Lipospheres in Drug Targets and Delivery

Approaches, Methods, and Applications

Lipospheres in Drug Targets and Delivery

Approaches, Methods, and Applications

EDITED BY

Claudio Nastruzzi

CRC PRESS

Boca Raton London New York Washington, D.C.

Library of Congress Cataloging-in-Publication Data

Lipospheres in drug targets and delivery : approaches, methods, and applications / edited by Claudio Nastruzzi.
p. ; cm.
Includes bibliographical references and index.
ISBN 0-8493-1692-8 (alk. paper)
1. Liposomes—Therapeutic use. 2. Microspheres (Pharmacy). 3. Nanoparticles. 4. Drug carriers (Pharmacy). 5. Drug targeting.
[DNLM: 1. Liposomes—therapeutic use. 2. Drug Delivery Systems—methods. 3. Lipids—therapeutic use. QU 93 L7658 2004] I. Nastruzzi, Claudio. II. Title.

RS201.L55L595 2004
615′.7--dc22 2004051936

Visit the CRC Press Web site at www.crcpress.com

No claim to original U.S. Government works
International Standard Book Number 0-8493-1692-8
Library of Congress Card Number 2004051936
Printed in the United States of America 1 2 3 4 5 6 7 8 9 0
Printed on acid-free paper

Preface

Colloidal drug carriers, such as liposomes and lipid nanoparticles, are able to modify the *in vivo* distribution of associated substances. They, therefore, can be used to improve the therapeutic index of drugs by increasing their efficacy or reducing their toxicity. If these delivery systems are carefully designed with respect to the target and route of administration, they help us to overcome some of the delivery problems posed by new classes of active molecules, such as peptides, proteins, genes, and oligonucleotides. They may also extend the therapeutic potential of established drugs, such as doxorubicin and amphotericin B.

This book describes the use of lipid-based nano- and microparticulate carriers in such applications. It presents innovative methods of delivering active biochemicals to different systems, discusses lipospheres as a technical solution to problems associated with controlled release of biochemicals, covers lipospheres as carriers for vaccines, and finally provides procedures for specific applications and describes biological systems.

With the identification, characterization, and cloning of specific growth factors, recombinant proteins are now widely used in the clinic. The use of recombinant hematopoietic growth factors has, for example, allowed the clinical manipulation of the hematopoietic system. Recombinant human granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor are now widely used to mobilize hematopoietic stem cells, thereby providing a source of hematopoietic stem cells for autologous or allogeneic transplantation.

However, one disadvantage associated with the use of most recombinant molecules is their rapid clearance. Because of this rapid clearance, recombinant molecules require repeated administration to achieve biological efficacy. Initially, continuous infusion was used to address this pharmacological deficiency. Continuous infusion has the advantage of delivering drugs in a controlled manner and is particularly appropriate when it is important to maintain constant plasma drug concentrations. However, the requirement for continuous venous access and the use of ambulatory pumps limits its use.

In this context, other approaches have been developed to improve the pharmacokinetic and pharmacodynamic properties of recombinant proteins *in vivo*. These have included the addition of polyethylene glycol to the recombinant molecules (PEGylation) and the use of sustained-release delivery systems. One goal of these approaches is to achieve clinical efficacy and lower the number of administrations, possibly to single injections, and thereby increase patient compliance. In addition to improving the pharmacokinetic and pharmacodynamic profile of recombinant molecules, sustained release may also increase the biological activity of specific molecules.

Moreover, it should be acknowledged that the use of an efficient carrier for nucleic acid–based medicines is considered to be a determinant factor for the successful

application of gene therapy. However, the drawbacks associated with the use of viral vectors, namely, those related to safety problems, have prompted investigators to develop alternative methods for gene delivery, with cationic lipid-based systems being the most representative systems. Despite extensive research in the last decade on the use of cationic liposomes as gene transfer vectors, and the development of elegant strategies to enhance their biological activity, these systems are still far from being viable alternatives to the use of viral vectors in gene therapy.

Finally, in this book considerations are made regarding the structure–activity relationships of cationic liposphere/DNA complexes, and the key formulations are presented and discussed in terms of their effect on biological activity.

Editor

Claudio Nastruzzi was born in Ferrara, Italy, on March 29, 1958. In 1983, he earned his undergraduate degree in pharmaceutical chemistry at the University of Ferrara, and in the late 1980s he was a fellow at the university's Department of Pharmaceutical Science, working on natural compound synthesis (prostaglandins and leukotrienes) and the characterization of isosazolic and isosazolinic nuclei reactivity. In 1988, he obtained his Ph.D. in pharmaceutical science with a dissertation on the synthesis and antitumor and antimetastatic activity of aromatic polyamidines.

During the late 1990s, Dr. Nastruzzi worked with Professor P. L. Luisi as a postdoctoral fellow at the Institute for Polymers at the Swiss Federal Institute of Technology in Zürich. As a postdoctoral fellow at the Department of Pharmaceutical Sciences at the University of Ferrara, he focused on the production and characterization of liposomes specially designed for retinoid delivery, as well as on biophysical studies and activity of *in vitro* cultured cell lines. In 1991, he obtained a researcher position in this department, where he devoted his energy to the production of microspheres, liposomes, and microemulsions for the controlled delivery of biological response modifiers.

Since 1998, Dr. Nastruzzi has been an associate professor in the Department of Chemistry and Pharmaceutical Technology at the University of Perugia, Italy. His main topics of interest include the production and characterization of innovative

dermatologic and cosmetic formulations for dermal and transdermal delivery (phospholipid-based microemulsion gels, cubic phases, liposomes, and niosomes); the production and characterization of microspheres and solid lipid microparticles; and liposome-based formulations for gene delivery.

Dr. Nastruzzi has published more than 90 papers in international journals and has presented more than 80 contributions to national and international congresses.

Contributors

Heike Bunjes
Department of Pharmaceutical Technology
Friedrich Schiller University of Jena
Jena, Germany

Rita Cortesi
Department of Pharmaceutical Sciences
University of Ferrara
Ferrara, Italy

Abraham J. Domb
Department of Medicinal Chemistry and Natural Products
Hebrew University of Jerusalem
Jerusalem, Israel

Elisabetta Esposito
Department of Pharmaceutical Sciences
University of Ferrara
Ferrara, Italy

Aviva Ezra
Department of Medicinal Chemistry and Natural Products
Hebrew University of Jerusalem
Jerusalem, Israel

Achim Göpferich
Department of Pharmaceutical Technology
University of Regensburg
Regensburg, Germany

Stephanie Könnings
Department of Pharmaceutical Technology
University of Regensburg
Regensburg, Germany

Karsten Mäder
Institute of Pharmaceutics and Biopharmacy
Martin Luther University of Halle
Halle, Germany

Wolfgang Mehnert
Department of Pharmaceutical Technology
Free University of Berlin
Berlin, Germany

Boaz Mizrahi
Department of Medicinal Chemistry and Natural Products
Hebrew University of Jerusalem
Jerusalem, Israel

R.H. Müller
Department of Pharmaceutics, Biopharmaceutics, and Biotechnology
Free University of Berlin
Berlin, Germany

Carsten Olbrich
Schering AG
Berlin, Germany

Monika Schäfer-Korting
Department of Pharmacology and Toxicology
Free University of Berlin
Berlin, Germany

Nadja Schöler
Schering AG
Berlin, Germany

Kerstin Tabatt
PenCef Pharma GmbH
Berlin, Germany

Sylvia A. Wissing
Drug Delivery Services GmbH
Kronshagen, Germany

Table of Contents

1 Solid Lipid Nanoparticles — Concepts, Procedures, and Physicochemical Aspects

Karsten Mäder and Wolfgang Mehnert

CONTENTS

0-8493-1692-8/05/$0.00+$1.50

1.1 SUMMARY

Solid lipid nanoparticles (SLN) have attracted increasing scientific and commercial attention during the last few years. This chapter highlights the main features of SLN, including the concept of SLN, different methods of production, and their applications. Special attention is paid to the relation among drug incorporation, the heterogeneity of the lipid particle, and the presence of other colloidal species. Strategies of SLN stabilization to avoid particle growth or gelation are discussed. The biological fate of the particles and the suitability of SLN for drug targeting are reviewed.

1.2 INTRODUCTION TO COLLOIDAL DRUG CARRIERS

Many existing drugs and drug candidates have poor solubility in biological fluids, which results in low and highly variable bioavailability and a high food dependency after oral administration. Intravenous injection of these drugs is not possible because of their low solubility. Appropriate formulation technologies might solve these problems. Colloidal drug carriers (CDC) have gained the most attention. The main therapeutic and commercial aims of CDC include:

- Enhancement of oral bioavailability
- Decrease in variability and food dependency
- Development of intravenous injectable formulations
- Drug targeting to specific tissues (with reduction of general toxicity)
- Life cycle management (protection by propriety formulation techniques)

CDC are defined only by their size (most scientists agree on sizes below 1 μm; others set 0.5 μm as the upper limit). CDC are very heterogeneous in all other aspects (e.g., thermodynamic stability, chemical composition, and the physical state, including solid, liquid, or liquid-crystalline dispersions) [1]. The most prominent examples are nanoparticles, nanoemulsions, nanocapsules, liposomes, nanosuspensions, (mixed) micelles, microemulsions, and cubosomes. Some CDC have reached the commercial market. Probably the best known example is the microemulsion preconcentrate of cyclosporine (Sandimmun-Neoral), which minimized the high variability of pharmacokinetics of the Sandimmun formulation. In addition, intravenous injectable CDC have been on the commercial market for many years. Examples include nanoemulsions of etomidate (Etomidat-Lipuro) and diazepam (Diazepam-Lipuro) [2–4], mixed micelles (Valium-MM, Konakion), and liposomes (AmBisome) [5].

However, overall only a very limited number of CDC has reached the marketplace. It is expected that this number will increase as a result of requirements for drug safety and of the increasing number of poorly soluble compounds in the pipeline. More and more molecules have to be formulated with a sophisticated drug delivery system to achieve predictable pharmacokinetics. Patents of other molecules (e.g., cyclosporine) have expired, and the first generics of cyclosporine microemulsion preconcentrates have entered the market. Companies develop strategies to protect or to get market shares based on formulation technology using CDC. Therefore, the modification of existing CDC (to circumvent existing patents) or the development

of new CDC (preferably with new advantages and patent protection) is considered crucial by many companies.

The main efforts to improve current CDC are related to:

- Increasing drug load
- Possibility of controlled release
- Possibility of drug targeting
- Increasing feasibility of large-scale production
- Increasing physical and chemical storage stability
- Minimizing overall costs

1.3 FROM NANOEMULSIONS TO NANOPELLETS: SLN — HISTORY AND SCOPE

Advantages of nanoemulsions include toxicological safety and a high content of the lipid phase as well as the possibility of large-scale production using high-pressure homogenization (HPH). However, controlled drug release from nanoemulsions is very unlikely because of the small size and the liquid state of the carrier. It has been estimated that retarded release requires very lipophilic drugs: Their octanol/water partition coefficient should be larger than 1,000,000:1 [6]. The use of solid lipids instead of liquid oils is a very attractive idea for achieving controlled drug release because drug mobility in a solid lipid should be considerably lower compared with a liquid oil. In addition, the stability of certain drugs might be higher in a solid matrix compared with in a liquid lipid. Therefore, solid lipid micro- and nanoparticles were developed as "frozen nanoemulsions" to realize controlled drug delivery and to increase the chemical stability of incorporated drugs.

The pioneer in this field was Speiser, who developed nanopellets for peroral administration [7]. These nanopellets were produced by dispersing melted lipids with high-speed mixers or with ultrasound. A relatively large amount of microparticles was present in these formulations, which might not be a serious problem for peroral administration, but they exclude an intravenous injection. Lipospheres, produced by high-shear mixing or ultrasound, were developed by Domb and represent similar systems [8–10]. They also contain large amounts of microparticles.

It was soon found that HPH is more effective for the production of submicron-sized dispersions of solid lipids than is high-shear mixing or ultrasound [11–13]. Dispersions obtained in this way are called SLN. Most SLN dispersions produced by HPH are characterized by an average particle size of around 100 to 200 nm and a low microparticle content. Alternative production procedures were also investigated, including the combination of organic solvents and HPH (HPH/solvent evaporation) [14], the dilution of microemulsions [15,16], and solvent injection [17].

It has been claimed that SLN combine the advantages of other colloidal carriers and avoid their disadvantages [18]. Proposed advantages include:

- Possibility of controlled drug release and drug targeting
- Increased drug stability
- High drug payload

- Feasible incorporation of lipophilic and hydrophilic drugs
- Lack of biotoxicity of the carrier
- Avoidance of organic solvents
- No difficulties with large-scale production and sterilization

1.4 SLN INGREDIENTS AND FORMULATION PROCESSES

1.4.1 Ingredients

General ingredients include the drug, solid lipids, emulsifiers, and water. Depending on the application, other ingredients might be present (osmotic agents, matrices for lyophilization, buffers, etc.).

The danger of acute and chronic toxicity resulting from the SLN lipids is rather low because, in general, physiological lipids are used (there are few exceptions, such as amphiphilic calixarenes [19]). The term "lipid" is generally used in a very broad sense and includes triglycerides (e.g., tristearin), partial glycerides (e.g., monostearate), fatty acids (e.g., stearic acid), steroids (e.g., cholesterol), and waxes (e.g., cetyl palmitate). More attention should be given to the physicochemical properties of the lipid and to classifying them in relation to their interactions with water, according to Small [20].

The choice of emulsifier depends on the administration route and is more limited for parenteral administrations. A large variety of ionic and nonionic emulsifiers of different molecular weight has been used to stabilize the lipid dispersion. The most frequently used compounds include different kinds of poloxamer, polysorbates, lecithin, and bile acids. In many cases, the combination of emulsifiers was more efficient at preventing particle agglomeration than was the use of a single surfactant.

1.4.2 Production Processes for SLN

1.4.2.1 High-Shear Homogenization and Ultrasound

High-shear homogenization and ultrasound were initially used for the production of solid lipid nanodispersions [7,8]. Both methods are widespread and easy to handle. However, in many cases, bimodal size distributions are obtained with one population in the micrometer range. In addition, metal contamination has to be considered if ultrasound is used.

Ahlin et al. used a rotor-stator homogenizer to produce SLN by melt-emulsification [21]. They investigated the influence of different process parameters — including emulsification time, stirring rate, and cooling conditions — on the particle size and the zeta potential. In most cases, average particle sizes in the range of 100 to 200 nm were obtained using stirring rates of 20,000 to 25,000 rpm for 8 to 10 min and controlled cooling with a stirring rate of 5,000 rpm.

1.4.2.2 High-Pressure Homogenization

HPH has emerged as a reliable and powerful technique for the preparation of SLN. HPH has been used for years for the production of nanoemulsions for parenteral nutrition. In contrast to other techniques, scaling up represents no or minor problems in most cases. High-pressure homogenizers push a liquid with high pressure (10 to

200 MPa) through a narrow gap (in the range of few microns). The fluid accelerates on a very short distance to very high velocity (over 1000 km/h). Very high-shear forces disrupt the particles down to the submicron range. Typical lipid contents range between 5 to 10% of the fluid and represent no problem to the homogenizer. Even lipid concentrations up to 40% have been homogenized to lipid nanodispersions [22].

Two general approaches of the homogenization step, the hot and the cold homogenization techniques, can be used for the production of SLN [13,23,24]. In both cases, a preparatory step involves incorporating the drug into the bulk lipid by dissolving or dispersing the drug in the lipid melt.

1.4.2.2.1 Hot Homogenization

Hot homogenization is carried out at temperatures above the melting point of the lipid and can therefore be regarded as the homogenization of an emulsion. A preemulsion of the drug-loaded lipid melt and the aqueous emulsifier phase (same temperature) is obtained by a high-shear mixing device (Ultra-Turrax). The quality of the preemulsion affects the quality of the final product to a large extent, and obtaining droplets in the size range of a few micrometers is desirable. HPH of the preemulsion is carried out at temperatures above the melting point of the lipid. In general, higher temperatures result in lower particle sizes because of the decreased viscosity of the inner phase [25]. However, high temperatures may also increase the degradation rate of the drug and the carrier. Furthermore, many surfactants have decreased solubilities and HLB values at a higher temperature, which might have a negative impact on homogenization efficacy. The homogenization step can be repeated several times. It should always be kept in mind that HPH increases the temperature of the sample (approximately 10˚C for 500 bar [26]). In most cases, 3 to 5 homogenization cycles at 500 to 1500 bar are sufficient. Increasing the homogenization pressure or the number of cycles often results in an increase of the particle size because of particle coalescence, which occurs as a result of the high kinetic energy of the particles [27].

The primary product of the hot homogenization is a nanoemulsion resulting from the liquid state of the lipid. Solid particles are expected to be formed by the cooling of the sample to room temperature or below. Because of the small particle size and the presence of the emulsifiers, lipid crystallization may be highly retarded, and the sample may remain as a supercooled melt (nanoemulsion) for several months [28]. Westesen and Bunjes found that purported "SLN" data published by another group were, in fact, measurements from supercooled melts [29].

1.4.2.2.2 Cold Homogenization

Cold homogenization is carried out with the solid lipid and can therefore by regarded as a high-pressure milling of a lipid suspension. Effective temperature control and regulation is needed to ensure the unmolten state of the lipid because of the increase in temperature during homogenization [26]. Cold homogenization has been developed to overcome the following three problems of the hot homogenization technique:

- Temperature-induced drug degradation
- Drug distribution into the aqueous phase during homogenization
- Complexity of the crystallization step of the nanoemulsion leading to several modifications or supercooled melts

The first preparatory step is the same as in the hot homogenization procedure and includes the solubilization or dispersing of the drug in the melt of the bulk lipid. However, different steps follow. The drug-containing melt is cooled very rapidly (e.g., by means of dry ice or liquid nitrogen). The high cooling rate favors a homogenous distribution of the drug within the lipid matrix. The solid, drug-containing lipid is milled by means of ball or mortar milling in the range of 50 to 100 μm. Low temperatures increase the fragility of the lipid and, therefore, favor particle disruption. The solid lipid microparticles are dispersed in a chilled emulsifier solution. The presuspension is subjected to HPH at or below room temperature. In general, compared with hot homogenization, larger particle sizes and a broader size distribution are observed in cold-homogenized samples [30]. The method of cold homogenization minimizes the thermal exposure of the sample, but it does not avoid it because of the melting of the lipid/drug mixture in the initial step. Most investigators use the hot homogenization process because of its higher efficacy and the avoidance of the cold milling process. It must be also mentioned that the rapid cooling of the lipid melt in the first step favors metastable lipid modifications (with higher drug loading capacity), which might transform with time into more stable polymorphs (with the expulsion of the incorporated drug).

1.4.2.3 SLN Prepared by Solvent Emulsification/Evaporation

Sjöström and Bergenståhl used a solvent emulsification/evaporation method to prepare solid lipid nanodispersions [14]. The lipophilic material is dissolved in a water-immiscible organic solvent (e.g., cyclohexane) that is emulsified in an aqueous phase to give an oil/water (o/w) emulsion. On evaporation of the solvent by reduced pressure, a solid lipid nanoparticle dispersion is formed. The mean diameter of the obtained particles was 25 nm, with cholesterol acetate as the model drug and using a lecithin/sodium glycocholate blend as the emulsifier. The reproducibility of these results was confirmed by Siekmann and Westesen [31], who also prepared nanoparticles of tripalmitin by dissolving triglyceride in chloroform. Mean particle sizes of the final particles ranged from 30 to 100 nm, depending on the lecithin/cosurfactant blend. The smallest particle diameters were obtained by using bile salts as cosurfactants. Comparable small-particle-size distributions are not achievable by melt emulsification of a similar composition. The mean particle size depends on the concentration of the lipid in the organic phase. Very small particles could only be obtained with low fat loads (5 w%) related to the organic solvent. With increasing lipid content, the efficiency of the homogenization declines because of the higher viscosity of the dispersed phase. The advantage of this procedure over the cold homogenization process described before is the avoidance of any thermal stress. A clear disadvantage is the use of organic solvents.

1.4.2.4 Solvent Injection Method

The production of polymeric nanoparticles by dilution of polymer solutions in water has been described by De Labouret et al. [32]. The particle size is critically determined by the velocity of the distribution processes. Nanoparticles were produced only with polar solvents, which distribute very rapidly into the aqueous phase (e.g.,

acetone, ethanol, isopropanol, methanol), whereas larger particle sizes were obtained with more lipophilic solvents. The process also can be easily used for the production of lipid nanodispersions [17]. A requirement is the solubility of the lipid in the polar organic solvent, which limits the application range of this procedure. A further disadvantage is the low concentration of the lipid nanoparticles (typically 1% or less). Higher amounts of the organic solvent increase the solubility of the lipid in the aqueous phase and lead to an increase in particle size resulting from Ostwald ripening. The main advantage of the method is the avoidance of thermal stress.

1.4.2.5 Microemulsion-Based SLN Preparations

SLN preparation techniques that are based on the dilution of microemulsions have been developed by Gasco [16]. It should be mentioned that there are different definitions and opinions about the structure and dynamics of microemulsion in the scientific community. An extended review has recently been published by Moulik and Paul [33].

Gasco and other scientists describe microemulsions as two-phase systems composed of an inner and outer phase (e.g., o/w microemulsions). Microemulsions are made by stirring at 65 to 70°C an optically transparent mixture that is typically composed of a low-melting fatty acid (e.g., stearic acid), an emulsifier (e.g., polysorbate 20, polysorbate 60, soy phosphatidylcholine, taurodeoxycholic acid sodium salt), coemulsifiers (e.g., butanol, sodium monooctylphosphate), and water. The hot microemulsion is dispersed in cold water (2 to 3°C) under stirring. Typical volume ratios of the hot microemulsion to cold water are in the range of 1:25 to 1:50. The dilution process is critically determined by the composition of the microemulsion. According to the literature [34,35], the droplet structure is already contained in the microemulsion, and, therefore, no energy is required to achieve submicron particle sizes.

In addition to the composition, the temperature gradient and the pH value are key parameters for the quality of the final lipid nanosuspension. High-temperature gradients facilitate rapid lipid crystallization and prevent aggregation [36,37]. Because of the dilution step, achievable lipid contents are considerably lower compared with the HPH-based formulations.

1.4.2.6 Comparison of Different Formulation Procedures

A reliable comparison between different formulation procedures can be made only by the same investigator, who has used the same batches of ingredients, the same storage conditions, and the same equipment for particle sizing. Otherwise, impurities in the ingredients and differences in particle-sizing technologies might lead to misleading results.

Siekmann and Westesen investigated the influence of the formulation procedure on the quality of tyloxapol- (1.5 w%) and soy lecithin– (1 w%) stabilized tripalmitin (3 w%) nanoparticles [27]. They demonstrated the principal possibility of obtaining size distributions in the range of 30 to 180 nm by ultrasonification. However, these small particle sizes required long sonication times (>15 min), which raises concerns about metal contamination of the product. Moreover, it is difficult to disperse higher

fat concentrations homogeneously by probe sonication, and, therefore, ultrasound is only of limited use. However, HPH proved to be a very effective dispersing technique in this study. A reduction of the average particle size from 474 to 155 nm was obtained after just the first homogenization cycle (800 bar). The maximum dispersing grade was observed after five homogenization cycles. Results reported by other investigators show similar dependences of the particle size from the homogenization pressure and the number of cycles [13,38].

The solvent emulsification/evaporation process was compared by Siekmann and Westesen to the melt-homogenization method [31]. In the case of lecithin/sodium glycocholate-stabilized tripalmitin dispersions, solvent emulsification yielded significantly smaller particles than melt-homogenization of similarly composed systems under the same production conditions. The mean particle size of the melt-homogenized tripalmitin nanoparticles was 124 nm, and that of the evaporated solvent only 28 nm. This result might be partly explained by the lower homogenization efficiency of the lecithin/sodium glycocholate blend in the emulsified tripalmitin melt compared to the organic solvent-in-water emulsion, as the mobility of phospholipid molecules is lower in the lipid melt than in the solvent. Moreover, the melt of the lipid/emulsifier blend is much more viscous than the solvent, so the homogenization requires more energy input. However, solvent emulsification is not always superior to melt homogenization with respect to the dispersing degree. In contrast, for systems stabilized by phospholipids and nonionic surfactants, melt homogenization produced smaller particles than the solvent emulsification procedure. These results show that the particle size heavily depends on the composition of the emulsifiers. Solvent emulsification is a suitable alternative method to prepare small, homogeneously sized lipid nanoparticle dispersions. An important advantage of that technique is the avoidance of any heat. However, solvent-emulsified suspensions are relatively diluted (0.5 to 2.5 w% tripalmitin) because of the limited solubility of the tripalmitin in organic solvents. Furthermore, it has to be considered that solvent emulsification may cause regulatory and toxicological problems arising from the solvent residues.

The dispersing grade depends on the power density and the power distribution in the dispersion volume. High-power densities result in more effective particle disruption, and high-pressure homogenizers reach by far the highest power densities (10^{12} to 10^{13} W/m^3). A homogeneous distribution of the power density is necessary to obtain narrow size distributions. Otherwise, particles localized in different volumes of the sample will experience different dispersing forces, and, therefore, the degree of particle disruption will vary within the sample volume. Inhomogeneous power distributions are observed in high-shear homogenizers and ultrasonifiers. High-pressure homogenizers are characterized by a homogenous power distribution because of the small size of the homogenizing gap (25 to 30 μm).

1.4.2.7 Influence of Ingredient Composition on Product Quality

1.4.2.7.1 Influence of the Lipid

Unfortunately, only a few investigators pay sufficient attention to detailed consideration of the lipid characteristics. A monoglyceride and a triglyceride will behave differently in an aqueous environment. The used lipids should be classified, according

to Small [20], into nonpolar and different classes of polar lipids. This classification is very helpful for understanding the interplay among drug, lipid emulsifier, and water. The general lipid composition (mixed chain lengths or triglycerides made from one fatty acid) will have different crystallinities and capacities for accommodating foreign molecules. In addition, pH levels may change the behavior of lipids considerably. Fatty acids are, in the protonated form (e.g., myristic acid), insoluble, nonswelling lipids, and they behave similarly to triglycerides. Unprotonated fatty acids (e.g., sodium myristate) can form micelles and are soluble amphiphiles with lyotropic mesomorphism. Around the pK_a (which varies in strong dependency of the environment), fatty acid–fatty acid salt complexes form lyotropic liquid crystalline lamellar phases and represent insoluble swelling lipids. The interaction between the fatty acid and the drug will be very different for each species.

Furthermore, even triglycerides with the same fatty acid composition will behave differently, depending on the localization of the fatty acid on the glycerol. For example, cacao butter has a rather sharp melting point because of the defined localization of the oleic (2 position) and palmitic and stearic acids (1 and 3 positions). Random localization of the fatty acids leads to a broadening of the melting point to a melting range, which means that a certain amount of liquid lipids will be present over a large temperature range.

Jenning and Gohla found that high crystallinity of lipid matrices was linked with good physical stability but a low degree drug incorporation, whereas lipid matrices with low crystallinity were able to accommodate higher amounts of drug and showed poor physical stability [39]. However, further parameters for nanoparticle formation will be different for different lipids. Examples include the melting point, the velocity of lipid crystallization, and the shape of the lipid crystals (and therefore the surface area). Higher-melting lipids led to an increase in particle size [11,30]. These results are in agreement with the general theory of HPH [26] and can be explained by the higher viscosity of the dispersed phase.

It is also noteworthy that most of the lipids used represent a mixture of several chemical compounds. The composition might, therefore, vary among different suppliers and might even vary for different batches from the same supplier. Small differences in the lipid composition (e.g., impurities) might have a significant effect on the quality of SLN dispersion (e.g., by changing the zeta potential or retarding crystallization processes). For example, lipid nanodispersions made with cetyl palmitate from different suppliers had different particle sizes and storage stabilities (A. Lippacher, personal communication).

The influence of lipid composition on particle size was also confirmed for SLN produced via high-shear homogenization [21]. The average particle size of Witepsol W35 SLN was found to be significantly smaller (117.0 ± 1.8 nm) than the average particle size of Dynasan 118 SLN (175.1 ± 3.5 nm). Witepsol W35 contains shorter fatty acid chains and considerable amounts of mono- and diglycerides, which possess surface-active properties.

Increasing the lipid content over 10% leads to larger particles (including microparticles) and broader particle size distributions [27,30]. Both a decrease of the homogenization efficiency and an increase in particle agglomeration cause this phenomenon.

1.4.2.7.2 Influence of the Emulsifier

The choice of the emulsifiers and their concentration has a great impact on the quality of the SLN dispersion [13,30]. Siekmann and Westesen determined that 2 w% tyloxapol was insufficient to stabilize a 10 w% tripalmitin dispersion. Increasing the tyloxapol concentration to 10 w% resulted in 85-nm particles with unimodal size distribution [27].

High concentrations of the emulsifier reduce the surface tension and facilitate the particle partition during homogenization. The decrease in particle size is connected with a tremendous increase in surface area. The increase of the surface area during HPH occurs very rapidly. Therefore, kinetic aspects have to be considered. The process of a primary coverage of the new surfaces competes with the agglomeration of uncovered lipid surfaces. The primary dispersion must contain excessive emulsifier molecules, which should rapidly cover the new surfaces. The excessive emulsifier molecules might be present in different forms, for example, molecularly solubilized or in the form of micelles or liposomes. The timescale of the redistribution processes of emulsifier molecules between particle surfaces, water-solubilized monomers, and micelles or liposomes is different. In general, SDS and other micelle-forming, low-molecular weight surfactants will rapidly achieve the new equilibrium. Redistribution processes will take a longer time for high-molecular weight surfactants (poloxamer) or stabilizers with poor solubility in water (lecithin).

However, it is not recommended that one use rapidly distributing surfactants exclusively because surfactant mixtures (e.g., Lipoid S75/poloxamer 188 [30] or tyloxapol/lecithin [27]) might lead to lower particle sizes and higher storage stability compared with formulations with only one surfactant. The addition of sodium glycocholate to the aqueous phase as coemulsifying agent decreases the particle size, too [27].

Different emulsifier compositions might require different homogenization parameters. For example, the maximal degree of dispersing was obtained with 500 bar and 3 cycles for poloxamer 188–stabilized systems [38]. Homogenization with pressures of 1000 or 1500 bar did not result in further reduction of the particle size. In contrast, pressures of 1500 bar proved to be the best for lecithin- (Lipoid S75) stabilized systems. A possible explanation for this observation is the different velocity of the coverage of the new lipid surfaces.

The choice of emulsifiers might also influence the crystallization behavior. For example, glycocholate was able to preserve the alpha polymorph of the lipid tripalmitin for long times, whereas transitions to more stable polymorphs occurred more rapidly for the other surfactants [40].

1.4.3 Sterilization

Parenteral administration requires sterile products. Aseptic production, filtration, γ-irradiation, and heating are normally used to achieve sterility. Filtration sterilization of dispersed systems requires high pressure and is not applicable to particles larger than 0.2 μm.

The impact of different sterilization techniques (steam sterilization at 121˚C [15 min] and 110˚C [15 min], γ-sterilization) on SLN characteristics has been

investigated [38,41,42]. Particle aggregation might occur as a result of the treatment. Critical parameters include sterilization temperature and SLN composition, and increased temperatures might significantly affect the mobility and the hydrophilicity of the emulsifier. Schwarz found that lecithin is a suitable surfactant for steam sterilization because there was only a minor increase in particle size and number of microparticles [38]. In contrast, steam sterilization induced a significant increase in particle size for poloxamer 188–stabilized Compritol SLN. For emulsifiers of this type, it is well known that increased temperatures lead to dehydration of the PEG-units and a decrease of the HLB value, which finally causes a decrease in the thickness of the protecting layer. It has been demonstrated by ^{1}H-NMR (nuclear magnetic resonance) spectroscopy on poloxamer-stabilized lipid nanoparticles that even a moderate temperature increase from room temperature to 37˚C decreases the mobility of the ethylene glycol chains on the particle surface [43].

Drug loading might have a large effect on sterilization-induced destabilization. Steam sterilization of 5% tetracaine–loaded poloxamer 188/Compritol SLN induced a broader size distribution and an increase in the mean particle size (from 160 to 260 nm) [41]. Even a larger increase in particle size (>500 nm) was observed for a higher tetracaine loading of 10%. These results indicate that drug-related phenomena contribute to destabilization processes in addition to changes of the emulsifier film. The destabilizing effect of tetracaine and etomidate has also been observed in steam sterilization of fat emulsions and is probably caused by the distortion of the mechanical properties of the surfactant film [44].

Experiments conducted by Freitas indicated that lowering the lipid content (to 2%), surface modification of the glass vials, and purging with nitrogen prevent the particle increase to a large extent and prohibit gelation [45]. In addition, it was observed that purging the sample with nitrogen showed a protective effect during sterilization. Cavalli et al. studied the influence of steam sterilization on particle size and zeta potential of SLN produced via microemulsions [46]. The lipid phase was made of stearic acid, behenic acid, or Acidan N12 (monostearate monocitrate diglyceride); and Epikuron 200 (soy phosphatidylcholine 95%) and taurodeoxycholate were used as stabilizers. SLN were dispersed in aqueous trehalose (2%) or poloxamer 188 solution (2%). Steam sterilization (121˚C, 15 min) did not change the average particle size of Acidan N12 SLN, but increased particle sizes were observed for SLN composed of behenic acid (from 70 to 135 nm) and of stearic acid (from 55 to 110 nm). After 1 year, increased particle sizes were observed for all systems (Acidan N12 SLN, 350 nm; behenic acid SLN, 120 nm; stearic acid SLN, 450 nm).

Particle sizes of diazepam-loaded SLN showed similar changes after sterilization as did drug-free systems. Steam sterilization (121˚C, 20 min) did not cause changes in particle size and zeta potential of azidothymidine palmitate–loaded SLN (trilaurin, phospholipid stabilized) [47].

An alternative method to steam sterilization for temperature-sensitive samples could be γ-irradiation. Comparative studies on SLN sterilization by steam and γ-rays were conducted by Schwarz and others [38,41,42]. Lecithin-stabilized SLN proved to be superior to poloxamer-stabilized SLN for the process of steam sterilization. However, this difference was not observed for γ-sterilized samples, which indicates the importance of heat-induced changes. Compared to steam sterilization at 121˚C,

the increase in particle size after γ-irradiation was lower, but was comparable to steam sterilization at 110°C.

Unfortunately, most investigators did not search for steam sterilization– or irradiation-induced chemical degradation. It should be kept in mind that degradation does not always cause increased particle sizes. In contrast, the formation of species like lysophosphatides or free fatty acids might even preserve small particle sizes, but might also cause toxicological problems (e.g., hemolytic activity). Detailed studies that involve the aspects of chemical stability are clearly necessary to permit valid statements of the possibilities of SLN sterilization.

1.4.4 Stabilization by Drying

It has been shown that particle sizes of aqueous SLN dispersions might be stable over 12 to 36 months [30]. However, this stability is not a general feature of SLN dispersions, and, in most cases, physical (increases of the particle size) or chemical instability (degradation reactions, hydrolysis) will be observed in a shorter period of time.

The particles must have a very narrow size distribution to avoid crystal growth by Ostwald ripening. The SLN formulation should be resistant to temperature changes that will occur during shipping. Furthermore, mechanical stress resulting from transport might lead to sudden gelation of the SLN dispersions. In addition, microbiological aspects also have to be kept in mind. A strategy to solve these problems is to remove the water and produce SLN powders, which can be stored for longer times. The addition of water just before administration should lead to the particle sizes of the SLN dispersion.

Lyophilization is a widely used process for water removal from sensitive samples. However, two additional transformations between the formulations are necessary and may be the source of additional stability problems. The first transformation, from aqueous dispersion to powder, involves the freezing of the sample and the evaporation of water under vacuum. Freezing of the sample might cause stability problems because of the freezing-out effect, which results in changes of the osmolarity and the pH. The second transformation, resolubilization, involves, at least in its initial stages, situations that favor particle aggregation (low water and high particle content and high osmotic pressure).

The protective effect of the surfactant can be compromised by lyophilization [48]. It has been found that, to prevent an increase in particle size, the lipid content of the SLN dispersion should not exceed 5%. Direct contact of lipid particles is decreased in diluted samples. Furthermore, diluted SLN dispersions will also have higher sublimation velocities and a higher specific surface area [49]. The addition of cryoprotectors will be necessary to decrease SLN aggregation and to obtain a better redispersion of the dry product. Typical cryoprotective agents are sorbitol, mannose, trehalose, glucose, and polyvinylpyrrolidone.

The best results occurred when the lyophilization of SLN was obtained with the cryoprotectors glucose, mannose, maltose, and trehalose in concentrations of 10 to 15% [50]. The observations come into line with the results of studies on liposome lyophilization, which indicated that trehalose was the most sufficient substance to prevent liposome fusion and leakage of the incorporated drug [51].

Mean particle sizes and size distributions of Compritol and Dynasan 112 SLN were only slightly increased after freeze-thaw experiments [50].

The results of unloaded SLN do not predict the quality of drug-loaded lyophilizates. Even low concentrations of 1% tetracaine or etomidate caused a significant increase in particle size, which excludes an intravenous administration [50].

Siekmann and Westesen investigated the lyophilization of tripalmitin SLN (surfactants: 4.5% tyloxapol and 3% soybean lecithin [Lipoid S100]) [52]. Glucose, sucrose, maltose, and trehalose were used as cryoprotective agents in concentrations of 5, 10, and 20%. Handshaking of redispersed samples was an insufficient method, whereas bath sonification produced better results. Average particle sizes of all lyophilized samples with cryoprotective agents were 1.5 to 2.4 times higher than the original dispersions. Cryoprotector-free samples showed very high particle aggregation. Samples with a lipid content below 10% showed less aggregation than more highly concentrated samples. The efficiency of the cryoprotectors decreases in the following order: trehalose > sucrose > glucose and maltose. Surprisingly, it was found that the time at which the cryoprotector is added influences the quality of the final formulation. Best results were obtained when the cryoprotector was added to the sample before homogenization. Under these circumstances, average particle size remained almost unchanged, though storage over 1 year caused significant increases in particle sizes. Average particle sizes were 4 to 6.5 times larger than in the original dispersion. In contrast to the lyophilizates, the aqueous dispersions of tyloxapol/phospholipid-stabilized tripalmitin SLN exhibited remarkable storage stability. The average particle size increased only very slightly, from 56 to 65 nm, over 1 year. The instability of the SLN lyophilizates can be explained by the sintering of the particles.

Cavalli et al. also observed increased particle sizes (2.1 to 4.9 times) after lyophilization [46]. A trehalose concentration of 2% was insufficient to prevent lyophilization-induced particle aggregation. Increasing the concentration of trehalose to 15% resulted in average particle sizes around 100 nm and in polydispersity indices of 0.25 after reconstitution.

Heiati compared the influence of four cryoprotectors (trehalose, glucose, lactose, and mannitol) on the particle size of azidothymidine palmitate–loaded SLN lyophilizates [47]. Trehalose was found to be the most effective cryoprotector for preventing aggregation during lyophilization and subsequent reconstitution of SLN. A sugar/lipid weight ratio of 2.6 to 3.9 was recommended.

The freezing process has an effect on the product quality. Rapid freezing in liquid nitrogen was suggested by Schwarz and Mehnert [50]. In contrast, other researchers observed the best results after a slow freezing process. Zimmermann et al. found that optimization of the lyophilization parameters results in formulations that are intravenously injectable, with regard to particle size [53]. Again, best results were obtained with samples of low lipid content and with the cryoprotector trehalose. In contrast to the results of Schwarz, slow freezing in a deep freeze (–70˚C) was superior to rapid cooling in liquid nitrogen. Furthermore, introduction of an additional thermal treatment to the frozen SLN dispersion (2 h at –22˚C followed by a 2-h temperature decrease to –40˚C) was found to improve the quality of the product.

Recent studies of Gasco's group indicate that drying with a nitrogen stream at low temperatures (3 to 10˚C) might be superior to lyophilization [54]. Compared to lyophilization, the advantages of this process are the avoidance of freezing and the energy efficiency resulting from the higher vapor pressure of water.

Spray drying might be an alternative procedure to lyophilization to transform an aqueous SLN dispersion into a dry product. This method has been used scarcely for SLN formulation, although spray drying is cheaper than lyophilization. By spray drying, Freitas and Müller obtained a redispersable powder that complies with the general requirements regarding particle size and selection of ingredients for intravenous injections [55]. Spray drying might potentially cause particle aggregation as a result of high temperatures, shear forces, and partial melting of the particles. Freitas and Müller recommend the use of lipids with melting points greater than 70˚C for spray drying. Furthermore, the addition of carbohydrates and low lipid contents favor the preservation of the colloidal particle size in spray drying. The melting of the lipid can be minimized by using ethanol/water mixtures as a dispersion medium instead of pure water because of the lower inlet temperatures. The best result was obtained with SLN concentrations of 1% in solutions of 30% trehalose in water or 20% trehalose in ethanol/water mixtures (10/90 v/v).

1.5 CHARACTERIZATION OF SLN

Appropriate characterization of the solid lipid nanodispersion is a necessary and very difficult task because of the submicron size of the particles and the complexity of the system, which also includes dynamic phenomena. The following statement of Laggner about lipids should always be kept in mind [56]:

> Lipids and fats, as soft condensed material in general, are very complex systems, not only in their static structures but also with respect to their kinetics of supramolecular formation. Hysteresis phenomena or supercooling can gravely complicate the task of defining the underlying structures and boundaries in a phase diagram (p. 334).

This is especially true for lipids in the colloidal size range. Many analytical tools do not permit direct measurement in the undiluted SLN dispersion. Therefore, sample preparation might cause artifacts (e.g., removal of emulsifier from particle surface by dilution, induction of crystallization processes, changes of lipid modifications). Pushing SLN dispersions though a syringe needle might result in the immediate transformation of the low, viscous SLN dispersion into a viscous gel. In this case, the artifact caused by sample preparation is clearly visible, although in many other cases it will not be.

The most important parameters include:

- Particle size
- Degree of crystallinity and lipid modification
- Coexistence of additional colloidal structures (micelles, liposomes, supercooled melts, drug nanoparticles) and timescale of distribution processes
- Zeta potential

Photon correlation spectroscopy (PCS) and laser diffraction (LD) are the most frequently applied techniques for measurements of particle size. PCS (also known as dynamic light scattering) measures the fluctuation of the intensity of the scattered light caused by particle movement and covers a size range from a few nanometers to about 3 μm. New developments (back scattering, cross polarization) permit measurement in undiluted or less diluted samples. Microparticles are not detected by PCS, but they can be visualized by means of LD measurements. This method is based on the dependence of the diffraction angle on the particle radius. Smaller particles cause more intense scattering at high angles than do larger ones. New developments of LD expanded the lower limit of measurable particle sizes from 40 to 100 nm.

However, despite this progress, it is highly recommended that both PCS and LD be used simultaneously. It should be kept in mind that both methods are not "measuring" particle sizes. Rather, they detect light-scattering effects that are used to calculate particle size. For example, uncertainties may result from nonspherical particle shapes and from the assumption of certain parameters that are used to calculate the particle size. Platelet structures commonly occur during lipid crystallization [57] and have also been observed for SLN [11,40,58]. The presence of several populations and other colloidal structures adds further difficulties.

The use of additional techniques is recommended. Light microscopy is not sensitive to the nanometer size range but gives a fast indication of the presence of microparticles. Electron microscopy provides, in contrast to PCS and LD, direct information on the particle shape. However, the investigator should pay special attention to possible artifacts that may be caused by the sample preparation. For example, solvent removal may cause modification changes that will influence the particle shape [57].

Atomic force microscopy (AFM) has also been applied to image the morphological structure of SLN [59]. The size of the visualized particles was of the same magnitude as the results of PCS measurements. The AFM investigations revealed the disklike structure of the particles. Dingler and others investigated cetyl palmitate SLN (stabilized by polyglycerol methylglucose distearate, Tegocare 450) by electron microscopy and AFM and observed spherical forms of the particles [60,61]. Different SLN shapes were reported by Westesen and others for SLN made of well-defined lipids of high purity (e.g., pure triglycerides) [11,62]. A disadvantage of AFM is the required fixation of the particles (by removal of water), which changes the status of the emulsifier and might also cause polymorphic transitions of the lipid.

The particle sizing by field flow fractionation (FFF) is based on the different effect of a perpendicular applied field on particles in a laminar flow [63–66]. The separation principle corresponds to the nature of the perpendicular field and may, for example, be based on different mass (sedimentation FFF), size (cross-flow FFF), or charge (electric-field FFF). Cross-flow FFF has been applied recently to investigate nanoemulsions, SLN, and nanostructured lipid carriers (NLC, particles composed of liquid and solid lipids) [58]. Although all samples had comparable particle sizes in PCS, their retention in the FFF was very different. Compared to the spherical droplets of the nanoemulsion, SLN and NLC were pushed more efficiently to the bottom of the channel because of their anisotropic shape. Their very different shapes have been confirmed by electron microscopy.

Special attention must be paid to the characterization of the degree of lipid crystallinity and the modification of the lipid, because these parameters are strongly related to drug incorporation and release rates. Both thermodynamic stability and lipid packing density increase, while drug incorporation rates decrease in the following order:

supercooled melt > alpha modification > beta′ modification > beta modification

Because of the small size of the particles and the presence of emulsifiers, lipid crystallization and modification changes might be highly retarded. For example, it has been observed that polymorphic transitions might occur very slowly and that Dynasan 112 SLN, if crystallization is not artificially induced, may remain a supercooled melt over several months [29,62]. Q_{10} nanodispersions remain also stable as supercooled melts over several months [1].

Differential scanning calorimetry and x-ray scattering are widely used to investigate the status of the lipid. Differential scanning calorimetry is based on the fact that different lipid modifications possess different melting points and melting enthalpies. By means of x-ray scattering, it is possible to assess the length of the long and short spacings of the lipid lattice. Measuring the SLN dispersions themselves is highly recommended because solvent removal will lead to modification changes. Sensitivity problems and long measurement times of conventional x-ray sources might be overcome by synchrotron irradiation [62]. In addition, this method allows for conducting time-resolved experiments and allows the detection of intermediate states of colloidal systems that will be undetectable by conventional x-ray methods [56]. Unfortunately, this source has limited accessibility for most investigators.

NMR is a very useful tool for investigating colloidal systems. NMR active nuclei of interest are ^{1}H, ^{13}C, ^{19}F, and ^{31}P. Because of the different chemical shifts, it is possible to attribute the NMR signals to particular molecules or their segments. Simple ^{1}H-NMR spectroscopy permits an easy and very rapid detection of supercooled melts caused by the low line widths of the lipid protons [67]. This method is based on the different proton relaxation times in the liquid and semisolid/solid states. Protons in the liquid state give sharp signals with high signal amplitudes, whereas semisolid/solid protons give weak and broad NMR signals under these circumstances. Simple ^{1}H-NMR spectroscopy also allows the characterization of lipid particles composed of solid and liquid lipids (NLC) [68,69]. The great potential of NMR, with its variety of different approaches (solid-state NMR, determination of self-diffusion coefficients, etc.), has scarcely been used in the SLN field, although it will provide unique insights into the structure and dynamics of SLN dispersions.

Electron spin resonance (ESR) is, as is NMR, a noninvasive method that does not require dilution of the sample. Paramagnetic spin probes are used as model drugs to investigate SLN dispersions. A large variety of spin probes is commercially available. The corresponding ESR spectra give information about the microviscosity and micropolarity. ESR permits the direct, repeatable, and noninvasive characterization of the distribution of the spin probe between the aqueous and the lipid phases. Experimental results demonstrate that storage-induced crystallization of SLN leads to an expulsion of the probe out of the lipid into the aqueous phase [43]. Furthermore,

by using an ascorbic acid reduction assay, it is possible to monitor the timescale of the exchange between the aqueous and the lipid phase. It recently has been shown that, by this method, lipophilic model drugs are not protected from the aqueous environment in NLC and SLN [69].

Similar results to the ESR experiments can be obtained with fluorescence measurements [69]. Further analytical methods include infrared and raman spectroscopy (to study the arrangement of the lipids chains) [70], rheometry (to investigate gel formation [22]), and measurement of the zeta potential (to predict storage stability of colloidal dispersion [71]).

1.6 ADVANTAGES AND DISADVANTAGES OF SLN AND NLC

Clear advantages of SLN include the ease of scaling up, the avoidance of organic solvents, and the high content of nanoparticles. Critical points relate to high pressure–induced drug degradation, the coexistence of different lipid modifications and different colloidal species, the low drug loading capacity, and the kinetics of distribution processes.

HPH might decrease the molecular weight of polymers [25]. High-molecular weight compounds and long-chain molecules are more sensitive than low-molecular weight drugs or molecules with a spherical shape. For example, it was found that HPH causes degradation of DNA and albumin [72].

Lipid crystallization is an important point for the performance of SLN carriers. Lipid crystallization might be delayed, and supercooled melts (nanoemulsions) remain. The crystallization will involve several polymorphic forms with different melting behaviors, different capacities to incorporate foreign molecules (drugs), and different particle shapes. In most cases, these polymorphic transitions have been observed to last over long periods of time and to continue during the storage of the sample. The handling of the sample (changes of temperature, mechanical vibration, or pushing it through a syringe) might catalyze polymorphic transitions and induce gelation of the sample.

The degree of drug incorporation is, in general, low as a result of the crystalline nature of the lipid, with even triglycerides with minor differences in chain length forming separated lipid crystals. In most cases, the investigators did not differentiate between drug incorporation or drug association with the lipid. Lukowski and Pflegel observed by electron diffraction that acyclovir is not molecularly dissolved in the lipid matrix [73]. Bunjes used NMR techniques to characterize the physicochemical state of diazepam, the lipid and the emulsifier [74]. Cryo transmission electron microscopy studies of SLN give clear evidence that PCS sizes of 130 nm correspond to only one to five lipid layers [58]. Therefore, a much higher amount of the drug will be localized directly on the surface of the particles, which is in conflict with the general aim of the SLN systems (drug protection and controlled release caused by the incorporation of the drug in the solid lipid).

NLC have been proposed as the SLN of a new generation, with higher drug loadings and controlled release properties [75,76]. Several structures were proposed, including the presence of oil droplets in a solid lipid matrix (which should combine

high drug loading caused by the liquid lipid and controlled release caused by the solid lipid). Unfortunately, the proposed structures were not backed up by experimental data. Recent studies demonstrate that NLC possess no advantages over nanoemulsions. It has been shown that the liquid lipid forms a half drop on the solid platelet [58,69].

The presence of several colloidal species is an important point that has been overlooked by many scientists. Stabilizing agents are not localized exclusively on the lipid surface but also in the aqueous phase in different forms, which might serve as an alternative location to host the drug molecules. Sometimes the amount of stabilizers exceeds the amount of the lipid phase. For example, stearic acid (as lipid phase), Epikuron 200 (lecithin), and taurocholate have been formulated in the ratio of 3:4:6 [54].

Published NMR spectra of diazepam indicate a high mobility of the drug, which indicates a localization of the drug in other colloidal species of high mobility [74] (an association with the solid lipid would cause extensive line broadening [69]).

Therefore, micelle-forming surfactant molecules (e.g., SDS) will be present in three different forms, namely, on the lipid surface, as micelles, and as monomeric surfactant molecules in solution. Lecithin will form liposomes, which have also been detected in nanoemulsions for parenteral nutrition [77]. Mixed micelles have to be considered in glycocholate/lecithin-stabilized and -related systems. Micelles, mixed micelles, and liposomes are known to solubilize drugs, and are therefore attractive alternative drug-incorporation sites (especially with respect to the low incorporation capacity of lipid crystals).

A more detailed investigation of the SLN and NLC — including the appropriate characterization of drug incorporation, the presence of other colloids, and their *in vivo* fate — is necessary to understand and judge the real potential of these colloids.

ACKNOWLEDGMENTS

We thank K. Jores (Novartis, Basle) and H. Bunjes for helpful discussions and scientific cooperation.

REFERENCES

[1] Westesen, K. (2000) Novel lipid-based colloidal dispersions as potential drug administration systems—expectations and reality. *Coll. Polym. Sci*. 278(7), 608.

[2] Davis, S.S., Washington, C., West, P. and Illum, L. (1987) Lipid emulsions as drug delivery systems. *Ann. N.Y. Acad. Sci*. 507, 75–88.

[3] Graham, C.W., Pagano, R.R. and Katz, R.L. (1977) Thrombophlebitis after intravenous diazepam—can it be prevented? *Anest. Analg*. 56, 409–413.

[4] Levy, M.Y. and Benita, S. (1989) Design and characterization of a submicronized o/w emulsion of diazepam for parenteral use. *Int. J. Pharm*. 54, 103–112.

[5] Janknecht, R., De Marie, S., Bakker-Woudenberg, I.A.J.M. and Crommelin, D.J.A. (1992) Liposomal and lipid formulations of amphotericin B. *Clin. Pharmacokinet*. 23, 279–291.

[6] Nishikawa, M., Takakura, Y. and Hashida, M. (1998) Biofate of fat emulsions. In: S. Benita (Ed.), *Submicron Emulsions in Drug Targeting and Delivery.* Harwood Academic Publishers, Amsterdam, pp. 99–118.
[7] Speiser, P. (1990) Lipidnanopellets als Trägersystem für Arzneimittel zur peroralen Anwendung. European Patent 0167825.
[8] Domb, A.J. (1993) Lipospheres for controlled delivery of substances. United States Patent 5,188,837.
[9] Domb, A.J. (1995) Long acting injectable oxytetracycline-liposphere formulation. *Int. J. Pharm*. 124, 271–278.
[10] Domb, A.J. (1993) Liposphere parenteral delivery system. *Proc. Int. Symp. Control. Rel. Bioact. Mater.* 20, 346–347.
[11] Siekmann, B. and Westesen, K. (1992) Submicron-sized parenteral carrier systems based on solid lipids. *Pharm. Pharmacol. Lett*. 1, 123–126.
[12] Müller, R.H. and Lucks, J.S. (1996) Arzneistoffträger aus festen Lipidteilchen, Feste Lipidnanosphären (SLN). European Patent 0605497.
[13] Müller, R.H., Mehnert, W., Lucks, J.S., Schwarz, C., zur Mühlen, A., Weyhers, H., Freitas, C. and Rühl, D. (1995) Solid lipid nanoparticles (SLN)—an alternative colloidal carrier system for controlled drug delivery. *Eur. J. Pharm. Biopharm*. 41, 62–69.
[14] Sjöström, B. and Bergenståhl, B. (1992) Preparation of submicron drug particles in lecithin-stabilized o/w emulsions. I. Model studies of the precipitation of cholesteryl acetate. *Int. J. Pharm*. 88, 53–62.
[15] Cavalli, R., Caputo, O. and Gasco, M.R. (1993) Solid lipospheres of doxorubicin and idarubicin. *Int. J. Pharm*. 89, R9–R12.
[16] Gasco, M.R. (1993) Method for producing solid lipid microspheres having a narrow size distribution. United States Patent 5,250,236.
[17] Schubert, M.A. and Müller-Goymann, C.C. (2003) Solvent injection as a new approach for manufacturing lipid nanoparticles—evaluation of the method and process parameters. *EJPB.* 55(1), 125.
[18] Müller, R.H. and Runge, S.A. (1998) Solid lipid nanoparticles (SLN®) for controlled drug delivery. In: S. Benita (Ed.), *Submicron Emulsions in Drug Targeting and Delivery.* Harwood Academic Publishers, Amsterdam, pp. 219–234.
[19] Gualbert, J., Shahgaldian, P. and Coleman, A.W. (2003) Interactions of amphiphilic calix[4]arene-based solid lipid nanoparticles with bovine serum albumin. *Int. J. Pharm*. 257(1–2), 69.
[20] Small, D.M., *The Physical Chemistry of Lipids: From Alkenes to Phospholipids*. Plenum Press, New York, 1986.
[21] Ahlin, P., Kristl, J. and Smid-Kobar, J. (1998) Optimization of procedure parameters and physical stability of solid lipid nanoparticles in dispersions. *Acta Pharm*. 48, 257–267.
[22] Lippacher, A., Müller, R.H. and Mäder, K. (2000) Investigation on the viscoelastic properties of lipid based colloidal drug carriers. *Int. J. Pharm*. 196, 227–230.
[23] zur Mühlen, A. and Mehnert, W. (1998) Drug release and release mechanism of prednisolone loaded solid lipid nanoparticles. *Pharmazie* 53, 552–555.
[24] zur Mühlen, A., Schwarz, C. and Mehnert, W. (1998) Solid lipid nanoparticles (SLN) for controlled drug delivery—drug release and release mechanism. *Eur. J. Pharm. Biopharm*. 45, 149–155.
[25] Lander, R., Manger, W., Scouloudis, M., Ku, A., Davis, C. and Lee, A. (2000) Gaulin homogenization—a mechanistic study. *Biotechnol. Prog*. 16, 80–85.

[26] Jahnke, S. (1998) The theory of high pressure homogenization. In: R.H. Müller, S. Benita and B. Böhm (Eds.), *Emulsions and Nanosuspensions for the Formulation of Poorly Soluble Drugs.* Medpharm Scientific Publishers, Stuttgart, pp. 177–200.

[27] Siekmann, B. and Westesen, K. (1994) Melt-homogenized solid lipid nanoparticles stabilized by the nonionic surfactant tyloxapol. I. Preparation and particle size determination. *Pharm. Pharmacol. Lett.* 3, 194–197.

[28] Bunjes, H., Siekmann, B. and Westesen, K. (1998) Emulsions of supercooled melts—a novel drug delivery system. In: S. Benita (Ed.), *Submicron Emulsions in Drug Targeting and Delivery.* Harwood Academic Publishers, Amsterdam, pp. 175–218.

[29] Westesen, K. and Bunjes, H. (1995) Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix? *Int. J. Pharm*. 115, 129–131.

[30] zur Mühlen, A. (1996) Feste Lipid-Nanopartikel mit prolongierter Wirkstoffliberation: Herstellung, Langzeitstabilität, Charakterisierung, Freisetzungsverhalten und -mechanismen, PhD thesis, Free University of Berlin.

[31] Siekmann, B. and Westesen, K. (1996) Investigations on solid lipid nanoparticles prepared by precipitation in o/w emulsions. *Eur. J. Pharm. Biopharm*. 43, 104–109.

[32] De Labouret, A., Thioune, O., Fessi, H., Devissaguet, J.P. and Puisieux F. (1995) Application of an original process for obtaining colloidal dispersions of some coating polymers—preparation, characterization, industrial scaling up. *Drug Dev. Ind. Pharm*. 21, 229–241.

[33] Moulik, S.P. and Paul, B.K. (1998) Structure, dynamics and transport properties of microemulsions. *Adv. Coll. Interf. Sci*. 78, 99–195.

[34] Gasco, M.R. (1997) Solid lipid nanospheres from warm microemulsions. *Pharm. Technol. Eur.* 9, 52–58.

[35] Boltri, L., Canal, T., Esposito, P.A. and Carli, F. (1993) Lipid nanoparticles—evaluation of some critical formulation parameters. *Proc. Int. Symp. Control. Rel. Bioact. Mater.* 20, 346–347.

[36] Cavalli, R., Marengo, E., Rodriguez, L. and Gasco, M.R. (1996) Effect of some experimental factors on the production process of solid lipid nanoparticles. *Eur. J. Pharm. Biopharm*. 43, 110–115.

[37] Gasco, M.R., Morel, S. and Carpigno, R. (1992) Optimization of the incorporation of desoxycortisone acetate in lipospheres. *Eur. J. Pharm. Biopharm*. 38, 7–10.

[38] Schwarz, C. (1995) Feste Lipidnanopartikel—Herstellung, Charakterisierung, Arzneistoffinkorporation und -freisetzung, Sterilisation und Lyophilisation. PhD thesis, Free University of Berlin.

[39] Jenning, V. and Gohla, S. (2000) Comparison of wax and glyceride solid lipid nanoparticles (SLN). *Int. J. Pharm*. 196, 219–222.

[40] Bunjes, H., Koch M.H.J. and Westesen K. (2003) Influence of emulsifiers on the crystallization of solid lipid nanoparticles. *J. Pharm. Sci*. 92, 1509–1520.

[41] Schwarz, C. and Mehnert, W. (1995) Sterilization of drug-free and tetracaine-loaded solid lipid nanoparticles (SLN). Proc. First World Meeting APGI/APV, Budapest, 485–486.

[42] Schwarz, C., Freitas, C., Mehnert, W. and Müller, R.H. (1995) Sterilization and physical stability of drug-free and etomidate-loaded solid lipid nanoparticles. *Proc. Int. Symp. Control. Rel. Bioct. Mater.* 22, 766–767.

[43] Liedtke, S., Zimmermann, E., Müller, R.H. and Mäder, K. (1999) Physical characterisation of solid lipid nanoparticles (SLNTM), *Proc. Int. Symp. Control. Rel. Bioact. Mater.* 26, 599–600.

[44] Lucks, J.S. (1993) Parenterale Fettemulsionen als Arzneistoffträger—Herstellung, Charakterisierung und Stabilität, PhD thesis, Christian Albrechts University of Kiel.

[45] Freitas, C. (1998) Feste Lipid-Nanopartikel (SLN): Mechanismen der physikalischen Destabilisierung und Stabilisierung, PhD thesis, Free University of Berlin.
[46] Cavalli, R., Caputo, O., Carlotti, M.E., Trotta, M., Scarnecchia, C. and Gasco, M.R. (1997) Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. *Int. J. Pharm*. 148, 47–54.
[47] Heiati, H., Tawashi, R. and Phillips, N.C. (1998) Drug retention and stability of solid lipid nanoparticles containing azidothymidine palmitate after autoclaving, storage and lyophilization. *J. Microencapsulation* 15, 173–184.
[48] Rupprecht, H. (1993) Physikalisch-chemische Grundlagen der Gefriertrocknung. In: D. Essig and R. Oschmann (Eds.), *Lyophilisation*. Band 35. Wissenschaftliche Verlagsgesellschaft, Stuttgart, pp. 13–38.
[49] Pikal, M.J., Shah, S., Roy, M.L. and Putman, R. (1990) The secondary drying stage of freeze drying—drying kinetics as a function of temperature and chamber pressure. *Int. J. Pharm*. 60, 203–217.
[50] Schwarz, C. and Mehnert, W. (1997) Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. *Int. J. Pharm*. 157, 171–179.
[51] Crowe, L.M., Crowe, J.H., Rudolph, A., Womersley, C. and Appel, L. (1985) Preservation of freeze-dried liposomes by trehalose. *Arch. Biochem. Biophys*. 242, 240–247.
[52] Siekmann, B. and Westesen, K. (1994) Melt-homogenized solid lipid nanoparticles stabilized by the nonionic surfactant tyloxapol. II. Physicochemical characterization and lyophilization. *Pharm. Pharmacol. Lett*. 3, 225–228.
[53] Zimmermann, E., Müller, R.H. and Mäder, K. (2000) Influence of different parameters on reconstitution of lyophilized SLN. *Int. J. Pharm*. 196, 211–213.
[54] Marengo, E., Cavalli, R., Rovero, G. and Gasco, M.R. (2003) Scale-up and optimization of an evaporative drying process applied to aqueous dispersions of solid lipid nanoparticles. *Pharm. Dev. Technol*. 8(3), 299–309.
[55] Freitas, C. and Müller, R.H. (1998) Spray-drying of solid lipid nanoparticles (SLN). *Eur. J. Pharm. Biopharm*. 46, 145–151.
[56] Laggner, P. (1999) X-ray diffraction of lipids. In: R.J. Hamilton and J. Cast (Eds.), *Spectral Properties of Lipids.* Sheffield Academic Press, Sheffield, pp. 327–367.
[57] Sato, K. (1988) Crystallization of fats and fatty acids. In: N. Garti and K. Sato (Eds.), *Crystallization and Polymorphism of Fats and Fatty Acids.* Marcel Dekker, New York, pp. 227–266.
[58] Jores, K., Mehnert, W., Drechsler, M., Bunjes H., Johann, C. and Mäder, K. (2004) Investigations on the structure of solid lipid nanoparticles (SLN) and oil-loaded solid lipid nanoparticles by photon correlation spectroscopy, field-flow fractionation and transmission electron microscopy. *J. Cont. Rel*. 95(2), 217.
[59] zur Mühlen, A., zur Mühlen, E., Niehus, H. and Mehnert, W. (1996) Atomic force microscopy studies of solid lipid nanoparticles. *Pharm. Res*. 13, 1411–1416.
[60] Dingler, A., Blum, R.P., Niehus, H., Müller, R.H. and Gohla, S. (1999) Solid lipid nanoparticles (SLN/Lipopearls)—a pharmaceutical and cosmetic carrier for the application of vitamin E in dermal products. *J. Microencapsulation* 16, 751–767.
[61] Dingler, A. (1998) Feste Lipid-Nanopartikel als kolloidale Wirkstoffträgersysteme zur dermalen Applikation, PhD thesis, Free University of Berlin.
[62] Westesen, K., Siekmann, B. and Koch, M.H.J. (1993) Investigations on the physical state of lipid nanoparticles by synchroton radiation x-ray diffraction. *Int. J. Pharm*. 93, 189–199.
[63] Anger, S., Caldwell, K.D., Niehus, H. and Müller, R.H. (1999) High resolution size determination of 20 nm colloidal gold particles by SedFFF. *Pharm. Res*. 16, 1743–1747.

[64] Anger, S., Caldwell, K.D. and Müller, R.H. (2000) Charakterisierung von Makromolekülen, Kolloiden und Partikeln mit der Field Flow Fractionation. *Pharm. Ind*. 62, 150–156.

[65] Anger, S., Mehnert, W., Caldwell, K.D. and Müller R.H. (1998) High resolution determination of adsorption layer mass on particles by sedimentation field-flow fractionation. Proc. Second World Meeting APGI/APV, Paris, 643–644.

[66] Anger, S., Caldwell, K.D., Mehnert W. and Müller, R.H. (1999) Coating of nanoparticles—analysis of adsorption using sedimentation field-flow fractionation (SdFFF), *Proc. Int. Symp. Control. Rel. Bioact. Mater.* 26, 599–600.

[67] Jenning, V. (1999) Feste Lipid Nanopartikel als Trägersystem für die dermale Applikation von Retinol, PhD thesis, Free University of Berlin.

[68] Zimmermann, E., Liedtke, S., Müller, R.H. and Mäder, K. (1999) H-NMR as a method to characterize colloidal carrier systems. *Proc. Int. Symp. Control. Rel. Bioact. Mater.* 26, 595–596.

[69] Jores, J., Mehnert, W. and Mäder, K. (2003) Physicochemical investigations on solid lipid nanoparticles and on oil-loaded solid lipid nanoparticles—a nuclear magnetic resonance and electron spin resonance study. *Pharm. Res*. 20(8), 1274–1283.

[70] Kobayashi, M. (1988) Vibrational spectroscopic aspects of polymorphism and phase transition of fats and fatty acids. In: N. Garti and K. Sato (Eds.), *Crystallization and Polymorphism of Fats and Fatty Acids*. Marcel Dekker, New York, pp. 139–188.

[71] Müller, R.H. (1996) *Zetapotential und Partikelladung in der Laborpraxis—Einführung in die Theorie, praktische Meßdurchführung, Dateninterpretation*. Wissenschaftliche Verlagsgesellschaft, Stuttgart.

[72] Weyers, H. (1995) Feste Lipid Nanopartikel (SLN) für die gewebsspezifische Arzneistoffapplikation—Herstellung und Charakterisierung oberflächenmodifizierter Formulierungen, PhD thesis, Free University of Berlin.

[73] Lukowski, G. and Pflegel, P. (1997) Electron diffraction of solid lipid nanoparticles loaded with aciclovir. *Pharmazie* 52, 642–643.

[74] Bunjes, H. (1998) Einflussnahme unterschiedlicher Faktoren auf Struktur und Eigenschaften von Nanopartikeln aus festen Triglyceriden, PhD thesis, Friedrich Schiller University of Jena.

[75] Müller, R.H., Radtke, M. and Wissing, S.A. (2002) Nanostructured lipid matrices for improved microencapsulation of drugs. *Int. J. Pharm*. 242(1–2), 121.

[76] Müller, R.H., Radtke, M. and Wissing, S.A. (2002) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv. Drug Deliv. Rev.* 54(Suppl. 1), S131–S155.

[77] Siekmann, B. and Westesen, K. (1998) Submicron lipid suspensions (solid lipid nanoparticles) versus lipid nanoemulsions—similarities and differences. In: S. Benita (Ed.), *Submicron Emulsions in Drug Targeting and Delivery.* Harwood Academic Publishers, Amsterdam, pp. 205–218.

2 Production of Lipospheres for Bioactive Compound Delivery

Elisabetta Esposito, Rita Cortesi, and Claudio Nastruzzi

CONTENTS

2.1 INTRODUCTION

One approach for increasing the beneficial action of drugs and decreasing systemic adverse effects is to deliver the necessary amount of drugs to the diseased sites, where they are most needed, for the appropriate period of time [1–3].

Although the drug delivery system concept is not new, great progress has recently been made in the treatment of a variety of diseases. Particulate carriers (e.g., polymeric nano- and microparticles, fat emulsion, and liposomes) possess specific advantages and disadvantages. For instance, in the case of polymeric microparticles, the degradation of the polymer might possibly cause systemic toxic effects through the impairment of the reticuloendothelial system [4] or by accumulation at the injection site [5]; cytotoxic effects have indeed been observed *in vitro* after phagocytosis of particles by human macrophages and granulocytes [6]. In addition, organic solvent residues deriving from the preparation procedures, such as the solvent evaporation technique often used for liposome [7] and polyester microparticles [8], can be present in the delivery system and could result in severe acceptability and toxicity problems [9].

0-8493-1692-8/05/$0.00+$1.50

To solve these adverse effects, lipid microspheres, often called lipospheres (LS), have been proposed as a new type of fat-based encapsulation system for drug delivery of bioactive compounds (especially lipophilic compounds). LS consist of solid microparticles with a mean diameter usually between 0.2 and 500 μm, composed of a solid hydrophobic fat matrix in which the bioactive compounds are dissolved or dispersed [10–12]. LS have some advantages over other delivery systems, such as good physical stability, low cost of ingredients, ease of preparation and scale-up, and high entrapment yields for hydrophobic drugs. Because of their large range in particle size, LS can be administered by different routes — such as orally, subcutaneously, intramuscularly, or topically — or they can be used in cell encapsulation, thus allowing them to be proposed for treatment of a number of diseases [13–15]. For instance, the *in vivo* distribution of LS demonstrated a high affinity to vascular wells (including capillaries), inflamed tissues, and granulocytes [16–17].

LS have been used for the controlled delivery of various types of drugs, including vasodilator and antiplatelet drugs, antiinflammatory compounds, local anesthetics, antibiotics, and anticancer agents; they have also been used successfully as carriers of vaccines and adjuvants [18].

This chapter will discuss (a) the production and characterization of LS formed by the melt dispersion technique, by the solvent evaporation method, and by the water/oil/water (w/o/w) double-emulsion method; (b) the influence of preparation parameters on liposphere morphology; and (c) the encapsulation efficiency and the release characteristics of two lipophilic model drugs, such as retinyl acetate and progesterone, and one hydrophilic drug, sodium cromoglycate (SCG), from the prepared LS.

For a biocompatible formulation suitable for human administration, triglycerides and monoglycerides have been chosen as the biomaterials for LS because of their high biocompatibility, high physicochemical stability, and drug delivery release. LS were prepared by two alternative approaches, namely, the melt dispersion and the solvent evaporation techniques (Figure 2.1 and Table 2.1).

2.2 MELT DISPERSION TECHNIQUE

The choice of the lipid matrix plays an important role in the morphology of the particles and in the possible formation of aggregates. In a first set of experiments, LS were prepared by the melt dispersion technique, using a lipid mixture constituted of cetyl alcohol and cholesterol (2:1, w/w) and gelatin as the stabilizer. Gelatin was selected from among eight natural or synthetic emulsifiers, namely, gelatin (200 Bloom), pectin, carrageenan κ, carrageenan ι, carrageenan λ, polyvinyl alcohol (PVA), polyoxyethylene 20 sorbitan trioleate (polysorbate 85, Tween 85), and lauryl sarcosine.

The lipidic mixture, both with and without a lipophilic model drug, was melted at 70°C and then emulsified into an external aqueous phase containing a suitable surfactant. The emulsion was mechanically stirred by a stirrer equipped with alternative impellers. Afterward, the emulsion was heated to the same temperature as the melted lipidic phase. The milky formulation was then rapidly cooled to about 20°C by immersing the formulation flask in a cool water bath without stopping the

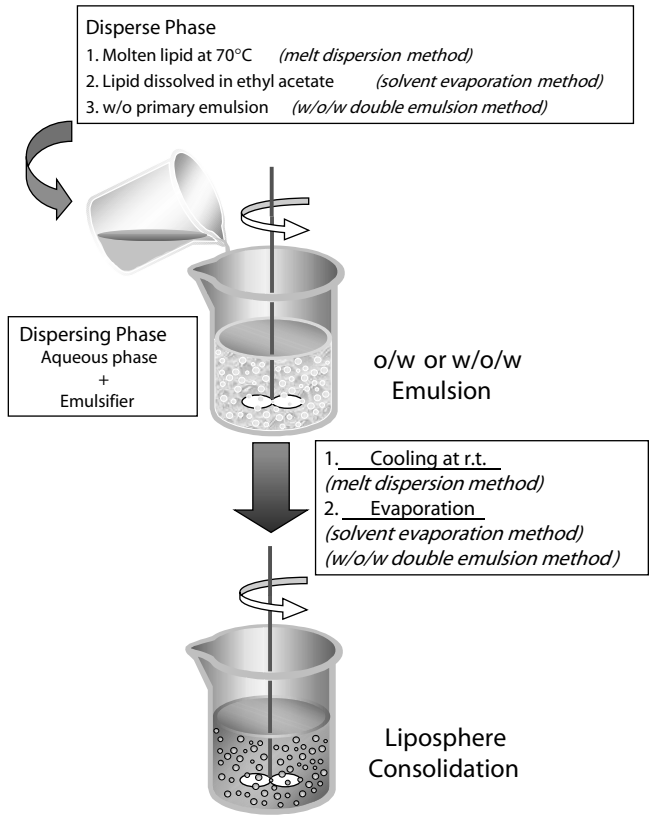

FIGURE 2.1 Schematic representation of the methods of production of LS: melt dispersion and solvent evaporation.

agitation to yield a uniform dispersion of LS. The obtained LS were then washed with water and isolated by filtration through a paper filter.

The morphology of LS was evaluated by optical microscopy (Nikon Diaphot inverted microscope) and scanning electron microscopy observations (Cambridge Stereoscan 360). Microsphere size distributions were determined by photomicrograph analyses, analyzing at least 300 microparticles per sample.

The resulting microparticles (Figure 2.2 and Table 2.2) were characterized by an irregular surface; in addition, some aggregates caused by the fusion of lipid droplets before solidification were present. An improvement of LS features was obtained in terms of recovery, mean diameter, and aggregate formation by decreasing the molecular weight of the gelatin used as a stabilizer. The viscosity of the dispersing phase was, in fact, progressively reduced, passing from gelatin 50 to gelatin 250 Bloom grades.

By adjusting the stirring speed during the emulsification process, it was possible to modify the size of the particles. Increasing the stirring speed from 500 to 1000 rpm, the mean diameter of particles progressively decreased (Table 2.3). With the aim of

TABLE 2.1
Overview of Liposphere Preparation Methods

Method	Process Duration (h)	Stirring Speed (rpm)	Dispersion Medium	Disperse Phase	Particle Isolation
Melt dispersion	1 with rapid cool up to 20°C	500, 750, 1000	Water (150 mL) plus stabilizer[a]	Melted lipid (5 g) at 70°C	Filtration through a glass filter[b]
o/w solvent evaporation	6–8 at room temperature	500, 750, 1000	Water (150 mL) plus polyvinyl alcohol as stabilizer	Dissolved lipids (5 g) in ethyl acetate (10 mL) at 50°C	Filtration through a glass filter[b]
w/o/w double emulsion	3–5 at room temperature	500, 750, 1000	Water (150 mL) plus 0.25% (weight/volume) polyvinyl alcohol as stabilizer	w/o emulsion of melted lipids (5 g) at 70°C stabilized with gelatin or poloxamer 407	Filtration through a glass filter[b]

[a] See Table 2.8.
[b] Glass filters with a maximum nominal pore size of 10–16 µm.

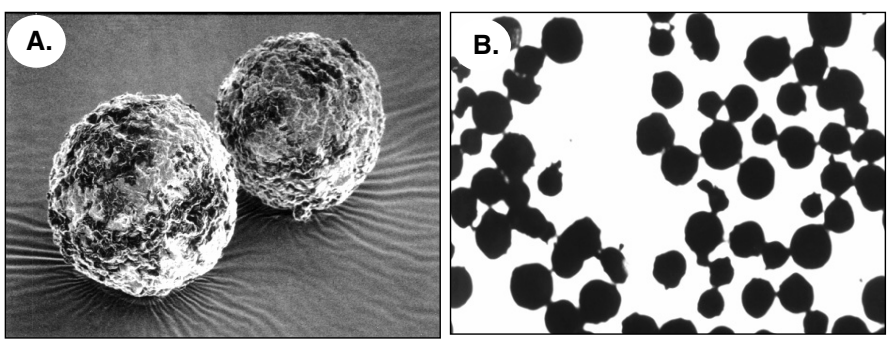

FIGURE 2.2 Effect of gelatin Bloom on morphology and particle size of cetyl alcohol: cholesterol 2:1 (w/w) lipospheres. Scanning electron microscopy **(A)** and optical micrographs **(B)** of microspheres produced with gelatin 200 Bloom grades. Bar corresponds to 76 and 381 µm in panels A and B, respectively.

further improving the characteristics of LS, alternative lipid compositions were considered. For instance, LS were prepared with apolar triglycerides, such as tristearin, tripalmitin, or tribehenin, in combination with other polar (more hydrophilic) lipids, including glyceryl monostearate, glyceryl monooleate, cetyl alcohol, and cholesterol (Table 2.4). As a general consideration, all formulations, apart from those including cholesterol and glyceryl monooleate, were satisfactory in terms of shape, recovery, and size (Figure 2.3 and Figure 2.4).

TABLE 2.2
Effect of Gelatin Type on Liposphere Characteristics

Gelatin (Bloom grades)	Lipid Composition (w/w ratio)	Recovery (%)[a]	Mean Diameter (μm)
250	Cetyl alcohol/cholesterol (2:1)	52	250 ± 12
200	Cetyl alcohol/cholesterol (2:1)	60	205 ± 15
150	Cetyl alcohol/cholesterol (2:1)	61	197 ± 8
50	Cetyl alcohol/cholesterol (2:1)	82	150 ± 21

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor; a 5% gelatin solution; and a 750-rpm stirring speed. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

TABLE 2.3
Effect of Stirring Speed on Characteristics of Cetyl Alcohol/Cholesterol Lipospheres

Stirring Speed (rpm)	Gelatin (Bloom grades)	Recovery (%)[a]	Mean Diameter (μm)
500	50	92	250 ± 14
750	50	82	150 ± 16
1000	50	82	80 ± 24

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor and a 5% gelatin solution. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

Other experiments were undertaken to evaluate the effect of glyceryl monostearate concentration on LS characteristics. Glyceryl monostearate was used in mixture with tristearin (Table 2.5) or tripalmitin (Table 2.6) at different weight ratios. Tables 2.5 and 2.6 report the results of such experiments, in which the percentage of glyceryl monostearate was varied from 0% up to 33% (w/w). In the case of tripalmitin, it was impossible to produce LS without the presence of at least 1% glyceryl monostearate (because of the formation of large blobs), whereas pure tristearin particles were obtained, even if they were of poor quality. By increasing the content of glyceryl monostearate, a progressive decrease in LS size was evident. On the contrary, no effect was detectable on LS recovery.

TABLE 2.4
Effect of Lipid Composition on the Production of Lipospheres

Lipid Composition (w/w ratio)	Recovery (%)[a]	Mean Diameter (μm)
Tristearin:monostearate (2:1)	90	170 ± 19
Tristearin:cetyl alcohol (2:1)	92	200 ± 26
Tristearin:cholesterol (2:1)	98	250 ± 16
Tripalmitin:monostearate (2:1)	82	250 ± 8
Tripalmitin:monooleate (2:1)		Fused mass
Tripalmitin:cholesterol (2:1)		Fused mass
Tripalmitin:cetyl alcohol (2:1)	96	300 ± 15
Tribehenin:monostearate (2:1)	75	200 ± 22

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor; a 1% polyvinyl alcohol solution; and a 750-rpm stirring speed. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

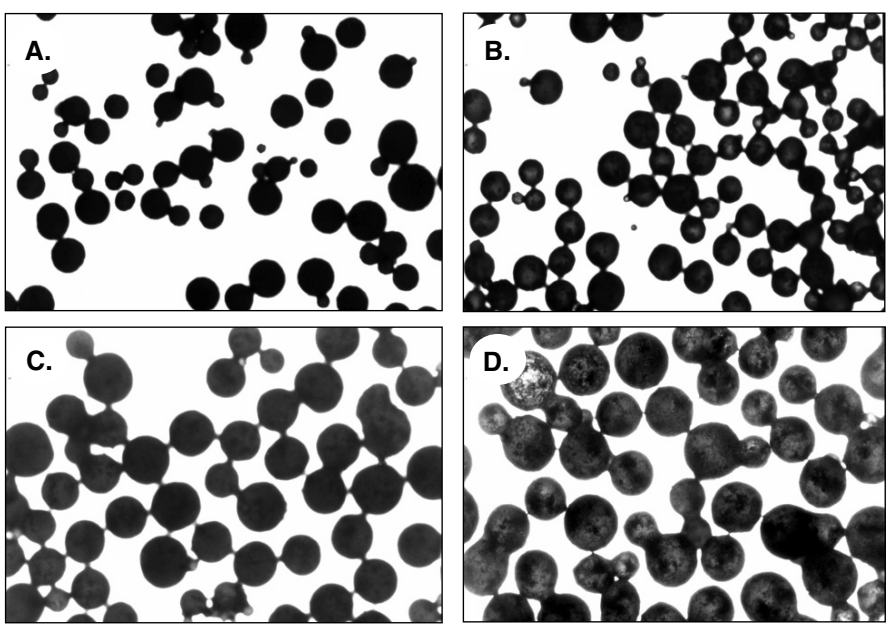

FIGURE 2.3 Effect of lipid composition on the morphology of lipospheres. Lipospheres were prepared with **(A)** cetyl alcohol:cholesterol 2:1 (w/w), **(B)** tripalmitin:glyceryl monostearate 2:1 (w/w), **(C)** tristearin:glyceryl monostearate 2:1 (w/w), and **(D)** tripalmitin:glyceryl monostearate 9:1 (w/w). Bar corresponds to 533, 812, 315, and 487 μm in panels A, B, C, and D, respectively.

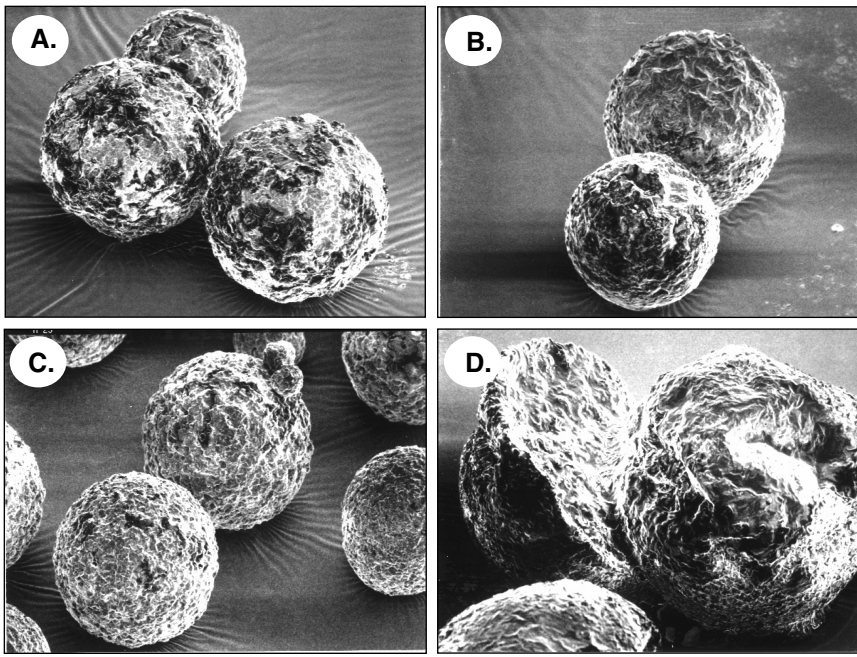

FIGURE 2.4 Scanning electron microscopy photographs showing the effect of lipid composition on the morphology of lipospheres. Lipospheres were prepared with **(A)** tristearin:glyceryl monostearate 2:1 (w/w), **(B)** tristearin:cetyl alcohol 2:1 (w/w), **(C)** tripalmitin:glyceryl monostearate 2:1 (w/w), and **(D)** tripalmitin:cetyl alcohol 2:1 (w/w). Bar corresponds to 67, 87, 101, and 76 μm in panels A, B, C, and D, respectively.

TABLE 2.5
Effect of Monostearate on the Production of Tristearin Lipospheres

Lipid Composition (w/w ratio)	Recovery (%)[a]	Mean Diameter (μm)
Tristearin:glyceryl monostearate 100:0	76	220 ± 31
Tristearin:glyceryl monostearate 98:2	93	200 ± 23
Tristearin:glyceryl monostearate 95:5	90	170 ± 18
Tristearin:glyceryl monostearate 90:10	89	160 ± 15
Tristearin:glyceryl monostearate 80:20	73	158 ± 28
Tristearin:glyceryl monostearate 66:33	90	170 ± 19

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor; a 750-rpm stirring speed; and 1% polyvinyl alcohol solution as the dispersing phase. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

TABLE 2.6
Effect of Monostearate on the Production of Tripalmitin Lipospheres

Lipid Composition (w/w ratio)	Recovery (%)[a]	Mean Diameter (μm)
Tripalmitin:glyceryl monostearate 100:0	Fused mass	
Tripalmitin:glyceryl monostearate 99:1	96	300 ± 9
Tripalmitin:glyceryl monostearate 95:5	75	300 ± 16
Tripalmitin:glyceryl monostearate 90:10	72	300 ± 31
Tripalmitin:glyceryl monostearate 66:33	82	250 ± 21

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor; a 750-rpm stirring speed; and 1% polyvinyl alcohol solution as the dispersing phase. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

The choice and the adjustment of the manufacturing parameters for the production of microspheres of defined size were performed in agreement with the following equation

$$d \propto K \frac{D_v R \upsilon_a \gamma}{D_s N \upsilon_o C_s} \tag{2.1}$$

where

d is the average particle size
K is a variable depending on the apparatus geometry (e.g., type and dimension of stirrer)
D_v and D_s are the diameter of the vessel and of the stirrer, respectively
R is the volume ratio between aqueous and oil phases
υ_a and υ_o are their respective viscosities
N is the stirring speed
γ is the surface tension between the two immiscible phases
C_s is the stabilizer concentration [19]

The influence of some parameters, such as stirring conditions and stabilizer type and concentration, was studied on morphology, mean diameter, dimensional distribution, and recovery of microparticles.

2.2.1 Effect of the Stirring Conditions

The effect of the stirring speed was considered on the production of LS (Table 2.7 and Figure 2.5). LS with dimensions between 90 and 170 μm were obtained by changing the stirring speed from 500 to 1000 rpm. In particular, particles obtained at 1000 rpm presented a spherical geometry with a narrow size distribution; in

TABLE 2.7
Effect of Stirring on Tristearin Liposphere Characteristics

Stirring Speed (rpm)	Impeller Type	Recovery (%)[a]	Mean Diameter (μm)
500	3-blade rotor	90	170 ± 35
750	3-blade rotor	78	120 ± 24
750	4-blade helicoidal rotor	64	50 ± 8
750	double-truncated cone rotor	77	55 ± 19
750	2-blade helicoidal rotor	elliptical particles and filaments	
1000	3-blade rotor	69	90 ± 19

Note: The dispersing phase was a 1% polyvinyl alcohol solution. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

addition, aggregation phenomena were almost absent. The recovery efficiency of particles produced at the higher stirring speed was 69%, whereas for those produced at 500 rpm, it was over 90%.

LS were produced by means of different impellers, namely, (a) a 3-blade rotor with a diameter of 55 mm (taken as reference impeller), (b) a 4-blade helicoidal rotor with a diameter of 50 mm, (c) a double-truncated cone rotor with a diameter of 50 mm, and (d) a 2-blade rotor with a diameter of 50 mm (Figure 2.5, lower panel). The use of rotors (b) and (c) allowed us to obtain smaller particles, with mean diameters of 50 and 55 μm, and a recovery of 64% and 77%, respectively (Table 2.7). However, the use of rotor (d) did not allow the production of lipid particles; in fact, this particular impeller caused the formation of elliptical particles and filaments.

2.2.2 Effect of the Stabilizer Type and Concentration

The effects of different stabilizers on particle morphology and recovery were tested (Table 2.8). As is clearly appreciable from the obtained results, the addition of emulsifiers leads to variable effects on the size of LS droplets during the emulsification step, thus influencing the final microspheres' size. In particular, LS obtained with natural polymers, such as gelatin, pectin, and carrageenans κ and ι allowed the production of spherical particles with an irregular surface and a mean diameter between 150 and 250 μm. In the case of carrageenan λ, it was not possible to isolate the particles because the high viscosity of the suspension did not allow the filtration process. On the contrary, in the cases of gelatin and other carrageenans, the high viscosity was compatible with the separation process, even if it was caused by a lower recovery efficiency (between 62 and 69%) compared to pectin (80%).

The use of synthetic emulsifiers gave different results; for instance, the use of 1% (w/w) of the polyoxyethylene–polyoxypropylene block copolymer Pluronic PE

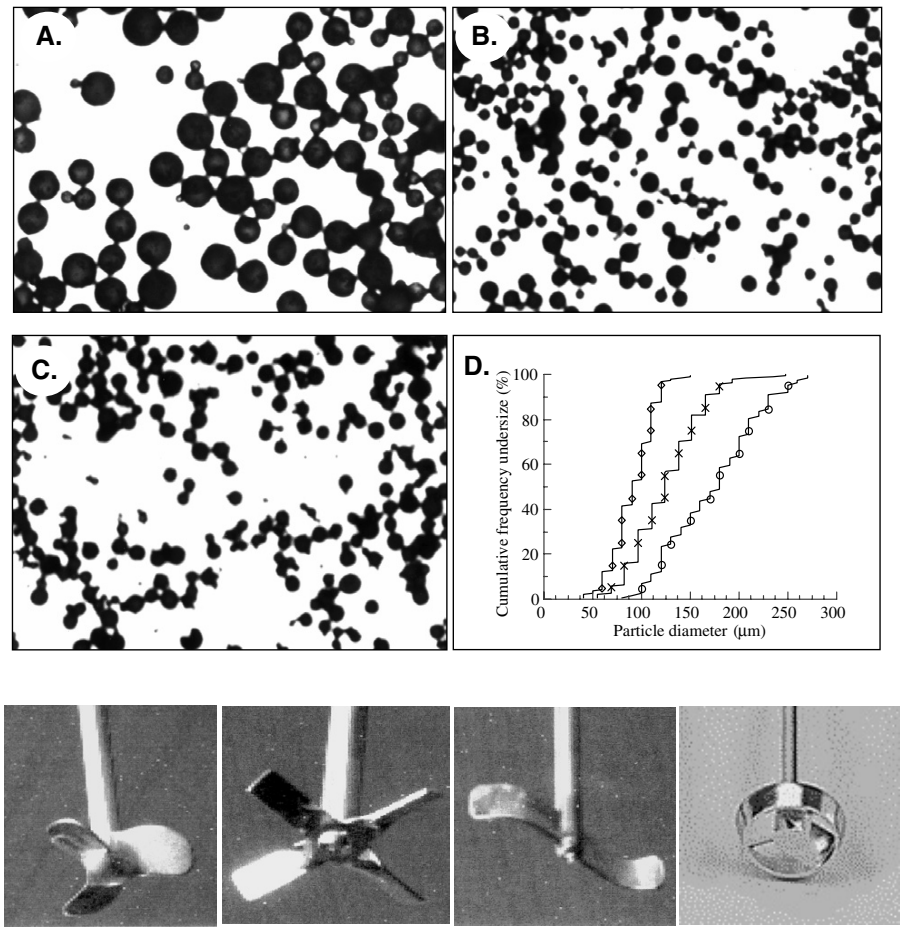

FIGURE 2.5 Optical microscopy photographs showing the effect of stirring speed on morphology and particle size of tristearin: monostearate 2:1 (w/w) produced at **(A)** 500 rpm, **(B)** 750 rpm, and **(C)** 1000 rpm. Bar corresponds to 650, 650, and 347 μm in panels A, B, and C, respectively. **(D)** Frequency distribution plot of microspheres produced at 500 rpm (○), 750 rpm (x), and 1000 rpm (◇). Data are the mean of three different microsphere batches. **Lower panel:** impellers employed for microsphere production, from left to right: a 3-blade rotor with a diameter of 55 mm (taken as reference impeller), a 4-blade helicoidal rotor with a diameter of 50 mm, a 2-blade rotor with a diameter of 50 mm, and finally a double-truncated cone rotor with a diameter of 50 mm.

8100 did not allow the stabilization of the o/w emulsion during the preparation, resulting in the formation of large lipid aggregates. The use of 1% polyoxyethylene sorbitan trioleate or PVA allowed the production of spherical particles with mean diameters of 190 and 120 μm and recoveries of 54% and 78%, respectively. Finally, lauryl sarcosine caused the formation of a very fine o/w emulsion, resulting in the final formation of very small particles (mean diameter 10 ± 4.1 μm) that were isolated

TABLE 2.8
Effect of Stabilizer on Tristearin Liposphere Characteristics

Stabilizer (%, w/v)	Recovery (%)[a]	Mean Diameter (μm)
Gelatin 200 Bloom (8)	69	150 ± 33
Pectin (0.5)	80	250 ± 17
Carrageenan κ (0.5)	62	200 ± 25
Carrageenan ι (0.5)	65	200 ± 31
Carrageenan λ (0.5)	Compromised separation	
Polyvinyl alcohol (1)	78	120 ± 26
Polyoxyethylene sorbitan trioleate (1)	54	190 ± 18
Pluronic PE 8100 (1)	Lipid aggregation	
Lauryl sarcosine (1)	—	10 ± 41

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor and a 750-rpm stirring speed. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

by centrifugation. Further studies are in progress to evaluate the experimental parameters for the production of lipid nanoparticles.

2.3 SOLVENT EVAPORATION TECHNIQUE

As an alternative to the melt dispersion technique, a solvent evaporation method was also tested for the production of LS (Figure 2.1). This approach was considered with the aim of possibly reducing the exposure to the high temperatures of thermolabile compounds, such as proteins and nucleic acids. The solvent evaporation method is based on the evaporation of the organic solvent in which lipids are dissolved, allowing the formation of solid microparticles. Through this technique, LS constituted of tristearin:glyceryl monostearate 2:1 w/w were produced, with 1% PVA as the emulsifier agent. In particular, the lipidic matrix dissolved in an organic solvent such as ethyl acetate at 50°C was emulsified in an external aqueous phase containing the surfactant agent. The resulting oil-in-water emulsion was stirred for 6 to 8 h under ambient conditions to allow the solvent evaporation. LS, after the water rose, were collected by filtration through a paper filter.

The obtained particles (Figure 2.6) were spherical and were characterized by their smaller size with respect to the particles of the same composition that were produced by the melt dispersion technique. Unfortunately, the produced LS showed some poor mechanical properties, including fragility, as well as a higher proportion of interparticellar bridges when compared with the melt dispersion technique.

With the aim of improving the mechanical properties of LS produced by solvent evaporation, as well as the aim of obtaining prolonged-release profiles, the possibility

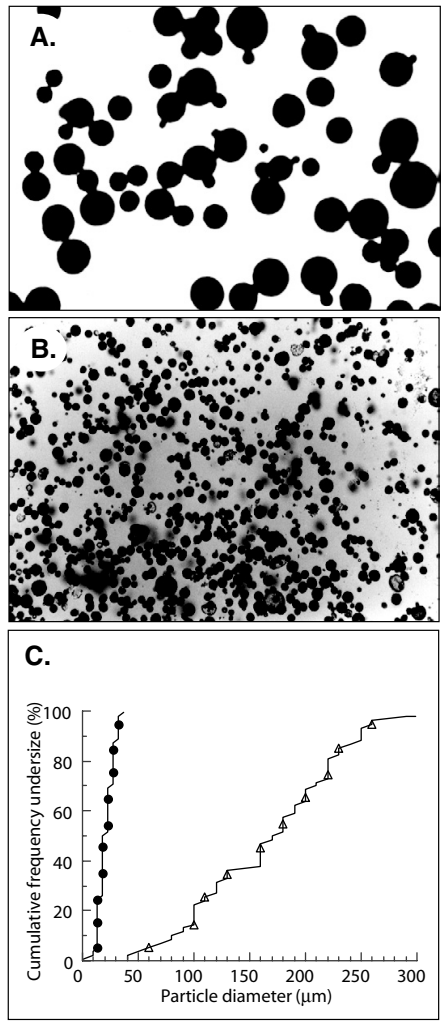

FIGURE 2.6 Effect of the type of the method of preparation on morphology and particle size of lipospheres. Optical micrographs of microspheres produced by **(A)** melt dispersion technique and **(B)** solvent evaporation technique. Lipospheres were constituted of tristearin:glyceryl monostearate 2:1 (w/w) and prepared in the presence of 1% polyvinyl alcohol. Bar corresponds to 292 and 162 μm in panels A and B, respectively. **(C)** Frequency distribution plot of microspheres produced by melt dispersion (△) and solvent evaporation (●) technique. Data are the mean of three different microsphere batches.

of producing particles with a mixed matrix was considered (Table 2.9). LS were produced using lipids in combination with different polymers, in a ratio of up to 20% with respect to the lipid components. Both biodegradable polymers, such as polylactic acid (PLA), and nonbiodegradable polymers, such as Eudragit RS 100, were used. The different polymers allowed the improvement of the mechanical characteristics of the LS and, particularly in the case of Eudragit RS 100, allowed

TABLE 2.9
Effect of Synthetic Polymers on the Production of Lipospheres by Solvent Evaporation

Microparticle Composition (w/w ratio)	Stabilizer (%, w/v)	Recovery (%)[a]	Stirring Speed (rpm)
Tristearin:monostearate (66:34)	PVA (1)	20 ± 4.4	750
Tristearin:monostearate (66:34)	PVA (1)	165 ± 6.2	500
Tristearin:monostearate (66:34)	PVA (0.5)	n.d.	250
Tristearin:monostearate (66:34)	PVA (0.25)	n.d.	250
Tristearin:monostearate:PLA (52:28:20)	PVA (1)	50 ± 11	750
Tristearin:monostearate:PLA (52:28:20)	PVA (0.1)	n.d.	250
Tristearin:monostearate:Eudragit RS (52:28:20)	PVA (1)	15 ± 3.6	750
Tristearin:monostearate:Eudragit RS (52:28:20)	PVA (1)	n.d.	250
Tristearin:monostearate:Eudragit RS (52:28:20)	PVA (0.1)	50 ± 12	500
Tristearin:monostearate:Eudragit RS (52:28:20)	PVA (0.1)	100 ± 9	250

Note: Common experimental parameter was a 55-mm, 3-blade turbine rotor. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation. n.d. = not determined; PVA = polyvinyl alcohol.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

the production of more spherical particles with a narrow size distribution and a mean diameter of 15 μm; in addition, interparticle fusion phenomena were almost absent.

2.4 DRUG-CONTAINING LS

Two hydrophobic compounds, such as retinyl acetate and progesterone, and one hydrophilic drug, SCG, were considered as model drugs. LS were produced by the melt dispersion technique.

LS containing hydrophobic retinyl acetate were yellow (because of the color of the drug) and spherical, with a slightly waved surface (Figure 2.7) and a narrow size distribution (184 ± 6.6 μm). Particles containing progesterone were very similar in shape (data not shown) and were white (mean diameter, 192 ± 11.6 μm).

The amount of encapsulated model drug (retinyl acetate or progesterone) per mg of dried LS was determined through solubilization of LS in ethyl acetate at 60°C. Following filtration, the solution was analyzed by reverse-phase high-performance liquid chromatography (HPLC) to find the drug content.

The HPLC determinations were performed using an HPLC system operating at 215 nm. Samples were chromatographed on a stainless steel C18 reverse-phase column eluted isocratically at room temperature, at a flow rate of 1 mL/min. The mobile phases were 180 mM ammonium acetate (pH 3.0)/methanol (4:96, v/v) for retinyl acetate [20], methanol/water (70:30, v/v) for progesterone [21], and phosphate buffer (pH 2.3)/methanol (50:50, v/v) for cromoglycate [22]. Drug detection was monitored at the λ_{max} characteristic of each compound.

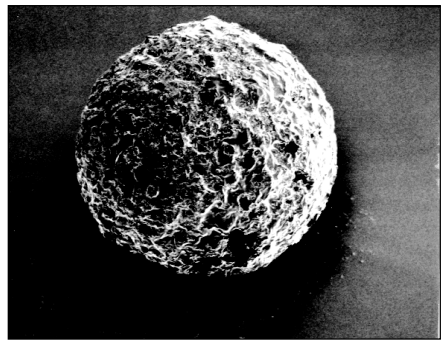

FIGURE 2.7 Scanning electron micrographs of tristearin:glyceryl monostearate 66:33 (w/w) lipospheres containing retinyl acetate. Bar corresponds to 48 μm.

TABLE 2.10
Drug Encapsulation Efficiency and Recovery of Lipospheres

Drug	Encapsulation Yield (%)	Recovery (%)[a]
Retinyl acetate	87.4 ± 1.5	85.4
Progesterone	70.7 ± 2.1	93.0
Sodium cromoglycate (o/w)	2.0 ± 0.6	67.0
Sodium cromoglycate (o/w)	3.0 ± 1.6	63.4
Sodium cromoglycate (w/o/w, gelatin)	22.0 ± 2.4	72.7
Sodium cromoglycate (w/o/w, gelatin)	50.0 ± 8.1	81.0
Sodium cromoglycate (w/o/w, poloxamer)	12.0 ± 3.2	77.0

Note: Experimental parameters were a 55-mm, 3-blade turbine rotor and a 500-rpm stirring speed. Data represent the average of 3 independent experiments on different microsphere batches ± standard deviation.

[a] Percentage (w/w) of liposphere production with respect to the total amount of lipids used for preparation.

As reported in Table 2.10, both LS types are characterized by high encapsulation and recovery efficiencies. In the case of the hydrophilic drug SCG, again the microparticles were morphologically almost identical (data not shown) (mean diameter 234 ± 14.8 μm), but the encapsulation efficiency was, on the contrary, unsatisfactory, being only 2%. This result was partially expected, as hydrophilic drugs can be less efficiently incorporated in LS with respect to hydrophobic ones. In fact, if the drug is too hydrophilic to be soluble in organic solvents, microcrystalline fragments of the drug could be incorporated in the microparticle. The water-soluble drug could then diffuse into the outer continuous aqueous phase, resulting in low trapping of the compound and inducing an initial rapid release of the drug known as "burst effect."

To improve the encapsulation of SCG, LS were produced using a w/o/w double-emulsion strategy, consisting of the solubilization of the drug to be encapsulated in the internal aqueous phase of a w/o/w double emulsion, along with a stabilizer that was able to prevent the loss of drug to the external phase during solvent evaporation [23]. In particular, an aqueous solution of the drug was emulsified in melted lipids at 70°C by an Ultra-Turrax. This primary emulsion was stabilized adding gelatin (250 Bloom grades) or the polyoxyethylene–polyoxypropylene block copolymer, poloxamer 407, as stabilizers solubilized in the aqueous phase. The primary emulsion was then dispersed at 70°C into an aqueous phase containing 0.25% (w/v) of PVA. The obtained double emulsion was stirred at 300 rpm by a four-blade turbine impeller. After 3 to 5 h, microparticles were isolated by filtration.

In particular, the effects of various stabilizers of the primary emulsion on the encapsulation of SCG were studied. Different hydrophilic polymers were employed; namely gelatin (250 Bloom grades) or the polyoxyethylene–polyoxypropylene block copolymer, poloxamer 407. To further optimize the encapsulation yield, some experimental contrivances have been performed: dispersion by turbine of the drug within the lipidic matrix, rapid emulsion cooling using an ice bath, and rapid separation of LS by filtration. The optimized procedure resulted in a final encapsulation of the drug of 50% (Table 2.10).

2.5 *IN VITRO* DRUG RELEASE

To obtain quantitative and qualitative information on drug release from the LS, and possibly to correlate the experimental data with the release mechanism, the complete release profile of LS encapsulated drugs was determined by placing a drug containing LS in a buffer under magnetic stirring at 150 rpm. The buffer was ethanol/water in a 30:70 ratio, with the addition of 0.5% (w/w) polysorbate 20. Following different lengths of time (0 to 8 h), samples of receiving buffer were filtered and analyzed for drug content by reverse-phase HPLC, as previously described.

In Figure 2.8, the release kinetics of retinyl acetate and progesterone (panel A) and of SCG (panel B) is reported. As is noticeable, the release of both hydrophobic drugs was much slower with respect to SCG, especially in the case of retinyl acetate containing LS. For these particles, the drug release efficacy within the first 8 h of release was 27% of the total amount of the drug. In the same period, the amount of progesterone released was 63%. This behavior could be ascribed to the physicochemical characteristics of the drugs, which, as expected, showed a high affinity for the oil phase instead of the aqueous one.

Concerning sodium cromoglycate containing LS, the release of the drug was largely influenced by the type of stabilizer used in the primary emulsion. In the case of LS produced in the presence of gelatin, the shape of the release has a sigmoid form, and, after 8 h, the release reaches 80% of the total amount of the drug. Conversely, LS produced in the presence of poloxamer 407 shows a drug release typified by a biphasic profile. The first part, characterized by rapid drug release, is followed by a slower release rate, during which the drug is released in an approximately

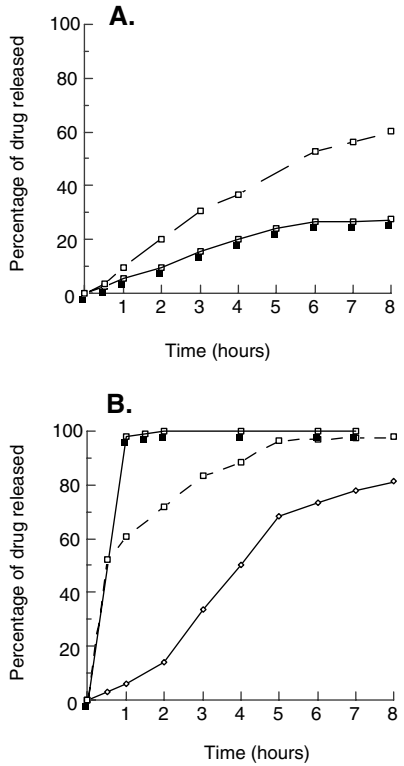

FIGURE 2.8 Release profiles of drugs encapsulated in lipospheres. **(A)** Lipophilic compounds, such as retinyl acetate (■) and progesterone (□). **(B)** Hydrophilic compounds, such as sodium cromoglycate, encapsulated using gelatin (◇) or poloxamer 407 (□) as the stabilizer. As a reference, the release of free SCG is also reported (■). The releases were determined by dialysis method. Data represent the average of five independent experiments on different microsphere batches.

linear mode. In addition, it should be emphasized that the release of cromoglycate reaches 100% of the total amount of the drug 5 h after the experiment begins.

2.6 CONCLUSIONS

Melt dispersion, solvent evaporation, and w/o/w double-emulsion methods enabled us to produce LS whose morphology and size were influenced by the experimental parameters employed.

In particular, in the case of LS prepared by melt dispersion, the use of different lipid mixtures, types of stabilizer, and stirring speeds affected both microparticle shapes and their size distribution. The use of lauryl sarcosine as the stabilizer allowed the formation of very small LS; further experiments will be performed to better investigate the experimental parameters involved in the production of very small LS.

LS, under appropriate experimental conditions, can entrap both hydrophobic and hydrophilic drugs and can control the release of the encapsulated drug. The encouraging results obtained in this study could propose LS for future *in vivo* studies, especially in the delivery of antiinfectives and hormones.

In an earlier paper, we described the production and characterization of biodegradable microparticles containing tetracycline, which were designed for periodontal disease therapy. Microparticles were made by using different preparation procedures and different polyesters: poly(L-lactide), poly(DL-lactide), and poly(DL-lactide-co-glycolide) 50:50. Selection of the appropriate preparation method and polyester enabled us to obtain biodegradable microparticles intended for sustained delivery of tetracycline to the periodontal pocket [23].

Lipid-based microspheres appear to be ideal candidates for administering antibacterial agents for periodontal therapy; because they are biodegradable, they do not have to be removed after the treatment period, and they possess mucoadhesive properties.

REFERENCES

[1] Domb, A.J. et al., Degradable polymers for site-specific drug delivery, *Polymer Adv. Technol.,* 3, 279, 1992.

[2] Barke, S.A. and Khossravi, D., Drug delivery strategies for the new millennium, *Drug Del. Technol.,* 6, 75, 2001.

[3] Mizushima, Y., Lipid microspheres (lipid emulsions) as a drug carrier: an overview, *Adv. Drug Del. Rev.,* 20, 113, 1996.

[4] Ravi Kumar, M.N.V., Nano and microparticles as controlled drug delivery devices, *J. Pharm. Pharmaceut. Sci.,* 3, 234, 2000.

[5] Gombotz, W. et al., Prolonged release of GM-CSF, United States Patent: 5,942,253, 1995.

[6] Wake, M.C. et al., Effects of biodegradable polymer particles on rat marrow-derived stromal osteoblasts *in vitro, Biomaterials,* 19, 1255, 1998.

[7] Cortesi, R. et al., Preparation of liposomes by reverse-phase evaporation using alternative organic solvents, *J. Microencapsul.,* 16, 251, 1999.

[8] Vyas, S.P., Singh, R. and Dimitrijevic, D., Development and characterization of nifedipine lipospheres, *Pharmazie,* 52, 403, 1997.

[9] Jenning, V., Thünemann, A.F. and Gohla, S.H., Characterization of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids, *Int. J. Pharm.,* 199, 167, 2000.

[10] Schwarz, C. and Mehnert, W., Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization, *J. Microencapsul.,* 16, 205, 1999.

[11] Müller, R.H., Mäder, K. and Gohla, S., Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art, *Eur. J. Pharm. Biopharm.,* 50, 161, 2000.

[12] Takenaga, M., Application of lipid microspheres for the treatment of cancer, *Adv. Drug Del. Rev.,* 20, 209, 1996.

[13] Yang, S.C., et al., Body distribution of camptothecin solid lipid nanoparticles after oral administration, *Pharm. Res.,* 16, 751, 1999.

[14] Inoue, K. et al., Ex vivo anti-platelet effects of isocarbacyclin methyl ester incorporated in lipid microspheres in rabbits, *Arzneim-Forsch/Drug. Res.,* 45, 980, 1995.
[15] Müller, R.H. et al., Solid lipid nanoparticles (SLN) as potential carrier for human use: interaction with human granulocytes, *J. Control. Rel.,* 47, 261, 1997.
[16] Schwarz, C. et al., Solid lipid nanoparticles (SLN) for controlled drug delivery: production, characterization and sterilization, *J. Control. Rel.,* 30, 83, 1994.
[17] Bodmeier, R., Chen, H. and Bhagwatwar, H., Polymer and wax microspheres prepared by emulsification techniques, *Bull. Tech. Gattefossé,* 1990, 83.
[18] zur Mühlen, A., Schwarz, C. and Mehnert, W., Solid lipid nanoparticles (SLN) for controlled drug delivery: drug release and release mechanism, *Eur. J. Pharm. Biopharm.,* 45, 149, 1998.
[19] Arshady R., Albumin microspheres and microcapsules: methodology of manufacturing techniques. *J. Control. Rel.,* 14, 111, 1990.
[20] Cortesi, R. et al., Liposome-associated retinoids: production and antiproliferative activity on neoplastic cells, *Eur. J. Pharm. Sci.,* 2, 281, 1994.
[21] Pereira, G.R., Marchetti, J.M. and Bentley M.V., A rapid method for determination of progesterone by reversed-phase liquid chromatography from aqueous media, *Anal. Lett.,* 33, 881, 2000.
[22] Radulovic, D. et al., HPLC determination of sodium cromoglycate in pharmaceutical dosage forms, *Farmaco,* 49, 375, 1994.
[23] Esposito, E. et al., Biodegradable microparticles for sustained delivery of tetracycline to the periodontal pocket: formulatory and drug release studies, *J. Microencapsul.,* 14, 175, 1997.

3 Characterization of Solid Lipid Nano- and Microparticles

Heike Bunjes

CONTENTS

3.1 GENERAL CONSIDERATIONS

To ensure the suitability of solid lipid nanoparticle dispersions for the intended type of application, and to enable a focused development of dispersions with specific properties, the characteristics of the dispersions have to be known in detail. A major point of interest is the question of sufficient colloidal stability of these thermodynamically labile systems. However, a detailed characterization of these systems is also very important when it is considered that the material properties of solid lipids, particularly the melting, crystallization, and polymorphic behavior of these lipids, may change dramatically when the substances are dispersed into particles in the lower nanometer size range. The behavior of lipid microparticles, which closely

0-8493-1692-8/05/$0.00+$1.50

resembles that of the bulk material, is less peculiar in this respect. The characterization of lipid nanoparticle formulations is often not an easy task because of the small size and sometimes low concentration of the dispersed particles. Moreover, colloidal dispersions of solid lipids are extremely complex systems. Several types of particles often coexist in the dispersions, and the solid state of the particles generally allows more complicated processes (such as polymorphism and variations in particle shape) than, for example, the liquid state of emulsion droplets. Sophisticated experimental techniques are thus required to address the different questions concerning the structure and behavior of these systems. The aim of this chapter is to introduce the methods commonly applied for the characterization of lipid nanosuspensions and to give an impression of their capabilities as well as their problems. This chapter focuses on nanosized dispersions based on solid lipids, but it also includes some considerations about the corresponding microparticle field.

3.2 PARTICLE SIZE

Particle size is one of the most important characterization parameters for solid lipid nanoparticle dispersions, and parameters relating to particle size are consequently reported in all studies on these systems. Particle size determinations are predominantly performed to confirm that the desired colloidal size range has been obtained during preparation and that it is retained upon storage or further processing (e.g., during freeze drying or sterilization).

Moreover, particle size can significantly affect the material properties of the nanoparticles and is important for their interaction with the biological environment (e.g., as concerns their ability to pass fine capillaries or to leave the vascular compartment via fenestrations after intravenous administration). Particle sizing results are thus crucial parameters in the development and optimization of preparation processes as well as in the evaluation of dispersion stability. Particle sizing, however, has also been employed for other purposes: for example, to evaluate the size dependence of the nanoparticle matrix properties [1] or to obtain additional information on the particle shape [2,3].

Almost all particle size determinations of solid lipid nanoparticle dispersions are performed by light-scattering methods (some information on the particle size can also be obtained by nanoscale microscopic methods, which will be described in Section 3.5). In spite of the practical convenience with which values of particle size and size distributions can be obtained with commercially available equipment, the light-scattering methods used are not without problems. The particle size of solid lipid nanoparticles can be in a rather difficult range for particle size analysis with light-scattering methods — it is sometimes in the upper range of photon correlation spectroscopy and is usually at the lower limit of laser diffraction. Moreover, the conventional theories for deriving particle sizes from light-scattering data assume suspensions of particles that are all of the same type, homogeneous and spherical. Neither of these prerequisites may apply for dispersions of solid lipid nanoparticles, which often contain anisometric and sometimes even inhomogeneous particles. In many cases, the dispersions also contain other types of colloidal particles (such as micelles or vesicles) in addition to the solid lipid particles of interest. Moreover, the

dispersions usually have to be diluted for particle sizing. Although this is usually not considered a practical problem, the redistribution of the components may alter the colloidal composition of the sample, and, in some cases, instabilities occurring on dilution can make a meaningful characterization of the particle size problematic. All these potential complications have to be taken into consideration when working with particle sizing methods. As a consequence, particle sizing results — or at least those obtained in routine analysis — should be regarded as approximations rather than as absolute values. Combining the information obtained from different particle sizing techniques (and sometimes also from visual inspection of the sample) can help the investigator to get a better impression of the "real" particle size range.

3.2.1 Photon Correlation Spectroscopy

The most widely used method to characterize the size of solid lipid nanoparticles is photon correlation spectroscopy (PCS). This method requires only very small amounts of sample and is rapid and easy to perform, and its range of operation (nominally between a few nanometers and a few micrometers) covers the relevant range for lipid nanoparticle suspensions. PCS analyzes the Brownian motion of the particles in the dispersion medium [4–7]. The randomly moving particles are irradiated with a laser beam, and the intensity of the light scattered from a small volume of the sample in a (usually) fixed or variable angle is recorded in dependence on time. The scattered light "flickers" as a result of the particle motion. Small particles lead to fast intensity fluctuations as a result of their high diffusion coefficient, whereas for larger particles, which move more slowly, the fluctuations are slower. The scattering intensity–time curve is analyzed via an autocorrelation function from which parameters relating to particle size and size distribution can be derived. The so-called *z*-average diameter (*z*-ave, sometimes also referred to as the effective diameter) and the polydispersity index (PI), as an indication of the width of the particle size distribution, can be derived directly from the autocorrelation function in a comparatively simple manner (method of cumulants) [8]. PCS particle sizes for solid lipid nanoparticles are usually reported using these parameters. The *z*-ave/PI values are quite robust and well suited to characterizing dispersions in a comparative way, provided that the PI is not too high, as this will preclude a meaningful interpretation of the results from the cumulant-based evaluation. Particle size results with fairly high (>0.3 to 0.4) PI values have occasionally been reported for solid lipid nanoparticle dispersions [9–14], but the corresponding parameter sets should rather be regarded as indications for "a very broad, heterogeneous dispersion" than as realistic parameters in an absolute way. The *z*-ave and PI are quite specific for this type of analysis and do not have much in common with the parameters normally used for the description of particle size distributions (such as volume or number diameter). The *z*-ave is intensity weighted and, as large particles scatter light much more strongly than smaller particles, gives a relatively large estimate of the particle size. Assuming, for example, a log normal particle size distribution, *z*-ave and PI can be "translated" into parameters characteristic of a monomodal intensity-, volume-, or number-weighted distribution [15]. In cases in which the PI is not extremely small, however, such a procedure may lead to highly artificial values because the

real particle size distribution may not be monomodal at all. Moreover, a meaningful transformation into a number or volume distribution usually requires information on the optical properties of the particles (such as their refractive index) unless they are very small.

The extraction of more complex particle size distributions from PCS data (which is not part of the commonly performed particle size characterization of solid lipid nanoparticles) remains a challenging task, even though several corresponding mathematical models and software for commercial instruments are available. This type of analysis requires the user to have a high degree of experience and the data to have high statistical accuracy. In many cases, data obtained in routine measurements, as are often performed for particle size characterization, are not an adequate basis for a reliable particle size distribution analysis.

Because PCS relies on the determination of the particle diffusion coefficient, it is not a direct method for the determination of particle sizes. Information on the particle size can be obtained via the Stokes–Einstein equation

$$D = kT/3\pi\eta d \tag{3.1}$$

where D is the diffusion coefficient, k is Boltzmann's constant, T is the absolute temperature, η is the viscosity of the dispersing liquid, and d is the particle diameter. Determination of the particle size thus requires a well-defined and exactly known temperature during the measurement and requires information on the viscosity of the dispersion medium. Usually, a spherical particle shape is assumed for data evaluation. In contrast to the situation with latex or lipid emulsion particles, this assumption may not be justified for solid lipid nanoparticles, which frequently crystallize in a platelet-like shape [1,2,16–20]. Because the diffusion coefficient of anisometric particles is larger than that of a sphere of the same volume [21,22], a larger hydrodynamic diameter is observed in PCS for these anisometric particles compared with corresponding emulsion systems in spite of the volume contraction on crystallization (Table 3.1).

Although PCS is very reliable in giving characteristic particle size values for narrow, monomodal distributions of particles in the nanometer range, it is not an optimal method to provide detailed information on dispersions with broad or multimodal particle size distributions, particularly when these dispersions contain a considerable fraction of particles in the upper nano- or micrometer size range. The presence of large particles or aggregates may severely disturb the measurement. Removal of such particles, for example, by filtration or the use of inbuilt electronic "dust filters" is not recommended because it will produce misleading results unless the separated large-particle fraction is characterized separately. However, samples that appear problematic from visual inspection as a result of the presence of larger aggregates may sometimes give surprisingly "nice" results in PCS cumulant analysis.

3.2.2 Laser Light Scattering

For broader size distributions and dispersions that contain a considerable amount of particles in the upper nanometer and/or the micrometer range, laser light scattering

TABLE 3.1
Examples of Differences in Photon Correlation Spectroscopy Particle Size as a Result of Different Particle Shapes in Lipid Nanosuspensions and Nanoemulsions

	Z-Average Diameter (Polydispersity Index)	
	Suspension (nm)	**Emulsion (nm)**
Trilaurin (Dynasan 112)	156 (0.16)	137 (0.15)
Trimyristin (Dynasan 114)	163 (0.13)	149 (0.13)
Tripalmitin (Dynasan 116)	165 (0.20)	129 (0.18)
Hard fat (Witepsol H42)	191 (0.12)	179 (0.13)
Hard fat (Witepsol W35)	145 (0.18)	129 (0.16)

Note: The samples (10% matrix lipid stabilized with 2.4% soybean phospholipid [Lipoid S100] and 0.6% sodium glycocholate) were obtained by high-pressure homogenization (Micron Lab 40, 5 cycles at 800 bars) above the melting temperature of the matrix lipid. The crystalline nanoparticles (obtained by cooling the particles to below 0°C in the case of the trilaurin dispersion) were transformed into emulsion droplets by heating the dispersion above their melting temperature before measurement.

(LS; also referred to as laser diffraction) with an adequate instrument is probably a better choice than PCS for getting an impression of the particle size distribution (narrow, monomodal distributions can, of course, also be characterized by LS). A laser diffractometer determines the angular distribution of the light scattered from the dispersion on irradiation with laser light by an array of detectors [23]. The geometric distribution of the scattered light arising from a particle depends on its size: Large particles, compared to the wavelength of the laser light, scatter predominantly in a forward direction, whereas very small particles emit a more spherelike "cloud" of scattered light. Analysis of the angular intensity distribution of the scattered light thus gives information on the particle size. For the calculation of particle size distributions, iterative processes are usually applied to fit a model distribution to the experimental data. The instrument gives an estimation of the particle size distribution in addition to characteristic particle size values (e.g., mean, mode, median diameter, diameter at 90 or 99% of the distribution) in a comparatively short time. The technique is well suited, for example, to characterizing lipid microparticles [24–27].

Originally, LS instruments were constructed for the investigation of particles in the micrometer and millimeter size range only, using the Fraunhofer approximation (which is valid for particles that are very large compared with the wavelength of the laser light) for data evaluation. Modern instruments are also capable of using the Mie theory as a basis for data evaluation, and thus they are also theoretically prepared to evaluate the scattering pattern in the nanometer region. The scattering information provided by traditional experimental setups is, however, insufficient for an adequate evaluation of small particles in the nanometer range. Some modern LS instruments are, therefore, equipped with "submicron" features that aim at the investigation of

nanometer-sized particle fractions in particular. Additional lamps with different wavelengths, wide-angle detectors, or special setups giving information on the interaction of the particles with polarized light provide complementary data on the nanoparticle fraction [28,29]. Such instruments have frequently been used for the characterization of lipid nanosuspensions [3,11,30–37]. As a drawback, applying this technique in the nanometer range requires knowledge of the optical parameters (the real and imaginary part of the refractive index at the corresponding wavelength) of the dispersed material. The calculated particle size distributions may depend highly on the optical model that is constructed on the basis of these parameters. Moreover, as in PCS, the models assume the particles to be spherical, which leads to further uncertainty in the results when nonspherical lipid particles are under investigation. For the characterization of dispersions that simultaneously contain particles in the nano- and micrometer size ranges, commercial instruments often apply data obtained from the application of different physical methods that require different theoretical models for evaluation. The experimentally and theoretically complex combination of these methods may cause additional uncertainties in the results.

3.2.3 Characterization of Microparticulate Contaminations

In particular, trace amounts of microparticles may be difficult to detect in solid lipid nanoparticle dispersions with light-scattering methods. The question of microparticulate contamination, which is of particular interest for dispersions developed with respect to parenteral administration, has hitherto been addressed only scarcely. Electrical zone sensing (the Coulter counter method) has been used to determine the absolute number of particles in the micrometer range for dispersions of lipid nanoparticles [11]. This method has also been applied for the particle size determination of solid lipid microparticles [38,39]. It is based on alterations of the electrical resistance of an aqueous salt solution within a pinhole when this pinhole is passed by a particle [40]. The technique was originally developed for counting cells but is also well accepted for the determination of particle size distributions in the micrometer range. In particular, for electrostatically stabilized colloidal particles, the need to be dispersed in a comparatively concentrated salt solution (e.g., 0.9% sodium chloride) is a major drawback, as the presence of ions may lead to the destabilization of the nanoparticles because of interference with the electric double layer. As an alternative, light-blockage or light-microscopic methods may be considered, though, except for a microscopic study in semisolid preparations, they hitherto have not been used for the characterization of suspensions of lipid nanoparticles with respect to microparticulate contaminations [35]. Although not suitable for the evaluation of particle size distributions of colloidal suspensions, light microscopy and optical imaging systems can be applied for particle size characterization of lipid microparticles [41,42].

3.3 ZETA POTENTIAL

Colloidal particles usually bear a surface charge as a result of the presence of ionized groups or of ion adsorption from the dispersion medium. These surface charges and the strength and extension of the electrical field around the particles play an important

role in the mutual repulsion of nanoparticles and thus in their stability against aggregation. For colloidal drug carriers, the surface charge also has an impact on their *in vivo* behavior. Because the surface potential of the particles cannot be measured directly, the zeta potential ζ (electrical potential at the surface of hydrodynamic shear around the colloidal particles) is usually determined as a characteristic parameter for the nanoparticle charge [4,43]. For measurement, a dilute suspension of the nanoparticles is subjected to a weak electric field, and the mobility of the particles is commonly determined by laser Doppler anemometry. This technique 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. As a result, the electrophoretic mobility μ (velocity of the particles/electric field strength) of the nanoparticles is obtained. For comparatively large particles in a weak electric field, the zeta potential can be derived from this value using the Helmholtz–Smoluchowski relation

$$\mu = \varepsilon\zeta/\eta \tag{3.2}$$

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

In the study of solid lipid nanoparticles, zeta potential determinations have mainly been employed with respect to conclusions about the physical stability or instability, respectively, of the formulations during storage or on interaction with electrolytes or (simulated) biological fluids. Zeta potential measurements were, for example, used in the investigation of gel formation phenomena [16,31,33] and to assess different compositions with respect to electrolyte and pH stability [35,44]. When drawing conclusions about stability issues from zeta potential measurements, however, it has to be taken into account that surface charge may not be the only stabilizing mechanism, particularly when pegylated or macromolecular compounds are employed for stabilization, as they add a steric component. Investigation of the effect of different surface-active agents on the zeta potential can provide information on the interaction of the particles with surface-active agents [45–49]. The effect of variations in preparation procedure [50] as well as the potential influence of drug loading [11,49,51] or further processing, such as freeze drying or sterilization, on the zeta potential of solid lipid nanoparticles has also been studied [49,52]. In the development of particulate carriers with good adsorbing capacity for oppositely charged molecules, such as DNA or peptides, zeta potential investigations are a very important tool that allows optimization of the surface charge and investigation of the interaction of the particles with the molecules to be adsorbed [25,53,54]. In many cases, however, the zeta potential simply serves as a "standard" parameter to characterize the properties of the dispersions [14,55–61].

3.4 CRYSTALLINITY AND POLYMORPHISM

When preparing lipid nano- and microparticles from solid, crystalline raw materials, it is usually expected that the lipid matrix of the particles is or will become solid after the dispersion step. It has, however, turned out that some matrix materials do not crystallize easily in the colloidally dispersed state after processing in the heat

as, for example, in melt-homogenization. Shorter chain triglycerides like tricaprin, trilaurin, or trimyristin are particularly problematic in this respect, but retarded crystallization has also been observed for dispersions of more complex glycerides [11,49,62–66]. Dispersions of such materials may require special thermal treatment after preparation to ensure the solid state of the particles. In addition to the lipid matrix composition, the solidification process can also be affected by the stabilizer composition or by the presence of a drug [67,68]. Because the expected advantages of solid lipid particles (e.g., modified release properties) essentially rely on the solid state of the particles, monitoring of the crystalline status is a crucial point in their characterization, particularly when novel compositions or preparation procedures are introduced.

Solidification of the particles may not be the final step in the formation process of solid lipid particles. Lipidic materials exhibit rich polymorphism [69,70], which may also occur in the dispersed state. In nanoparticles, the polymorphic behavior of the matrix lipids may, however, differ distinctly from that in the bulk material. Polymorphic transitions are usually accelerated in the nanoparticles compared with the bulk lipids [2,62]. In some cases, polymorphic forms not observable in the corresponding bulk materials were detected in lipid nanoparticles [1,65]. Because polymorphism can affect pharmaceutically relevant properties of the particles, such as the drug incorporation capacity [65], corresponding investigations should also be included in the characterization process. As long as polymorphic or other crystal-aging phenomena have not terminated, the particle matrix cannot be regarded as "static," and alterations of the particle properties may still occur.

Differential scanning calorimetry (DSC) and x-ray diffraction (XRD) are the techniques most widely used for the characterization of crystallinity and polymorphism of solid lipid particles. Although DSC is usually more sensitive in detecting crystalline material, XRD is much more reliable in determining the type of polymorph present in the dispersions because it provides structural data. In contrast, DSC can detect the type of polymorph only indirectly via the transition temperatures and enthalpies. Because these parameters may be different from those observed in the bulk material, particularly for small colloidal particles [1,62], assignment of polymorphic forms in DSC curves should be supported by x-ray data.

Modern DSC and XRD equipment is usually capable of analyzing lipid particles in their native, dispersed state, which is the preferred mode of investigation unless the final formulation is a dry product (e.g., in the case of microparticles or freeze-dried powders for injection). If problems with sensitivity arise, for example, when the samples are highly diluted — the samples may have to be concentrated before measurement. In this case, great care has to be taken not to change the properties of the particles during the sample preparation procedure (e.g., by causing an increase in particle size or by application of temperatures that may lead to phase transitions). Freeze drying or air drying of samples, for example, may lead to changes in transition temperatures, crystallinity, and polymorphism [11,71–73] and should, therefore, be avoided if possible. In some special cases, such procedures may be inevitable (e.g., drying samples to be checked for the presence of high-melting drug crystals by DSC [52,74]), but the potential alterations of the sample caused by the preparation technique have to be considered on data interpretation.

3.4.1 DSC

In DSC the sample is subjected to a controlled temperature program, usually a temperature scan, and the heat flow to or from the sample is monitored in comparison to an inert reference [75,76]. The resulting curves — which show the phase transitions in the monitored temperature range, such as crystallization, melting, or polymorphic transitions — can be evaluated with regard to phase transition temperatures and transition enthalpy. DSC is thus a convenient method to confirm the presence of solid lipid particles via the detection of a melting transition. DSC recrystallization studies give indications of whether the dispersed material of interest is likely to pose recrystallization problems and what kind of thermal procedure may be used to ensure solidification [62–65,68,77].

DSC is also well suited to monitor physical changes: for example, those caused by polymorphism or an increase in crystallinity upon storage. In the case of polymorphic matrix lipids, the determination of an absolute value for the crystallinity requires the unambiguous assignment of the DSC transitions to the different crystal forms. Comparison with the thermal values described or observed for the bulk material may be inadequate for this purpose, particularly when very small particles are under investigation, as dispersing the matrix materials into the colloidal state may affect their melting behavior. A decreased melting temperature has frequently been observed, particularly in small-size lipid nanoparticles — in some cases to such an extent that confusion of the melting transition with that of a less stable polymorph could be possible [1,50,62]. Eutectic behavior with incorporated compounds such as oils or drugs may also lead to a shift in the peak positions [37,68]. Parallel XRD studies are very helpful in determining the type of polymorph present. If an absolute value for the crystallinity is not required (e.g., to simply monitor changes over storage), the value observed for the bulk material could be used as a reference even without knowledge of the crystalline form of the nanoparticles. In this case, "crystallinity" values above 100% have to be expected in cases in which the particles transform into a more stable polymorph than that present in the bulk material. The degree of crystallinity of solid lipid nanoparticles has been investigated with respect to its influence on a variety of application-related parameters such as gelation tendency, enzymatic degradation, or occlusive properties [32,78,79]. An increase in crystallinity during storage has been reported for particles prepared by melt homogenization, depending on composition [2,32,62]. The melting enthalpy of glyceride nanoparticles decreases with particle size [1]. When prepared from monoacid triglycerides, smaller nanoparticles (<150 to 200 nm) display a very peculiar size-dependent melting behavior with multiple transitions that are not caused by polymorphism, as confirmed by XRD (Figure 3.1). Such complications are not expected in lipid microparticles, which can be characterized with respect to polymorphism (depending on preparation procedure and composition) more easily with DSC by comparing the position and size of the transitions with those of the bulk material [39,80].

DSC has also been used to evaluate interactions with incorporated drugs. These interactions can be reflected in changes of the melting and recrystallization temperatures or in differences in melting enthalpy [52,68,81]. Direct evidence of the

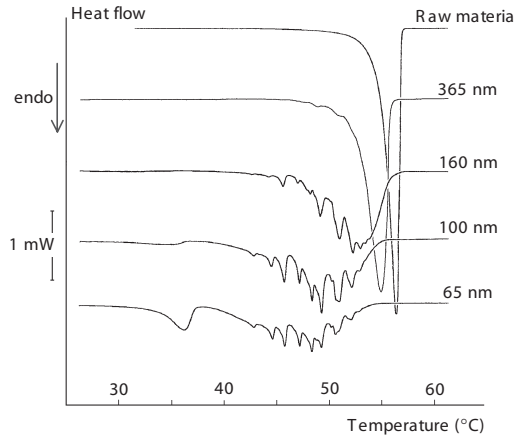

FIGURE 3.1 Differential scanning calorimetry melting curves (scan rate 0.04°C/min) of trimyristin dispersions (10% triglyceride stabilized with different concentrations of tyloxapol) with different mean photon correlation spectroscopy *z*-average diameters. The raw material was dispersed in an aqueous phase containing 1% tyloxapol. (Adapted from [1]. Copyright 2000, American Chemical Society. With permission.)

presence of crystalline drugs in the particles or dispersions as reflected by the occurrence of a melting transition has only scarcely been reported [26,82]. The absence of drug-melting transitions may point to a preferably amorphous or molecularly dispersed state of the incorporated drug [52,68,74], but the detection of very small amounts of crystalline material can be complicated by effects such as a large width and the superimposition of transitions.

The presence and state of solid lipid nanoparticles incorporated into semisolid formulations have also been investigated by DSC [77,83,84]. Using this method, de Vringer and de Ronde were able to draw conclusions on the preparation-dependent distribution of the matrix lipid of their particles in the different phases of a cream formulation [77].

3.4.2 X-Ray Diffraction

The interaction of a monochromatic x-ray beam with the crystal lattices in randomly oriented powder or suspension particles gives rise to a set of reflections that can be detected with a film, a moving counter tube, or position-sensitive detectors [85]. The resulting diffractogram, displaying the intensity of the reflections with respect to their angular position related to the incident x-ray beam, is specific for the crystal structure under investigation and can be used to identify substances or their different crystal forms. In the characterization of solid lipid nanoparticles, the major points of interest are usually the confirmation of the solid, crystalline state of the particles and the identification of the polymorphic form of the lipid matrix [10,37,65,68,73,81,86–88]. This can be done by comparison to literature data or to measurements of the corresponding bulk materials. The x-ray diffractograms of the

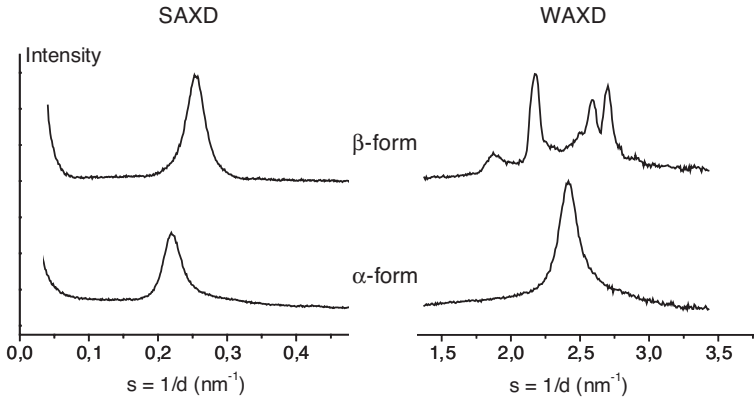

FIGURE 3.2 Small-angle (SAXD) and wide-angle (WAXD) x-ray diffractograms of tripalmitin nanoparticles in the α- and β-modification. s = scattering vector; d = d-value.

lipid matrix materials usually display only few major reflections characteristic of the packing of the alkyl chains. The lipids crystallize in lamellar organizations, with the alkyl chains packed side by side in different arrangements and oriented either perpendicular to or tilted toward the plane of the single layers. Typically, these arrangements display a strong small-angle reflection, indicating the repeating distance of the single layers. The packing of the alkyl chains within the layer is reflected in the occurrence of one or more reflections in the wide-angle region, where the reflections of crystalline drugs also are to be expected. As the wide-angle reflections for a specific polymorphic form are usually very similar within a given class of lipids, these reflections are particularly suitable for identifying the polymorphic form of the sample (Figure 3.2).

With the help of XRD, the differences in polymorphism between glyceride nanoparticles and the corresponding bulk material were clearly demonstrated [2,65,86]. Moreover, this method has been applied to evaluating the factors that affect the rate of polymorphic transitions in glyceride nanoparticles such as the type and chain length of the lipid, the presence of drug or liquid oil, the type of surfactant, or the particle size [1,64,65,67,68,89,90]. Microparticles were analyzed by x-ray diffraction to evaluate the influence of composition and preparation procedure on the resulting polymorphic form [39]. Temperature-dependent x-ray scans have proven to be very useful in addressing more complex questions such as the time course of polymorphic transitions during the crystallization process on cooling or the processes underlying complex DSC melting curves. For continuous scans, the use of synchrotron radiation as a source of intense x-radiation is usually necessary to minimize the exposure time for the single diffractograms and to provide the required time resolution (Figure 3.3).

The x-ray reflections of solid lipid nanoparticles are usually much broader than those of the bulk material as a result of the small particle size and, potentially, also of a decrease in crystalline order. Fine details may thus not be recognizable in the resulting, more diffuse diffractograms. Assignment of polymorphs may become increasingly difficult with the complexity of the material under investigation (e.g.,

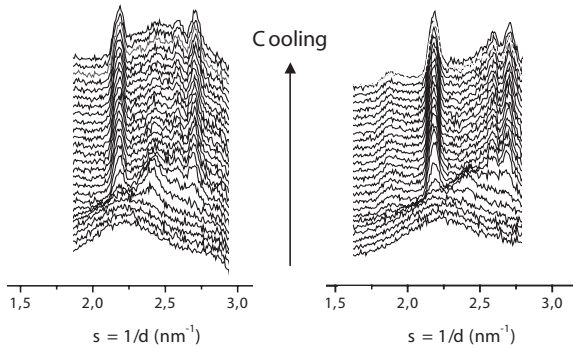

FIGURE 3.3 Evolution of the wide-angle x-ray diffraction patterns of differently stabilized tripalmitin dispersions (10% tripalmitin prepared with 3.2% soybean lecithin and 0.8% sodium laureate [left] or 2% soybean lecithin and 2% Tween 20 [right]) during the crystallization process. The samples were cooled with ~0.3˚C/min, and the diffractograms represent the situation every other minute. The emulsion present at the beginning of the scan only displays diffuse scattering. The sample to the left retains a considerable fraction of α-modification beside the developing β-modification, whereas the scattering pattern of the dispersion to the right quickly transforms into that of the β-modification.

complex glyceride mixtures), particularly in cases in which there is also very limited knowledge about the structure and behavior of the bulk material.

On incorporation of ubidecarenone into tripalmitin nanoparticles, the presence of the drug was reflected indirectly in a change in the rate of polymorphic transitions and in differences in the width of the small-angle reflection, but not in the formation of signals caused by the crystalline drug, which was confirmed to be amorphous by complementary investigations [68]. In general, x-ray signals caused by the presence of incorporated drugs have usually not been reported for solid lipid nanoparticles [10,65,73]. This may point to the presence of molecularly dispersed or amorphous drug. However, the detection of a crystalline drug that has phase separated from the nanoparticles is problematic when using the methods commonly employed for the diffractometric investigation of solid lipid nanoparticle dispersions, as demonstrated in measurements on dispersions containing microscopically visible drug crystals [91]. In particular, when comparatively few large drug crystals are formed, the drug particles may sediment out of the beam during measurement or may simply provide too few adequately oriented reflection planes to be detected.

The particle size–dependent line-broadening effect was used to assign the single transitions in complex DSC heating curves of small triglyceride nanoparticles to particle fractions of different, distinct thickness via a sophisticated analysis of the line shape of the corresponding x-ray reflection [19]. In some diffractograms of triglyceride nanoparticle preparations, the occurrence of additional small-angle reflections has been observed, indicating a very large repeating unit. The reflections are caused by the reversible formation of superstructures in the form of stacks of the platelet-shaped particles [92,93]. These examples illustrate that XRD has much broader applicability in the characterization of lipid nanoparticle dispersions than in the simple detection of crystalline material and assignment of crystal polymorphs

and that XRD can be used to reveal surprising details of the ultrastructure of lipid nanosuspensions, even in a quantitative manner. First approaches to characterize the fine structure of lipid nanoparticle dispersions with neutron scattering have also been reported [92].

3.5 PARTICLE MORPHOLOGY AND ULTRASTRUCTURE OF THE DISPERSIONS

Because of the small size of the structures of interest, electron microscopic techniques in particular have been employed to characterize the overall structure and shape of solid lipid particles. Particles with a spherical shape would offer the highest potential for controlled release and protection of incorporated drugs, as they provide minimum contact with the aqueous environment, as well as the longest diffusion pathways. Compared to particles with any other shape, spherical particles will also require the smallest amount of surface-active agent for stabilization because of their small specific surface area. However, more anisometric particles could have advantages when active agents are to be incorporated into the surfactant layer or adsorbed onto the surface. Particle shape may thus influence drug loading and release characteristics of solid lipid nanoparticles. In addition, with respect to the interpretation of particle sizing data, knowledge about the particle shape is very useful, as anisometric particle shape may affect the results, especially those from PCS.

Any conclusions about the organization of different components within the dispersions should take the ultrastructure of the systems into consideration. The surface-active agents that act as stabilizers for the nanoparticles are often able to form additional colloidal structures, such as vesicles or micelles, by self assembly. In addition to a potential importance in the formation and stability of the dispersions, such structures contain lipophilic domains that may represent alternative compartments for the localization of incorporated drugs. As a consequence, their presence may affect drug incorporation and release.

3.5.1 Transmission Electron Microscopy

Transmission electron microscopy (TEM) can provide valuable information on particle size, shape, and structure, as well as on the presence of different types of colloidal structures within the dispersion. As a complication, however, all electron microscopic techniques applicable for solid lipid nanoparticles require more or less sophisticated specimen preparation procedures that may lead to artifacts. Considerable experience is often necessary to distinguish these artifacts from real structures and to decide whether the structures observed are representative of the sample. Moreover, most TEM techniques can give only a two-dimensional projection of the three-dimensional objects under investigation. Because it may be difficult to conclude the shape of the original object from electron micrographs, additional information derived from complementary characterization methods is often very helpful for the interpretation of electron microscopic data.

Staining techniques employing heavy metal compounds (e.g., uranyl acetate or phosphotungstic acid) as contrast agents have often been used to demonstrate the

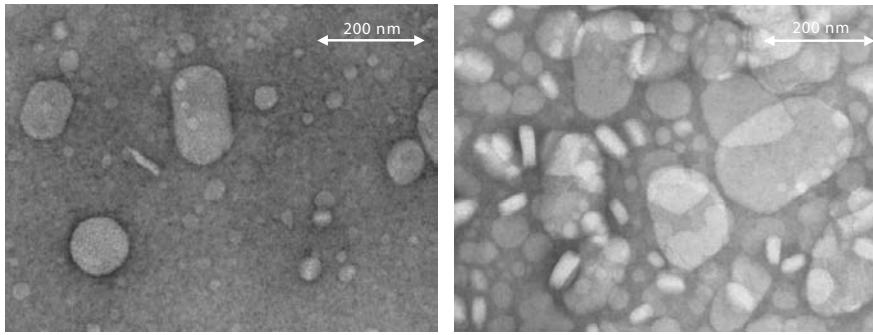

FIGURE 3.4 Transmission electron micrographs of a trimyristin suspension (10% trimyristin stabilized with 4% soybean lecithin and 1% sodium glycocholate) after staining with uranyl acetate solution. In the specimen with lower particle concentration (left), structures that are circular to elongated dominate the image. These structures can also be identified in a higher concentrated specimen (right). The smaller particles of this appearance, in particular, are often of almost circular shape. Because, however, a considerable amount of short, very bright, rodlike structures of a length comparable to the diameter of the circular structures can also be observed, it may be concluded (in good agreement with results from other electron microscopic techniques [Figures 3.5, 3.6]) that at least a fraction of the circular structures corresponds to disks that have adsorbed to the grid with their circular surface. Because of its high phospholipid content, however, the sample may also contain liposomes that cannot clearly be identified in these images.

presence of small particles in dispersions of solid lipids [12,50,52,55,94–99]. Several techniques can be used to place the nanoparticles on the electron microscopic grid (e.g., spraying or passive adsorption from a drop of sample) and to bring them into contact with the staining solution [100]. In any case, the samples have to be dried after staining before they can be viewed microscopically.

Staining techniques have the advantage of being very fast and comparatively simple to use. Moreover, they do not require sophisticated preparation equipment. Unfortunately, the resulting images are often of low resolution, which complicates interpretation. As a further disadvantage, the dispersions do not remain in their original state during preparation because the suspension liquid has to be removed. Drying often leads to artifacts. When preparation relies on diffusion-driven adsorption of the dispersed structures to the surface of the electron microscopic grid, the adsorption process may be selective and the resulting specimen not representative of the whole sample. Anisometric particles (like disks or platelets) may attach in a preferred orientation allowing maximum contact with the grid, and thereby distorting the impression of their real three-dimensional shape (Figure 3.4).

To obtain more detailed information on the ultrastructure of lipid dispersions and the morphology of the particles, electron microscopy is usually performed on replicas of freeze fractured or on frozen hydrated samples. These techniques aim to preserve the liquid-like state of the sample and the organization of the dispersed structures during preparation. By using special devices, the sample is frozen so quickly that all liquid structures, including the dispersion medium, solidify in an amorphous state.

For freeze fracturing, a very small volume of the dispersion is sandwiched as a thin film between two thin metal holders and rapidly frozen with the aid of a cryogen (e.g., liquid propane or melting nitrogen) [101]. Under cooling, the frozen sample is transferred into a high-vacuum chamber, where it is fractured. The fracture plane is usually not completely smooth, as the sample tends to break preferably at the sites with low binding forces. The remaining relief-like structure may be further elaborated by evaporating a small amount of the frozen water surrounding the particles (freeze etching). The sample surface is shadowed with a carbon/platinum mixture at a certain angle (e.g., 45°), and the resulting thin film is stabilized by the vertical deposition of a thicker film of pure carbon. After the specimen is removed from the freeze fracturing device, all of the original dispersion is removed by rinsing the film with solvents. Only a replica of the surface structure remains, which is viewed in the electron microscope.

Freeze fracture electron micrographs give information not only about the size and shape of the colloidal structures in the sample but also about their internal structure; for example, solid triglyceride nanoparticles frequently appear as sharply edged isometrical structures with distinct internal layers that can be attributed to the molecular ordering of the crystalline matrix lipids. In contrast, emulsion droplets are imaged as circular structures with an unstructured core, reflecting the liquid state of the matrix. Liposomes, which may be formed by excess phospholipid in phospholipid-stabilized dispersions, are often fractured along the lipid area of their bilayer and therefore appear as small bulbs standing out from the overall plane. In the characterization of lipid nanosuspensions, freeze fracture electron micrographs are very helpful in elucidating the shape and structure of the particles (e.g., demonstrating the crystallinity and anisometric, platelet-like shape of different types of solid triglyceride nanoparticles) (Figure 3.5) [1,2,16–20]. Changes in particle structure and morphology caused by polymorphic transitions as well as intraparticulate phase separation between matrix triglyceride and incorporated drug were also visualized with this technique [2,68,90]. Moreover, freeze fracture electron microscopy has been used to investigate the formation of superstructures in colloidal dispersions of solid triglycerides, such as the formation of gels in phospholipid-stabilized dispersions [48] or the stacking of particles in sufficiently concentrated suspensions [92,93]. The morphology and localization of solid paraffin nanoparticles in semisolid preparations has also been investigated by this technique [77].

When concluding on the particle size and shape from images obtained by freeze fracture TEM, the fact that the particles are fractured randomly with respect to the localization of the fracture site within the particle and to orientation toward the fracture plane has to be taken into consideration. Most of the particle remains "hidden" from observation. Therefore, a sufficiently large number of particles have to be investigated to obtain a realistic impression. The determination of a particle size distribution will not be possible when the particles are of anisometric shape.

Cryoelectron microscopy makes it possible to have a direct view into the frozen sample without additional preparation [100]. With the aid of a cryogen (e.g., liquid nitrogen–cooled liquid ethane), the sample is plunge frozen as a very thin aqueous film prepared on a microscopic grid. Subsequently, the vitrified specimen is directly transferred into a precooled electron microscope. Because the specimens are usually

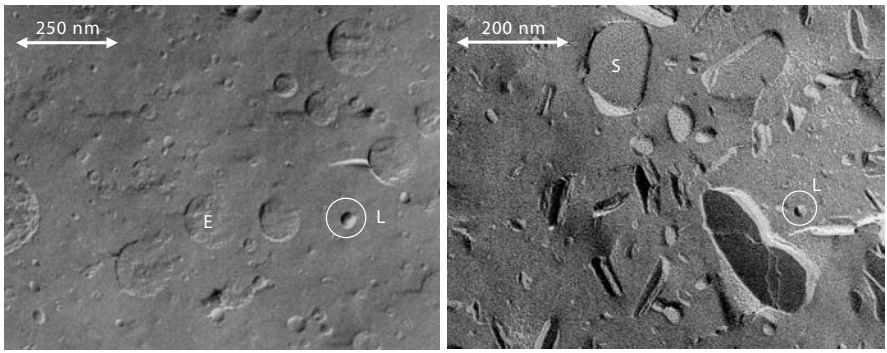

FIGURE 3.5 Transmission electron micrographs of a freeze fractured trimyristin dispersion (10% trimyristin stabilized with 2.4% soybean lecithin and 0.6% sodium glycocholate, prepared by melt-homogenization) stored at room temperature (left) and stored at refrigerator temperature (right). The image to the left has the typical appearance of an emulsion (circular particles with unstructured core [E]) because the matrix lipid does not recrystallize under these conditions. A few liposomes (L) can also be detected. In contrast, the micrograph of the corresponding suspension system (right) displays predominantly anisometric, platelet-like particles with a layered internal structure (S). Liposomal structures are also present in the suspension. (Adapted from [2].)

very sensitive to the electron beam, they have to be viewed under low-dose conditions, avoiding long exposure times. In contrast to freeze fracture electron microscopy, the overall contours of the particles are visible in the plane of projection.

Contrast, which is often quite low for lipid dispersions, depends on the electron optical properties of the structures and on their thickness. As in staining electron microscopy, the three-dimensional particles are projected in a two-dimensional way (e.g., platelet-like crystalline triglyceride particles can be seen as circular to elongated edged particles with low contrast in top-view, or as dark, needle-like structures when viewed edge-on; Figure 3.6). In contrast, spherical emulsion droplets appear as circular, rather dark structures in all orientations [2,3,102]. Unilamellar phospholipid vesicles that may be formed by excess phospholipid can easily be recognized by their ringlike appearance, arising from the phospholipid bilayer [2,48]. Cryoelectron microscopy is thus very well suited to study the coexistence of different colloidal structures such as matrix-type particles and vesicles in a dispersion, as well as to obtain information on the presence of nonspherical particles [2,48,103]. Cryoelectron microscopy has also been used to visualize the presence of different compartments within single nanoparticles in the case of glyceride particles loaded with liquid drug or oil (Figure 3.6) [3,68]. However, it is usually problematic to draw definitive conclusions on the size distribution of the particles under investigation because cryoelectron micrographs tend to be strongly biased toward small particles. This is a result of the preparation technique, which aims at leaving only an extremely thin film on the microscopic grid. Structures distinctly larger than the thickness of this film are either removed during specimen preparation or relocated to thicker film areas (which are usually too sensitive to the electron beam to be reliably investigated)

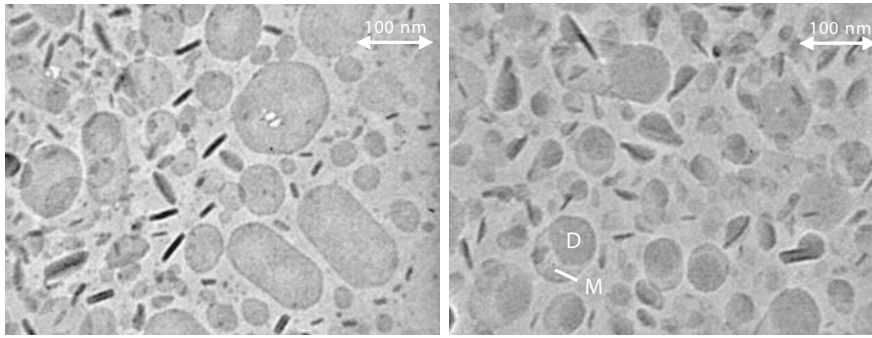

FIGURE 3.6 Cryoelectron micrographs of tripalmitin dispersions (10% lipid phase [triglyceride + drug] stabilized with 2% soybean lecithin and 2% tyloxapol, prepared by melt homogenization) loaded with 10% (left) and 50% (right) ubidecarenone with respect to the dispersed phase. In the dispersion with low drug load (left), the solid lipid particles appear to be circular to elongated, in some cases edged structures with low contrast in top view, and as dark, rodlike structures when projected edge-on. The image also shows indications of size fractionation as a result of differences in film thickness with smaller/thinner structures to the right and larger/thicker, and in some areas superimposed, structures to the left. The particles in the dispersion with high drug load (right) represent two-phase structures: In addition to the structure typical of the crystalline matrix lipid (M), each particle contains a droplet of phase-separated, liquid drug on one of its surfaces (D). These droplets appear as circular areas of higher contrast in top-view and as "cap-like" structures in side-view. (Adapted from [68].)

during this process. Anisometric particles with only one or two large dimensions — such as platelets — may be accommodated by being stretched out parallel to the film surface so that they cannot be viewed edge-on.

3.5.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) seems to have been used only scarcely for the characterization of solid lipid–based nanoparticles [104]. This method, however, is routinely applied for the morphological investigation of solid lipid microparticles (e.g., to study their shape and surface structure also with respect to alterations in contact with release media) [24,38,39,41,42,80,105]. For investigation, the microparticles are usually dried, and their surface has to be coated with a conductive layer, commonly by sputtering with gold. Unlike TEM, in SEM the specimen is scanned point by point with the electron beam, and secondary electrons that are emitted by the sample surface on irradiation with the electron beam are detected. In this way, a three-dimensional impression of the structures in the sample, or of their surface, respectively, is obtained.

3.5.3 Optical Microscopy

Solid lipid microparticles can also be analyzed by optical microscopy (e.g., with respect to particle size or presence of drug crystals within the particles [41,42,106]),

whereas the structures in solid lipid nanoparticle dispersions are usually too small to be investigated this way. In spite of this restriction, optical microscopy can be a helpful tool in the study of nanoparticle dispersions, particularly for the detection of phase-separated crystalline drugs [65,81], as well as for the characterization of microparticulate contaminations, as outlined above.

3.5.4 Atomic Force Microscopy

To date, there are only few reports on the use of atomic force microscopy (AFM) for the investigation of the morphology of solid lipid nanoparticle preparations [54,107–109]. The size and shape, in particular, and also the mechanical properties of the sample surface were under investigation in these studies. In contrast to the situation in electron microscopy, information on the three-dimensional extension of single solid particles can be obtained by AFM. Although it is, in principle, possible to investigate hydrated samples with AFM, most research reported so far has been performed on dried specimens. In any case, the particles have to be fixed on a very smooth surface for investigation, usually by adsorption. The sample is investigated by scanning with a very fine probe and monitoring the forces acting on the probe that are caused by interaction with the sample surface. This can be done with the probe either in contact or not in contact with the sample surface (contact of the probe tip with the sample has been reported to cause artifacts because of mechanical interference with the sample).

3.6 INTERACTION WITH INCORPORATED DRUGS

In spite of a rapidly increasing amount of literature data on drug-loaded solid lipid nanoparticle dispersions, there is comparatively little systematic knowledge about the interaction of drugs with these complex systems (e.g., with respect to the state and localization of the drug within the dispersions). One reason may be that the type of interaction is expected to be quite specific for each drug/particle matrix combination, and this interaction may also depend on the general composition of the dispersion and the preparation procedure.

It has to be assumed that the crystalline matrix in solid lipid nanoparticles is a rather unfavorable localization for the incorporation of at least larger amounts of drugs because drug incorporation will disturb the order of the crystal lattice. The incorporation capacity will depend not only on the type of matrix material (e.g., pure triglycerides are assumed to provide a lower incorporation capacity than complex lipids) and its state with respect to degree of crystallinity and polymorphic form, but also on the characteristics of the drug to be incorporated. Some drugs may more favorably interact with the solid matrix than others. Excess drug that cannot be accommodated within the crystalline matrix may adsorb to the surface of the nanoparticles or separate from the particles, either in pure form (e.g., as drug crystals or droplets [64,65,81]) or redistribute to other colloidal structures that may be present in the dispersion (e.g., micelles or phospholipid vesicles) or into the aqueous phase. Many analytical investigations of drug loading of solid lipid nanoparticles were performed on the whole dispersion, often after drying [46,56–58,74,94,97,110–112].

Investigations such as these yield valuable data on the drug concentration of the overall dispersion but this concentration is the sum of drug incorporated in or adsorbed to the nanoparticles, potentially phase separated drug or drug solubilized in additional colloidal structures, and drug dissolved in the aqueous phase (the latter may be accounted for after separation from the dispersion; e.g., by ultrafiltration). In some cases, additional processing steps, such as washing by centrifugation or ultrafiltration, that are performed before analysis may remove much of the drug present in locations other than in the particles, but the overall value still cannot give information about how much of the drug is actually associated with the nanoparticles or about the type of interaction. Such information may, however, be very important to understand the performance of the dispersions *in vitro* as well as *in vivo*.

The detection of a drug that has phase separated from the nanoparticles may be comparatively easy when large crystals that can be detected by light microscopy are formed [64,65,81], but it could be problematic if the drug does not crystallize or if it forms nanoparticles on its own. Assessing the drug distribution between the aqueous phase and the nanoparticles usually requires a separation of the two phases (e.g., by ultrafiltration, centrifugation, or gel chromatography) [11,36,49,72,96,113,114]. Depending on the separation technique, additional colloidal structures may or may not contribute to the amount of drug detected in the aqueous phase. Indications for the association of incorporated drug with the lipid nanoparticles were, for example, obtained via alterations of the thermal properties of the matrix lipid, such as the melting and recrystallization temperature [52,68,81]. The association of ubidecarenone with triglyceride nanoparticles was also reflected in a change of their polymorphic behavior and their density [68].

Interaction with such properties does, however, not allow the investigator to answer the question of where the drug is localized in the particles (on the surface or in the crystal lattice). Thermal interactions were also observed when an incorporated drug or a second type of triglyceride formed a separate phase within the nanoparticles [3,37,64,68]. Drug release studies can provide supportive information on the accessibility of the drug to the aqueous phase [72,108], but separation of the effects from the nanoparticles from those of additional colloidal structures — if present — may be difficult.

The use of spectroscopic techniques is a very promising approach for evaluating the interaction of drugs with solid lipid nanoparticle dispersions on a molecular level. For example, it was shown by nuclear magnetic resonance (NMR) spectroscopy, using trimyristin dispersions as model systems, that low amounts of the model drugs diazepam, menadione, and ubidecarenone are more strongly immobilized in solid lipid nanoparticle dispersions than in corresponding emulsion systems [65]. This result demonstrated that the drugs under investigation were indeed associated with the nanoparticles. It was, however, not possible to decide whether the drug molecules were incorporated within the crystalline particle matrix or adsorbed onto the particle surface. Similar observations were made on incorporation of triglyceride oil into lipid nanoparticle suspensions [34,37]. In principle, NMR investigations should allow even more detailed insights into the type of interaction of the drug with the nanoparticulate system, but the full potential of this technique still remains to be exploited. As a drawback, highly immobile molecules, as expected on incorporation

into the solid particles, do not give an analyzable signal in conventional high-resolution NMR spectroscopy. Investigation by magic angle or off–magic angle spinning methods may help overcome this limitation [115].

Electron spin resonance (ESR) spectroscopy, also called electron paramagnetic resonance spectroscopy, has also been used to study the interaction of lipid nanoparticle dispersions with incorporated model compounds. In contrast to NMR spectroscopy, which is able to provide information on conventional drug or excipient molecules via the investigation of signals arising from commonly occurring nuclei such as ^{1}H (in particular, but also, e.g., ^{13}C, ^{31}P, and ^{19}F), an ESR signal can be observed only in substances containing unpaired, paramagnetic electrons. ESR spectroscopy thus requires incorporation of a paramagnetic probe molecule, usually containing a nitroxide radical group, as a spin label. Consequently, the information obtained refers to the molecule carrying this spin label and its local environment, not to a "real" drug-containing system. Experiments using spin probes of different structures as model drugs can, however, provide very valuable basic information on the possible types of interaction of foreign substances with lipid nanoparticle dispersions, for example, with regard to their distribution within the systems and local environment [37,60,116]. Because the position of the signal-giving radical within the carrier molecule can be varied, the local environment of the spin label can be investigated with respect to its localization within the molecules [116]. A simple assay based on the reduction of the nitroxide radical by ascorbic acid contained in the aqueous phase can be used to study the accessibility of a spin-labeled model drug in solid lipid nanoparticle dispersions from the aqueous phase, depending, for example, on the structure and lipophilicity of the spin probe and composition of the particle matrix [37,60,61,116].

3.7 CONCLUSION

A wide variety of techniques has been employed to study the characteristics of solid lipid nanoparticle dispersions. Because of the complexity of these systems, a combination of different characterization techniques is the most promising approach to obtaining a realistic image of the sample properties. Unfortunately, the physicochemical characterization of solid lipid nanoparticle dispersions is often rather limited — particularly in studies closer to application (e.g., *in vivo* investigations). As the characteristics of a dispersion may depend on its exact composition and on the method of preparation, it is often difficult to conclude the behavior of one system from that of another. Even though the knowledge of solid lipid nanoparticle dispersions has increased considerably during recent years, there is still a high demand for more detailed investigations, particularly with respect to the interaction with incorporated drugs, but also concerning the effect of different preparation procedures and dispersion compositions. Because variations may affect the properties of the dispersions in unexpected ways, the introduction of new compositions and preparation procedures should always be accompanied by intensive structural investigations.

REFERENCES

[1] Bunjes H., Koch M.H.J., and Westesen K., Effect of particle size on colloidal solid triglycerides, *Langmuir,* 16, 5234, 2000.

[2] Westesen K., Drechsler M., and Bunjes H., Colloidal dispersions based on solid lipids, in *Food Colloids: Fundamentals of Formulation,* Dickinson E. and Miller R., eds., Royal Society of Chemistry, Cambridge, 2001, 103.

[3] Jores K. et al., Investigations on the structure of solid lipid nanoparticles (SLN) and oil loaded solid lipid nanoparticles by photon correlation spectroscopy, field-flow fractionation and transmission electron microscopy, *J. Control. Rel.,* 95, 217, 2004.

[4] Washington C., Photon correlation spectroscopy, in *Particle Size Analysis in Pharmaceutics and Other Industries,* Ellis Horwood, New York, 1992, 135.

[5] Finsey R., Particle sizing by quasi-elastic light scattering, *Adv. Colloid Interface Sci.,* 52, 79, 1994.

[6] Allen T., Photon correlation spectroscopy, in *Particle Size Measurement,* Vol. 1, Allen T., ed., Chapman & Hall, London, 1997, 426.

[7] Tscharnuter W., Photon correlation in particle sizing, in *Encyclopedia of Analytical Chemistry,* Meyers R.A., ed., Wiley, Chichester, 2000, 5469.

[8] Koppel D.E., Analysis of macromolecular polydispersity in intensity correlation spectroscopy: the method of cumulants, *J. Chem. Phys.,* 57, 4814, 1972.

[9] Gasco M.R., Cavalli R., and Carlotti M.E., Timolol in lipospheres, *Pharmazie,* 47, 119, 1992.

[10] Cavalli R. et al., Study by x-ray powder diffraction and differential scanning calorimetry of two model drugs, phenothiazine and nifedipine, incorporated into lipid nanoparticles, *Eur. J. Pharm. Biopharm.,* 41, 329, 1995.

[11] Schwarz C. and Mehnert W., Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles, *Int. J. Pharm.,* 157, 171, 1997.

[12] Pellizzaro C. et al., Cholesteryl butyrate in solid lipid nanospheres as an alternative approach for butyric acid delivery, *Anticancer Res.,* 19, 3921, 1999.

[13] Santos Maia C., Mehnert W., and Schäfer-Korting M., Solid lipid nanoparticles as drug carriers for topical glucocorticoids, *Int. J. Pharm.,* 196, 165, 2000.

[14] Kristl J. et al., Effect of colloidal carriers on ascorbyl palmitate stability, *Eur. J. Pharm. Sci.,* 19, 181, 2003.

[15] Thomas J.C., The determination of log normal particle size distributions by dynamic light scattering, *J. Colloid Interface Sci.,* 117, 187, 1987.

[16] Siekmann B. and Westesen K., Submicron-sized parenteral carrier systems based on solid lipids, *Pharm. Pharmacol. Lett.,* 1, 123, 1992.

[17] Siekmann B. and Westesen K., Melt-homogenized solid lipid nanoparticles stabilized by the nonionic surfactant tyloxapol. II. Physicochemical characterization and lyophilization, *Pharm. Pharmacol. Lett.,* 3, 225, 1994.

[18] Siekmann B. and Westesen K., Investigations on solid lipid nanoparticles prepared by precipitation in o/w emulsions, *Eur. J. Pharm. Biopharm.,* 43, 104, 1996.

[19] Unruh T. et al., Observation of size-dependent melting in lipid nanoparticles, *J. Phys. Chem. B,* 103, 10373, 1999.

[20] Friedrich I. and Müller-Goymann C.C., Characterization of solidified reverse micellar solutions (SRMS) and production development of SRMS-based nanosuspensions, *Eur. J. Pharm. Biopharm.,* 56, 111, 2003.

[21] Cantor C.R. and Schimmel P.R., *Biophysical Chemistry: Techniques for the Study of Biological Structure and Function,* Part II, Freeman, New York, 1980, 539.

[22] Perrin F., Mouvement Brownien d'un ellipsoide. II. Rotation libre et dépolarisation des fluorescences. Translation et diffusion de molécule ellipsoidales, *J. Phys. Radium,* 7, 1, 1936.
[23] Washington C., The angular distribution of scattered light, in *Particle Size Analysis in Pharmaceutics and Other Industries,* Ellis Horwood, New York, 1992, 109.
[24] Del Curto M.D. et al., Lipid microparticles as sustained release system for a GnRH antagonist (Antide), *J. Control. Rel.,* 98, 297, 2003.
[25] Erni, C. et al., Evaluation of cationic solid lipid microparticles as synthetic carriers for the targeted delivery of macromolecules to phagocytic antigen-presenting cells, *Biomaterials,* 23, 4667, 2002.
[26] Frederiksen H.K., Kristensen H.G., and Pedersen M., Solid lipid microparticle formulations of the pyrethroid gamma-cyhalothrin-incompatibility of the lipid and the pyrethroid and biological properties of the formulations, *J. Control. Rel.,* 86, 243, 2003.
[27] Masters D.B. and Domb A.J., Liposphere local anesthetic timed-release for perineural site application, *Pharm. Res.,* 15, 1038, 1998.
[28] Wedd M.W., Particle sizing in the sub-micron range by laser diffraction, in *Particle and Surface Characterization Methods,* Müller R.H. and Mehnert W., eds., Scientific Publishers, Stuttgart, 1997, 57.
[29] Xu R., Improvements in particle size analysis using light scattering, in *Particle and Surface Characterization Methods,* Müller R.H. and Mehnert W., eds., Scientific Publishers, Stuttgart, 1997, 27.
[30] Freitas C. and Müller R.H., Spray-drying of solid lipid nanoparticles (SLN), *Eur. J. Pharm. Biopharm.,* 46, 145, 1998.
[31] Freitas C. and Müller R.H., Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN) dispersions, *Int. J. Pharm.,* 168, 221, 1998.
[32] Freitas C. and Müller R.H., Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase, *Eur. J. Pharm. Biopharm.,* 47, 125, 1999.
[33] Freitas C. and Müller R.H., Stability determination of solid lipid nanoparticles (SLN) in aqueous dispersion after addition of electrolyte, *J. Microencapsulation,* 16, 59, 1999.
[34] Jenning V., Mäder K., and Gohla S.H., Solid lipid nanoparticles (SLN) based on binary mixtures of liquid and solid lipids: a ^{1}H-NMR study, *Int. J. Pharm.,* 205, 15, 2000.
[35] Jenning V., Schäfer-Korting M., and Gohla S., Vitamin A-loaded solid lipid nanoparticles for topical use: drug release properties, *J. Control. Rel.,* 66, 115, 2000.
[36] Wang J.-X., Sund X., and Zhang, Z.R., Enhanced brain targeting by synthesis of 3,5-dioctanoyl-5-fluoro-2-deoxyuridine and incorporation into solid lipid nanoparticles, *Eur. J. Pharm. Biopharm.,* 54, 285, 2002.
[37] Jores K., Mehnert W., and Mäder K., Physicochemical investigations on solid lipid nanoparticles and on oil-loaded solid lipid nanoparticles: a nuclear magnetic resonance and electron spin resonance study, *Pharm. Res.,* 20, 1274, 2003.
[38] Reithmeier H., Herrmann J., and Göpferich A., Lipid microparticles as parenteral controlled release device for peptides, *J. Control. Rel.,* 73, 339, 2001.
[39] Reithmeier H., Herrmann J., and Göpferich A., Development and characterization of lipid microparticles as a drug carrier for somatostatin, *Int. J. Pharm.,* 218, 133, 2001.
[40] Washington C., Electrical zone sensing, in *Particle Size Analysis in Pharmaceutics and Other Industries,* Ellis Horwood, New York, 1992, 79.

[41] Savolainen M. et al., Evaluation of controlled-release polar lipid microparticles, *Int. J. Pharm.,* 244, 151, 2002.
[42] Cortesi R. et al., Production of lipospheres as carriers for bioactive compounds, *Biomaterials,* 23, 2283, 2002.
[43] Hunter R.J., Electrokinetics of particles, in *Encyclopedia of Surface and Colloid Science,* Hubbard, A.T., ed., Marcel Dekker, New York, 2002, 1907.
[44] Zimmermann E. and Müller R.H., Electrolyte- and pH-stabilities of aqueous solid lipid nanoparticle (SLN) dispersions in artificial gastrointestinal media, *Eur. J. Pharm. Biopharm.,* 52, 203, 2001.
[45] Cavalli R. et al., Albumin adsorption on stealth and non-stealth solid lipid nanoparticles, *S.T.P. Pharm. Sci.,* 9, 183, 1999.
[46] Zara G.P., Intravenous administration to rabbits of non-stealth and stealth doxorubicin loaded solid lipid nanoparticles at increasing concentrations of stealth agent: pharmacokinetics and distribution of doxorubicin in brain and other tissues, *J. Drug Targeting,* 10, 327, 2002.
[47] Bocca C. et al., Phagocytic uptake of fluorescent stealth and non-stealth solid lipid nanoparticles, *Int. J. Pharm.,* 175, 185, 1998.
[48] Westesen K. and Siekmann B., Investigation of the gel formation of phospholipid stabilized solid lipid nanoparticles, *Int. J. Pharm.,* 151, 35, 1997.
[49] Lim S.-J. and Kim C.-K., Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid, *Int. J. Pharm.,* 243, 135, 2002.
[50] Trotta M., Debernardi F., and Caputo O., Preparation of solid lipid nanoparticles by a solvent emulsification-diffusion technique, *Int. J. Pharm.,* 257, 153, 2003.
[51] Pinto J.F. and Müller R.H., Pellets as carriers of solid lipid nanoparticles (SLN) for oral administration of drugs, *Pharmazie,* 54, 506, 1999.
[52] Cavalli R. et al., Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles, *Int. J. Pharm.,* 148, 47, 1997.
[53] Cui Z.R. and Mumper R.J., Topical immunization using nanoengineered genetic vaccines, *J. Conrol. Rel.,* 81, 173, 2002.
[54] Olbrich C. et al., Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA, *J. Control. Rel.,* 77, 345, 2001.
[55] Yang, S.C. et al., Body distribution of camptothecin solid lipid nanoparticles after oral administration, *Pharm. Res.,* 16, 751, 1999.
[56] Miglietta A. et al., Cellular uptake and cytotoxicity of solid lipid nanospheres (SLN) incorporation doxorubicin or paclitaxel, *Int. J. Pharm.,* 210, 61, 2000.
[57] Bargoni A. et al., Transmucosal transport of tobramycin incorporated in solid lipid nanoparticles (SLN) after duodenal administration to rats. II. Tissue distribution. *Pharmacol. Res.,* 43, 497, 2001.
[58] Zara G.P. et al., Pharmacokinetics and tissue distribution of idarubicin-loaded solid lipid nanoparticles after duodenal administration to rats, *J. Pharm. Sci.,* 91, 1324, 2002.
[59] Olbrich C. et al., Stable biocompatible adjuvants — a new type of adjuvant based on solid lipid nanoparticles: a study on cytotoxicity, compatibility and efficacy in chicken, *ATLA,* 30, 443, 2002.
[60] Ahlin P. et al., The effect of lipophilicity of spin-labeled compounds on their distribution in solid lipid nanoparticle dispersions studied by electron paramagnetic resonance, *J. Pharm. Sci.,* 92, 58, 2003.
[61] Kristl J. et al., Interactions of solid lipid nanoparticles with model membranes and leukocytes studied by EPR, *Int. J. Pharm.,* 256, 133, 2003.

[62] Siekmann B. and Westesen K., Thermoanalysis of the recrystallization process of melt-homogenized glyceride nanoparticles, *Colloids Surf. B,* 3, 159, 1994.
[63] Westesen K. and Bunjes H., Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix?, *Int. J. Pharm.,* 115, 129, 1995.
[64] Bunjes H., Westesen K., and Koch M.H.J., Crystallization tendency and polymorphic transitions in triglyceride nanoparticles, *Int. J. Pharm.,* 129, 159, 1996.
[65] Westesen K., Bunjes H., and Koch M.H.J., Physicochemical characterization of lipid nanoparticles and evaluation of their drug loading capacity and sustained release potential, *J. Controlled Rel.* 48, 223, 1997.
[66] Bunjes H., Siekmann B., and Westesen K., Emulsions of supercooled melts: a novel drug delivery system, in *Submicron Emulsions in Drug Targeting and Delivery,* Benita S., ed., Harwood Academic Publishers, Amsterdam, 1998, 175.
[67] Bunjes H., Koch M.H.J., and Westesen K., Effects of surfactants on the crystallization and polymorphism of lipid nanoparticles, *Prog. Colloid Polym. Sci.,* 121, 7, 2002.
[68] Bunjes H. et al., Incorporation of the model drug ubidecarenone into solid lipid nanoparticles, *Pharm. Res.,* 18, 287, 2001.
[69] Garti N. and Sato K., *Crystallization and Polymorphism of Fats and Fatty Acids,* Marcel Dekker, New York, 1988.
[70] Kaneko F., Polymorphism and phase transitions of fatty acids and acylglycerols, in *Crystallization Processes in Fats and Lipid Systems,* Garti N. and Sato K., eds., Marcel Dekker, New York, 2001, 53–97.
[71] Schwarz C. and Mehnert W., Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization, *J. Microencapsulation,* 16, 205, 1999.
[72] zur Mühlen A., Schwarz C., and Mehnert W., Solid lipid nanoparticles (SLN) for controlled drug delivery: drug release and release mechanism, *Eur. J. Pharm. Biopharm.,* 45, 149, 1998.
[73] Jenning V. and Gohla S., Comparison of wax and glyceride solid lipid nanoparticles (SLN), *Int. J. Pharm.,* 196, 219, 2000.
[74] Cavalli R. et al., Solid lipid nanoparticles as carriers of hydrocortisone and progesterone complexes with b-cyclodextrins, *Int. J. Pharm.,* 182, 59, 1999.
[75] Ford J.L. and Timmins P., *Pharmaceutical Thermal Analysis: Techniques and Applications,* Ellis Horwood, Chichester, 1989.
[76] Giron D., Thermal analysis of drugs and drug products, in *Encyclopedia of Pharmaceutical Technology,* Vol. 3, Swarbrick J. and Boylan J.C., eds., Marcel Dekker, New York, 2002, 2766.
[77] de Vringer T. and de Ronde H.A.G., Preparation and structure of a water-in-oil cream containing lipid nanoparticles, *J. Pharm. Sci.,* 84, 466, 1995.
[78] Olbrich C., Kayser O., and Müller R.H., Lipase degradation of Dynasan 114 and 116 solid lipid nanoparticles (SLN): effect of surfactants, storage time and crystallinity, *Int. J. Pharm.,* 237, 199, 2002.
[79] Wissing S.A. and Müller R.H., The influence of the crystallinity of lipid nanoparticles on their occlusive properties, *Int. J. Pharm.,* 242, 377, 2002.
[80] Eldem T., Speiser P., and Altdorfer H., Polymorphic behavior of sprayed lipid micropellets and its evaluation by differential scanning calorimetry and scanning electron microscopy, *Pharm. Res.,* 8, 178, 1991.
[81] Jenning V. and Gohla S., Encapsulation of retinoids in solid lipid nanoparticles (SLN), *J. Microencapsulation,* 18, 149, 2001.

[82] Cavalli R. et al., The effect of the components of microemulsions on both size and crystalline structure of solid lipid nanoparticles (SLN) containing a series of model molecules, *Pharmazie,* 53, 392, 1998.
[83] Wissing S.A. and Müller R.H., A novel sunscreen system based on tocopherol acetate incorporated into solid lipid nanoparticles, *Int. J. Cosm. Sci.,* 23, 233, 2001.
[84] Wissing S.A. and Müller R.H., The influence of solid lipid nanoparticles on skin hydration and viscoelasticity: *in vivo* study, *Eur. J. Pharm. Biopharm.,* 56, 67, 2003.
[85] Suryanarayanaran R. and Rastogi S., X-ray powder diffractometry, in *Encyclopedia of Pharmaceutical Technology,* Vol. 3, Swarbrick J. and Boylan J.C., eds., Marcel Dekker, New York, 2002, 3005.
[86] Westesen K., Siekmann, B., and Koch, M.H.J., Investigations on the physical state of lipid nanoparticles by synchrotron radiation x-ray diffraction, *Int. J. Pharm.,* 93, 189, 1993.
[87] Aquilano D., Cavalli R., and Gasco M.R., Solid lipospheres obtained from hot microemulsions in the presence of different concentrations of cosurfactant: the crystallization of stearic acid polymorphs, *Thermochim. Acta,* 230, 29, 1993.
[88] Lukowski G. et al., Crystallographic investigation of cetyl palmitate solid lipid nanoparticles, *Int. J. Pharm.,* 196, 201, 2000.
[89] Jenning V., Thünemann A.F., and Gohla S.H., Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids, *Int. J. Pharm.,* 199, 167, 2000.
[90] Bunjes H., Koch M.H.J., and Westesen K., Influence of emulsifiers on the crystallization of solid lipid nanoparticles, *J. Pharm. Sci.,* 92, 1509, 2003.
[91] Bunjes H., Einflussnahme unterschiedlicher Faktoren auf Struktur und Eigenschaften von Nanopartikeln aus festen Triglyceriden. PhD thesis, Friedrich Schiller University of Jena, 1998.
[92] Unruh T. et al., Self-assembly of triglyceride nanocrystals in suspension, *Langmuir,* 18, 1796, 2002.
[93] Illing A., Unruh T., and Koch M.H.J., Investigation on particle self-assembly in solid lipid based colloidal drug carrier systems, *Pharm. Res.,* 21, 592, 2004.
[94] Ugazio E., Cavalli R., and Gasco M.R., Incorporation of cyclosporin A in solid lipid nanoparticles (SLN), *Int. J. Pharm.,* 241, 341, 2002.
[95] Cavalli R., Gasco R.M., and Morel S., Behaviour of timolol incorporated in lipospheres in the presence of a series of phosphate esters, *S.T.P. Pharm. Sci.,* 2, 514, 1992.
[96] Chen D.B. et al., In vitro and in vivo study of two types of long-circulating solid lipid nanoparticles containing paclitaxel, *Chem. Pharm. Bull.,* 49, 1444, 2001.
[97] Hu F.Q. et al., Preparation of solid lipid nanoparticles with clobetasol propionate by a novel solvent diffusion method in aqueous system and physicochemical characterization, *Int. J. Pharm.,* 239, 121, 2002.
[98] Cui Z.R. and Mumper R.J., Coating of cationized protein on engineered nanoparticles results in enhanced immune responses, *Int. J. Pharm.,* 238, 229, 2002.
[99] Cui Z.R. and Mumper R.J., Genetic immunization using nanoparticles engineered from microemulsion precursors, *Pharm. Res.* 19, 939, 2002.
[100] Harris J.R., *Negative Staining and Cryoelectron Microscopy: The Thin Film Techniques,* Bios Scientific, Oxford, 1997.
[101] Severs N.J. and Shotton D.M., eds., *Rapid Freezing, Freeze Fracture and Deep Etching,* Wiley-Liss, New York, 1995.

[102] Siekmann B. and Westesen K., Preparation and physicochemical characterization of aqueous dispersions of coenzyme Q10 nanoparticles, *Pharm. Res.,* 12, 201, 1995.
[103] Sjöström B. et al., Structures of nanoparticles prepared from oil-in-water-emulsions, *Pharm. Res.,* 12, 39, 1995.
[104] Sjöström B., Bergenståhl B., and Kronberg B., A method for the preparation of submicron particles of sparingly water-soluble drugs by precipitation in oil-in-water-emulsions. II. Influence of the emulsifier, the solvent, and the drug substance, *J. Pharm. Sci.,* 82, 584, 1993.
[105] Demirel M. et al., Formulation and in vitro-in vivo evaluation of piribedil solid lipid micro- and nanoparticles, *J. Microencapsulation,* 18, 359, 2001.
[106] Amselem S., Domb A.J., and Alving C.R., Lipospheres as vaccine carrier systems: effects of size, charge, and phospholipid composition, *Vaccine Res.,* 1, 383, 1992.
[107] zur Mühlen A. et al., Atomic force microscopy studies of solid lipid nanoparticles, *Pharm. Res.,* 13, 1411, 1996.
[108] zur Mühlen A. and Mehnert W., Drug release and release mechanism of prednisolone loaded solid lipid nanoparticles, *Pharmazie,* 53, 552, 1998.
[109] Dingler A. et al., Solid lipid nanoparticles (SLN/Lipopearls): a pharmaceutical and cosmetic carrier for the application of vitamin E in dermal products, *J. Microencapsulation,* 16, 751, 1999.
[110] Cavalli R. et al., Duodenal administration of solid lipid nanoparticles loaded with different percentages of tobramycin, *J. Pharm. Sci.,* 92, 1085, 2003.
[111] Cavalli R. et al., Solid lipid nanoparticles (SLN) as ocular delivery system for tobramycin, *Int. J. Pharm.,* 238, 241, 2002.
[112] Fundaro A. et al., Non-stealth and stealth solid lipid nanoparticles (SLN) carrying doxorubicin: pharmacokinetics and tissue distribution after i.v. administration to rats, *Pharmacol. Res.,* 42, 337, 2000.
[113] Yang S.C. et al., Body distribution of intravenously injected camptothecin solid lipid nanoparticles and targeting effect on the brain, *J. Control. Rel.,* 59, 299, 1999.
[114] Sznitowska M. et al., Bioavailability of diazepam from aqueous-organic solution, submicron emulsion and solid lipid nanoparticles after rectal administration in rabbits, *Eur. J. Pharm. Biopharm.,* 52, 159, 2001.
[115] Mayer C. and Lukowski G., Solid state NMR investigations on nanosized carrier systems, *Pharm. Res.,* 17, 486, 2000.
[116] Ahlin P. et al., Influence of spin probe structure on its distribution in SLN dispersions. *Int. J. Pharm.,* 196, 241, 2000.

4 Lipospheres as Delivery Systems for Peptides and Proteins

Stephanie Könnings and Achim Göpferich

CONTENTS

4.1 INTRODUCTION

Delivery systems are designed to protect an incorporated drug from the environment during delivery and to provide a controlled release. The goal may be either to deliver a drug locally to specific sites in the body or to prepare a drug carrier system that acts as a reservoir at the site of injection over a certain time period [1].

In recent years, a growing number of potential peptide and protein drugs has been discovered as a result of progress in biotechnology and genetic engineering. Unfortunately, protein drugs are subject to numerous chemical and physical instability mechanisms and rapid enzymatic degradation; therefore, they often show low

0-8493-1692-8/05/$0.00+$1.50

bioavailabilities and have short *in vivo* half-lives, thus necessitating parenteral delivery [2]. To sustain therapeutic effects, these drugs have to be administered by infusion or via frequent injections. It is obvious that there is an urgent need for suitable delivery systems capable of preserving protein stability and improving administration frequencies, and thus lessening the strain on patients.

Particulate drug carriers that have been investigated for this purpose are oil/water (o/w) emulsions, liposomes, microparticles, and nanoparticles based on synthetic polymers or natural macromolecules [3]. Successful long-term delivery of peptide and protein drugs has been achieved by using biodegradable polymers such as copolymers of lactide and glycolide [4,5]. Use of synthetic materials, however, often goes along with biocompatibility problems, residual solvents, and detrimental effects on the incorporated drug during the manufacturing procedure or during polymer degradation after application [6].

Therefore, alternative carrier substances have been investigated in recent years. Among them, lipidic materials have garnered growing attention. Successful peptide or protein incorporation and delivery has been reported for liposomes [7], multivesicular liposome preparations [8], cubic phase gels [9], hollow lipid microparticles [10], hollow lipid microcylinders [11], microparticles [12,13], and solid lipid nanoparticles (SLN) for intravenous applications [14,15].

Lipospheres were first reported by Domb, who described them as water-dispersible solid microparticles of a particle size between 0.2 and 100 μm in diameter, composed of a solid hydrophobic fat core stabilized by a monolayer of phospholipid molecules embedded in the microparticles' surface [1]. Using this definition, liposphere size is on the nanometer scale. Usually, nanoscale particles consisting of a solid lipid core are termed SLN [16], though sometimes inconsistent nomenclature can be found. Unlike SLN, lipospheres are restricted to the stabilizing material of a phospholipid layer because of their definition [1]. This chapter focuses on research results obtained for peptide and protein formulations termed lipospheres, and it does not consider SLN literature at large.

Lipospheres have successfully been used to incorporate and deliver a variety of substances, including antiinflammatory compounds [17], local anesthetics [18], antibiotics [1], insect repellants [19], vaccines, and adjuvants [20]. The number of publications concerning protein delivery, though, is still limited. To the best of our knowledge, only a few peptide and protein drugs have been incorporated into lipospheres and characterized for release behavior to date (Table 4.1). Prerequisites for the use of any carrier for drug delivery are sufficient drug load, physical stability of the aqueous dispersion, and optimized drug release profiles [21]. This chapter will try to point out the special demands and difficulties associated with peptide and protein drugs when aiming at the realization of these prerequisites. Discussion of issues such as particle characterization and biocompatibility can be found elsewhere in this publication.

Proteins are challenging substances to formulate because of their many instabilities and, most often, high hydrophilicity [22]. The latter is one of the main obstacles encountered when designing delivery systems, as potential carriers most often consist of lipophilic materials, thus complicating preparation procedures and impeding high drug loading. Often, proteins are exposed to detrimental conditions

TABLE 4.1
Examples of Peptides and Proteins Incorporated into Lipospheres

Peptide/Protein Drug	Matrix Material	Preparation Method	Reference
Antigen	Waxes, fatty alcohols, paraffins, hard fat	Melt method, solvent technique	[1]
[D-Trp-6]-LHRH	Stearic acid	w/o/w multiple microemulsion	[38]
Thymopentin	Stearic acid	w/o/w multiple microemulsion, o/w multiple microemulsion	[39]
R32NS1 Malaria antigen	Tristearin, Polylactide, Polycaprolactone	Melt dispersion	[20]
Somatostatin	Triglycerides	Cosolvent–solvent evaporation	[37]
Triptorelin, Leuprolide	L-PLA, PLGA 50:50, PLGA 75:25	Cosolvent–solvent evaporation	[35]
Hydrophilic model drug	Triglycerides, PLA, Eudragit RS 100	Melt dispersion, solvent evaporation, w/o/w double emulsion	[36]

Note: LHRH = lutenizing hormone-releasing hormone; L-PLA = L-poly(lactic acid); PLGA = poly(lactic-co-glycolic acid); PLA = poly(lactic acid); w/o/w = water/oil/water; o/w = oil/water.

in the manufacturing procedure, and there are several publications dealing with stability issues during microparticle formulation [6,23]. We give an overview of protein stability issues before discussing preparation procedures for peptide- and protein-loaded lipospheres.

4.2 PROTEIN STABILITY

4.2.1 General Considerations

Peptide and protein stability is highly dependent on amino acid composition and sequence and, for proteins, on the formation of higher-order structures, which means that every protein has to be considered as a special case. Given a certain sequence, external factors such as pH, ionic strength, temperature, pressure, and the existence of interfaces can also have a tremendous impact on peptide and protein integrity [24].

There are two main degradation pathways:

- Physical or noncovalent degradation, which leads to changes in secondary tertiary structures
- Chemical inactivation, which results from changes in primary structure [6]

The term "stability" can have different meanings in the context of protein formulations. A stable pharmaceutical product according to the U.S. Food and Drug Administration definition is one that deteriorates no more than 10% in 2 years [25]. Conformational and physical stability of a protein are defined as the ability of the protein to retain its tertiary structure [6]. Noncovalent degradation is relevant mainly for proteins having higher order structures, rather than peptides. Native structure is maintained by a balance of noncovalent interactions such as hydrogen bonds,

van der Waals interactions, salt bridges, and hydrophobic interactions [26]. Classic conditions leading to loss of conformational stability, called denaturation, are elevated temperature, extremes of pH, denaturants, and adsorption to hydrophobic surfaces [6]. Proteins can unfold locally and globally, which may lead to inactive forms. In biochemistry, this inactivity is expressed by the magnitude of the change in Gibbs free energy between the folded and the unfolded states of the protein. The larger the free energy change, the more stable the protein. For most proteins, the unfolded state is insoluble and favors aggregation [24].

Considering chemical stability, even alterations at single amino acids or the peptide bond can be detrimental [6]. Chemical reactions having an impact on protein stability include hydrolysis of the peptide bond, deamidation, oxidation, β-elimination, isomerization, and disulfide bond breakage and formation. The extent to which they occur is mainly influenced by the temperature and pH value of the solution [24].

Bearing in mind that proteins react sensitively to the above-mentioned environmental conditions, preparation procedures for protein pharmaceuticals have to be chosen very carefully to preserve protein integrity and functionality.

4.2.2 Protein Stability during Formulation Procedures

Protein stability during encapsulation in biodegradable polymer microparticles has been reviewed in detail [6,23,27]. In comparison, little information is available on lipid materials. However, conditions causing stability problems are not specific for polymer microparticle formulations. Lipids, being a hydrophobic material like many biodegradable polymers, may involve similar processing parameters [22,28].

When formulating lipophilic materials, techniques often involve the use of organic solvents, interfaces with aqueous solutions, and high-shear forces [6]. One of the most often used techniques to encapsulate proteins is the water/oil/water (w/o/w) double-emulsion solvent evaporation technique, in which an aqueous protein solution is emulsified into an organic solution of the matrix material. This primary emulsion is added to an outer aqueous phase in which particles start to harden as the organic solvent evaporates. Alternatively, the solid protein can be added directly to the organic solution in a solid/oil/water (s/o/w) emulsion method [23].

Upon contact of an organic solvent with an aqueous protein solution, the solvent can diffuse into the water phase, alter its ionic strength, or bind directly to the protein, all favoring the exposure of the protein's hydrophobic regions, which can lead to the formation of soluble and insoluble aggregates [6]. Some organic solvents are capable of solubilizing lyophilized proteins without denaturing them. They are generally protic and hydrophilic [6]. An important factor in influencing protein solubility is the pH of the aqueous solution before lyophilization [29].

Upon addition of proteins to aprotic, hydrophobic solvents, the increased intramolecular interactions of the lyophilized protein result in restricted conformational mobility of the protein, thus restricting activity [30]. However, proteins display increased thermostability in anhydrous organic solvents as a result of the reduced conformational mobility [31], and water-free methods may help avoid aggregation processes that occur when using the double-emulsion technique, in which the protein is conformationally mobile.

During emulsification, a large, hydrophobic surface is formed. Exposure to air, which has a high hydrophobicity that favors unfolding, is considered a main cause of protein inactivation during the emulsification processes [6]. Proteins can adsorb strongly to both hydrophilic and hydrophobic materials. However, although the former adsorption is typically reversible, the latter results in irreversible conformational changes. Adsorption is strongest at the isoelectric point of the protein [6].

Methods employed for emulsification, such as homogenization or ultrasonication, will introduce large pressure gradients, shear forces, and heat development in the emulsion, thus speeding up unfolding and denaturation [28]. In addition, ultrasound has been proven to produce free radicals that can initiate chemical reactions [32].

Before evaluating protein stability during liposphere preparation, a summary of the different approaches for peptide and protein encapsulation will be given. Table 4.1 shows an overview of relevant publications arranged according to their publication dates.

4.3 PREPARATION OF PEPTIDE- AND PROTEIN-LOADED LIPOSPHERES

4.3.1 Preparation Methods

Lipospheres can contain a biologically active agent in the core, in the phospholipid, adhered to the phospholipid, or a combination of the two [1]. Since the emergence of lipospheres, a number of research teams have conducted studies to investigate relevant production parameters such as the effects of different compositions, ratio of ingredients, drugs, and preparation procedures on encapsulation efficiency, size distribution, and release characteristics [20,33–36]. Within this chapter, only results relating to peptide and protein drugs shall be considered, and the reader is referred to the literature and the other chapters in this book for a complete overview.

Two preparation methods for drug-loaded lipospheres can be used: a solvent technique or a melt technique [1]. For the solvent technique, organic solvents are employed to dissolve the active agent, the solid carrier, and the phospholipid component. After evaporating the solvent, warm buffer solution is mixed with the resulting solid until a homogeneous dispersion of lipospheres is obtained.

In contrast, the melt method, where the lipophilic agent is melted together with the lipid core material or dissolved in melted core material, is described as the preferred technique. The phospholipid, together with warm aqueous medium, is added as a solid, followed by mixing (mechanical shaking or stirring, fine mixing using homogenization and sonication) and rapidly cooling the preparation to solidify the liquid core.

It has been suggested that hydrophilic antigens should be dissolved in aqueous buffer and added to the molten mixture of vehicle and phospholipid [1]. For the preparation of R32NS1 malaria antigen lipospheres, the lipid components at a 1:1 molar ratio were dissolved in chloroform in a round-bottom flask. After evaporation of the organic solvent, the lipid mixture was heated from 40 to 80˚C to melt the fat. Warm phosphate-buffered saline containing the antigen was added, and the formulation

was mixed until a homogeneous dispersion was obtained. Cooling was performed by immersion of the flask in a dry ice–acetone bath for several seconds while shaking. Antigen encapsulation was found to be more than 80% [20].

Although lipospheres are primarily designed for the incorporation of lipophilic substances, Domb suggests approaches for processing a water-soluble agent [1]. Because the inner core of the liposphere is hydrophobic, it is recommended that the water solubility of the agent be decreased before liposphere preparation. Possible methods suggested are using a water-insoluble salt or base, a complex, or an insoluble precursor form of the agent, or preparing an aqueous medium in which the agent is less soluble (e.g., by adjusting the pH or ionic strength or by adding salts or additives).

4.3.1.1 Preincorporation into Lipophilic Carriers

Alternatively, the hydrophilic agent can be preincorporated into liposomes or microparticles that can be used as hydrophobic agent particles and incorporated into lipospheres with a matrix having a lower melting point [1]. This was demonstrated for tetracaine; however, no example exists for peptide or protein incorporation. Successful reports about model peptide incorporation into lipid microparticles can be found in Reithmeier: A solvent evaporation and a melt dispersion were compared for insulin, somatostatin, and thymocartin [12,13,37]. For the solvent evaporation method, the peptide drug was added as a solid or an aqueous solution to an organic lipid solution, which was then dispersed in an outer aqueous phase and stirred for evaporation of the organic solvent. For the melt dispersion method, the peptide drug was added as a solid or an aqueous solution to a lipid melt, which was subsequently poured into a cooled outer aqueous phase and stirred until solidification of the particles occurred.

Domb presents an example of liposphere encapsulation of tetracaine/tristearin microparticles having a size of less than 38 μm. The particles were suspended in molten ethyl stearate containing lecithin at 40˚C. The melting point of tristearin is 65 to 72˚C, so the microparticles remained solid during liposphere preparation. Warm phosphate buffer was added and the formulation was mixed and cooled. The resulting lipospheres had a particle size of 50 μm [1].

Domb further describes a method of incorporating antigens into lipospheres where the antigen, together with lipid A, an adjuvant, was first incorporated into multilamellar liposomes. Ethyl stearate and L-alpha-lecithin were heated to 40˚C to melt the ethyl stearate. Warm liposome dispersion was then added and the formulation shaken and cooled as described for the melt method before [1].

4.3.1.2 Multiple Microemulsion

A different approach of protein encapsulation is reported by Morel, Gasco, and Cavalli [38]. These authors describe a method of applying a warm multiple microemulsion in which the peptide is dissolved in an aqueous solution and added to a mixture of melted stearic acid, egg lecithin, and butyric acid at 70˚C. This primary microemulsion is then added at 70˚C to an aqueous solution of egg lecithin, butyric acid, and taurodeoxycholate sodium salt. Addition of warm multiple microemulsions to water at 2˚C leads to precipitation of the lipid phase, forming solid lipospheres.

This method resulted in an encapsulation efficiency of 90% and in particles having an average diameter of 300 nm [38]. Müller reports that large-scale experiments at Vectorpharma in Italy are employing this method [16].

The same group of authors has reported encapsulation of thymopentin, again using the warm w/o/w multiple microemulsion and additionally introducing an o/w method in which the distribution coefficient of thymopentin is altered by forming a salt with a lipophilic counter ion, sodium hexadecyl phosphate (SHDP) [39]. The peptide was thus contained in a stearic acid melt that was mixed with an aqueous solution of egg phosphatidylcholine, taurodeoxycholate sodium salt (TDC), and butanol. TDC, like sodium hexadecyl phosphate, has the potential to act as a counter ion for the peptide. Determination of the distribution coefficient revealed that it showed only a minor effect and even reduced SHDP efficiency. In this preparation, though, TDC is supposed to be occupying the interface and thus not interfering with the salt formation between peptide and SHDP.

Particles resulting from the o/w method were found to have a size of 100 nm. After washing, an incorporation of 5.2% peptide was obtained, with recovery being 47% compared to 1.7% incorporated peptide, and 63% recovery with the w/o/w method; particle size was 200 nm. Release experiments with lipospheres containing a lipid core have shown sustained release ranging from a few hours to several days. The preferred core material for delayed release, according to Domb, is a polymer such as polylactide [1].

To create lipospheres using polymers, the same melt dispersion as described above has successfully been applied for the formation of antigen-loaded lipospheres using a 1:1 (w/w) ratio for phospholipid and polymer [20].

4.3.1.3 Cosolvent Method

A new approach using a cosolvent–solvent evaporation method for peptide-loaded lipospheres having a polymer core has been described by Rasiel and coworkers [35], who investigated solvents suitable for dissolving the polymers and at the same time mixing with a protein solution in an organic solvent as well. The final preparation consisted of poly(lactic acid) (PLA) and hydrogenated soybean phosphatidylcholine (HSPC) dissolved in chloroform and mixed with peptide dissolved in N-methylpyrollidone to create a clear solution. This solution was then added to 0.25% aqueous PVA solution by vortex mixing, to form the hydrophobic core. After adding this solution to a larger amount of 0.1% polyvinyl alcohol (PVA), the system was stirred for 30 min.

An attempt was made to prepare peptide-loaded lipospheres according to Domb's description of antigen encapsulation [1], where a thin film of polymer, phospholipid, and drug is formed after evaporation of organic solvent, and lipospheres are created by adding warm buffer solution and mixing. This resulted only in the formation of large particles at low yield.

Several organic solvents were investigated, including dichloromethane, chloroform, ethyl acetate, acetone, methylethylketone, tetrahydrofuran, acetonitrile, and mixtures thereof, but only water-insoluble solvents were suited for dissolving polymer and phospholipid in high concentrations and forming spherical particles in good yield.

Polymers with a molecular weight above 50,000 Da did not form uniform particles, and therefore L-poly(lactic acid) (L-PLA, Mw 2000), poly(lactic-co-glycolic acid) (PLGA, 75:25, 15,000 Da), and PLGA (50:50, 23,000 Da) were chosen for further investigation. Only L-PLA showed good entrapment efficiencies (80% for triptorelin and >50% for leuprolide). PLGA failed to entrap more than 10% in both cases. In comparison, microspheres were produced that differ from the liposphere preparation only in that the solid hydrophobic core of the lipospheres is stabilized by a monolayer of phospholipid molecules embedded in its surface. All liposphere particle diameters were smaller compared to those of the microspheres.

Another group having done extensive studies on the influence of preparation procedure on liposphere characteristics is Cortesi et al. [36]. Strictly speaking, they were not investigating particles as described by Domb, who states in the patent that phospholipids may be replaced only in part with surfactants such as Tween, Span, and PEG surfactants. Steroids cannot function alone but may be incorporated, and amphiphiles can be added to the phospholipid coating to alter the surface charge [1].

Cortesi et al. worked completely without phospholipids and used cholesterol, cetyl alcohol, monostearate, and oleate as polar lipids in combination with triglycerides as apolar components, but they still termed the resulting particles "lipospheres" [36]. For the encapsulation of proteins, they suggest a solvent evaporation method to avoid high-temperature exposure during melt method preparation. Consisting of tristearin/glyceryl monostearate 2:1 (w/w), particles proved their poor mechanical properties, being fragile and having formed an increased number of interparticular bridges as compared to through the melt method. Thus, they investigated mixed matrices constituted of lipids in combination with polymers up to 20%. Both biodegradable (PLA) and nonbiodegradable polymers (Eudragit RS 100) were used, and they allowed an improvement of mechanical characteristics. Unfortunately, there is no datum published about the incorporation of proteins in context with this composition. For the hydrophilic model drug sodium cromoglycate, a melt dispersion and a w/o/w double emulsion were compared for a tristearin/glycerol monostearate formulation. The melt dispersion resulted in 2% encapsulation efficiency, which could be improved by the double-emulsion method by up to 50% encapsulation efficiency.

4.3.2 Influence of Preparation Parameters on Drug Encapsulation

Apart from different preparation procedures, factors determining the loading capacity of a drug in lipid carriers have been found to be the matrix composition and thus the solubility of drug in melted lipid, the miscibility of drug melt and lipid melt, the chemical and physical structure of the solid lipid matrix, and the polymorphic state of the lipid material [16].

4.3.2.1 Preparation Method

Melt method procedures are reported to show higher incorporation efficiencies [18,37]. However, a problem arising from the use of molten lipid phase is a different crystallization behavior than that exhibited during solvent processes. Reithmeier

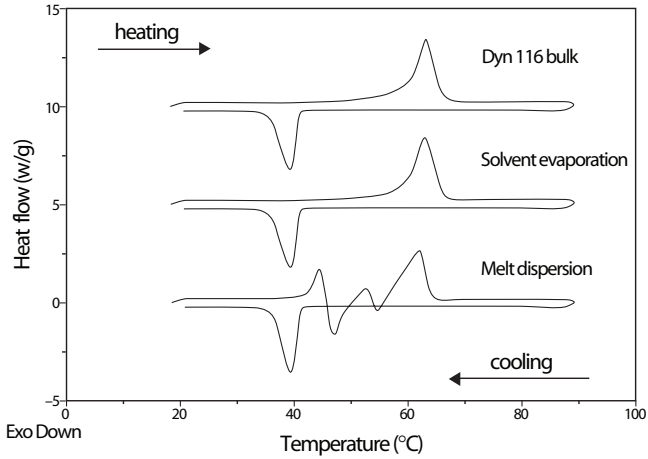

FIGURE 4.1 Differential scanning calorimetry heating and cooling curves of glyceryl tripalmitate (Dyn 116) bulk material, with microparticles prepared by solvent evaporation and microparticles prepared by melt dispersion 1 d after the preparation. The plots are displaced vertically for better visualization. (Adapted from [13] with permission from Elsevier.)

reports about differential scanning calorimetry investigations of microparticles prepared by the melt and the solvent evaporation method [13]. Whereas lipid bulk material and microparticles prepared by solvent evaporation show only one single endothermic peak that results from the melting of the stable crystalline form (β-modification), for microparticles prepared by melt dispersion, three peaks were detected [13]. The first endothermic peak represents the melting of the α-modification, which crystallizes subsequently in the β′-modification, resulting in an exothermic peak. The second endothermic peak corresponds to the melting of β′-modification, and the third to melting the stable β-modification (Figure 4.1). Melt dispersion techniques most often comprise a fast congealing step in which only the unstable α-modification is formed, whereas slow diffusion of organic solvent into the outer aqueous phase allows for slow solidification and arrangement of the molecules in a stable β-modification [13]. Higher drug-loading capacities have been reported for unstable modifications with lower crystalline order [40], as less perfect crystals with many imperfections offer more space to accommodate drugs. During storage, however, a transformation of unstable modifications takes place and the formation of more stable modifications has shown to promote drug expulsion, which can result in burst release behavior [41].

The presence of surfactant is also reported to lead to reduced crystallinity [16] being another possible reason — apart from drug solubilization — for higher incorporation efficiencies into lipid carriers.

4.3.2.2 Phospholipid Content

The influence of phospholipid content on drug encapsulation and release has been examined both for classic lipospheres having a lipid core and for polymer lipospheres having a polymer core [35,37].

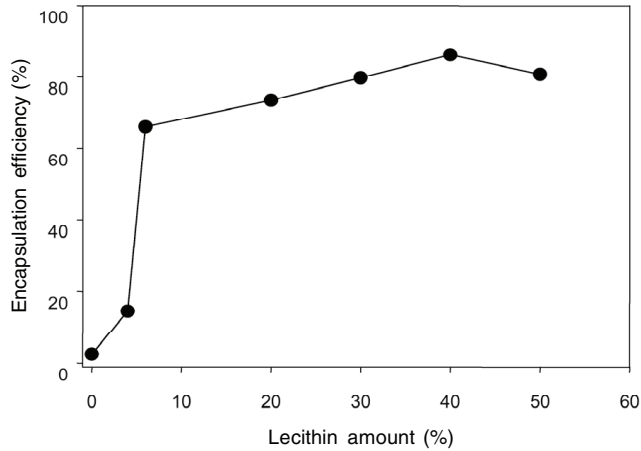

FIGURE 4.2 Influence of added lecithin amount on encapsulation efficiency of somatostatin. Solvent evaporation method: solvent hexane, cosolvent methanol, theoretical loading 2%. (Adapted from [37] with permission from Elsevier.)

Reithmeier investigated the influence of fat/phospholipid ratio to improve drug encapsulation efficiency into microparticles prepared by the solvent evaporation method [37]. A cosolvent–solvent evaporation method like the one described above for the preparation of polymer lipospheres [35] was used. Here, somatostatin as a model peptide was dissolved in methanol and added to a solution of the lipid components in hexane [37].

Above a phospholipid content of 6% the encapsulation efficiency showed a large increase (Figure 4.2). Reithmeier suggests that increased stability of the primary emulsion or electrostatic interactions between peptide and lecithin are possible reasons for this increase [37]. Rasiel compared different phospholipids in varying concentrations [35]. Unlike in Reithmeier's experiments, phospholipids did not stabilize the polymer emulsion, and PVA had to be added to the formulation as a further surfactant. The phospholipids were judged for their ability to interact with polymers regarding free phospholipid content in the supernatant. Strong phospholipid–polymer interactions were found to result in decreased particle size (HSPC) compared with weaker interactions (egg phosphatidylcholine [EPC]). A ratio of 1:6 was suggested to be most favorable because of an optimal liposphere shape. Different phospholipid/polymer ratios were assessed for their release behavior, which will be discussed later; no effects of phospholipid content on drug-loading capacity were discussed.

For the melt method, a phospholipid/triglyceride ratio of 1:4 was found to result in the best yield of drug-free lipospheres when compared with ratios of 1:2, 1:3, and 1:6 [33]. Domb [42] investigated different phospholipid/fat ratios with respect to the phospholipid content on the liposphere surface. At a phospholipid/triglyceride ratio of 1:2 to 1:4, 70 to 90% of the phospholipid was located at the liposphere surface. Increasing the phospholipid content resulted in the formation of other phospholipid structures, such as liposomes.

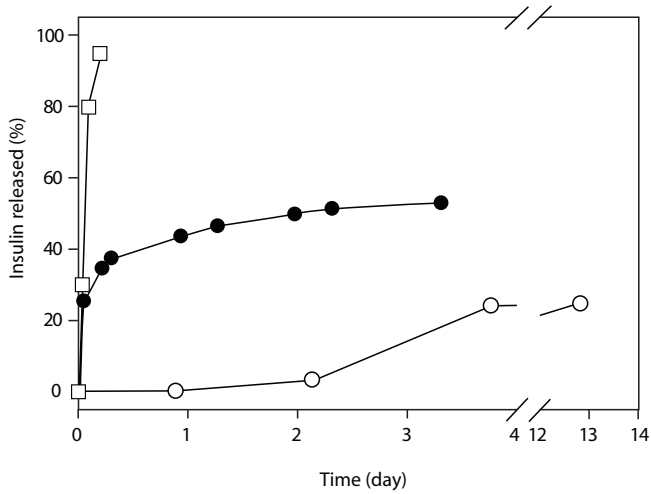

FIGURE 4.3 *In vitro* release of insulin (release medium: PBS buffer, pH 7.4, 10 mmol, assessment of the residual insulin in the microparticles), (solid circles) microparticles washed with water (drug loading 2.3%), (open circles) microparticles washed with 0.01 M HCl, (open squares) control (insulin powder). (Adapted from [12] with permission from Elsevier.)

The aspect of by-products functioning as alternative drug incorporation sites is most often neglected in liposphere experiments. Domb observed unincorporated bupivacaine in tristearin formulations in the form of dispersible microparticles composed of the solid drug and of phospholipids [42].

Mehnert implicates micelles, mixed micelles, liposomes, and drug-nanoparticles, depending on composition, as possible structures resulting from SLN preparation methods, apart from the main particulate carrier. He calls for control samples such as a liposome formulation prepared under identical conditions [40]. Often, liposphere preparation procedures include a washing step with phosphate-buffered saline (PBS) to remove unencapsulated drug which possibly partly removes by-products as well.

Reithmeier reports about a decrease in the drug loading of microparticles after a washing step. When washed particles were compared with nonwashed particles, a significant decrease in burst release phenomena could also be found (Figure 4.3). This was explained by removal of surface-located drug crystals that formed during solidification of the lipid carrier [12].

4.3.3 Stability of Protein Drugs during Preparation

To our knowledge, no explicit studies of protein stability during liposphere preparation and release have been conducted, and protein stability has to be estimated considering what is generally known about detrimental effects during preparation procedures, as described above. Domb suggests that the carrier have a low melting temperature to avoid antigen exposition to high temperatures to preserve the antigenicity during preparation [1]. Antigen functionality was indirectly assessed by immunization of test animals and monitoring of IgG production using an enzyme-linked

immunosorbent assay. An immune response comparable to liposome carriers — and better — could be detected [20].

In regard to microemulsions, it should be pointed out that there are different opinions about the structure of these systems [40]. Microemulsions are defined as clear, thermodynamically stable dispersions obtained by mixing surfactant, cosurfactant, oil, and water [34]. Gasco, in agreement with other scientists, understands them as two-phase systems composed of an inner and an outer phase. Microemulsions proved to be more stable than emulsions [38], sometimes termed "critical solutions" (see [16]), thus obviating the need for high-shear emulsification methods that could exert detrimental effects on a protein drug. Still, it is desirable that microemulsions be further characterized in terms of phasing, to have a better understanding of the organization of a microemulsion system and, thus, critical parameters for protein stability.

To investigate whether the high temperature needed for melting the lipid components was harmful, Morel assessed thymopentin stability by observation in water heated to 70˚C for 1.5 h (three times as long as it takes for microemulsion preparation) without detecting degradation products [39]. It has been found, however, that the thermal stability of proteins in microemulsions can differ from their stability in water. Although in some cases it was found that protein micellar solutions were stable, physicochemical properties of proteins and thermal protein stability are described as being highly dependent on the water content of a microemulsion system [43].

Rasiel claims that liposphere preparation with the use of N-methylpyrrolidone can no longer be considered to be a double-emulsion formulation because there is no use of aqueous inner phase to dissolve the drug. Instead, this preparation is considered to be an o/w emulsion, which is less sensitive to stability problems [35].

Several research groups employ high-performance liquid chromotography (HPLC) analytics to monitor release [35,38,39]. Possible degradation products could result in altered retention behavior, but no such observations have been published for peptides or proteins released from lipospheres.

4.4. RELEASE OF PEPTIDE AND PROTEIN DRUGS FROM LIPOSPHERES

Apart from a sufficient drug load and formulation stability, which have been discussed above, an optimized drug release profile is another prerequisite for a drug delivery system [21]. Drug release of a hydrophilic substance from a lipophilic matrix material can depend on several factors, such as matrix material composition [44], properties of the incorporated drug (solubility in lipid and aqueous medium, molecular weight, interactions with the carrier) [41,45], drug loading [46,47], presence of surfactants [37], particle size [48], and preparation method [49], which will be discussed in the following section.

4.4.1 Classic Lipospheres

Domb claims in his patent that the release rate of incorporated substances is controlled by both the phospholipid coating and the carrier [1]. The first peptide-loaded

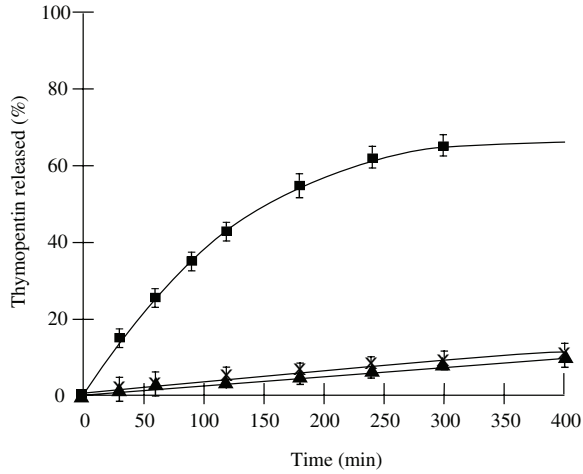

FIGURE 4.4 Percentage release of thymopentin from (filled triangles) lipospheres obtained by o/w microemulsion, from (X) lipospheres obtained by w/o/w microemulsion and diffusion from an (filled squares) aqueous solution. (Adapted from [39] with permission from Elsevier.)

lipospheres to be investigated, malaria antigen–loaded lipospheres prepared by the melt method, were only characterized *in vivo*, where they induced a superior immune response compared to that evoked by liposomes. No *in vitro* data were presented [20].

Liposphere preparations of luteinizing hormone-releasing hormone (LHRH) prepared with the multiple-emulsion method were characterized for release behavior for 8 h. A pseudo zero-order release of 10% drug loading was observed [38]. Thymopentin lipospheres prepared by the same method released 10% of their loading in 6 h, again following pseudo zero-order kinetics [39]. These results were obtained by placing lipospheres and a blank protein solution for comparison in the donor phases in a multicavity microdialysis cell. This result can only be interpreted taking into account the diffusion data from protein solution, which was 50% in 8 h for LHRH and 65% in 6 h for thymopentin (Figure 4.4). Unfortunately, the experiments were not continued, so the complete release potential can only be estimated. No difference between preparation with counter ion or w/o/w method was found.

Particles in the nanometer size range often show burst release phenomena as a result of large surface area and short diffusion distance of the drug [48]. Zur Mühlen showed a direct correlation between microparticle size range and the extent of burst release and the release profile for Compritol microparticles loaded with tetracaine [48]. That is probably one reason why Domb demands that the particles to be greater than 1 µm for controlled drug delivery [1].

Reithmeier produced particles below 5 µm [37]. When comparing microparticles obtained with different triglyceride/phospholipid ratios, only particles with a lecithin content below 6% showed a sustained release behavior for 3 d (Figure 4.5). Higher amounts of lecithin having shown a high increase in encapsulation efficiency lead to a burst effect, as depicted for a 1:1 ratio, which was even more pronounced for higher lecithin contents (not shown). A microparticle preparation without the use of

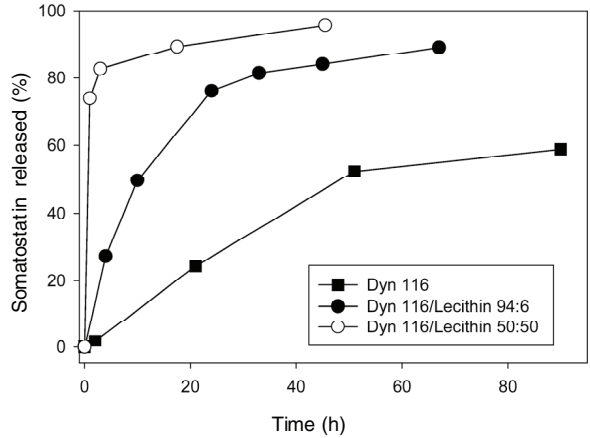

FIGURE 4.5 Influence of lecithin amount added to glyceryl tripalmitate (Dyn 116) on somatostatin release (particles washed with bidestilled water). (Adapted from [37] with permission from Elsevier.)

lecithin is shown as a comparison. No burst release could be observed; not all peptide was released from the particles though. Incomplete release can result from the loading of a drug in amounts that are too small for the formation of a network of pores throughout the whole matrix, through which the drug can diffuse out. Another reason for incomplete release is the interactions of a released substance with the carrier material as it has been observed for insulin [12].

The accelerating effect of the phospholipid on release from lipid microparticles might be even more pronounced *in vivo*, where it is reported that lipid particles degrade faster in the presence of surfactant, which enables the contact with lipases [3].

4.4.2 Polymer Lipospheres

For matrices made from biodegradable polymers, longer release periods have been reported. When loaded with malaria antigen, one single injection was sufficient to induce an immune response without the help of adjuvant. Polycaprolactone proved to be superior to polylactide, which was explained by polycaprolactone's slower degradation behavior [20].

Polymer matrices represent a powerful tool of controlling release rates. Different profiles can be obtained by varying molecular weight and copolymer composition. For example, PLA matrices made from one single stereoisomer are more resistant to degradation than are racemic polymers. Increasing the amount of glycolic acid in PLGA leads to an increase of degradation rate [50].

Rasiel investigated triptorelin release profiles from lipospheres made from L-PLA, PLGA 50:50, and PLGA 75:25 [35]. Although both PLGA polymers showed a burst release within the first 24 h, L-PLA released the peptide for over 30 d (Figure 4.6).

The effect of two different phospholipids in different concentrations was investigated: EPC showed only weak interactions with PLA, and HSPC showed strong

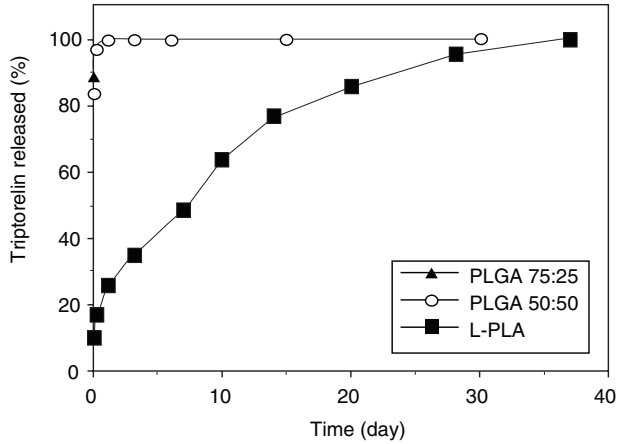

FIGURE 4.6 Effects of polymer type on the cumulative release of triptorelin from lipospheres. Lipospheres were prepared from L-PLA (solid squares), PLGA 50:50 (open circles), or PLGA 75:25 (filled triangles) with HSPC in a 1:6 phospholipid/polymer ratio. Triptorelin (4 mg) was dissolved in N-methylpyrrolidone (500) μL and mixed with a chloroformic solution of L-PLA and HSPC (1 mL). The release experiment was performed in pH 7.4 phosphate buffer, at 37˚C, and analyzed by HPLC. (Adapted from [35] with permission from Wiley & Sons.)

interactions. Leuprolide release was fastest from lipospheres prepared from PLA and EPC compared with those prepared from PLA/HSPC lipospheres and with microspheres prepared without phospholipid (Figure 4.7). No difference was found when varying EPC ratios were used, and 80% of the drug was released within the first 48 h (data not shown), whereas preparations of HSPC/L-PLA showed sustained release for up to 30 d. No clear correlation between phospholipid content and release profile can be deducted, as for lipid lipospheres. Surprisingly, polymer microspheres without the presence of phospholipid showed a faster release profile than lipospheres.

Drug release was observed to depend on the kind of protein as well, for both polymer lipospheres (Figure 4.8A) and lipid microparticles (Figure 4.8B). As for the peptide and protein release profile, polymer lipospheres are superior to lipid lipospheres if one is aiming at long duration. On degradation and erosion however, the physicochemical environment inside a polymer matrix constantly changes, and peptide protein drugs may be exposed to detrimental effects, including low pH and acylation by degradation products. Triglyceride matrices, in contrast, have been shown to preserve the integrity and bioactivity of encapsulated model peptides and seem to be a promising alternative to polymer matrices [41].

4.5 CONCLUSION

Lipospheres have successfully been used to deliver a variety of substances, among them peptide drugs. Lipospheres seem to fulfill the basic requirements of a carrier for drug delivery. A sufficient drug load can be achieved, depending on the incorporation methods. Attempts have been made to investigate procedures, limiting

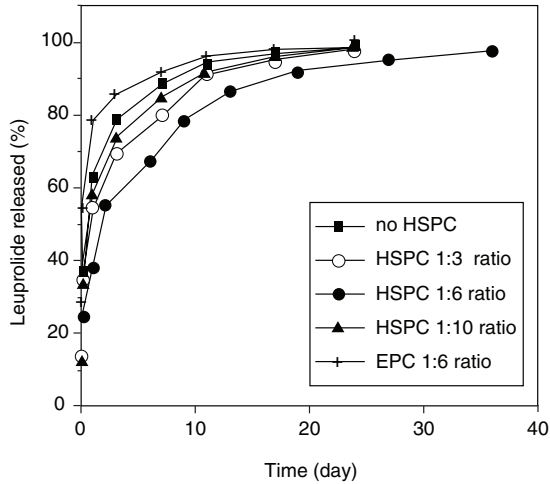

FIGURE 4.7 Effects of phospholipid/polymer ratio on the cumulative release of leuprolide from lipospheres. Lipospheres were prepared from L-PLA and EPC (1:6, pluses), hydrogenated soybean phosphatidylcholine (1:3, open circles), HSPC (1:6, filled circles), or HSPC (1:10, filled triangles), as described in Figure 4.6. Microspheres release data (filled squares) are presented for comparison. (Adapted from [35] with permission from Elsevier.)

detrimental preparation steps for peptide and protein drugs, though there is still a need for further optimization.

Lipospheres are often praised for their good physical stability and dispersibility in aqueous solution [1]. For increased storage stability of protein drugs, they can be freeze-dried and reconstituted before use. They show potential for being used to target peptide and protein drugs to the site of action while avoiding systemic site effects. Drug release profiles between days and several weeks can be adjusted by the choice of matrix material; classic lipospheres, however, are more suitable for shorter time periods, necessitating the use of a polymer core for sustained release of more than 3 d.

To estimate the future prospect of lipospheres as a drug delivery system for peptides and proteins, more investigations with a wider peptide and protein spectrum are desirable, as the available data are still restricted to only a few publications. Stability during preparation and long-term storage has not yet been dealt with in detail for peptide and protein drugs, but it is one of the prerequisites for successful protein pharmaceutics.

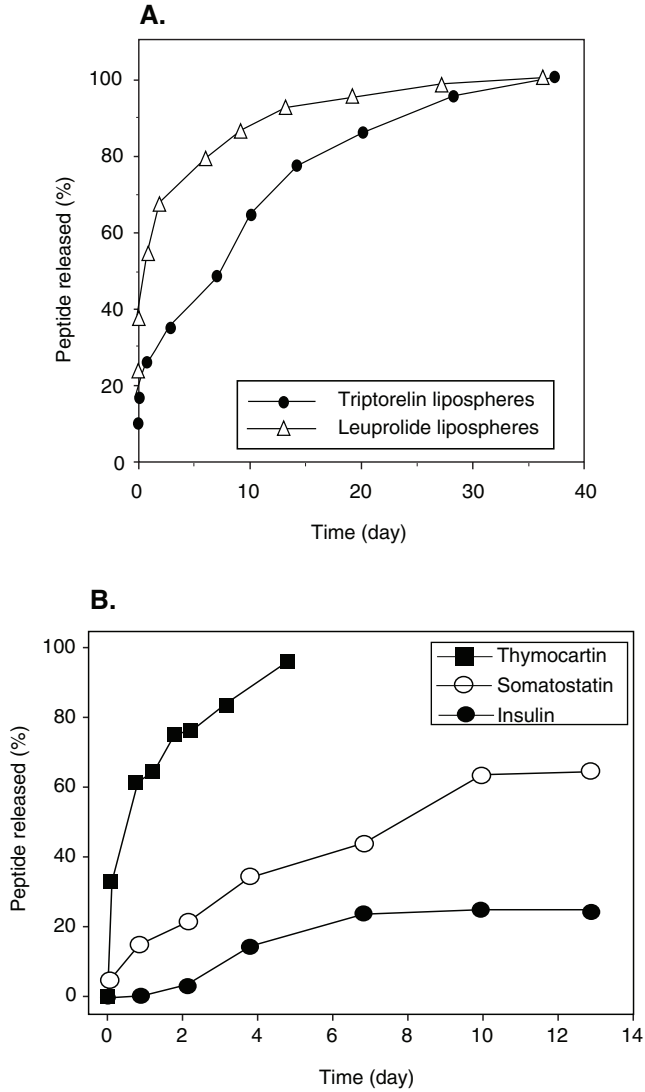

FIGURE 4.8 (A) Cumulative release of triptorelin and leuprolide from lipospheres. Lipospheres were prepared from L-PLA and HSPC, as described in Figure 4.6. The release experiment was performed in pH 7.4, phosphate buffer, at 37˚C, and analyzed by HPLC for both formulations. (Adapted from [35] with permission from Elsevier.). **(B)** Comparison of release profiles of thymocartin (loading 9.0%), somatostatin (loading 9.3%), and insulin (loading 6.83%) from glyceryl tripalmitate microparticles. (Adapted from [37] with permission from author.)

REFERENCES

[1] Domb, A.J. and Maniar, M., Lipospheres for controlled delivery of pharmaceuticals, pesticides, and fertilizers. Nova Pharmaceutical Corp. 90-US6519[9107171], 79. 8-11-1990.

[2] Pitt, C.G., The controlled parenteral delivery of polypeptides and proteins, *International Journal of Pharmaceutics,* 1990, 59, 173–196.

[3] Müller, R.H. and Hildebrand, G.E., Eds., 2003. *Pharmazeutische Technologie: Moderne Arzneiformen*. 2. erweiterte Aufl., Wissenschaftliche Verlagsgesellschaft, Stuttgart.

[4] Cohen, S. et al., Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres, *Pharmaceutical Research,* 1991, 8, 713–720.

[5] Lewis, D.H., Controlled release of bioactive agents from lactide/glycolide polymers, *Drugs and the Pharmaceutical Sciences,* 1990, 45, 1–41.

[6] Schwendeman, S.P. et al., Stability of proteins and their delivery from biodegradable polymer microspheres, *Drugs and the Pharmaceutical Sciences,* 1996, 77, 1–49.

[7] Crommelin, D. et al., Liposomes: vehicles for the targeted and controlled delivery of peptides and proteins, *Journal of Controlled Release,* 1997, 46, 165–175.

[8] Ye, Q. et al., DepoFoam technology: a vehicle for controlled delivery of protein and peptide drugs, *Journal of Controlled Release,* 2000, 64, 155–166.

[9] Shah, J.C., Sadhale, Y. and Chilukuri, D.M., Cubic phase gels as drug delivery systems, *Advanced Drug Delivery Reviews,* 2001, 47, 229–250.

[10] Bot, A.I. et al., Novel lipid-based hollow-porous microparticles as a platform for immunoglobulin delivery to the respiratory tract, *Pharmaceutical Research,* 2000, 17, 275–283.

[11] Meilander, N.J. et al., Lipid-based microtubular drug delivery vehicles, *Journal of Controlled Release,* 2002, 71, 141–152.

[12] Reithmeier, H., Herrmann, J. and Göpferich, A., Lipid microparticles as a parenteral controlled release device for peptides, *Journal of Controlled Release,* 2001, 73, 339–350.

[13] Reithmeier, H., Herrmann, J. and Göpferich, A., Development and characterization of lipid microparticles as a drug carrier for somatostatin, *International Journal of Pharmaceutics,* 2001, 218, 133–143.

[14] Almeida, A.J., Runge, S. and Müller, R.H., Peptide-loaded solid lipid nanoparticles (SLN): influence of production parameters, *International Journal of Pharmaceutics,* 2001, 149, 255–265.

[15] Garcia-Fuentes, M., Torres, D. and Alonso, M.J., Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules, *Colloids and Surfaces, B: Biointerfaces,* 2003, 27, 159–168.

[16] Müller, R.H., Mäder, K. and Gohla, S., Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art, *European Journal of Pharmaceutics and Biopharmaceutics,* 2000, 50, 161–177.

[17] Khopade, A.J. and Jain, N.K., Long-circulating lipospheres targeted to the inflamed tissue, *Pharmazie,* 1997, 52, 165–166.

[18] Masters, D.B. and Domb, A.J., Liposphere local anesthetic timed-release for perineural site application, *Pharmaceutical Research,* 1998, 15, 1038–1045.

[19] Domb, A.J. et al., Insect repellent formulations of N,N-diethyl-m-toluamide (DEET) in a liposphere system: efficacy and skin uptake, *Journal of the American Mosquito Control Association,* 1995, 11, 29–34.

[20] Amselem, S., Alving, C.R. and Domb, A.J., Lipospheres for vaccine delivery, *Drugs and the Pharmaceutical Sciences,* 1996, 77, 149–168.
[21] Schwarz, C. and Mehnert, W., Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization, *Journal of Microencapsulation,* 1999, 16, 205–213.
[22] Weiner, A.L., Developing lipid-based vehicles for peptide and protein drugs. I. Selection and analysis issues, *BioPharm*, 1990, 3, 27–32.
[23] Perez, C. et al., Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers, *Journal of Pharmacy and Pharmacology,* 2002, 54, 301–313.
[24] Wang, W., Instability, stabilization, and formulation of liquid protein pharmaceuticals, *International Journal of Pharmaceutics,* 1999, 185, 129–188.
[25] Cleland, J.L. and Langer, R., Formulation and delivery of proteins and peptides: design and development strategies, *ACS Symposium Series,* 1994, 567, 1–19.
[26] Creighton, T.E., 1993. *Proteins: Structures and Molecular Properties,* 2nd ed., W.H. Freeman, New York.
[27] van de Weert, M., Hennink, W.E. and Jiskoot, W., Protein instability in poly(lactic–co-glycolic acid) microparticles, *Pharmaceutical Research,* 2000, 17, 1159–1167.
[28] Weiner, A.L., Lipid-based vehicles for peptide and protein drugs. II. Manufacturing variables, *BioPharm*, 1990, 3, 16–21.
[29] Chin, J.T., Wheeler, S.L. and Klibanov, A.M., On protein solubility in organic solvents, *Biotechnology and Bioengineering,* 1994, 44, 140–145.
[30] Hartsough, D.S. and Merz, K.M., Jr., Protein dynamics and solvation in aqueous and nonaqueous environments, *Journal of the American Chemical Society,* 1993, 115, 6529–6537.
[31] Colombo, G., Ottolina, G. and Carrea, G., Modelling of enzyme properties in organic solvents, *Monatshefte fuer Chemie,* 2000, 131, 527–547.
[32] Zorina, O.M. and El'piner, I.E., Protein degradation by ultrasound, *Biofizika,* 1972, 17, 322–324.
[33] Iannuccelli, V. et al., The effect of the triglyceride/phospholipid ratio in liposphere preparation, *Proceedings of the 27th International Symposium on Controlled Release of Bioactive Materials,* 2000, 353–354.
[34] Aquilano, D., Cavalli, R. and Gasco, M.R., Solid lipospheres obtained from hot microemulsions in the presence of different concentrations of cosurfactant: the crystallization of stearic acid polymorphs, *Thermochimica Acta,* 1993, 230, 29–37.
[35] Rasiel, A. et al., Phospholipid coated poly(lactic acid) microspheres for the delivery of LHRH analogues, *Polymers for Advanced Technologies,* 2002, 13, 127–136.
[36] Cortesi, R. et al., Production of lipospheres as carriers for bioactive compounds, *Biomaterials,* 2002, 23, 2283–2294.
[37] Reithmeier, H., Entwicklung und Charakterisierung von Lipidmikropartikeln als parenterale Depotarzneiform für Peptide. Ph.D. Thesis, Fakultät für Chemie und Pharmazie der Uni Regensburg, 1999.
[38] Morel, S., Gasco, M.R. and Cavalli, R., Incorporation in lipospheres of [D-Trp-6]LHRH, *International Journal of Pharmaceutics,* 1995, 119, 126.
[39] Morel, S. et al., Thymopentin in solid lipid nanoparticles, *International Journal of Pharmaceutics,* 1996, 132, 259–261.
[40] Mehnert, W. and Mäder, K., Solid lipid nanoparticles: production, characterization and applications, *Advanced Drug Delivery Reviews,* 2001, 47, 165–196.
[41] Maschke, A. et al., Lipids: an alternative material for protein and peptide release. *ACS Symposium Series,* 2000, 879, 176–196.

[42] Domb, A.J., Bergelson, L. and Amselem, S., Lipospheres for controlled delivery of substances, *Drugs and the Pharmaceutical Sciences,* 1996, 73, 377–410.
[43] Battistel, E., Luisi, P.L. and Rialdi, G., Thermodynamic study of globular protein stability in microemulsions, *Journal of Physical Chemistry,* 1988, 92, 6680–6685.
[44] Vogelhuber, W. et al., Monolithic glyceryl trimyristate matrices for parenteral drug release applications, *European Journal of Pharmaceutics and Biopharmaceutics,* 2003, 55, 133–138.
[45] Vogelhuber, W. et al., Monolithic triglyceride matrices: a controlled-release system for proteins, *Pharmaceutical Development and Technology,* 2003, 8, 71–79.
[46] Bodmer, D., Kissel, T. and Traechslin, E., Factors influencing the release of peptides and proteins from biodegradable parenteral depot systems, *Journal of Controlled Release,* 1992, 21, 129–137.
[47] Zhu, G. and Schwendeman, S.P., Stabilization of proteins encapsulated in cylindrical poly(lactide-co-glycolide) implants: mechanism of stabilization by basic additives, *Pharmaceutical Research,* 2000, 17, 351–357.
[48] zur Mühlen, A., Schwarz, C. and Mehnert, W., Solid lipid nanoparticles (SLN) for controlled drug delivery: drug release and release mechanism, *European Journal of Pharmaceutics and Biopharmaceutics,* 1998, 45, 149–155.
[49] El Shanawany, S., Sustained release of nitrofurantoin from inert wax matrixes, *Journal of Controlled Release,* 1993, 26, 11–19.
[50] Göpferich, A., Mechanisms of polymer degradation and erosion, *Biomaterials,* 1996, 17, 103–114.

5 Lipospheres for Vaccine Delivery

Abraham J. Domb, Aviva Ezra, and Boaz Mizrahi

CONTENTS

5.1 INTRODUCTION

The tremendous advances of genetic engineering, and the ability to obtain many synthetic recombinant protein antigens derived from parasites, viruses, and bacteria, have revolutionized the development of new generation vaccines. Although the new, small, synthetic antigens offer advantages in the selection of antigenic epitopes and safety, a general drawback of small antigens is poor immunogenicity. Unfortunately, the body's immune system does not respond strongly to small peptides. In particular, macrophages do not readily ingest and process the small antigens, resulting in low antibody titers and the need for repeated immunizations. This lack of immunogenicity has created an acute need to identify pharmaceutically acceptable delivery systems for these new antigens.

One approach to enhancing the bioavailability and effectiveness of peptide-based vaccines is the use of microparticles as vaccine carriers. Several reports describing the

0-8493-1692-8/05/$0.00+$1.50

improvement of immune response achieved by the association of antigens with lipid carriers such as liposomes [1,2] or microparticles like polymeric biodegradable microcapsules [3,4] have been published. The ability of these delivery systems to enhance immunogenicity was related to the physicochemical characteristics of the particles.

Significant attention has been given to developing formulations taken orally that induce immunization [5]. An appropriate microparticulate carrier may provide protection of the encapsulated peptide vaccine from enzymatic and environmental degradation, reduction of nonspecific interactions with food proteins, and facilitation of uptake by the gut-associated lymphoid tissue (GALT). Improved uptake of vaccine-loaded particles by the GALT can result in enhanced absorption across the intestinal epithelium and in avoidance of first-pass metabolism by the liver. In addition, the GALT has been shown to function in a manner that is analogous to lymph nodes, sampling antigenic and particulate material entering the gastrointestinal tract and mounting an immune response [6].

Particle size is a key factor, and it appears that particles of certain compositions in the size range of 50 to 3000 nm are capable of uptake and translocation [7,8]. Uptake increases with decreasing particle size. Surface hydrophobicity has a direct correlation with the immune response. Hydrophilic surfaces have little effect, as shown by confocal microscopy studies using polystyrene nanoparticles. Polystyrene was effectively targeted to the M-cell surface of the Peyer's patches, whereas absorbing poloxamers on these polystyrene particles resulted in a complete loss of the gastrointestinal absorption [9]. Further uptake can be induced by adding targeting ligands, such as monoclonal antibodies with specificity for M cells, onto the particle surface.

Lipospheres are fat-based encapsulation particulate systems developed for parenteral drug delivery [10–12] that also have been used successfully as carriers of vaccines and adjuvant [13,14]. Lipospheres have been used for topical applications, including with insect repellents and moisturizers with extended action. Lipospheres consist of water-dispersible solid microparticles composed of a solid hydrophobic fat core stabilized by one layer of phospholipid molecules embedded in their surface. Manufacture of liposphere–vaccine formulations is accomplished by gently melting neutral fat in the presence of phospholipid and dispersing the mixture in an aqueous solution containing the antigen by vigorous shaking. Upon cooling of this mixture, a phospholipid-stabilized solid hydrophobic fat core containing the antigen forms spontaneously.

Although the lipospheres seem to fit very well in vaccine formulations provided by injection or by oral intake, apparently not much has been published in this field since our last review [12]. This chapter is an update of the that review, with an emphasis on the possible use of lipospheres for oral immunization.

5.2 PREPARATION OF LIPOSPHERES

5.2.1 Formulations

In contrast to certain oil emulsions (including Incomplete Freund's Adjuvant), the liposphere approach uses pharmaceutically acceptable biodegradable constituents.

The internal hydrophobic core of lipospheres is composed of fats and biodegradable polymers, mainly triglycerides and lactide-based polymers, whereas the surface activity of liposphere particles is provided by the surrounding lecithin layer, composed of phospholipid molecules.

The neutral fats used in the preparation of the hydrophobic core of the several liposphere-vaccine formulations described here included tricaprin and tristearin, stearic acid, and ethyl stearate. The phospholipids used to form the surrounding layer of lipospheres were egg phosphatidylcholine and dimyristoyl phosphatidylglycerol. Polymeric biodegradable lipospheres were prepared from low molecular weight polylactide (PLA) and polycaprolactone-diol (PCL).

Liposphere formulations are prepared by solvent or melt processes. In the melt method, the active agent is dissolved or dispersed in the melted solid carrier (i.e., tristearin or polycaprolactone) and a hot buffer solution is added at once, along with the phospholipid powder. The hot mixture is homogenized for about 2 to 5 min, using a homogenizer or ultrasound probe, after which a uniform emulsion is obtained. The milky formulation is then rapidly cooled down to about 20°C by immersing the formulation flask in a dry ice–acetone bath, while homogenization is continued to yield a uniform dispersion of lipospheres.

Alternatively, lipospheres might be prepared by a solvent technique. In this case, the active agent, the solid carrier, and the phospholipid are dissolved in an organic solvent such as acetone, ethyl acetate, ethanol, or dichloromethane. The solvent is then evaporated and the resulting solid mixed with warm buffer solution, and mixing is continued until a homogeneous dispersion of lipospheres is obtained.

In a typical preparation, the active agent (200 mg), trilaurin (400 mg), and propylparaben (5 mg) are added to a 50-mL round-bottom glass flask. The flask is heated at 45°C to melt the triglyceride-active agent mixture, and hot 0.1 M phosphate buffer solution (pH 7.4, 45°C, 9.3 g) is added, along with egg phosphatidylcholine (100 mg). The mixture is homogenized for 2 min until a uniform milk-like formulation is obtained. The hot formulation is rapidly cooled to below 20°C by immersing the flask in a dry ice–acetone bath with continued mixing to yield a white, thin dispersion. If needed, the pH of the formulation is adjusted to 7.4 with a 1N HCl solution. The formulation may contain antioxidants such as tocopherol and preservatives such as parabens. Submicron-sized lipospheres are prepared by passing (four times) the liposphere formulation by extrusion through a submicron series of filters at a temperature 5°C above the melting point of the liposphere core composition. Particle size may be reduced to about 200 nm.

Polymeric biodegradable lipospheres can also be prepared by solvent or melt processes. The difference between polymeric lipospheres and the standard liposphere formulations is the composition of the internal core of the particles. Standard lipospheres, such as those previously described, consist of a solid hydrophobic fat core composed of neutral fats like tristearin, whereas in the polymeric lipospheres, biodegradable polymers such as polylactide or polycaprolactone were substituted for the triglycerides. Both types of lipospheres are thought to be stabilized by one layer of phospholipid molecules embedded in their surface. As lipospheres loaded with short peptides, luteinizing hormone-releasing hormone (LHRH) analogs have been recently reported [15]. In this study, peptides were incorporated into PLA lipospheres

by dissolving the peptide, the polymer, and the egg phospholipid in a 1:9 mixture of N-methylpyrrolidone (NMP) and dichloromethane. The solution was then evaporated to almost dryness, and buffer solution was added at 37°C and homogenized to form a uniform microdispersion of a particle size below 10 μm. The final formulation may contain traces of NMP in the aqueous medium; NMP is considered safe for injection. LHRH was constantly released from this formulation for more than 30 d. It is expected that vaccine peptides of similar molecular weight can be fabricated into a similar formulation and can provide extended action.

Sterile liposphere formulations are prepared by sterile filtration of the dispersion in the hot stage during preparation through a 0.2-μm filter at a temperature 5°C above the melting point of the liposphere core composition. Heat sterilization using a standard autoclave cycle decomposed the formulation. Gamma-irradiation sterilization of liposphere formulations did not affect their physical properties. Liposphere formulations of 1:4:2 and 2:4:2 bupivacaine:tristearin:phospholipid (w/w% ratio) were irradiated with a dose of 2.33 Mrad, and the samples were analyzed for particle size, bupivacaine content, *in vitro* release characteristics, and *in vivo* activity. The irradiated formulations had similar particle size, bupivacaine content, release rate, and anesthetic effectiveness in the rat paw analgesia model to those of a bupivacaine HCl solution (Marcaine). However, a more careful analysis of the formulation ingredients should be performed because phospholipids may degrade during irradiation [16].

Nanosized lipospheres have been prepared by homogenization by using a serial filter of reduced pore size, as described above. However, this method is limited to vaccines that are either soluble in the carrier systems or that are presented in a nanosize particle size. An alternative method for the preparation of nanosized lipospheres of particle size below 100 nm was recently developed using a dispersible concentrated oil system [17]. In this system, the drug, triglyceride, phospholipid, and other additives are dissolved in a mixture of surfactants (Tween and Span), and an organic solvent that is miscible with all components (propylene glycol, low–molecular weight polyethylene glycol, NMP, Cremophor, and polyethylene glycol–conjugated a-tocopherol). This clear, anhydrous solution spontaneously forms nanoparticles when gently mixed in buffer solution. Cationic or anionic nanolipospheres can be obtained when adding a cationic or anionic lipid, such as stearyl amine, phosphatidilethanolamine, stearic acid, or phosphatidic acid, to the solution. This concept has been applied for various water-insoluble drugs and peptides [17]. Solid lipid nanospheres, which are essentially nanosized lipospheres, have also been suggested for peptide and drug delivery, when phospholipid is used [18].

5.2.2 Antigens

The feasibility of developing a human malaria sporozoite vaccine was demonstrated in a clinical trial by using irradiated sporozoites as antigens. Protection against sporozoite infection apparently can be achieved by inducing a high titer of anti-sporozoite antibodies. It is currently presumed that it is only during the brief period (a few minutes or hours) when the sporozoite resides in the blood that antibodies

can gain access to the sporozoite and prevent continuation of the malaria infection. Therefore, a major goal of a sporozoite immunization scheme is to maintain a high titer of antibodies at the time of transfer of the organism from the mosquito to the host. A major challenge is to induce a high antisporozoite antibody titer that is also long-lived, hopefully with a protective duration of several months or more.

The major sporozoite antigen that is responsible for inducing protective immunity is a protein, the circumsporozoite (CS) protein, that covers the outer surface of the sporozoite. A region containing repeating tetrapeptides in the middle of the CS protein is thought also to be capable of inducing protective immunity. It is widely believed that high titers of antibodies to the CS protein can interrupt the life cycle of the sporozoite stage and provide protection against infection.

The two malaria antigens used in this study, R32NS1 and R32LR, were supplied under a Cooperative Research and Development Agreement by SmithKline Beecham Pharmaceuticals (King of Prussia, Pennsylvania). R32NS1 is a fusion protein with the following amino acid sequence: $[MDP(NANP)_{15}NVDP(NANP)_{15}NVDP]NS1_{81}$. The R32 refers to the 32 repeats of the tetrapeptide NANP interspersed with two tetrapeptide NVDP repeats from the immunodominant repeat region of the CS protein of the human malaria parasite (*Plasmodium falciparum*), and $NS1_{81}$ refers to 81 amino acids from the nonstructural protein of influenza virus. $NS1_{81}$ is added because it is thought to include human T helper cell epitopes and to function as a carrier protein [19]. In the case of R32LR, R32 is linked to the first two amino acids, leucine and arginine (LR), from a nonsense reading of the tetracycline gene of the vector [20]. The R32LR was used as capture antigen in the enzyme-linked immunosorbent assay (ELISA) because it contains the same repeating units as the R32NS1 antigen used for immunization [21].

The liposphere platform formulation for vaccines and adjuvants can be applied for a range of vaccination agents, for either oral or injectable administration. Bacterial toxins such as the cholera toxin and its nontoxic receptor binding B subunit have been formulated for oral intake and showed strong stimulation with mucosal secretory immunoglobin A and plasma immunoglobin G (IgG) antitoxin responses that last for months in the intestine. However, this adjuvant may induce toxicity and nonspecific stimulation of the immune system [5,22]. DNA plasmids that code for antigenic proteins have been recently considered for immunization. The antigen is synthesized *in vivo* directly from the protein coding sequences. An advantage of this approach is that the vector is unlikely to become toxic. However, one should consider the need for an effective transfection agent that will allow safe and efficient presentation of the plasmid into the nucleus of a selected cell or tissue. The complex of the anionic DNA with a cationic lipid or polymeric carriers might be sufficient for both delivery and transfection, with no need for liposphere carrier.

5.2.3 Adjuvants

It is widely believed that optimal methods for immunization against certain synthetic antigens may require the use of adjuvants, and this belief has stimulated a considerable amount of research aimed at developing new or improved adjuvants. The

most widely used adjuvants consist of aluminum compounds, particularly aluminum hydroxide (alum), which is used in diphtheria and tetanus toxoid vaccines [23].

A variety of lipid adjuvants and protein mediators have also been shown to influence the immune response to antigens encapsulated in liposomes. The most widely used examples of such adjuvants for practical immunization procedures are endotoxin (including lipid A and lipopolysaccharide) and numerous types of lipophilic derivatives of muramyl dipeptide.

Lipid A is the portion of Gram-negative bacterial lipopolysaccharide. In addition to containing nearly all of the endotoxic activity of lipopolysaccharide, lipid A is responsible for numerous other biological activities that are ordinarily associated with lipopolysaccharide [24]. Because of its potent endotoxic activities, lipid A by itself has had limited applicability as an adjuvant for use in human vaccines. Lipid A isolated from Salmonella Minnesota R595 (obtained from List Biological Laboratories, Campbell, California) was used as adjuvant in some of the liposphere-vaccine formulations. Alum has also been used as an additional adjuvant in some of the liposphere-R32NS1 formulations.

All liposphere formulations prepared remained stable during the 3-month period of the study, and no phase separation or appearance of aggregates were observed. The difference between polymeric lipospheres and the standard liposphere formulations is the composition of the internal core of the particles. Standard lipospheres, such as those previously described, consist of a solid hydrophobic fat core composed of neutral fats like tristearin, whereas, in the polymeric lipospheres, biodegradable polymers such as polylactide or polycaprolactone were substituted for the triglycerides. Both types of lipospheres are thought to be stabilized by one layer of phospholipid molecules embedded in their surface.

5.3 IMMUNOGENICITY OF LIPOSPHERES

5.3.1 Effect of Liposphere Fat Composition

The effect of the type of fat used in the preparation of liposphere on their immune response to encapsulated antigen was tested. Mice were immunized twice at weeks 0 and 4 with lipospheres containing R32NS1 malaria antigen. For all liposphere formulations, the first immunization at week 0 caused a very small immune response. However, after the boost injection, a very marked increase of mean IgG antibody levels was observed. For most of the six liposphere–vaccine formulations tested, the immune response obtained remained at very high levels of IgG antibody titers, even after the 12-week period of the experiment. The most immunogenic liposphere formulation was the one made of ethyl stearate, where lipospheres made of stearic acid showed the lowest IgG ELISA titers. The complete order of immunogenic activity of the six liposphere formulations tested was: ethyl stearate > olive oil > tristearin > tricaprin > corn oil > stearic acid. No correlation between liposphere particle size or chemical characteristics and immunogenicity was found. It is worth noting that the IgG antibody ELISA titers obtained on immunizing rabbits with LS (R32NS1) were superior to those obtained following similar immunizations with

the free antigen absorbed to alum, which showed no antibody activity at the same antigen concentrations. It was previously shown that this antigen was also poorly immunogenic in humans when injected alone as an aqueous solution or when adsorbed on alum [14,19].

5.3.2 Influence of Phospholipid Composition

Incorporation of a negatively charged phospholipid, dimyristoyl phosphatidylglycerol, in the liposphere lipid phase caused a significant increase in the antibody response to the encapsulated R32NS1 antigen [13]. Enhancement of immunogenicity by inclusion of charged lipids has also been observed with certain antigens in liposomes. Negatively charged liposomes produced a better immune response to diphtheria toxoid than positively charged liposomes [25]. However, when liposomes were prepared with other antigens, positively charged liposomes worked equally as well as those bearing negative charge [26,27]. Further studies are needed to determine whether negative charges in lipospheres have a general ability to enhance immunogenicity or whether, as with liposomes, charge effects are dependent on individual antigen composition.

5.3.3 Influence of Fat/Phospholipid Molar Ratio

An interesting correlation was observed between the liposphere fat to phospholipid (F/PL) molar ratio, particle size, and immunogenicity. Low F/PL ratios (0.75) were found to induce the formation of lipospheres of small particle size (70% less than 10 μm in diameter), and this apparently resulted in increased antibody titers [13]. Among the ratios tested, a maximal level of IgG antibody production was obtained at a F/PL ratio of 0.75, whereas at larger ratios, decreased antibody production was observed. Although the reason for this phenomenon is unknown, a possible explanation may be the occurrence of better antigen orientation and epitope exposures in the small lipospheres because of higher surface curvature. It may be relevant to note that small liposomes have also been reported to generate higher antibody titers against encapsulated antigen than large liposomes [28].

5.3.4 Effect of Particle Size Distribution

Two populations of particles usually coexist in liposphere formulations: one in the size range of 1 to 10 μm in diameter (population A), and a second population with a diameter between 10 and 80 μm (population B). As mentioned in the previous section, the particle size distribution of lipospheres depends on the F/PL molar ratio, and the immune response to liposphere-encapsulated R32NS1 was also dependent on the F/PL ratio. The average size of the particles increases with increasing F/PL molar ratio. Under conditions in which the F/PL ratio is high (2.5), the large-particle population is predominant (approximately 80% of the particles had an average size of 73 μm), whereas for F/PL ratios of 0.75, most of the lipospheres have a diameter of less than 10 μm [13].

5.3.5 Effect of Route of Administration

To examine the influence of different routes of administration of lipospheres on their immunogenicity, rabbits were immunized orally or parenterally (by subcutaneous, intraperitoneal, intramuscular, and intravenous routes) with lipospheres made of tristearin and lecithin (1:1 molar ratio) and containing the malaria antigen. The immune response obtained was followed with time for a period of 12 weeks postimmunization.

No antibody activity was found after oral immunization in any of the individual rabbits immunized with liposphere R32NS1–vaccine formulation. However, rabbit immunization by all parenteral routes tested resulted in enhanced immunogenicity, with increased antibody IgG levels over the entire postimmunization period. The individual rabbit immune response shows that immunization by subcutaneous injection was the most effective vaccination route among all parenteral routes of administration tested.

5.4 LIPOSPHERES AS CARRIERS OF ADJUVANTS

5.4.1 Adjuvant Activity of Lipid A

The adjuvant activity of lipid A on the immunogenicity of lipospheres was investigated. Lipid A was included in the lipid phase of lipospheres because it has been used effectively by many laboratories to enhance humoral immunity to a wide range of antigens because of its adjuvant properties [2]. The adjuvant activity of liposomal lipid A has been recently investigated [2], and it has been found that liposomes can serve as a vehicle that allows expression of the adjuvant activity of lipid A and simultaneously can reduce certain unwanted side effects of lipid A. It has been established that incorporation of lipid A into liposomes greatly reduces many of the toxic effects normally associated with endotoxin as pyrogenicity and neutropenia with no significant reduction of its adjuvant activity [2].

A successful human trial of alum-adsorbed liposomes containing monophosphoryl lipid A recently demonstrated that a formulation consisting of a combination of oil/water and adsorbent adjuvants can have considerable safety and efficacy and may be useful in the development of a potential vaccine against *Plasmodium falciparum* [29].

A comparison was made between 100-μg injection of R32NS1 malaria antigen incorporated in lipospheres lacking lipid A and R32NS1 entrapped in lipospheres containing lipid A, with both formulations administered in the absence of alum. Incorporation of lipid A in lipospheres significantly increased the immune response to R32NS1 malaria antigen, resulting in double IgG levels compared with R32NS1 lipospheres lacking the lipid A. The adjuvant effect of lipid A incorporated in lipospheres was observed even after 1600-fold dilution of the rabbit sera.

The adjuvant effect of different doses of lipid A in lipospheres was also examined by immunizing rabbits with lipospheres containing R32NS1 and prepared at different final concentrations of lipid A. The ELISA titers of the individual rabbit groups immunized, as determined by dilution of serum obtained at 6 weeks after primary immunization, have shown a gradual increase in IgG antibody titer with increasing

lipid A dose. The strongest antibody activity was obtained with lipospheres containing 150 μg of lipid A/rabbit. At a higher lipid A dose (200 μg/rabbit), a decrease in ELISA units was observed.

5.4.2 Effect of Alum

The effect of alum as adjuvant was also tested in the liposphere–vaccine formulation. In the presence of lipid A, enhanced immune response is obtained even in the absence of alum. This observation is very important because there is increasing concern about the toxic side effects of alum in the long term. Research has suggested a link between aluminum and diseases of the brain, including Alzheimer's disease.

5.5 POLYMERIC BIODEGRADABLE LIPOSPHERE VACCINES

Over the last decade, the use of polymeric materials for the administration of pharmaceuticals and as biomedical devices has increased dramatically. The most important biomedical applications of biodegradable polymers are in the form of implants and devices for surgical dressings and are in the area of controlled drug delivery systems. Several articles have been published describing the adjuvant effect achieved by the association of antigens with biodegradable polymeric microparticulate delivery systems, showing controlled release of several immunogens [30–34].

The improvement of the efficiency of essential vaccines by the combination of new immunological adjuvants and advanced delivery systems based on controlled release technology is actually one of the major priorities of the World Health Organization Program for Vaccine Development, as announced by the World Health Organization (WHO) [35]. The general objective is to improve vaccine immunogenicity and to simplify delivery trough conversion of multiple-dose vaccines to single-dose vaccines, with an emphasis on controlled release systems to induce a protective immune response as soon as possible after first immunization with delayed boost of immunity. The preparation and use of polymeric biodegradable lipospheres as a potential vehicle for the controlled release of vaccines was studied. The recombinant R32NS1 malaria antigen was incorporated in biodegradable polymeric lipospheres in the absence or presence of lipid A as an adjuvant.

The immunogenicity of polymeric lipospheres composed of PLA or PCL was tested in rabbits after intramuscular injection of the formulations [14]. High levels of specific IgG antibodies were observed in the sera of the immunized rabbits up to 12 weeks after primary immunization, using a solid-phase ELISA. PCL lipospheres containing the malaria antigen were able to induce sustained antibody activity after one single injection in the absence of immunomodulators. PCL lipospheres showed superior immunogenicity compared to PLA lipospheres, with the difference being attributed to the different biodegradation rates of the polymers.

The important factors in PLA biodegradation are the molecular weight and polydispersity, as well as the crystallinity and morphology of the polymers [36]. Others factors that may affect PLA degradation include chemical and configurational structure, fabrication conditions, site of implantation, and degradation conditions.

Biodegradation of the aliphatic polyesters occurs by bulk erosion. The lactide/glycolide polymer chains are cleaved by random nonenzymatic hydrolysis to the monomeric lactic and glycolic acids and are eliminated from the body through the Krebs cycle, primarily as carbon dioxide and in urine.

On the basis of the differences in the biodegradation profiles of PLA and PCL, it can be assumed that the higher degradation rate of PLA results in faster release of a R32NS1 malaria antigen from the lipospheres, causing the observed temporary increase in antibody activity followed by a gradual time-dependent decrease in IgG ELISA titers. In contrast, PCL, which is known to biodegrade at a slower rate, is probably released from the lipospheres in a more sustained way over a longer period of time, resulting in prolonged immunogenicity.

The adjuvant effect of lipid A on the immunogenicity of polymeric lipospheres was also tested [14]. Incorporation of lipid A in PCL lipospheres had no effect on the IgG ELISA titers. However, in the case of PLA lipospheres, lipid A significantly increased the immune response to R32NS1 malaria antigen, resulting in IgG levels similar to those obtained with PCL lipospheres. The adjuvant effect of lipid A incorporated in PLA lipospheres was observed even after 1600-fold dilution of the rabbit sera [14].

Most vaccines require two or three primary immunizations, followed by a booster for optimum immune response. If one injection of the immunization schedule is missed, it leads to manifold loss of effective antibody titers. According to WHO statistics, more than 30% of the patients do not return for the next injection at each period of the immunization schedule. The effect of noncompliance is most severe in third world countries, where more than a million children die each year from vaccine-preventable diseases.

The ideal method for substantial improvement of current vaccines is to develop formulations that would provide time-released doses of immunogens that could replace the need for multiple visits and booster shots. Controlled release vaccines would be particularly advantageous in the third world, where a repeated immunization of the vaccine by health-care personnel is difficult to achieve [37].

The data described here showed sustained high levels of IgG antibody production following one single immunization of rabbits immunized with biodegradable lipospheres containing malaria antigen. These results are very promising, with the expectation that biodegradable polymeric lipospheres might be very useful in the conversion of multiple-dose vaccines to single-dose vaccinations, avoiding the need for repeated immunizations.

5.6 CONCLUSIONS

The results presented in this chapter demonstrate that enhanced immunogenic efficacy can be achieved by using liposphere-based formulations, indicating the potential usefulness of lipospheres in the formulation of human and veterinary vaccines. The liposphere approach employs the fat–lipid environment to achieve several goals: to serve as a carrier to protect the antigen, to serve as a "depot," and to provide a surface interphase necessary for adjuvant activity. The ability to provide different surface

properties to the lipospheres, in addition to reducing the particle size to below 100 nm, makes lipospheres attractive for oral immunization.

It is reasonable to presume that the immunogenic and adjuvant activity of lipospheres may be the result of a combination of factors. These factors may include a focused and enhanced delivery of the antigen to an antigen-presenting cell (macrophage) and protection of the antigen from metabolic destruction at other sites in the body that do no participate in the immune response.

The binding of the antigen to a surface, or the presentation of a special type of surface for antigen adsorption, appears to be critical for the biological activities of many agents that are reported to have adjuvant activities [38]. The data obtained with the liposphere-encapsulated antigen in this study confirm the proposed relationship that exists between physicochemical properties of surface-active systems and their ability to serve as adjuvants. It has been proposed that the ability of surfactants to act as adjuvants is dependent on their capability to concentrate adjuvants and immunogens on hydrophobic surfaces, where they are more effectively presented to cells of the immune system [39].

The liposphere delivery system as a fat-based adjuvant formulation may both provide the surface interface necessary for solubilization and proper orientation of the adjuvant-active material, and provide potential carriers for vaccines, which may allow better position for processing and presentation of the incorporated antigens, resulting in enhanced immunogenicity.

The feasibility of polymeric biodegradable lipospheres as carriers for the controlled release of a recombinant malaria antigen was also demonstrated. Polymeric lipospheres containing R32NS1 malaria antigen were able to induce very high levels of antibody activity after a single injection, in the absence of immunomodulators.

Polymeric lipospheres prepared with a copolymer mixture of PCL–PLA, as well as other biodegradable polymers, can also be prepared using the same procedure described here. An advantage of the copolymer lipospheres delivery system is the ability to control the time or rate at which the incorporated immunogen is released. In the case of vaccines, this allows for better scheduling of the antigen release in such a manner as to maximize the antibody response following a single administration, thus avoiding the need for repeated vaccinations.

REFERENCES

[1] Alving, C.R., Liposomes as Carriers of Vaccines, in *Liposomes: From Biophysics to Therapeutics,* M.J. Ostro, ed., Marcel Dekker, New York, 1987, 195.

[2] Alving, C.R., Liposomes as Carriers of Antigens and Adjuvants, *Journal of Immunological Methods.* 140, 1, 1991.

[3] Eldridge, J.H. et al., Biodegradable Microspheres as a Vaccine Delivery System, *Molecular Immunology.* 28, 287, 1991.

[4] Cohen S. and H. Bernstein, *Microparticulate Systems for the Delivery of Proteins and Vaccines,* Marcel Dekker, New York, 1996.

[5] Hillery, A.M., Microparticulate Delivery Systems: Potential Drug/Vaccine Carriers via Mucosal Routes, *Pharmaceutical Science and Technology Today.* 1, 69, 1998.

[6] McGhee, J.R. et al., The Mucosal Immune System: from Fundamental Concepts to Vaccine Development, *Vaccine.* 10, 75, 1992.
[7] Hillery, A.M., P.U. Jani, A.T. Florence, Comparative, Quantitative Study of Lymphoid and Nonlymphoid Uptake of 60 nm Polystyrene Particles, *Journal of Drug Targeting.* 2, 151, 1994.
[8] Jani, P. et al., The Uptake and Translocation of Latex Nanospheres and Microspheres after Oral-Administration to Rats, *Journal of Pharmacy and Pharmacology.* 41, 809, 1989.
[9] Hillery, A.M. and A.T. Florence, The Effect of Adsorbed Poloxamer 188 and 407 Surfactants on the Intestinal Uptake of 60-nm Polystyrene Particles after Oral Administration in the Rat, *International Journal of Pharmaceutics.* 132, 123, 1996.
[10] Domb, A.J., L. Bergelson, S. Amselem, Lipospheres for Controlled Delivery of Substances, in *Microencapsulation, Methods and Industrial Applications,* Marcel Dekker, 1996, 377.
[11] Maniar, M. et al., Characterization of Lipospheres: Effect of Carrier and Phospholipid on the Loading of Drug into the Lipospheres, *Pharmaceutical Research.* 8, 1991.
[12] Amselem, S., Delivery Systems for Vaccines, in *Microparticulate Systems for Drug Delivery,* S. Cohen and H. Bernstein, eds., Marcel Dekker, New York, 1996, 149.
[13] Amselem, S., A.J. Domb, C.R. Alving, Lipospheres as Vaccine Carrier Systems: Effects of Size, Charge, and Phospholipid Composition, *Vaccine Research.* 1, 383, 1992.
[14] Amselem, S., C.R. Alving, A.J. Domb, Polymeric Biodegradable Lipospheres as Vaccine Delivery Systems, *Polymers for Advanced Technologies,* 3, 351, 1992.
[15] Rasiel, A. et al., Phospholipid Coated Poly(Lactic Acid) Microspheres for the Delivery of LHRH Analogues, *Polymers for Advanced Technologies.* 13, 127, 2002.
[16] Marathe, D. and K.P. Mishra, Radiation-Induced Changes in Permeability in Unilamellar Phospholipid Liposomes, *Radiation Research.* 157, 685, 2002.
[17] Bekerman, T., J. Golenser, A. Domb, Cyclosporin Nanoparticle Lipospheres for Oral Administration, *Journal of Pharmaceutical Sciences,* 93, 1264, 2004.
[18] Müller, R.H., K. Mäder, S. Gohla, Solid Lipid Nanoparticles (SLN) for Controlled Drug Delivery: A Review of the State of the Art, *European Journal of Pharmaceutics and Biopharmaceutics.* 50, 161, 2000.
[19] Hoffman, S.L. et al., Plasmodium-Falciparum: Sporozoite Boosting of Immunity Due to a T-Cell Epitope on a Sporozoite Vaccine, *Experimental Parasitology.* 64, 64, 1987.
[20] Verma, J.N. et al., Phagocytosis of Liposomes by Macrophages: Intracellular Fate of Liposomal Malaria Antigen, *Biochimica et Biophysica Acta.* 1066, 229, 1991.
[21] Edelman, R., Vaccine Adjuvants, *Reviews of Infectious Diseases.* 2, 370, 1980.
[22] Holmgren, J., N. Lycke, C. Czerkinsky, Cholera-Toxin and Cholera-B Subunit as Oral Mucosal Adjuvant and Antigen Vector Systems, *Vaccine*. 11, 1179, 1993.
[23] Baylor, N.W., W. Egan, P. Richman, Aluminum salts in vaccines: US perspective, *Vaccine*. 20, S18, 2002.
[24] Takada, H. and S. Kotani, Structural Requirements of Lipid-a for Endotoxicity and Other Biological Activities, *CRC Critical Reviews in Microbiology.* 16, 477, 1989.
[25] Allison, A.C. and G. Gregoria, Liposomes as Immunological Adjuvants, *Nature*. 252, 252, 1974.
[26] Heath, T.D., D.C. Edwards, B.E. Ryman, Adjuvant Properties of Liposomes, *Biochemical Society Transactions*. 4, 129, 1976.
[27] Vanrooijen, N. and R. Vannieuwmegen, Endotoxin Enhanced Adjuvant Effect of Liposomes, Particularly When Antigen and Endotoxin Are Incorporated within the Same Liposome, *Immunological Communications*. 9, 747, 1980.

[28] Shek, P.N., B.Y.K. Yung, N.Z. Stanacev, Comparison between Multilamellar and Unilamellar Liposomes in Enhancing Antibody Formation, *Immunology.* 49, 37, 1983.
[29] Fries, L.F. et al., Liposomal Malaria Vaccine in Humans: A Safe and Potent Adjuvant Strategy, Proceedings of the National Academy of Sciences of the United States of America. 89, 358, 1992.
[30] Singh, M., A. Singh, G.P. Talwar, Controlled Delivery of Diphtheria Toxoid Using Biodegradable Poly(D, L-Lactide) Microcapsules, *Pharmaceutical Research*. 8, 958, 1991.
[31] Ohagan, D.T. et al., Biodegradable Microparticles as Controlled Release Antigen Delivery Systems, *Immunology.* 73, 239, 1991.
[32] Kreuter, J., Nanoparticle-Based Drug Delivery Systems, *Journal of Controlled Release*. 16, 169, 1991.
[33] Hunziker, I.P. et al., Perspectives: Towards a Peptide-Based Vaccine against Hepatitis C Virus, *Molecular Immunology.* 38, 475, 2001.
[34] Raghuvanshi, R.S. et al., Improved Immune Response from Biodegradable Polymer Particles Entrapping Tetanus Toxoid by Use of Different Immunization Protocol and Adjuvants, *International Journal of Pharmaceutics*. 245, 109, 2002.
[35] Classified announcements, *Nature.* 353, 1991.
[36] Moynihan, J.S. et al., A Novel Microencapsulated Peptide Vaccine against Hepatitis B, *Vaccine*. 19, 3292, 2001.
[37] Bloom, B.R., Vaccines for the Third World, *Nature*. 342, 115, 1989.
[38] Woodard, L.F., Surface Chemistry and Classification of Vaccine Adjuvants and Vehicles, in *Bacterial Vaccines,* A. Mizrahi, ed., Alan R. Liss, New York, 281, 1990.
[39] Hunter R., S.F., F. Kezdy, The Adjuvant Activity of Nonionic Block Polymer Surfactants: I. The Role of Hydrophile-Lipophile Balance, *Journal of Immunology.* 127, 1244, 1981.

6 Solid Lipid Nanoparticles: Interaction with Cells, Cytokine Production, and Enzymatic Degradation

Carsten Olbrich, Kerstin Tabatt, Sylvia A. Wissing, Nadja Schöler, and R.H. Müller

CONTENTS

0-8493-1692-8/05/$0.00+$1.50

6.1 INTRODUCTION

Solid lipid nanoparticles (SLN) were introduced as novel drug carriers to the literature in the 1990s and have since been investigated intensively. Various parameters such as structure of the particles, physical stability of the dispersions, entrapment efficiency for different drugs, chemical stabilization of incorporated drugs, sterilization, lyophilization, spray drying, *in vitro* and *in vivo* release, and penetration have been studied and reviewed [1–3]. This chapter describes the interaction of SLN with cells — their effect on cytokine production after parenteral administration as a measure for cytotoxicity — and reviews the mechanism of enzymatic degradation found for SLN.

The cytotoxicity of SLN, expressed as viability, was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and an enzyme-linked immunosorbent assay for the determination of the amount of interleukin 6 (IL-6) secreted by peritoneal mouse macrophages after incubation with SLN was used [4]. IL-6 is a proinflammatory cytokine produced by macrophages and granulocytes after stimulation and possesses both a local and a systemic effect [5]. Other members of this group of cytokines are IL-1, IL-6, IL-8, IL-12, IL-18, and TNF-α and TNF-β (tumor necrosis factor alpha and beta) [6]. They initiate and support the acute inflammatory reaction and regulate the lymphocytic immune response. Among other effects, these cytokines also induce fever as endogenous pyrogenes [7]. The interplay of all proinflammatory cytokines results in an inflammation reaction. Overproduction of cytokines can lead to exaggerated and detrimental effects in the body, as described for lipopolysaccharides acting as effectors [8]. The endotoxin-mediated production of proinflammatory cytokines through macrophages can lead to a detrimental reaction, called sepsis with hypotensive shock. This possible toxic reaction is produced in living beings by foreign material in the bloodstream. Thus, colloidal drug carriers like SLN, recognized by monocytes and macrophages as foreign to the human body, can provoke this reaction [9,10]. Depending on the formulation, up to 90% of the injected dose will be taken up by Kupffer cells (macrophages) of the liver. For SLN, their *in vitro* phagocytosis by granulocyte-like cells (retinoic acid–differentiated human promyelocytic cells [HL-60]) was determined using a luminescent assay [11]. With this technique, it was shown that the extent of phagocytosis depends on the particle size and the surfactant used.

The cytotoxic effects of colloidal carriers can take place both on the outside of the cell or after ingestion in the interior of the cell. Both surfactants on the particle surface and matrix material can potentially lead to toxic effects. Cytotoxic effects can be provoked alternatively by degradation products of the carriers, like formaldehyde in the case of polycyanoacrylate nanoparticles [12]. Studies using polymethylcyanoacrylate nanoparticles showed that a perforation of the cell membrane has taken place after incubation of macrophages with the particles [13]. It was shown that the nanoparticles adhere at the cell membrane and that degradation products can impair the membrane function, which results in cytotoxic effects. The cytotoxic effects of polyalkylcyanoacrylate nanoparticles depend mainly on the length of the aliphatic side chains and are more pronounced for shorter (methyl-) than for longer (butyl-) chain lengths [13–15]. Further cytotoxic effects may result from the internalization of the colloidal carriers with the consecutive production of cytotoxic

degradation products. It could be demonstrated in hepatocytes that polyalkylcyanoacrylate nanoparticles are able to provoke cytotoxic effects after internalization, as well [16]. This kind of cytotoxic effect toward murine peritoneal macrophages could be reduced by the covalent coupling of polyethylene glycol on the particle surface, creating "stealth" nanoparticles [17,18]. Nanoparticles from polylactic or polylactic–polyglycolic acid, biocompatible materials for depot implants (e.g., Zoladex), or depot microparticles (e.g., Decapeptyl Depot, Parlodel LAR) also showed pronounced cytotoxic effects on the cellular level when formulated as nanoparticles. The viability of human granulocytes is reduced to 50% after incubation with 0.2% of these nanoparticles, whereas incubation with 0.5% leads to complete cell death [19,20]. Because nanoparticles are internalized and microparticles (50 to 100 μm) and implants are not, and the degradation products cause cytotoxic effects, this effect is less pronounced in slowly degrading polylactic acid nanoparticles [21,22].

In comparison with the polymeric colloidal carriers mentioned so far, SLN are much more compatible with phagocytic cells. SLN composed of different lipids and surfactants do not exert any cytotoxic effects up to concentrations of 2.5% lipid, and even concentrations up to 10% led to a viability of 80% with human granulocytes [20]. Similar results were obtained in retinoic acid–differentiated HL-60 cells, whereas polymethylcyanoacrylate and polyhexylcyanoacrylate nanoparticles at concentrations of 0.05% or 0.35% led to complete cell death. In this chapter, the results of cytotoxicity testing of SLN on freshly isolated peritoneal mouse macrophages are presented.

Apart from the determination of cytotoxicity, SLN were tested for their degradation behavior. Therefore, SLN were incubated with a mixture of porcine pancreatic lipase and colipase to mimic the gastrointestinal lipolysis degradation of lipids, and as a model for lipolytic degradation in general. The degradation products (free fatty acids) were determined using the Nefa C test kit, a colorimetric test for the assessment of free fatty acids in serum and plasma [11]. Knowledge about the degradation of lipid nanoparticles is of great importance because of the possible impact on cytotoxicity and on the release of active ingredients. Therefore, a lipase–colipase assay was used and the degradation parameters were determined using an enzymatic test kit from Wako (Nefa C test kit).

6.2 INFLUENCE OF THE PARTICLE SIZE

The size of colloidal carriers may have an impact on both the cytotoxicity and the biodegradation of colloidal carriers. The cytotoxicity can be caused by ingested nanoparticles, and their size is one of the factors that determine the cell uptake of the particles. Larger particles are degraded more slowly, and an incorporated drug will be released more slowly than from smaller particles if the drug release is guided by matrix erosion.

6.2.1 On Cytotoxicity

The effect on cytotoxicity and IL-6 production of Dynasan 114 SLN with different surfactants of different sizes was studied using freshly isolated peritoneal mouse

TABLE 6.1
Photon Correlation Spectroscopy Diameters of Solid Lipid Nanoparticles

Composition and Size	Diameter (μm) ± Standard Deviation	Polydispersity Index
Dynasan 114/poloxamer 188		
Small	0.245 ± 0.127	0.196 ± 0.023
Medium	0.523 ± 0.211	0.299 ± 0.054
Large	1.532 ± 0.976	0.456 ± 0.100
Dynasan 114/Polysorbate 80		
Small	0.262 ± 0.126	0.107 ± 0.008
Medium	0.705 ± 1.011	0.202 ± 0.012
Large	3.191 ± 2.540	—
Dynasan 114/Lipoid S75		
Small	0.218 ± 0.115	0.123 ± 0.013
Medium	0.428 ± 0.283	0.305 ± 0.044
Large	1.430 ± 1.322	0.452 ± 0,117
Dynasan 114/Cetylpyridinium chloride (CPC)		
Small	0.123 ± 0.058	0.175 ± 0.034
Medium	0.465 ± 0.123	0.199 ± 0.096
Large	2.231 ± 1.121	0.487 ± 0.166
Controls		
Soybean oil/MCT (1:1)/Lipoid S75		
Small	0.232 ± 0.124	0.118 ± 0.011
Medium	0.530 ± 0.429	0.349 ± 0.166
Large	4.808 ± 1.842	0.523 ± 0.318
Polystyrene beads		
Small	0.250	—
Medium	0.512	—
Large	5.100	—

Note: Produced with Dynasan 114 as matrix lipid and different surfactants. From all formulations, three sizes (small, medium, large) were produced to assess the size effect on cytotoxic effects toward peritoneal mouse macrophages. Both 10% lipid and 1% surfactant were used.

macrophages. Each of the formulations was produced in three sizes — small, medium, and large — by varying the production parameters. A soybean oil/medium-chain triglyceride (MCT) emulsion (parenteral fat emulsion) and nondegradable polystyrene beads were used as controls (Table 6.1). No statistically significant changes in viability (Figure 6.1) or cytokine production (Figure 6.2) between the same SLN of different sizes could be found. Incubation of SLN stabilized with cetylpyridinium chloride as surfactants led to massive cell death at the 0.1% concentration, whereas the poloxamer 188–, Tween 80–, and Lipoid S75–stabilized SLN led only to minor reductions of the viability and the IL-6 secretion. The IL-6 production decreased with increasing cytotoxicity of the particles, independent of particle size. The controls did not affect either the cytotoxicity or the IL-6 production of the cells [5,23].

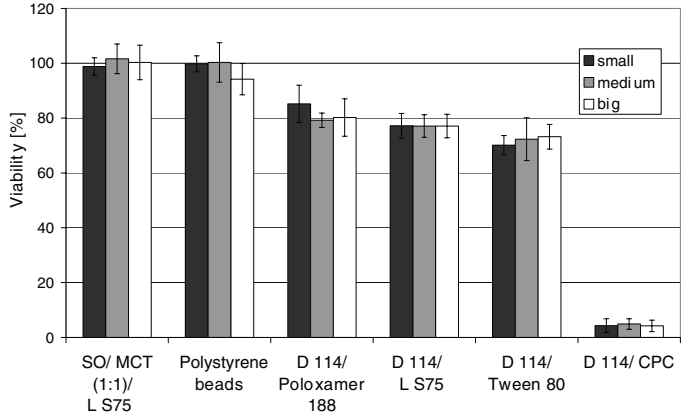

FIGURE 6.1 Viability (MTT assay) of mouse peritoneal macrophages after 20 h of incubation with SLN, polystyrene particles, or control emulsion (1:1 mixture of soybean oil and MCT stabilized with Lipoid S75) of different sizes at a concentration of 0.1% (means ± standard deviation, n = 3). The viability of untreated cells is 100%, and the IL-6 secretion is 1.0. D 114, Dynasan 114; L S75, Lipoid S75; CPC, cetylpyridinium chloride; SO, soybean oil.

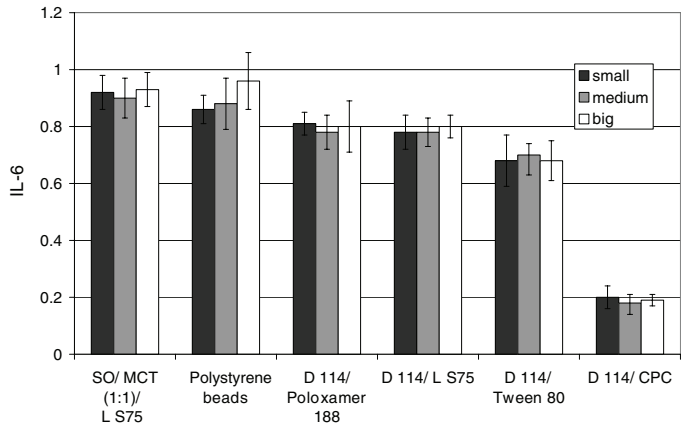

FIGURE 6.2 IL-6 secretion of mouse peritoneal macrophages after 20 h of incubation with SLN, polystyrene particles, or control emulsion (1:1 mixture of soybean oil and MCT stabilized with Lipoid S75) of different sizes at a concentration of 0.1% (means ± standard deviation, n = 3). The viability of untreated cells is 100%, and the IL-6 secretion is 1.0.

6.2.2 On Biodegradation

To determine the effect of the particle size on the biodegradation of Dynasan 114 SLN, stabilized with sodium cholate, a degradation-enhancing surfactant and poloxamer 407 (a surfactant that hinders the enzymatic biodegradation [11]) were used to produce SLN of different sizes (Table 6.2). Sodium cholate–stabilized SLN are in the size range of 182 to 304 nm and do not show any differences in their

TABLE 6.2
Photon Correlation Spectroscopy Diameters of Solid Lipid Nanoparticles

Composition and Size	Diameter (μm) ± Standard Deviation	Polydispersity Index
Dynasan 114/poloxamer 407		
Small	0.287 ± 0.085	0.202 ± 0.013
Medium	0.344 ± 0.052	0.299 ± 0.054
Large	0.389 ± 0.0031	0.326 ± 0.016
Dynasan 114/Sodium cholate		
Small	0.182 ± 0.002	0.171 ± 0.015
Medium	0.304 ± 0.016	0.202 ± 0.012
Large	0.803 ± 0.0075	0.586 ± 0.041

Note: Produced with Dynasan 114 as matrix lipid and poloxamer 407 and sodium cholate as surfactants. The formulations were produced in three sizes (small, medium, large) to assess the influence of the size on the degradation of the nanoparticles. Both 5% lipid and 0.5% surfactant were used.

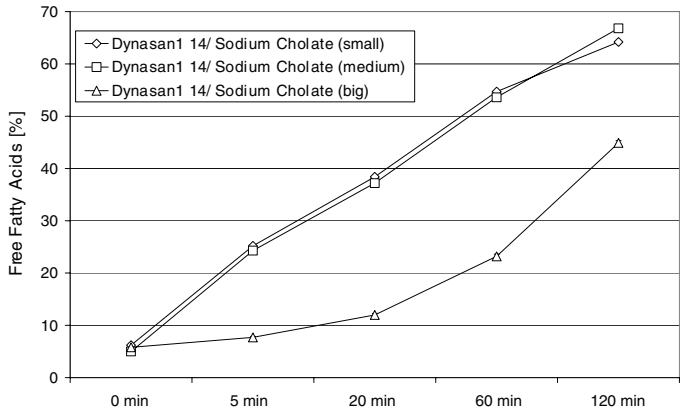

FIGURE 6.3 Degradation profile of sodium cholate–stabilized Dynasan 114 SLN of different sizes. The percentage of free fatty acids after complete hydrolysis is 66%.

degradation behavior, but the degradation velocity is decreased drastically when SLN of 803 nm were used. Not only is the percentage of free fatty acids per time lower than for the smaller particles but also the overall extent of the free fatty acids after 120 min is significantly lower (Figure 6.3). For poloxamer 407–stabilized SLN, the overall extent of biodegradation is reduced compared with the sodium cholate–stabilized particles. Particles of 344 and 389 nm possess similar degradation characteristics, whereas the small particles (287 nm) are degraded significantly faster, and to a greater extent, after 120 min (Figure 6.4). Sodium cholate influences the degradation to a lower extent when particle sizes different than poloxamer 407 are

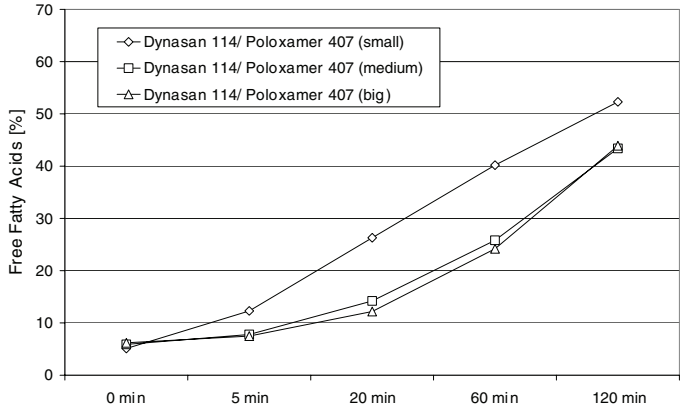

FIGURE 6.4 Degradation profile of poloxamer 407–stabilized Dynasan 114 SLN of different sizes. The percentage of free fatty acids after complete hydrolysis is 66%.

used; small differences in the particle sizes lead to differences in the degradation behavior.

6.3 INFLUENCE OF THE MATRIX LIPID

6.3.1 On Cytotoxicity

The influence of the matrix lipid on cytotoxicity depends on the cell line used. With HL-60 cells, no cytotoxic effect of the matrix lipid could be found [24]. Up to concentrations of 1.5%, SLN made from Dynasan 114 (triglyceride of myristic acid), Compritol ATO 888 (glycerol behenate), or the wax cetyl palmitate, all stabilized with Lipoid S75 (soy lecithin), did not show any reduction in viability [24,25]. On murine peritoneal macrophages, additional matrix lipids were tested [23]. Formulations with 10% lipid and 1% poloxamer 188 as surfactant were used. In addition to the very good tolerability of Compritol ATO 888, Dynasan 114, and paraffin, the matrix lipids Dynasan 118 and cetyl palmitate showed slight concentration-dependent effects and stearic acid showed strong cytotoxic effects (Table 6.3 and Figure 6.5). Because Dynasan 118 is a triglyceride of stearic acid, the reduced viability at the highest concentration (0.1%) can be explained by the enzymatic degradation of the lipids within the cells, leading to fast release of free fatty acids. Solid paraffin, which is not biodegradable, shows a good tolerability, but at the 0.1% concentration, there is a reduction of the viability to about 60%, as well. The parenteral lipid emulsion Lipofundin does not show any reductions, indicating the good tolerability of this formulation. The determination of IL-6, secreted in the cell supernatants of SLN-treated cells showed a reduction in a concentration-dependent manner only for the Dynasan 118– and stearic acid–treated cells, whereas the supernatants of all other formulations did not show reduced amounts of IL-6 (Figure 6.6). Stearic acid formulated as nanoparticles is not cytotoxic to these cells when used in a 0.001%

TABLE 6.3
Photon Correlation Spectroscopy Diameters of Solid Lipid Nanoparticles Produced with Different Matrix Lipids (10% Lipid, 1% Poloxamer 188) to Assess the Influence of the Matrix Lipid on the Cytotoxicity toward Peritoneal Mouse Macrophages

Composition	Diameter (nm) ± Standard Deviation	Polydispersity Index
Compritol 888	314 ± 3	0.38 ± 0.03
Dynasan 114	232 ± 1	0.13 ± 0.03
Dynasan 118	274 ± 3	0.10 ± 0.05
Cetyl Palmitate	360 ± 2	0.13 ± 0.03
Solid Paraffin	234 ± 1	0.14 ± 0.01
Stearic Acid	360 ± 2	0.13 ± 0.03

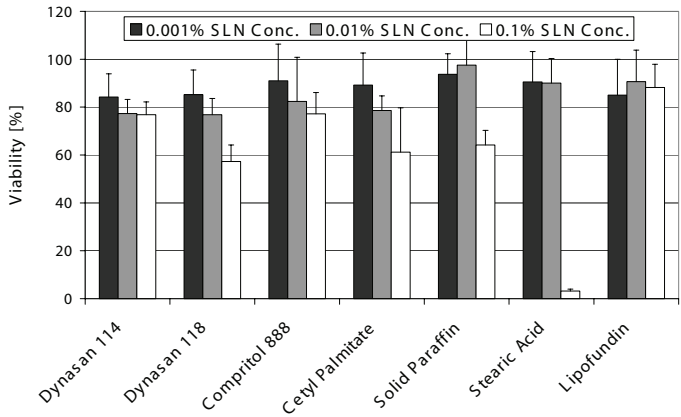

FIGURE 6.5 Viability of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different lipid matrices and poloxamer 188) or Lipofundin MCT in different concentrations (means ± standard deviation, n = 3, untreated cells have a viability of 100%).

concentration, whereas higher concentrations lead to a massive reduction of the viability (0.01% lipid) or to complete cell death. The complete cell death of the highest concentrations is represented by the absence of detectable IL-6 after the incubation with the stearic acid nanoparticles.

6.3.2 On Biodegradation

To study the effect of different lipid matrices SLN of cetyl palmitate, Dynasan 116 (glycerol tripalmitate) and Dynasan 118 (glycerol tristearate) were used (Table 6.4). The amounts of free fatty acids produced after 120 min incubation as a measure of the degradation are shown (Figure 6.7). SLN made from cetyl palmitate showed the lowest degradation. Because cetyl palmitate is a wax and therefore not an optimal

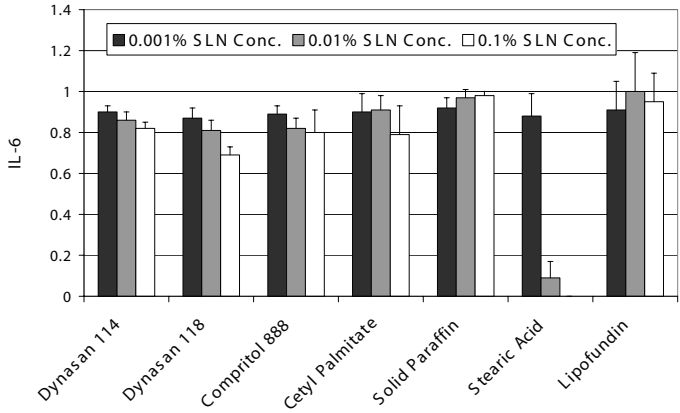

FIGURE 6.6 IL-6 production of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different lipid matrices and poloxamer 188) or Lipofundin MCT in different concentrations (means ± standard deviation, n = 3, untreated cells have a IL-6 secretion of 1).

TABLE 6.4
Photon Correlation Spectroscopy Diameters of Solid Lipid Nanoparticles Produced with Different Lipids and Surfactants (5% Lipid, 0.5% Surfactant) to Assess the Influence of the Matrix Lipid on the Biodegradation of the Corresponding Solid Lipid Nanoparticles

Lipid and Surfactant	Diameter (nm) ± Standard Deviation (%)	Polydispersity Index
Cetyl Palmitate		
Sodium Cholate	170 (2.31)	0.166
Lipoid E 80	431 (2.00)	0.273
Poloxamer 407	303 (3.02)	0.200
Tween 80	543 (2.52)	0.220
Dynasan 116		
Sodium Cholate	253 (8.02)	0.185
Lipoid E 80	350 (1.03)	0.235
Poloxamer 407	388 (7.21)	0.298
Tween 80	315 (1.53)	0.223
Dynasan 118		
Sodium Cholate	283 (7.21)	0.187
Lipoid E 80	385 (2.14)	0.232
Poloxamer 407	422 (6.43)	0.321
Tween 80	314 (4.23)	0.245

substrate for the lipase/colipase, the reduced degradation can be explained. However, even with this matrix material, the degradation is modulated by the surfactants used. Poloxamer 407 leads to the most pronounced reduction as a result of a possible

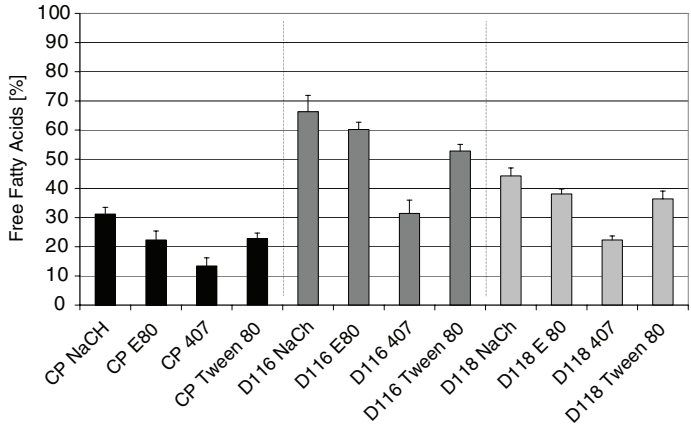

FIGURE 6.7 Lipase/colipase degradation of SLN with different matrix lipids: CP, cetyl palmitate; D116, Dynasan 116; D118, Dynasan 118; NaCh, sodium cholate; E80, Lipoid E80; 407, poloxamer 407; Tween 80, polysorbate 80. The lipid concentration was 5% and the surfactant concentration 0.5%. The values for free fatty acids after 120 min of incubation are given.

hindrance of the enzymes anchoring to the particle surface because of the steric stabilization of the polyethylene oxide chains of the poloxamer. Even when sodium cholate, a degradation-promoting surfactant, was used, only about 30% free fatty acids could be found. Lipoid E80, a phospholipid and natural ingredient of food, leads to a slightly reduced degradation, and the results for Tween 80 were comparable. SLN made from Dynasan 116 show significantly higher extents of degradation compared with cetyl palmitate because this is an optimal substrate for the enzymes. The tendency of the influence of the different surfactants is the same as that described for cetyl palmitate, and the same tendency is valid for the Dynasan 118 SLN, whereas the degradation of these surfactants is significantly reduced compared with the Dynasan 116 SLN. The reason for this is that the enzymatic degradation of fatty acids in triglycerides is slower for longer fatty acid chains than for shorter chains [11].

6.3.3 On *In Vitro* Transfection Efficacy

Recent studies revealed the good *in vitro* transfection efficacy of cationic SLN [26,27]. These cationic SLN are formulated from a matrix lipid: surfactants to stabilize the formulation and cationic lipids to charge the SLN surface positively. The cationic lipids employed are the same used in cationic liposomes for transfection [28]. Formulation optimization studies revealed that both the cationic lipid and the matrix lipid influence transfection activity [27]. Comparable results were found for the oil component in cationic nanoemulsions for transfection [29]. Several formulations made from different cationic lipids and matrix lipids were tested for *in vitro* transfection efficiency (Figure 6.8). The SLN Cp DOTAP made from the wax cetyl palmitate and the cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium

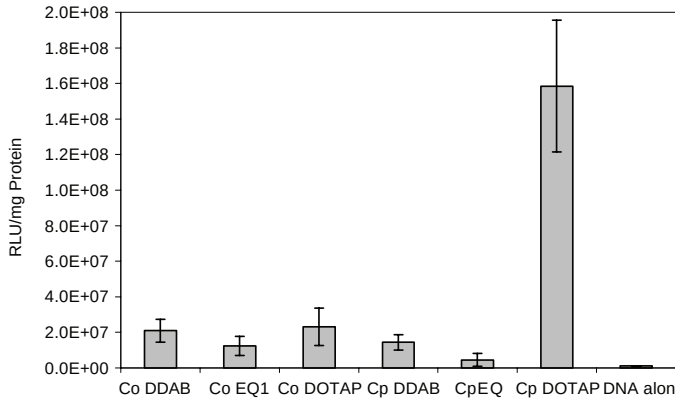

FIGURE 6.8 Transfection efficiency (quantified as "relative light units" [RLU]/mg protein) of six different SLN formulations (Co DDAB: 4% Compritol ATO 888, 1% dimethyldioctadecylammonium bromide = DDAB; Co EQ: 4% Compritol, 1% N,N-di-(β-stearoylethyl)-N,N-dimethyl-ammonium chloride = EQ; Co DOTAP: 4% Compritol, 1% N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride = DOTAP; Cp DDAB: 4% cetyl palmitate, 1% DDAB; Cp EQ: 4% cetyl palmitate, 1% EQ; Cp DOTAP: 4% cetyl palmitate, 1% DOTAP); all formulations were stabilized with 2% [7:3] Tween 80; Span 85). The experiments were performed on Cos-1 cells (African green monkey kidney). The transfection efficiency of naked plasmid (DNA alone) served as control. All tested SLN–DNA complexes showed a statistically significant increase of transfection activity compared to naked plasmid. Cp DOTAP was 10 times more potent then the other formulations.

chloride (DOTAP) showed 10 times higher activities than that SLN also made from DOTAP but containing the matrix lipid Compritol ATO 888 (tribehenate). The superiority of cetyl palmitate SLN appeared only in combination with the cationic lipid DOTAP and not with the other tested cationic lipids. Therefore, for formulation of cationic SLN for transfection, the choice of the matrix lipid in combination with the cationic lipid is a very important factor.

6.4 INFLUENCE OF THE SURFACTANT

6.4.1 On Cytotoxicity

Influencing the cytotoxic potential of SLN surfactants can be considered an important factor. To assess this factor, SLN from Dynasan 114 (glycerol trimyristate) were produced using different surfactants (Table 6.5). Nonionic surfactants, poloxamer 188, poloxamer 407, and poloxamine 908; anionic surfactants sodium dodecyl sulphate and sodium cholate; and cationic agent CPC (cetylpyridinium chloride) were employed. The nonionic and anionic surfactants are generally well tolerated and show no or only slight cytotoxic effects when incorporated in SLN in concentrations up to 0.01% (total surfactant concentration in the incubation medium). Only the cationic CPC leads to massively reduced viabilities of the macrophages even at the

TABLE 6.5
Photon Correlation Spectroscopy Diameters of Dynasan 114 Solid Lipid Nanoparticles with Different Surfactants (10% Lipid, 1% Surfactant) to Assess the Influence of Different Surfactants on the Cytotoxicity toward Peritoneal Mouse Macrophages

Composition	Diameter (nm) ± Standard Deviation	Polydispersity Index
Dynasan 114/poloxamer 407	252 ± 4	0.21 ± 0.02
Dynasan 114/Sodium cholate	254 ± 2	0.19 ± 0.03
Dynasan 114/poloxamine 908	253 ± 1	0.14 ± 0.03
Dynasan 114/poloxamer 188	181 ± 1	0.18 ± 0.01
Dynasan 114/sodium dodecyl sulfate	167 ± 3	0.19 ± 0.01
Dynasan 114/CPC	160 ± 1	0.19 ± 0.01

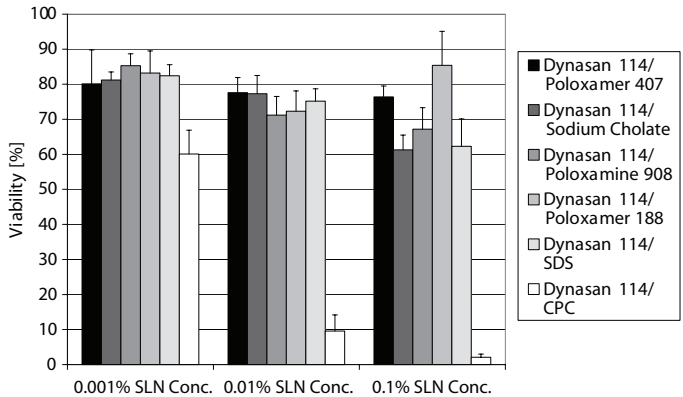

FIGURE 6.9 Viability of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different surfactants and Dynasan 114) in different concentrations (means ± standard deviation, n = 3, untreated cells have a viability of 100%).

0.01% concentration (SLN concentration, 0.001% surfactant concentration) and to complete cell death at 0.1% SLN (0.01% surfactant) concentration (Figure 6.9). CPC at the same concentration, present in the 0.1% SLN dispersion (0.01%), does not show any cytotoxic effects in the MTT assay when given as a solution (Figure 6.10). Corresponding results are obtained when the IL-6 secretion is taken into consideration. The secretion is reduced to zero in the case of CPC-SLN when the highest SLN concentration of CPC-stabilized SLN is used, whereas CPC in solution at ambient concentrations does not lead to these fatal findings (Figure 6.11 and Figure 6.12). It is likely that the toxicity of CPC is related to the simultaneous administration of Dynasan 114 nanoparticles. The enrichment of the cationic surfactant on the particle surface may lead to locally higher concentrations on the cell surface, leading to membrane damages, or else the cytotoxic effect is affected by the surfactant after ingestion in the cell. Because the free solution does not show these effects, free CPC

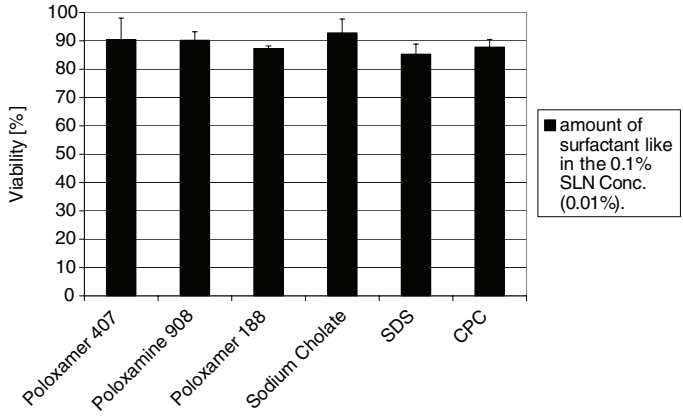

FIGURE 6.10 Viability of peritoneal macrophages of the mouse after 20 h of incubation with different surfactant solutions (0.01%). This corresponds to the surfactants present in the 0.1% formulation (means ± standard deviation, n = 3, untreated cells have a viability of 100%).

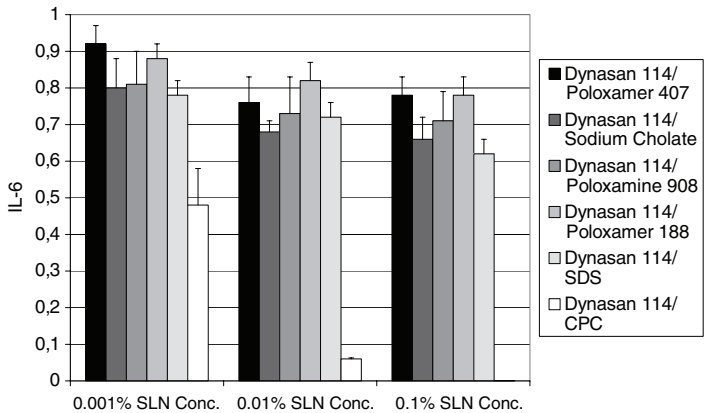

FIGURE 6.11 IL-6 production of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different surfactants and Dynasan 114) in different concentrations (means ± standard deviation, n = 3, untreated cells have an IL-6 secretion of 1).

is not cytotoxic toward mouse macrophages up to a concentration of 0.01%. These findings are important when considering SLN as delivery agents for negatively charged material like DNA or proteins (antigens). For this purpose, the use of other cationic surfactants is recommended.

It is important to keep in mind that cytotoxic effects of surfactants differ depending on the cell line used because of metabolic abilities (e.g., the presence special of enzymes) and capabilities (e.g., phagocytosis) of the cells [30]. In experiments with murine peritoneal macrophages the viability was, in comparison with the pure surfactant solution, slightly reduced when incubated with the SLN [5]. On HL-60, and human peripheral blood granulocytes, a severe reduction of toxicity of the surfactants when incorporated in SLN was observed [24]. Here the cytotoxicity of

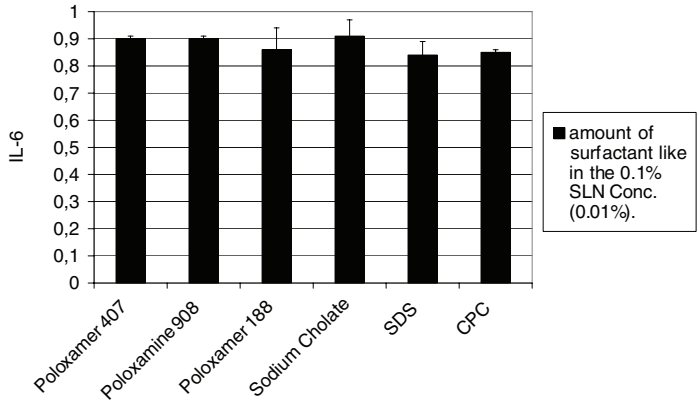

FIGURE 6.12 IL-6 production of peritoneal macrophages of the mouse after 20 h of incubation with different surfactant solutions (0.01%). This corresponds to the surfactants present in the 0.1% formulation (means ± standard deviation, n = 3, untreated cells have an IL-6 secretion of 1).

the surfactants was strongly reduced through the incorporation of Tween 80, poloxamer 184, poloxamer 235, poloxamer 335, and sodium dodecyl sulfate in SLN. Studies with RAW 264.7 macrophages showed an increase of viability for anionic surfactants when incorporated in SLN, as well [31] (Figure 6.13).

Although uncharged or anionic surfactants in SLN are well tolerated, cationic ones might lead to cytotoxicity (Figure 6.13A). That is not so surprising because even solutions of cationic surfactants induce cytotoxicity in a dose-dependent manner (Figure 6.13). For cationic liposomes for transfection, a dose-dependent cytotoxicity is well known [32,33], and cationic polymer nanoparticles [34] show cytotoxicity too. Some cationic amphiphiles, like benzalkonium chloride or CPC, are used as preservatives in concentrations of 0.001 to 0.01% [35]. The proposed mechanisms of the cytotoxicity are electrostatic interactions of these cationic amphiphile molecules, with the anionic phospholipids of the cell membrane leading to membrane damage [36,37]. In contrast to uncharged and anionic surfactants, the cytotoxicity of cationic surfactants in solution is not reduced when incorporated in a SLN formulation [5,31]. Figure 6.13 shows that the cytotoxicity of cationic surfactants in SLN is even increased in comparison to the cationic surfactant solutions, which might be explained by the local increase of concentration on the SLN surface [5].

The extent of cytotoxicity is strongly dependent on the concentration of the cationic lipid in the cell culture medium and on the molecular structure of the cationic lipid used. The ratio of cationic lipid to matrix lipid in the SLN formulation has only a slight effect. On Cos-1 cells (African green monkey kidney cells), SLN made from cationic surfactants with two lipophilic tails (Figure 6.14, 4–6) show cytotoxic effects only when the SLN are in very high concentrations (Figure 6.15). They are, for example, well tolerated in concentrations required for effective transfection (12.5 to 25 µg/mL in cell culture medium, corresponding to 0.00125 to 0.0025% surfactant in cell culture medium). SLN formulated from cationic tensides with only

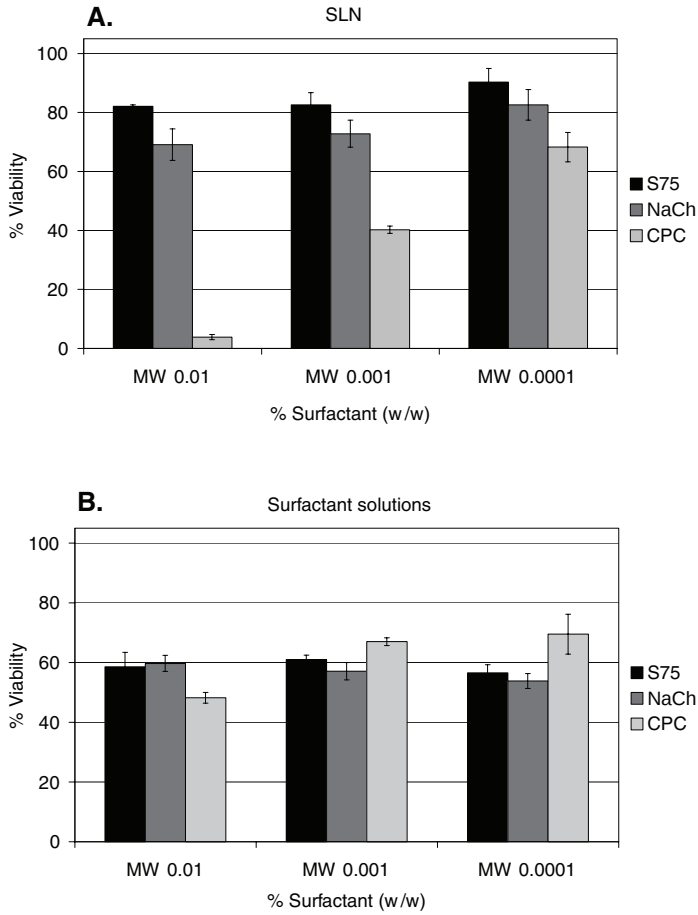

FIGURE 6.13 **(A)** Viability of RAW 264.7 macrophages treated with different SLN formulations compared with cells treated with pure medium (100% viability). Viability is related to the total surfactant concentration in the cell culture medium and was quantified by an MTT assay. All SLN contained 10% Dynasan 114 and 1% of different surfactants (the anionic surfactants sodium cholate [NaCh] or lecithin [S75] or the cationic surfactant cetylpyridinium chloride [CPC]). **(B)** Viability of RAW 264.7 macrophages treated with different surfactant solutions (the anionic surfactants sodium cholate [NaCh] or lecithin [S75] or the cationic cetylpyridinium chloride [CPC]) compared to cells treated with pure medium (100% viability). Viability is related to the total surfactant concentration in the cell culture medium and was quantified by an MTT assay.

one lipophilic tail (Figure 6.14, 1–3) were highly cytotoxic, even in low concentrations. The same observation was made for cationic liposomes [38]. These differences, depending on the molecular structure, might be explained by different interactions with the anionic phospholipids of the cell membrane caused by different arrangements on the SLN surface. When choosing the right two-tailed cationic surfactants and

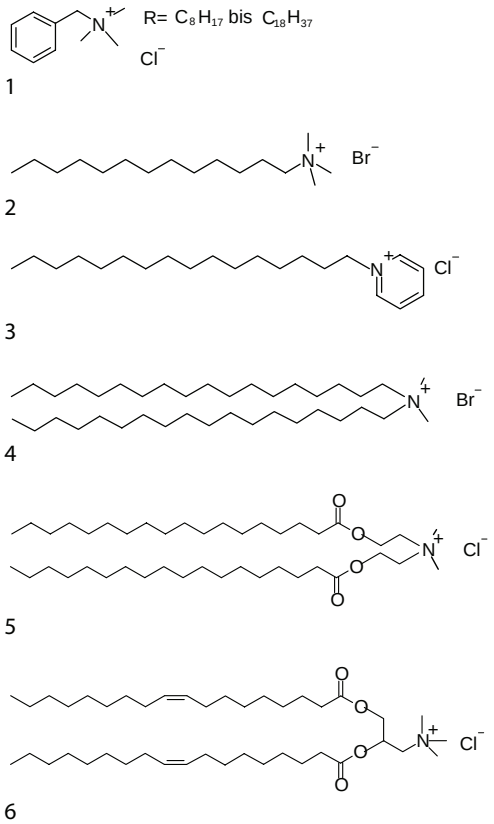

FIGURE 6.14 Organic formulas of the following cationic lipids: **(1)** benzalkonium chloride (alkyldimethylbenzylammonium chloride, BA); **(2)** cetrimide (tetradecyltrimethylammonium bromide, CTAB); **(3)** cetylpyridinium chloride (hexadecylpyridinium chloride, CPC); **(4)** dimethyldioctadecylammonium bromide (DDAB); **(5)** N,N-di-(β-stearoylethyl)-N,N-dimethyl-ammonium chloride (Esterquat 1EQ); **(6)** N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP).

optimizing their concentrations for the required purpose (e.g., electrostatic stabilization or transfection), even cationic SLN with good tolerability can be formulated.

6.4.2 On Biodegradation

The influence of different surfactants on the enzymatic degradation of SLN was studied using Dynasan 114 SLN made from different surfactants. The compositions and the sizes are given in Table 6.6. From photon correlation spectroscopy diameters, it can be assumed that the differences in degradation velocity are related to the surfactants and not to size effects, because all sizes are almost in the same range of about 165 to 279 nm. Only the size of the poloxamine formulation is increased (335 nm). By comparing the degradation data over 120 min, it can clearly be seen

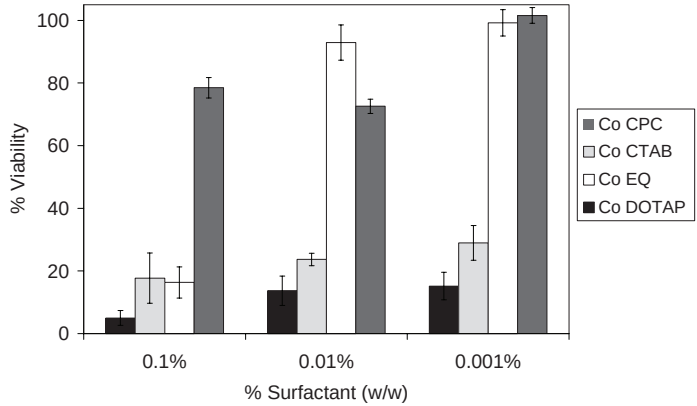

FIGURE 6.15 Viability of Cos-1 cells (African green monkey kidney) after incubation with different SLN formulations. All were made from the matrix lipid Compritol ATO 888 (4% w/w), the surfactants Tween 80/Span 85 (7:3, 2% w/w), and 1% of different cationic lipids. CPC, cetylpyridinium chloride; CTAB, tetradecyltrimethylammonium bromide; EQ, N,N-di-(β-stearoylethyl)-N,N-dimethyl-ammonium chloride; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride. Cytotoxic effects were determined by quantifying the lactate dehydrogenase release in the cell culture medium after incubation with the particles. Lactate dehydrogenase is a intracellular enzyme. The lactate dehydrogenase amount in the medium correlates with cell membrane damage. As one can see, the two-tailed cationic lipids DOTAP and EQ are well tolerated, whereas the one-tailed cationic lipids CPC and CTAB show severe cytotoxicity even in low concentrations.

TABLE 6.6
Photon Correlation Spectroscopy Diameters of Dynasan 114 Solid Lipid Nanoparticles with Different Surfactants (10% Lipid, 1% Surfactant) to Assess the Influence of Different Surfactants on the Enzymatic Degradation (Lipase/Colipase) of Solid Lipid Nanoparticles

Composition	Diameter (nm) ± Standard Deviation	Polydispersity Index
Dynasan 114/Sodium cholate	210 ± 9.9	0.254 ± 0.056
Dynasan 114/poloxamer 188	258 ± 2.3	0.226 ± 0.054
Dynasan 114/poloxamer 407	279 ± 0.71	0.162 ± 0.005
Dynasan 114/poloxamine 908	335 ± 1.84	0.171 ± 0.015
Dynasan 114/Sodium dodecyl sulfate	177 ± 0.71	0.175 ± 0.004
Dynasan 114/CPC	165 ± 1.84	0.263 ± 0.004

that CPC seems to hinder the degradation after 60 min compared with sodium dodecyl sulfate or poloxamer 188, but after 120 min all three surfactants lead to the same extent of degradation, with free fatty acids in the range of 62 to 66%, which means complete degradation to the 2-monoglyceride (Figure 6.16). The degradation of Dynasan 114 SLN seems to be significantly inhibited by poloxamine 908, but because of the large nanoparticles in this formulation, the possibility that this is a

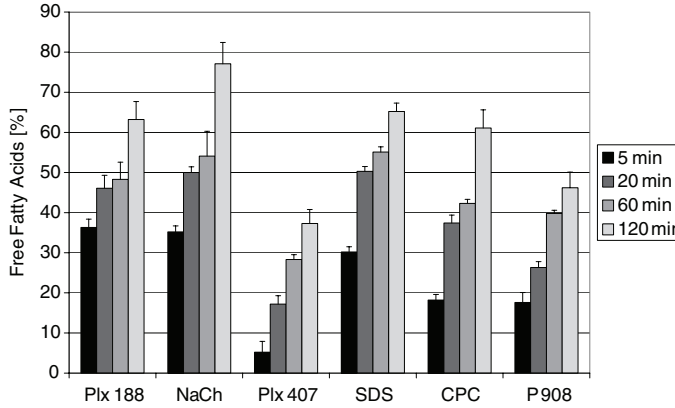

FIGURE 6.16 Degradation of different Dynasan 114 formulations to assess the influence of different surfactants. Plx 188, poloxamer 188; NaCh, sodium cholate; Plx 407, poloxamer 407; SDS, sodium dodecyl sulfate; CPC, cetylpyridinium chloride; P 908, poloxamine 908.

size effect cannot be excluded. Regarding the poloxamer 407 formulation, it is clear that the inhibition of the degradation is an effect of the surfactant. Poloxamer 407 is reported to be an inhibitor of lipolytic processes, as sodium cholate is a promoter of enzymatic degradation of finely dispersed triglycerides.

In further experiments, the effect of mixtures of these two surfactants on the degradation of SLN was studied (Table 6.7). As a measure, the values of degradation after 120 min were recorded. For this purpose, SLN from Dynasan 114, Dynasan 116, and Dynasan 118 were prepared using different ratios of the two surfactants (Figure 6.17). When using Dynasan 114 as the matrix lipid, it could be found that the degradation could not be influenced by the surfactant mixtures, because only the pure poloxamer 407 led to an inhibited degradation. A totally different result was obtained studying the Dynasan 116 SLN. With this lipid, it was possible to inhibit the degradation gradually to obtain a stepwise different degradation after 120 min. Interestingly, it was necessary to have at least 75% (wt/wt) poloxamer 407 present in the surfactant mixture to have a significant effect. Whereas the Dynasan 116 formulation was completely degraded after 120 min, Dynasan 118 was not. With this lipid, the influence of the length of the fatty acid chain in the triglycerides can be seen because longer chains are more difficult to cleave for the lipase/colipase complex. With this lipid, there seems to be a threshold of 75% poloxamer 407 to achieve an inhibition to an extent that cannot be more pronounced even when using 100% poloxamer 407.

6.4.3 On *In Vitro* Transfection Efficacy

Transfection efficiency of cationic SLN is strongly dependent on the matrix lipid and the cationic lipid used [27]. Figure 6.8 shows the transfection efficiencies of six SLN formulations made from three different cationic lipids and two matrix lipids. All six formulations increased transfection activity significantly compared with naked DNA, but only SLN made from the combination of the cationic lipid DOTAP

TABLE 6.7
Photon Correlation Spectroscopy Diameters of Dynasan 114, 116, and 118 Solid Lipid Nanoparticles Stabilized with Mixtures of Cholic Acid Sodium Salt (NaCh) and poloxamer 407 (5% Lipid, 0.5% Surfactant) to Assess the Influence of Different Surfactant Mixtures on the Enzymatic Degradation (Lipase/Colipase Assay) of Solid Lipid Nanoparticles

Composition	Diameter (nm) ± Standard Deviation	Polydispersity Index
Dynasan 114/NaCh 100%	292 ± 8.2	0.231 ± 0.074
Dynasan 114/NaCh 50%/Plx 407 50%	322 ± 7.3	0.163 ± 0.024
Dynasan 114/NaCh 25%/Plx 407 75%	348 ± 5.3	0.182 ± 0.031
Dynasan 114/Plx 407 100%	451 ± 4.3	0.195 ± 0.023
Dynasan 116/NaCh 100%	253 ± 8.0	0.185
Dynasan 116/NaCh 50%/Plx 407 50%	353 ± 19.3	0.211
Dynasan 116/NaCh 25%/Plx 407 75%	364 ± 11.4	0.284
Dynasan 116/Plx 407 100%	388 ± 7.21	0.298
Dynasan 118/NaCh 100%	283 ± 7.2	0.187
Dynasan 118/NaCh 50%/Plx 407 50%	346 ± 2.1	0.220
Dynasan 118/NaCh 25%/Plx 407 75%	368 ± 1.3	0.235
Dynasan 118/Plx 407 100%	422 ± 6.4	0.321

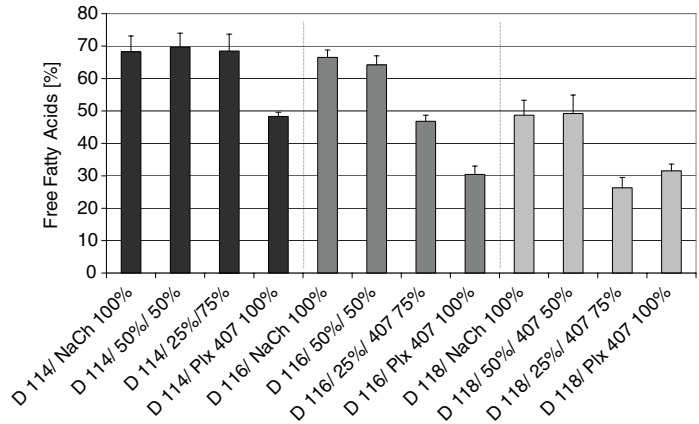

FIGURE 6.17 Degradation of different lipids with mixtures of sodium cholate (NaCh) and poloxamer 407 (Plx 407). D 114, Dynasan 114; D 116, Dynasan 116; D 118, Dynasan 118.

and the matrix lipid cetyl palmitate showed 10 times higher efficiencies. Neither the other SLN, made from the same matrix lipid but from other cationic lipids, nor the SLN, made from the same cationic lipid but from a different matrix lipid, were as effective. For high *in vitro* transfection activities, good combinations of cationic

lipids and matrix lipids are required. With these optimized SLN formulations, transfection activities comparable to cationic liposomes are obtained [39].

6.5 INFLUENCE OF PRESERVATIVES ON CYTOTOXICITY

SLN might be produced aseptically with or without a final sterilization step [40,41]. If no sterilization follows the aseptic production, the addition of a preservative is required because the aqueous continuous phase is susceptible to microbial contamination. For biological stabilization of SLN, thiomersal has been used so far [4]. To determine whether the addition of thiomersal to SLN induces cytotoxicity, three different SLN formulations were stabilized with 0.002% (w/w) thiomersal and tested in different concentrations for their tolerability on murine peritoneal macrophages. The same formulations without thiomersal were taken as control. In comparison to the SLN without thiomersal, no increase in cytotoxicity could be detected.

6.6 MECHANISM OF UPTAKE AND FATE IN THE CELL

There are two main ways SLN might be taken up in cells: phagocytosis and endocytosis. Which way dominates is highly dependent on the type of cells used. For cells that are able to perform phagocytosis, this will surely be the main uptake mechanism, but in mammals, the phagocytotic cells are only macrophages, neutrophile leukocytes, monocytes, and microglia cells.

The particles internalized by phagocytosis may be almost as large as the phagocytotic cell [42]. First the particle becomes opsonized through serum proteins. This opsonization facilitates the binding of the phagocyte to the particles through special membrane receptors. This is the stimulus for the phagocyte to develop pseudopodia that surround the particle. Then it will be internalized in an intracellular phagosome. This phagosome fuses rapidly with lysosomes, which contain enzymes for degradation [42]. In regard to parenteral application, phagocytosis is the most limiting factor for site-specific delivery. After intravenous application, non-surface-modified SLN are mostly internalized by phagocytes of the mononuclear phagocytic system, which mainly means the Kupffer cells in the liver [11]. Thus, targeting of SLN to non-mononuclear phagocytic system cells requires special tricks [43]. By coating with hydrophilic, high–molecular weight polymers such as, for example, poloxamine 908 or poloxamer 407, the surface becomes hydrophilic and low charged, which minimizes serum protein adsorption [44]. The uptake of such surface-modified SLN by human granulocytes and HL-60 is reduced to approximately 8 to 15% compared with the phagocytosis of hydrophobic polystyrene particles [43].

Nonphagocytic cells might take up particles by clathrin-dependent (ligand-mediated) or by clathrin-independent endocytosis. For liposomes, a direct membrane fusion is also discussed, but surely not the main entrance mechanism [45]. The size limit below which particles can be engulfed by nonphagocytotic cells *in vitro* is approximately 150 nm, though some authors claim it can be up to 1 µm [46–48]. Figure 6.18 describes the proposed mechanism of non–ligand mediated endocytosis for cationic solid lipid nanoparticles [31]. The contact of the particle to the cell

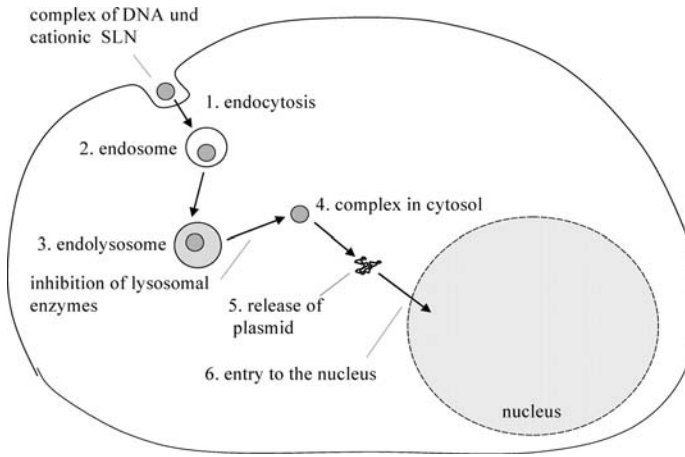

FIGURE 6.18 Proposed cell uptake mechanism for cationic solid lipid nanoparticles. **(1)** The slightly cationic complex of SLN and DNA interacts electrostatically with the anionic cell surface and is internalized by endocytosis; **(2)** the complex is localized in the endosome; **(3)** after fusion of the endosome with lysosomes, the complex is within a endolysosome, which contains nucleases; **(4)** through inhibition of endolysosomal nucleases (rising of the pH by chloroquine) or rising of the osmotic pressure, the complex is released to the cytoplasm ("endosomal escape"); **(5)** by electrostatic interactions with cytoplasmatic proteins, the plasmid is released from the complex; **(6)** the plasmid enters the nucleus through core pores — this step might be facilitated by nuclear localization signals.

surface is mediated by unspecific electrostatic interactions (cationic SLN) or simply sedimentation (in cell culture). The cell internalizes the particle in an endosome, which fuses rapidly with enzyme-containing lysosomes [49]. Transfection experiments with cationic SLN showed that for cationic SLN, endocytosis might be the mechanism of uptake [27,39]. Here, the addition of chloroquine phosphate, which is taken up in lysosomes, becomes protonated, raises the intralysosomal pH, and thus inhibits the pH-dependent nucleases and enhances the transfection efficiency that is dependent on the formulation 10- to 30-fold (Figure 6.19). These nucleases are not present in the cytoplasm but in the endolysosomes. Thus, if SLN were taken up by direct fusion with the cell membrane, the inhibition of endolysosomal nucleases would not have any effect on transfection efficiency, but the addition of chloroquine had a strong effect. Thereofore, at least for the slightly cationic complexes of SLN and DNA, endocytosis is proposed to be the main entrance mechanism. For cationic liposomes and complexes of cationic polymers with DNA (polyplexes), which are not taken up as receptor-mediated, endocytosis is also thought to be the main uptake mechanism [50–52].

Further enhancement of transfection efficiency may be achieved by combination of cationic SLN with nuclear localization signals like the arginine-rich motive of the HIV-1 TAT protein (TAT_2) [39]. The small cationic peptide facilitates nuclear DNA uptake through binding to a special core transporter and through mediation of

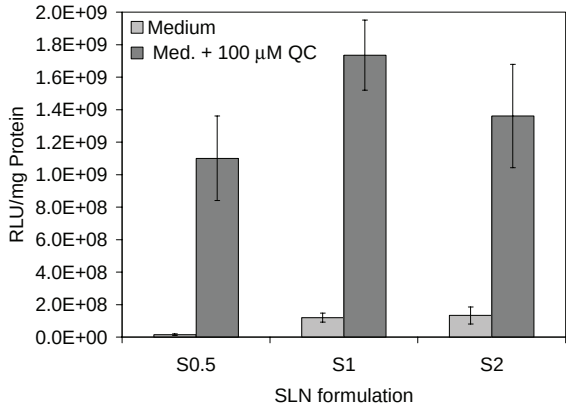

FIGURE 6.19 Transfection efficiency (quantified as "relative light units" [RLU]/mg protein) of three different SLN formulations (all made from 4% cetyl palmitate, 2% Tween 80/Span 85 [7:3], and 0.5% [S0.5], and 1.0% [S1] or 2.0% [S2] of the cationic lipid DOTAP) without medium (Medium) and with medium and 100 µM chloroquine phosphate (Med. + 100 µM QC). The chloroquine addition enhanced transfection activity for all tested SLN in different extends.

an active import [53]. The entrance of the plasmid into the core is considered to be one of the most limiting steps for transfection [54,55].

6.7 CONCLUSION

SLN — especially when they are composed of optimized matrix lipid and surfactant/stabilizer — are in general well-tolerated carrier systems. In particular, compared with many polymeric particles, they possess a lower cytotoxicity and their degradation products are of a physiological nature (fatty acids). Based on these facts, exploitation of SLN in delivery systems for various routes and entry of products for the patients is feasible.

REFERENCES

[1] Müller, R.H. et al., Solid lipid nanoparticles (SLN): an alternative colloidal carrier system for controlled drug delivery, *Eur. J. Pharm. Biopharm.*, 41, 62, 1995.
[2] Müller, R.H., Mäder, K., and Gohla, S., Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art, *Eur. J. Pharm. Biopharm.*, 50, 161, 2000.
[3] Mehnert, W. and Mäder, K., Solid lipid nanoparticles: production, characterization and applications, *Adv. Drug Del. Rev.*, 47, 165, 2001.
[4] Schöler, N. et al., Preserved solid lipid nanoparticles (SLN) at low concentrations do cause neither direct nor indirect cytotoxic effects in peritoneal macrophages, *Int. J. Pharm.*, 196, 235, 2000.
[5] Schöler, N. et al., Surfactant, but not the size of solid lipid nanoparticles (SLN), influences viability and cytokine production of macrophages, *Int. J. Pharm.*, 221, 57, 2001.

[6] Liles, W.C. and Van Voorhis, W.C., Review: nomenclature and biologic significance of cytokines involved in inflammation and the host immune response, *J. Infect. Dis.*, 172, 1573, 1995.
[7] Janeway, C.A. and Travers, P., Immunabwehr von Infektionen, in: *Immunologie*, Janeway, C.A. and Travers, P. (Eds.), Spektrum Akademischer, Heidelberg, 1997.
[8] Hahn, H. et al., *Medizinische Mikrobiologie und Infektiologie*, Springer, Heidelberg, 1999.
[9] Grislain, L. et al., Pharmacokinetics and distribution of biodegradable drug carrier, *Int. J. Pharm*., 15, 335, 1983.
[10] Müller, R.H., *Colloidal Carriers for Controlled Drug Delivery and Targeting: Modification, Characterization and* in vivo *Distribution*. Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1991.
[11] Müller, R.H. and Olbrich, C., Solid lipid nanoparticles: Phagocytic uptake, *in vitro* cytotoxicity and *in vitro* biodegradation, *Pharm. Ind*., 61, 462, 1999.
[12] Vezin, W.R. and Florence, A.T., *In vitro* heterogeneous degradation of poly(n-alkylcyanoacrylates), *J. Biomed. Mater. Res.*, 14, 93, 1980.
[13] Kante, B. et al., Toxicity of polyalkylcyanoacrylate nanoparticles. I. Free nanoparticles, *J. Pharm. Sci.*, 71. 786, 1982.
[14] Gipps, E.M. et al., The effects of polyalkylcyanoacrylate nanoparticles on human normal and malignant mesenchymal cells *in vitro*, *Int. J. Pharm*., 40, 23, 1987.
[15] Lherm, C. et al., Cytotoxicity of polyalkylcyanoacrylate particles of increasing alkyl chain length, *Proc. 5th Int. Conf. Pharm. Technol*. (APGI), 3, 81, 1989.
[16] Bräuer, A., Hepatozytenzellkulturen In-vitro-Testsystem zur Optimierung neuer Arzneiformulierungen, Ph.D. thesis, Christian Albrechts University of Kiel, 1991.
[17] Allen, T.M., Long-circulating, sterically stabilized liposomes for targeted drug delivery, *Trends in Pharm. Sci.,* 15, 215, 1994.
[18] Peracchia, M.T. et al., Pegylated nanoparticles from a novel methoxypolyethylene glycol cyanoacrylate-hexadecyl cyanoacrylate amphiphilic copolymer, *Pharm. Res.*, 15, 550, 1998.
[19] Maaβen, S. et al., Comparison of cytotoxicity between polyester nanoparticles and solid lipid nanoparticles, *Proc. Int. Symp. Control. Rel. Bioact. Mater*., 20, 490, 1993.
[20] Müller, R.H. et al., Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles, *Int. J. Pharm.,* 138, 85, 1996.
[21] Maaβen, S., Solid Lipid Nanoparticles (SLN): Vergleichende Phagozytose- und Zytotoxizitätsuntersuchungen an humanen Granulozyten, Ph.D. thesis, Christian Albrechts University of Kiel, 1994.
[22] Opravil, M. et al., Once-weekly administration of dapsone/pyrimethamine vs. aerosolized pentamidine as a combined prophylaxis for *Pneumocystis carinii* pneumonia and toxoplasma encephalitis in human immunodeficiency virus–infected patients, *Clin. Infect. Dis.*, 20, 531, 1995.
[23] Schöler, N. et al., Effect of lipid matrix and size on solid lipid nanoparticles (SLN) on the viability and cytokine production of macrophages, *Int. J. Pharm*., 231, 167, 2002.
[24] Müller, R.H. et al., Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant, *Pharm. Res.*, 14, 458, 1997.
[25] Rühl, D., Oberflächenmodifizierte Nanopartikel: In-vitro-Phagozytose und Zytotoxizität, Ph.D. thesis, Free University of Berlin, 1997.
[26] Olbrich, C. et al., Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA, *J. Contr. Rel.*, 77, 345, 2001.
[27] Tabatt, K. et al., Effect of cationic lipid and matrix lipid composition on SLN-mediated gene transfer, submitted to *Eur. J. Pharm. Biopharm.*, 2003.

[28] Felgner, P.L. et al., Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, *Proc. Acad. Sci. USA*, 84, 7412, 1987.
[29] Chung, H. et al., Oil components modulate physical characteristics and function of the natural oil emulsion as drug or gene delivery system, *J. Contr. Rel.*, 71, 339, 2001.
[30] Lehrm, C. et al., Alkylcyanoacrylate drug carriers. II. Cytotoxicity of cyanoacrylate nanoparticles with different alkyl chain lengths, *Int. J. Pharm*., 84, 13, 1992.
[31] Tabatt, K., Olbrich, C., and Müller, R.H., Cytotoxicity testing of solid lipid nanoparticles on RAW 264.7 macrophages, *Proc. 4th World Meeting Pharm. Technol. Biopharm, Pharm. Biotechnol.*, Florence, 699, 2002.
[32] Leventis, R. and Silvius, J.R., Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphililes, *Biochim. Biophys. Acta*, 1023, 124, 1990.
[33] Filion, M.C. and Philips, N.C., Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells, *Biochim. Biophys. Acta*, 1329, 345, 1997.
[34] Hoffmann, F. et al., Preparation, characterization and cytotoxicity of methylmethacrylate copolymer nanoparticles with a permanent positive surface charge, *Int. J. Pharm*., 157, 189, 1997.
[35] Burger, A. and Wachter, H., *Hunnius Pharmazeutisches Wörterbuch*, 7th ed., Walter de Gruyter, Berlin, 1993, 196 and 308.
[36] Roberts, W.R. and Addy, M., Comparison of the *in vivo* and *in vitro* antibacterial properties of antiseptic mouthrinses containing chlorhexidine, alexidine, cetylpyridinium chloride and hexetidine: relevance to mode of action, *J. Clin. Periodontol*., 8, 295, 1981.
[37] Lappalainen, K. et al., Comparison of cell proliferation and toxicity assays of two cationic liposomes, *Pharm. Res*., 11, 1127, 1994.
[38] Pinnaduwage, P., Schmitt, L., and Huang, L., Use of quaternary ammonium detergents in liposome mediated DNA transfection of mouse L-cells, *Biochim. Biophys. Acta*, 985, 33, 1989.
[39] Tabatt, K., Pharmazeutisch-biotechnologische Anwendungen von Festen Lipidnanopartikeln (SLN): Vakzinadjuvantien und Gentransfervehikel, Ph.D. thesis, Free University of Berlin, 2003.
[40] Schwarz, C., Feste Lipidnanopartikel: Herstellung, Charakterisierung, Arzneistoffinkorporation und –freisetzung, Sterilisation und Lyophilisation, Ph.D. thesis, Free University of Berlin, 1995.
[41] Schwarz, C. and Mehnert, W., Sterilization of drug-free and tetracaine-loaded solid lipid nanoparticles (SLN), *Proc. 1st World Meeting Pharm. Technol., Biopharm., Pharm. Biotechnol*., Budapest, 485, 1995.
[42] Alberts, B. et al., *Molekular Biologie der Zelle*, 1st ed., Wiley-VCH, Weinheim, 1986.
[43] Müller, R.H. et al., Phagocytic uptake and cytotoxicity of solid lipid nanoparticles (SLN) sterically stabilized with poloxamine 908 and poloxamer 407, *J. Drug Target*., 4, 161, 1996.
[44] Lück, M. et al., Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics, *J. Biomed. Mat. Res*., 39, 478, 1998.
[45] Li, S. and Huang, L., Nonviral gene therapy: promises and challenges, *Gene Ther.*, 7, 31, 2000.
[46] Zuhorn, I.S. et al., Phase behavior of cationic amphiphiles and their mixtures with helper lipid influences lipoplex shape, DNA translocation, and transfection efficiency, *Biophys. J.*, 83, 2096, 2002.

[47] Almofti, M.R. et al., Lipoplex size determines lipofection efficiency with or without serum, *Mol. Membr. Biol*., 20, 35, 2003.
[48] Almofti, M.R. et al., Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization, *Arch. Biochem. Biophys*., 15, 410, 246, 2003.
[49] Bally, M.B. et al., Biological barriers to cellular delivery of lipid-based DNA carriers, *Adv. Drug Del. Rev*., 38, 291, 1999.
[50] Zabner, J. et al., Cellular and molecular barriers to gene transfer by cationic lipid, *J. Biol. Chem*., 270, 18997, 1995.
[51] Wagner, E. et al., Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes, *Proc. Natl. Acad. Sci. USA,* 89, 6099, 1992.
[52] Plank, C., Zauner, W., and Wagner, E., Application of membrane-active peptides for drug and gene delivery across cellular membranes, *Adv. Drug Del. Rev*., 34, 21, 1998.
[53] Keminer, O. and Peters, R., Permeability of single nuclear pores, *Biophys. J*., 77, 217, 1999.
[54] Capecci, M.R. et al., High efficiency transformation by direct microinjection of DNA into cultured mammalian cells, *Cell*, 22, 479, 1980.
[55] Escriou, V. et al., Cationic lipid-mediated gene transfer: analysis of cellular uptake and nuclear import of plasmid DNA, *Cell. Biol. Toxicol*., 14, 95, 1998.

7 Delivery of Lipophilic Compounds with Lipid Nanoparticles — Applications in Dermatics and for Transdermal Therapy

Monika Schäfer-Korting and Wolfgang Mehnert

CONTENTS

0-8493-1692-8/05/$0.00+$1.50

7.1 INTRODUCTION

Human skin (cutis) consists of three different layers: the superficial stratum corneum (horny layer; about 10 µm), viable epidermis (50 to 100 µm), and dermis (1 to 2 mm). The outermost stratum corneum is a buildup of dead cells (corneocytes) surrounded by extremely hydrophobic epidermal lipids, mainly consisting of ceramides, cholesterol, and long-chain (predominantly C22 and C24) free fatty acids, but also cholesteryl sulfate. The water content is low (20%). In healthy skin, epidermal lipids form lamellae of orthorhombic packing but also a liquid phase and build up an efficient barrier against excessive water loss and harmful environmental effects (for review, see [1]). Hair follicles reaching from the dermis or even the subcutis to the skin surface are the most important appendages of human skin; the orifices of the hair follicles cover 0.1% of the surface. Hair follicles occur much more frequently in the skin of furry animals.

The horny layer also forms an efficient barrier against the uptake of drugs topically applied for skin diseases or intended for transdermal therapy. Drugs penetrate and permeate the skin via the intercellular pathway between the corneocytes, in addition to using the orifices of hair follicles. Hair follicles passing through the epidermis allow direct access of the xenobiotics to the blood vessels. Because the orifices cover only about 0.1% of the surface of human skin, the impact of the follicles on cutaneous uptake in general is limited [2], yet follicular uptake may be more relevant with rigid particulate carriers [3] with a size of 3 to 10 µm [2,4]. However, follicular uptake is neglected with respect to regulatory human studies [4].

Despite the less efficient barrier function resulting from many skin diseases (e.g., atopic eczema and psoriasis), piercing through the horny layer is still an important challenge in pharmaceutical technology. This is because conventional drug carrier systems such as creams and ointments result in drug uptake of only a small percentage. A rather high interindividual variation of uptake rates may be the consequence.

At present, research in the field of pharmaceutical technology focuses on micro- and nanoparticulate systems to improve or control uptake rates and induce drug targeting to the skin or even to its substructures, and thus allow for an improved benefit/risk ratio of drug therapy. In addition to innovative carrier systems, the formation of prodrugs facilitating the use of carrier systems may add to the improvement of topical therapy of skin diseases.

In the following text, we describe the production and characterization of solid lipid nanoparticles (SLN, Nanopearls) to be used in dermatotherapy and cosmetics. This is an innovative drug carrier system first developed for application via the

intravenous route. In recent years its suitability for other administration routes (e.g., peroral and transdermal application) has been intensively investigated. SLN are an alternative to polymer nanoparticles, liposomes, and nanoemulsions [5–10]. The solid state of the particle matrix should influence the fate of incorporated compounds. Chemically labile agents should be protected from degradation, and the release profile of drugs can be modulated.

7.2 GENERAL INGREDIENTS OF SLN

SLN for the topical application to the skin are made up from lipids such as glyceryl behenate (Compritol 888 ATO), glyceryl monostearate (Imwitor 900), glyceryl palmitostearate (Precirol ATO 5), triglycerides (trimyristin, tripalmitin, tristearin), or the wax cetyl palmitate. Nanodispersions contain 5 to 40% lipid; the higher-concentrated preparations have a semisolid appearance. These nanodispersions are cosmetically acceptable as they are, whereas the fluid nanodispersions with lower lipid content should be incorporated into, for example, a cream that facilitates the application.

Mean particle size ranges 50 to 1000 nm. Depending on the type and concentration of the lipid, 0.5 to 5% emulsifier (surfactant) has to be added for physical stabilization. For dermal use, surfactants are very often poloxamer 188, polysorbate 80, lecithin, tyloxapol, polyglycerol methylglucose distearate (Tegocare 450), sodium cocoamphoacetate (Miranol Ultra C32), or saccharose fatty acid ester.

7.3 PREPARATION OF SLN

7.3.1 High-Shear Homogenization and Ultrasound

Lipid microparticles and "nanopellets for oral use" were first described by Speiser [11]. Nanopellets are prepared by dispersing melted lipids with high-speed mixers or via ultrasound techniques. Lipospheres developed by Domb are also prepared from dispersed lipids by stirring and sonication [12]. These preparations may contain a high degree of microparticles, which thus excludes an intravenous injection. For other routes of application (e.g., peroral administration), these microparticles might not be a serious problem. Furthermore, the dispersions may be contaminated by metal shed. With optimized conditions, however, mean particles sizes of 100 to 200 nm are possible [13].

7.3.2 Microemulsion-Based SLN Preparations

Lipid particles can also be prepared by dispersing a hot microemulsion in cold water (2 to 3°C) under stirring. Drawbacks of this process are the frequent need for organic solvents and the relative low particle concentration as a result of the dilution with water [14].

7.3.3 SLN Prepared by Solvent Emulsification/Evaporation

Moreover, nanoparticles can be prepared by precipitation in oil/water (o/w) emulsions [15]. Lipids dissolved in a water-immiscible organic solvent (e.g., cyclohexane) are

emulsified in water. Through evaporation of the solvent, lipid nanoparticles precipitate in the remaining aqueous phase. Depending on the surfactant, mean particle sizes vary from 25 to 100 nm. Although a heating step is avoided, which protects thermolabile drugs, the need for organic solvents is disadvantageous.

7.3.4 High-Pressure Homogenization

Organic solvents are avoidable for the production of SLN if high-pressure homogenization is used. Because of the production conditions, these dispersions are characterized by an average particle size below 500 nm and a low microparticle content. The drug has to be dissolved or dispersed in the melted lipid, and lipids sufficiently dissolving the drug are suitable for nanoparticles prepared according to this technique.

7.3.5 Hot Homogenization

The drug dissolved or dispersed in the melted lipid is poured into an aqueous emulsifier phase of the same temperature. By means of a rotor-stator homogenizer (e.g., an Ultra-Turrax), an o/w preemulsion is prepared and is then homogenized at high pressure and at a temperature at least 10°C above the melting point of the lipid. In most cases, nanoemulsion arises after only three to five homogenization cycles at 500 bar. Nanoparticles are formed by cooling the nanoemulsion to room temperature.

7.3.6 Cold Homogenization

The drawback of hot homogenization is that hydrophilic drugs may diffuse into the aqueous phase during the dispersion step. Moreover, labile drugs may degrade as a result of the thermal load. With these drugs, however, nanoparticles may be prepared by cold homogenization, which includes a very rapid cooling step of the drug–lipid melt that is then milled at reduced temperature. The resulting microparticles of about 50 to 100 μm are suspended in a cold emulsifier solution. The microparticle suspension is subjected to high-pressure homogenization at or below room temperature. In general, larger particle sizes and a broader size distribution are observed in cold homogenized samples than in hot homogenization. A common feature of these dispersing techniques is that mean particle size and particle size distribution depend not only on production procedures but also on the type and amount of lipid as well as on the emulsifier.

7.4 ALTERNATIVE SYSTEMS: NANOSTRUCTURED LIPID CARRIERS

A major drawback of SLN is the frequently low drug incorporation. In addition to the physicochemical features of the drug, the composition of the lipid influences the capacity for drug incorporation. Because of the limited space in the forming crystal lattice, drug expulsion from the matrix during lipid crystallization may occur. This is most relevant with homogenous lipids, which form lattices almost free of any imperfections (the lack of which impedes drug incorporation). In contrast, mixed

lipids — such as mixtures of mono-, di-, and triglycerides or polyacid triglycerides — form less densely packed lattices, which should favor drug incorporation. Numbers of imperfections should increase also in mixtures of lipids that are solid and liquid at room temperature. Moreover, drug solubility is often higher in an oil phase. On the basis of these considerations, nanostructured lipid carriers (NLC) recently have been developed [10]. Three different types of NLC are discussed here: imperfect crystal type NLC (type I), solid amorphous NLC (type II), and multiple NLC (type III).

Drug incorporation increases with type I and type II NLC because of a reduced or lacking crystallinity, whereas drugs dissolve in oily nanocompartments located in the solid matrix of type III particles. The distinct advantage of NLC compared with SLN should be the higher drug loading capacity.

7.5 PHYSICOCHEMICAL CHARACTERIZATION OF LIPID NANOPARTICLES

Lipid nanodispersions (SLN and NLC) are complex, thermodynamically unstable systems. The colloidal size of the particles alters physical features (e.g., increasing solubility and the tendency to form supercooled melts). The complex structured lipid matrix may include liquid phases and various lipid modifications that differ in the capacity to incorporate drugs. Lipid molecules of variant modifications may differ in their mobility. Moreover, the high amount of emulsifier used may result in liposome or micelle formation in addition to the nanoparticles.

Therefore, extensive characterization is required, as the physicochemical properties of lipid nanodispersions influence not only drug incorporation and release but also the physical stability of the preparation: for example, drug localization in the matrix. Several methods have to be combined for characterization to allow detection of dynamic processes such as changes in lipid modifications, particle aggregation, and the formation of nanostructures of other kinds.

7.5.1 Measurement of Particle Size, Particle Shape, and Zeta Potential

Both photon correlation spectroscopy and laser diffraction are used for the routine measurement of particle size. Because photon correlation spectroscopy measures particle sizes of about 3 nm to 3 μm, it is most suitable to characterizing nanoparticles. Larger particles, however, are to be detected only by laser diffraction, which covers a broader range of particle size (40 nm to 2000 μm). Difficulties arise with dispersions containing populations of different size. Furthermore, uncertainties may result from nonspherical particle shapes. Platelet structures commonly occur during lipid crystallization and have also been suggested for lipid nanoparticles [6]. Therefore, additional procedures have to be used. Light microscopy allows a rapid detection of microparticles. In addition, it is possible to differentiate between microparticles of unit form and microparticles emerging from an aggregation of nanoparticles. Particle shapes are shown by electron microscopy [16], yet the investigator has to

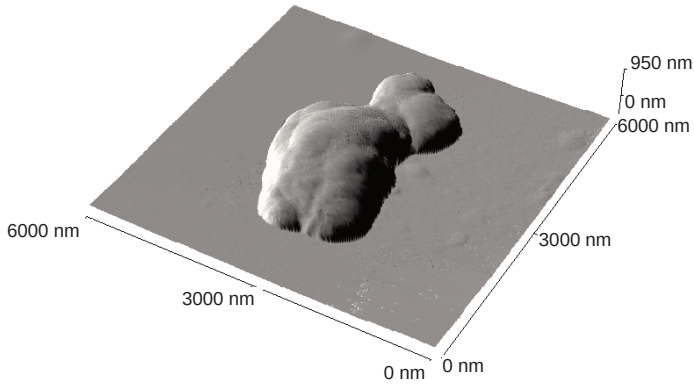

FIGURE 7.1 Atomic force microscopy image of prednisolone-loaded Compritol nanoparticles produced by cold homogenization. Imaging was performed by using the noncontact mode. The formulation is composed of 5% Compritol, 1% prednisolone, 2.5% poloxamer 188, and 92.5% water. (From zur Mühlen, A. and Mehnert, W., *Pharmazie,* 53, 552–55, 1998. With permission.)

be aware of possible artifacts caused by the sample preparation. Atomic force microscopy (Figure 7.1) is another increasingly important technique to depict particle morphology and surface features [17–19]. Quantification of the zeta potential allows us to predict physical stability of a colloidal dispersion. Because of electrical repulsion, aggregation is less likely to occur when the zeta potential exceeds –30 mV.

7.5.2 Determination of Crystallinity and Lipid Modifications

In addition to particle size, the degree of crystallinity and the modification of the lipid are of relevance for drug incorporation and release. Lipid crystallization and a change of the modification can be delayed with very small particles and in the presence of emulsifiers [20,21].

Differential scanning calorimetry and x-ray scattering are well established as means to characterize the crystallinity of lipids [22,23]. Differential scanning calorimetry is based on the fact that different lipid modifications possess different melting points and melting enthalpies. X-ray scattering allows us to determine the length of the long and short spacing of the lipid lattices as well as the influence of storage conditions (temperature, time) on this parameter. The colloidal size of the particles and the addition of emulsifiers may change the features of the nanodispersion considerably as compared to the bulk lipid. Characteristics of lipid particles are influenced by surface-related phenomena and lipid–surfactant interactions [24]. As compared to thermodynamically stable modifications, lipid molecules of unstable modifications are of higher mobility. Because of the lower packing density of the lipid molecules, guest molecules (e.g., drugs) can be incorporated more easily. During storage, unstable lipid modifications may change to their thermodynamically

more stable form, possibly leading to an expulsion of the incorporated drug. These processes can follow water evaporation and may result in an increased cutaneous penetration if a lipid nanodispersion is applied to the surface of skin [25].

To detect dynamic features of colloidal preparations, additional methods are required. Nuclear magnetic resonance spectroscopy allows a rapid, repeatable, and noninvasive measurement of the physical parameters of lipid matrices without sample preparation (e.g., dilution of the probe) [26,27]. Decreased lipid mobility results in a remarkable broadening of the signals of lipid protons, which allows the differentiation of SLN and supercooled melts. Because of the different chemical shifts, it is possible to attribute the nuclear magnetic resonance signal to particular molecules or their segments.

Less frequently used at present is electron spin resonance spectroscopy, which is based on the use of spin probes as model compounds or covalent spin labeling of drugs. Microviscosity and micropolarity of the molecular environment of the probe can be derived from electron spin resonance spectra. Moreover, the spectra allow us to differentiate isotropic and anisotropic movements, which result from the incorporation of the probe into liposomal structures. Quantitative distribution of the spin probes between the internal lipid layer, the surfactant, and the external water phase is to be determined noninvasively. On the basis of the chemical degradation of drugs released from the lipid compartment, agents with reductive features (e.g., ascorbic acid) allow us to measure the exchange rate of the drugs between lipophilic compartments and the water phase [27,28].

7.6 DRUG RELEASE

Drug release from SLN has been evaluated using local anesthetics and steroids as model drugs. Tetracaine and etomidate have been released spontaneously from the solid lipid matrix [29], and a release over 6 weeks has been observed with prednisolone [30]. The preparation (cold and hot homogenization) and the ingredients of the nanodispersion (lipid, emulsifier) influence the release profile. As shown with prednisolone, emulsifier and higher temperature–enhancing drug solubility in the aqueous phase favor the enrichment of the steroid in superficial layers of the nanoparticles during cooling of the preparation and crystallization of the lipid. Superficially entrapped prednisolone is available for the initial burst release (Figure 7.2). The solubility of the drug determines whether the crystallization of the melt results in a monolithic solid solution or a solid dispersion containing the drug in a homogenous distribution or in clusters. Depending on the melting point of the lipid and the drug, as well as its solubility and concentration, the compound may be enriched in the core or in the more outer layers of the particle. A drug-enriched core will be formed if the drug precipitates before the lipid recrystallizes. In summary, the particle structure obtained (Figure 7.3) is a function of the ingredients and the production conditions.

The interpretation of *in vitro* drug release profiles also has to take the specific *in vivo* environment into account. Then a possible enzymatic degradation of lipid particles may be influenced to a relevant extent by the composition of the particles [31].

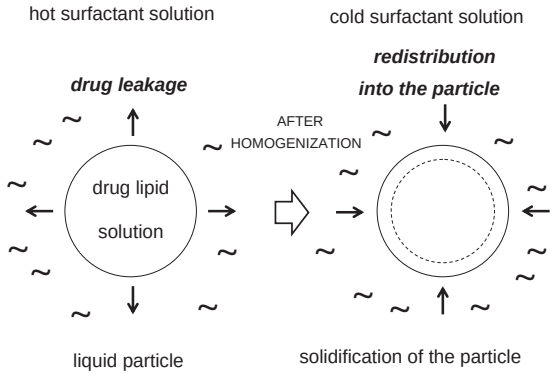

FIGURE 7.2 Distribution processes of drug, occurring during and after the production of prednisolone-loaded SLN, applying the hot homogenization. During the production process, the drug dissolved in the hot aqueous phase. After the production, the solubility in the aqueous phase decreases and the drug is enriched in the outer particle shell. (From zur Mühlen, A. and Mehnert, W., *Pharmazie,* 53, 552–55, 1998. With permission.)

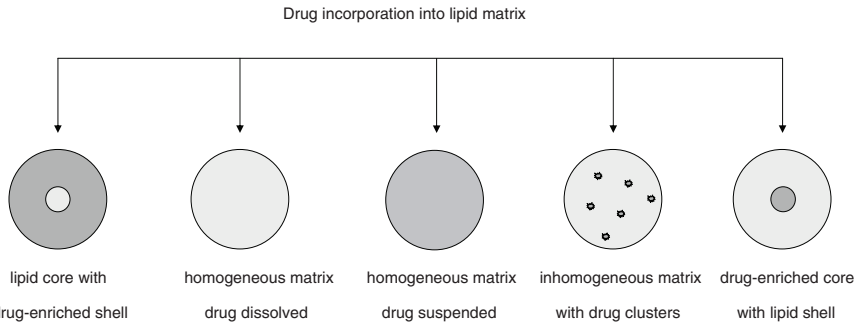

FIGURE 7.3 Models of drug incorporation into solid lipid nanoparticles.

7.7 STORAGE STABILITY

Although nanoparticles and nanoemulsions are closely related with respect to ingredients and production method, SLN should not be regarded just as colloidal lipid dispersions of solidified droplets. Both drug carrier systems may contain additional colloidal structures, such as micelles, mixed micelles, liposomes, or drug nanoparticles, that can influence the physical stability of the lipid dispersion. Other colloidal structures may make up additional distribution compartments for the drug that compete with the intended carrier system. With respect to SLN, the situation is even more complex, as supercooled melts, different lipid modifications, and nonspherical particle shapes may also occur and influence the stability of the colloidal dispersion. Moreover, particle aggregation and gelation of the dispersion are frequently found

with drug-loaded and drug-free SLN preparations. Gelation appears to depend on exposure to light, temperature, and air as well as on density of particle and electrolyte concentration. The reason for this unwanted gelation process, however, is not fully understood yet [32]. Finally, the amount of the lipid in crystalline form and changes in lipid modifications influence physical stability [33–35].

7.8 INCORPORATION OF LIPID NANODISPERSIONS INTO DERMATICS FOR TOPICAL USE

The generally low lipid content and the poor viscosity of lipid nanodispersions make these preparations, as they are, less suitable for dermal drug application. The handling of the preparation by the patient is improved by SLN incorporation into ointments, creams, and gels. Alternatively, ready-to-use preparations may be obtained by one-step production, increasing the lipid phase to at least 30%. However, increasing the lipid frequently results in an unwanted increase in particle size. Surprisingly, it has been found that very concentrated (30 to 40%) semisolid cetyl palmitate formulations preserve the colloidal particle size [10].

If SLN are incorporated into vehicles, interactions with the vehicle constituents may induce physical instabilities such as dissolution or aggregation of lipid particles. Therefore, during storage, particle sizes and the solid character of the particles have to be followed.

7.9 METHODS FOR EVALUATING CUTANEOUS UPTAKE

Because the measurement of clinical efficiency is very expensive and time consuming, less laborious procedures have been tested for comparing multiple formulations of a drug. With respect to topical dermatics, plasma levels are not to be followed because they are generally very low and the drug passes through the target organ skin before entering the blood stream. Alternatives to be used in humans include the tape-stripping procedure to determine drug levels in the horny layer, which has to be passed through by any topical dermatic for the drug to become active. Drug levels in the horny layer are reported to parallel drug levels in the viable epidermis and dermis. This way, bioequivalence can be determined with many like topical formulations (e.g., cream vs. cream). Glucocorticoid formulations are compared by the blanching assay, based on the surrogate parameter vasoconstriction, which is closely correlated with efficiency (for review, see [4]). The test protocol is given by a Food and Drug Administration guidance document released in 1995 [36].

For *in vitro* testing the Organisation for Economic Cooperation and Development (OECD) approved in 2004 test guideline 428 [37], which currently advocates the use of human, rat, and pig skin to measure cutaneous absorption by a vertical diffusion system (Franz cell). Drug concentrations are followed in an acceptor fluid separated by the skin from the donor vehicle, which is applied to the external surface of the skin. Instead of human or animal skin, human skin models could be used as soon as the equivalence of their results are proven. Comparative studies indicate a correlation of penetration data *in vitro* and in humans [38].

7.10 FEATURES OF SLN DISPERSIONS FOR TOPICAL USE

7.10.1 Improved Chemical Stability

The solid matrix of SLN protects the drug from hydrolysis and oxidation. Chemical stability of tocopherol and retinol improves considerably [17,39], with tocopherol improving by 57% compared with an aqueous dispersion. The degree of retinol stabilization depends on the nature of lipid and surfactant [39]. For each drug, the optimal preparation has to be defined individually.

7.10.2 Occlusive Properties

Following the evaporation of water from the lipid nanodispersion applied to the skin surface, lipid particles form an adhesive layer, applying occlusion to the surface [17,40]. Therefore, the hydration of the stratum corneum may increase, which can facilitate drug penetration into deeper skin strata and even systemic availability of the drug. Occlusive effects are strongly related to particle size. Nanoparticles have turned out 15-fold more occlusive than microparticles [17], and particles smaller than 400 nm in a dispersion containing at least 35% high-crystallinity lipid proved to be most potent [41].

7.10.3 Influence of Carrier Systems on Epidermal Input and Skin Permeation

At present, *in vitro* and *in vivo* studies with SLN-based dermatics are clearly lower in number than studies with liposome applications to the skin. Therefore, to describe particle effects, liposome preparations also will be referred to in the following text. Investigations into the mode of cutaneous uptake indicate that liposomal drug application, in general, results in the use of the transepidermal pathway [1,2]. Other studies, however, indicate the follicular pathway [42,43] to be more relevant than the minor coverage of skin surface by the hair follicle orifices indicates. High lipophilicity apparently promotes follicular deposition [44]. Liquid-state vesicles pertubating the lipid organization in the deeper horny layer are more effective than gel-state liposomes in increasing skin permeation. The most elastic Transfersomes appear especially active with respect to skin permeation — even with peptides and proteins. Obviously interacting with the more superficial horny layer, gel-state particles appear to inhibit permeation, whereas liposome size and lamellarity appear less relevant (for review, see [1]).

Because of the rigid nature of SLN, cutaneous drug uptake should be closer to the cutaneous penetration of drugs from gel-state liposomes. Indeed, an increase in glucocorticoid uptake over a conventional cream and a relative increase in drug concentrations in human stratum corneum plus epidermis as compared to the dermis has been reported [45]. This fits nicely with the interaction of skin and rigid liposomes. The improved uptake may result from occlusive effects, as described. Moreover, the small particle size and the intense dispersion of the drug with the particle or drug adsorption to the particle surface favor the contact of the active agent with the skin surface. This may be of relevance, too.

7.10.4 Colloidal Carriers to Improve Eczema Therapy

Lipid-based carrier systems have been investigated to improve treatment of inflammatory skin diseases such as atopic eczema and psoriasis by glucocorticoids and T-cell inhibitors such as ciclosporin and tacrolimus.

Indeed, glucocorticoids were the first drugs tested as liposomal preparations for cutaneous application [46,47]. The improved uptake of liquid-state liposomes as compared to gel-state particles described above was observed with glucocorticoids, as well (for review, see [1]). Liposomes made up from epidermal lipids more efficiently delivered glucocorticoid to the deeper skin than did phospholipid-based vesicles [48]. We could demonstrate an improved prednicarbate uptake by human skin *in vitro* if the steroid is applied as a SLN dispersion or a cream containing prednicarbate-loaded SLN. Most importantly, a prednicarbate targeting to the epidermis occurred [45]. This is most relevant with respect to eczema treatment, as it is the glucocorticoid in the dermis that is most important for induction of skin atrophy. Complete regeneration of skin thickness after the application of potent conventional glucocorticoids takes time [49] and may not be complete before the next exacerbation of disease requires another glucocorticoid treatment [50]. Because targeting is not seen with drug-free SLN added to prednicarbate cream, particle association of the drug is an essential feature [45]. Further clinical studies in patients with repeated eczema eruptions have to show whether the SLN-improved kinetics of prednicarbate and possibly other topical glucocorticoids transfers into benefits for the chronically ill patient. Despite the clearly lower atrophogenicity of prednicarbate, there is still place for a further increase in the benefit/risk ratio.

Although ciclosporin and tacrolimus applied systemically improve psoriatic lesions, they are clearly less active when applied topically. Therefore, liposomal preparations have been developed. Indeed, ciclosporin penetrates deeper strata of rodent and human cadaver skin more efficiently when incorporated into liposomes [51]. Moreover, tacrolimus concentrations in murine skin have increased ninefold, and skin graft survival prolonged, if the drug is liposome encapsulated [52]. This indicates that topical psoriasis therapy with tacrolimus may become possible. At present, topical tacrolimus is confined to the less recalcitrant forms of mild eczema.

7.10.5 Antiacne Drugs and Cosmetics

Liposome-encapsulated tretinoin has been tested in hairless mice as well as in man. The animal experiment has demonstrated the favorable uptake of the retinoid, whereas the liposomal lipids appear to be more retained in the horny layer [53]. Moreover, with phospholipid-based liposomes belonging to the gel-state type, tretinoin penetration in murine skin appears to be confined to the epidermis [54] and, thus, is close to prednicarbate penetration described above. In patients with acne vulgaris, we could demonstrate a better tolerability of liposomal tretinoin as compared to a commercial gel while efficiency remains the same [55].

Not infrequently, retinol is used for mild acne, and the more active retinoids such as retinoic acid, isotretinoin, and adapalene serve for the treatment of the more severe forms. Retinol incorporated into Compritol-based SLN has been released more rapidly and to a higher extent compared with conventional vehicles and with a

nanoemulsion [25,56]. This effect appears to result from a burst release from the solid particles following water evaporation on the skin surface, as well as from the change of lipid modification.

7.10.6 Transdermal Application of High–Molecular Weight Agents

Investigations by Yarosh over almost two decades have proven that liposomal carriers allow uptake of a DNA repair enzyme into the skin [57]. This uptake significantly reduces the number of new actinic keratoses and new lesions of basal cell carcinoma in patients with xeroderma pigmentosum who were treated for 12 months [58]. Moreover, in a mice model, transdermal vaccination by antigen incorporation into liposomes has also been demonstrated [59,60].

On the basis of the phagocytotic activity of human keratinocytes [61], liposomes also appear to be of interest with respect to gene therapy. Cationic lipids are especially interesting because they spontaneously form complexes with DNA. In hairless mice, skin liposomal lipids have been tested as a nonviral transfer system for gene therapy using the Franz cell system for application. Reporter genes could be expressed in viable epidermis [62]. Serious side effects (T-cell leukemia) of retroviral vectors possibly as a result of proto-oncogen activation [63,64] are of high relevance.

Therefore, liposomes, and also nanoparticles, may allow for the development of needle-less vaccination systems. Studies on mice inoculated with influenza DNA vaccine complexes with liposomes and SLN already demonstrated a clear T-cell (predominantly Th1-type) response. Therefore, the immune response appears to be mediated by Langerhans cells, which is the immune competent cell in the skin (for review, see [65]).

Particles from cationic lipids may also be useful for antisense therapy of skin disease — a nontoxic increase in the oligonucleotide uptake by cultivated keratinocytes and a sebocyte cell line has been reported [66]. Moreover, cationic dendrimers also efficiently transfer reporter gene DNA to human keratinocytes cultivated *in vitro*. In the skin of hairless mice, *in vivo* transfection was possible with complexes, yet reporter gene expression was localized to perifollicular areas. Transfection, however, failed with the naked plasmid. For prolonged contact, biodegradable membranes coated with dendrimer/DNA complexes were used [67]. This hints at a follicular uptake of these complexes and indicates that gene transfection also may be possible with human skin, which has a thicker stratum corneum compared with mouse skin (eight to ten vs. two to three layers [58]).

7.10.7 Colloidal Carriers in Cosmetics

Nonloaded and loaded SLN were already investigated with respect to use in cosmetics. Although adequate controls are difficult to prepare, first experiments indicate an increase in skin hydration and a reduction in wrinkle depth following SLN application [68]. Moreover, cetyl palmitate-nanodispersions act both as particulate ultraviolet (UV) blockers themselves and as carriers for UV absorbing agents (e.g., 2-hydroxy-4-methoxy benzophenone; Eusolex 4360). This results in a threefold

increase in UV protection, which allows a reduction in the concentration of the UV absorber. This is particularly important as UV absorbers are currently in discussion because of their possible estrogenic activity and long-term effects in the environment [69,70]. SLN may also be suitable for long-lasting perfume and insect repellent formulations (for review, see [10]). As with drugs, an improved uptake of cosmetic agents (Q_{10}, tocopherol) into the horny layer has been described. The relation of cutaneous penetration to particle size indicates that the increase is caused by an occlusive effect.

7.11 OUTLOOK

The efficiency of liposomes as drug carriers to the skin has been investigated for two decades. Until today, their limited physical stability is the major drawback of this system. SLN and NLC developed to overcome the stability problems of liposomes should result in a higher number of approved drugs with improved stability, high and constant cutaneous absorption, or even drug targeting to specific skin layers or appendages in the near future. Improved skin levels may be due to enhanced contact of drug and skin resulting from the large particle surface, film formation, and skin hydration. Improved uptake may also be due to the interaction of the particle lipids and surfactants with epidermal lipids or sebum. This interaction has to be evaluated in detail because these carrier systems should present remarkable therapeutic progress. Moreover, SLN and NLC may also favorably influence the effects of active compounds in cosmetics.

REFERENCES

[1] Bouwstra, J. A., et al., Structure of the skin barrier and its modulation by vesicular formulations. *Prog. Lipid Res.,* 42, 1–36, 2003.
[2] Barry, B. W., Drug delivery routes in skin: a novel approach. *Adv. Drug Deliv. Rev.,* 54 (Suppl. 1), S31–40, 2002.
[3] Hoffman, R. M., Topical liposome targeting of dyes, melanins, genes, and proteins selectively to hair follicles. *J. Drug Target.,* 5, 67–74, 1998.
[4] Shah, V. P., Progress in methodologies for evaluating bioequivalence of topical formulations. *Am. J. Clin. Dermatol.,* 2, 275–80, 2001.
[5] Müller, R. H. and Lucks, J. S., Arzneistoffträger aus festen Lipidteilchen, Feste Lipidnanosphären (SLN). European Patent, EP 0605497, 1996.
[6] Siekmann, B. and Westesen, K., Sub-micron sized parenteral carrier systems based on solid lipids. *Pharm. Pharmacol. Lett.,* 1, 123–26, 1992.
[7] Müller, R. H., et al., Solid lipid nanoparticles (SLN): an alternative colloidal drug carrier system for controlled drug delivery. *Eur. J. Pharm. Biopharm.,* 41, 1995.
[8] Müller, R. H., Mäder, K. and Gohla, S., Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of the art. *Eur. J. Pharm. Biopharm.,* 50, 161–77, 2000.
[9] Mehnert, W. and Mäder, K., Solid lipid nanoparticles: production, characterization and applications. *Adv. Drug Deliv. Rev.,* 47, 165–96, 2001.

[10] Müller, R. H., Radtke, M. and Wissing, S. A., Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv. Drug Deliv. Rev.,* 54 (Suppl. 1), S131–55, 2002.

[11] Speiser, P., Lipidnanopellets als Trägersystem für Arzneimittel zur peroralen Anwendung. European Patent, EP 0167825, 1990.

[12] Domb, A. J., Lipospheres for controlled delivery of substances. United States Patent, US 5,188,337, 1993.

[13] Ahlin, P., Kristl, J. and Smid-Kobar, J., Optimization of procedure parameters and physical stability of solid lipid nanoparticles in dispersions. *Acta Pharm.,* 48, 257–67, 1998.

[14] Gasco, M. R., Solid lipid nanospheres from warm microemulsions. *Pharm. Technol. Eur.,* 9, 52–58, 1997.

[15] Sjöström, B. and Bergenståhl, B., Preparation of submicron drug partides in lecithin-stabilized o/w emulsions. I. Model studies of the precipitation of cholesteryl acetate. *Int. J. Pharm.,* 88, 53–62, 1992.

[16] Siekmann, B. and Westesen, K., Electron-microscopic characterization of melt-homogenized solid lipid nanoparticles. *Eur. J. Pharm. Biopharm.,* 2, 190, 1994.

[17] Dingler, A., et al., Solid lipid nanoparticles (SLN/Lipopearls): a pharmaceutical and cosmetic carrier for the application of vitamin E in dermal products. *J. Microencapsul.,* 16, 751–67, 1999.

[18] Dubes, A., et al., Scanning electron microscopy and atomic force microscopy imaging of solid lipid nanoparticles derived from amphiphilic cyclodextrins. *Eur. J. Pharm. Biopharm.,* 55, 279–82, 2003.

[19] zur Mühlen, A., et al., Atomic force microscopy studies of solid lipid nanoparticles. *Pharm. Res.,* 13, 1411–16, 1996.

[20] Westesen, K. and Bunjes, H., Do nanoparticles prepared from lipids solid at room temperature always possess a solid lipid matrix? *Int. J. Pharm.,* 115, 129–31, 1995.

[21] Bunjes, H., Koch, M. H. J. and Westesen, K., Influence of emulsifiers on the crystallization of solid lipid nanoparticles. *J. Pharm. Sci.,* 92, 1509–20, 2003.

[22] Siekmann, B. and Westesen, K., Thermoanalysis of the recrystallization of melt-homogenized glyceride nanoparticles. *Colloids Surf. B.,* 3, 159–75, 1994.

[23] Laggner, P., X-ray diffraction of lipids, in: *Spectral properties of lipids*, Hamilton, R. J. and Cast, J., Eds., Sheffield Academic Press, Sheffield, 1999, 327–67.

[24] Unruh, T., et al., Observation of size-dependent melting in lipid nanoparticles. *J. Phys. Chem. B,* 103, 10373–77, 1999.

[25] Jenning, V., et al., Vitamin A loaded solid lipid nanoparticles for topical use: occlusive properties and drug targeting to the upper skin. *Eur. J. Pharm. Biopharm.,* 49, 211–18, 2000.

[26] Jenning, V., Mäder, K. and Gohla, S. H., Solid lipid nanoparticles (SLN) based on binary mixtures of liquid and solid lipids: a ^{1}H-NMR study. *Int. J. Pharm.,* 205, 15–21, 2000.

[27] Jores, K., Mehnert, W. and Mäder, K., Physicochemical investigations on solid lipid nanoparticles and on oil-loaded solid lipid nanoparticles: a nuclear magnetic resonance and electron spin resonance study. *Pharm. Res.,* 20, 1274–83, 2003.

[28] Ahlin, P., Kristl, J. and Sentjurc, M., EPR study of loading capacity of spin-labelled lipophilic substance in different SLN. *Proc. Int. Symp. Control. Release Bioact. Mater.,* 25, 334–35, 1998.

[29] zur Mühlen, A., Schwarz, C. and Mehnert, W., Solid lipid nanoparticles (SLN) for controlled drug delivery: drug release and release mechanism. *Eur. J. Pharm. Biopharm.,* 45, 149–55, 1998.

[30] zur Mühlen, A. and Mehnert, W., Drug release and release mechanism of prednisolone loaded solid lipid nanoparticles. *Pharmazie,* 53, 552–55, 1998.
[31] Olbrich, C. and Müller, R. H., Enzymatic degradation of SLN-effect of surfactant and surfactant mixtures. *Int. J. Pharm.,* 180, 31–39, 1999.
[32] Freitas, C. and Müller, R. H., Stability determination of solid lipid nanoparticles (SLN) in aqueous dispersion after addition of electrolyte. *J. Microencapsul.,* 16, 59–71, 1999.
[33] Bunjes, H., Westesen, K. and Koch, M. H. J., Crystallization tendency and polymorphic transitions in triglyceride nanoparticles. *Int. J. Pharm.,* 129, 159–73, 1996.
[34] Freitas, C. and Müller, R. H., Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase. *Eur. J. Pharm. Biopharm.,* 47, 125–32, 1999.
[35] Westesen, K. and Siekmann, B., Investigation of the gel formation of phospholipid-stabilized solid lipid nanoparticles. *Int. J. Pharm.,* 151, 35–45, 1997.
[36] FDA Guidance: Topical dermatologic corticosteroids: *in vivo* bioequivalence, available online at http://www.fda.gov/cder/guidance/old098fn.pdf, June 2, 1995.
[37] OECD, Skin absorption: *in vitro* method. OECD Test Guideline 428, Paris, 2004.
[38] Wagner, H., et al., Drug distribution in human skin using two different *in vitro* test systems: comparison with *in vivo* data. *Pharm. Res.,* 17, 1475–81, 2000.
[39] Jenning, V. and Gohla, S. H., Encapsulation of retinoids in solid lipid nanoparticles (SLN). *J. Microencapsul.,* 18, 149–58, 2001.
[40] de Vringer, T. and de Ronde, H. A. G., Preparation and structure of a water-in-oil cream containing lipid nanoparticles. *J. Pharm. Sci.,* 84, 466–72, 1995.
[41] Wissing, S. A., Lippacher, A. and Müller, R. H., Investigations on the occlusive properties of solid lipid nanoparticles (SLN). *J. Cosmet. Sci.,* 52, 313–24, 2001.
[42] Weiner, N., Targeted follicular delivery of macromolecules via liposomes. *Int. J. Pharm.,* 162, 29–38, 1998.
[43] Otberg, N., et al., Visualization of topically applied fluorescent dyes in hair follicles by laser scanning microscopy. *Laser Phys.,* 13, 761–64, 2003.
[44] Grams, Y. Y. and Bouwstra, J. A., Penetration and distribution of three lipophilic probes *in vitro* in human skin focusing on the hair follicle. *J. Control. Release,* 83, 253–62, 2002.
[45] Santos Maia, C., et al., Drug targeting by solid lipid nanoparticles for dermal use. *J. Drug Targeting,* 10, 489–95, 2002.
[46] Mezei, M. and Gulasekharam, V., Liposomes: a selective drug delivery system for the topical route of administration: gel dosage form. *J. Pharm. Pharmacol.,* 34, 473–74, 1982.
[47] Mezei, M. and Gulasekharam, V., Liposomes — a selective drug delivery system for the topical route of administration: lotion dosage form. *Life Sci.,* 26, 1473–77, 1980.
[48] Fresta, M. and Puglisi, G., Survival rate improvement in a rat ischemia model by long circulating liposomes containing cytidine-5I-diphosphate choline. *Life Sci.,* 61, 1227–35, 1997.
[49] Korting, H. C., Vieluf, D. and Kerscher, M., 0.25% prednicarbate cream and the corresponding vehicle induce less skin atrophy than 0.1% betamethasone-17-valerate cream and 0.05% clobetasol-17-propionate cream. *Eur. J. Clin. Pharmacol.,* 42, 159–61, 1992.
[50] Schäfer-Korting, M., Schmid, M. H. and Korting, H. C., Topical glucocorticoids with improved risk-benefit ratio: rationale of a new concept. *Drug Saf.,* 14, 375–85, 1996.
[51] Egbaria, K., Ramachandran, C. and Weiner, N., Topical application of liposomally entrapped cyclosporin evaluated by *in vitro* diffusion studies with human skin. *Skin Pharmacol.,* 4, 21–28, 1991.

[52] Erdogan, M., Wright, J. R., Jr. and McAlister, V. C., Liposomal tacrolimus lotion as a novel topical agent for treatment of immune-mediated skin disorders: experimental studies in a murine model. *Br. J. Dermatol.,* 146, 964–67, 2002.

[53] Masini, V., et al., Cutaneous bioavailability in hairless rats of tretinoin in liposomes or gel. *J. Pharm. Sci.,* 82, 17–21, 1993.

[54] Michel, C., et al., Penetration of spin-labeled retinoic acid from liposomal preparations into the skin of SKH1 hairless mice: measurement by EPR tomography. *Int. J. Pharm.,* 98, 131–39, 1993.

[55] Schäfer-Korting, M., Korting, H. C. and Ponce-Pöschl, E., Liposomal tretinoin for uncomplicated acne vulgaris. *Clin. Investig.,* 72, 1086–91, 1994.

[56] Jenning, V., Schäfer-Korting, M. and Gohla, S., Vitamin A-loaded solid lipid nanoparticles for topical use: drug release properties. *J. Control. Release,* 66, 115–26, 2000.

[57] Yarosh, D., et al., Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomised study. *Lancet,* 357, 926–29, 2001.

[58] Yarosh, D. B., Liposomes in investigative dermatology. *Photodermatol. Photoimmunol. Photomed.,* 17, 203–12, 2001.

[59] Paul, A., Cevc, G. and Bachhawat, B. K., Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes. *Vaccine,* 16, 188–95, 1998.

[60] Baca-Estrada, M. E., et al., Effects of IL-12 on immune responses induced by transcutaneous immunization with antigens formulated in a novel lipid-based biphasic delivery system. *Vaccine,* 18, 1847–54, 2000.

[61] Schaller, M., Korting, H. C. and Schmid, M. H., Interaction of cultured human keratinocytes with liposomes encapsulating silver sulphadiazine: proof of the uptake of intact vesicles. *Br. J. Dermatol.,* 134, 445–50, 1996.

[62] Birchall, J. C., et al., Gene expression in an intact ex-vivo skin tissue model following percutaneous delivery of cationic liposome-plasmid DNA complexes. *Int. J. Pharm.,* 197, 233–38, 2000.

[63] Williams, D. A. and Baum, C., Medicine: gene therapy — new challenges ahead. *Science,* 302, 400–01, 2003.

[64] Hacein-Bey-Abina, S., et al., LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science,* 302, 415–19, 2003.

[65] Choi, M. J. and Maibach, H. I., Topical vaccination of DNA antigens: topical delivery of DNA antigens. *Skin Pharmacol. Appl. Skin Physiol.,* 16, 271–82, 2003.

[66] Fimmel, S., et al., Development of efficient transient transfection systems for introducing antisense oligonucleotides into human epithelial skin cells. *Horm. Res.,* 54, 306–11, 2000.

[67] Bielinska, A. U., et al., Application of membrane-based dendrimer/DNA complexes for solid phase transfection *in vitro* and *in vivo*. *Biomaterials,* 21, 877–87, 2000.

[68] Wissing, S. A. and Müller, R. H., The influence of solid lipid nanoparticles on skin hydration and viscoelasticity: *in vivo* study. *Eur. J. Pharm. Biopharm.,* 56, 67–72, 2003.

[69] Schreurs, R., et al., Estrogenic activity of UV filters determined by an *in vitro* reporter gene assay and an *in vivo* transgenic zebrafish assay. *Arch. Toxicol.,* 76, 257–61, 2002.

[70] Schlumpf, M., et al., *In vitro* and *in vivo* estrogenicity of UV screens. *Environ. Health Perspect.,* 109, 239–44, 2001.

8 Cationic Lipospheres as Delivery Systems for Nucleic Acid Molecules

Rita Cortesi, Elisabetta Esposito, and Claudio Nastruzzi

CONTENTS

8.1 INTRODUCTION

Gene delivery, or the release of exogenous genetic material into cells or tissues at a pathological state, has recently received much attention as a therapeutic methodology for a number of acquired and inherited diseases, including cancer [1–6]. Several diseases can, in fact, be traced back to defective or missing genes. Thus, bringing an appropriate gene into the appropriate cells could prevent or mitigate manifestations of the disease. *In vivo* cell transfection with foreign genes could be a promising pharmacologic treatment for a variety of diseases, including cancer, cystic fibrosis [7], and viral infections [6]. A large number of abnormally expressed genes have, in fact, been cloned and identified, allowing the prevention or the treatment of many human diseases [8], thus making gene therapy an active field that has progressed rapidly into clinical trials.

Because polydeoxyribonucleotides do not penetrate easily into the cell by themselves, the use of gene delivery vehicles has been proposed to facilitate the transport of genes to cellular targets. Gene transfer vehicles can be divided into viral and nonviral systems [9,10]. In the first case, the virus uses normal tactics to enter the cell and to begin to transcript its genome, but the absence of a key gene prevents

0-8493-1692-8/05/$0.00+$1.50

the packaging of more infectious viral particles. At the same time, the gene of interest gets expressed by the cell and compensates for the lack of cellular proteins related to genetic defects. Viral delivery systems, such as retroviruses, adenoviruses, lentiviruses, and so forth, provide high transfection efficiency, and despite the deletion of vital genes, viruses can induce immune responses that are able to abolish transgenic expression. Moreover, viral vectors, because of their viral tropism, can infect only certain cells expressing receptors for proper replication, thus limiting their activity to specific tissues or compartments. In addition, engineered viruses may induce an immune response that compromises transfection resulting from subsequent injections and that lacks target specificity.

Obviating these problems, a large array of nonviral transfection agents have emerged as potential safe and effective gene vectors for *in vitro* applications. These nonviral systems include cationic lipids, peptides, glycopeptides, liposomes, micelles, glycosylated polymers, dendrimers, and micro- and nanoparticles [10–18]. The efficiency of nonviral transfection systems has, in general, improved several orders of magnitude in the last decade, even if none of them has yet proven to be efficient enough *in vivo* [10–18].

Thus, the key to success for any gene therapy strategy is to design a vector able to provide safe and efficient gene transcription of the transgene in a variety of cells and tissues. In this view, the development of protocols aimed at obtaining optimal and efficient genetic transfer has been studied [19–21] and has led to the production of many delivery vehicles that are able to bind to DNA. The optimal carrier has to accumulate at sites of diseases such as infections, inflammations, and tumors and has to be a small, neutral, and highly serum-stable particle. Moreover, it has to be not readily recognized by the fixed and free macrophages of the reticuloendothelial system.

Among nonviral systems, particulate carriers (e.g., polymeric nano- and microparticles, fat emulsion, liposomes) possess specific advantages and disadvantages. For instance, the relatively slow degradation of polymeric particles might possibly cause systemic toxic effects by impairment of the reticuloendothelial system or by accumulation at the injection site; cytotoxic effects have been indeed observed *in vitro* after phagocytosis of particles by macrophages and human granulocytes [20]. In addition, organic solvent residues deriving from preparation procedures, such as the solvent evaporation technique often used for liposome and polyester microparticles, could result in severe acceptability and toxicity problems [22].

However, with respect to other delivery systems, microparticles could maintain their physicochemical characteristics unaltered for long periods, allowing long-term storage; they can be administered through different ways (orally, intramuscularly, or subcutaneously), depending on their composition; and they are suitable for industrial production [23,24].

All together, these findings have encouraged the development of neutral and cationic lipospheres (CLS) as nonviral DNA-mediated gene transfer techniques because CLS enable the extemporaneous production of pharmaceutical formulations. Like emulsions and liposomes, lipospheres (LS) consist of physiologically well-tolerated ingredients that have often already been approved for pharmaceutical use

in humans [25]; in addition, similar to polymeric nanoparticles, their solid matrix can protect drugs against chemical degradation and allow modulation of drug release profile [24]. Thus, LS combine the advantages of polymeric nanoparticles, fat emulsions, and liposomes, avoiding some of their typical disadvantages, such as cytotoxic effects after phagocytosis, toxic effects of organic residues after the production of polymers, and lack of large industrial-scale production.

LS and CLS are solid microparticles with a mean diameter usually between 0.2 and 500 μm, composed of a solid hydrophobic fat matrix in which (in the case of LS) the bioactive compound or compounds are dissolved or dispersed. Because of their large range in particle size, LS can be administered by different routes, such as orally, subcutaneously, intramuscularly, or topically, or they can be used for cell encapsulation, thus allowing them to be proposed for treatment of a number of diseases [26–28]. The *in vivo* distribution of LS demonstrated a high affinity to vascular wells (including capillaries), to inflamed tissues, and to granulocytes [29,30].

In addition, LS have several advantages over other delivery systems: good physical stability, low cost of ingredients, ease of preparation and scale-up, and high entrapment yields for hydrophobic drugs. Moreover, LS have been successfully used both for the controlled delivery of various types of drugs and as carriers of vaccines and adjuvants [29,31,32].

This chapter will discuss the production and characterization of CLS obtained by different techniques, the formation of the complex between CLS and DNA, and, finally, the *in vitro* cytotoxicity on different cell lines and the transfection efficiency of CLS.

8.2 NUCLEIC ACID STABILITY: GENERAL CONSIDERATIONS AND IMPROVEMENT

In the past few years, many types of nucleic acid molecules, such as synthetic oligonucleotides complementary to viral or eukaryotic RNA, have been reported to inhibit viral replication in cell culture as well as the *in vitro* and *in vivo* expression of genes [33,34], thus indicating the use of synthetic antisense DNA for the modulation of specific gene expression as a novel pharmacological approach for pathological states deriving from an altered expression of a gene or genes. In this respect, viral infections, including AIDS as well as neoplastic diseases, could represent possible targets for oligonucleotide therapeutics [35]. Moreover, other DNA molecules — such as triple helix–forming oligonucleotides and double-stranded polymerase chain reaction–generated DNA fragments, mimicking genomic regulatory regions recognized by transcriptional factors — could be efficiently employed molecular tools to study and modulate gene transcriptional activity [36].

Nevertheless, in spite of these interesting pharmacobiological properties, nucleic acid molecules (i.e., oligonucleotides and polymerase chain reaction–generated DNA fragments) are, in general, rapidly degraded by cellular and extracellular nucleases. It is thus necessary that, to carry on their pharmacobiological activities, nucleic acid molecules should remain stable after *in vivo* administration, retaining an appreciable

half-life in the extracellular environment. The stability problems of this class of compounds have been approached largely through chemical modification of the oligonucleotides, and mainly through substitution of the natural phosphodiester linkage with alternative chemical groups [37]. Although many backbone-modified oligonucleotides have been synthesized and proposed, methylphosphonates and phosphorothioates are the most studied and used nuclease-resistant compounds [37,38].

An alternative approach to chemical modification of oligonucleotides is offered by attaching a "pendant" group to the 5- or 3-ends of the oligonucleotide [33]. A large variety of pendant groups has been designed and synthesized, both to study oligonucleotide cellular uptake and compartmentalization, such as fluorochrome, and to improve or modify the biological activity of the oligonucleotides. This latest class comprises intercalating agents playing different roles, such as stabilizing intercalating agents, cleaving or photo-induced cleaving reagents, and photo-induced cross linkers [39], as well as lipophilic groups such as fatty acid or cholesterol moieties [40].

The potential of the chemically modified nucleic acid molecules has been proven by *in vitro* studies; however, the *in vivo* therapeutic applicability of these molecules seems to be unsatisfactory because of their possible toxic effects (largely unknown) and adverse bioavailability. In this view, both antisense and transfection technologies require reliable and efficient systems for their delivery into target cells. On the basis of this consideration, the development of an efficient nucleic acid delivery system represents one of the key steps for these therapeutic agents, which are necessary for a practical clinical utilization of natural or unnatural oligonucleotides.

In this respect, an interesting approach to reduce degradation and possible toxicity problems related to nucleic acid use *in vivo* is offered by their encapsulation in or association to microcarrier systems, such as neutral or cationic liposome and polymeric microparticles [41–44].

Microparticles can, in principle, vehiculate nucleic acids in two ways: DNA can be physically entrapped in the polymeric matrix of the particle, or DNA can be bound through electrostatic interactions to the positively charged surface of cationic particles. The surface of these carriers could be positively charged as a result of the presence of quaternary ammonium cationic lipids in the liposome composition, such as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) [43,45], DEBDA (diisobutyl-cresoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium hydroxide) [46], or CTAB (cetyltrimethylammonium bromide) [47]. In this way, negatively charged nucleic acids are able to complex the surface of preformed cationic carrier. The association of DNA molecules to preformed cationic microparticles could provide two important benefits. First, DNA is not exposed to the chemical, thermal, or mechanical stresses often present in the production of microparticles, and second, the association of DNA to microparticles can be performed extemporarily immediately before their administration [45,48]. In this way, both microspheres and DNA can be maintained separately in sterile lyophilized forms, thus avoiding possible long-term instability problems. To confer a positive charge to the microparticle surface, different approaches can be proposed, such as a polymeric mixture

that is constituted of an uncharged copolymer and a cationic one, or a not entirely polymeric mixture constituted of a neutral copolymer plus a cationic lipid [49,50].

As above reported, among microparticles, CLS have been proposed as a new type of fat-based encapsulation system developed for drug delivery of bioactive compounds.

8.3 PREPARATION OF CLS

8.3.1 Materials and Preparation Methods

CLS can potentially be used for gene delivery and have shown to be less rapidly cleared from the circulation than negatively charged particles [51]. In addition, the choice of the lipid matrix also plays an important role on the morphology of the particles and on the possible formation of aggregates that are important for the circulations of this carrier [52]. As described in many papers [53–58], LS, and thus also CLS, can be prepared in a variety of ways by using a wide range of chemical ingredients [26]. To obtain a formulation suitable for human administration, triglycerides and monoglycerides have been chosen as LS biomaterials because of their high biocompatibility, high chemicophysical stability, and drug delivery release. However, all preparation methods make use of surfactants, and the resulting particles are characterized by an overall positive, neutral, or negative surface charge, which is determined by the composition, influencing the aggregation tendency in suspension. The simplest and more used preparation methods are typified by the emulsion–melt dispersion and the solvent evaporation technique [53].

It has to be underlined that, in comparison to LS containing nucleic acid molecules inside the particle, the production of CLS may be performed obviously without considering the stability problems of nucleic acid molecules. In this view, some preparation procedures are not considered, such as the microemulsion technique [55] that represents a favorable method when working with substances unstable because of the high mechanical stress produced by high-pressure homogenization.

The production of CLS by the melt dispersion technique is based on the melting of the lipid core material together with the lipophilic agent (i.e., phospholipids). Afterward, a warm aqueous solution is added to the molten material and is mixed by various methods (i.e., mechanical stirring, shaking, sonication, homogenization). Then the preparation is rapidly cooled until lipid solidification and the formation of particle dispersion. This method was used by Olbrich et al. [19] to produce the cationic solid lipid nanoparticles to use as novel transfection agent.

The solvent evaporation technique is based on the use of organic solvents as dissolving agents for the lipid matrix (i.e., phospholipids or triglycerides and monoglycerides) and the subsequent evaporation of the solvent within an aqueous medium until a CLS dispersion is obtained.

However, Erni et al. [50] have prepared LS with a method established in their laboratory using a solvent extraction. In particular, the method is based on the dissolution of the triglyceride (i.e., tripalmitin) and the cationic lipid in the organic solvent (i.e., dichloromethane), and on the addition of an aqueous polyvinyl alcohol (PVA)

solution (0.5% w/w) used as extraction fluid. The solution and the extraction fluid are pumped into a static microchannel mixer, leading to the production of an O/W (oil in water) emulsion. The mixing leads to the production of fine lamellae, which subsequently disintegrate into droplets, allowing the formation of solid lipid microparticles dispersed in the extraction aqueous medium.

In a recent set of experiments [56], LS composed of triglycerides and monoglycerides were alternatively produced by melt dispersion technique, by solvent evaporation, or by the w/o/w double-emulsion method. The influence of preparation parameters, such as type and amount of lipids, presence and concentration of surfactants, stirring speed, and type of stirrer was studied. In the case of LS prepared by melt dispersion, the use of a lipid composition of cetyl alcohol/cholesterol (2:1, w/w), a 5% (w/w) gelatin solution (50 Bloom grades), and a 1000-rpm stirring speed resulted in the production of spherical particles with a high percentage of recovery (82%, w/w), a mean diameter of 80 μm, and a narrow size distribution. In the case of LS prepared by solvent evaporation, the best results in terms of LS morphology, recovery, and size distribution were obtained by the use of a lipid composition of tristearin/monostearate (66:34, w/w), a 1% (w/w) PVA solution, a 750-rpm stirring speed, and a 55-mm three-blade turbine rotor.

The solvent evaporation method resulted in the production of LS characterized by a smaller size (20 μm mean diameter) but poor mechanical properties in respect to particles with the same composition that were obtained by the melt dispersion technique (170 μm mean diameter). The use of a combination of lipids and a methacrylic polymer (Eudragit RS100) overcame this problem, resulting in the production of spherical particles with a narrower size distribution and good mechanical properties [53,56].

Concerning the SLN produced by hot homogenization as described by Olbrich et al. [19], as lipidic matrix Compritol ATO 888 or paraffin were used, as tenside a mixture of Tween 80 and Span 85 was used, and as charge carrier either EQ1 [*N*,*N*-di-(β–steaorylethyl)-*N*,*N*-dimethylammonium chloride] or cetylpyridinium chloride were used. The resulting particles were characterized by size between 101 and 105 nm and showed zeta potentials around 40 mV at pH 7.4.

In contrast, solid lipid microparticles consisting of a tripalmitin matrix and cationic lipids prepared using the micromixer-based solvent extraction process as described by Erni et al. [50] were of monomodal size, showing a narrow size distribution in the submicrometer range (Table 8.1).

8.3.2 Formation of CLS/DNA Complex

To our knowledge, only a few papers have been recently published concerning CLS for gene therapy. In particular, to investigate the ability of CLS to bind nucleic acids, different nucleic acid molecules were considered as models, namely, two double-stranded plasmid DNA [19,50] and defibrotide (DFT, Mw 26,200 Da), a single-stranded polydeoxyribonucleotide (DNA) sodium salt extracted from mammalian organs [56]. This last molecule was chosen because it is included in a pharmaceutical product presently on the market as an antithrombotic agent, it is a low–molecular weight nucleic acid mimicking the behavior of polymerase chain reaction products

TABLE 8.1
Composition, Particle Size, Zeta Potential, and Loading Efficiency of Nucleic Acid of the Cationic Lipospheres Considered in the Chapter

Cationic Lipospheres Composition (% of component)	Method of Preparation	Particle Size (nm)	Zeta Potential (mV)	Reference
Solid paraffin (4%), Tween 80/Span 65 (7:3) (4%), cetyltrimethylammonium bromide (0.5%)	Melt dispersion	103 ± 2.3	+41.2 ± 0.9	[19]
Compritol (4%), Tween 80/Span 65 (7:3) (4%), cetyltrimethylammonium bromide (0.5%)	Melt dispersion	105 ± 1.7	+40.2 ± 0.5	[19]
Compritol (4%), Tween 80/Span 65 (7:3) (4%), N,N-di-(β-steaorylethyl)-N,N-dimethylammonium chloride (1%)	Melt dispersion	101 ± 2.3	+42.3 ± 1.0	[19]
Stearylamine (1.8%), cetyltrimethyl-ammonium bromide (1.2%), Trimyristin (1.8%), Polysorbate 80 (19.0%)	Melt dispersion (unwashed)	189.5 (bimodal)	+17.9	[54]
Stearylamine (1.8%), cetyltrimethyl-ammonium bromide (1.2%), Trimyristin (1.8%), Polysorbate 80 (19.0%)	Melt dispersion (dialyzed)	211.7	10.1	[54]
Tripalmitin (95%), cetyltrimethyl-ammonium bromide (5%), PVA (0.5%)	Solvent extraction	1890	+21.4 ± 7.3	[50]
Tripalmitin (95%), $DDAB_{12}$ (5%) PVA (0.5%)	Solvent extraction	5150	+24.9 ± 7.9	[50]
Tripalmitin (95%) DDAB18 (5%) PVA (0.5%)	Solvent extraction	2370	+30.9 ± 8.7	[50]
Tripalmitin (95%) PVA (0.5%)	Solvent extraction	6830	8.4 ± 2.3	[50]
Tristearin (66%) Glyceryl monostearate (33%) cetyltrimethyl-ammonium bromide (5%) PVA (1%)	Melt dispersion	1260 ± 150	n.d.[a]	[56]
Tristearin (66%) Glyceryl monostearate (33%) $DDAB_{12}$ (5%) PVA (1%)	Melt dispersion	1650 ± 90	n.d. [a]	[56]
Tristearin (66%), Glyceryl monostearate (33%), $DDAB_{18}$ (5%), PVA (1%)	Melt dispersion	1420 ± 210	n.d. [a]	[56]

[a] n.d.: not determined

and synthetic oligonucleotides, and its complex with cationic liposomes is a patented pharmaceutical formulation.

The formation of the CLS/nucleic acid complex was performed by mixing an aqueous suspension of CLS with a solution containing the nucleic acids [19,50,56].

In particular, Erni et al. [50] used a gentle shaking in phosphate-buffered salt with pH 7.4 or Dulbecco's Modificatin of Eagle's Medium (DMEM) for 2 h at 41°C, whereas Olbrich et al. [19] prepared the CLS/DNA complexes by mixing 20 mg/mL of plasmid in 200 mL of 25 mM Hepes (pH 7.4).

In the first case [50], the loading efficiency was in the range of 65 to 95% for CLS and was up to a maximum of 56% for neutral particles, showing that adsorption was enhanced by the cationic surface of the particles. In addition, it was proven that loading efficiency was further dependent on the composition of the medium used for dispersing the particles. With respect to the second case [19], the binding of the CLS to polyanionic DNA was studied by analysis of the electrophoretic mobility of the DNA within an agarose gel, the so-called electrophoretic mobility shift assay. Addition of the tenside mixtures or the cationic modifier molecules alone did not result in a change of DNA migration during electrophoresis of the cationically modified CLS; however, one mixture (SII-13) resulted in a shift in DNA mobility, whereas the other CLS formulations (SII-4, SII-5, SII-9, SII-10), as well as the surfactant mixture (SII-17), were not able to immobilize detectable amounts of DNA at w/w ratios up to 10,000. At ratios above 50, 100% of the DNA was shifted to a higher apparent molecular weight or was even completely immobilized within the wells. Twenty to 50 weight equivalents were sufficient to bind most of the DNA, and five or fewer weight equivalents of SII-13 were practically inactive.

Concerning DFT association to CLS (third case) [56], Figure 8.1 shows the ability of the different types of CLS to ionically bind the nucleic acid when used at a different positive to negative molar charge ratio (+/–) comprised between 1:1 to 16:1. In particular, CLS containing $DDAB_{18}$ (dioctadecyl-dimethylammonium bromide) prepared at 500 rpm in the presence of PVA (white diamond) or gelatin 50 Bloom (black diamond), both at 1% w/v, were considered. As reported in Figure 8.1A, the association of DFT to CLS of both types showed a similar trend, reaching at the highest molar charge ratio an association around 90%, namely, 87.3% and 90.4% for CLS obtained with PVA and gelatin 50 Bloom, respectively. The association ability of CLS containing $DDAB_{12}$ (didodecyl-dimethylammonium bromide) and CTAB were evaluated only on lipoparticles obtained in the presence of PVA. As demonstrated by the data of Figure 8.1B for $DDAB_{12}$ and CTAB, the association capacity of CLS was only scarcely affected by their size; in fact, smaller particles (mean size 0.66 μm) displayed an association profile almost identical to that of larger particles (mean size 0.87 μm).

Cortesi et al. [56] have conducted a study to evaluate the strength of the interaction occurring between DFT and CLS and to evaluate whether different cationic detergents could cause a variation in binding strength. Briefly, CLS containing increased concentrations of cationic lipid were incubated with DFT for 10 min, and then samples were electrophoresed to determine the electrophoretic migration of DNA complexed to liposome. The results reported in Figure 8.2 indicate that DFT migration is only slightly retarded by low amounts of CLS, whereas higher CLS concentration (especially in the case of $DDAB_{18}$) causes the formation of high–molecular weight complexes with DFT molecules that precipitated within the well. These nonmigrating complexes were attributed to interparticle bridges formed by DNA molecules [59].

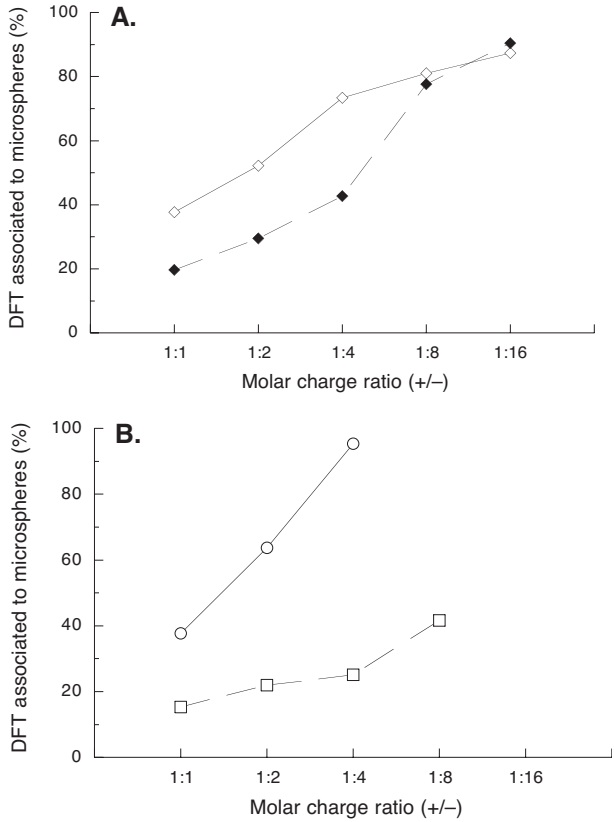

FIGURE 8.1 Percentages of DFT association to CLS containing as cationic lipid. **(A)** $DDAB_{18}$ prepared in the presence of PVA (open diamonds) or gelatin (closed diamonds); **(B)** $DDAB_{12}$ (open circles) or CTAB (open squares).

8.4 CYTOTOXICITY OF NUCLEIC ACIDS FROM CLS

To evaluate the cytotoxic activity of CLS, different types of experiments were performed. For instance, Erni et al. [50] tested the cytotoxicity of the different cationic lipids used for the preparation of CLS in two different cell lines (293 embryonic kidney cells and RAW macrophages) for compromised membranes of dead cells indicating necrosis [60]. All cationic lipids displayed comparable concentration-dependent cytotoxicity profiles, all being nontoxic at concentrations up to 2 mg/well. Slightly increased cytotoxicities were observed with the short alkyl chain–length $DDAB_{12}$. Interestingly, a lower necrosis was observed in the RAW macrophage cell line as compared to the 293 cell line. Significant cytoxicity was found in primary macrophages after adding CLS or cationic lipid at amounts that did not cause detectable cytotoxicity in 293 cells or RAW macrophages. In addition, the cytotoxicity of the various CLS formulations turned out to be comparable to the

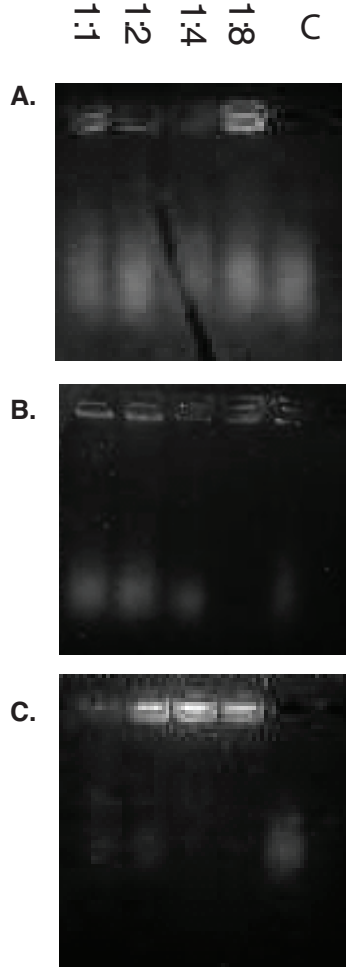

FIGURE 8.2 Effect of CLS complexation on the electrophoretic migration of DFT. **(A)** $DDAB_{18}$, **(B)** $DDAB_{12}$, **(C)** CTAB. The following CLS/DFT charge molar ratios, namely, 1:1, 1:2, 1:4, and 1:8 mol/mol, were used. C: control untreated DFT.

respective soluble cationic lipid, with negligible effects at concentrations less than 2 mg/well (referring to the amount of cationic lipid incorporated in the formulation). Neutral lipid particles prepared under identical conditions without the addition of cationic lipid did not show any detectable cytotoxicity when added to the cells in comparable amounts.

Olbrich et al. [19] tested *in vitro* the cytotoxicity of the transfection agents, considering the viability of Cos-1 cell monolayers. Both the initial perturbation of cell integrity during the 4-h incubation and the influence on the cellular activity after 48 hours were assessed by measuring the release of lactate dehydrogenase and the mitochondrial conversion of the tetrazolium salt WST-1. Unmodified paraffin particles (SII-4) did not show cytotoxicity in the LDH (lactate dehydrogenase) release

assay. The same behavior was observed for paraffin particles modified with EQ1 (SII-5), whereas those containing cetylpyridinium resulted in 50% LDH release at 120 µg/mL. Similar observations were made for modified Compritol-based particles with no detectable LDH release after incubation with up to 3 mg/mL SII-13 (EQ1 modified) but a LD_{50} of 150 µg/mL for SII-10, which contains the modifier cetylpyridinium chloride. Interestingly, the mixture of Tween/Span and EQ1 (SII-17) was more toxic when it was not bound to a suitable particulate matrix, as in batch SII-13. Assessment of the cell viability after 48 h, using the WST-1 test, was generally more sensitive than the LDH release assay. However, it produced the same overall correlations: cetylpyridinium-containing SLN were toxic, and EQ1-modified particles were less problematic. Moreover, modification of paraffin-based SLN with EQ1 reduced their negative effect on cell viability, resulting in IC_{50} values of 700 mg/mL instead of 100 mg/mL. The pure tenside mixture (SII-17) showed similar IC_{50} values of 150 mg/mL.

To determine the cytotoxic activity of cationic microparticles, Cortesi et al. [56] performed an *in vitro* study treating human leukemic K562 and murine macrophagic J774 cell lines with different amounts of CLS. After 6 d in cell culture, cells were electronically counted. Figure 8.3 reports the cytotoxic activity of CLS containing, alternatively, $DDAB_{18}$, $DDAB_{12}$, and CTAB (with an amount between 0 and 300 µg/mL corresponding to a cationic lipid concentration between 0 and 250 µM). The obtained data demonstrated that cationic lipoparticles are only slightly cytotoxic, especially when compared with other cationic formulations used for gene therapy, such as liposomes [49], indicating that $DDAB_{18}$-based lipoparticles could be safely used in *ex vivo* experiments. To assess whether cationic lipoparticles can be efficiently internalized by *in vitro* cultured cells, an experiment was also conducted with J774 murine macrophages. Cells were cultured in the presence of CLS and, after 5 min, were fixed with glutaraldehyde and observed by scanning electron microscopy analysis. As clearly evident in Figure 8.4, CLS are efficiently internalized (probably by phagocytosis) in J774 cells, indicating that they could be used as a delivery system for at least *ex vivo* experiments.

Erni et al. [50] have demonstrated that neutral lipid particles consisting of a tripalmitin matrix can be efficiently phagocytosed by primary macrophages *in vitro*. In particular, complete intracellular degradation was observed within 24 h, making neutral lipid particles a suitable carrier for the immediate delivery of therapeutics to antigen-presenting cells. However, CLS also adsorbed plasmid DNA and triggered the cellular internalization of the macromolecules by phagocytic macrophages. Surprisingly, the CLS also triggered the internalization of these molecules by nonphagocytic 293 cells. This was probably a result of the detachment of nanocomplexes formed of cationic lipid and DNA from the surface of DNA-loaded CLS and their subsequent uptake into the cells.

8.5 TRANSFECTION EFFICIENCY

Erni et al. and Olbrich et al. [19,50] have data about transfection performed with CLS as carriers. For instance, Erni et al. tested the transfection efficiency in primary macrophages and in RAW macrophages. No transfection was observed during 72 h

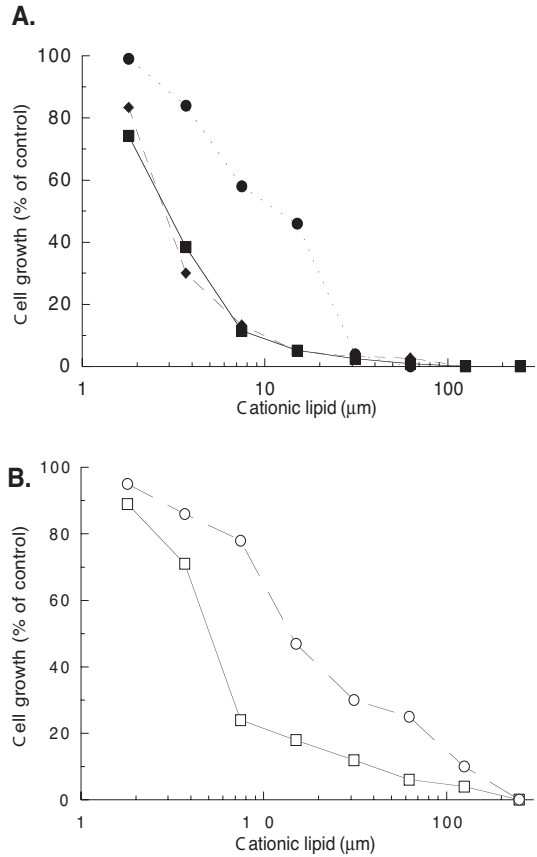

FIGURE 8.3 Cytotoxic activity of $DDAB_{18}$ (circle), $DDAB_{12}$ (square), and cetyltrimethylammonium bromide (diamond) cationic lipoparticles on the cultured human K562 **(A)** and murine macrophagic J774 **(B)** cell lines. Data represent the percentage of cell number per milliliter compared with untreated control K562 cells.

in either type of cells. However, the authors demonstrated that transfection efficiency of the DNA-loaded CLS was most pronounced in nonphagocytic cells and was not detected in the macrophage cell line or in primary macrophages. Further studies have revealed that cytotoxic effects of CLS were more pronounced in the phagocytic cells, because of the very rapid uptake and degradation of the CLS in these cells. In particular, free cationic lipid equivalent to the amount of cationic lipid contained in the tested CLS was mixed with DNA and administered to the various cell cultures as controls. Detectable transfection in 293 cells was exclusively found with $DDAC_{18}$. Its level was about threefold higher than with CLS containing $DDAC_{18}$ and was in the range of the commonly used transfectant DOTAP. CTAB and $DDAC_{12}$ failed to induce cell transfection. Thus, transfection mediated by CTAB or $DDAC_{12}$ was only feasible when enhanced with CLS. However, when CLS containing $DDAC_{18}$ were separated by filtration, and the supernatant subsequently mixed with DNA and further

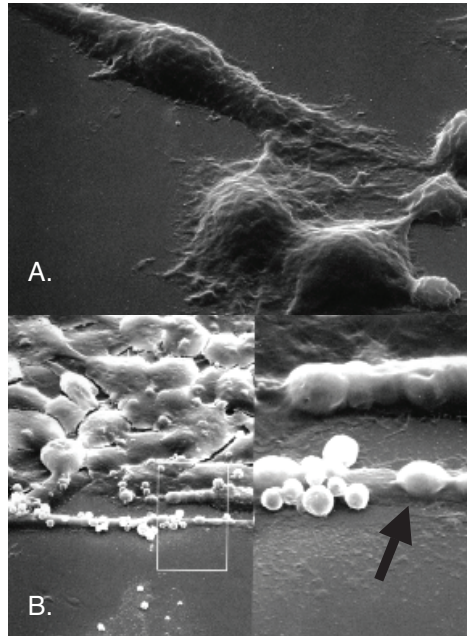

FIGURE 8.4 Scanning electron micrograph of J774 cells alternatively untreated **(A)** and treated **(B)** with cationic lipospheres containing $DDAB_{18}$. Black arrows indicate internalized submicron particles. Bar corresponds to 10 and 30 μm in panels A and B, respectively.

used for transfection, no measurable transfection occurred. This may indicate the absence of significant amounts of free $DDAC_{18}$ in the dispersion medium that are available for complex formation with DNA that induces cell transfection.

As previously stated, Olbrich et al. [19] tested both cytotoxicity and transfection efficiency on Cos-1 cells *in vitro*. In particular, the ability of CLS to transfect the pCMVb reporter gene plasmid at a fixed concentration into Cos-1 cells was tested in the absence or in the presence of 100 mM of the endosomolytic agent chloroquine. The obtained results demonstrated that CLS were able to promote transfection in a wide window of CLS/DNA ratios, whereas incubation with uncomplexed DNA did not result in detectable levels of β-galactosidase expression in this experimental setup. In the absence of chloroquine, the strongest reporter gene expression was observed when the DNA was complexed with 60 weight equivalents of CLS, although, when the overall transfection efficiency of CLS is compared to established transfection agents such as poly-L-lysine or polyethylenimine, it has to be ranked only moderate. Nevertheless, the controls, SII-10 (a CLS not able to immobilize plasmid DNA in the agarose gel) and SII-17 (the pure cationic modifier unable to stably bind DNA), were not able to retransfect Cos-1 cells. In all cases, the β-galactosidase activity was within the background range.

Another important aspect of transfection agents, especially for nonviral systems, was the efficiency/toxicity ratio. The CLS described by Olbrich et al. [19] showed only moderate transfection efficiencies compared with the established polymers

poly-L-lysine and polyethylenimine. However, this transfection activity was accompanied only by a low degree of cytotoxicity. A number of possibilities exist for optimizing the activity of the system, including choice of matrix lipid, size, and modifier.

8.6 CONCLUSIONS

During recent years, solid lipid nanoparticles have attracted increasing attention. However, only a few studies that have been aimed to obtain innovative nonviral transfection systems for gene therapy have been performed on CLS. In the last decade, the efficiency of nonviral transfection systems has improved several orders of magnitude. Although as yet none has proven to be effective enough *in vivo*, new developments are still ongoing. Among nonviral transfection systems, colloidal carriers such as CLS represent an alternative drug delivery system to emulsions, liposomes, and polymeric particles. From the analysis of the results reported in this chapter, it emerges that CLS may provide a new, efficient means for the immediate intracellular delivery of therapeutic macromolecules. Nevertheless, caution is warranted for cationic carriers, which may accentuate cytotoxic effects in the phagocytic cells.

REFERENCES

[1] Tomlinson, E., and Rolland, A.P., Controllable gene therapy: pharmaceutics of nonviral gene delivery systems, *Journal of Controlled Release,* 1995, 39, 357–372.

[2] Bennett, M.J., Aberle A.M., Balasubramaniam R.P., Malone, J.G., Malone, R.W., and Nantz, M.H., Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with *in vivo* transfection activity, *Journal of Medicinal Chemistry,* 1997, 40, 4069–4078.

[3] Wasan, E.K., Fairchild, A., and Bally, M.B., Cationic liposome-plasmid DNA complexes used for gene transfer retain a significant trapped volume, *Journal of Pharmaceutical Science,* 1998, 87, 9–14.

[4] Ropert, C., Liposomes as a gene delivery system, *Brazilian Journal of Medicine and Biology Research,* 1999, 32, 163–169.

[5] Tseng, W.C., and Huang, L., Liposome-based gene therapy, *Pharmaceutical Science and Technology Today,* 1998, 1, 9–11.

[6] Ishii Morita, H., Agbaria, R., Mullan, C.A., Hirano, H., Koeplin, D.A., Ram, Z., Oldfield, E.H., Johns, D.G., and Blaese, R.M., Mechanism of "bystander effect" killing in the herpes simplex thymidine kinase gene therapy model of cancer treatment, *Gene Therapy,* 1997, 4, 244–251.

[7] Sharma, A., and Sharma, U.S., Liposomes in drug delivery: progress and limitations, *International Journal of Pharmaceutics,* 1997, 154, 123–140.

[8] Anderson, W.F., Human gene therapy, *Nature,* 1998, 392, 25–30.

[9] Smith, J., Zhang, Y.L., and Niven, R., Toward development of non-viral gene therapeutics, *Advanced Drug Delivery Reviews,* 1997, 26, 135–150.

[10] Kneuer, C., Sameti, M., Bakowsky, U., Schiestel, T., Schirra, H., Schmidt, H., and Lehr, C.M., Surface modified silica-nanoparticles can enhance transfection *in vitro*: a novel class of non-viral DNA vectors, *Bioconjugate Chemistry,* 2000, 11, 926–932.

[11] Filion, M.C., and Philips, N.C., Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells, *Biochimica et Biophysica Acta,* 1997, 1329, 345–356.

[12] Zelphati, O., Nguyen, C., Ferrari, M., Felgner, J., Tsai, Y., and Felgner, P.L., Stable and monodisperse lipoplex formulations for gene delivery, *Gene Therapy,* 1998, 5, 1272–1282.

[13] Gottschalk, S., Sparrow, J.T., Hauer, J., Mims, M.P., Leland, F.E., Woo, S.L.C., and Smith, L.C., A novel DNA–peptide complex for efficient gene transfer and expression in mammalian cells, *Gene Therapy,* 1996, 3, 448–457.

[14] Takagi, T., Hashiguchi, M., Hiramatsu, T., Yamashita, F., Takakura, Y., and Hashida, M., Effect of cationic liposomes on intracellular trafficking and efficacy of antisense oligonucleotides in mouse peritoneal macrophages, *Journal of Drug Targeting,* 2000, 7, 363–371.

[15] Miller, A.D., The problem with cationic liposome/micelle-based non-viral vector systems for gene therapy, *Current Medicinal Chemistry,* 2003, 10, 1195–1211.

[16] Haensler, J., and Szoka Jr., F.C., Polyamidoamine cascade polymers mediate efficient transfection of cells in culture, *Bioconjugate Chemistry,* 1993, 4, 372–379.

[17] Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.P., A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine, *Proceedings of the National Academy of Sciences,* United States of America, 1995, 92, 7297–7301.

[18] Blessing, T., Remy, J.S., and Behr, J.P., Monomolecular collapse of plasmid DNA into stable virus-like particles, *Proceedings of the National Academy of Sciences,* United States of America, 1998, 95, 1427–1431.

[19] Olbrich, C., Bakowsky, U., Lehr, C.M., Müller, R.H., and Kneuer, C., Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA, *Journal of Controlled Release,* 2001, 77, 345–355.

[20] Luo, D., and Saltzman, W.M., Synthetic DNA delivery systems, *Nature Biotechnology,* 2000, 18, 33–37.

[21] Truong, V.L., Walsh, S., Schweibert, M.E., Mao, H.Q., Guggino, W.B., August, J.T., and Leong, K.W., Gene transfer by DNA–gelatin nanospheres, *Archives in Biochemistry and Biophysics,* 1999, 361, 47–56.

[22] Cortesi, R., Esposito, E., Gambarin, S., Telloli, P., Menegatti, E., and Nastruzzi, C., Preparation of liposomes by reverse-phase evaporation using alternative organic solvents, *Journal of Microencapsulation,* 1999, 16, 251–256.

[23] Walter, E., Dreher, D., Kok, M., Thiele, L., Kiama, S.G., Gehr, P., and Merkle, H.P., Hydrophilic poly(dl-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells, *Journal of Controlled Release,* 2001, 76, 149–168.

[24] Reithmeier, H., Herrmann, J., and Göpferich, A., Lipid microparticles as a parenteral controlled release device for peptides, *Journal of Controlled Release,* 2001, 73, 339–350.

[25] Müller, R.H., Ruhl, D., Runge, S., Schulze-Forster, K., and Mehnert, W., Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant, *Pharmaceutical Research,* 1997, 14, 458–462.

[26] Müller, R.H., K., Mäder, S., Gohla, Solid lipid nanoparticles (SLN) for controlled drug delivery: a review of the state of art, *European Journal of Pharmaceutics and Biopharmaceutics,* 2000, 50, 161–177.

[27] Takenaga, M., Application of lipid microspheres for the treatment of cancer, *Advanced Drug Delivery Review,* 1996, 20, 209–219.

[28] Yang, S.C., Zhu, J., Lu, Y., Liang, B., and Yang, C., Body distribution of camptothecin solid lipid nanoparticles after oral administration, *Pharmaceutical Research,* 1999, 16, 751–757.

[29] Inoue, K., Aoki, Y., Hayashi, M., Kitahara, S., Tanabe, H., Kiyoki, M., and Araki, H., Ex vivo anti-platelet effects of isocarbacyclin methyl ester incorporated in lipid microspheres in rabbits, *Arzneim-Forsch/Drug Research,* 1995, 45, 980–984.

[30] Müller, R.H., Maassen, S., Schwarz, C., and Mehnert, W., Solid lipid nanoparticles (SLN) as potential carrier for human use: interaction with human granulocytes, *Journal of Controlled Release,* 1997, 47, 261–269.

[31] Olbrich, C., Kayser, O., Müller, R.H., and Grubhofer, N., Solid lipid nanoparticles (SLN) as vaccine adjuvant: study in sheep with a mycoplasma bovis antigen and stability testing, *International Symposium of Controlled Release and Bioactive Material,* 2000, 27, 293–294.

[32] Olbrich, C. and Müller, R.H., Tabatt, K., Kaiser, O., Schulze, C., and Schade, R., Stable biocompatible adjuvants — a new type of adjuvant based on solid lipid nanoparticles: a study on cytotoxicity, compatibility and efficacy in chicken, *Alternatives to Laboratory Animals,* 2002, 30, 443–458.

[33] Neckers, L., Whitesel, L., Rosolen, A., and Geselowitz, D.A., Antisense inhibition of oncogene expression, *Critical Reviews in Oncogenesis,* 1992, 3, 175–231.

[34] To, R.Y., and Neiman, P.E., The potential for effective antisense inhibition of retroviral replication mediated by retroviral vectors. In: Erickson R.P., Izant J.G., eds., *Gene Regulation: Biology of Antisense RNA and DNA.* Raven Press, New York, 1992, pp. 261–271.

[35] Chirila, T.V., Rakoczy, P.E., Garrett, K.L., Lou, X., and Constable, I.J., The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides, *Biomaterials,* 2002, 23, 321–342.

[36] Lysik, M.A., and Wu-Pong, S., Innovations in oligonucleotide drug delivery, *Journal of Pharmaceutical Sciences,* 2003, 92, 1559–1573.

[37] Crooke, R.M., *In vitro* toxicology and pharmacokinetics of antisense oligonucleotides, *Anti-Cancer Drug Design,* 1991, 6, 609–646.

[38] Uhlmann, E., and Peyman, A., Antisense oligonucleotides: a new therapeutic principle, *Chemical Reviews,* 1990, 90, 543–584.

[39] Helene, C., Control of gene expression by antisense and antigene oligonucleotide-intercalator conjugates. In: Erickson R.P., Izant J.G., eds., *Gene Regulation: Biology of Antisense RNA and DNA.* Raven Press, New York, 1992, pp. 109–118.

[40] Mackellar, C., Graham, D., Will, D.W., Burgess, S., and Brown, T., Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups, *Nucleic Acid Research,* 1992, 20, 3411–3417.

[41] Janoff, A.S., Lipids, liposomes, and rational drug design, *Laboratory Investigation,* 1992, 66, 655–658.

[42] Gregoriadis, G., *Liposome as Drug Carriers: Recent Trends and Progress,* Wiley, Chichester, 1988.

[43] Thierry, A.R., Rahman, A., and Dritschilo, A., Liposomal delivery as a new approach to transport antisense oligonucleotides. In: Erickson, R.P., Izant, J.G., eds., *Gene Regulation: Biology of Antisense RNA and DNA,* Raven Press, New York, 1992, pp. 147–161.

[44] Cortesi, R., Esposito, E., Menegatti, E., Gambari, R., Nastruzzi, C., Gelatine microspheres as a new approach for controlled delivery of synthetic oligonucleotides and PCR-generated fragments, *International Journal of Pharmaceutics,* 1994, 105, 181–186.

[45] Felgner, P.L., Particulate systems and polymers for *in vitro* and *in vivo* delivery of polynucleotides, *Advanced Drug Delivery Reviews,* 1990, 5, 163–187.

[46] Ballas, N., Zakay, N., Sela, I., and Loyter, A., Liposome bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplast, *Biochimica et Biophysica Acta,* 1988, 939, 8–18.

[47] Pinnaduwage, P., Schmitt, L., and Huang, L., Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells, *Biochimica et Biophysica Acta,* 1989, 985, 33–37.

[48] Hug, P., and Sleight, R.G., Review: liposomes for the transformation of eukaryotic cells, *Biochimica et Biophysica Acta,* 1991, 1097, 1–15.

[49] Esposito, E., Sebben, S., Cortesi, R., Menegatti, E., and Nastruzzi, C., Preparation and characterization of cationic microspheres for gene delivery, *International Journal of Pharmaceutics,* 1999, 189, 29–41.

[50] Erni, C., Suard, C., Freitas, S., Dreher, D., Merkle, H.P., and Walter E., Evaluation of cationic solid lipid microparticles as synthetic carriers for the targeted delivery of macromolecules to phagocytic antigen-presenting cells, *Biomaterials,* 2002, 23, 4667–4676.

[51] Gregoriadis, G., and Neerunjun, D.E., Control of the rate of hepatic uptake and catabolism of liposome-entrapped proteins injected into rats: possible therapeutic applications, *European Journal of Biochemistry,* 1974, 47, 179–185.

[52] zur Mühlen, A., Schwarz, C., And Mehnert, W., Solid lipid nanoparticles (SLN) for controlled drug delivery: drug release and release mechanism, *European Journal of Pharmaceutics and Biopharmaceutics,* 1998, 45, 149–155.

[53] Cortesi, R., Esposito, E., Luca, G., and Nastruzzi, C., Production of lipospheres as carriers for bioactive compounds, *Biomaterials,* 2002, 23, 2283–2294.

[54] Heydenreich, A.V., Westmeier, R., Pedersen, N., Poulsen, H.S., and Kristensen, H.G., Preparation and purification of cationic solid lipid nanospheres: effects on particle size, physical stability and cell toxicity, *International Journal of Pharmaceutics,* 2003, 254, 83–87.

[55] Gasco, M.R., Solid lipid nanospheres from warm microemulsions, *Pharmaceutical Technology Europe,* 1997, 9, 52–58.

[56] Cortesi, R., Esposito, E., Magnan, M., Campi, F., and Nastruzzi, C., Cationic lipospheres for nucleic acid delivery, manuscript in preparation.

[57] Hou, D.Z., Xie, C.S., Huang, K.J., and Zhu, C.H., The production and characteristics of solid lipid nanoparticles (SLNs), *Biomaterials,* 2003, 24, 1781–1785.

[58] Dingler, A., and Gohla, S., Production of solid lipid nanoparticles (SLN): scaling up feasibilities, *Journal of Microencapsulation,* 2002, 19, 11–16.

[59] Sternberg, B., Sorgi, F.L., and Huang, L., New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy, *FEBS Letters,* 1994, 356, 361–366.

[60] Walter, E., and Merkle, H.P., Microparticle-mediated transfection of non-phagocytic cells *in vitro*, *Journal of Drug Targeting,* 2002, 10, 11–21.