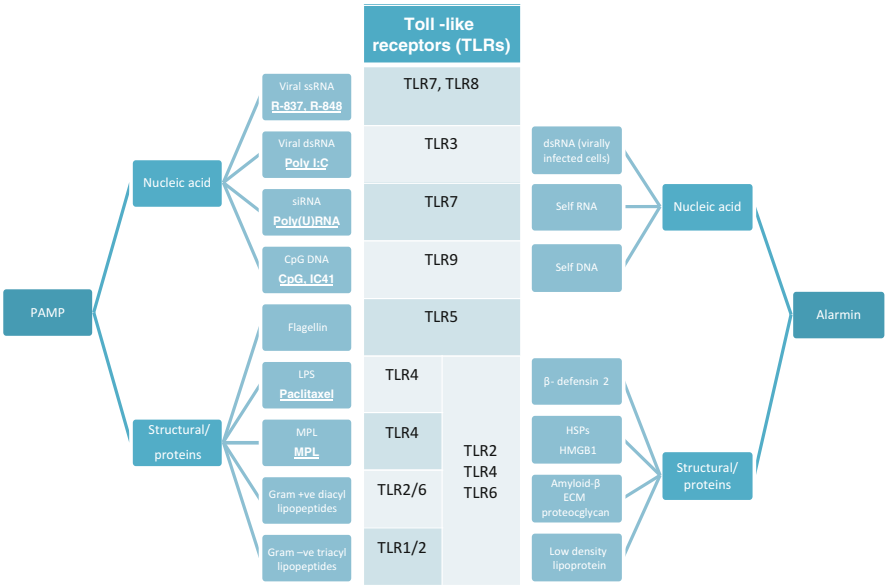

Fig. 17.4 Flow chart showing pathogen-associated molecular patterns (PAMP) and alarmins which are known to bind Toll-like receptors (TLRs). Synthetic analogues known to target specific TLRs are bold underlined. *R-837* Imiquimod, *R-838* resiquimod, *HSP* heat shock protein, *HMGB1* high motility group box 1, *ECM* extracellular matrix. Data obtained from a range of sources including (Bianchi 2007; Dockrell and Kinghorn 2001; Monie et al. 2009; O'Hagan and Gregorio 2009)

have also been administered with vaccines experimentally [see (Boyaka and McGhee 2001; Egan and Israel 2002; Heath and Playfair 1992) for review articles], as well as TLR-signalling adjuvants.

The final two adjuvantal categories relate more to the physical structure of the adjuvant and its physical localisation in vivo. As discussed previously, there is much diversity in the structures of adjuvants, although many of them are based on emulsions or vesicular structures. The benefits of vesicular structures are twofold; firstly they can be used to 'carry' antigen, either by entrapment or adsorption; secondly vesicles are naturally occurring and appropriately sized structures that can be endocytosed by cells depending on their composition and size, making them ideal intracellular delivery systems. This paradigm of assisting antigen uptake is the fourth adjuvantal category and principally concerns liposomes, nanoparticles, microspheres, virosomes, emulsions, and ISCOMs, all of which are able to package antigen into a delivery vehicle. The final concept is the idea that the antigen held at the injection site for a long period of time results in lengthy presentation of the antigen to innate immune cells. Known as the depot-effect, it refers to localisation of antigen (with or without adjuvant) at the injection site and not in the lymphoid organs (although increased presentation of antigen in the lymphoid organs may be a direct consequence of the depot-effect and is often the desired effect). The depot-effect is therefore dependent on numerous factors such as the route of injection, the tissue found at the injection site, and characteristics associated with the formulation itself such as viscosity and particulate size.

17.3.4 Defining the Ideal Adjuvant

From this analysis of different 'types' of adjuvants, it is clear that there are still many overlaps between groupings; however, the system devised by Schijns does help to define the mechanisms by which adjuvants work. With this in mind, it is possible to envisage how the perfect adjuvant would work. Firstly, it must contain a sufficient amount of PAMPs to alert the immune system of danger but without causing hyper-immunostimulation which may result in anaphylactic shock or local tissue damage by excess of inflammatory mediators. Too few PAMPs or PAMPs without sufficient toxicity may not stimulate sufficient production of pro-inflammatory molecules or chemokines which are vital to alert circulating APCs. It may just be that a certain degree of local tissue damage is also beneficial as it allows the release of alarmins, further promoting APC influx to the injection site (Shi et al. 2000). The adjuvant should also ideally stay associated with the antigen until uptaken by APCs so that the immune system can make a collective association of both components. Free antigen, in the form of nucleic material or small peptide antigens, is rapidly degraded by extracellular enzymes and many subunit protein vaccine antigens are themselves immunogenically inert. Protein and peptide antigens undergo rapid removal via the circulatory or lymphatic system and without any danger signals attached, the protein may simply be removed before antigen uptake and presentation can occur.

17.4 Nano-Enabled Vaccine Formulations

Given the key characteristics outlined above, it is no surprise that nanosystems have been extensively considered and exploited as potential vaccine adjuvants, with a wide range of nanomaterials being considered. Examples of such include nanoparticles, virosomes, immune-stimulating complexes (ISCOMS), liposomes, and bilosomes among others. By associating antigens with such nanosystems, the antigen can be protected from the extracellular milieu thereby limiting peptide/protein and nucleic antigen breakdown by enzymes, as well as preventing the rapid removal of such small compounds by the mononuclear phagocytic system (MPS).

17.4.1 Liposomes, Niosomes, and Other Vesicular-Based Adjuvants

The immunological role and adjuvant properties of liposomes were first identified by Allison and Gregoriadis (1974), where the ability of negatively charged liposomes (prepared with the inclusion of dicetyl phosphate) to deliver and potentiate immune responses against diphtheria toxoid (DT) was demonstrated. Since then the

immunological adjuvanticity of liposomes has been well recognised and liposomes have been extensively investigated as potential vaccine adjuvants for more than 20 years and for a number of antigens, including, e.g. tetanus toxoid (Davis and Gregoriadis 1987), *Leishmania major* antigen (Kahl et al. 1989), hepatitis B surface antigen (Brunei et al. 1999), DNA vaccines (Perrie et al. 2001, 2003), and tuberculosis vaccines (Davidsen et al. 2005; Smith Korsholm et al. 2007), with some liposomal-based vaccines (i.e. virosomes) having been licensed for human use (i.e. Inflexal vaccine for influenza).

Yet while there is a wealth of research dating from the 1980s and 1990s based on improving immune responses with the aid of liposomes, generally more attention was given to the ability of the formulations to effectively deliver the vaccines through enhancing protection and APC uptake of the antigen. However, given current understanding of adjuvants, more recently the focus has turned not just to delivery attributes but also immunomodulation, therefore research has focussed on combining the delivery attributes of liposomes with adjuvant properties through the inclusion of immunomodulating molecules. For example, Olsen et al. (2001) showed an increase and an induction of protective immunity against tuberculosis when isolated protein antigens, found in mycobacterial culture filtrates, were incorporated within liposomal vesicles combined with dimethyldioctadecylammonium (DDA; when hydrated in an aqueous environment this cationic lipid self-assembles into closed bilayers) (Olsen et al. 2001). Yet these identified proteins possess low inherent immunogenicity when injected alone (Andersen 1994). The authors reached a conclusion that in order for immune protection to remain high over an extended period, a depot must have been formed at the injection site by DDA (Holten-Andersen et al. 2004; Olsen et al. 2001). Holten-Andersen and co-workers conclude DDA may act to increase antigen and immunostimulator uptake into APC when it forms the depot.

The immunostimulatory properties of cationic lipids were originally documented in a screening study by Gall (1966). Within this study a wide range of compounds were investigated for their ability to adjuvant diphtheria or tetanus toxoids in guinea pigs. Compounds included non-ionic, anionic, and cationic surface-acting agents, amines, guanidines, benzamidines, thioureas, thiosemicarbazides, thiosemicarbazones, thiouroniums, and various nitrogenous bases. With regard to the group of surface-acting agents, or more commonly termed surfactants, Gall observed increased adjuvanticity for those expressing cationic quaternary ammonium head groups and long alkyl chains (Gall 1966). Within this group was DDA, a synthetic amphiphile, which contains a quaternary ammonium group with two 18-carbon-long alkyl chains forming the hydrophobic moiety and two methyl groups, which together with the ammonium group form the polar head group (Fig. 17.5). The positively charged head group carries a monovalent counter ion, typically bromide or chloride. Due to its amphiphilic character DDA can form liposomal structures when dispersed in aqueous media at temperatures above its gel-to-liquid phase transition temperature (~47 °C) (Davidsen et al. 2005). DDA is known to induce cell-mediated immunity and delayed-type hypersensitivity (Snippe et al. 1982), and along with its cationic nature and surfactant properties, has been shown to be an effective adjuvant

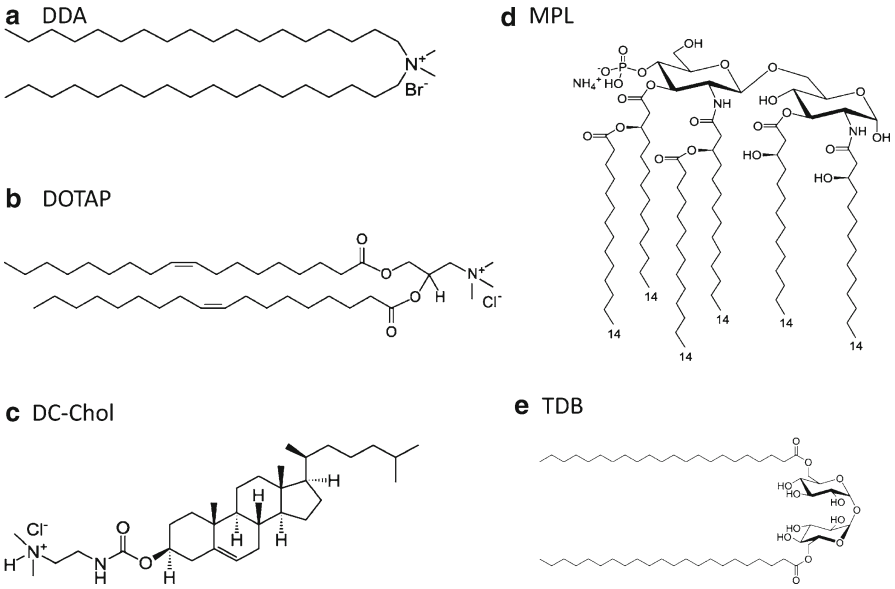

Fig. 17.5 Examples of lipids currently investigated in potential liposomal adjuvant systems (images courtesy of Avanti Polar lipids inc., http://avantilipids.com/)

in numerous applications, including mucosal immunisation (Klinguer et al. 2001), gene delivery (Esposito et al. 1999), and subunit vaccine delivery (Lindblad et al. 1997; Brandt et al. 2000; Holten-Andersen et al. 2004; Rosenkrands et al. 2005).

The mechanism of action behind the adjuvant effect of DDA has been attributed to its positive surface charge and its ability to associate antigens (Hilgers et al. 1985). This was recently confirmed and further elaborated by using ovalbumin (OVA) as a model antigen (Korsholm et al. 2007). Stimulation of immature bone marrow-derived dendritic cells (BMDCs) with fluorescently labelled OVA showed that adsorption of OVA onto DDA enhanced the cellular acquisition of the antigen. Further inhibition of active cellular processes by OVA stimulation at 4 °C or by the addition of cytochalasin D reduced the cellular uptake, suggesting that active actin-dependent endocytosis is the predominant uptake mechanism (Korsholm et al. 2007). DDA-mediated OVA uptake was further associated with a functional enhancement of the APCs. This was shown by measuring the increase in IFN-gamma production and cellular proliferation of purified autologous DO11.10 T-cells transgenic for a T-cell receptor recognising a major histocompatibility complex (MHC) class II–restricted OVA-epitope (OVA323-339). Both proliferation and IFN-gamma production were increased upon interaction with either murine BMDCs or purified B-cells, stimulated with OVA adsorbed to DDA (Korsholm et al. 2007; Christensen et al. 2009). More recent studies replacing DDA with a neutral lipid, DSPC, further demonstrate the role of the cationic lipid in the liposomal adjuvant

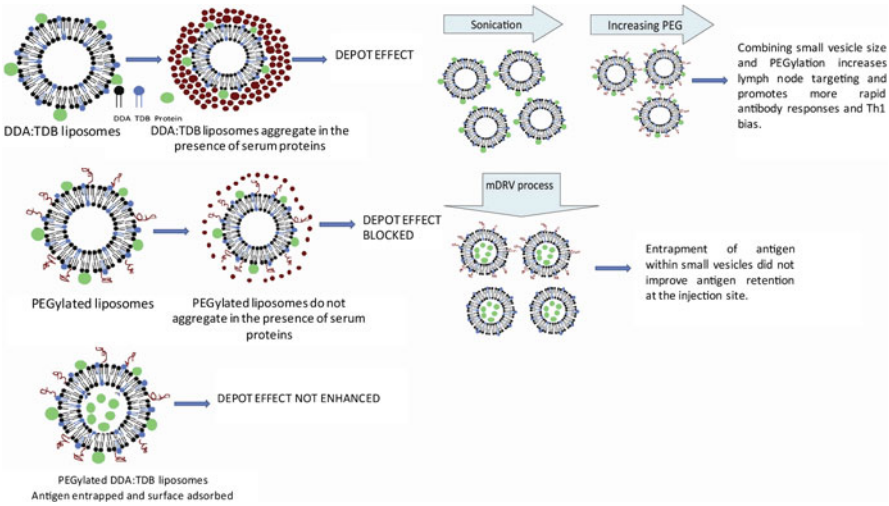

Fig. 17.6 Summary of the outcomes of manipulation of DDA:TDB liposomes in terms of vesicle size, surface pegylation, and antigen location (Kaur et al. 2012a, b)

by showing that neutralisation of the surface charge of the liposomes changes the biodistribution profile and diminishes their immunogenicity (Henriksen-Lacey et al. 2010).

The role of charge in the depot-effect of cationic liposomes has been further demonstrated by masking of the cationic charge in combination with manipulation of vesicle size and antigen location (Fig. 17.6); results from our laboratory have shown that PEGylation of DDA-based liposomes (which contained an immunostimulatory lipid trehalose dibenate) with polyethylene glycol (PEG) at 25 mol% was able to significantly inhibit the formation of a liposome depot and also severely limit the retention of antigen at the site, resulting in a faster drainage of the liposomes from the site of injection (SOI). This change in biodistribution profile was reflected in the immunisation response, where lower levels of IgG2b antibody and IFN-γ and higher levels of IL-5 cytokine were found. Additional studies investigated the impact of a combination of reduced vesicle size and surface pegylation on the biodistribution and adjuvanticity of the formulations, in a bid to further manipulate the pharmacokinetic profiles of these adjuvants. From these biodistribution studies, it was found that with small unilamellar vesicles, 10 % PEGylation of the formulation could influence liposome retention at the injection site after 4 days, while higher levels (25 mol%) of PEG blocked the formation of a depot and promoted clearance to the draining lymph nodes. Interestingly, while the use of 10 % PEG in the small unilamellar vesicles did not block the formation of a depot at the SOI, it did result in earlier antibody response rates and switch the type of T cell responses from a Th1 to a Th2 bias, suggesting that the presence of PEG in the formulation not only controls the biodistribution of the vaccine, but also results in different types of interactions with innate immune cells (Kaur et al. 2012a, b).

Table 17.2 Clinically approved virosome products

Product name		Formulation	Clinical indications
Epaxal	Inactivated Hepatitis A virus	These vesicles are known as virosomes as the bilayer structures are built from phosphatidylcholne, cephalin, and purified influenza virus surface antigens. The subunit antigens are incorporated within the bilayer lipid membrane and the vesicles are ~150 nm	Hepatitis A vaccine
Inflexal V	Influenza haemagglutinin glycoprotein and neuraminidase	Similar to Epaxal, these are virosomes prepared from phosphatidylcholine. The subunit antigens are incorporated within the bilayer lipid membrane	Influenza vaccine

Yet it is important to note that the efficacy of DDA may not be purely electrostatically driven as substitution of DDA with other cationic lipids including 3_-[*N*-(*N*′,*N*′-dimethylaminoethane)carbomyl] cholesterol (DC-Chol; Fig. 17.5) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP; Fig. 17.5) were considered: While all three cationic liposomes facilitated increased antigen presentation by antigen-presenting cells, the monocyte infiltration to the SOI and the production of IFN-γ upon antigen recall was markedly higher for DDA and DC-Chol-based liposomes which exhibited a longer retention profile at the SOI. A long-term retention and slow release of liposome and vaccine antigen from the injection site hence appears to favour a stronger Th1 immune response (Henriksen-Lacey et al. 2011). Similarly a modification of the hydrophobic backbone of DDA to a lower transition temperature lipid (dimethyldioctadecylammonium bromide; DODA) demonstrated that the antigen would more readily dissociate from the less rigid bilayer, DODA-based liposomes and these liposomes were also rapidly removed from the SOI. This resulted in lower up-regulation of co-stimulatory CD40 and CD86 molecules on adjuvant-positive antigen-presenting cells (Christensen et al. 2012).

Furthermore, the adjuvant properties of these DDA systems can be additionally supplemented by the use of a second lipid which can act as an immunomodulator. Table 17.2 highlights example immunomodulators that can be incorporated into liposomal delivery systems. For example, α,α′-trehalose 6,6′-dibehenate (TDB; Fig. 17.5) is a synthetic analogue of trehalose 6,6′-dimycolate (TDM) with two saturated fatty acid chains of 22 carbons (behenyl), each replacing the branched mycobacterial mycolic acids of >70 carbons. These two behenyl chains are linked by ester bonds to carbon number 6 of each of the two glucopyranose rings making up the trehalose head group. TDB has been shown to retain much of the bioactivity of the native form, while showing less toxicity as a result of the shorter fatty acid chains (Pimm et al. 1979; Olds et al. 1980). The combination of the DDA with TDB was first studied by Holten-Andersen et al. (2004). Using ESAT-6 as a possible TB antigen they investigated the ability of seven different immunostimulators to increase the protective efficacy of DDA, which included four mycobacteria-derived immunostimulators.

DDA combined with MPL and/or TDB induced an effective IFN-gamma response and protection in mice was equivalent to that provided by BCG vaccination (Christensen et al. 2009). The adjuvant activity of DDA:TDB when combined with Ag85B-ESAT-6 was also compared to aluminium hydroxide, an adjuvant approved for human use (Davidsen et al. 2005). $CD4^+$ T cells in mice secreted high levels of IFN-gamma and low levels of interleukin-5 (IL-5) in response to DDA:TDB, whereas the opposite pattern was observed for aluminium hydroxide (Davidsen et al. 2005; Christensen et al. 2009). Although high levels of IgG1 antibody titres were seen with both DDA:TDB-adjuvated vaccine and aluminium hydroxide-adjuvated vaccine, higher levels of IgG2 antibody titres were seen with DDA:TDB (Davidsen et al. 2005; Agger et al. 2008). DDA:TDB has also been shown to induce a multi-functional $CD4^+$ T-cell populations expressing several cytokines, mainly tumour necrosis factor-alpha (TNF-alpha)$^+$, IL-2^+, and IFN-gamma$^+$, TNF-alpha$^+$, IL-2^+. In mice such a population is maintained for at least 1 year and thus are long-lived (Lindenstrom et al. 2009).

In addition to liposomes there are a wide range of alternative vesicle constructs and many of these have been investigated as potential adjuvants; these include niosomes (e.g. Baillie et al. 1985), surfactant polymers (e.g. polymersomes (Okada et al. 1995, see reference within Mann et al. 2006)), vesicles incorporating bile salts to improve stability (e.g. bilosomes (Conacher et al. 2001)), or virus components (e.g. virosomes (Almeida et al. 1975)) to name but a few. Many of these systems use alternatives to phospholipids to circumvent potential issues related to storage instabilities and cost (e.g. synthetic-based systems), others to improve stability within harsh biological environments (e.g. bilosomes and polymersomes), or alternatively to modulate the properties of the vesicles in terms of immunological efficacy (e.g. virosomes).

In terms of niosome use for antigen delivery, the combination of 1-monopalmitoyl glycerol, cholesterol, and dicetyl phosphate (DCP) is often employed (Bramwell and Perrie 2005). The inclusion of DCP within these systems helps vesicle formation and is also reported to enhance stability due to the electrostatic repulsive forces between the vesicles which restrict aggregation (Bayindir and Yuksel 2010). The anionic nature of the vesicles has been reported to aid in uptake when delivering antigens via the oral route (Eldridge et al. 1990). Niosomes prepared from 1-monopalmitoyl glycerol, cholesterol, and DCP at a 5:4:1 M ratio incorporating bovine serum albumin (Brewer and Alexander 1992), ovalbumin (Brewer et al. 1996, see reference within Bramwell and Perrie (2005)), or a synthetic peptide containing a known T-cell epitope (Brewer et al. 1996) were shown to stimulate higher levels of IgG2a compared with Freud's complete adjuvant, but the vesicle formulations were shown to be weak stimulators for IgG1. In addition, the adjuvant activity of niosomes was wholly dependent on the model antigen being entrapped within the vesicles; mixing free antigen with the preformed vesicles was unable to elicit a significant immune responses (Brewer and Alexander 1992). This was attributed to the ability of niosomes to retain the antigen for a prolonged period and promoting APC uptake through active or passive targeting to cells (Brewer and Alexander 1992; Conacher et al. 2001). 1-monopalmitoyl glycerol (MPG)-based niosomes have also been considered for the delivery of DNA vaccines. MPG-based niosomes

incorporating cationic surfactants (DC-Chol) rather than anionic surfactants have been shown to offer an increased stability and increased plasmid DNA retention in the presence of competitive anions when compared to similarly formulated PC-based liposomes and engender transgene-specific immune responses comparable with their liposomal counterparts (Obrenovic et al. 1998; Perrie et al. 2002, 2004).

As with the liposome systems a range of immunostimulatory agents have been considered in the design of niosomes as vaccine adjuvants. For example, MPG-based vesicles incorporating both DDA and TDB were developed by Vangala et al. (2006), which resulted in an increase in the vesicle size due to the hydrophilicity of the surfactants, without altering the zeta potential of the vesicles compared to DDA:TDB vesicles. These systems were used to deliver two malarial antigens (Merozoite surface protein 1 (MSP1) and glutamate rich protein (GLURP)), and the MPG-based vesicles, in comparison to DDA liposomes, gave similarly strong Th2 humoural responses when analysing IgG1 titres; however, the MPG-based vesicles also showed high IgG2b titres unlike the DDA:TDB systems (Vangala et al. 2006).

In a further modification of non-ionic-based vesicles, bile acids have been included in the formulation. These systems, known as bilosomes, have been developed to promote the oral delivery of vaccines by offering protection to antigens from the enzymes present in the GIT and acting as immunological adjuvants. This is achieved by incorporating bile salts such as sodium deoxycholate into the formulation thereby increasing the stability of the carrier, thus preventing premature release of the antigen via the oral route. It has been proposed that by incorporating bile salts into the vesicles this offers resistance against degradation and disruption from the digestive enzymes, therefore making the formulation more stable (Schubert et al. 1983). Studies using bilosomes incorporating several antigens have proven to be successful in various animal models, e.g. the A/panama (Mann et al. 2004), tetanus toxoid (Mann et al. 2006), and hepatitis B (Shukla et al. 2010).

17.4.2 Virosomes

Virosomes, in terms of general structural attributes, resemble liposomal systems and are often considered within the general area of liposomes. They are unilamellar vesicles (with a mean diameter <150 nm) built from phospholipids, but in addition, virosomes incorporate functional viral envelope glycoproteins, such as influenza haemagglutinin. This promotes heamaglutinin-receptor binding, cell fusion, and immunostimulation. Of all the variations of liposomal systems discussed, currently only virosomes have been developed as clinical products. Two examples of virosome-based vaccines are Epaxal® and Inflexal® (Table 17.3), which are licensed in over 40 countries for clinical use.

Table 17.3 A selection of immunomodulators which can enhance the immunogenicity of liposomal systems

Immunomodulator	Description
MPL (monophosphoryl lipid A)	Induces the synthesis and secretion of various cytokines and is effective at potentiating mucosal and systemic immune responses to the incorporating antigen. This adjuvant has no observed side effects, other than minor irritation at the injection site Thoelen et al. (1998)
MPD (muramyl dipeptide)	Derived from bacterial cell walls and activates macrophages thus regulates the immune system Murata et al. (1997)
TDM (trehalose 6,6′-dimycolate)	Cord factor, which is a glycoprotein present on the cell membrane surface of *M. tuberculosis*. Activates macrophages and synthesis of cytokines, to drive a Th1 immune response. It is extremely toxic as it induces hypersensitivity granulomas, complex inflammatory events, and apoptosis Yamagami et al. (2001)
TDB (trehalose 6,6′-dibehenate)	An analogue of trehalose 6,6′-dimycolate (TDM) but consists of a shorter fatty acid chains, therefore is considered to be less toxic Davidsen et al. (2005). Very immunogenic as a co-adjuvant for eliciting protective immunity against tuberculosis Holten-Andersen et al. (2004); Davidsen et al. (2005)

17.4.3 Immune-Stimulating Complexes

Immune-stimulating complex particles (ISCOMs) were initially described by Morein et al. (1978) as novel structures that facilitated antigenic presentation of membrane proteins. ISCOMs are cage-like particles approximately 40 nm in size that incorporate protein antigen through hydrophobic interactions and due to their particulate nature (Myschik et al. 2006). ISCOMs generally consist of a mixture of Quil A saponins, cholesterol, and phospholipids. Therefore, while this system contains lipids, their structural attributes are very different from those of liposomes, and it is the addition of the saponin to the phosophipid/cholesterol mixture that drives the change in structure of these nanosystems. ISCOMs can also be prepared to offer a cationic charge when DC–cholesterol replaces cholesterol, or the substitution of PC with dioleoyl-trimethyl-ammonium-propane. The cationic complexes formed are similar to the classical anionic ISCOMs and allow a more diverse range of antigens to be used in their formulation (Lendemans et al. 2005, see reference within Lendemans et al. 2007). Orally, ISCOMs have shown promising systemic immune responses by eliciting Th1, Th2, and MHC-restricted cytotoxic T-cell responses in addition to local induction of IgA (Mowat et al. 1993). As a result, the introduction of immunostimulatory agents has been shown to be useful to enhance immune responses to sub-unit vaccines and offer a promising platform for further studies.

17.4.4 Solid Nanoparticles as Adjuvants

Solid nanoparticles have also been extensively investigated for their potential as vaccines adjuvants. Like the vesicle type systems already described, they can be

prepared in a range of sizes, with a choice of surface characteristics and include a selection of immunomodulators. Polymeric nanoparticles are generally formulated from natural or synthetic polymers with the most commonly studied polymers being those which are biodegradable such as poly(lactide-co-glycolide) (PLGA), poly(lactic acid) (PLA), poly(caprolactone) (PCL), and polysaccharides (particularly chitosan). The advantage of these polymers is that they are well characterised and used in a range of clinical products, particularly PLGA. Alternatively, nanoparticles can be prepared from solid (high melting point) lipids dispersed in an aqueous phase. Examples of lipids used can include solid triglycerides, saturated phospholipids, and fatty acids which are well tolerated by the body. Due to their composition, they are sometimes described as 'solidified' o/w emulsions in which the oil globule is replaced by solidified lipids. Much like the solid polymeric nanoparticles, solid lipid nanoparticles can be used as vaccine adjuvants by the antigen being incorporated within the lipid matrix of the particle or by attaching it to the lipid nanoparticle surface. Lipid particles normally start from around 50 nm in size and can be prepared in a large-scale by homogenisation to disperse the lipid into an aqueous environment.

The use of polymer-based particulates as vaccine adjuvants has been strongly investigated, but it has been more recent work that has refocused investigations into the potential advantage of using these systems in the nano range. For example, work by Stano et al. (2012) demonstrated that by increasing the size of their polypropylene sulphide nanoparticles from 30 to 200 nm, the antigen was more effectively delivered into both MHC class I and MHC class II-presentation pathways. Enhancing the targeting of nanoparticles to dendritic cells has also been considered, e.g. the addition of a recombinant fusion protein to the surface of PLGA nanoparticles was shown to promote a twofold increase in DC uptake in vitro, and in vivo studies demonstrated these formulations promoted enhanced antigen-specific IgG and IgG subclasses, and higher cytokine responses.

17.4.5 Emulsion-Based Adjuvants

While not all emulsion-based systems fall within the nanoscience definition, it is useful to consider the role of emulsions in general in vaccine formulation. In 1997 the first emulsion adjuvant MF59® (Novartis, Italy) was licensed in Europe in the influenza vaccine Fluad® (O'Hagan and Gregorio 2009). MF59® is an oil-in-water (o/w) emulsion composed of 5 % squalene (naturally occurring oil) combined with surfactants sorbitan trioleate (Span 85) and poly(oxyethylene) sorbitan monooleate (Tween 80). Despite concerns regarding its safety due to the occurrence of autoimmune dysfunctions in rats (Carlson et al. 2000), the adjuvant has an established safety profile in humans of good-health and immunocompromised populations (Black et al. 2010; Donatoa et al. 1999). It is interesting to note that MF59® is not the first emulsion to be licensed for human use; the well-known experimental adjuvant Incomplete Freund's adjuvant (ICF) (Freund et al. 1948) was once used in

human influenza vaccines (Chang et al. 1998). In contrast to MF59®, ICF is a water in oil (w/o) emulsion composed of a light mineral oil (such as Bayol F) and the emulsifier mannide monooleate (Aracel A™), combined in a 9:1 volume ratio (Lindblad 2000). While it has been withdrawn from human use due to occasional serious local reactions, it remains an experimental gold-standard adjuvant in immunisation protocols. The success of MF59® has been attributed to the rendering of soluble antigen to particulate form, improved cell recruitment to the injection site, and antigen uptake with transport to local lymph nodes (Mosca et al. 2008). Similar to aluminium salts, no direct activation of dendritic cells has been noted, although improved trafficking and antigen uptake by macrophages and DCs, respectively, has been noted, and expression of soluble activation factors may indeed lead to indirect DC activation (Mosca et al. 2008).

17.4.6 The Influence of Vaccine Formulation and Delivery Route on Vaccine Performance

In terms of potential advantages of these solid nanoparticles over the bilayer type systems, there has been few direct comparisons made, as it is difficult to control the number of different parameters between the systems. Similarly little work has been undertaken to understand the impact of the route of administration of such these adjuvants on the type and strength of immune response promoted. However, in a recent multi-centre study, the difference in immune responses generated in mice vaccinated by the subcutaneous, intradermal, intramuscular, and intralymphatic routes was considered with ovalbumin-loaded liposomes, *N*-trimethyl chitosan (TMC) nanoparticles, and poly(lactide-co-glycolide) (PLGA) microparticles, all with and without specifically selected immune-response modifiers were directly compared (Mohanan et al. 2010). Interestingly, neither the route of administration nor the presence of immunomodulators within the formulations made a notable difference to induced IgG1 antibody responses. However, the administration route had a strong impact both on the kinetics and magnitude of the IgG2a response: a single intralymphatic administration of all the evaluated vaccine formulations (liposomes, nanoparticles, and microspheres) generated a strong IgG2a response, whereas subcutaneously, only the adjuvanted nanoparticles were able to promote notable IgG2a responses, and the intradermal and intramuscular routes generated intermediate IgG2a responses (Mohanan et al. 2010). The benefit of the intralymphatic administration route for eliciting a Th1-type response was confirmed in terms of IFN-gamma production. This study demonstrated that the IgG2a associated with Th1-type immune responses is sensitive to the route of administration, whereas IgG1 response associated with Th2- type immune responses was relatively insensitive to the administration route of the particulate delivery systems. Therefore, consideration of the vaccine formulation in combination with the route of administration should be considered when planning and interpreting preclinical research or development on vaccine delivery systems.

17.5 Clinically Relevant Nanosystem-Based Vaccines

Given the range of clinically approved nanotechnology-based pharmaceutical products already available, it is interesting to note that currently only virosomal vaccines [Epaxal® and Inflexal® V (Crucell)] are clinically approved for use. However, until the desirable characteristics for a nanosystem-based vaccine adjuvant are fully elucidated, it is difficult to identify key attributes we should be focusing on other than final in vivo performance, which is a lengthy and expensive marker to rely on. Similarly there continues to be issues regarding regulation. For example the WHO stipulates that liposomal adjuvants must be licensed as a vaccine formulation, and not as an adjuvant which could be combined with various antigens post-licensing. This adds further complications to the development and licensing processes. However, progress continues with cationic nanosystems such as the DDA:TDB formulation developed by Staten Serum, Institute, and the cationic Adjuvant IC31® (Intracell, Austria).

17.6 Concluding Remarks

Overall, liposomes offer a strong potential as vaccine adjuvants by combining the ability to deliver antigens to the correct cells, and also appropriately interact and stimulate such cells. While progress remains limited to-date, new advances in the understanding of effective vaccine systems and in the production and regulation of liposomes in a cost-effective manner should enhance their progress into the clinical setting.

Problem Box

1. Identify the various vaccine types currently clinically available. Discuss their respective advantages and disadvantages.

   Answer: Vaccines can be divided into three basic groups: (1) live vaccines, (2) inactivated vaccines, (3) toxoids and subunit systems.

   Live attenuated vaccines consist of live microorganisms or viruses that have mutated so that it has a reduced ability to grow in human cells. They are still able to infect their target cells, but the infection is mild and replication is limited. These vaccines generally give good protection, but there is a risk of reversion to virulence, and this type of vaccine is not considered safe for use in immunocompromised individuals.

   Inactivated Vaccines consist of microorganisms or viruses that have their ability to be infectious removed while retaining immunogenicity. In terms of advantages they tend to have improved safety profiles compared

(continued)

Problem Box (continued)

with live system, they are generally less effective than live attenuated vaccines, usually only stimulating humoural immunity and often requiring booster doses.

The third group consists of part of the infectious agent, such as excreted toxoids or subunit proteins. Subunit vaccines initiate strong immune responses using a small part of the organism. The advantage of subunit vaccines is their good safety profile; however, the immune response induced by such vaccines is short-lived and thus several boosts are required to achieve protection.

2. Describe the structural attributes of virosomes and discuss, with the use of examples, how these are able to be used as vaccines.

Answer: Virosomes are bilayer vesicles around 150 nm in size. They are prepared from phospholipids and incorporate functional viral envelop glycoproteins, such as influenza haemagglutinin. This promotes heamaglutinin-receptor binding, cell fusion, and immunostimulation. For example, Epaxal is a virosome vaccine clinically indicated for Hepatitis A immunisation. By incorporating the subunit antigen for Hepatitis A, the antigen can be protected from the extracellular milieu, thereby limiting antigen breakdown by enzymes, as well as preventing the rapid removal of such small compounds by the MPS. This ensures the antigen carried within the virosome delivery system to the appropriate immunological cells, and subsequently the antigen is taken by the cells due to the action of the glycoproteins.

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Chapter 18
Gene and Ribonucleic Acid Therapy

Jayanant Iemsam-Arng, Xiao Kong, Andreas G. Schätzlein, and Ijeoma F. Uchegbu

Abstract Gene and small interfering ribonucleic acid (siRNA) therapies are applicable to a wide range of diseases; diseases where a mutated gene is the basis for the disease, e.g. cystic fibrosis, haemophilia and cancer. However, there are some difficulties with gene and siRNA therapy, i.e. low gene and siRNA internalisation into the cells, instability of the nucleic acid molecule in the cell and the lack of specific gene targeting to the nucleus. Although physical methods exist to deliver naked deoxyribonucleic acid (DNA) into cell nuclei, these methods are inefficient and hence nucleic acid vectors are used. These delivery vectors are viruses, cationic polymers and dendrimers or cationic liposomes; with the synthetic vectors all possessing a mandatory amine functional group. Viral vectors are the most efficient gene vectors and there are currently three licenced products available: Rexin G for the treatment of malignant solid tumours, Gendicine for the treatment of head and neck cancer and Glybera for the treatment of lipoprotein lipase deficiency; these three are gene therapies involving viral vectors. Other nucleic acid delivery vectors that are still in development include amine polymers, dendrimers and liposomes prepared from amine phospholipids.

18.1 Introduction

A gene is a unit of deoxyribonucleic acid (DNA) which contains the information for the synthesis of functional proteins or non-coding ribonucleic acids (RNAs) (Pesole 2008). Structurally, DNA and RNA consist of nitrogenous bases (adenine, cytosine,

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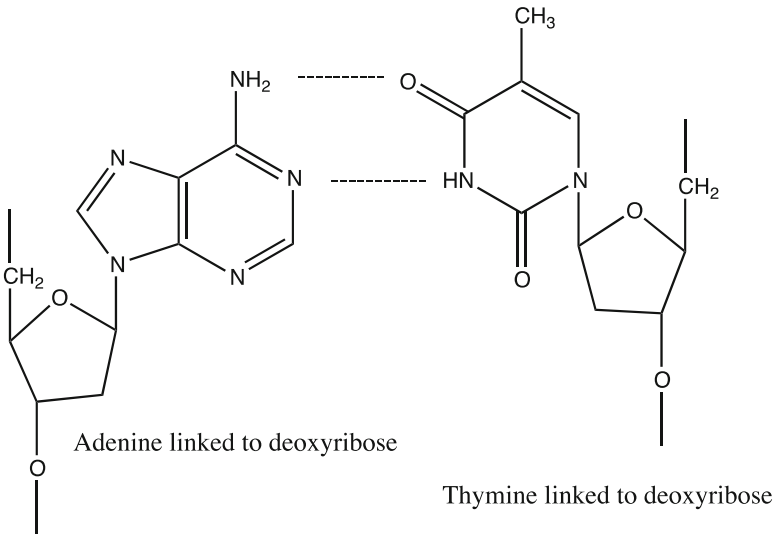

Fig. 18.1 Adenine and thymine nucleosides as constituents of DNA

guanine and thymine for DNA; and adenine, cytosine, guanine and uracil in place of thymine for RNA), a sugar (2′-deoxyribose for DNA and ribose for RNA) and phosphate groups. These components are called the nucleotide subunit (Kingston 1989) (e.g. Fig. 18.1).

Genes are expressed by transcription of DNA into messenger RNA (mRNA) and by the translation of mRNA into a functional protein (Fig. 18.2). The specific region for the expression of primary protein structure is known as the *open reading frame* (ORF). This region encodes particular codon sequences for the amino acid sequence in a protein (Bunz 2008). Gene mutation leads to the expression of a non-functional protein or to the expression of a disease-causing protein. Mutations may alter gene function and affect protein structure by changing the codons. The alteration of the codon sequences disturbs RNA splicing and mutates the promoter elements, transcriptional initiation sites, initiation codons, polyadenylation sites and termination codons (Bunz 2008).

The concept of gene therapy was first introduced in the 1970s (Weatherall 1995). Gene therapy is defined as a procedure aimed at replacing, manipulating or supplementing non-functional genes with healthy genes. Candidate diseases for gene therapeutics include cystic fibrosis (Rosenecker et al. 1998), haemophilia (Murphy and High 2008) and cancer (El-Aneed 2004).

The strategies used in gene therapy include gene replacement, gene reprogramming, gene repair and gene supply (Verma and Somia 1997; Driskell and Engelhardt 2003). However gene replacement is the most common form of gene therapy. Gene replacement therapy aims to supplement or replace the mutated gene with the wild type gene. The three licenced gene therapies: Gendicine (Peng 2005), Rexin G (Gordon et al. 2007) and Glybera (Bruno 2010) are all gene replacement therapeutics.

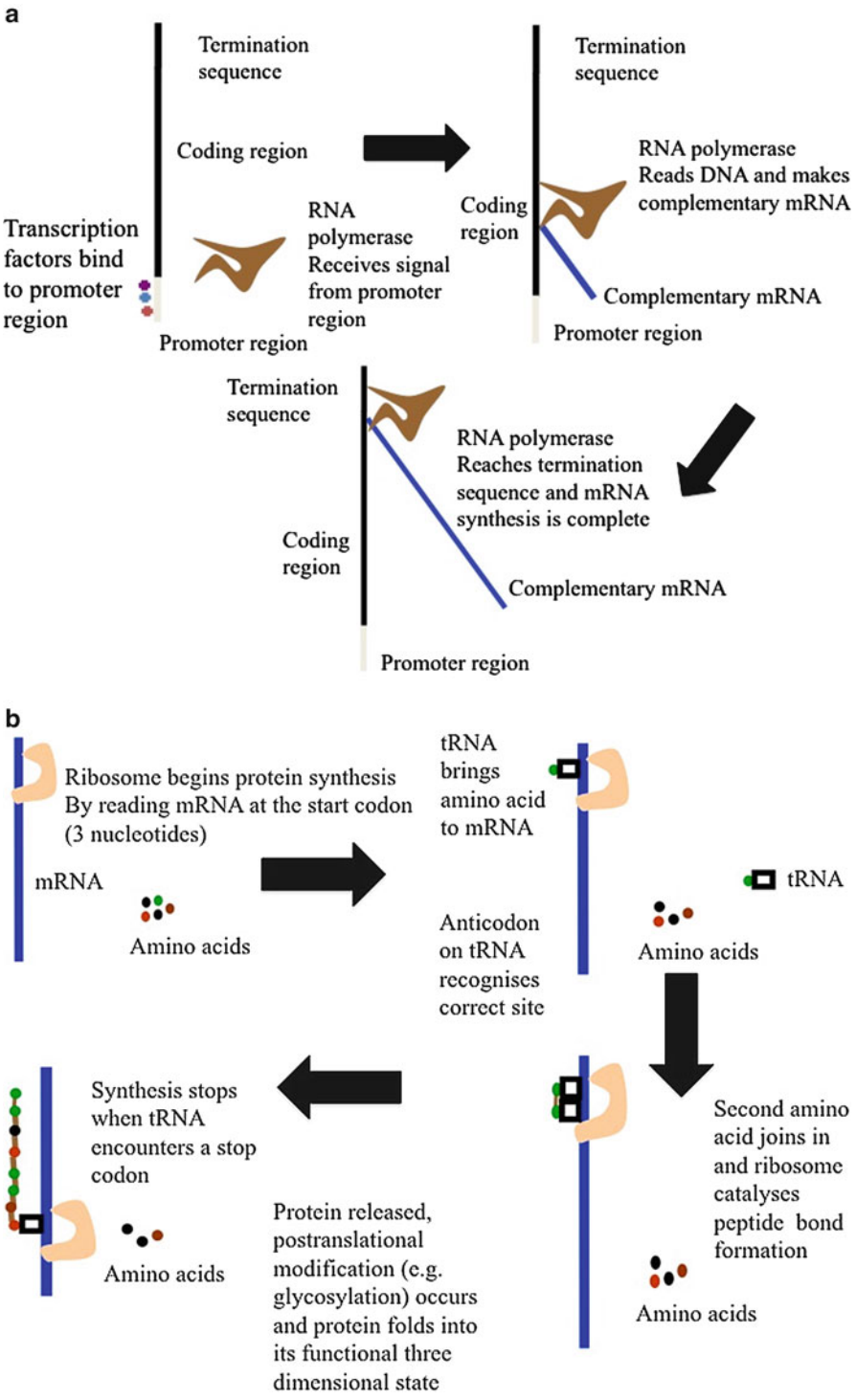

Fig. 18.2 (**a**) Transcription, synthesis of mRNA, takes place in the cell nucleus. (**b**) Translation, protein synthesis, takes place in the cell cytoplasm

Initially, gene therapy was focused on the treatment of the monogenic disorders (a disorder in which a mutation in one gene gives rise to a disease). Examples of monogenic diseases include cystic fibrosis and sickle cell anaemia. However, the application of gene therapy has broadened to polygenic disorders (Verma and Somia 1997). As such there have been several attempts to restore cellular and metabolic function in Parkinson's disease, diabetes, reverse degenerative vascular disorders and to treat cancer using gene therapy (Touchefeu et al. 2010). The first successful clinical gene therapy cure was the treatment of X-linked severe combined immunodeficiency (X-SCID) in 2000 (Cavazzana-Calvo et al. 2000). While these studies demonstrated the potential of gene therapy, they were associated with fatal insertional mutagenesis (Howe et al. 2008). Further gene therapy treatments have followed the X-SCID trial such as the gene therapy of chronic granulomatous disease (CGD); however, this therapeutic intervention was also associated with the development of myelodysplasia in the patients (Stein et al. 2010). While the potential of gene therapy to overcome fatal and currently incurable diseases is not in doubt, the pace of introduction of gene therapies has been slow partly because of the tragedies encountered in the X-SCID trial and the sudden death of Jesse Gelsingher. Jesse Gelsingher was an 18-year-old boy, who suffered from a genetic disease, ornithine transcarbamylase deficiency. He was entered into a gene therapy trial run by the University of Pennsylvania and died as a result of the trial. Jesse Gelsingher was injected with an adenovirus therapy and suffered a fatal immune response.

Another reason for the slow commercialisation of the many preclinical gene therapy findings lies in the lack of efficient delivery systems for these nucleic acid-based therapies (Brown et al. 2001). DNA is a molecule which is easily degraded by plasma nucleases and because of its large size (several thousand base pairs ~3,000,000–6,000,000 Da) and possession of ionised phosphate groups at physiological pH (Fig. 18.1); DNA is not taken up by cells and translocation to the nucleus; and its site of action is difficult (Brown et al. 2001). Although there are physical methods to deliver naked DNA, such as the intratumoural injection of DNA (Wolff et al. 1990; Huang et al. 2005), the use of electroporation (Sukharev et al. 1992; Somiari et al. 2000), rapid large volume injections which cause the large volume to be rapidly pumped to the liver in order to protect the heart (Yang et al. 2002; Budker et al. 2006; Herweijer and Wolff 2007) and ultrasound (Manome et al. 2000; Miller et al. 2002; Guo and Huang 2012); ultimately successful gene therapy is associated with the employment of vectors. The vectors are nano-sized in nature and have so far been drawn from viruses, polymers, dendrimers or liposomes. Viral vectors enable DNA to be translocated to the nucleus for eventual transcription and translation (Wilkinson and Lowenstein 1994; Weiss et al. 2001), while synthetic vectors package the DNA in a tight complex (Zinselmeyer et al. 2002; Schatzlein et al. 2005) and prevent its degradation in the plasma, ultimately facilitating uptake by the endosomes and translocation of some of the DNA to the nucleus, principally when the nucleus is dividing; making these therapies especially relevant to the malignant phenotype.

Small interfering RNA sequences siRNA inhibit translation (Fig. 18.3) by combining with the RNA-induced silencing complex (RISC) to selectively and

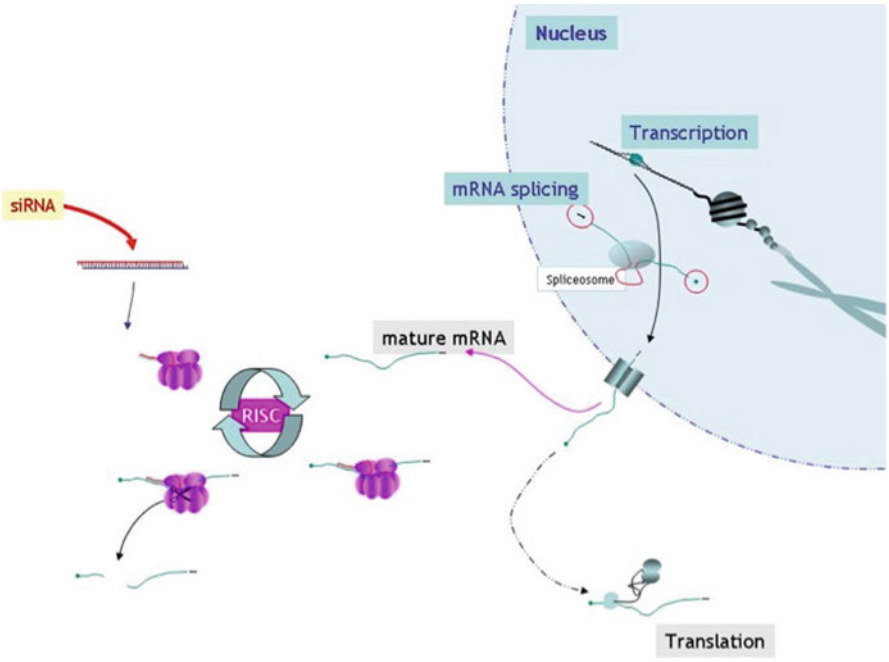

Fig. 18.3 Gene silencing with siRNA

specifically cause the hydrolytic degradation of mRNA (Zamore et al. 2000). The RISC precursor complex (~250 kDa) is transformed in the presence of adenosine triphosphate (ATP) into the active 100 kDa complex that cleaves the substrate mRNA in regions homologous to the siRNA template. siRNA therapies are still largely experimental and there are no siRNA therapeutics on the market at present. The plasma degradation of siRNA and its inefficient cellular uptake also make the delivery of siRNA therapies problematic (Gary et al. 2007). Hence polymer nanoparticles are used to package siRNA by again exploiting the electrostatic attraction between the phosphate groups on siRNA and the amine groups of the polymer, in order to achieve siRNA uptake by the cell and gene silencing in the cytoplasm, with ultimate clinical benefit (Davis et al. 2010).

18.2 Viral Delivery of Genes

Viral gene therapy is a very efficient method for delivering genes (Waehler et al. 2007). Viruses infect cells and are able to express the genes of interest using the cell's transcription machinery. Adenoviruses were one of the first viruses used for clinical studies and they were used in cystic fibrosis patients with limited success

(Wilmott et al. 1994). Adenoviruses are non-enveloped viruses containing linear, double-stranded DNA encapsulated in an icosahedral protein capsid (Zhang 1999). The antenna-like fibre on the outer capsid of the adenovirus is the key structural component in the infection process as it attaches to the cell surface during infection (Knipe et al. 2001). Adenoviruses are able to carry large genes (Kennedy and Parks 2009). The transfection of the adenoviral vectors is transient since the DNA genome does not permanently integrate into the genetic material of the host cell (Kelly 1984). Adenoviruses have been widely used for cancer therapy and they serve as the basis of the gene medicine — Gendicine (Peng 2005). This was the world's first licenced gene therapeutic. Gendicine was licenced in China in 2003 and launched in 2004. Gendicine is a p53 apoptosis-forming gene delivered in an adenoviral vector. The E1-deleted adenovirus is replication incompetent and Gendicine is indicated for the treatment of head and neck cancer. The gene therapeutic must be administered intratumourally as an adjunct to radiotherapy. Patients receive one injection per week for 4–8 weeks and each injection consists of 10^{12} viral particles in 1 mL of water for injection containing glycerol. Adenoviruses are known to generate unexpected adverse events (Smith 2002); however, the side effects reported for Gendicine are mild and limited to flu-like symptoms (Peng 2005). When the adenovirus is injected into the tumour, the virus gains entry to the cell using the Coxsackie Adenovirus Receptor.

Retroviral vectors are RNA viruses which consist of a glycoprotein envelope and a linear, single-stranded, RNA genome of 7–13 kilobases (Maeda et al. 2008). These viruses use reverse transcription to synthesise DNA; this viral DNA is then used by the cell to make viral proteins and copies of the virus. The viral genomes contain four genes: *gag*, *pro*, *pol* and *env* (from the *cis* 5′ to 3′), encoding the structural proteins, proteases (operating during virion maturation), reverse transcriptase and viral surface protein, respectively (Kay et al. 2001; Maeda et al. 2008). The stable incorporation of retroviral DNA into the host DNA genome is the basis of the long-term expression. The retroviral DNAs are transmitted to subsequent generations, maintained in the cell permanently and produce sufficient viral titres for gene transfer (Yi et al. 2011). However, the non-specific incorporation of the retroviral DNA to the host genome may cause the abnormal expression in the adjacent cell and cause a mutation at the site of incorporation (Yi et al. 2011), i.e. insertional mutagenesis. Retroviral vectors include the Moloney Murine Leukaemia Viruses (Wu et al. 2003) and the Lentiviruses (Persons 2010). The clinical gene therapy cure of X-SCID was carried out using a retroviral vector in 2000 (Cavazzana-Calvo et al. 2000). However, insertional mutagenesis subsequently led to the development of cases of fatal leukaemia (Howe et al. 2008).

Subsequently a greatly attenuated retrovirus, little more than a retroviral envelope, has been successfully used in gene therapy. Rexin G is derived from a Moloney Murine Leukaemia Virus and expresses the human Cyclin G1 gene (Gordon et al. 2007). Rexin G is licenced in the Philippines for the treatment of metastatic tumours. Rexin G has also been entered into clinical trials in the USA for the treatment of breast cancer and pancreatic cancer (Uchegbu and Siew 2013). The product consists of the viral envelope and the therapeutic gene and is said to be devoid of all viral

genes. Rexin G is also free of all replication competent retroviruses. The Cyclin G1 gene causes apoptosis of the tumour cells (Gordon et al. 2001) and is anti-angiogenic to the tumour neovasculature (Gordon et al. 2007). Targeting is accomplished by a ligand derived from von Willibrand factor which enables the viral particles to accumulate at metastatic regions (Gordon et al. 2007).

The adeno-associated viruses (AAVs) are single-stranded DNA viruses with the small gene capacity of 4.7 kilobases (Kotin et al. 1991). AAVs are non-pathogenic viruses which can target broad cell types for viral infection. They have shown a high level of gene expression with no related viral side effects during the viral infection (Buning et al. 2008). The third gene therapeutic to be licenced, Glybera, comprises an AAV virus vector and the lipoprotein lipase gene (Gaudet et al. 2010). Lipoprotein lipase deficiency is a rare disease that presents as a build up of chylomicrons in the blood (hyperchylomicronaemia and hypertriglyceridaemia), especially after a fatty meal and leads to episodes of pancreatitis. Clinical trials with Glybera showed a significant reduction in plasma triglyceride levels (Ross et al. 2004). Glybera, manufactured by UniQure is the first gene therapeutic to be approved in Europe.

Viral vectors because of their efficient gene transfer ability have delivered the first generation of commercialised gene therapeutics. There are of course problems associated with their use such as the intratumoural administration required by Gendicine (Peng 2005) and the multiple (30–70) intramuscular injections (Gaudet et al. 2010) required for Glybera.

18.3 Polymer and Dendrimer Delivery of Genes

Amine polymers and dendrimers, which have a cationic charge at physiological pH form an electrostatic complex with plasmid DNA (Brown et al. 2001). This complex protects the DNA from plasma degradation and enables cell uptake of the plasmid and eventual gene expression of the therapeutic protein (Brown et al. 2001). The gene transfection efficiency depends on the molecular weight, surface charge, charge density, hydrophillicity and structure of the cationic polymers (Uchegbu et al. 2004; Sun and Zhang 2010). Currently, there are a number of polymers and dendrimers used preclinically in gene therapy, including poly-L-lysine (PLL), poly(ethylenimine) (PEI), chitosan, poly(amidoamine) dendrimers and poly(propylenimine) dendrimers (Fig. 18.4).

18.4 Poly(L-Lysine) and Poly(L-Ornithine)

The poly(amino acids)—poly(L-lysine) and poly(L-ornithine) are linear cationic polymers which form electrostatic complexes with DNA (Brown et al. 2000). Although, the internalisation of the complex into the cells is rapid, the transfection

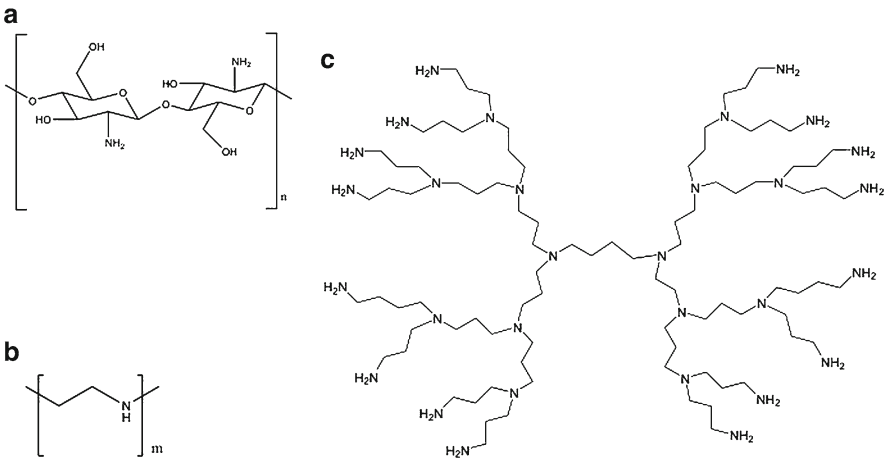

Fig. 18.4 Poly(ethylenimine), poly(propylenimine), PLP, chitosan

efficiency of the polyplexes is poor, due to the lack of endosomal escape and the low dissociation of DNA from the complex (Midoux and Monsigny 1999; Ward et al. 2001). These polymers are also relatively toxic to cells (Brown et al. 2000), although the toxicity at the cellular level may be modulated by forming amphiphilic derivatives such as *N*-palmitoyl-poly(L-lysine)-graft-*N*-poly(ethylene glycol) (Brown et al. 2000, 2003); however, this does not make these poly(L-amino acids) any more efficient at gene transfection.

18.5 Chitosan

Chitosan (Fig. 18.4a) is a natural polymer, derived from the deacetylation of chitin, a key component of shrimp and crab shells (Lamarque et al. 2007). It is a 1-4 poly(glucosamine) polymer and it forms an electrostatic nanoparticle complex with DNA (Peng et al. 2009). Chitosan and its amphiphilic derivatives demonstrate good biocompatibility against cell lines (Uchegbu et al. 2004) (Reitan et al. 2009) and these molecules are able to transfer plasmids into cells. The physicochemical properties, the transfection efficiency and cell cytotoxicity of the complexes depend on the molecular weight, salt form, degree of deacetylation of chitosan and the pH of environment (Kim et al. 2007). However, when the transfection efficacy of a series of chitosan derivatives were compared, the overriding driver of transfection was found to be the degree of polymerisation of the chitosan backbone (Uchegbu et al. 2004). Generally chitosan is not a very efficient gene transfer system.

18.6 Poly(Ethyleninime)

The PEIs (e.g. Fig. 18.4b) are highly cationic polymers with comparatively efficient gene transfer properties (Patil et al. 2005). Both linear PEI (Fig. 18.4b) and branched PEI are effective gene carriers (Florea et al. 2002; Lungwitz et al. 2005). PEIs are efficient due to their ability to facilitate endosomal escape on uptake of the complex by endocytosis; the polymer buffers the acidic endosome causing a resultant increase in hydrogen ions within the endosome, accompanying chloride counter ions and as such water molecules; the water to maintain osmotic homeostasis. The end result is endosomal rupture and the buffering of the endosome that leads to endosomal rupture is known as the proton sponge effect (Godbey et al. 1999). PEI has both high transfection efficiency and is also toxic to the cells (Brownlie et al. 2004). As a result, safety concerns have been raised due to the serious toxicity of PEI. PEIs toxicity means that in vivo transfection with PEI is not possible unless the molecule is derivatised, for example, with poly(ethylene glycol) (Ogris et al. 1999). Once derivatised with poly(ethylene glycol) and transferrin, tumouricidal activity is observed with mice intravenously administered the tumour necrosis alpha gene complexed with PEI.

18.7 Dendrimers

Dendrimers are polycationic synthetic star-shaped polymers (e.g. Fig. 18.4c) of a defined molecular weight (Dufes et al. 2006; Chooi et al. 2010). There are two types of dendrimers that have been used predominantly in gene delivery: the poly(amidoamine) dendrimers (Lee et al. 2003) and the poly(propylenimine) dendrimers (Zinselmeyer et al. 2002) (Fig. 18.4c). These dendrimers form electrostatic complexes with DNA at low nitrogen to phosphate ratios and yield 200–500 nm sized dendriplexes (Zinselmeyer et al. 2002). The high charge densities of these molecules (Fig. 18.4c) are said to be responsible for their excellent transfection efficiency of (Kukowska-Latallo et al. 1996). The higher molecular weight entities are less biocompatible than the lower molecular weight entities (Dufes et al. 2005a, b; Duncan and Izzo 2005). The haemolytic and cytotoxic behaviour of these cationic dendrimers have been attributed to the surface amine groups (Haensler 1993; Malik et al. 2000).

Poly(propylenimine) dendrimers, unlike cationic liposomes and cationic polymers (Bragonzi et al. 1999) do not accumulate in the lung and transfection in healthy animal models is seen predominantly in the liver (Schatzlein et al. 2005). This is hypothesised to be due to the fact that there is less aggregation of these dendriplexes on injection into the blood, when compared to conventional polyplexes or lipoplexes. The low molecular weight of the dendrimers (MW generation 3 poly(propylenimine) dendrimer = 1,687 Da) is believed to be implicated in this phenomenon and the

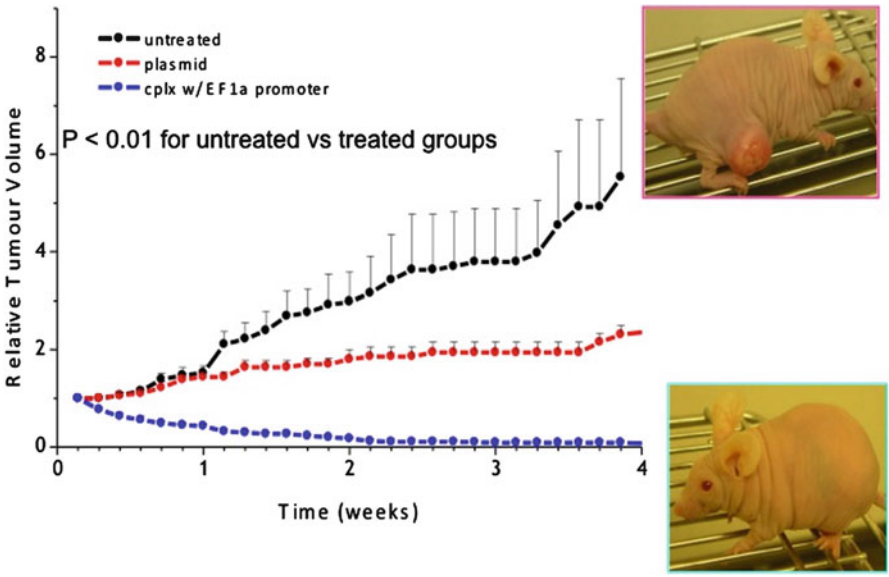

Fig. 18.5 The tumouricidal activity of a generation 3 poly(propylenimine) dendrimer—tumour necrosis factor alpha complex in nude mice bearing the A431 tumours (Dufes et al. 2005a)

biodistribution of transfection with poly(propylenimine) dendriplexes away from the lung has been termed the lung avoidance hypothesis (Schatzlein et al. 2005). Ultimately this lack of lung accumulation in the first capillary bed encountered on intravenous injection has led to the targeting of genes to solid tumour xenografts in preclinical studies (Chisholm et al. 2009). This gene targeting to tumours is accompanied by tumour eradication in tumour-bearing mice on intravenous injection with tumour necrosis alpha gene — poly(propylenimine) dendriplexes (Fig. 18.5) (Dufes et al. 2005a). Incidentally the generation 3 poly(propylenimine) dendrimers are also intrinsically anti-proliferative (Dufes et al. 2005a).

18.8 Liposomal Gene Delivery

Cationic liposomes were first used for gene therapy in 1987 (Felgner et al. 1987). Cationic liposomes are the result of the self-assembly of polar lipids, wherein the polar lipid head group comprises an amine group to give the resulting liposome its cationic character at physiological pH, e.g. [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane] (DOTAP) (Zuidam and Barenholz 1998), *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and dioctadecylamidoglycylspermine (DOGS) (Balazs and Godbey 2011; Even-Chen et al. 2012). Cationic liposomes form an electrostatic complex with DNA, termed

the lipoplex (Radler et al. 1997; Koltover et al. 1998). The advantages of using liposomes are their low immunogenicity as well as the ability to protect the gene from nucleases (Godbey and Mikos 2001). While lipoplexes have been used in gene therapy clinical trials, e.g. for the treatment of cystic fibrosis (Caplen et al. 1995; Gill et al. 1997; Porteous et al. 1997), they have not progressed to a clinical product.

18.9 siRNA Delivery

siRNA treatments are not yet licenced for use and there are only a few clinical stage treatments (Davis et al. 2010). There are a number of preclinical studies demonstrating in vivo down regulation with either siRNA or with small hairpin RNA silencing (in which a plasmid for siRNA is administered and the silencing RNA is produced in situ) (Bell et al. 2007). For example, the down regulation of the oncogene polo-like kinase 1 has been achieved using siRNA specific for polo-like kinase 1 complexed with a cholesterol-derived cationic lipid and methoxy poly(ethylene glycol)-block-poly(lactic acid), with tumour cell apoptosis and tumour cell regression resulting (Yang et al. 2012). Recently, in a ground breaking study, clinical gene silencing was demonstrated in refractory melanoma patients (Davis et al. 2010) (Table 18.1). These patients were administered a cyclodextrin-based construct bearing a transferrin-targeting ligand (Fig. 18.6) and siRNA against the M2 subunit of ribonucleotide reductase (RRM2), a DNA repair enzyme which enables cancer cells to proliferate. Tumour biopsies showed concrete evidence of particle uptake, reduced levels of RRM2 mRNA, reduced levels of RRM2 and RRM2 mRNA cleavage (Table 18.1) (Davis et al. 2010).

Table 18.1 Outputs from the CALAA-01 clinical trial with refractory melanoma patients (Davis et al. 2010)

	Patient dose (mg kg^{-1})		
Clinical findings	18	24	30
Nanoparticles detected in tumour biopsies using gold-labelled adamantane—PEG conjugates	No	Yes	Yes
Reduction in the M2 subunit of ribonucleotide reductase (RRM2) mRNA measured in tumour biopsies by quantitative reverse transcriptase PCR	Yes	Yes	Yes
Reduction in RRM2 protein expression measured by western blotting	Not determined	Not determined	Yes
Reduction in RRM2 protein expression analysed by immunohistochemistry	Not determined	Not determined	Yes
mRNA cleavage fragment detected by a modified PCR technique	No	No	Yes

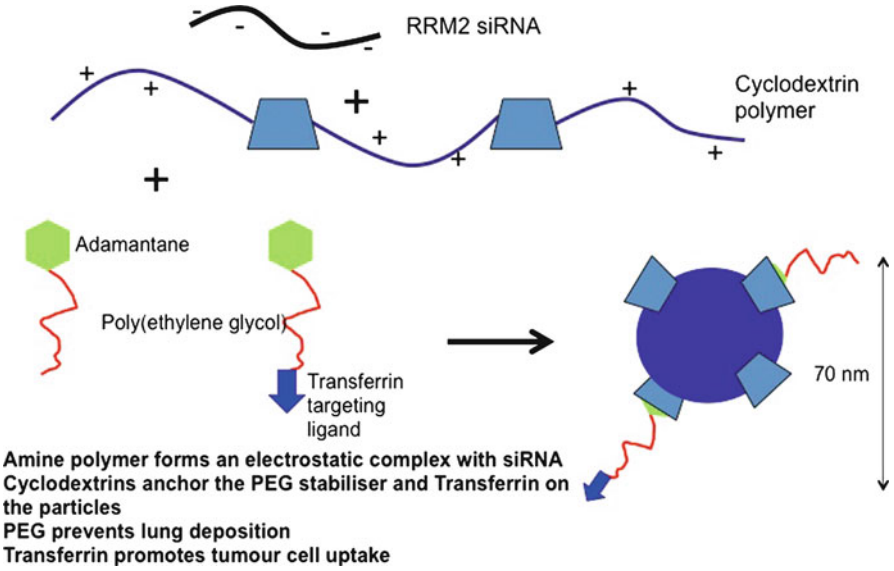

Fig. 18.6 A schematic representation of the siRNA gene silencing experimental therapeutic CALAA-01 (Davis et al. 2010)

18.10 Conclusion

Pharmaceutical nanoscience has played a central role in the development of gene and siRNA therapies, as these medicinal agents require delivery vectors to effect their pharmacology. While the fact that there are three marketed gene therapeutics currently available is evidence of progress, this low number of therapeutics is testament to the difficulties encountered in developing gene and siRNA medicines. All three licenced gene medicines are delivered using viruses. Polymers, dendrimers and polar lipids, all with amine functional groups have been used to prepare DNA-based self-assemblies, with the self-assemblies bearing a cationic charge at physiological pH. Such self-assemblies have been demonstrated to facilitate gene transfer both in preclinical and in some cases even some clinical studies, although there are no licenced gene therapy products which utilise synthetic vectors. siRNA-based gene silencing has been accomplished in the clinic using amine polymers bearing targeting ligands which self-assemble in the presence of siRNA to yield nanoparticles. These siRNA nanoparticles are taken up by the cell and demonstrate clinical gene silencing. Undoubtedly the road to a wide plethora of gene therapeutics has been winding and unpredictable in places, with fatalities associated with the world's first gene therapy cure; however, these early studies have laid the foundation for a new generation of gene medicines, all facilitated by pharmaceutical nanoscience.

Problem Box

Q1: (a) Use no more than three short sentences to explain what is meant by replacement gene therapy; illustrate your answer with examples.

(b) Name the three gene therapeutics (along with their respective indications) that have been approved for use in humans.

Answer

(a) Replacement gene therapy is the administration of a gene (e.g. a wild type gene) to enable the expression of a functional protein. Replacement gene therapy is administered when there is a mutation of the wild type gene to produce a non-functioning variant or a variant with clinically relevant lower activity. Glybera is a replacement gene therapeutic, containing lipoprotein lipase and is indicated for the treatment of lipoprotein lipase deficiency.

(b) Rexin G approved in the Philippines for the treatment of metastatic solid tumours, e.g. metastatic pancreatic tumours. Gendicine approved in China for the treatment of head and neck cancers Glybera approved in Europe for the treatment of lipoprotein lipase deficiency (LPLD).

Q2: (a) What are the main delivery barriers associated with the formulation of gene therapeutics?

(b) Using examples from approved and experimental gene therapeutics, explain how these gene delivery barriers may be overcome.

Answer

(a) Genes are hydrophilic macromolecules that are susceptible to enzymatic degradation; they also have to reach a nucleus of a target cell. The main biological barriers are the membrane barriers as genes are large and charged and so do not diffuse easily across lipid membranes. They thus do not easily cross epithelial membranes and so may not be given by non-parenteral means and do not easily cross the endothelial membranes when given intravenously. Even if they do extravasate then genes do not cross the plasma membrane easily to gain access to the cell. When in the plasma, extracellular fluid or even within the cell, genes are susceptible to degradation by nucleases giving genes very short plasma half-lives. Genes do not have access to the nucleus because of the nucleolar membrane unless the cell is dividing.

(b) Genes may be protected from degradation before reaching their site by being injected directly to the site of pathology as in the case of Gendicine where the dose is injected intratumourally or Glybera, which is injected

(continued)

Problem Box (continued)

intramuscularly, as the muscle is a main site of lipoprotein lipase production. Genes may be protected from degradation by packaging into nanoparticles with dendrimers. They may be protected from degradation by packaging into viruses as in the case of Rexin G. Gene entry into the cell may be facilitated with adenoviruses as in the case of Gendicine in which the virus enters tumour cells using the coxsackie adenovirus receptor. Genes cannot be given by non-parenteral routes as they are unable to cross the various mammalian epithelia. Access to the nucleus is facilitated in dividing cells such as cancer cells; hence, selecting a neoplasm as a disease overcomes this delivery limitation.

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Chapter 19
Peptides, Proteins and Antibodies

Aikaterini Lalatsa

Abstract Peptide and protein therapeutics are increasingly able to address a growing range of clinical pathologies and their high specificity and potency combined with low toxicity of metabolic products and minimal potential for drug–drug interactions makes them attractive candidates for clinical development. The pharmaceutical industry is today more in need of delivery technologies that are able to stabilise and effectively deliver therapeutic peptides and proteins across physiological barriers and particularly via non-parenteral routes. Nanoparticulate delivery has the potential to stabilise peptide and protein therapeutics from physical and enzymatic degradation, reduce clearance via the kidneys, prolong plasma half-lives and even target these molecules to the tissue of interest. Nanoparticulate technologies have enabled the delivery of peptide therapeutics via the oral, nasal and pulmonary route and numerous preclinical nano-delivery systems such as polymeric nanoparticles, lipidic nanoparticles and drug–polymer conjugates have been investigated for the delivery of protein therapeutics. In this chapter, a description of these delivery systems and their applications will be discussed.

19.1 Peptide and Proteins as Novel Therapeutics and the Necessity of Novel Delivery Technologies

The emerging field of peptide and protein therapeutics is responsible for a new therapeutic revolution. Peptides regulate most physiological processes acting as endocrine or paracrine signals and also act as neurotransmitters or growth factors. Peptides are already used therapeutically in areas such as endocrinology and haematology.

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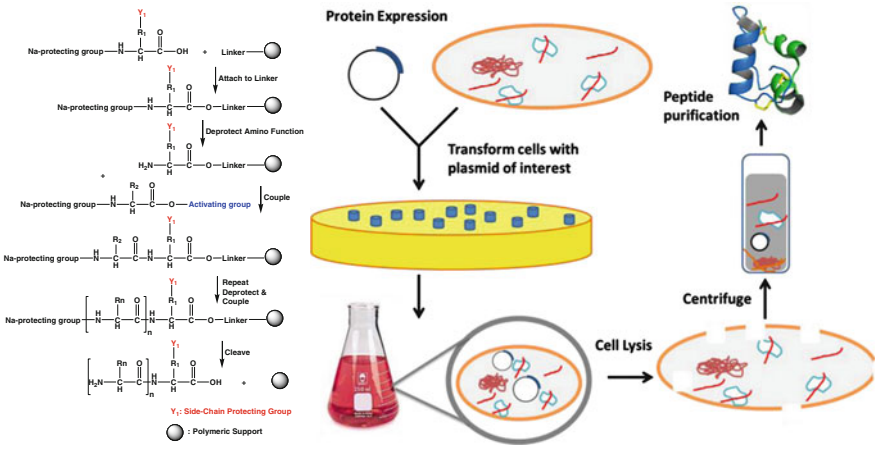

Fig. 19.1 Basic steps of solid phase peptide synthesis (left) and recombinant peptide synthesis (right) schematic diagram

The therapeutic role of peptides is attractive due to their high specificity and potency combined with the low toxicity of their metabolic products and their minimal potential for drug–drug interactions. The advantages of using endogenous or structurally similar peptides as drug candidates is that there is a substantially reduced risk of unforeseen side effects, which gives the product a good probability of regulatory approval when compared to low molecular weight active pharmaceutical ingredients (Lax 2010).

The first peptide and protein drugs approved for clinical use were insulin and factor VIII and these were approved from the 1920s onwards (Van der Walle 2011). Advances in genetic engineering and recombinant technologies have led to the genetic engineering of proteins and some peptides and there is no longer a requirement for extraction and purification of these agents from animal or human tissue (Fig. 19.1). Further advances have led to the routine chemical manufacture of peptides. This technological progress has offered therapies for previously untreatable conditions and has made peptide and protein therapeutics one of the fastest growing segments in the pharmaceutical industry.

The second major advance responsible for increasing the acceptability of peptides, in particular, as drug candidates is the introduction of polymer technologies which allowed the development of controlled release long-acting formulations of peptides encapsulated in biodegradable polymers, e.g. gonadotropin releasing hormone (GnRH) (Lax 2010). The average number of new peptide therapeutics entering clinical trials every year has steadily increased from 1.2 per year in the 1970s, 4.6 per year in the 1980s, 9.7 per year in the 1990s and 16.8 per year in the 2000s (Reichert 2010). Peptides now account for more than 10 % of the overall sales for the pharmaceutical industry.

As peptide therapeutics are increasingly able to address a growing range of medical challenges, the pharmaceutical industry is today more in need of technologies able to stabilise and effectively deliver therapeutic peptides across physiological barriers. Peptides are almost inevitably unable to survive the stomach and intestinal environment and possess oral bioavailabilities of less than 1 % (Mahato et al. 2003). The low oral bioavailability of peptides is a direct result of their physical (degradation by the acidic environment of the stomach) and metabolic instability (degradation by pepsin, intestinal enzymatic degradation, brush-border enzymatic degradation) and their very poor permeation across biological barriers in the absence of specific transport systems. This poor permeation is due to their hydrophilicity, charge and relatively high molecular weight (>500 Da). These attributes limit permeation of peptide therapeutics across the transcellular route, while the presence of tight junctions limits their permeation across the paracellular route. Efflux proteins such as P-glycoprotein contribute in reducing even further the intestinal permeation of peptide therapeutics, as peptides are often substrates for efflux proteins. Even if an adequate amount of the peptide/protein therapeutic has managed to reach the bloodstream, the peptide would still be subject to liver metabolism, degradation by plasma enzymes and would be cleared via the kidneys [cutoff molecular weight for glomerular filtration is reported to be between 30 and 50 kDa (Ruggiero et al. 2010)]. The later challenges are also applicable to parenteral peptide therapeutics.

Protein therapeutics suffer the same problems as peptides but to an even worse degree. For example protein drugs are not bioavailable via the oral route and must usually be administered parenterally. On parenteral administration, they are also rapidly degraded and cleared by the kidneys.

In order to produce controlled release technologies for protein and peptide delivery via parenteral routes (e.g. intramuscular) and in an attempt to increase patient compliance, novel technologies have been developed for delivery across parenteral and non-parenteral routes. Traditional drug development of proteins and peptides has relied on parenteral injection of liquid formulations as the fastest and often least expensive route to commercialisation. The key drivers for selecting a protein or peptide delivery method for commercial development include: patient convenience and compliance, requirement for local or topical delivery, systemic toxicity or other safety issues as well as market competition. As a result a number of new peptide and protein therapeutics have been launched, e.g. insulin, human growth hormone, interferon and erythropoietin. These have been launched to meet clinical needs and various delivery systems have been developed to tackle the issue of bioavailability and patient compliance, the latter particularly among the elderly and among children.

Patient compliance could be increased by using the nasal, pulmonary and oral route for systemic therapies and the dermal and ocular routes, when local therapy is the desired objective. The selection and development of an appropriate delivery system and route of administration for peptides and proteins depends on various factors: (1) therapeutic dose and release profile required (Table 19.1), (2) duration of treatment, (3) disease conditions and target patient population (intravenous injections/infusions for hospitalised patients and non-parenteral dosage forms for outpatients, Fig. 19.2), (4) the impact of processing conditions on stability and bioactivity of

Table 19.1 Therapeutic requirements for protein delivery method choice

Dose	Local delivery	Systemic delivery
High (>2 mg/kg)		
Bolus—Immediate release	IV/IM/SC	IV/IM/SC (devices)[a], intranasal, oral
Sustained	Depots, pumps	Depots, pumps, intranasal, oral
Pulsatile	IM/SC	IM/SC (devices)
Medium (0.05–2 mg/kg)		
Bolus—Immediate release	IV/IM/SC/pulmonary	IV/IM/SC/pulmonary, intranasal, oral, sublingual
Sustained	Depots, pumps	Depots, pumps, intranasal, oral, sublingual
Pulsatile	IV/IM/SC/pulmonary, pumps	IV/IM/SC/pulmonary
Low (<0.05 mg/kg)		
Bolus—Immediate release	IV/IM/SC/pulmonary, intranasal, ocular	IV/IM/SC/pulmonary, intranasal, oral, transdermal
Sustained	Depots, pumps, transdermal	Depots, pumps, intranasal, oral, sublingual, transdermal
Pulsatile	IV/IM/SC/pulmonary, pumps	IV/IM/SC/pulmonary, pumps

IM intramuscular, *IV* intravenous, *SC* subcutaneous
[a]Devices may be useful for IM/SC administration of solutions or depots

peptides and proteins (in order to avoid an increase in immunogenicity or loss of efficacy) and (5) the bioavailability for the particular route and delivery system chosen.

A challenge in developing depot delivery systems for peptides and proteins is the difficulty in establishing good in vitro/in vivo correlations that are able to guide the development process. Additionally, the lack of animal chronic models to test long-term delivery systems (e.g. longer than a week) is a further complication.

19.2 Parenteral Peptide and Protein Delivery

It is safe to assume that over 90 % of the protein/peptide therapeutics approved today are injectable products since parenteral administration avoids some of the challenges associated with physical and enzymatic degradation in the gut. Parenteral administration involves the: intravenous, intramuscular, subcutaneous, epidural and intrathecal administration routes. Although these routes are invasive and not preferred by patients, they ensure that the therapeutic agent reaches specific target areas of the body via the blood and lymphatic systems with predictable pharmacodynamics consequences. Additionally, as most animal research studies and early clinical trials are performed by direct injections, the development of injectable dosage forms or injection devices is more likely to succeed commercially than non-parenteral routes of delivery. However, extensive preformulation studies are required to stabilise peptide and protein therapeutics as proteins, for example, have complex tertiary

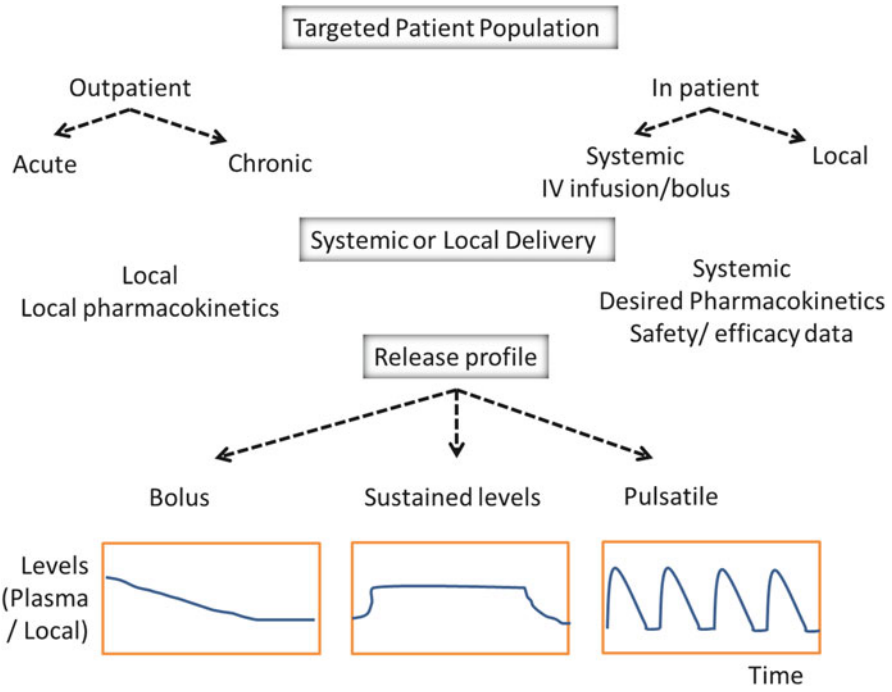

Fig. 19.2 Selection of a protein delivery method: disease indication, duration of treatment and mechanism of action are important parameters in the selection, with the target patient population being the primary concern (inpatient or outpatient). Non-parenteral routes are important for outpatients and necessary novel technologies to enhance compliance and convenience and ensure efficacy, which relies on the achieved pharmacokinetic profile

structures, high molecular weight, and are highly purified, heat-unstable molecules that have the propensity to aggregate, undergo chemical changes and are generally unstable. Peptides may also easily undergo chemical degradation. Intravenous solutions are required to be sterile and free from particles or aggregates in order to avoid the risk of infection, thromboembolism or local tissue reactions such as thrombophlebitis and tissue necrosis (Banerjee et al. 1991) associated with repeated administration.

Peptides and proteins administered intravenously have to cross capillary endothelial barriers to reach various organs to elicit their effects or to be effectively cleared by the body. The structural properties and the permeability of the capillary endothelium varies in different organs and is classified as continuous (in brain, muscles, connective tissue, lungs, heart), fenestrated (in intestinal mucosa, renal glomeruli and endocrine and exocrine glands), and sinusoidal (in liver, spleen and bone marrow). Most of the continuous capillaries in the periphery are permeable to proteins of up to 70 kDa in molecular weight (~7 nm) (Sarin 2010), with the notable exception of the blood–brain barrier (BBB) which is characterised by tight junctions between adjacent cells, the absence of fenestrations and is impermeable to polar

molecules (Lalatsa et al. 2011). Capillaries possessing fenestrations (openings of 24–60 nm) (Grotte 1956; Grotte et al. 1960; Banerjee et al. 1991) are only permeable to macromolecules with a particle size of 15 nm, while sinusoidal capillaries (>100 nm pores) (Naito and Wisse 1978; Wisse et al. 2008) do not pose a barrier to effective peptide/protein delivery. Thus, understanding the impact of the molecular size of the protein, in particular, is critical in the design of an effective delivery method. Particles greater than 7 μm in size accumulate in the lung capillaries, while particles of between 0.1 and 7 μm in size, tend to be cleared via the reticuloendothelial system (which can be detrimental to the in vivo half-life of the peptide); while particles smaller than 0.1 μm tend to accumulate in the bone marrow (Banerjee et al. 1991).

In order to ensure that the peptide or protein reaches its desired location in a timely manner, active targeting strategies may be adopted and these are covered elsewhere Chap. 13 of the book.

As peptides and proteins are very rapidly deactivated by proteolytic enzymes, prolonged activity is highly desirable if chronic treatments are required, making implants capable of releasing peptides or proteins in a controlled manner and for the desired length of time clinically important systems. However, zero-order release kinetics are not always the most desirable kinetics as due to the periodic down-regulation of biological receptors, pulsatile or self-regulated release may be required. Parenteral delivery systems for peptides and proteins can be broadly divided into the following three major groups: (1) immediate release systems, (2) sustained or controlled release systems and (3) pulsatile and self-regulated systems (Fig. 19.2). Non-degradable or degradable systems have both been used to prepare both immediate and sustained release systems and there are four major delivery technologies: implants, microspheres, nanoparticles and injectable hydrogels. These systems have been reviewed in detail previously (Heller et al. 1983, 2000; Heller 1993; Cleland et al. 2001). Nanoparticle technologies for peptide and protein delivery, that are able to elicit sustained release or are self-regulated systems, are emerging but they are mostly in the preclinical phase of development.

Nanoparticle protein or peptide technologies (liposomes, polymeric nanoparticles, solid lipid nanoparticles) can ensure controlled and or sustained release, improve biodistribution and may be passively or actively targeted in vivo to sites of clinical interest. Furthermore a reduction in protein clearance will necessitate a lower dose of the therapeutic to be administered and reduce the frequency of administration; both elements will enhance patient compliance and treatment outcomes. Additionally nanoparticles enable peptides to cross the BBB (Lalatsa et al. 2012b; Mazza et al 2013).

19.2.1 Polymeric Nanoparticles

Polymeric nanoparticles prepared from biodegradable polymers enhance the biological half-life of peptides after intravenous administration, protecting them from

proteolytic degradation. Several reports have illustrated the ability of polymeric nanoparticles to enable delivery across the BBB, a notable barrier to the treatment of central nervous system diseases. Please see the section on biological barriers, elsewhere in this volume. Quaternary ammonium palmitoyl glycol chitosan (GCPQ) nanoparticles delivered leucine^5-enkephalin and its lipidic prodrug (Tyrosyl palmitate leucine^5-enkephalin—TPLENK) across the BBB and the result was an improvement in brain peptide pharmacokinetics and a prolonged and sustained pharmacological effect (Lalatsa et al. 2012b). The ability to elicit a short lived enhanced pharmacological effect after the intravenous administration of poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80 loaded with a synthetic enkephalin (Dalargin) has also been shown. A polysorbate 80 coating was critical for dalargin entry into the brain as it enables the particles to adsorb alipoprotein E from the blood plasma onto the nanoparticle surface (Kreuter et al. 2002). Recently, brain delivery of a neuroprotective octapeptide has been reported using actively targeted polyethylene glycol and poly(lactic-*co*-glycolic acid) nanoparticles (Li et al. 2013).

Other applications of polymeric nanoparticles include the use of amphiphilic engineered peptides able to form core-shell nanoparticles which have been shown to possess enhanced antimicrobial activity in a *Staphylococcus aureus* in vivo meningitis model (Liu et al. 2009).

Finally poly(ethylene glycol)-*block*-poly(lactic-*co*-glycolic acid) nanoparticles encapsulating AC2-26 (an Annexin A1 N-terminal 25 amino acid long peptide mimetic) has been tested in an in vivo inflammation model and found to be active, making this experimental formulation a possible treatment of atherosclerosis (Kamaly et al. 2013) (Table 19.2).

19.2.2 Lipid-Based Nanocarriers

Self-assembled lipids are also employed for delivery of peptides (e.g. Camurus' Fluid Crystal technology) (Tiberg and Johhnson 2010) (Table 19.2). Camurus' FluidCrystal® nanoparticle delivery system is based on lipid nanoparticles comprising liquid crystal nanostructures with the interior of the particles featuring both hydrophilic and lipophilic domains (Joabsson and Tiberg 2013). The lipid content of liquid crystal nanoparticles is high (50–80 % w/w) allowing a high surface area per gram which can be used to entrap high payloads (up to 50 % w/w) of amphiphilic bioactive agents such as peptides and proteins (Joabsson and Tiberg 2013). Products can be formulated as ready-to-use aqueous formulations in pre-filled syringes (the high drug payload enables small injection volumes) or preconcentrates for dilution. Formulations may be sterilised using autoclaving or sterile filtration. The plasma peptide half-life may be extended to several hours with this Fluid Crystal technology; however, the applicability of this technology for the delivery of proteins or antibodies needs to be established.

Table 19.2 Examples of nanoparticle parenteral peptide/protein formulations

Nanoparticulate technology	(a) Formulation (b) Drug release characteristics	(a) Peptide/protein (b) Marketed formulations	Pharmacokinetic and/or pharmacodynamic advantage	References
Nanofibres	(a) In situ gel formed from self-assembled peptide nanofibres of lanreotide acetate 10 % w/w (b) Bulk erosion	(a) Lanreotide (b) Somatuline Autogel	Can be given by monthly injections for the chronic treatment of acromegaly	Valery et al. (2003)
Nanofibres	(a) pDal nanofibre dispersion (b) Nanofibres yield dalargin on demicellisation and attack by endogenous esterases	(a) Dalargin (b) No marketed formulations	An anti-nociceptive response is achieved with the formulation and pDal is detected in the brain on intravenous administration	Mazza et al. (2013)
Quaternary ammonium palmitoyl glycol chitosan nanoparticles (GCPQ)	(a) Suspension of polymer—peptide or polymer and peptide prodrug self-assembled nanoparticles (b) Demicellisation and release of leucine^5-enkephalin on attack by endogenous esterases	(a) Leucine^5-enkephalin or Tyrosyl^1 Palmitate-Leucine^5-enkephalin (b) No marketed products	Both types of nanoparticles produce a sustained anti-nociceptive effect on intravenous administration and enhanced levels of leucine^5-enkephalin are detected in the brain	Lalatsa et al. (2012a, b)
Poly(butyl cyanoacrylate) (PCBA) nanoparticles coated with polysorbate 80	(a) PCBA nanoparticles coated with polysorbate 80 or apolipoproteins (b) Bulk erosion and diffusion	(a) Dalargin (b) No marketed products	Nanoparticles found in the brain on intravenous administration and an anti-nociceptive response was observed	Olivier et al. (1999), Kreuter et al. (2002)

Poly(ethyl)ene glycol 3400 Da–block-poly(lactide-*co*-glycolide) 40,000 Da (PEG-PLGA) nanoparticles	(a) PEG-b-PLGA coated with a phage display peptide—TGN (b) Release mechanism not stated	(a) NAP(NAPVSIPQ) [highly active fragment of activity-dependent neuroprotective protein (ADNP)] (b) No marketed products	Mice showed an improvement in spatial learning in a Morris water maze test after the intravenous administration of NAP-loaded nanoparticles coated with TGN and no amyloid beta plaques were found the hippocampus and cortex of treated mice.	Li et al. (2013)
PEG-PLGA nanoparticles	(a) PEG-b-PLGA AC_2-26 nanoparticles (b) Release mechanism not stated	(a) AC_2-26 (b) No marketed products	Intravenous administration of AC_2-26 PEG-b-PLGA coated with a targeting peptide (KLWVLPKGGGC) significantly blocks tissue damage in a hind-limb ischemia reperfusion injury	Kamaly et al. (2013)
Amphiphilic peptide nanoparticles	(a) CGGGRRRRRRTAT peptide core-shell nanoparticles (b) Release mechanism not stated	(a) CGGGRRRRRRTAT peptide (b) No marketed products	Nanoparticles show activity in an in vivo *S. aureus*-induced meningitis model	Liu et al. (2009)
Liquid crystal (Fluid Crystal NP Technology)	(a) Liquid crystal cubosome and hexosome nanoparticles (b) Release mechanism not stated	(a) Glucagon-like peptide-1 (b) No marketed products	Cubosomes possess extended plasma circulation half-lives	Tiberg and Johhnson (2010), Joabsson and Tiberg (2013)

AC2-26 Annexin A1 N-terminal mimetic peptide, *CGGGRRRRRRTAT* Cysteine–Glycine–Glycine–Glycine–Arginine–Arginine–Arginine–Arginine–Arginine–Arginine–TAT peptide, *KLWVLPKGGGC* Lysine–Leucine–Tryptophan–Valine–Leucine–Proline–Lysine–Glycine–Glycine–Glycine–Glycine, *NAPVSIPQ* Asparagine–Alanine–Proline–Valine–Serine–Isoleucine–Proline–Glutamine, *PD* pharmacodynamics, *PK* pharmacokinetics, *S. aureus Staphylococcus aureus*, *TAT peptide* Tyrsoine–Glycine–Arginine–Lysine–Lysine–Lysine–Arginine–Arginine–Glutamine–Arginine–Arginine–Arginine, *TGN* phase display peptide

19.2.3 Peptide Nanofibres

Nanofibres prepared from the self-assembly of an active endogenous peptide have been licensed in the USA (Somatuline Autogel) to deliver the endogenous peptide, lanreotide, as an in situ formed hydrogel after subcutaneous delivery. Lanreotide forms dimers via hydrogen bonding resulting in supramolecular structures (b-sheet fibres) built from an alternated stacking of antiparallel peptides (Valery et al. 2003). This self-assembly prolongs the half-life of the parent peptide and the gel follows first order release kinetics, with release driven by the enzymatic hydrolysis of the nanofibres.

Furthermore peptide nanofibres prepared from Tyrosinyl^{1}palmitate-dalaragin (pDal) deliver dalargin to the brain, with the peptide nanofibres being detected in the brain and pDal being detected in the brain, plasma and liver (Mazza et al. 2013). The nanofibre formulation leads to a sustained anti-nociceptive effect on intravenous administration. No activity, or indeed dalargin itself, is detected when dalargin is administered alone as it is degraded so rapidly.

19.2.4 Protein Conjugates: Pegylation

Although protein entrapment in polymeric matrices, microspheres, liposomes and nanoparticles has been successfully carried out in the laboratory, a very successful and commercially relevant approach has been the covalent coupling of polymers such as poly(ethylene glycol) (PEG) to proteins and antibodies known as "pegylation". PEG-asparaginase and PEG-adenosine deaminase were the first pegylated proteins to be approved for human use (Veronese and Harris 2002). However, in the last 20 years there has been a dramatic expansion in the use of pegylated proteins, resulting in clinical products such as: PEG-granulocyte colony stimulating factor, PEG-human growth hormone antagonist, PEG-alpha interferon 2b (PEG-Intron®), PEG-alpha-interferon 2a (Pegasys®) (Rajender Reddy et al. 2002) and pegylated eryrthopoietin [Epogen, Aranesp (darbepoetin) and Mircera (sustained release)] (Veronese and Harris 2002). Attachment of PEG to proteins results in a unique set of properties, including the absence of toxicity, immunogenicity, and antigenicity, low elimination via the kidneys, high solubility in water and certain organic media, enhanced stability and a resistance to proteolysis. Improvements in protein pharmacokinetics in terms of absorption and volume of distribution also result from pegylation. The various methods for attachment of PEG chains to proteins have been recently reviewed (Veronese 2001; Kinstler et al. 2002; Sato 2002; Balan et al. 2007) and site-specific pegylation is critical in order to avoid the many isomers that can result from nonspecific chemistries. This is particularly important for antibodies, as random pegylation can dramatically reduce their binding affinity for the target antigen (Chapman 2002). Site-specific pegylation has dramatically improved antibody circulation half-life (King and Adair 1999).

19.2.5 Protein Conjugates: Glycosylation

Similar to pegylation or in combination with pegylation, glycosylation has been employed for the stabilisation of protein therapeutics and has resulted in clinically available products such as dextran-streptokinase (licensed in Russia for thrombolytic therapy) (Torchilin et al. 1982; Sola and Griebenow 2009). While many protein pharmaceuticals have been successfully formulated by employing stabilising mutations, excipients, and pegylation, the use of pegylation, point mutations and other excipients can sometimes be problematic due to the following factors: predicting the stabilising nature of amino acid substitutions, protein or excipient phase separation upon freezing, cross-reactions between some excipients and the proteins, acceleration of certain chemical (e.g. aspartate isomerization) and physical (e.g. aggregation) instabilities by some excipients (e.g. sorbitol, glycerol, sucrose), interferences caused by some sugar excipients during various protein analysis methods, and safety concerns regarding the long-term use of pegylated proteins due the possibility of PEG-induced immunogenecity and the chronic accumulation toxicity resulting from its reduced degradation and clearance rates (Sola and Griebenow 2009).

Glycosylation offers stability to: (1) proteolytic degradation, such as in the case of thyroid-stimulating hormone (Thyrogen®, Genzyme) (Weintraub et al. 1983) or ribonuclease (Onconase®, Alfacell) (Rudd et al. 1994) by steric hindering the access of proteases to the peptide backbone adjacent to the glycosylation sites, (2) oxidation of amino acid side chains (e.g. histidine, methionine, cysteine, tryptophan and tyrosine) with an example being the enhanced stability of glycosylated erythropoietin to tryptophan oxidation as tryptophan oxidation can lead to a loss of bioactivity (Epogen®—Amgen, Procrit®—Ortho) (Uchida et al. 1997), (3) avoiding chemical cross-linking and protein aggregation caused by disulphide cross-linking as in the case of glycosylated interferon beta (Rebif®—Pfizer/Serono or Avonex®—Biogen) (Runkel et al. 1998) and (4) physical instabilities such as precipitation, aggregation, pH denaturation and chemical, thermal and kinetic denaturation (Sola and Griebenow 2009).

19.2.6 Protein Conjugates: Albumin Conjugates

Conjugation of proteins to biopolymers such as albumin is also another strategy employed to enhance the circulation half-life of protein therapeutics. Recently this strategy has been employed in the delivery of domain antibodies [(the variable domain of an antibody heavy chain (VH domain) or the variable domain of an antibody light chain (VL domain)] (Holt et al. 2003). A single domain antibody (sdAb) is a peptide chain of about 110 amino acids in length, comprising a variable domain (VH) of a heavy chain antibody, or of a common IgG (Holt et al. 2003). These proteins have similar affinity to antigens as whole antibodies, but are more heat-resistant (up to 90 °C in some cases) (van der Linden et al. 1999) and are stable towards both

detergents and high urea concentrations; while stability towards gastric acid and proteases depends on the amino acid sequence. The comparatively low molecular weights of sdAbs leads to better tissue permeability, but also to short plasma half-lives and renal excretion (Harmsen and De Haard 2007), while unlike whole antibodies, sdAbs seem to have a limited ability to activate the complement system as they lack an Fc region. The delivery of toxins or radioisotopes to diseased tissues would be an ideal function for a sdAb (with their comparatively shorter plasma half-lives) as this could provide specific delivery of the toxin to the tumour while minimising the length of time that the toxin could cause damage to healthy cells in the blood (Holt et al. 2003). However, for some applications such as in the treatment of rheumatoid arthritis or cancers, the target antigens need to be available for binding in the bloodstream for prolonged periods (Holt et al. 2003). PEGylation and conjugation or fusion to serum albumin (Smith et al. 2001) has been used to prolong serum half-life (Holt et al. 2003). Site-specific conjugation of a Fab fragment to serum albumin by maleimide cross-linking or generation of a bispecific Fab with specificity for serum albumin in one arm increased the plasma half-life of the Fab fragment in rats (Smith et al. 2001). Fusion of peptides with high affinity for serum albumin also extended the half-life of the anti-tissue factor Fab leading to extended serum half-life of 37-fold in rabbit and of 26-fold in mouse (Holt et al. 2003).

19.3 Noninvasive Peptide Delivery

Technologies able to ensure noninvasive delivery of peptide and protein therapies are being investigated in order to make the administration of daily therapies more convenient for patients. In this chapter we will focus on the oral, nasal, pulmonary routes although ocular and transdermal administration technologies are under development.

19.3.1 Oral Peptide and Protein Delivery

The preference for drug delivery is usually oral administration due to the convenience and increased patient acceptability and compliance. Peptides are, not unjustifiably, considered by the pharmaceutical industry to be poor oral drug candidates as they possess low oral bioavailability (<1 %) (Mahato et al. 2003). This low oral bioavailability is a direct result of their physical (degraded by the low acidic pH of the stomach) and metabolic instability (degraded by pepsin, intestinal soluble and brush-border enzymes) and their very poor permeation across biological barriers in the absence of a specific transport system. The hydrophilicity, charge and high molecular weight (>500 Da) of peptides all serve to preclude their absorption in the gut. Peptides routinely violate the majority or all of Lipinski's predictors for good absorption and bioavailability (Olivier et al. 1999).

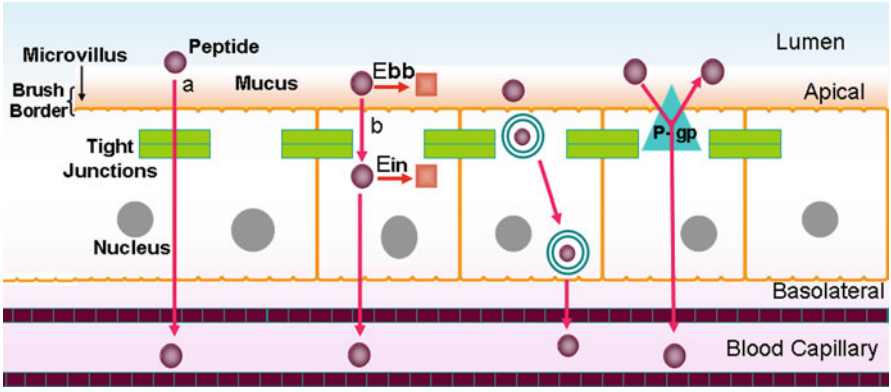

Fig. 19.3 The intestinal mucosa as a barrier. The physical barrier limits peptide flux to the (a) paracellular and (b) transcellular pathways. The biochemical barrier reduces transport of peptides through the intestinal mucosa by brush-border (Ebb: brush-border enzymes) and/or intracellular (Ein: intracellular enzymes) metabolism and by an apically polarised efflux system

Paracellular permeation of the peptides is driven by the electrochemical potential and hydrostatic pressure between the two sides of the epithelium (Madara 2000) (Fig. 19.3). The dimensions of the paracellular space lie between 10 and 50 Å, suggesting that solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route (Rubas, Cromwell et al. 1996). Considering the large surface area of the intestinal epithelium (more than 2×10^6 cm^2) with the paracellular surface area ranging from 200 to 2,000 cm^2, paracellular permeation will only ever be a minor route of entry but even minute quantities (plasma levels in the pM–nM range) of a peptide drug may be sufficient to exert the required biological effect (Li et al. 2013). Examples of peptide drugs that have been shown to permeate the intestinal mucosa via the paracellular route and in combination with a penetration enhancer include octreotide, vasopressin analogues, thyrotropin releasing hormone and salmon calcitonin. However, epithelial tight junctions limit paracellular permeation of peptides and as they are negatively charged overall, they appear selective for positively charged permeants (Li et al. 2013).

The transcellular pathway involves transport across the apical cell membrane, through the cytoplasm of the cell and finally, across the basolateral membrane, with lipophilicity, hydrogen bonding potential and size being the most important controlling factors (Fig. 19.3). For peptide passive absorption to occur, energy is required to break water-peptide hydrogen bonds allowing the peptide to enter the cell membrane. Thus, due to hydrophilicity, hydrogen bonding and large molecular size, passive diffusion of peptide therapeutics is minimal unless a more lipophilic active derivative is engineered. Transcellular transport can involve carrier-mediated uptake or by vesicle-mediated processes which are important for those peptides that are too large to be absorbed by the di- or tripeptide transporters. Efflux transporters (such as the P-glycoprotein, multidrug resistance-associated protein 2 and breast cancer

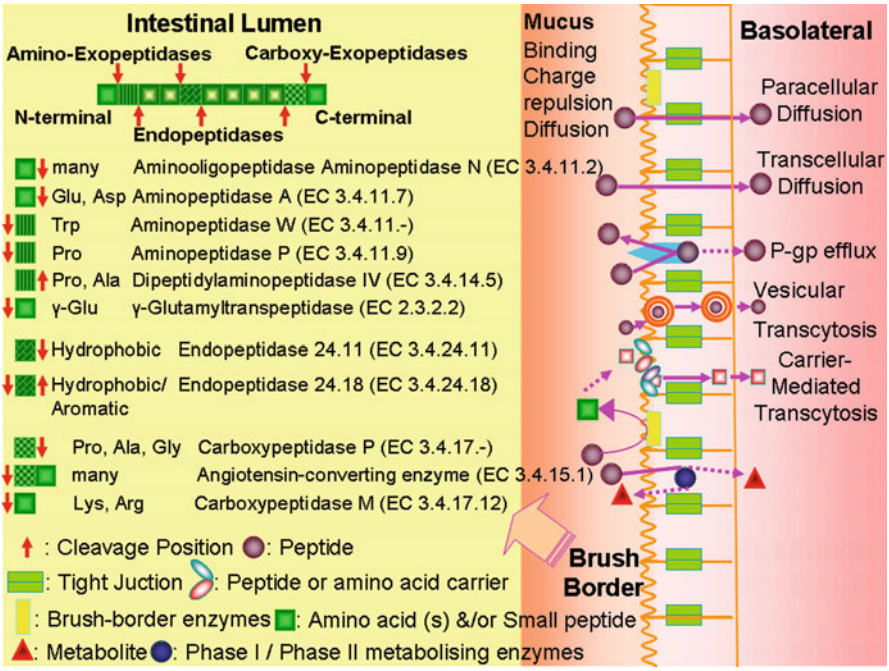

Fig. 19.4 Degradation and absorption processes of peptides and proteins in the intestine: Important brush-border membrane peptidases and their substrate requirements. Squares are indicative of different amino acids. Modified from (Pal et al. 2005)

resistance protein) expressed on the apical membrane of intestinal epithelia can reduce intracellular accumulation, as in the case of cyclosporine, valinomycin and gramicidin D (Zhang et al. 2009).

The first hurdle that a peptide faces upon oral delivery is the high salt and acidic environment in the stomach, which is able to denature the therapeutic peptide (e.g. insulin) (Sweeney and Walker 1993; Rekha and Sharma 2013). Pepsin, an aspartic proteinase optimally active at a pH ~3, is the main proteolytic gastric enzyme hydrolysing peptide bonds but sparing non-peptide amide bonds and ester linkages. As digestion progresses, the gastric juices are emptied within the intestinal lumen where the administered peptides are faced with luminally secreted pancreatic proteases (trypsin, chymotrypsin, elastase and carboxypeptidase A and B) (Langguth, Bohner et al. 1997; Bernkop-Schnurch and Krajicek 1998) able to hydrolyse a peptide to amino acids or small di- or tripeptides (Fig. 19.4). Complete peptide degradation to amino acids can be accomplished by the action of brush-border peptidases (endo as well as amino- and carboxypeptidases) or even, after absorption has taken place, by intracellular enzymes (cytosolic and lysosomal peptidases), limiting further the amount of the peptide reaching the blood circulation (Langguth, Bohner et al. 1997). Another factor that hinders oral absorption is the presence of the mucus layer coating the epithelial surfaces of the intestine. The mucus barrier is a

constantly changing mix of many secretions mostly consisting of mucins, high molecular weight glycoproteins cross-linked via disulphide bonds, and is 400–450 μm in thickness and narrower (100–200 μm) in the duodenum and jejunum. Due to the sialic and sulphonic acid functional groups, the mucus layer is negatively charged which means that it can interact with positively charged peptides or functional groups slowing their diffusion across this layer and thus slowing or reducing their absorption while possibly allowing increased brush-border enzymatic metabolism. Even if a peptide is able to escape physical and gastrointestinal enzymatic degradation and is absorbed in the blood in adequate amounts it is still subject to first-pass metabolism by liver enzymes, degradation by plasma enzymes and clearance via the kidneys. Although the enhanced potency of the peptides means that only very small amounts need to bind to the receptor of interest to result in a pharmacological response or cascade, the very low oral bioavailabilities cause larger doses to be administered, essentially increasing development costs.

The major challenge is enhancing the oral bioavailability of peptides from less than 1 % to at least 10–20 % and if possible to 30–50 % (Acke et al. 2009). Thus designing a successful nanomedicine strategy for an orally bioavailable peptide therapeutic must involve enhancing peptide stability and promoting gut permeation.

Today, only two peptides are marketed as oral products; desmopressin acetate (DDAVP®) approved for the treatment of diabetes insipidus and cyclosporine A (Neoral®) licensed as an immunosuppressant (Brown 2005) (Table 19.3). Both are cyclic peptides whose structural features protect them from intestinal enzymatic degradation. Recently, oral peptide delivery was effected utilising the RapidMist™ device for the delivery of aerosolised insulin particles (<7 μm) generated using GRAS excipients (permeation enhancers, non-chlorofluorocarbon propellant and stabilisers) that are absorbed by the buccal mucosa. This product has been commercialised in India and Ecuador and is currently in phase III studies in the USA.

Strategies to enhance the oral absorption of peptide therapeutics can be divided in two broad groups: one involving chemical modifications of the peptide structure (Borchardt et al. 1997; Bernkop-Schnurch and Thaler 2000) and the other involving formulating the peptide together with other compounds aimed in modifying or masking the unfavourable physicochemical characteristics that limit absorption or are responsible for enhancing enzymatic degradation, respectively. Peptide delivery technologies that are in clinical development involve co-administrating or formulating the peptide with other compounds to enhance the peptide's oral bioavailability such as: (1) co-administration of enzyme inhibitors like aprotinin (natural inhibitor of trypsin) (Yamamoto et al. 1994), ethylenediaminetetraacetic acid (EDTA) (Bernkop-Schnurch and Krajicek 1998), sodium glycocoholate, camostat mesylate (Yamamoto et al. 1994; Tozaki et al. 1997), or bacitracin (Kramer et al. 1991), (2) formulation with absorption enhancers [such as low molecular weight surfactants, bile salts, calcium ion chelators or cyclodextrins (Kanwar et al. 2011)] which modify the epithelial lining of the gastrointestinal tract in order to allow for improved trans- and paracellular transport by interfering with the mucus layer, modulating tight junctions or affecting membrane components (Shaji and Patole 2008), (3) altering the gastrointestinal retention time using mucoadhesive polymers such as

Table 19.3 Oral peptide nanomedicines

Technology	Peptide/protein	Clinical phase	Outcome	References
HDV Insulin Technology [Encapsulation in liposomes with hepatocyte-targeting molecules (biotin) conjugated to the lipids]	Insulin	II/III	Oral administration before meals (15–30 min) produces a 20 % reduction in plasma glucose levels, but was inferior to subcutaneous insulin	Huang et al. (2009)
Oradel™ technology [Carbohydrate-based polymeric nanoparticles coated with vitamin B12]	Insulin/TNF blockers	I	Preclinical studies in streptozotocin-induced diabetic rats showed an 80 % response rate with a 75–80 % reduction in plasma glucose levels lasting 5 h	Petrus et al. (2007), Kamaly et al. (2013)
Oshadi Oral Insulin [Enteric-coated capsules containing a mixture of silica nanoparticles, branched polysaccharides and peptide suspended in an oil core]	Insulin	I	Dose-dependent reduction in blood glucose levels lasting 9–12 h	Sabel and Schroeder (1997)
NOD Tech [Bio-adhesive-enteric-coated nanoparticles]	Insulin (Nodlin) Glucagon-like peptide 1 (GLP-1) agonist (Nodexen)	I Preclinical	Phase I studies in four-way cross-over randomised trial of healthy volunteers versus subcutaneous insulin	NOD Technology (2012)
PLGA—polyanhydride nanoparticles	Zinc insulin	Preclinical	11.4 % of the efficacy of intraperitoneal zinc insulin	Carino et al. (2000)
PLGA—HP55 (hydroxypropyl methylcellulose phthalate) nanoparticles	Insulin	Preclinical	The relative bioavailability of PLGA and PLGA-HP55 compared with subcutaneous insulin was 3.7 and 6.3 % respectively	Cui et al. (2007)
Polycaprolactone—Eudragit RS	Insulin	Preclinical	Decreased fasted glycaemia in a dose dependant manner Increased serum insulin levels and improved glycaemic response to an oral glucose challenge	Damge et al. (2007, 2010)

Poly(methylmethacrylate) PMMA—Chitosan [pH-sensitive carboxylated chitosan grafted poly(methyl methacrylate)] nanoparticles	Insulin	Preclinical	pH controlled release rate that increases at physiological pH. Oral bioavailability at a dose of 25 IU/kg was 9.7 %	Cui et al. (2009)
PACA [poly(alkylcyanoacrylates)] nanoparticles or nanocapsules	Insulin, calcitonin, octreotide	Preclinical	Insulin and calcitonin nanocapsules (insulin, calcitonin) did not enhance peptide absorption however blood glucose was reduced by 25 % with the insulin nanocapsules in diabetic rats Octreotide nanocapsules resulted in a reduction in prolactin secretion in oestrogen treated rats	Damge et al. (1997, 1988), Lowe and Temple 1994; Vauthier et al. (2003), Mesiha et al. (2005), Graf et al. (2009)
Dextran—Vitamin B12 nanoparticles	Insulin	Preclinical	A 70–75 % reduction in blood glucose levels, blood glucose reduction was detectable 54 h after dosing	Chalasani et al. (2007a, b)
Dextran—Alginate—Chitosan nanoparticles	Insulin	Preclinical	A 72 % reduction in plasma glucose levels	Pinto Reis et al. (2006), Reis et al. (2008), Woitiski et al. (2010)
Dextran—Chitosan nanoparticles	Insulin	Preclinical	A 35 % reduction in plasma glucose levels	Sarmento et al. (2007a)
Chitosan-alginate	Insulin	Preclinical	A 40 % reduction in serum glucose levels	Sarmento et al. (2007b)
Chitosan/poly(gamma-glutamic acid) nanoparticles or nanocapsules	Insulin	Preclinical	An insulin bioavailability of 20 % and a prolonged reduction in blood glucose levels	Sonaje et al. (2010a, b)
GCPQ (Quaternary ammonium palmitoyl glycol chitosan) nanoparticles	Leucine^5—Enkephalin and TPLENK (Tyrosyl^1 palmitate leucine^5-enkephalin—a lipidised prodrug)	Preclinical/Due to enter Phase I trials in 2015	Increases brain peptide levels by 67 % Sustained anti-nociceptive response lasting 8 h with no activity with Leucine^5—enkephalin alone	Lalatsa et al. (2012a, b)

Cyclosporine A formulations are excluded as cyclosporine A is a gut stable peptides

Table 19.4 Oral protein therapeutics

Technology	Peptide/protein	Clinical phase	Outcome	References
PLGA—PEG nanoparticles	Tetanus toxoid	Preclinical	Enhanced ^{125}I-Tetanus toxoid levels in lymph node tissue after oral administration	Vila et al. (2002)
PLGA—chitosan nanoparticles	Tetanus toxoid and elcatonin (eel calcitonin analogue)	Preclinical	Modest enhancement in ^{125}I-Tetanus toxoid levels in lymph node tissue after oral administration	Kawashima et al. (2000), Vila et al. (2002)
			Reduction in blood Ca^{++} levels with calcitonin nanoparticles	

chitosans (Khutoryanskiy 2011; Lalatsa et al. 2012a) and (4) loading or conjugating the peptide to a suitable carrier [such as lipidic carriers (Griffin and O'Driscoll 2011; Li et al. 2012), microparticles or nanoparticles (Acke et al. 2009; Trapani et al. 2010; Lalatsa et al. 2012a)] (Table 19.3).

Even though there are several technologies enabling peptide delivery via the oral route, very few have progressed beyond the proof-of concept stage to clinical trials. The only oral peptide nanomedicines that have progressed to Phase I clinical trials are all designed for the oral delivery of insulin (Table 19.3) and involve: (1) biotinylated phosphatidylethanolamine liposomes encapsulating insulin (HDV insulin technology) able to target insulin loaded liposomes to the liver, mimicking the physiological location of this peptide in vivo (Huang et al. 2009), (2) carbohydrate nanoparticles surface modified with vitamin B12 able to entrap insulin (ratio of insulin to excipients 1:36,000) that enables carrier-mediated transport of the loaded particles via the natural transport system available for vitamin B12 (Oradel™ technology in which 1 μg of attached B12 can deliver 160 mg of insulin) and elicits blood glucose levels that are lowered to 75–80 % of baseline values (Petrus et al. 2007; Kamaly et al. 2013), (3) bio-adhesive nanoparticles able to entrap macromolecules creating unidirectional release kinetics, enhanced permeation across physiological barriers and protection against enzymatic degradation (NOD Tech) and (4) a nanoparticle strategy, composed of a non-covalent mixture of silica nanoparticles, branched polysaccharides and insulin suspended in an oil phase within enteric-coated capsules (Oshadi).

Proteins administered via the oral route are subjected to all the limitations described above and while some progress has been made with oral peptide delivery, particularly the delivery of insulin, the oral delivery of proteins has not been accomplished and the only documented site of protein uptake is in the Peyer's patches, making oral vaccination the only way in which this uptake mechanism may be exploited (Table 19.4). Significant transcytosis of polypeptides has been described

for microfold cells (M-cells) located in the follicle-associated epithelium of the Peyer's patches as these Peyer's patches possess the unique ability to sample antigen from the intestinal lumen and deliver it to antigen presenting cells and lymphocytes located in a unique pocket-like structure on their basolateral side (Liu et al. 2009). The reduced levels of enzymatic activity compared to epithelial cells makes this route of entry attractive for peptide containing nanoparticles, even though Peyer's patches constitute about 1 % of the intestinal surface and the relative sparse nature of the glycocalyx facilitates the adherence of both microorganisms and inert particles to their surfaces (Liu et al. 2009).

19.3.2 Nasal Peptide/Protein Delivery

Nasal delivery is a promising alternative to intravenous injection for the delivery of peptides and proteins as the large surface area and high vascularity of the nasal cavity favours fast absorption of therapeutic molecules into the systemic circulation. However, peptide and protein intranasal delivery is considerably less effective than after intravenous administration (Illum et al. 2001). Peptide bioavailability is limited by pre-systemic elimination due to enzymatic degradation or mucociliary clearance, and by poor mucosal membrane permeability of large polar substrates (Irwin et al. 1994). Increasing interest in the development of nasal delivery systems is still fuelled by the rapid onset of action achieved, the accessibility of this route and the avoidance of parenteral administration. Simple nasal drops, a nasal spray or a nasal gel may be used.

Exploitation of the nasal route for the delivery of drugs and macromolecules to the brain via the olfactory region has been explored as the olfactory region of the nose can be a major site for entry of viruses into the brain (Reiss et al. 1998; Illum 2000). In order for a peptide to travel from the olfactory region in the nasal cavity to the CSF or the brain parenchyma, it has to traverse the nasal olfactory epithelium and, depending on the pathway followed, also the arachnoid membrane surrounding the subarachnoid space. Three different pathways can be envisaged across the olfactory epithelium; (1) a transcellular pathway, especially across the sustentacular cells, most likely by receptor-mediated endocytosis, fluid phase endocytosis or by passive diffusion (unlikely for peptides), (2) a paracellular pathway through tight junctions between the sustentacular cells and olfactory neurones and (3) via the olfactory nerve pathway where the drug is taken up into the neurons by endocytotic or pinocytotic mechanisms and transported by intracellular axonal transport to the olfactory bulb (Illum 2000). The transneuronal pathway is very slow and agents reach the CNS as late as 24 h after administration hence transport via neuronal routes cannot explain the rapid appearance of drug in the CSF that is seen for a range of low molecular weight compounds (Fig. 19.5) (Illum 2000). Hence, at least in animal models, a therapeutic molecule with moderate lipophilicity, i.e. one that is not so lipophilic so as to give rapid transport into the systemic circulation, will show a higher CSF and olfactory bulb concentration after nasal administration than after parenteral administration (Illum 2000).

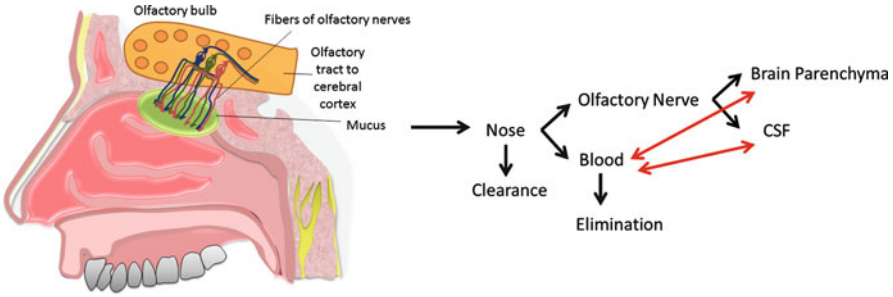

Fig. 19.5 Left: Structure of the nasal cavity and the olfactory bulb and olfactory nerve, Right: Nose to brain transport routes

While lipophilic small molecules are well absorbed from the nasal cavity, the permeability is usually very low for small polar molecules and large molecular weight peptides and proteins. The degree of drug uptake is dependent on the molecular weight (<1,000 Da), the degree of lipophilicity and the degree of dissociation (Illum 2003). Thus, the most likely pathway followed by such drugs will be by passive diffusion across the epithelium (more lipophilic drugs) or through the tight junction of the cells (more hydrophilic drugs) or endocytotic transport resulting in very low permeability for small polar molecules and high molecular weight peptides and proteins (Illum 2003).

Various strategies have been employed to enhance peptide and protein permeation across the nasal cavity. Permeation enhancers such as bile salts, phospholipids and cyclodextrins (dimethyl-β-cyclodextrin) have been used in nasal formulations to enhance absorption especially of macromolecules. It is also common to co-administer a protease inhibitor such as bestatin, puromycin or boroleucine (Agu et al. 2002; Banga 2006) or to co-administer protease inhibitors and membrane permeation enhancers such as amastatin with EDTA (Hussain et al. 1990; Sayani et al. 1993; Quan et al. 1999). A careful selection of permeation enhancers is very important if one is to avoid any local or systemic toxicity. Finally, chemical modification of the peptide to enhance its metabolic stability is also a commonly used strategy. Mucociliary clearance can be reduced by depositing the drug in the anterior part of the nasal cavity. Mucoadhesive polymers such as chitosan are less cleared by mucocilliary clearance and have been widely explored as they provide a prolonged residence time and a comparatively steeper concentration gradient for passive peptide and protein uptake (Illum 2003; Banga 2006). Finally, microparticles and nanoparticles have been successful in delivering peptides or proteins across the nasal mucosa. The nasal epithelium has an area of approximately 150 cm^2, and this will limit the dose range given by this route. Drug delivery technologies producing higher bioavailability are thus needed for this route.

Peptides such as calcitonin, desmospressin and buserelin are commercially available for nasal administration (Banga 2006) and many peptide and proteins are in clinical trials (phase I and II) such as human growth hormone, parathyroid hormone,

glucagon-like peptide 1, growth hormone releasing hexapeptide, leuprolide, erythropoietin and interferon beta (Illum 2012). Vaccines delivered via the nasal route are very effective in stimulating local and systemic immune responses since the nasal mucosa has abundant lymphoid tissue (Illum 2003). Nasal vaccines are very useful for the development of immunity against respiratory infections where the nasal mucosa is the first site of contact with pathogens or antigens. This has been demonstrated for respiratory syncytial virus (RSV), where nasal immunisation was superior to an intramuscular or oral vaccination (Yu et al. 2008). For particulate delivery, the particle size for nasal delivery is important as the normal diameter of the tight junctions is in the order of 3.9–8.4 Å, and even with the application of an absorption enhancer the diameter would most likely be smaller than 15 nm (Illum 2000). Hydrophilic (carboxylated) particles of 10 nm were able to be transported paracellularly, while larger or more hydrophobic particles with surface amines were restricted to the transcellular route (Illum 2007). Chitosan nanoparticles are highly researched as intranasal peptide and vaccine delivery systems but studies are still at a preclinical stage (Table 19.4). In some studies chitosan nanoparticle peptide or protein formulations did not appear to improve the activity of the peptide when compared to the peptide in a solution of chitosan (Illum 2007). However, other research has shown that the activity of peptide and protein antigens for vaccines is increased with nanoparticle formulations (Table 19.5).

Most studies investigating drug transport across the nasal mucosa have been performed in the rat model, although studies in mice, rabbits and monkeys have also been reported. It is important to note that the olfactory region of the rat, for example, and other commonly used animal models is comparatively large, whereas in humans the olfactory epithelium covers only a small area in the roof of the nasal cavity (Illum 2000). Hence, it is most likely that the olfactory transport of drugs will be much more pronounced in rats than in humans. The supine position used during application (either by anaesthesia and placing the animal on its back or holding the animal in a supine position for 15 s) will most likely contribute to the efficient bathing of the olfactory region and thereby an enhanced drug transport (Illum 2000).

19.3.3 Pulmonary Peptide Delivery

The lung is an attractive target for peptide delivery due the fact that access to the lung is noninvasive and delivery to the lung can be used to achieve systemic or local exposure to the drug. Lung delivery avoids hepatic first-pass metabolism and enables a rapid onset of therapeutic action. The delivery of proteins such as antibodies is challenging, as doses are high and in excess of 2 mg/kg for proteins with excellent bioavailability (Cleland et al. 2001). Most of the pulmonary delivery activity has been focused on the delivery of peptides and hence the account below reflects this focus.

Nanocarrier systems in pulmonary drug delivery offer many advantages such as: (1) a relatively uniform distribution of the drug dose within the alveoli, (2) the

Table 19.5 Nasal nanoparticle peptide and protein systems

Technology	Peptide/protein	Clinical phase	Outcome	References
PLGA—Chitosan nanoparticles	Tetanus toxoid	Preclinical	^{125}I-Tetanus toxoid blood levels were higher with a chitosan coating at 2 h post-nasal administration	Janes et al. (2001)
PLGA-SBPVA [sulphobutylated poly(vinylalcohol)-graft-poly(lactide-*co*-glycolide)	Tetanus toxoid	Preclinical	IgA titres were higher than intraperitoneally injected controls IgG titres were inferior to intraperitoneally injected controls	Jung et al. (2001)
PLA-PEG nanoparticles	Tetanus toxoid	Preclinical	^{125}I-Tetanus toxoid blood and tissue (lymph, live, spleen, small intestine) levels recorded	Tobio et al. (1998), Vila et al. (2004)
Chitosan nanoparticles	Tetanus toxoid	Preclinical	Higher IgG titres with ^{125}I-Tetanus toxoid chitosan nanoparticles when compared to tetanus toxoid alone	Vila et al. (2002)
Chitosan nanoparticles	Ovalbumin	Preclinical	Higher IgA levels than an intraperitoneally administered control Similar IgG levels to an intraperitoneally administered control	Nagamoto et al. (2004)
Chitosan nanoparticles	Insulin	PC	85 % Reduction in plasma glucose levels	Calvo et al. (1997)
Trimethyl chitosan—PEG nanoparticles	Insulin	PC	No different from an insulin solution with respect to blood glucose levels	Mao et al. (2005)
Chitosan—PEG nanoparticles	Insulin	PC	Enhanced insulin absorption compared to insulin solution	Zhang et al. (2008)
Chitosan—alginate nanoparticles	Insulin	PC	Insulin absorption recorded	Goycoolea et al. (2009)
Amine modified polyesters [Formed between poly(vinyl 3-(diethyl) propylcarbamate-co-vinyl acetate-*co*-vinyl alcohol) cationic backbone and the short (L-lactic acid) side chain polyester] nanocomplexes	Insulin	PC	8.32 % insulin bioavailability in a nondiabetic rat model compared to a bioavailability of 2.82 % for insulin solution	Simon et al. (2005)

sustained release of peptides which consequently reduces the dosing frequency, (3) a decreased incidence of side effects, (4) improved patient compliance and (5) peptide internalisation by the alveolar cells (Sung et al. 2007; Bailey and Berkland 2009). Comparing the pulmonary and nasal delivery for peptides, it is not clear which route is able to elicit higher systemic peptide levels (Cleland et al. 2001). Permeation enhancers are not typically used for pulmonary delivery, but their use might prove beneficial for enhancing the tracheal absorption of proteins leading to greater overall bioavailability (Morimoto et al. 2000b). Peptide bioavailability on pulmonary delivery in animals has been reported to be up to 50 % depending on the protein (Patton 2000).

Exubera® was the first marketed inhaled insulin pharmaceutical product and was composed of insulin microspheres (White et al. 2005). Although, Exubera was a breakthrough in inhaled peptide delivery, Exubera was withdrawn from the market quite soon after launch, due to low sales (Keegan 2007).

Insulin is an important example for which the delivery of the peptide to the lungs has been shown to be beneficial. The pulmonary delivery of peptides such as insulin may be limited by proteases in the lung which reduce the overall bioavailability (Shen et al. 1999). Studies of pulmonary insulin delivery suggest that the overall bioavailability in humans is in the order of 10–15 %, while it is 20–30 % relative to a subcutaneous injection. This compares unfavourably to published bioavailability levels of 57 % in animal models, indicating that animal studies are not entirely predictive of human responses (Patton 2000).

Rapid peptide adsorption results from pulmonary delivery and there is a fast achievement of peak serum levels; this is a major problem with peptide pulmonary delivery (Patton 2000) as in some cases a rapid onset of action may not be preferred, e.g. in the case of insulin administration prior to meals. Fast peak protein serum levels can result in unwanted adverse effects and require more than once daily administration. Slower dissolving particles (such as insulin crystals) (Havelund 2002) or controlled release formulations have been used in this respect as biodegradable nanospheres (Kawashima et al. 1999) or microspheres (Morimoto et al. 2000a). Insulin PLGA nanoparticles administered to guinea pig lungs resulted in a significant reduction in blood glucose levels, when compared to insulin solution (Kawashima et al. 1999). Prolonging drug presence can be manipulated by using mucoadhesive polymers, such as the biodegradable polysaccharide chitosan. Yamamoto et al. produced poly(lactic-*co*-glycolic acid) nanoparticles surface modified with chitosan and encapsulating the peptide calcitonin (Yamamoto et al. 2005). Nanoparticles delivered to the lungs of guinea pigs produced significant reductions in blood calcium levels relative to the initial calcium concentrations, with prolonged effects up to 24 h. This was significantly longer than results obtained with unmodified nanoparticles. The chitosan-modified nanoparticles were shown to be eliminated more slowly than the unmodified nanoparticles, which suggest that the sustained effects were the result of particle retention within the lungs.

In order to overcome the issues of storage and delivery of nanoparticles to the lungs, nanoparticles can be incorporated into micron-scale structures (Sung et al. 2007). By formulating nanoparticles into larger hollow or porous microparticles

Table 19.6 Pulmonary peptide/protein nanoparticles

Technology	Peptide/protein	Clinical phase	Outcome	References
PLGA—PVA nanoparticles	Insulin	Preclinical	A reduction in blood glucose recorded	Kawashima et al. (1999)
Poly(butyl cyanoacrylic acid) PBCA—Dextran	Insulin	Preclinical	A reduction in blood glucose recorded	Zhang et al. (2001)
PLGA—Chitosan	Elcatonin (analogue of eel calcitonin)	Preclinical	A reduction in blood calcium levels recorded	Yamamoto et al. (2005)

using spray-drying, the dry powders can be delivered more easily into the lungs with a simple inhaler, with the matrix of the microparticle consisting exclusively of nanoparticles or additional inert pharmaceutical excipients such as amino acids, sugars or phospholipids. When exposed to moisture within the humid environment of the lung or the lung lining fluid, the matrix of the microparticles dissolves and allows the release of the nanoparticles (Tsapis et al. 2002; Sung et al. 2007).

The pulmonary delivery of peptides is limited to therapeutics that require low doses and do not cause side effects when high peak serum levels are reached. Current nanoparticle peptide and formulations are still in preclinical development (Table 19.6).

19.4 Future Perspectives and Conclusion

The increasing number of new molecules of biological origin such as peptides, monoclonal antibodies, hormones and vaccines, makes peptide and protein delivery an important area of research. Nanoparticle-based protein and peptide delivery has evolved over the years into systems that are able to achieve relatively high bioavailabilities. Parenteral administration strategies are still dominating the peptide and protein delivery market with novel injection devices and controlled release formulations abounding and there are very few attempts to deliver proteins via non-parenteral routes. Nanoparticles are ideal carriers for the controlled delivery of peptides via parenteral and non-parenteral routes and there has been a reasonable amount of activity in this area. Enhanced stability to physical and enzymatic peptide degradation, increased permeation across biological barriers, and the ability to target peptide pharmaceuticals to the tissue of interest are possible with engineered nanomedicines. However, a great deal of research and development is still required to make most of these technologies feasible for clinical translation.

Problem Box

Question 1

Question 1: Insulin is parenterally self-administered for the treatment of diabetes. Describe the advantages of an oral insulin nanomedicine and give an example of a nanotherapeutic under clinical development

Answer 1: Oral insulin nanomedicines are able to protect insulin from physical degradation (low gastric pH and high salt content) and enzymatic degradation in the gastrointestinal tract (pepsin, pancreatic enzymes). Oradel™ technology is based on the entrapment of insulin within a nanoparticle matrix. These nanoparticles (100–200 nm) are made up of a carbohydrate-based protective polymer which is coated with vitamin B12. Vitamin B12 binds to haptocorrin (R factor) in the stomach and when the complex enters the duodenum, pancreatic enzymes digest haptocorrin allowing B12 in the more alkaline environment of the intestine to bind to the intrinsic factor and to be endocytosed by endothelial cells within which it binds to transcobalamin II and is packaged into chylomicrons that are transported via the lymphatic circulation into the systemic circulation and the liver. This delivery strategy thus exploits this natural transport system for delivering insulin while the carbohydrate nanoparticle matrix protects the peptide from enzymatic digestion and acid denaturation in the stomach. An advantage of this technology is the near 100 % entrapment efficiency of insulin. In the formulation 1 μg of B12 can deliver 160 mg of insulin. This is important as B12 uptake is limited to 1–2 μg per dose received by humans. Another advantage involves the transport of the peptide loaded nanoparticles via the lymphatic circulation, which can allow for protection of the peptide when it reaches the systemic circulation. Positive outcomes in preclinical studies of Oradel technology have been reported and the technology has completed successfully a phase Ib human study for the oral delivery of a TNF-alpha blocker for the treatment of rheumatoid arthritis.

Question 2

Briefly describe a clinically successful strategy that has been used to enhance the plasma circulation half-life of peptide and protein therapeutics.

Answer 2: Although protein entrapment in polymeric matrices, microspheres, liposomes and nanoparticles has been successful, a very successful approach that has been widely used in clinical development of peptide therapeutics has been the covalent coupling of polymers such as PEG to proteins and antibodies, a process known as “pegylation”. This strategy can be used alone or in combination with nanoparticle technologies. Attachment of PEG to proteins results in a unique set of properties, including an absence of toxicity, immunogenicity, and antigenicity, low elimination via the kidney, high solubility in

(continued)

Problem Box (continued)

water and certain organic media, enhanced stability and resistance to proteolysis. Improvements in protein pharmacokinetics in terms of absorption and volume of distribution are also effected by pegylation. Pegylation is a versatile strategy that has been used for delivery or proteins and antibodies, and advances in site-specific pegylation have resulted in multiple clinically licensed protein therapeutics. Combining this technology with pre-filled syringes has enabled the development of patient friendly dosage forms.

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Chapter 20
Nanoparticles in Medical Imaging

Mazen El-Hamadi and Andreas G. Schätzlein

Abstract Research output in the area of medical and molecular imaging has grown exponentially in the last decade. A key driver of this endeavour stems from the insight that a much more detailed understanding of the biological basis of disease and the body's interactions with therapeutics will be required to drive the next stage of medical and therapeutic development. Medical and molecular imaging techniques today increasingly allow us to study biological process in the individual in real time. Nanoparticles have proven to be highly versatile structures that can be engineered to change their biological properties systematically. In this chapter we will examine how the use of nanoparticles as imaging agents (contrast agents, dyes, probes, or labels) can help to improve the imaging output. The key properties of the most important in vivo imaging modalities applicable to pre-clinical and clinical use will be examined and key examples of the application of nanoparticles discussed.

20.1 Introduction

Medical imaging aims to acquire spatial, temporal, and functional information about structures and processes inside of the body, which are not normally accessible to direct visual observation without invasive intervention. Historically, medical imaging started with the discovery of X-rays by Gustav Röntgen in Germany in

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1895. For the first time, images revealed the structure of bones, i.e. the hand of his wife Anna Bertha, in a non-invasive fashion, a discovery for which Röntgen was awarded the Nobel Prize in Physics in 1901.

Since then, the development of novel imaging technologies has largely been driven by the medical need for improved diagnostics, i.e. as a means to inform diagnosis and therapeutic decision-making. More recently, an important additional impetus has come from the increasing need for sophisticated biological information in the process of drug development.

Historically, the need for accurate and detailed spatial information about the body has been a key objective for the development of imaging technologies. As the spatial information is actually of a 3D nature its projection on to a screen, film, or similar medium brings challenges in terms of interpretation of the data. This in turn has driven the desire to acquire the spatial information in a 3D form. This need is exemplified by the development of computer tomographic techniques in the context of X-rays, techniques in which the simple radiographic film image is complemented by the ability to detect and ultimately visualise 3D information.

The information sought includes whole body anatomical information, e.g. about the location of bone fractures to inform orthopaedic interventions, or the location of a tumour to aid surgical removal. Increasingly, however, there is also a need to gain access to information at the histological and molecular level, e.g. the distribution of particular receptors such as HER2 in breast cancer, in order to guide therapeutic decisions in medical oncology or the location of an inflammatory reaction, etc. (Weber et al. 2008). In either case, there is a drive for an ever-increasing level of detail that leads to the constant search for imaging capabilities at higher resolution and with more rapid acquisition times.

Increases in resolution directly feed into an increase in the amount of information that needs to be gathered. Typically, this will also lead to a concomitant increase in the time required to acquire an image. Rapid image acquisition is therefore also important, as long image acquisition times can become impractical. For example, in the case of long magnetic resonance imaging (MRI) protocols the limited access to patients in the scanner can create clinical challenges. Rapid image acquisition also becomes important when repeat images are acquired. In this case rapid image acquisition capabilities increase the time-resolution of an imaging technique and limit motion artefacts that may arise from involuntary movement, such as in the chest during breathing. Thus, rapid image acquisition also allows dynamic processes, such as the performance of the heart using echocardiography, to be studied in real time.

The non-invasive nature of imaging also allows monitoring of phenomena in longitudinal studies, i.e. in the same individual. It thus becomes possible to assess the pharmacodynamic effects of a pharmacological intervention, such as the effects of treatment of an experimental tumour, over time and in the same individual. This approach creates much more informative experiments while at the same time minimising the number of experimental animals used.

20.2 Imaging Nanoparticles

The role of nanoparticles in medical imaging is twofold: nanoparticles offer unique biological, pharmaceutical, and therapeutic applications that are discussed in detail in other chapters of this book. One motivation for the imaging of nanoparticles is therefore the wish to study the behaviour and fate of these particles in animal models or in clinic applications. On the other hand, nanoparticles also offer some unique properties that may be exploited to enhance the capabilities of an existing imaging modality or to create new diagnostics only made possible by using the imaging properties of nanoparticles. In the latter case nanoparticles are used because of their enhanced diagnostic capabilities due to either their biological behaviour (e.g. lack of extravasation allowing enhanced imaging of blood vessels) or their unique signal. For example iron oxide particles create a strong and clear T_2 contrast that requires the presence of a crystalline matrix of at least nanometre-sized dimensions; similarly quantum dots create distinct and stable fluorescent signals.

We will not specifically review the multitude of papers which report studies involving the imaging of nanoparticles. The remaining chapters of this book and reviews e.g. for the various types of nanoparticles, their modifications, and multitude of applications (Hahn et al. 2011; Minchin and Martin 2010), provide a comprehensive overview. Instead we will discuss the important general principles of medical imaging and highlight the most important medical imaging modalities relevant to nanoparticle imaging.

20.3 Principles of Medical Imaging with Nanoparticles

Imaging of nanoparticles for medical purposes relies on the ability to detect the nanoparticles. This typically requires the particles to carry a unique and detectable tag or to have unique properties that allow detection of the unmodified particle. Depending on the imaging modality, such agents are frequently described by terms such as: contrast agents, labels, dyes, and probes. Collectively these will be called imaging agents throughout this chapter.

20.3.1 Active and Passive Imaging Agents

Imaging agents can be *active*, i.e. the agent is the source of the signal, as would be the case with radioisotopes for positron emission tomography (PET) or single photon emission computed tomography (SPECT) (see below). Alternatively, imaging agents can be *passive*, i.e. they have the ability to change the incident imaging signal in a specific, reproducible, and predictable fashion. These types of agents are typically known as contrast agents. Examples include X-ray contrast agents which increase

contrast by efficient absorption of the X-ray beam energy, MRI contrast agents which enhance the relaxivity of the surrounding water protons, or fluorescent dyes which absorb incident photons to emit lower energy photons. Some agents have an intermediate status, e.g. fluorescent labels first absorb external energy provided by the incident excitation photons and then emit photons with changed spectral properties. In the case of luminescent compounds the energy driving the emission can also come from chemical energy (e.g. the luciferin—luciferase signal).

20.3.2 Tissue Penetration

All medical imaging information has to travel through at least part of the body to reach the observer. Thus, the ease with which the information travels through the body and how it interacts with other structures on the way are key parameters that define the utility of any imaging modality. Tissue penetration therefore is important in determining the type of studies that an imaging modality will be useful for. It is vital to know whether a signal from the inner organs of a human can be detected or whether detection is limited to smaller animals such as a mouse, where penetration of only a few centimetres of tissue is required (see below).

There are only a few imaging techniques for which the signal comes directly from the label used. In terms of practical relevance for clinical imaging, only active imaging agents such as those used in radioisotope-based imaging modalities provide a signal by themselves that can be detected outside the body. For many other imaging modalities the information from which the image is derived is actually not created directly by the label but rather based on information that is produced in the body in response to stimulation or interaction of the imaging agent with external energy. For example, an X-ray image is produced by the variable absorption of external electromagnetic waves (X-rays), while ultrasound images require the reflection of the external pulsed ultra sound waves to be detected. For these types of imaging modalities the ease of penetration of the external stimulating energy is equally important as that of the imaging signal itself.

20.3.3 Biological Differences in Tissues Lead to Signal Variations and Image Contrast

A broad range of information can be obtained from in vivo imaging, depending on the modality employed. In the simplest case the imaging modality used only reveals inherent properties of tissues and organs. Examples of this approach include X-ray imaging and MRI, which reveal anatomical information based on the different tissue densities and water content, respectively. Such inherent differences between tissues and tissue states, i.e. physiological vs. pathological, can produce a wealth

of information and are routinely used to locate a bone fracture for instance or to diagnose tumours and monitor their response to cytotoxic therapy. However, the use of signal modulating agents (contrast agents) can enhance these capabilities considerably.

20.3.4 The Imaging Agent Signal and its Distribution Change in Response to the Environment

The use of imaging agents such as contrast agents, dyes, and probes adds enormous additional possibilities as the image not only reflects inherent tissue properties, but is also based on the interplay between the external agent and the tissues. This interplay modifies the distribution or response observed in the imaging process. For example, the interaction with the body can change the biodistribution of the external imaging agent because of access or because of its affinity for specific tissues, e.g. probes with receptor specific ligands may accumulate in sites with high receptor concentration. The presence of the imaging agent could also be detected as changes in the imaging signal observed: for example, changes in environmental conditions will modulate the intensity and spectrum of many fluorescent dyes.

20.3.5 Plasma Half-Life and Clearance

The binding of plasma proteins can dramatically alter particle characteristics and this may manifest as a change in particle surface charge, hydrophilicity, and particle size. Binding of specific proteins could potentially even lead to a re-targeting of particles as has been observed for Tween 80-coated particles, which, after binding to plasma Apolipoprotein E, show some brain targeting (Gessner et al. 2001; Kreuter et al. 2002). Commonly used strategies to minimise such interactions include the use of poly(ethylene glycol) (PEG) polymers which minimise binding by creating a steric barrier; the steric barrier prevents the adsorption of a protein layer, which could be up to 15 nm thick (Choi et al. 2007). Such polymers frequently have molecular weights of a few thousand Dalton and therefore may add considerably to the size of the particle. Approaches for less "bulky" coatings include the use of small PEGs of only 550 Da as well as zwitterionic and neutral coatings with amphiphilic and multidentate polymer ligands showing some promise (Kairdolf et al. 2013).

Particle size is also critical in terms of access to tissues and the plasma half-life of particles. For example, the endothelial lining of the blood vessels does not normally permit permeation of large macromolecules or particles. For the endothelial lining of the blood vessels the average "pore" size has been estimated to be around 5 nm, whereas for the lymphatic vessels a comparable but slightly higher value of

7 nm has been suggested (Longmire et al. 2008). The threshold for filtration of particles in the kidney is less than 6 nm. For particles larger than 8 nm no filtration occurs, whereas for particles with a size between 6 and 8 nm filtration depends on specific physical properties, e.g. cationic > neutral > anionic (Choi et al. 2007; Longmire et al. 2008). For individual polymer molecules the filtration threshold depends on the actual hydrodynamic size, but materials with a molecular weight (MW) of less than 20 kDa are expected to be filtered out, whereas larger molecules such as albumin (MW > 60 kDa) would normally be retained.

This makes particle size one of the most important factors in determining biological behaviour. In addition to tissue access and renal filtration, size also affects how particles can be used for diagnostic purposes. For example, to exploit the enhanced permeation and retention (EPR) of macromolecules and particles in tumours based on the sieve functionality of the leaky tumour neovasculature, particles must be of 10–400 nm in diameter in order to accumulate in the tumour interstitium (Maeda et al. 2013).

The surface chemistry is not only important in terms of physical chemistry and consequent non-specific binding, but will also determine the options available for the attachment of specific targeting ligands.

20.3.6 Scale and Resolution

Medical imaging techniques span a broad range of possible resolutions and diagnostic details; imaging resolution scales from micrometres (μm) to millimetres (mm) (Table 20.1). When considering these numbers it is important to bear in mind that many practical factors will affect the actual achievable resolution. This fact may be illustrated by considering that the theoretical resolution of an imaging technique such as microscopy is dependent on the wavelength used and is typically in the order of half of the wavelength, i.e. hundreds nanometres. However, when optical techniques are applied in medical imaging the interaction with the tissue, i.e. the scattering of light, leads to a pronounced loss in resolution and for near-infrared fluorescence (NIRF), for example, the resolution is just 1–3 mm.

Another important consideration is the signal to noise ratios that are achieved—typically an increase in the signal will reduce uncertainty due to noise. Due to the risk of damage related to increases in the amounts of energy tissues are exposed to as a result of a boost of signal, there typically are practical limitations to safe increases of the signal strength. Other limitations stem from costs and practicality consideration. It is known for instance that the homogeneity and strength of the magnetic field correlates directly with the achievable resolution, but it would be very difficult and costly indeed to try and reach the field strength available in preclinical microbore MRI instruments (e.g. 11 T) with a clinical scanner, which has a field strength of typically just 1.5 T.

Table 20.1 Summary of common imaging modalities used in conjunction with nanoparticles

Technique	Typical NP label	Signal measured	Resolution	Depth	Sensitivity (mole label)	Throughput	Cost	Main limitation
NIRF	QDs, dye-doped NPs, upconverting NPs, SWNTs, and other carbon-based nanomaterials	Light, particularly in the near-infrared	1–3 mm	<1 cm	$1.E^{-12}$	High	Low	Poor depth penetration
MRI	Iron oxide NPs, Gd(III)-doped NPs, NP-based CEST, and hyperpolarized probes (e.g. ^{129}Xe)	Alterations in magnetic fields	50 μm	No limit	$1.E^{-09}$ to $1.E^{-06}$	Low	High	Low sensitivity, cannot follow many labels
PET	NPs incorporating radioisotopes (e.g. ^{18}F, ^{11}C, ^{64}Cu, $1^{24}I$)	Positron from radionuclides	1–2 mm	No limit	$1.E^{-15}$	Low	High	Can detect only one radionuclide, requires radioactivity
SPECT	NPs incorporating radioisotopes (e.g. ^{99}mTc, ^{111}In)	γ-rays	1–2 mm	No limit	$1.E^{-14}$	Low	High	Requires radioactivity
CT	Iodinated NPs, gold NPs, iron oxide-doped nanomaterials	X-rays	50 μm	No limit	$1.E^{-06}$	Low	High	Poor resolution of soft tissues
US	Micro-bubbles, nano-emulsions, silica NPs, polystyrene NPs	Sound	50 μm	Several cm	$1.E^{-08}$	High	Low	Poor image contrast, works poorly in air-containing organs
PAI	Gold nanoshells, gold nanocages, gold nanorods, gold NPs, SWNTs, dye-doped NPs	Sound	50 μm	<5 cm	$1.E^{-12}$	High	Low	Information processing and machines still being optimised

Source: Modified from Hahn et al. (2011)

20.4 Medical Imaging Modalities and Imaging Agents

In this chapter we will focus on imaging modalities with a more established pre-clinical in vivo and clinical role. This in general means that in order to provide non-invasive information in the most commonly used small laboratory animals, the image generating signal has to be able to penetrate at least tens of millimetres. This precondition precludes many useful imaging techniques which are being developed and are routinely applied in the research context at the cellular level or are being applied to understand and characterise nanoparticles; these works are beyond the scope of this chapter. For example, optical microscopic techniques will not be covered in any detail as light penetration in tissues is typically less than 100 μm. Using three-photon fluorescence which has very favourable spectral properties (see below), it has been possible to image the cortical vasculature in mice up to a depth of around 1 mm (Schaffer and Xu 2013). Even based on the latest advances in multiphoton imaging probe and microscopy design, these techniques are thought to be limited to depths of up to 3 mm at best (Zagorovsky and Chan 2013).

20.4.1 Near-Infrared Fluorescence

Photon penetration in living tissues is limited by absorbance and scattering. Light scattering is a function of the wavelength of light used, relative to the size of the scattering centre encountered. By contrast, i.e. shorter wavelength will scatter more absorption depends on the chemical nature (electrons) of the material the photons pass through but again shorter wavelengths are more likely to be absorbed. Biological tissues are made up from a highly complex mixtures of organic molecules, organelles, materials, cells, and fluids that are rich in conjugated bonds, and as such delocalised electrons, which may interact with photons to absorb their energy. These different elements combine to give a gradual loss of signal intensity that is uneven across the spectrum. The majority of biologically relevant molecules absorb strongly at a lower wavelengths, i.e. in the ultra violet (UV) region but less so at longer wavelengths. However, biological chromophores such as haemoglobin are present in high concentrations and absorb quite strongly even at higher visible light wavelengths (<600 nm). On the other hand, lipids and water, although optically transparent, absorb strongly in the infrared region (>1,000 nm). These factors thus combine to create a window of low absorption in the near-infrared region (~600–100 nm) in which tissue penetration is relatively higher. A further advantage of the near-infrared spectral region stems from the fact that tissue autofluorescence from biological molecules and materials, in particular from elastin and collagen, is also relatively low at these longer wavelengths.

20.4.2 Near Infrared Fluorescence (NIRF) Imaging Agents

20.4.2.1 NIRF Dyes

NIRF dyes have been developed for clinical use (Hilderbrand and Weissleder 2010): Indocyanine green (ICG) allows the monitoring of cardiac function, hepatic output, and retinal angiography, while methylene blue (MB) can be used as a marker for the identification of sentinel lymph nodes or colon polyps. These types of dyes may be incorporated in nanoparticles in order to improve some of their properties. For example ICG is an amphiphilic molecule and has a tendency for binding to albumin and many plasma proteins and lipoproteins. After injection it is rapidly cleared from the blood (2–4 min) and excreted via the hepatobiliary pathway (Desmettre et al. 2000). Various groups are working on the encapsulation of this and other NIRF dyes in various nanoparticles such as, e.g. PEG carrying nanocapsules (Bahmani et al. 2013). These PEG nanocapsules are predicted to enjoy a prolonged plasma half-life.

20.4.2.2 Semi-Conductors Quantum Dots

Quantum dots are highly efficient and photostable sources of fluorescent light made from binary heavy metal alloys such as cadmium selenide or cadmium sulphide (Kairdolf et al. 2013). The materials are arranged as semi-conductor crystal lattices constrained in a very small space. Light of a suitable, relatively broad spectrum can be used to excite the valence electrons in such semi-conductor dots. When these return to their ground state they emit fluorescent light. The efficiency of this process and the specific wavelength of the emission is determined by the dots dimensions, with larger particles emitting red light while smaller particles emitting light more towards the blue end of the spectrum. This allows quantum dot fluorescence spectral characteristics to be tuned by controlling their size, i.e. typically particles in the order of 10–30 nm will show fluorescence in the 500–650 nm range (Kairdolf et al. 2013). Research is underway to create quantum dots with a broader range of wavelengths and more even brightness by working with various ternary alloys instead of CdSe. This strategy allows the tuning of the emission from 700–2,000 nm without requiring changes to particle size.

A key factor in determining the suitability of such nanoparticles for biological applications is their biocompatibility. The materials used in quantum dot production are toxic and strategies to alleviate this problem are focused on the creation of stable, protective coatings and the production of particles smaller than around 5.5 nm, which can be cleared via the kidney (Choi et al. 2007). Given the large surface area of quantum dot nanoparticles, their propensity for interaction with other molecules and surfaces will impact their biological behaviour.

20.4.3 Magnetic Resonance Imaging

MRI is based on the ability of nuclei in a magnetic field to absorb and emit electromagnetic radiation at a specific resonance frequency: The nuclei of isotopes with an odd number of protons such as water possess a spin or magnetic moment which can be either "up" or "down." In the presence of a strong magnetic field, these nuclei will tend to align with the field, resulting in nuclei having a low or a high-energy state. When exposed to electromagnetic waves with the energy, i.e. wavelength, matching the energy difference between these energy states magnetic resonance absorption occurs. The resonance frequency depends, among other factors, on the strength of the magnetic field that the nucleus is exposed to. In a variable magnetic field, differences in the resonance frequency of the nuclei can therefore be linked to their location within that field.

In the MRI scanner the nuclei—typically water protons—are first aligned in the constant magnetic field with a magnetic field strength of between 1 and 10 T. Then a series of electromagnetic field pulses at the appropriate resonant radiofrequency are applied to change the alignment of the nuclei in a systematically varying magnetic field. Once this varying external field is switched off, the nuclei "relax" and return to the basic aligned state by emitting an electromagnetic signal that can be detected by receiver coils. The relaxation has two components based on independent physical phenomena, i.e. the spin–lattice relaxation ("T_1") and the spin-spin relaxation ("T_2"). While the time needed for T_1 relaxation depends on the interactions of the nuclei with their surroundings, T_2 depends on the magnetic field variations caused by interaction with other protons. Typical timescales for relaxation are smaller for T_2 than T_1 and in clinical scanners range from 40 ms (T_2, liver or muscle) to more than ~2 s (T_1).

20.4.3.1 MRI Contrast Agents

The intrinsic tissue contrast in MRI depends among other factors on three key tissue specific intrinsic properties, i.e. the proton density (e.g. water vs. fat), as well as the T_1 and T_2 relaxation times. Image sequences using these intrinsic properties already allow a detailed assessment of many anatomical, pathological, and clinical medical imaging needs: MRI images using intrinsic contrast are highly sensitive but fairly non-specific. The use of contrast agents increases the utility of MRI in a number of important areas and it has been estimated that currently already around half of all MRI studies are carried out with contrast enhancement (Bellin and van der Molen 2008).

MRI contrast agents work by shortening the T_1 and T_2 relaxation times; consequently the electromagnetic energy of the nuclei is released more rapidly leading to an increase in the signal intensity, specificity, and signal to noise ratios. As an individual contrast agent molecule can act on multiple neighbouring protons, the contrast agent can be used in trace amounts. However, the signal intensity is not necessarily directly dependent on the concentration of the contrast agent as many other factors play into the signal generated.

20.4.3.2 Paramagnetic Contrast Agents

Metal ions with unpaired electrons, e.g. Gd^{3+} or Mn^{2+}, have a permanent magnetic moment and are paramagnetic. Their magnetic field can interact with the field of neighbouring water protons to reduce both T_1 and T_2 relaxation times ($T_1 > T_2$). The relaxivity value (r_1 for T_1 and r_2 for T_2) allows the comparison of the relative efficiency of various contrast agents. The ratio of r_1 to r_2 indicates the potential application as either a T_1 or a T_2 contrast agent. In their simplest form such MRI contrast agents contain a paramagnetic metal ion, most commonly Gd^{3+}. Typically, the metal is chelated by a linear or macrocylic polyamino-carboxylate/or phosohonate, to reduce toxicity and unfavourable biodistribution (Geraldes and Laurent 2009). Nine monovalent agents of this type using different chelating moieties are in clinical use (Bellin and van der Molen 2008). These agents rapidly distribute throughout the body and reach the interstitium of all organs with the exception of those organs that have specific barriers such as the blood–brain barrier.

The stability of these chelates is considered to be crucial as free Gd^{3+} is highly toxic: The ionic radius of Gd^{3+} is very similar to that of Ca^{2+} and free Gd^{3+} can therefore act as a blocker of many voltage-gated calcium channel-dependent processes. Furthermore, in recent years the use of gadolinium contrast agents in patients with kidney problems has been linked to a nephrogenic systemic fibrosis syndrome that affects the skin and organs (Bellin and van der Molen 2008).

This monovalent architecture has been expanded upon by linking multiple chelating groups to polymers and dendrimers to create polyvalent gadolinium contrast agents (Bryson et al. 2012). Using general nanoparticle modification approaches, such systems can also be adapted to encapsulation in or linking to nanoparticles and liposomes (Mulder et al. 2006). Nanoparticulate systems also allow the use of targeting approaches, e.g. using antibodies, and have recently been shown to allow detection of environmental pH: encapsulation of a Gd^{3+} chelator complex in which the coordination of the metal changes in a pH-dependent fashion allows pH measurements in the physiologically relevant pH ranges (5.5–7.3) (Gianolio et al. 2012). While it remains to be seen as to whether such experimental systems will progress into the clinic, their utility for experimental medicine and drug development studies is clear.

20.4.3.3 Superparamagnetic Contrast Agents

Superparamagnetic agents consist of nanoparticles—typically in the size range of 10–100 nm which contain a crystalline core (1–10 nm) of iron oxide or a similar magnetic crystallite. Particles of more than 50 nm in diameter are known as superparamagnetic iron oxide nanoparticles (SPIONs), while particles of less than 50 nm in diameter are known as ultra-small superparamagnetic iron oxide nanoparticles (USPIONs) (Geraldes and Laurent 2009). Such iron particles, e.g. magnetite (Fe_3O_4), can be made by physical methods such as gas phase deposition or electron beam lithography, a range of solution-based chemical methods including coprecipitation,

and microbial methods whereby iron-reducing bacteria produce iron oxide from precursors under anaerobic conditions. Similar to the semi-conductor quantum dot efforts, there is also work underway to produce particles with enhanced properties, by doping of the iron oxide crystal with guest metals such as Mn, Ni, Co (Reddy et al. 2012).

In contrast to the paramagnetic materials the magnetic fields of the individual agents once aligned in a field do not cancel each other out but rather combine, resulting in a permanent magnetic field that is very strong compared to that of the individual ions. These particles have T_1 and T_2 relaxation-enhancing properties with T_1 relaxation depending more on surface interactions with neighbouring protons and T_2 more on the strength of the particles' magnetic field. The strength of the magnetic field of a particle is proportional to the size of its crystalline core. As the core size increases, the relative proportion of atoms on the surface decreases. Consequently the ratio of r_2/r_1 increases with the size of the particle (Reddy et al. 2012). While larger particles (>10 nm) are predominately used as T_2 contrast agents, recent work has highlighted the potential of very small particles (<10 nm), which may be used as T_1 contrast agents.

Iron oxide particles are the only metal oxide nanoparticles currently approved in for clinical use. Such particles are thought to be metabolised using the body's pre-existing iron metabolism pathways; in fact, magnetite nanoparticles occur naturally in organisms. Various studies suggest low toxicity responses for doses below 100 μg/mL and in humans the side effects reported for dextran-coated SPIONs are short lived and mild (urticaria, nausea, diarrhoea) (Singh et al. 2010). The typical dose administered is in the order of 1–5 % of the body's total iron store and so repeat administration could have a noticeable biological effect. Nevertheless, it is also clear that high local concentrations of iron oxide particles could lead to some toxicity which often can be linked to the oxidative stress and formation of reactive oxygen species (ROS) from the Fenton reaction and direct DNA damage has also been demonstrated (Singh et al. 2010). The details of iron oxide particle metabolism and toxicity will furthermore strongly depend on the nature of the specific particle. As is the case for all nanoparticles, the chemical composition as well as the physical properties such as size, surface coating, and surface potential will change the biological properties of iron oxide nanoparticles and these factors are thus likely to modulate the iron oxide's metabolism and toxicity.

As clinically used contrast agents, a number of diagnostic applications for USPIONs have emerged that take advantage of their excellent visibility in T_2 weighted MRI images. All of these applications take advantage of the biological properties of such nanoparticles (Reddy et al. 2012). After intravenous application SPIONs are rapidly taken up by macrophages in the spleen and liver. Liver tumours and metastases show less uptake and can thus be diagnosed. USPIONs do not show this accumulation and circulate much longer in the blood stream, e.g. Ferumoxtran-10 (30 nm in size) has a half-life of 24–36 h in humans (Reddy et al. 2012). These particles gain access to the interstitium of tissues where they can be taken up by local inflammatory macrophages.

20.4.4 X-Ray and Computed Tomography

The electromagnetic spectrum continues from visible light (>400 nm, <3 eV) and UV light (>10 nm, 124 eV) to soft X-rays and medical X-rays, which have shorter wavelength and higher energy (~100–10 pm and ~10 eV–100 keV).

X-rays are used in projectional radiography, i.e. imaging of the 3D structure of the body on to a planar surface, frequently a film, but higher energy X-rays are also important for various X-ray computed tomography (CT) scans. Here the X-ray source and the detector rotate around the body to generate a continuous 360° radiological signal representative of a specific "slice" of the body; the information from a series of consecutive "slices" is then used to tomographically reconstruct the 3D information of the body which can then be studied in various cross sections. It is a widely available, relatively cheap and rapid imaging procedure that, since its discovery and first clinical application by Hounsfield in 1972, has become one of the most widely used routine medical procedures with more than 80 million CT scans being carried out in the US in 2010 alone (Lee et al. 2013). CT is able to provide 3D anatomical images with high resolution and to do this fast enough to even allow imaging of the heart.

X-rays are produced by collision of very fast electrons produced by acceleration from an anode, e.g. a tungsten anode. Scattering of these electrons by the electric field of the anode nuclei creates a continuous spectrum of high-energy photons known as a Bremsstrahlung radiation, or if inner shell electrons are ejected the line spectrum of the "characteristic radiation" is observed (Lee et al. 2013). X-ray photons of sufficient energy are able to penetrate deeply and through a range of materials. The image is produced based on the differential contrast of various materials, which leads to different levels of attenuation of the source X-rays. The underlying mechanisms involved are coherent scattering, Compton scattering, and the photoelectric effect. The relative importance of these effects varies with X-ray energy, with higher energy X-rays exhibiting dramatically reduced attenuation. In CT imaging, the photoelectric effect is the most relevant source of attenuation and contrast. When the energy of the incident X-ray beam is sufficient to allow ejection of inner shell electrons, materials show characteristic discontinuous increases in absorption (K-edge). Due to the mechanism of attenuation, the contrast provided for denser materials such as bones is excellent. However, the differentiation of subtle differences between similar soft tissues remains a challenge and more than half of all CT scans are reported to involve contrast agents of some form (Lee et al. 2013).

20.4.4.1 X-Ray and CT Contrast Agents

The CT contrast in the body spans a range of around 2,000 Houndsfield Units (HU, e.g. bone vs. air), whereas contrast in soft tissues typically ranges from 40 to 80 HU. Detection of small changes or regions rapidly approaches the detection limits of the technique (Lee et al. 2013).

Contrast agents have been in use since the 1920s, with iodine-based agents being most relevant. With sodium iodide being too toxic the use of triiodinated benzene derivatives has been central to the development of these types of agents. The need to be able to achieve high concentrations while minimising osmotic effects has been a key objective of this research. Furthermore, it is desirable to reduce general extravasation and increase plasma half-life. The use of various macromolecular and nanoparticle-based agents is a promising strategy to achieving these goals.

20.4.4.2 Iodine-Based Nanoparticle Contrast Agents

Iodine-based nanoparticle contrast agents have been developed either by formulating the specific hydrophilic or hydrophobic contrast agents into a nanosized-drug delivery system or through the synthesis of polyvalent covalent conjugates that then assemble into nanoparticles.

Examples for the use of hydrophobic contrast agents in nanoparticles include micellar systems for which the colloidal stability and resistance to dilution (low critical micellar concentration) are critical in avoiding toxicity; these micelles enable payloads of up to 130 mg/mL and a half-life of around 3 h in mice (Lee et al. 2013). Similarly, the use of emulsions is an equally well established method of formulating hydrophobic materials and this has been applied to the formulation of hydrophobic iodinated contrast agents. Lipiodol, a formulation using ethyl esters of iodized fatty acids of poppy seed oil, was one of the earliest contrast agents to be developed. Various groups have worked on incorporating this agent in nano-emulsions by using stabilising surfactants such as the Pluronics (Lee et al. 2013). Similarly iodinated triglycerides have been formulated as nano-emulsions for hepatobiliary and vascular contrast enhancement (Henning et al. 2008). In contrast, N1177 is a surfactant stabilised suspension of milled (260 nm) nanoparticles of an iodinated aroyloxyl ester, which has been shown to allow visualisation of atherosclerotic plaques in a rabbit model due to the high macrophage activity at those sites (Van Herck et al. 2010).

Liposomes can be used to encapsulate water-soluble iodinated contrast agents such as iohexol in the aqueous core and can be used as blood pool agents. When they are coated with PEG to achieve enhanced half-lives, liposomes can be used to visualise experimental tumours (Maeda et al. 2013).

Covalent linking of the commonly used triiodinated benzene derivatives to a micelle forming polymer backbone is an alternative approach, which completely abrogates any risk of leaking of the contrast agent (Torchilin 2002). Similar covalently linked iodine-based nanoparticles can be synthesised by emulsion polymerisation of iodinated monomers (2-mathacryloyloxyethyl(2,3,5,-triiodobenzoate), MAOETIB) (Aviv et al. 2009).

Materials, which provide good X-ray contrast, have high atomic numbers and approaches, which utilise such materials, are an active area of research. Lanthanides such as Gd have beneficial contrast properties but because of their toxicity require stabilisation through chelators. Consequently, many of the

chemical and nanoparticle formulation strategies used in making effective and non-toxic X-ray contrast agents are similar to those examined for the lanthanide MRI contrast agents.

20.4.4.3 Gold Nanoparticle CA

In addition to the approaches above, which essentially utilise general nanoparticle know-how for the formulation of X-ray opaque materials, an alternative research strand is focused on creating nanoparticle contrast agents using materials that have optimum X-ray contrast. One important example of those materials is gold which has a high atomic number and suitable k-edge value which provides superior contrast for high-energy X-rays such those used in CT (Lee et al. 2013).

While ultra-small gold particles of ~2 nm can be excreted by the kidneys, particles are frequently much larger (~30 nm) because they require surface modifications such as PEG chains to become colloidally stable. Such particles frequently show high uptake in the liver similar to many other larger nanoparticles and could be used to distinguish between phagocytotically active normal liver and a hepatoma (Kim et al. 2007).

20.4.5 Radioisotope-Based Imaging Methods

Radioisotope imaging methods are based on the detection of radioisotope decay. In principle these events can result in other types of radiation (e.g. α, β) but in practical terms the use of radioisotopes for imaging is limited to methods based on γ-ray detection, as this is the only form easily detectable from outside the body.

20.4.5.1 Single Photon Emission Computed Tomography (SPECT)

SPECT is based on the detection of individual γ photons emitted from a radioisotope. Similar to X-rays, where a screen or film can be used to capture a flat image, in SPECT a gamma camera can produce a simple 2D image. However, frequently tomographic techniques are used to create 3D information about the distribution of the radionuclide in the body from a series of 2D images taken at different angles. Suitable γ-emitting radionuclides are quite easily available and with half-lives in the order of days allow on-site storage rather than requiring on-site production via a cyclotron, as is the case for PET imaging (see below). In order to allow the precise location of emitted photons, sophisticated collimators or pinholes are used in front of the SPECT camera to exclude all but those incident photons that travel in a straight line. The more restrictive the collimator the more precise the location can be detected, but equally the more γ photons will need to be accumulated for the image. The fact that it is possible to detect 10^{-14} mol of a label makes this one of the

most sensitive imaging techniques and with a resolution on the scale of millimetres, it is well suited to whole body imaging. Commonly used isotopes include pure gamma emitters such as ^{99m}Te (decay half-life = 6 h), ^{111}In (decay half-life = 2.8 days), ^{123}I (decay half-life = 13 h), ^{67}Ga (decay half-life = 78 h), or ^{125}I (decay half-life = 60 days) but also mixed β/γ emitters.

20.4.5.2 Positron Emission Tomography

Some radioisotopes also produce positrons on decay, i.e. the anti-matter counterpart of an electron, which will rapidly collide with surrounding electrons. This collision leads to the annihilation of the particles and the production of two 511 keV γ-ray photons that travel in opposite directions and can be detected simultaneously (coincident). A back triangulation along the line of sight allows calculations to pinpoint the location of the source in the body. A series of scans can thus be combined to allow tomographic construction of the 3D distribution of the radioisotope. The isotopes used in PET imaging typically have much shorter half-lives ^{18}F (decay half-life = 109 min) than those used for SPECT imaging. This allows the use of a higher dose as a rapid decay reduces the overall exposure, but this use of short half-life isotopes also limits the applicability of the technique for longer-term studies. The production of suitable isotopes typically requires significant infrastructure (e.g. a cyclotron). Some isotopes, in addition to the positrons, also emit γ-rays (^{68}Ga, decay half-life = 68 min), which can reduce the selectivity of co-incidence detection.

20.4.5.3 Radioisotope Imaging Agents

Commonly these radioisotopes are not used in their free form but are linked covalently, e.g. iodine is linked to the amino acid tyrosine. Alternatively compounds such as Te or In can be non-covalently bound by chelation, e.g. using 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA); chelators of this type provide multiple coordination sites in a ring form and can be covalently and non-covalently linked to other molecules or to nanoparticles.

A range of soft (liposomes, micelles) and dense (iron, gold) nanoparticles have been prepared for SPECT imaging (Psimadas et al. 2012) typically aimed at diagnostic use, e.g. for cancer diagnosis in conjunction with a targeting ligand. Other typical applications involve pre-clinical studies aimed at studying the mechanism and biodistribution of labelled nanoparticles (Areses et al. 2011). SPECT imaging is also used clinically to assess lung blood flow by using ^{99m}Te macroaggregated albumin with a particle size of 10–90 μm, as such particles are trapped in the lung. Non-obstructing alternatives based on cationic ^{99m}Te labelled nanoparticles are in development as an alternative (Lobov et al. 2013).

The use of PET in nanoparticle imaging applications follows similar lines of research, but labelling strategies tend to be more challenging. For example the non-covalent adsorption of phospholipid-PEG conjugates of the macrocyclic chelating agent DOTA to single-walled carbon nanotubes (SWNTs) has been reported, with

the nanotubes then being imaged using the positron emitting radionuclide ^{64}Cu (decay half-life = 12.7 h) (Liu et al. 2007). Rapid and convenient covalent-linking approaches with ^{124}I for nanoparticles targeted to the endothelial cells have potential advantages in terms of the stability of the conjugate (Simone et al. 2012). The use of click chemistry is another example of this type of rapid covalent linking; in this case using ^{18}F (Devaraj et al. 2009). In a direct comparison of PET and SPECT imaging in animal models the relative ease of labelling of particles using chelators (e.g. ^{99m}Te) rather than covalent linkage (e.g. ^{18}F) was considered particularly advantageous and both techniques led to comparable imaging results, despite the higher sensitivity of the PET system (Cheng et al. 2010).

SPECT and PET imaging approaches allow specific imaging of radioisotope localisation, but do not provide anatomical whole body information. A combination of these techniques with imaging modalities such as CT or MRI provides very useful information when imaging with both systems is co-registered, i.e. taken at the same time, so that detailed whole body anatomical information can be overlaid with the radionuclide distribution information.

20.4.6 Sound Wave-Based Imaging

20.4.6.1 Contrast Enhanced Ultrasound

Ultrasound is one of the most frequently applied imaging techniques, based on sound waves with frequencies in the range of MHz rather than the less than 20 kHz that the human ear can hear. The higher the frequency of the sound the shorter the wavelength; sound with shorter wavelength can resolve smaller structures, but with the increased scatter and deflection will not penetrate as deep into the body. A piezoelectric transducer that is in direct contact with the body produces the sound waves that can be focused to different depths. As they travel the sound waves interact with the body and each transition in density leads to a partial reflection of the sound wave (echo), which can be received and detected by the transducer. An image of the location of various sounds is reconstructed based on the time of travel, the position of the sound focus, and strength and frequency of the echo.

Contrast enhanced ultrasound (CEUS) takes advantage of the dramatic density change (and strong reflection) between gas and liquid phases, using stabilised gas bubbles as contrast agents and a number of agents based on this approach are in clinical application. In general these agents facilitate the detection and quantification of blood flow (Qin et al. 2009). These micro-bubbles typically contain a gas such as nitrogen or a typically liquid perfluorocarbon surrounded by a shell made from, e.g. albumin, phospholipids, or polymers with sizes in the micrometre range (Correas et al. 2001). Micro-bubbles that use ligands to target receptors and structures typically aim for intravascular targets (Caskey et al. 2011). Similar imaging agents with sizes in the nanometre range have been developed in order to be able to access extravascular targets. Perfluorocarbon-filled nanodroplets and emulsions are relatively less easy to detect in solution compared to after binding and accumulation

at a target (Dayton et al. 2006). Interestingly, ultrasound in combination with nanoparticles is also being been explored as a means to enhance delivery. Ultrasound-triggered destruction of micro-bubbles creates mechanical forces which can create transient disturbances in surrounding cells to enhance gene delivery (Watanabe et al. 2010).

20.4.6.2 Photoacoustic Imaging

While ultrasound images are based on the mechanical properties of tissues and particles, photoacoustic imaging (PAI) images the light absorption. PAI uses light waves that penetrate into and are absorbed by tissues to induce sound waves based on the photoacoustic effect (Wang and Hu 2012). The photoacoustic effect describes the photothermal property of materials, i.e. the thermoelastic expansion due to the heating effect of the absorbed light energy; when absorption occurs from a series of light pulses this expansion translates into pulsed pressure changes or waves which are transmitted through the tissue and which can be received using microphones or transducers. By using arrays of transducers in combination with a tomographic rendering of the signal, the origin of the pulse and thus of the light absorption can be imaged in three dimensions. The technique offers a number of advantages, in particular relating to resolution and depth of imaging. Optical microscopic techniques are severely limited by the light diffusion depth (~1 mm) at which multiple scattering of photons will make focusing impossible. Optical tomographic techniques (e.g. NIRF) can probe deeper into tissues (up to a depth of cm), but their resolution is much more limited (~1/3 of the depth) (Wang and Hu 2012). Sound waves scatter around three orders of magnitude less than light. By translating light absorption into sound, PAI signals can travel through tissues much more easily. The imaging depth and lateral resolution are inversely correlated (e.g. an imaging depth of 3 mm gives a lateral resolution of 45 μm and an imaging depth of 4 cm gives a lateral resolution of 560 μm).

The differential light absorption of biological molecules in tissues is used to create the image contrast. Typical endogenous molecules with strong absorption include haemoglobin, melanin, cytochromes, or carotenoids.

PAI typically strongly absorb light either due to the presence of dye molecules or due to surface plasmon effects (Yang et al. 2009). Ideally these are tuned such that they absorb light in the tissue's "optical window" range of 600–900 nm, where light penetration is maximal and adsorption minimal. Strongly light absorbing exogenous molecules and nanoparticles can be used as contrast or imaging agents. Suitable dyes include Evans blue, ICG, lymphazurin, and methylene blue. Nanoparticles such as those based on modified silica (ormosil) can encapsulate 23,000 dye molecules per particle (Yang et al. 2009). While free dyes have a very short half-life in the blood, encapsulation in nanoparticles can lead to a significantly prolonged circulation time. Surface plasmon-based light absorption is based on absorption of light by a metallic surface when the wavelength/frequency of the incident light matches the natural resonance frequency of the surface electrons. In nanoparticles this is

usually exploited as a local phenomenon (localised surface plasmon resonance, LSPR). The resonant light frequency and, consequently wavelength is highly dependent on the dimensions and properties of the surface layer which makes such particles highly tunable. The prototypical PAI nanoparticle thus is based on a dielectric core surrounded by a conductive nanometre thick shell, frequently gold. LSPR can also be exploited by particles made from only gold if the particle shape is "tuned." Specifically, high axial ratio gold nanoparticles (nanorods) can be made much smaller than typical nano shells (Yang et al. 2009). More complex shapes such as cages, stars, and multipods can be created to adjust these properties further (Nehl and Hafner 2008). Covalent modification of the gold nanoparticle surface allows the attachment of various ligands to allow particle targeting and even quantitative assessment of binding. As such antibody-targeted nanoparticles have been used for in vivo imaging and have given an output in in vivo situations similar in information depth to that obtained with histopathology techniques and without the need to extensive tissue preparation (Cook et al. 2013).

20.5 Multifunctional Nanoparticles

One of the key attractions of nanoparticles is the high degree of flexibility that they offer in terms of engineering, making them adaptable for various purposes. Changes to the particles may involve their size, surface properties, drug loading, and drug release characteristics. In addition nanoparticles are frequently engineered to change their biodistribution and achieve some form of targeting; for example, the targeting of tumours actively, with ligands, or passively, via the EPR effect (please see a further discussion of the EPR effect elsewhere in this volume). Consequently, many of the NPs developed as imaging agents can be modified to accommodate these drug carrying or targeting functionalities. One related active area of research is the development of theranostic nanoparticles, i.e. particles with dual diagnostic and therapeutic functionality (Mura and Couvreur 2012; Lammers et al. 2011). While we will not be reviewing these functional combinations in detail, we will briefly summarise the development of multimodal imaging agents, i.e. nanoparticles that may be used with more than one imaging modality.

One of the key motivations behind these developments stems from the fact that among the various in vivo imaging modalities there is not one that could serve as panacea for all potential imaging needs. Each modality has a unique combination of relevant properties including imaging depth, resolution, speed of acquisition, selectivity and specificity, price etc. with the top line data summarised in Table 20.1. Thus, there are always scientific or diagnostic questions that will need to be addressed with more than one technique.

A typical example for the combination of two imaging techniques in a single instrument is based on the use of imaging techniques useful for anatomical imaging such as CT or MRI with imaging modalities such as PET or SPECT which are most suitable for the sensitive detection of tracers, i.e. radionuclides. The combination of

these modalities facilitates co-registration of both types of imaging information, thus providing a detailed anatomical location information for the tracer signals (Delbeke et al. 2009).

Another important motivation for the use of multimodal imaging techniques relates to resolution and scale. For example, it may be desirable to detect distribution of a nanoparticle in the body by MRI and then to be able to also image the distribution either at higher resolution or in the context of other tissue. Clinically this could involve diagnostic imaging of tumour lesions in the whole body followed by imaging guided surgery, e.g. to help localise tumour margins or draining lymph nodes for resection. The NIH's molecular imaging database provides many examples of multimodal combinations (MICAD, Chopra et al. 2012). These include combinations of PET/SPECT radionuclide and fluorescently labelled nanoparticles, MRI (Gd^{3+}) and fluorescence labels, iron oxide in combination with fluorescence or radionuclide, combinations of optical and near-infrared fluorescent dyes, quantum dots with Gd^{3+} to name but a few.

In addition there are imaging agents, which lend themselves easily to use in more than one imaging modality without the need for extensive changes. Gold nanoparticles for example are being explored as IA that provide contrast in X-ray/CT, but their ability for local surface plasmon resonance means they also have useful properties in photoacoustic and optical applications (Kim et al. 2007; Nehl and Hafner 2008; Zhang et al. 2009).

20.6 Concluding Remarks

The dramatic increase in medical imaging capabilities since the discovery of X-rays is driven by the need for high-resolution detailed and quantifiable information about structures and processes in the body. The more imaging information becomes available the more obvious it becomes that the need for the kind of detailed real time information only available from imaging will continue to increase. Medical imaging is a central part of the biological revolution of healthcare that is currently unfolding. At the core of many of these developments are two insights:

1. Network pharmacology. Many disease pathologies do not depend on a single pathological trigger, but develop in the context of complex molecular pathways and networks; understanding of this rich biological context should drive the development of new therapies.
2. Personalised medicines. Inter-individual differences in the molecular drivers of disease define the appropriate therapeutic target for the individual. Understanding of these differences allows the stratification of patient populations so that medicines can be prescribed to those most likely to benefit in order to maximise therapeutic success and minimise side effects.

Medical imaging has become one of the most important tools that allow scientists and clinicians to obtain this type of information. Nanoparticles have attracted considerable attention as tools for medical imaging because of their specific

biological properties, e.g. their ability to accumulate in tumours based on the EPR effect, and because of the ease with which they can be engineered to fulfil multiple functions. This multifunctional capability is particularly relevant to the future development of medical imaging. Apart from allowing simultaneous use of multiple imaging modalities, e.g. to explore different imaging scales or to obtain multiple types of information from the same location, multifunctional capabilities will also be important to the development of theranostics, i.e. the combination of diagnostic and therapeutic in one procedure, the ability to continuously image as well as to monitor and measure dynamic changes in the body, e.g. using reactive probes. The drive to develop increasingly sophisticated multifunctional nanoparticles will need to be accompanied by similar efforts to further understand the underlying biological interactions between such carriers and the body as well as the need to be able to produce these novel nano-devices safely and economically.

Problem Box

Question I

Imaging modalities can be divided into those which work by modifying an incident signal and those that do not require an external source. For one of the nanoparticle imaging techniques discussed in this chapter briefly describe the nature of the signal detected and its functional relationship to external sources where applicable.

Answer 1

NIRF imaging depends on a source of external light to provide excitation photons that can transfer energy to electrons of the absorbing fluorophore. When the electrons return to the ground state, they emit energy in the forms of photons, typically at a longer wavelength.

MRI uses a strong external magnetic field to align the average magnetic moment of water protons and electromagnetic pulse sequences to expose them to a varying electromagnetic field at their resonant frequency to be absorbed. This results in flip of the spin of these protons and their spontaneous return to equilibrium is associated with a relaxation radiofrequency signal which is detected to produce the image.

Radionuclides: SPECT detects γ-rays produced from the decay of suitable radioisotopes and does not require any additional external energy. Similarly, PET imaging does not depend on an external signal, but relies on the production of two coincident γ-ray photons as the result of the annihilation of an electron and positron. The absorption of these γ-rays in the tissue is negligible.

CT uses tomographic techniques to create a 3D image of the relative absorption of X-rays in tissues. X-rays from an external source are absorbed differentially in tissues based on their properties and the X-ray energy. The

(continued)

Problem Box (continued)

imaging agent absorbs proportionally stronger and thus creates a differential contrast compared to neighbouring areas.

Ultrasound waves from an external source travel as pressure waves through the body. The attenuation of the signal is inversely proportionally to wavelength of the sound used favouring penetration of longer wavelengths. At the transition to tissues/media with a different density, the sound waves are partly reflected. It is this reflected signal that carries information in the form of time of travel, intensity, and frequency that can be used to reconstruct the image. In the case of PAI the detected signal is not based on the reflection of external sound, but on the thermal expansion of tissues due to the absorption of a pulse of light energy (photons).

Question 2

Give examples of biological properties of nanoparticles that are being exploited in medical imaging.

Answer 2

Nanoparticles have some typical properties that determine their biological fate which can be exploited in imaging applications. In contrast to small molecules imaging agents are not easily filtered by the kidney and tend to be retained in the circulation. However, depending on exact surface properties and size such particles have a tendency to be recognised by macrophage cells, in particular in the liver and spleen, but also at other sites of macrophage cell accumulation, e.g. inflammation or atherosclerotic plaques. When macrophage uptake is reduced, such particles can circulate for extended periods of time typically in the vascular bed, making them useful blood pool imaging agents. While particles typically would not be able to extravasate they can leave the vascular bed in tumours because of defects in the wall of these blood vessels. This can lead to the accumulation and targeting of particles in tumours. Other means of targeting which can be exploited rely on the presence of ligands such as antibodies with high affinity to receptors or other structures of biological relevance.

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Chapter 21
Nanotechnology-Based Therapeutic Product Development and Commercialisation

Gregor Cevc

Abstract The chapter describes briefly, but as much as is reasonably possible specifically, the key steps in therapeutic product development, starting with an invention and its patent protection and ending with the resulting product pricing and commercialisation. The chapter also addresses certain nanotechnology-specific aspects of such development, and draws attention to the importance of quality control and quality assurance in the process. Explaining the rationale for and current good practices in the tests conducted with animals and human test subjects leads to a concise description of the regulatory framework for developing and launching new (nano)therapeutic products. The chapter closes with some remarks on risk management and general recommendations.

21.1 Introduction

Neglecting the rare combination of stroke of genius and luck, every realised innovation results from farsightedness, hard work, and perseverance; and teamwork; and adequate funding; and experience; and … Such realisation also tolerates only a few, if any, shortcuts, yet typically follows a tortuous path. If the decision makers knew, and were willing to observe, some basic facts and rules, the path to success would be straighter.

Scientific logic defines the key facts about an innovative therapeutic product. The project development logic dictates some pertinent-related rules. Many other rules are just regulatory decrees, but accepted by many as laws. This may be convenient but is not always wise, as one can reinterpret and, with good arguments, change most man-made rules. It is equally unwise to follow (too) low regulatory

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standards, as growing ethical or public safety concerns are permanently raising the regulatory bar, which one should also be able to cross in the future; one should therefore always adhere to the highest actual and reasonable international standards.

In this brief chapter, I summarise some facts, procedures, as well as personal experiences that are useful for development of mammalian therapeutics. I differentiate between medical products and medical devices, where necessary, but deal with the nanotechnological products specifics in just one separate section, given that most guidance and rules apply to the traditional as well as to nanotechnological therapeutics.

21.2 From Scientific Finding to a Therapeutic Product Idea

One major attraction of science is its relative freedom: a scientist can follow her/his vision and even pursue whims ad libitum, sufficient funds provided. This notwithstanding, more and more scientists argue with and think about the potential applicability of their research findings. This can seduce a scientist to keep research findings secret for too long, aiming to secure intellectual protection before publication, which then becomes obsolete, or to start with applicative work too early, i.e. before having properly understood the new drug, procedure, or mechanism of action. Ultimately, one must pay for any such timing deficiency dearly. Poor timing can even destroy an interesting scientific lead or kill an otherwise viable project. To me, the only good justification for an *academic research* project is accumulation of knowledge and better understanding of natural laws and interactions. If a result of such endeavour turns out to be practically valuable, so much the better. It is naïve, however, to target an invention, as any new finding must be unexpected (i.e. non-obvious) to be patentable, and a non-patented invention has normally little *commercial* value. To make it defendable, one must moreover back the resulting invention-describing patent application by sufficiently unambiguous evidence of priority (most often in the form of a hardbound, paginated, and signed laboratory journal containing the claim supporting information that is, ideally, witnessed).

In turn, one should contemplate practical utility of any novel and non-obvious finding in due time. If a finding seems to be useful (and thus fulfils the third proviso for a new idea patentability, i.e. for the "freedom to file"), one should file at least a provisional application. It is essential to do this prior to disclosing the finding to any third party,[1] as a non-confidential disclosure brings the invention into public domain and thus makes it non-patentable!

A US provisional patent application is relatively inexpensive (the cost in 2013: 130 $US for a small entity, excluding any professional advisor fees). It secures the

[1] Notable exceptions are such third parties that have signed a non-disclosure agreement with the inventor *in spe*, and lawyers.

filing party's priority right for at least 12 months, if nobody else has filed an earlier related invention. During the year following the provisional patent application, its inventor can gather further data. Up to the day 365 after the filing, at latest, the inventor can then decide between letting the filing lapse and transforming it into an official patent application.[2] The latter must be enabling (i.e. contain enough information to allow "a person skilled in the art"[3] to reproduce all the described embodiments) but may make broader claims, supported by embodiments as well as inventor's reasonable extrapolations. A patent office does not require from an inventor to understand fully the mechanism of his claimed invention at its filing date.

Before investing much effort, or money, into commercial pursuit of an invention, an "inventor to become a business person" should always check the "freedom to operate" (Krattiger et al. 2007). Strictly speaking, this is a legal opinion by patent counsel on whether the making, using, selling, or importing of a specified product, in a given geographic market, at a given time, is free from the potential infringement of third-party intellectual property or tangible property rights. To start with, a savvy scientist can conduct a less formal, independent check, however, by searching at least one cumulative patents data bank (such as Google Patents, http://www.google.com/?tbm=pts) and at least a few pertinent commercial/company websites, by employing sensible keyword combinations and alternatives and then determine the number of hits. When dealing with a potential therapeutic product, the searcher should also check and quantify the major unmet medical needs in the field, which will also help her/him to assess the commercial opportunity associated with the new idea. The more general basis is knowing, and at least fundamentally understanding, the process of new drug development in the developed countries. Figure 21.1 illustrates this lengthy—and correspondingly costly—process in the USA, which resembles the related processes in most other developed countries.

21.3 Assessing Commercial Value of a New Therapeutic Concept

All good scientists wish and know how to gauge merits of a new scientific finding or concept. In contrast, only few really desire and are familiar with scrutinising its commercial value and the value determining factors. In the nutshell and in the simplest approximation, the commercial value of a new treatment for a medical condition correlates with the daily const of treatment × the average annual duration of

[2] The cost for filling a full fledged patent application with 50 pages, five independent and ten dependent claims was in the USA in 2012 around 1,518 $US; the corresponding cost of an international, PCT, application was 2,755 $US, in either case excluding lawyers and examination fees.

[3] The European patent office defines this as "a skilled practitioner in the relevant field of technology, who is possessed of average knowledge and ability and is aware of what was common general knowledge in the art at the relevant date".

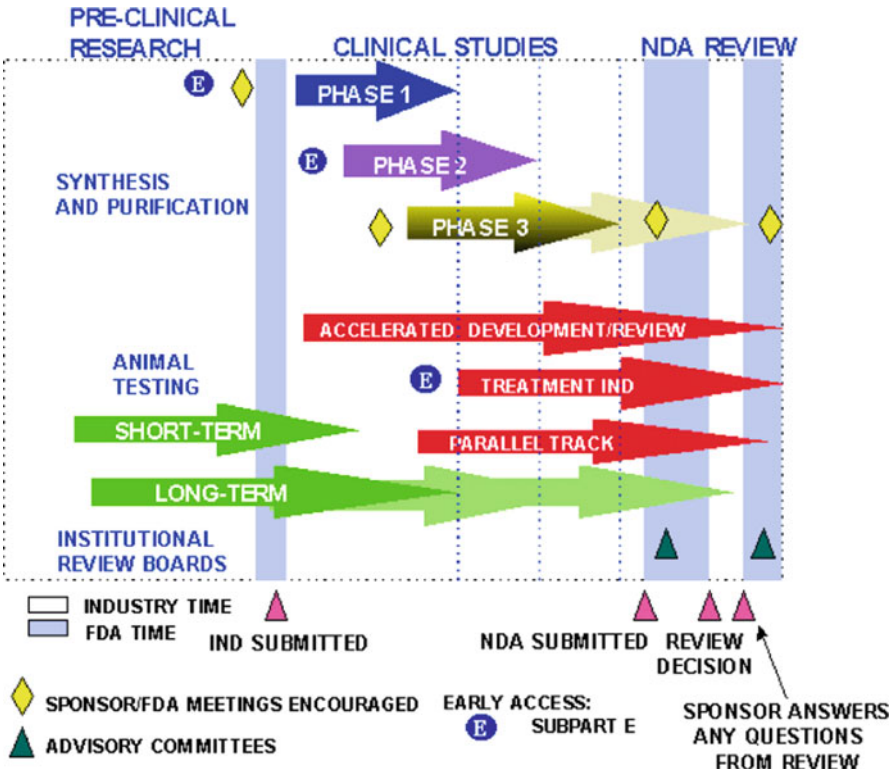

Fig. 21.1 Schematic representation of the new drug development process in the USA (from FDA 2013)

treatment × the number of patients treated, summed over all countries of interest.[4] Scientific publications and public data sources can normally provide the needed information (see e.g. various databanks of WHO, or OECD, EU (e.g. Eurostat/Eurobarometer), the USA (e.g. the US government or Centre for Disease Control and Prevention statistics, etc.) directly or indirectly, e.g. through a combination of prevalence and population data (see, e.g. the world fact book of CIA). The diversity or even inconsistency of, occasionally changing, printed or electronic data on health and treatment statistics makes the corresponding (re)search both time-consuming and cumbersome[5]; it may therefore be necessary to employ professional help for such (re)search.

[4] The latter number is proportional but not identical to the given condition prevalence, which is the proportion of the total number of cases to the total population, but often includes an estimated number of non-diagnosed cases.

[5] An official course on finding and using the US health statistics is available at http://www.nlm.nih.gov/nichsr/usestats/index.htm.

To estimate more quantitatively the net commercial value of a new finding,[6] and of the resulting therapeutic products, under zero risk assumption[7] one must furthermore factor-in at least the cost of development[8] and the time value of money, i.e. the annual discount rate. (Anyone familiar with the compounding effect of an annual interest rate will realise the importance of the latter, as the discount rate used in such calculations in the pharmaceutical industry is typically 9–18 %! This foretells halving of the future income value within 9–5.5 years, and makes any delay in a product development extremely costly, even after exclusion of the competition danger and of the limited duration of patent protection, which is presently 20 years post application filing).[9]

The situation is even more complex for a new platform technology, such as any new nanotechnological delivery method, that is useful for a number of targets (different drugs, various indications, etc.). In such a situation, one must compare, and at least semi-quantitatively prioritise, different options by considering not only the individual indication/market opportunity but also the overall cost and time for the corresponding therapeutic development, the competitive situation, the possible/tentative commercial partners for any contemplated product development, etc. A convenient tool for such comparative analysis is the four-square diagram, popular with consulting agencies.

21.4 Adopting Appropriate Laboratory, Manufacturing, and Testing Standards

Researchers value intuition highly and often resort to improvisation. In contrast, for developers' data reliability and documentation are high on the priorities list. In 1972, this prompted the introduction of good laboratory practice (GLP) that now

[6] The technical term for this is the Net Present Value (NPV), which can moreover be adjusted for risk.

[7] Zero risk assumption is ultimately not tenable and can be overcome by multiplying the risk-free NPV with the specific risk factor for the given product at the given development stage. Table 21.2 specifies the development risk typical of the latter for the clinical stage medicinal products.

[8] Most of the overall therapeutic development costs originate from clinical, especially from the large phases II and III, studies. With some experience, one can extrapolate the likely cost of the planned study by multiplying the cost per patient, which depends on the study design, with the scheduled number of patients in the study, which are derivable/estimatable from the published best practice/previous comparable approval studies.

[9] Product regulators can afford some extra protection time. Examples are the product-related data protection in EU (presently 10 years for an innovative EMA approved product, plus 2 years for a paediatric indication) and in the USA the corresponding Hatch/Waxman or New Drug Product Exclusivity under sections 505(c)(3)(E) and 505(j)(5)(F) of the Federal Food, Drug, and Cosmetic Act (presently 5 years for a new chemical entity (NCE) and for significant changes in already approved drug products, such as a new use). For a drug product that contains an active moiety that has been previously approved but was then approved again based on new clinical investigations (other than bioavailability studies) the Agency grants 3 years of extra exclusivity under sections 505(b)(1), 505(b)(2) of the Act.

represents the minimum standard expected by industrial partners and regulators to be met by any product developer and tester. In brief, OECD defines the GLP as "a quality system concerned with the organisational process and the conditions under which non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported" (OECD 1998, 2013). Regulatory agencies universally require performance of all key (such as toxicity) studies according to OECD/GLP protocols, and thus automatically exclude the results of the academic research that does not adhere to such protocols.

21.4.1 GLP Principles and Their Implementation

GLP specifically applies to: organisation and personnel, to regulate responsibilities of management, sponsor, study director, principal investigator(s), and study personnel; quality assurance (QA), to specify some general rules and QA personnel responsibilities and authority; facilities, including test-system facilities, facilities for handling test and reference items, archive facilities, and waste disposal; apparatus, material, and reagents; test-system, whether chemical, physical, or biological; test and reference items, most notably to assure proper receipt, handling, sampling and storage, as well as characterisation of such items; standard operating procedures (SOPs), which are documented procedures describing how to perform tests or activities normally not specified in detail in study plans or test guidelines; performance of a study, which deals with study plan as well as conduct; reporting of results; and storage and retention of records and materials.

GLP implementation starts with raising all participants' awareness for its importance and with appropriate and regular personnel training. For the purpose, SOPs must be in place for writing summaries of each involved person qualification and experience (typically in the form of a job description) as well as for their individual training plan and its record. Further SOPs relating to the test and reference substances must cover at least receipt, identification, labelling, handling, sampling, and storage of such substances. The SOPs relating to the measuring and environmental control apparati, at minimum, should regulate use, maintenance, cleaning, and calibration of any such equipment, whereas the reagents related SOPs should specify at least reagents preparation, record keeping, reporting, storage, and retrieval. Table 21.1 lists the basic GLP requirements applying to apparati, materials, and reagents.

Any research group striving to implement GLP at least partially will therefore have to review and typically reorganise/refurbish its facilities, for example, to separate physically the instruments and chemicals[10] used for product development from those

[10] GLP also requests receipt and storage of the test items and reference items in separate rooms and areas that are also different from the space for mixing of the test items with a vehicle, to prevent contamination or mix-ups.

Table 21.1 Some basic GLP requirements applying to apparati, materials, and reagents

(Qualified) apparatus	Material and reagents
Appropriate design	
Adequate capacity	
Suitable location	
Regular	Adequate labelling indicating
Inspection	Source
Cleaning	Identity (incl. concentration, if appropriate)
Calibration	Receipt or preparation date
Maintenance	Expiry date[a]
	Storage instructions/stability
Not interference	
Appropriate label	
Acceptable back-up	
Complete information management	

[a]The expiry date may be extended on the basis of documented evaluation or analysis

used for research. The group will have to assure that only qualified apparati[11] and validated[12] as well as suitable[13] test procedures are being used for the designated therapeutic product characterisation; it will have appropriately to re-educate and begin regularly and differently to supervise its personnel and documenting practices.

I am listing just a few, mundane, examples. The path to GLP is still long, if one does not *routinely* clean and *calibrate* pipettes, pH electrodes, and other sensors, routinely measure reference test spectra and reconfirm the sensitivity of a spectrometer, etc., routinely prove absence of germs in a biolaboratory and if one has not proven

[11] An analytical instrument (or apparatus) qualification (AIQ) is the collection of documented evidence that an apparatus performs suitably for its intended purpose. It is typically performed by the apparatus manufacturer and must be repeated partially (→ Change Control) after any moving of the apparatus to a new location, even within the same room. More generally, AIQ comprises design qualification, installation qualification, operational qualification, and performance qualification, the last of which must be repeated periodically. If an apparatus relies on software, one must also validate the latter (and, in case, the network of which the software is a part). Changes to instruments, including software, or defects correction are subject to Change Control, a formal process ensuring that changes of/to an apparatus are introduced in a controlled and coordinated manner that does not adversely affect the results of measurements with the apparatus. It is also necessary to regularly inspect, clean, calibrate, and generally well maintain any apparatus used in the GLP setting.

[12] Test validation is the collection of documented evidence that a test procedure is suitable for its intended use. It involves assessment of accuracy, precision, specificity, detection and quantification limits, linearity, range, and robustness. Regulators accept only results of the fully validated tests and measurements in the registration relevant (i.e. typically Ph III and toxicity) data sets. Prevalidated tests are acceptable for Ph I/II studies, however.

[13] Before actually starting to use certain test or method, one must confirm, by conducting a method suitability test, that the product does not obstruct the method and that the method is working for the product.

that the cleaned lab ware is really free of the residual material, routinely confirm identity of each used compound or reagent (with the specified receipt and expiration datum!) and also account for each single withdrawal of material from its store, etc.

Full GLP implementation and maintenance thus crucially depend on the existence of, and cooperation between, both quality assurance (QA; OECD 1999) and quality control (QC). The former focuses on planning and documenting the quality assuring processes, such as quality plans and inspection as well as test plans; it is essentially a system for evaluating performance, service, or the quality of a product against a system, standard, or specified requirement. Quality control, in contrast, focuses on the actual verification of the products conformity with such plans and their targets, via inspections, measurements, etc.; it is essentially the process involved within the system to ensure job management, competence and performance during the manufacturing of the product, or service to ensure it meets the quality plan as designed.

The scope and plurality of GLP goals suggests that establishing and maintaining facilities and operations compliant with GLP principles does not come for free. Indeed, laboratory personnel must typically spend some 15–25 % of their working time to fulfil the GLP requirements, the efforts associated with implementation of new test and activities excluding. This makes full GLP implementation and academic research nearly incompatible. It may therefore be advisable, or even necessary, to outsource certain quality confirming tests and activities from an academic originator group to specialised, and audited, laboratories in due time. In any case, people trained in GLP as well as people who must assure that GLP principles are adequately applied should be engaged in any therapeutic product development.

21.4.2 Good Manufacturing Practice

Good manufacturing practice (GMP) should also help ensure a quality product and is a collection of general principles applicable during product manufacturing and the associated testing (EudraLex 2013; WHO 2013), rather than a set of binding prescriptions or manufacturing instructions.[14] All national and international GMP regulations require at least that any manufacturing process be conducted exclusively by skilled and properly supervised personnel, following clearly defined, prescribed (in the form of SOPs), justified, controlled, and recorded manufacturing routine in which any change/deviation is properly evaluated/scrutinised, as well as notified. The manufacturing records thus allow permanent, complete, and unambiguous back tracing of all steps and procedures used to make and characterise a product (related to the traceability of all materials under GLP). The routinely updated, i.e. current, GMP moreover regulates product recalls and proper examination of complaints about marketed drugs.

[14] Since 1999, the International Conference on Harmonisation (ICH) issues GMP recommendations for Active Pharmaceutical Ingredients that by now apply in the countries signatories to ICH (the EU, Japan, and the USA) as well in some other countries (e.g. in Australia, Canada, Singapore) that adopt ICH guidelines for the manufacture and testing of active raw materials.

21.5 Special Considerations Pertaining to the Nanotechnological Products

Each nanomaterial may have its specific characteristics and pose its particular challenges. This notwithstanding, one can characterise and test most nanomaterials with the existing test methods and instruments, but not with each of them. In some cases, one must adapt sample preparation and measuring conditions; in other cases one must select one out of several generally available methods as the most, if not the only, suitable one for certain kind of characterisation.[15] In the selection process, one must bear in mind the non-linear dependence, for example, of absorbance (e.g. cave: heating!), emission (e.g. cave: fluorescence enhancement *or* quenching (Kang et al. 2011)), and/or reactivity of particles (Berman 2009) on the average size in nano-range and on particles concentration.

Owing to its focus on small size, the development of each nanotechnological therapeutic product must involve some kind of size determination, e.g. based on the dynamic or static light scattering and possibly computer-assisted electron microscopy. Alternative methods are available, however (Anderson et al. 2013). (Owing to the shape effect on nanoparticles bio-processing (Champion et al. 2007; Caldorera-Moore et al. 2010; Decuzzi et al. 2010), knowing just the average diameter may be insufficient. Solid nanoparticles are in this respect more problematic than the soft and/or deformable small entities). If any other nanotechnological product feature[16] is of the essence, testing of any such specific feature must become a part of the characterisation armamentarium.

Ensuring and checking suitability and reproducibility of the given drug release from a nanotechnological product is another essential component of such product characterisation. The release / underlying molecular process details gain importance with diminishing particle size and complexity. Often, if not always, one must therefore modify the drug release measurements used for characterising conventional products if one deals with a nanotechnological product. When using a classical dissolution vessel supplemented with a membraneous compartment filled with the tested formulation, for example, one needs a set of at least five to seven differential equations (Vierl and Cevc, to be published) to allow for the major factors, at least, that can affect a drug release kinetics from the tested single-shell nanocapsule / unilamellar bilayer vesicle. Relying for the purpose on a simple logarithmic or square-root time-plot of such system data can consequently lead to utterly misleading conclusions.

In light of the ongoing, and sometimes justified, concerns about the potential biotoxicity of some nanoparticles, animal tolerance studies will remain a constant of any nanotechnological product development, at least for a while. Interpretations of such study results must bear in mind that the frequency of blood passages through an organ increases with decreasing body weight of the involved animal species (being around

[15] Observing GLP principles automatically ensures an appropriate selection, as it requires confirmation of any experimental method *suitability* for the given tests set or purpose.

[16] Possible examples include, but are not limited to, binding to certain receptor, special mechanical or electromagnetic properties, etc.

50 times higher in murine than in human body, for example). One should therefore not forget the difference that is practically more consequential for particles than for the smaller molecules, which have generally bigger biodistribution volume in vivo.

OECD reviews the existing methodologies from time to time to identify and implement the necessary changes needed for their application to nanomaterials. Before deciding on which methods to use for the given nanomaterial or nanotechnological therapeutic product, its developers should therefore check the state of the art and pertinent guidelines. This should include safety and biotolerance limits.

21.6 Designing and Conducting Studies with Animals and Humans

To ensure safety and efficacy of the newly developed compounds, each such compound or each known compound tested for a new indication must undergo rigorous, placebo-controlled animal and clinical studies. Standing out amongst the former are animal toxicity tests, which must involve at least two animal species (typically rats + a larger mammal) and cover time spans corresponding to the anticipated duration of the tested therapeutic use (for chronic use: 2 years).

Good Clinical Practice. All human tests are subject to good clinical practice guidelines, which in their always current form (cGCP) summarise the international quality rules provided by ICH, an international body that defines standards, which governments can transpose into regulations for clinical trials involving human subjects (ICH 2013). cGCP helps to standardise the conduct of clinical trials, and defines the roles and responsibilities of clinical trial sponsors, clinical research investigators, and monitors.[17] cGCP should also protect the studied subjects' human rights in clinical trials.

A set of underlying SOPs must back any preclinical or clinical study to comply with GLP, GMP, and GCP. At minimum, the corresponding test-system related SOPs must regulate preparation of room and environmental room conditions for the test-system, procedures for receipt, transfer, proper placement, characterisation, identification, and care of the test-system. An addition, SOPs should regulate test-system preparation and any examination and observation before, during, and at the end of a study. It is also necessary to specify in appropriate SOPs how to deal with the test-system individuals who became sick or died during the study, and how to collect, identify, and handle any specimen including necropsy and histopathology. Overarching are the SOPs regulating quality assurance procedures operation of quality assurance personnel in performing and reporting study audits, inspections, and final study report reviews.

21.6.1 Study Plan

In short, a preclinical/clinical study is a carefully and ethically designed experiment to answer a precisely framed question having scientific/therapeutic relevance. Solid

[17] The term 'Monitor' is equivalent to 'Clinical Research Associate'.

scientific evidence and/or a clear study hypothesis co-dictate the proposed study (the Study) design, to be described (together with all other relevant details) sufficiently clearly in the Study specific plan—fully compliant with the current GLP, GMP, and GCP principles. The Study plan[18] must thus, inter alia, specify the Study purpose (including the primary and, in case, secondary objectives), the Study design and population (including the inclusion and exclusion criteria, as well as the statistically justified number of test subjects), the Study medication and subject treatments (including justification of the dosage regimen and treatment period, as well as packaging and labelling plus storage information, etc.), list the Study visits (including a chart of trial procedures), describe administrative procedures and safety monitoring, and provide plans for data review, database management, and final data analysis. A qualified professional should at least review and approve any Study plan not authored by an experienced person.

21.6.2 Study Conduct

Before the Study start, appropriate SOPs must specify the Study coding, data collection, indexing and handling systems (including use of any electronic media and storage), as well as reports preparation. Representatives of the carefully selected Study centres (ideally, investigators planned to examine not less than ~12 and not more than ~32 test subjects each) and all other relevant Study participants (e.g. monitors, team members) should be well educated about the Study goals and motivated to reach them. Good Study plan, tight but not narrow-minded project management, proactivity combined with hands-on approach all contribute to the Study final success. In addition to the Study monitors, other experienced team members should frequently visit the Study centres to gain personal impressions about the Study progress, the test subjects' feedback, and any potential issues (which, if emerging, should be dealt with as fast as is practically possible). Likewise, experienced and proactive data managers should be involved in the study conduct at all times and, most important, after the data-bank closure and before the final statistical analysis of the Study data. The allowed statistical pre-tests diminish the danger of a preventable failure of the Study.

21.6.3 Preclinical to Clinical Phase III Studies Cost and Success Probability

Each test has certain failure probability, but the likelihood of therapeutic product failure is unusually high. DiMasi et al. (2010) recently analysed both public and private data sources to estimate clinical phase transition and clinical approval probabilities for drugs in the development pipelines of the 50 largest pharmaceutical firms (by sales) from the time point at which they first entered clinical testing

[18] The Study plan is also called the Clinical Study Protocol.

Table 21.2 Risks associated with the clinical development of human therapeutic products

Development step/ clinical study phase	Small/NCE %	Cummulated %	Large/NBE %	Cummulated %
Preclinical → Phase 1	100	100	100	100
Phase 1 → Phase 2	*71*; 63	*71*; 63	*84*; 84	*84*; 84
Phase 2 → Phase 3	*44*; 38	*31*; 24	*56*; 53	*47*; 45
Phase 3 → NDA/BLA	*69*; 61	*22*; 15	*64*; 74	*30*; 33
NDA/BLA → Appr.	91	13	96	32

Based on the data of DiMasi et al. (2003) and DiMasi and Grabowski (2007) or DiMasi et al. (2010). Preclinical → Phase 1 in this table assumed 100 %

(1993–2004) through June 2009. Excluding the preclinical failure rate of >99.5 % (Preziosi 2004), this revealed that the US clinical approval success rate was 16 % for self-originated drugs during both the 1993–1998 and the 1999–2004 subperiods. For all compounds (including licenced-in and licenced-out drugs in addition to self-originated drugs), the clinical approval success rate for the entire study period was slightly higher, 19 % (being 32 % for the large and 13 % for the small molecules), which is consistent with the decades long, historical 13–20 % success range. For more detail, see Table 21.2.

The same research group earlier estimated the overall cost of preclinical (i.e. in vitro and animal) tests to contribute some 30 % to the total therapeutic development cost.[19] This includes all failed developments, however, which never got into a human. Considering merely the preclinical cost of the finally successful product developments, this percentage would be much lower. There is no doubt, however, that the overall cost of the later stage clinical development is absolutely and relatively higher: the Ph 2/Ph 1 cost ratio is ~1.5–1.9 and the Ph 3/Ph 2 cost ratio is around 6 (DiMasi et al. 2003). The larger number of test subjects and the more extensive investigations in later clinical stages of development explain the difference. Subsequent, independent, analysis of the time and cost of biopharmaceutical therapeutics development made clear that new biochemical entities (NBEs) pass through the clinical development stage no faster than NCEs and have similar cost/success probability ratio (see Table 21.3).

21.7 Regulatory Framework for Developing and Launching a New (Nano)Therapeutic

Laws strictly regulate the use of any therapeutic product on vertebrate animals: one must not test any substance, formulation, or a new procedure on vertebrae without official permission. Successful tests are a prerequisite for any new therapeutic

[19] The average time from synthesis of a compound to initial human testing is according to DiMasi et al. 2003 for self-originated drugs 52 months. The corresponding analysis of clinical phase lengths and phase gaps and overlaps indicates a period of around 70 months for the overall duration of clinical development.

Table 21.3 Average phase times and clinical period capitalised costs for, preclinically successful, investigational compounds of different types (new chemical entities, NCEs; new biological entities, large molecules, NBEs)

Testing phase	Mean			Phase cost, capitalised (M$US)				
NCEs[a]	Phase length	*t* to next phase	Success probability	Capitalised 2000		*SD*	Capitalised 2013	
	Months	Months	%	Mean	Expected	%	Mean	Expected
Long-term animal	*36.5*	33.8	31.4	9.5	3	*92*	29.1	9.2
Phase I	21.6	12.3	100	30.5	30.5	*84*	93.5	93.5
Phase II	25.7	26	71.0	41.6	29.5	*94*	127.5	90.4
Phase III	30.5	33.8	31.4	119.2	37.4	*70*	365.4	114.7
Approval	18.2[b]		21.5[b]					
Total	**96.0**			**201**	**100**		**616**	**308**
NBEs[b]				Capitalised **2005**			Capitalised **2013**	
Preclinical	*52.0*		100	185.6	185.62		369.9	369.9
Phase I	19.5		100	71.8	71.78		143.0	143.0
Phase II	29.3		83.7	67.3	56.32		134.1	112.2
Phase III	32.9		47.1	129.4	60.98		257.8	121.5
Approval	16.0		36.1					
Total	**97.7**			**454**	**375**		**905**	**747**
NBEs/NCEs	*1.0*		*1.7*	*2.4*	*3.8*		*1.5*	*2.5*

[a]Based on the data of DiMasi et al. (2003)
[b]Based on the data of DiMasi and Grabowski (2007)
Extrapolation to year 2013 presuming 9 % annual cost increase

product commercialisation, however. In preclinical experiments, an innovator should therefore check whether: first, an investigational drug, formulation, or procedure is sufficiently bioactive to justify further tests; second, the product is safe enough for initial use in humans. With viable candidates for further development, the sponsoring innovator then should focus on collecting the data and information necessary to establish that the product will not expose humans to unreasonable risks when used in limited, early-stage (i.e. phase I) clinical studies. Subsequent clinical studies (phases II to IV) then address efficacy (as a function of the applied product dose) in a progressively broader (and typically more international) target patient population.

21.7.1 Animal Studies

Any official European body authorised to issue a licence for animal testing (typically an Ethical Review Committee) requests a priori and adequate information about reasons for conducting the proposed test. The Committee also wants to learn about any relevant prior art, the detailed experimental plan (including statistical justification), etc. The person responsible for the planned tests must therefore submit all such information to the Committee in a written application document. The ensuing discussion can lead to the proposed experimental protocol revision and refinement. Based on an issued licence for animal testing, properly trained and

supervised personnel in the pre-specified and certified facility may do the proposed experiments, within certain periods, subject to recording any animal manipulation within the period minutely and in an auditable form. In the USA, the FDA has similar rules (in 21CFR58) for GLP relating to preclinical tests with animals needed prior to starting any clinical research in humans.

21.7.2 Human Studies

Clinical studies of any phase (see Sect. 21.6) also require pre-approval by an independent ethics committee (also called Institutional Review Board (IRB)). The Board is responsible for protecting the study subjects from any potential psychological or physical harm, and therefore routinely analyses the study protocols from the risk–benefit perspective. The Board may, and often does, require modifications of the proposed study (the "Study") prior to approval, or disapproves it.

In Europe, the Board bases its decisions on the proposed Clinical Study Protocol and the corresponding Investigational Medicinal Product Dossier (IMPD). In the USA, a project-specific working group of the Food and Drug Administration (FDA) scrutinises the regularly updated collection of all relevant documents in the Investigational New Drug (IND) Application (INDA)[20] or the Biologic License Application (BLA, used for biologicals).[21] Numerous non-EU/non-US countries accept either IMPD or IND as a basis for conducting clinical studies in their territory.

The study sponsor must prepare and submit either IMPD or IND to the pertinent authority, which then considers all the scientific, ethical, and regulatory aspects of the Investigational Medicinal Product (IMP)[2] or Investigational Medicinal Drug (IND)[22] relevant for the proposed study (the "Dossier").

The IMPD includes summaries of information related to the quality, manufacture and control of the Product, and data from non-clinical studies and from its clinical use. The Dossier must assess the overall risk–benefit ratio and critically analyse the non-clinical and clinical data in relation to the potential risks and benefits of the Study. (A simplified IMPD can suffice, if clinical studies with the Product have already been approved, or else if the Product has already been authorised as a medicinal product, in one of the EU Member States.) A valid Clinical Study Protocol (cf. Sect. 21.6.1) must back any clinical study with an IMP.

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[20] Under the USA law, a drug may be transported or distributed across state lines only with an approved marketing application. The IND is the means through which the sponsor obtains an exemption from this law.

[21] Biological products are approved for marketing under the provisions of the Public Health Service (PHS) Act. The Act requires a firm who manufactures a biologic for sale in interstate commerce to hold a licence for the product. A biologics licence application is a submission that contains specific information on the manufacturing processes, chemistry, pharmacology, clinical pharmacology, and the medical affects of the biologic product.

[22] In this chapter, I collectively use the term "Product" for both IMP and IND.

The INDA provides information in three broad areas: (a) animal pharmacology and toxicology,[23] (b) manufacturing,[24] and (c) clinical protocols and investigator qualification and obligations.[25] In an INDA, the sponsor also commits itself to obtain informed consent from the research subjects, to obtain review of the study by an IRB, and to adhere to the IND regulations. The submitted IND can fall in two categories (commercial and research/non-commercial) and be of one of the three possible IND types: (a) treatment IND,[26] (b) an investigator IND,[27] and (c) an emergency use IND.[28]

Following an IND submission, the sponsor must wait 30 calendar days before initiating any clinical trial. (During the waiting period, FDA can review the IND for safety, to preclude exposure of the research subjects to unreasonable risk, in a process illustrated in Fig. 21.2.) The sponsor must generally conduct all clinical studies according to the current Good Clinical Practice (cGCP).

One can, and ideally should, discuss all important planned clinical study protocols with the national and/or international regulating bodies (e.g. EMA, FDA). In the USA, this can lead to a Special Protocol Assessment (SPA), i.e. to a (non-binding) declaration from the FDA that a proposed Phase III clinical study design, clinical endpoints, and statistical analyses are acceptable for FDA approval.

21.7.3 Marketing Authorisation

Marketing a medicinal product or a medical device generally requires some form of authorisation by a competent regulatory body, which in the western world starts with submission of the Common Technical Document (CTD, Molzon 2003),

[23] Preclinical data that permit an assessment of the Product likely safety for initial testing in humans, including any previous experience with the drug in humans and not excluding foreign use.

[24] This means esp. information pertaining to the composition, manufacturer, stability, and controls used for manufacturing the drug substance and the drug product. The U.S. FDA assesses this information to ensure that the sponsor can guarantee adequate production of the Product and supply consistent batches of the drug.

[25] Detailed protocols for proposed clinical studies allowing an assessment of whether the initial-phase trials will expose subjects to unnecessary risks. The clinical part of IND must also provide information on the qualifications of clinical investigators overseeing the Product administration allowing the FDA to assess whether they are qualified to fulfil their clinical trial duties.

[26] One submits such an IND for promising experimental drugs in clinical testing for serious or immediately life-threatening conditions while the final clinical work is conducted and the FDA review takes place.

[27] A physician who both initiates and conducts an investigation, and under whose immediate direction the investigational drug is administered or dispensed, is entitled to submit such an IND, for example, with the intent to study an unapproved drug, or an approved product for a new indication or in a new patient population.

[28] Such IND allows the FDA to authorise use of an experimental drug in an emergency that does not allow time for submission of a regular, treatment IND. An emergency IND is also useful for patients who do not meet the criteria of an existing study protocol, or if an approved study protocol does not exist.

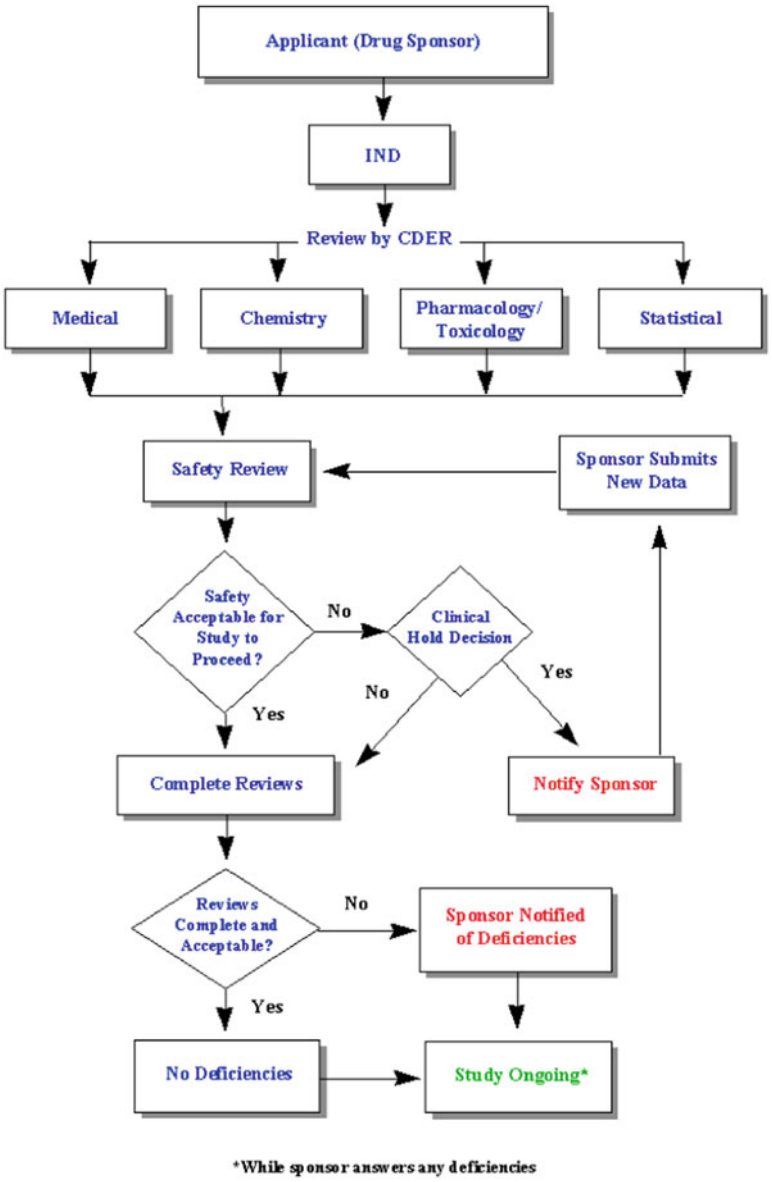

Fig. 21.2 IND submission and review process by the US Food and Drug Agency (from FDA CDER Handbook: New Drug Development and Review Process 2013)

designed by ICH, plus a region-specific module 1. Figure 21.3 schematically illustrates the corresponding document.

For a European medicinal product, the submission can result in a national marketing authorisation or a European marketing authorisation. For a medical device,

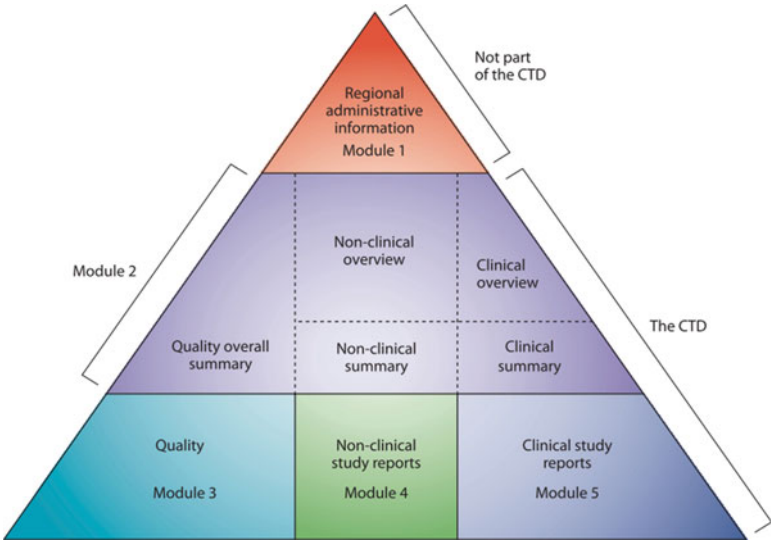

Fig. 21.3 The Common Technical Document (CTD) *triangle*, which illustrates the document structure and content (from Molzon 2003). International Conference on Harmonisation; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/CTD/CTD_triangle.pdf

a single CE marking is acceptable for the entire EU, Iceland, Liechtenstein, Norway, and Switzerland. Some countries in the Middle East, North Africa, Asia-Pacific, and parts of Latin America also recognise and accept CE marking.

To obtain a national marketing authorisation, one can use the national procedure and the mutual recognition procedures. Pursuing the national procedure with a national authority [such as the Budesinstitut für Arzneimittel und Medizenprodukte (BfArM), Medicines Evaluation Board (MEB), Medicines and Healthcare Products Regulatory Agency (MHRA), or Medical Products Agency (MPA)] opens only the respective national market to the medicinal product. Following a mutual recognition procedure,[29] in contrast, allows placing the medicinal product on any or all of the European countries market.

Applicants wishing to obtain a pan-European marketing authorisation from the European Commission must follow the centralised procedure, coordinated by EMA, and submit a Community Application for Marketing Authorisation (CAMA, see further text). In the ensuing process, scientific consultations between the member states take place in the European Committee for Medicinal Products for Human Use (CHMP).

[29] This includes the "Decentralised procedure" and the "Mutual recognition procedure" and relies on the principle that member states recognise marketing authorisations issued in another (EU) member state. The assessment report of the country that had granted the first marketing authorisation for the medicinal product in question is thus made available to the other member states. Mutual recognition procedures are coordinated by the Coordination group for Mutual recognition and Decentralised procedures (CMD(h)).

All CHMP members can in turn assess new medicinal products, acting as a (co-) rapporteur for the Committee. The CHMP finally submits a consolidated opinion to the European Commission, which in the case of a positive outcome grants a marketing authorisation for the Product valid in all EU member states.

EMA (and its rapporteur) and/or most national regulators are open to discuss an uncompleted regulatory procedure and/or dossier, i.e. are willing to offer scientific advice prior to a submission. To gain a marketing authorisation, a company must submit a dossier in certain number of printed copies (and in electronic form) for evaluation of the underlying medicinal product.

The European dossier needs to meet current requirements concerning content and layout. It presently consists of five modules: nr. 1 containing administrative data, including the Summary of Product Characteristics (SPC), the package leaflet and the labelling text, thus providing the most important scientific data about the medicinal product to doctors and pharmacists; nr. 2 including the summaries of the chemical–pharmaceutical, pharmacological–toxicological and the clinical–pharmacological dossiers; nr. 3 consisting of the chemical–pharmaceutical data, i.e. all data about the composition and preparation, as well as quality control of a medicinal product; nr. 4 comprise pharmacological toxicological data, including all animal experiments results relating to toxicity and the mechanism of action of a medicinal product; nr. 5 presenting all the available clinical–pharmacological data, most notably the results from (normally)[30] at least two independent, placebo-controlled, phase III studies.

The US New Drug Application (NDA) resembles, but is not identical to, the European CAMA dossier.[31] Most notably, the U.S. FDA additionally wants to receive the complete set of clinical data in the prescribed electronic form, for independent statistical analysis by the Agency. Figure 21.4 illustrates NDA submission and the ensuing review process.

The goals of the NDA (including the corresponding, regularly updated, IND) are to provide enough information to permit FDA reviewers to reach the following key decisions: (a) is the drug safe and effective in its proposed use(s), and the benefits of the drug outweigh the risks; (b) is the drug's proposed labelling (package insert) appropriate, and what it should contain; (c) are the methods used in manufacturing the drug and the controls used to maintain the drug's quality adequate to preserve the drug's identity, strength, quality, and purity.

The EMA has well defined and relatively short (210 days) time lines for processing an application for gaining the European marketing authorisation, the time needed by the applicant to respond to the agency's queries excluding. FDA has partially longer proposed time lines. However, according to a recent study (Downing et al. 2012), the average time taken by the FDA to approve a drug product was 322 days,

[30] In Europe, one can exceptionally submit a dossier with (strong) positive clinical results from just one study for a new medicinal product with a well known drug (already approved in a different formulation or for a different indication). One positive clinical study generally suffices to support CE marking of a medical device, however.

[31] Rules and procedures used by the Health Canada (HC) resemble more closely FDA than EMA practices.

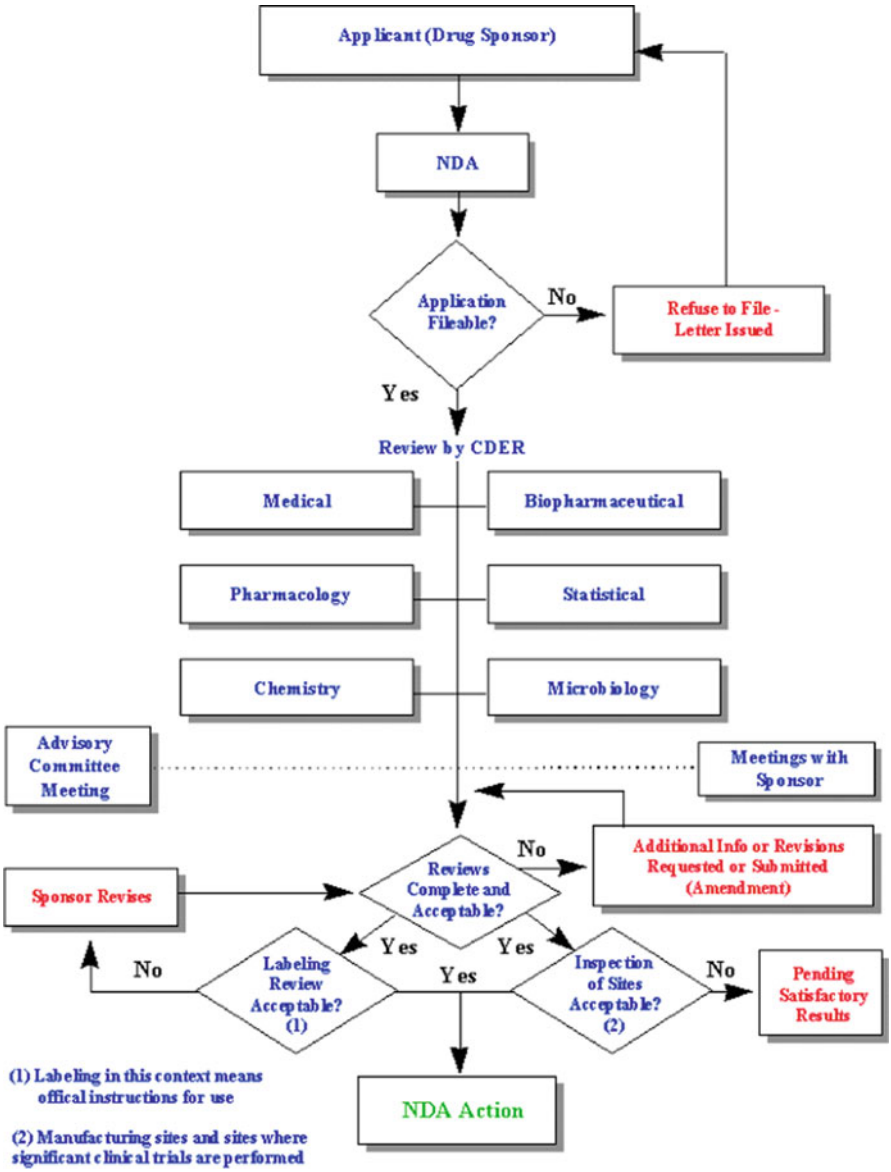

Fig. 21.4 NDA submission and review process by the US Food and Drug Agency (from FDA CDER Handbook: New Drug Development and Review Process 2013)

while EMA took 366 days and Health Canada needed 409 days. FDA also appears to have the lowest threshold, and potentially approves a drug for sale faster than any other Western World regulator. (Novartis' anti-cancer drug Gleevec, for example, obtained marketing authorisation from FDA in less than 80 days.) In turn, FDA can also be the slowest product approver: some of its reviews took up to 4 years.

21.8 Pricing and Reimbursement

For a long time, the originator of a newly registered, innovative therapeutic product was free to set the product price at its reasonable will; the treated patient's insurers then paid the price for the product. Increasing cost of health care and diseases has brought health economics more into the focus of policy makers, and prompted the introduction of cost-checking institutions, such as the UK National Institute for Health and Clinical Excellence (NICE), the Institute for Quality and Efficiency in Healthcare (IQWiG, Germany), Haute Autorité de Santé (HAS, France), etc., which are all expected to determine "community effectiveness" of any new drug and more generally to diminish health care cost. An even more common cost-decreasing measure is price tagging, i.e. setting the price for a product in one country at the lowest (or around the lowest two) price level(s) found elsewhere. It is also no longer certain that the public health insurer will reimburse the cost of any new therapeutic drug. To qualify for a reimbursement, the product developer must convince the price governing bodies that the product cost–efficiency and cost–benefit ratios are acceptable. This created an unprecedented barrier to new therapeutic products entry into a market, by posing the question of the product's *effectiveness* after the relevant public regulator (EMA, FDA, …) had already judged positively about the product safety and *efficacy*.

To face, and ideally overcome, challenges to a new product price, the product developer can prepare a pharmacoeconomic dossier (Osterhaus and Townsend 2004).[32] Such dossier reviews the condition(s) to be treated, describes the currently prevailing treatments and their risks, and analyses the total related costs and benefits.[33] Positive or negative changes in the treated patients' quality of life are important factors in any health economic evaluation too.

Health economic evaluation is the comparative analysis of alternative courses of action in terms of their costs *and* health consequences. Such evaluation should thus answer the key question: "Is the new therapeutic product worth using—cost-effective = efficient—compared with the other things doable with the same money?" The new drug is cost efficient if its cost plus the average other treatment-associated (direct and indirect) costs are lower than the total cost of the current best treatment.

A thorough health economic evaluation moreover weighs the net cost of new treatment against its overall effects. (Does the new therapeutic product reach more responders or ensures longer therapeutic response; is the new therapeutic product less toxic; does it increase survival or quality of life compared to previous treatments; etc.?)

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[32] Pharmacoeconomic research is the process of identifying, measuring, and comparing the costs, risks, and benefits of programmes, services, or therapies and determining which alternative produces the best health outcome for the resource invested (Trask 2011).

[33] A health economic evaluation normally includes direct medical costs (esp. cost of medications, supplies, laboratory tests, healthcare professionals' time, or hospitalisation) and direct nonmedical costs (e.g. for transportation, food, family care, home aides), indirect costs (such as lost wages due to morbidity), or income forgone (owing to premature death), intangible costs (such as pain and suffering, inconvenience, grief), and opportunity costs (such as lost opportunity, forgone revenue) (Trask 2011).

"Quality of life" quantification relies on calculation of QALY = "Quality Adjusted Life Years", and then compares (e.g. in a four-square diagram) "Total treatment cost" with "Total health effect", expressed in terms of QALY. Either the data necessary for QALY determination can originate from phase III studies (with the Study Plan supplemented with the appropriate questionnaire) or from phase IV studies (which better mimic realistic environments). Retrospective data analysis is possible, but can be burdened with the selection bias that needs to be compensated/overcome by using a suitable decision tree model, e.g. of Markov type. It is important, however, to validate any model and thus to make it credible and reliable. Sensitivity analysis is an essential part of any validation.

21.9 Risk Minimisation

In the simplest version, risk management is comprised of risk assessment, business impact analysis, hazard prevention, and deterrence plus risk mitigation. If well conducted it is transparent, dynamic, iterative, and responsive to changes. In therapeutic products development risk management is of the essence, owing to extensive regulations likely to cause problems, to the high development cost and to low overall success probability (see Fig. 21.5).

Risk assessment starts with potential hazards identification and characterisation (including their probability and magnitude determination) and continues with the analysis of the consequences of certain hazard occurrence and vulnerability of critical assets to specific threats. In the subsequent process of business impact analysis one determines the potential impacts of any given hazard, including the interruption of time sensitive or critical business processes. Hazard prevention thus includes,

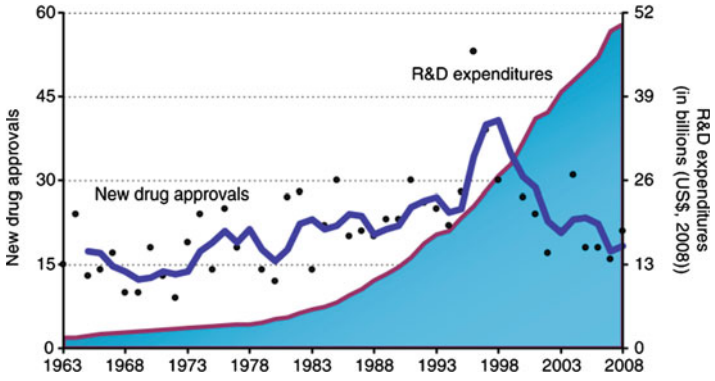

Fig. 21.5 New drug approvals (*dots*), represented on the left vertical axis, and pharmaceutical research and development expenditures in the USA (*shaded area*), represented on the right vertical axis in terms of constant 2008 dollar value. The trend line is a 3-year moving average (from Kaitin 2010)

at minimum, identification of ways to reduce the identified risks, such as taking measures of precaution and establishing schemes for an early recognition of upcoming problems and disturbances. Risk mitigation includes most notably elaboration of fall back plans, procurement or lease of fall back equipment and apparati, plus establishment of contingency procedures, as well as continuity assurance plans. It is prudent to reassess risk at least quarterly, ideally, through a combination of bottom-up (done by line function) and top-down (done by staff function) approaches. One must prioritise all resulting activities. Identification of a highly probable problem with a high potential impact calls always for an immediate action, however.

21.10 General Recommendations

Always strive to understand your product, and its development process as much as possible, but avoid all non-decisive tests and experiments.

Identify the best person for each job, and let her/him do it (cave micromanagement).

Try to base all important decisions on quantitative information, following the "four-eyes" principle.

Mistrust optimistic scenarios ("of the things that can go wrong at least some will go wrong"), but do not fret negative results ("there is no eternal 'down'").

Think the development through starting from its end.

Plan for the time after (a successful) completion of the next step (including the time post the product launch).

Seek and establish cooperations as soon as possible.

Be ready for surprises: assess and try to mitigate risk at all stages of development.

Never despair!

Glossary

BfArM Bundesinstitut für Arzneimittel und Medizinprodukte (Germany)
CFR Code of federal regulations (USA)
CTD Common technical document
CAMA Community application for marketing authorisation
CHMP Committee for medicinal products for human use (EMA)
EMA European Medicines Agency (until 2009: EMEA)
FDA U.S. Food and Drug Administration
GCP Good clinical practice (also known as current good clinical practice, cGCP)
GLP Good laboratory practice
GMP Good manufacturing practice

HAS Haute Autorité de Santé
ICH International Conference on Harmonisation
IMPD Investigational medicinal product dossier (EMA)
IND Investigational new drug (USA)
INDA Investigational new drug (IND) application (USA)
IQWiG Institute for Quality and Efficiency in Healthcare (Germany)
IRB Institutional Review Board/ethical review board (see subsection)
MEB Medicines Evaluation Board (The Netherlands)
MHRA Medicines and Healthcare Products Regulatory Agency (UK)
MPA Medical Products Agency (Sweden)
NDA New drug application to FDA (USA)
NICE National Institute for Health and Clinical Excellence
NPV Net present value
SPC/SmPC Summary of Product Characteristics (EMA)
SPA Special Protocol Assessment (by the FDA, USA)
QALY Quality Adjusted Life Years

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