4 Lipospheres as Delivery Systems for Peptides and Proteins

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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

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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

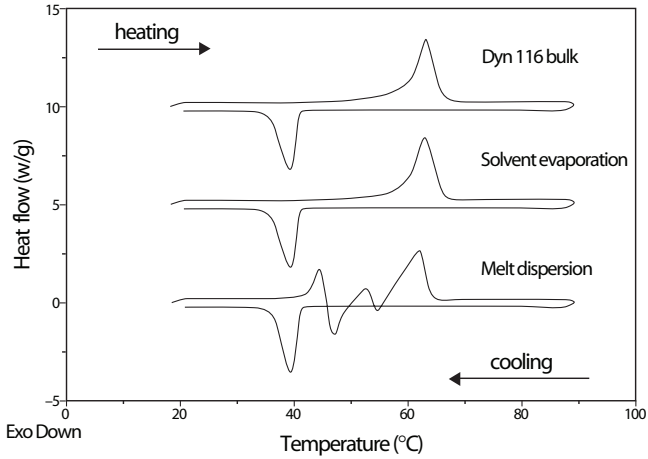

FIGURE 4.1 Differential scanning calorimetry heating and cooling curves of glyceryl tripalmitate (Dyn 116) bulk material, with microparticles prepared by solvent evaporation and microparticles prepared by melt dispersion 1 d after the preparation. The plots are displaced vertically for better visualization.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

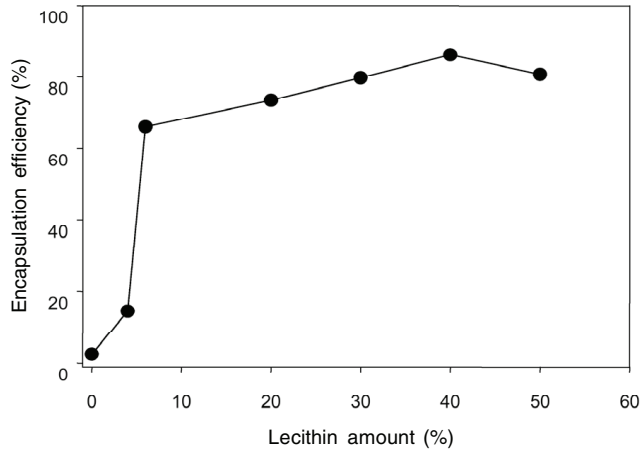

FIGURE 4.2 Influence of added lecithin amount on encapsulation efficiency of somatostatin. Solvent evaporation method: solvent hexane, cosolvent methanol, theoretical loading 2%. (Adapted from [37] with permission from Elsevier.)

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.

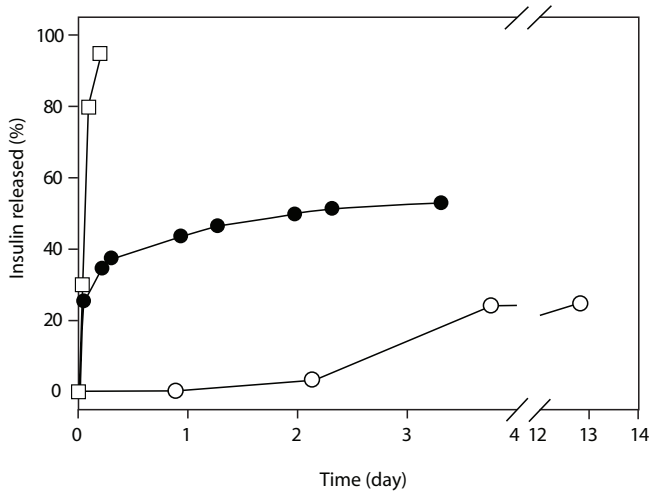

FIGURE 4.3 *In vitro* release of insulin (release medium: PBS buffer, pH 7.4, 10 mmol, assessment of the residual insulin in the microparticles), (solid circles) microparticles washed with water (drug loading 2.3%), (open circles) microparticles washed with 0.01 M HCl, (open squares) control (insulin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

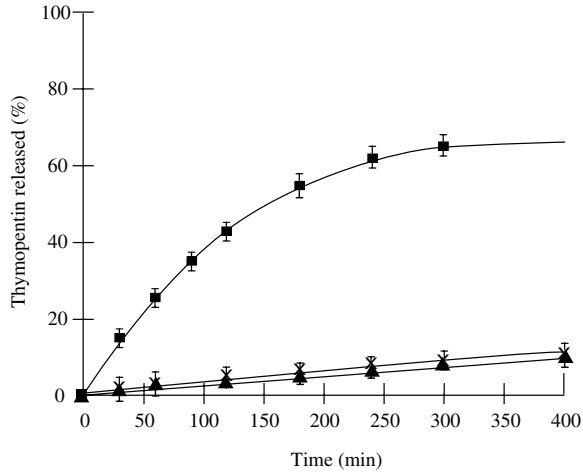

FIGURE 4.4 Percentage release of thymopentin from (filled triangles) lipospheres obtained by o/w microemulsion, from (X) lipospheres obtained by w/o/w microemulsion and diffusion from an (filled squares) aqueous solution. (Adapted from [39] with permission from Elsevier.)

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

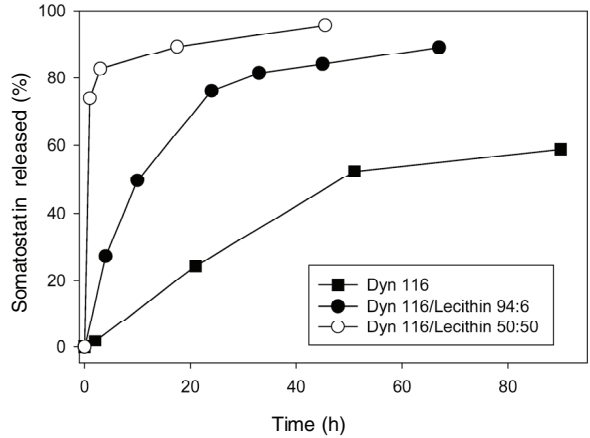

FIGURE 4.5 Influence of lecithin amount added to glyceryl tripalmitate (Dyn 116) on somatostatin release (particles washed with bidestilled water). (Adapted from [37] with permission from Elsevier.)

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

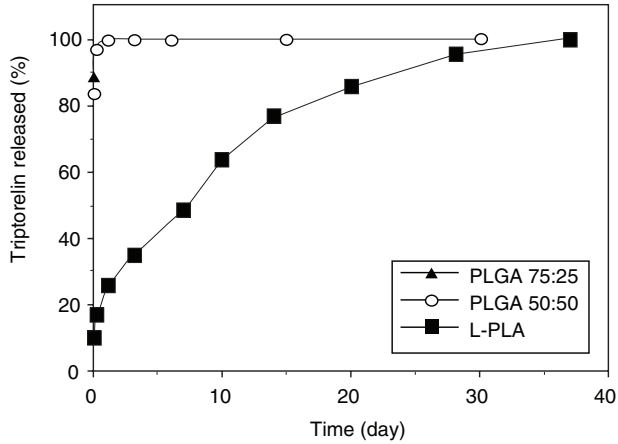

FIGURE 4.6 Effects of polymer type on the cumulative release of triptorelin from lipospheres. Lipospheres were prepared from L-PLA (solid squares), PLGA 50:50 (open circles), or PLGA 75:25 (filled triangles) with HSPC in a 1:6 phospholipid/polymer ratio. Triptorelin (4 mg) was dissolved in N-methylpyrrolidone (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

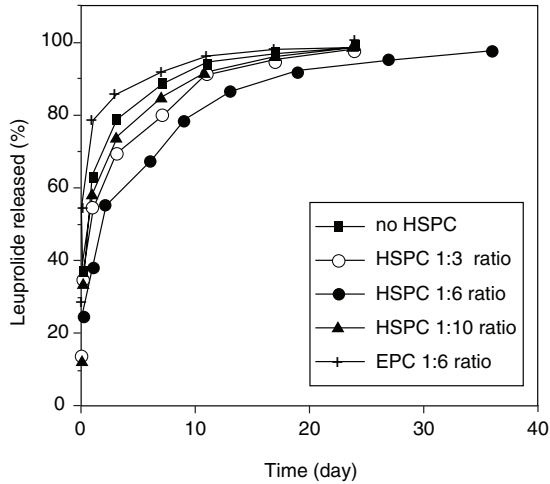

FIGURE 4.7 Effects of phospholipid/polymer ratio on the cumulative release of leuprolide from lipospheres. Lipospheres were prepared from L-PLA and EPC (1:6, pluses), hydrogenated soybean phosphatidylcholine (1:3, open circles), HSPC (1:6, filled circles), or HSPC (1:10, filled triangles), as described 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.

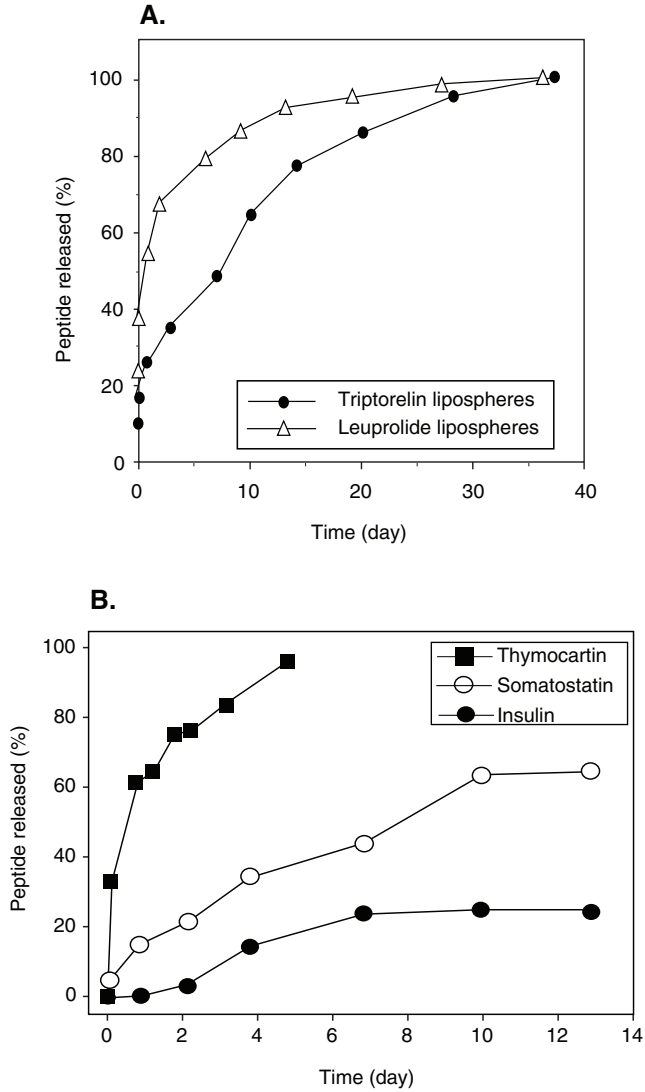

FIGURE 4.8 (A) Cumulative release of triptorelin and leuprolide from lipospheres. Lipospheres were prepared from L-PLA and HSPC, as described in Figure 4.6. The release experiment was performed in pH 7.4, phosphate buffer, at 37˚C, and analyzed by HPLC for both formulations. (Adapted from [35] with permission 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.