

# Lipid-based colloidal carriers for peptide and protein delivery – liposomes versus lipid nanoparticles

Susana Martins<sup>1</sup>  
Bruno Sarmiento<sup>1</sup>  
Domingos C Ferreira<sup>1</sup>  
Eliana B Souto<sup>1,2</sup>

<sup>1</sup>Department of Pharmaceutical Technology, Faculty of Pharmacy University of Porto, Porto, Portugal;

<sup>2</sup>Faculty of Health Sciences, Fernando Pessoa University, Porto, Portugal

**Abstract:** This paper highlights the importance of lipid-based colloidal carriers and their pharmaceutical implications in the delivery of peptides and proteins for oral and parenteral administration. There are several examples of biomacromolecules used nowadays in the therapeutics, which are promising candidates to be delivered by means of liposomes and lipid nanoparticles, such as solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). Several production procedures can be applied to achieve a high association efficiency between the bioactives and the carrier, depending on the physicochemical properties of both, as well as on the production procedure applied. Generally, this can lead to improved bioavailability, or in case of oral administration a more consistent temporal profile of absorption from the gastrointestinal tract. Advantages and drawbacks of such colloidal carriers are also pointed out. This article describes strategies used for formulation of peptides and proteins, methods used for assessment of association efficiency and practical considerations regarding the toxicological concerns.

**Keywords:** peptides, proteins, liposomes, solid lipid nanoparticles, SLN, nanostructured lipid carriers, NLC, oral, parenteral

## Introduction

In the last decades, several new pharmaceutically active peptides and proteins have been developed as a consequence of the progress of biotechnological techniques and genetic engineering. These new therapeutic biomolecules are usually characterized by a large size, short plasma half-life, high elimination rate (easy to be degraded by enzymatic and body fluids), limited ability to cross cell membranes, and poor bioavailability through intestinal administration. Therefore, frequent injection of drug over a long therapeutic period, are generally required when such biomolecules are used for therapeutic purposes (Hu et al 2004).

Traditional administration routes for these drugs are the oral and parenteral administration. The orally administered drug is absorbed from the gut and enters the blood stream, diffuses from the enteral absorption sites to blood and tissue, whereas a parenterally administered drug is injected directly into the blood, which is typically only possible in form of a drug solution or emulsion. After administration, the drug will be present as molecules in solution (ie, blood). It distributes in the body according to its physicochemical properties, eg, its partitioning coefficient. The presence as molecular solution turns these molecules easily accessible to degrading factors (eg, water, enzymes), and then they cannot be directed (targeted) to their desired site of action. One approach to overcome these problems would be the entrapment of those drugs into a particulate carrier system. Incorporation will protect them against degradation in vitro and in vivo, the release rate can be modified, offering as well targeting approaches. Examples of such particulate carrier systems are the polymeric nanoparticles (Speiser 1973; Kreuter 1978; Marty et al 1978; Couvreur et al 1979;

Correspondence: Eliana B Souto  
Fernando Pessoa University, Faculty  
of Health Sciences Department of  
Pharmaceutical Technology, Gab. S3 Rua  
Carlos da Maia, Nr. 296 P-4200-150 Porto  
Portugal  
Tel + 351 225 074630  
Fax ++351-225-074637  
Email eliana@ufp.pl

Luck et al 1998), microemulsions (Bangham 1993), liposomes (Bangham 1993), solid lipid nanoparticles (SLN) (Morel et al 1996; Almeida et al 1997; García-Fuentes et al 2002; Muller et al 2002a, 2006; Ugazio et al 2002; Gualbert et al 2003; Carsten et al 2004; Hu et al 2004; Garcia-Fuentes, Prego et al 2005; Garcia-Fuentes, Torres et al 2005; Schubert and Muller-Goymann 2005; Pedersen et al 2006; Souto and Muller 2006; Trotta et al 2006; Zhang et al 2006), nanostructured lipid carriers (NLC) (Muller et al 2002a; Garcia-Fuentes, Prego, et al 2005; Garcia-Fuentes, Torres, et al 2005) and self-emulsified drug delivery systems (SEDDS) (Gursoy and Benita 2004; Robert 2004; Zheng and Fulu 2006). Despite being a very smart idea, examples of such concept never reached the pharmaceutical market. It has been tried with polymeric nanoparticles, nevertheless, reasons for this are the regulatory issues and the scaling up problems. In fact, polymers used (eg, polylactic acid (PLA) and its co-polymer with glycolic acid (PLGA) (Luck et al 1998)) are accepted by the regulatory authorities for parenteral administration as implants (eg, Zoladex<sup>®</sup>) and microparticles (eg, Decapeptyl<sup>®</sup>, Parlodel LA<sup>®</sup>, Enantone Depot<sup>®</sup>), but not in the nanoparticulate form. In contrast to microparticles, the nanoparticles can be internalized (phagocytosed) by cells, intracellular degradation can cause cytotoxic effects. In addition, there is a lack of large scale production methods (Muller and Keck 2004).

Various approaches have been examined to overcome the delivery problems of peptides and proteins by means of colloidal carriers. The use of the above-mentioned lipid-based carrier systems seems to be promising.

Microemulsions are thermodynamically stable isotropic dispersions, transparent, of low viscosity, consisting of oil and water stabilized by an interfacial film of surfactant molecules, typically in conjunction with a co-surfactant. Usually, the inner phase either oil (o/w emulsions) or water (w/o emulsions) has sizes ranging from 5 to 100 nm. Microemulsions (o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides. Hydrophilic drugs of this kind can be successfully incorporated into the dispersed aqueous phase of w/o microemulsion droplets where it affords some protection against enzymatic degradation when administered orally. Sandimmune Neoral<sup>®</sup>, an example of a marketed formulation, is a microemulsion pre-concentrate containing a surfactant, lipophilic and hydrophilic solvents and ethanol. The presence of a surfactant and in some case a co-surfactant, and medium chain diacylglycerols is related in many cases, to the increase of membrane permeability, thereby increasing the drug uptake. Recently, considerable dosage form development activity has focused on the formulation of lecithin-based microemulsions.

Lecithin is a naturally occurring, non-toxic and safe material. It is also a biological surfactant and a major component of membrane lipids (Cilek et al 2005).

Lipid nanoemulsions are fine o/w dispersions, having droplets covering the size range between 50 and 200 nm. Nanoemulsions are also referred as mini-emulsions (Bouchemal et al 2004). Nanoemulsions were introduced during the 50ies for the purpose of parenteral nutrition. Fatty vegetable oils (eg, soy oil) or middle chain triacylglycerols are used as lipid phase, being typically 10%–20% of the emulsion. Further ingredients include phospholipids (stabilizers, 0.6%–1.5%) and glycerol (osmolarity regulation, 2.25%). During recent years it has been recognized that these systems might also be used as drug carriers for lipophilic drugs and several formulations are nowadays commercialized. Examples include etomidate (Etomidat-Lipuro<sup>®</sup>) and diazepam (Diazepam-Lipuro<sup>®</sup>). In comparison to previous solubilization-based formulations of these drugs, a reduction of the local and systemic side effects (eg, pain during injection) has been found. The hemolytic activity of sodium oleate is decreased in lipid emulsions because the lytic agent is restricted at the interface and in the lipophilic core, and thus the direct contact with erythrocyte membranes is hindered (Jumaa and Muller 2000). However, an important drawback related to nanoemulsions is the limited controlled release properties, due to the small size and the liquid state of the carrier. For most drugs, a rapid release will be observed. It has been estimated, that retarded drug release requires very lipophilic drugs, ie, the  $K_{o/w}$  should be larger than  $10^6:1$ . Advantages of nanoemulsions include toxicological safety and a high content of the lipid phase, as well as the possibility of large scale production by high pressure homogenization (Mehnert and Mader 2001).

Peptides and proteins associated to microemulsions and nanoemulsions are, for example, cyclosporine A (Robert 2004), immunoglobulin G (Gerhardt and Dungan 2002; Flanagan and Singh 2006), insulin (Watanasirichaikul et al 2000; Watanasirichaikul et al 2002; Cilek et al 2005, 2006),  $\alpha$ -lactalbumin (Rohloff et al 2003; Flanagan and Singh 2006) and fusion protein vaccine (Ge et al 2006).

Particular examples of nanoemulsions are the SEDDS, defined as isotropic mixtures of an oil, surfactant, co-surfactant and drug (Constantinides 1995; Gursoy and Benita 2004). These components form fine o/w nanoemulsions when introduced into aqueous media under mild agitation. The digestive motility of the stomach and intestine provides the agitation required for self-emulsification in vivo. Factors controlling the in vivo performance of SEDDS include their

ability to form small droplets of oil and the polarity of the oil droplets to promote faster drug release into the aqueous phase. The smaller oil droplets provide a large interfacial area for pancreatic lipase to hydrolyze triacylglycerols and thereby to promote the rapid release of the drug and/or formation of mixed micelles of the bile salts containing the drug. The surfactants used in these formulations are known to improve the bioavailability by various mechanisms including: (i) improved drug dissolution (Constantinides 1995), (ii) increased intestinal epithelial permeability (Swenson and Curatolo 1992), (iii) increased tight junction permeability (Lindmark et al 1995), and (iv) decreased/inhibited P-glycoprotein drug efflux (Yu et al 1999). A marketed formulation of cyclosporine (Sandimmune Neoral®), a microemulsion pre-concentrate with self-emulsifying properties, is reported to improve oral bioavailability and reduce inter- and intra-subject variability in cyclosporine pharmacokinetics. A few other studies have reported enhancement in the bioavailability of poorly soluble drugs when formulated as SEDDS (Kommuru et al 2001; Gursoy and Benita 2004). Since a relatively high concentration of surfactants is generally employed in the SEDDS formulation, toxicity of the surfactant being used should be taken into account. In fact, a compromise must be reached between the toxicity and self-emulsification ability of the surfactant that is considered for use. Currently, several formulations have been developed to produce modified emulsified formulations as alternative to conventional SEDDS. These include, for example, self-microemulsion formulations (Vondercher and Meizner 1994), surfactant dispersions (Aungustan et al 1994), pre-formulated freeze-dried emulsions (Tsuji et al 1996), microencapsulated emulsions (Jizomoto et al 1993), lipid/cross-linked polymeric matrices (Boltri et al 1997), self-emulsifiable pellets (Newton et al 2001), and solid self-emulsifying systems (self-emulsifying tablets) (Attama et al 2003). SEDDS typically produce emulsions with a droplet size between 100 and 300 nm, while self-microemulsifying drug delivery system (SMEDDS) form transparent microemulsions with a droplet size of less than 50 nm. When compared to emulsions, which are sensitive and metastable dispersed forms, SEDDS are physically stable formulations that are easy to manufacture. Thus, for lipophilic drug compounds that exhibit a dissolution rate limited absorption, these systems may offer an improvement in the rate and extent of absorption and result in more reproducible blood/time profiles (Gursoy and Benita 2004). In order to produce peptides- or proteins-loaded SEDDS, these bioactives are first formulated in a w/o microemulsion pre-concentrate, and then dispersed into an aqueous phase to

form a w/o/w microemulsion prior to administration (Zheng and Fulu 2006). The few commercial products related to these formulations are actually nonaqueous microemulsions, also known as microemulsion pre-concentrate, where the polar solvent is ethanol and not water. Upon contact with aqueous media, such as gastrointestinal fluids, the nonaqueous microemulsion spontaneously forms a fine dispersion or aqueous microemulsion (Robert 2004). Examples of peptides and proteins associated to SEDDS intended for oral administration are the cyclosporine A (Gursoy and Benita 2004; Robert 2004) and leuprolide (Zheng and Fulu 2006).

Liposomes are spherical vesicles composed of one or more phospholipid bilayers (in most cases phosphatidylcholine). Lipophilic drugs can be incorporated within the lipid bilayers while hydrophilic drugs are solubilized in the inner aqueous core (Gregoriades et al 1993). Drug release, in vivo stability, and biodistribution, are determined by the size of the vesicles, their surface charge, surface hydrophobicity, and membrane fluidity (Senior 1987). Membrane permeability can be adapted by the selection of the phospholipids composition, and by the presence of additives, such as cholesterol molecules. It is possible to avoid a rapid reticuloendothelial uptake of the liposomes by the incorporation of natural compounds (eg, gangliosides) or by the use of chemical modified polyethylene glycols (PEG's) (Gabizon et al 1994). The development of such sterically stabilized systems ('stealth liposomes') allows the development of drug targeting strategies (eg, by incorporation of specific antibodies) (Martin and Papahadjopoulos 1982). Liposomes also allow the intravenous injection of lipophilic drugs with very low water solubility, eg, amphotericin B (AmBisome®) (Janknecht et al 1992), reducing therefore the toxicity of such drugs. However, chemical and physical stability problems have been described leading to liposome aggregation and drug degradation during storage, compromising therefore the performance of liposome as intravenous drug carriers (Couvreur et al 1995; Mehnert and Mader 2001).

Nanoparticles based on solid lipids (SLN, NLC) have been proposed as an alternative colloidal drug delivery system to polymeric nanoparticles, emulsions and liposomes (Muller, Lippacher et al 2000; Muller, Mader et al 2000). They are composed of solid lipids stabilized with an emulsifying layer in an aqueous dispersion, ie, they resemble the nanoemulsions by replacing the inner liquid lipid with a solid lipid. The use of solid lipids instead of oils is a very attractive idea to achieve controlled drug release, because drug mobility in a solid lipid should be considerably lower compared with an oily phase. SLN are composed of solid lipids and show a submicron colloidal size between 50 and 1000 (Muller,

Dingler et al 1997; Muller, Weyhers et al 1997). Advantages of such carriers include the composition (physiological compounds), the fast and effective production process, including the possibility of large scale production, the avoidance of organic solvents in the production procedures, and the possibility to produce high concentrated lipid suspensions. Disadvantages include low drug loading capacities (the drug loading capacity of conventional SLN is limited because of the formation of a perfect lipid crystal matrix) (Wissing et al 2004), the presence of alternative colloidal structures (micelles, liposomes, mixed micelles, drug nanocrystals) in the aqueous dispersion, the complexity of the physical state of the lipid (transformation between different modifications, possibility of supercooled melts) which cause stability problems during storage or administration (eg, gelation, particle size increase, drug expulsion). Sample dilution or water removal might significantly change the equilibrium between the different colloidal species and the physical state of the lipid (Mehnert and Mader 2001). The solid core contains drug dissolved or dispersed in the high melting fat solid matrix. The hydrophobic chains of phospholipids are embedded in the fat matrix. Depending on the type and concentration of the lipid, 0.5 to 5% emulsifier (surfactant) is added for the physical stabilization of the system (Mehnert and Mader 2001). In particular, poloxamer 188, polysorbate 80, lecithin, polyglycerol methylgluco distearate, sodium cocoamphoacetate or saccharose fatty acid esters are very often employed.

The second generation of lipid nanoparticles has been introduced with the NLC (Luck et al 1998; Muller and Dingler 1998). These particles are prepared not from solid lipids only but from a blend of a solid lipid with a liquid lipid (oils) in such a proportion that the mixture needs to be solid at least at 40 °C. Advantages of this delivery system are the accommodation of higher amounts of drugs due to the formation of a less ordered lipid matrix with many imperfections (Muller et al 2002a, 2002b, 2004; Muller and Wissing 2003). The lipid matrix gives more flexibility for modulation of drug release, increasing the drug loading and preventing its leakage.

Similarly to polymeric nanoparticles, the solid matrix of SLN and NLC protects the incorporated drugs against chemical degradation, allowing the modulation of release profiles. Lipid nanoparticles have been tested for several administration routes (Mehnert and Mader 2001), such as oral and peroral (Yang et al 1999), pulmonary, ocular (Muller et al 2004), topical, dermal and transdermal administration (Souto et al 2004a, 2004b), as well as for gene therapy (Tabatt et al

2004), as new adjuvants for vaccines (Olbrich et al 2002), and for parenteral delivery. Lipid nanoparticles of suitable composition have been shown to be well tolerated *in vitro*, as well as after bolus injection into mice and rats (Olbrich et al 2001). Nanoparticles can accumulate in target tissues based on their natural host cell tropisms and on their biophysical properties (passive targeting). In practice, active targeting to cells other than the mononuclear phagocytic system (MPS) cells is often insufficient for rapid and specific accumulation in target tissues. Further improvement of tissue selectivity can be achieved by engineering the surface of lipid carriers with hydrophilic polymers (Goppert and Muller 2003; Göppert and Müller 2004) or coupling targeting ligands (Muller et al 2005). Lipid nanoparticles have been coated with PEG or chitosan, in order to be able to enter the Caco-2 cell monolayers (Garcia-Fuentes, Prego et al 2005).

The delivery of peptides and proteins intended for therapeutic purposes might cause special difficulties regarding their encapsulation, especially because most of them have a significant hydrophobic component and thus show a tendency to adsorb onto surfaces, such as glass and plastic. Such adsorption behavior can lead to distinct losses in the amount of macromolecule available for delivery (Duncan et al 1995). Furthermore, the amount of peptide or protein to be incorporated within the system is dependent on their physicochemical properties and on the production procedure (Hu et al 2004). The present review describes in detail the use of colloidal carriers composed of lipid materials for the delivery of peptides and proteins, particularly for oral and parenteral administration.

## State of the art in peptide and protein delivery

The convenience and acceptability of the oral route for drug administration means that it has received much attention for the delivery of macromolecules, such as peptides and proteins. It has already been shown that the nanoencapsulation of biomacromolecules in colloidal particles protects them against the harsh environment of the gastrointestinal tract, and enhances their transmucosal transport (Mathiowitz et al 1997; Tobío et al 1998). This ability of the colloidal carriers to enhance the transport of the associated macromolecules has been attributed to different mechanisms depending on the nanocarrier composition. These mechanisms are (Garcia-Fuentes, Prego et al 2005): (i) mucoadhesion, (ii) particle internalization phenomenon, and (iii) permeation enhancing effect. Various approaches have been used in an attempt to overcome these barriers and to increase the

oral bioavailability of such bioactives including the use of polymeric particulates. The encapsulation within such particulate delivery systems can protect peptides and proteins from proteolytic enzymes. Moreover, if the size of the particles is sufficiently small ( $<1\ \mu\text{m}$ ), they may pass across the intestinal mucosa and thus facilitate the absorption of the bioactive drugs from the gut lumen (Watanasirichaikul et al 2000). Hydrophobic nanoparticles are preferentially transported through the gut associated lymphoid tissue, whereas particles with a more hydrophilic nature are transported across the regular enterocytes (Garcia-Fuentes, Prego et al 2005). Under such circumstances, the material used for the preparation of the particulate carrier must be biodegradable (Watanasirichaikul et al 2000). Examples of biocompatible and physiological materials are the lipids used for the production of lipid-based colloidal carriers. Hydrophilic polymers such as polyacrylate derivatives and chitosan are examples of mucoadhesive materials used to coat these colloidal carriers. Regarding parenteral administration, macromolecules have been delivered by means of fat emulsions and liposomes (Robert 2004). In the 60ies, the first safe parenteral fat emulsion (Intralipid®) was developed by Wretling for parenteral nutrition (Muller et al 1993; Muller and Heinemann 1994a, 1994b). This was the beginning of a new delivery system for lipophilic drugs, which can be incorporated easily into the oil droplets. Other interesting parenteral carrier systems are the liposomes. Major obstacles for the development of liposomal formulations intended for parenteral drug delivery, are the limited physical stability of the dispersions, drug leakage, low activity due to no specific tumor targeting, non specific clearance by the (MPS) and difficulties in scaling up the production procedure (Arulsudar et al 2004; Tabatt et al 2004).

## Lipid-based colloidal carriers

### Liposomes

Liposomes are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer mainly composed of natural or synthetic phospholipids. Liposomes are formed when thin lipid films or lipid cakes are hydrated and stacks of liquid crystalline bilayers become fluid and swell. During stirring, hydrated lipid sheets detach and self associate to form vesicles, which prevent interaction of water with the hydrocarbon core of the bilayer at the edges. Depending on the method of preparation, lipid vesicles can be multi-, oligo- or unilamellar, containing many, a few, or one bilayer shell(s), respectively. The diameter of the lipid vesicles may vary between about 20 nm and a few

hundred micrometers (Walde and Ichikawa 2001). Small unilamellar vesicles (SUVs) are surrounded by single lipid layer (25–50 nm), whereas several lipid layers separated by intermittent aqueous layer surround large unilamellar vesicles (LUV) (100–200  $\mu\text{m}$ ). Giant unilamellar vesicles (GUV) have a mean diameter of 1–2  $\mu\text{m}$ , multilamellar vesicles (MLV) have a mean diameter between 1  $\mu\text{m}$  and 2  $\mu\text{m}$  (10 layers). Multivesicular vesicles are liposomes with lots of vesicles inside. Liposomes are characterized in terms of size, surface charge and number of bilayers (Walde and Ichikawa 2001).

Enveloped viruses, such as influenza or HIV, carry fusogenic proteins on their surface by which they enter the host cell (Torchilin et al 2001). Membrane destabilization and fusion is accomplished by a short amphiphilic sequence, the fusion peptide, located either at the top or in an internal region of the protein, which gets exposed upon receptor recognition or at acid pH upon endocytosis. Anchoring the peptide to the lipid bilayer via a myristic chain was shown to greatly enhance the fusogenic action and may provide a convenient way of incorporating the peptides into vesicular carriers.

An alternative, more cost-effective and less immunogenic approach relies on the adsorption of pH-titrable polymers to the liposomal surface. Poly-histidine and polylysine, for example, become positively charged upon lowering the pH and have been shown to destabilize and fuse negatively charged membranes *in vitro*. Polyanions, bearing carboxyl groups, are useful for destabilizing and fusing both negatively charged as well as uncharged membranes, due to their hydrophobic character at low pH. Promising pH-sensitive polyanions are, for example, poly(acrylic acid) derivatives and succinylated poly(glycerol)s. Altogether, there remains much potential to be explored with synthetic polymers and many open questions concerning their behavior in combination with liposomal carriers *in vivo* (Anne 2002). Peptides and proteins associated to liposomes are summarized in Table 1.

The methods below have been used for the encapsulation of peptides and proteins in liposomes. If natural or synthetic long-tailed phospholipids are dispersed in aqueous solution they form large liposomes, ie, multilamellar vesicles (MLV). Several methods have been developed to form unilamellar vesicles from multi-bilayers (Segota and Tezak 2006).

In the dry lipid hydration method, the vesicle-forming amphiphiles are first dissolved in an organic solvent (often chloroform) and the solvent is completely removed by rotatory evaporation and high vacuum drying in a round

bottom flask. In this way, a thin, dry lipid film is formed at the inner surface of the flask walls, to which an aqueous solution containing the peptide or protein to be entrapped is added. Vigorous shaking with the help of a vortex mixer above the phase transition temperature ( $T_m$ ) of the lipids leads to a dispersion of the lipid multi-layers in the aqueous solution, which results in the formation of a heterogeneous population of liposomes. The resulting size distribution and lamellarity of the MLV is highly heterogeneous, but sophisticated procedures have been developed to produce uniformly sized liposomes (Frkanec et al 2003; Takeuchi et al 2003; Takeuchi et al 2005; Thongborisute et al 2006; Brgles et al in press).

With the extrusion method, a controlled reduction in size and lamellarity of MLV can be achieved by using track-etch polycarbonate filters which contain almost cylindrical pores of a defined size. The MLV suspension is passed under moderate pressure repetitively (usually 10 times) through these filters above the  $T_m$  which leads to a mechanical transformation of the large vesicles into smaller ones. Usually, all the extrusion is started with filters containing relatively large pores (mean diameter, eg, 400 nm), followed by a filtration through smaller pores (200 nm and often finally 100 nm) (Torchilin et al 2001; Visser et al 2005).

With the freezing and thawing method, upon repetitively freezing the MLV suspension in liquid nitrogen (at  $-195^\circ\text{C}$ ),

**Table 1** Examples of peptides and proteins associated to liposomes, the production procedure and the association efficiency

Protein/peptide	Production procedure	Association efficiency (%)	References
Adamantyltripeptides	Dry lipid hydration	-	(Frkanec et al 2003)
Anti-ovalbumin antibodies	Dry lipid hydration	-	(Brgles et al in press)
Basic fibroblaste growth factor	Freeze-thawing extrusion	75–80	(Plum et al 2000; Luo et al 2003)
Bovine serum albumin	Reversal evaporation	25–71	(Dai et al 2005, 2006;
	Double emulsification	43–71	Murakami et al 2006)
	Freeze-thawing	20–45	
Calcitonin	Dry lipid hydration	20	(Takeuchi et al 2003, 2005;
			Thongborisute et al 2006)
Enkephalin	Double emulsification	50–85	(Ye et al 2000)
Epidermal growth factor receptor	Freeze-thawing extrusion	20–30	(Kullberg et al 2005)
Haptides	-	~100	(Gorodetsky et al 2004)
Hemoglobin	Dry lipid hydration extrusion	37–62	(Arifin and Palmer 2003;
			Patton and Palmer 2005;
			Teiji et al 2005; Xi and
			Guo, in press)
Horseadish peroxidase	Extrusion	2–5	(Visser et al 2005)
Human gamma-globulin	Dehydration-rehydration	30–31	(García-Santana et al 2006)
Insulin	Reverse phase	30–82	(Zhang et al
	evaporation		2005; Goto et al 2006)
	Freezing-thawing		
Leishmania antigen	Freeze-thawing extrusion	-	(Badiee et al 2007)
Leridistim	Double emulsification	>70	(Langston et al 2003)
Leuprolide	Dry lipid hydration	37–76	(Guo et al 2003;
	Reverse phase	33–72	Arulsudar et al 2004;
	evaporation		Shahiwala and Misra 2005;
			Carafa et al 2006)
Nerve growth factor	Reverse phase	24–34	(Xie et al 2005)
	evaporation		
Octreotide	Double emulsification	50–85	(Ye et al 2000)
Progenipoietin	Double emulsification	80–90	(Ramprasad et al 2003)
Recombinant malaria protein antigens	-	-	(Amselem et al 1992)
Superoxide dismutase	Dry lipid hydration	1–13	(Ruzica et al 2002)
	Dehydration-rehydration	2–3	
	Pro-liposome	39–65	
TAT <sup>a</sup> peptide	Extrusion	-	(Torchilin et al 2001)

<sup>a</sup>Transactivating transcriptional activator.

it is then thawed at a temperature above  $T_m$ . The vesicle suspension may undergo certain physicochemical changes thereby often equilibrating the internal and external aqueous phases of the vesicles, resulting in an increased association efficiency and possibly leading to the formation of an increased population of multivesicular vesicles (MVV) and to an elimination by means of fusion processes of very small vesicles eventually present. Typically, freezing-thawing cycles are repeated 3 to 6 times (Luo et al 2003; Kullberg et al 2005; Badiie et al 2007).

During the sonication method, MLV are treated, for a long period, with a probe type or bath type sonicator above the  $T_m$  under an inert atmosphere (usually nitrogen or argon) which leads to the defragmentation of MLV into small (sonicated) unilamellar vesicles (SUV) of diameters usually below 50 nm. The size of the SUV depends mainly on the sonication conditions and on the vesicle membrane composition (eg, cholesterol content).

In the dehydration-rehydration method, dehydrating preformed vesicles followed by a controlled rehydration above the  $T_m$  leads to the fusion of small preformed vesicles, resulting in multilamellar vesicles with high association efficiency. After rehydration, the new vesicles formed are considerably larger than the initially present SUV and MLV (García-Santana et al 2006).

In reverse-phase evaporation method, a water-immiscible organic solvent of low boiling point is used in which the vesicle-forming amphiphiles are soluble, eg, diethyl ether, isopropyl ether, or mixtures of these ethers with chloroform or methanol. The amphiphiles are first dissolved in the solvent, possibly with the help of chloroform or methanol if the solubility is too low. After adding the aqueous peptide or protein solution, the system is vortexed and briefly sonicated in a bath-type sonicator until a relatively stable (reverse) emulsion is formed. On the removal of solvent under reduced pressure, the reverse emulsion transforms into an aqueous vesicle dispersion, which contains a considerable amount of the originally present peptide or protein entrapped within the vesicles. The association efficiency obtained with this procedure is usually rather high (Xie et al 2005).

In the so-called 'pro-liposome' method, an initial mixture containing vesicle-forming amphiphiles, ethanol and water, is converted into vesicles by a simple dilution step (Walde and Ichikawa 2001; Anne 2002; Segota and Tezak 2006).

The double emulsification procedure involves the formation of a simple w/o emulsion. This first emulsion is then mixed with a second aqueous solution to form a

multiple w/o/w emulsion (Ye et al 2000; Langston et al 2003; Ramprasad et al 2003; Dai et al 2006).

## Lipid nanoparticles

### Solid lipid nanoparticles

For the location of drugs into SLN, the literature describes three different models according to Müller, Mader et al (2000) and Mehnert and Mader (2001): (i) the homogeneous matrix model, (ii) the drug-enriched shell model, and (iii) the drug-enriched core model. The morphological differences between those models depend mainly on the composition of the formulation itself, ie, the chemical nature of the bioactive, lipid, and surfactant, as well as on the production (Souto 2005). The matrix model is defined as a homogeneous lipid matrix with molecularly dispersed drug or drug being present in amorphous clusters. It is described for SLN prepared by the cold high pressure homogenization (HPH, described below) technique or when incorporating very lipophilic drugs in SLN when applying the hot HPH technique. When using the cold HPH, the bulk lipid contains the dissolved drug in molecularly dispersed form. Mechanical breaking of solid milled, drug-loaded bulk lipid by HPH leads to nanoparticles having the homogeneous matrix structure. The same will happen when the oil droplets produced by the hot HPH are cooled, crystallize, and no phase separation between lipid and drug occurs. This model is assumed for entrapped drugs that can show prolonged release from SLN (Müller, Mader et al 2000). The drug-enriched shell model is described by an outer shell enriched with drug, which covers a lipid core. This model is obtained when phase separation occurs during the cooling process from the liquid oil droplet to the formation of SLN when applying the hot HPH. The lipid precipitates first forming an almost drug-free lipid core. At the same time, the concentration of active compound in the remaining liquid lipid increases continuously. Finally, the compound-enriched shell crystallizes. Once the drug molecules are enriched in the outer shell of the particles, this model is assumed for drugs that release very fast from SLN. The drug-enriched core model is formed when the opposite mechanism as described for the former model occurs. In this case, the drug precipitates first and the lipid shell formed around this core will have distinctly less drug. This leads to a membrane-controlled release governed by Fick's law of diffusion (Müller et al 2002a). This model is formed when the drug concentration is close to its saturation solubility in the melted lipid. It has been reported that drugs can also be linked with the outer layer of SLN composed of phospholipids and steric stabilizers. Distribution of the drug depends both on

its physicochemical characteristics and on the components of the SLN. It is, however, most influenced by the partition coefficient of the drug molecules (Cavalli et al 1999; Souto and Muller 2006). Examples of peptides and proteins associated to SLN are summarized in Table 2.

The literature describes four main methods for the incorporation of peptides and proteins into SLN. The HPH can be performed at elevated temperature (hot HPH technique) or at or below room temperature (cold HPH technique) (Zara et al 1999; Fundaro et al 2000; Miglietta et al 2000; Muller et al 2002a, 2002b). The particle size is decreased by cavitation and turbulences. For the hot HPH, the lipid is heated at approx. 5–10°C above its melting point, and further added the bioactive to this melted lipid phase. This mixture is then combined with an aqueous surfactant solution heated at the same temperature. A hot pre-emulsion is formed by high speed stirring. This hot pre-emulsion is converted into a nanoemulsion when processed in a temperature controlled high pressure homogenizer, generally applying between 3 to 5 cycles at 500 bar. The obtained nanoemulsion recrystallizes upon cooling down to room temperature forming SLN (Carsten et al 2004). The cold HPH is a suitable technique for processing temperature labile drugs or hydrophilic drugs. Here, the lipid and drug are melted together and then rapidly ground under liquid nitrogen forming solid lipid microparticles. A pre-suspension is formed by high speed stirring of the particles in a cold aqueous surfactant solution. This pre-suspension is then homogenized at or below room temperature forming SLN, the homogenizing conditions are generally 5 cycles at 500 to 800 bar (Almeida et al 1997).

In the solvent diffusion method, the lipid is first dissolved in an organic phase, such as ethanol and acetone, using a water bath at 50 °C. An acidic aqueous phase is required in order to adjust the zeta potential to induce lipid coacervation, which leads to formation of SLN. The particles are then easily separated by centrifugation (Hu et al 2002).

In the w/o/w double emulsion method, the hydrophilic drug is encapsulated, along with a stabilizer to prevent drug partitioning to the external water phase during solvent evaporation, in the internal water phase of a w/o/w double emulsion (García-Fuentes et al 2002; Garcia-Fuentes, Prego et al 2005; Garcia-Fuentes, Torres et al 2005; Trotta et al 2006; Zhang et al 2006).

The group of Gasco has developed and optimized a suitable method for the preparation of SLN via microemulsions, which has been adapted and/or modified by different labs (Schwarz and Mehnert 1999; Fundaro et al 2000; Hossain et al 2000; Igartua et al 2002). Firstly, a warm microemulsion is prepared by stirring, containing typically 10% of melted solid lipid, 15% of surfactant and up to 10% of co-surfactant. This warm microemulsion is then dispersed under stirring in excess cold water at approx. 2 to 3 °C (typical ratio 1:50) using an especially developed thermostated syringe. The excess of water is removed either by ultra-filtration or by lyophilization in order to increase the particle concentration (Wissing et al 2004; Pedersen et al 2006).

### Nanostructured lipid carriers

As mentioned before, the main difference between SLN and NLC is the fact the concept of the latter is performed by

**Table 2** Examples of peptides and proteins associated to SLN, the production procedure and the association efficiency

Protein/peptide	Production procedure	Association efficiency (%)	References
Bovine serum albumin	Hot HPH <sup>a</sup> technique Microemulsion technique	~100	(Gualbert et al 2003; Schubert and Muller-Goymann 2005; Pedersen et al 2006)
Calcitonin	w/o/w double emulsion	75–90	(García-Fuentes, Prego et al 2005; García-Fuentes, Torres et al 2005)
Cyclosporine A	Microemulsion technique Hot HPH technique	6.0–13 96	(Muller et al 2002a, 2006; Ugazio et al 2002)
Gonadorelin	Solvent diffusion	50–69	(Hu et al 2004)
Human recombinant EPG <sup>b</sup>	Microemulsion technique	-	(Pedersen et al 2006)
Insulin	w/o/w double emulsion	27–68 35–45	(García-Fuentes et al 2002; Trotta et al 2006; Zhang et al 2006)
Lysozime	Cold HPH technique	~100	(Almeida et al 1997)
Streptavidin	Microemulsion technique	~100	(Pedersen et al 2006)
TAT <sup>c</sup> peptide	Hot HPH technique	-	(Carsten et al 2004)
Thymopentin	Microemulsion technique	2–5	(Morel et al 1996)

<sup>a</sup>High Pressure Homogenization; <sup>b</sup>Epidermal Growth Factor; <sup>c</sup>Transactivating Transcriptional Activator.



nanostructuring the lipid matrix, to increase the drug loading and to prevent drug expulsion. This could be realized in three ways: (i) the imperfect type, (ii) the multiple type, and (iii) the amorphous type.

Spatially different lipids, eg, acylglycerols composed of different fatty acids are mixed. Using spatially different lipids leads to larger distances between the fatty acid chains of the acylglycerols and general imperfections in the crystal and thus to more room for the accommodation of the drugs. The highest drug load could be achieved by mixing solid lipids with small amounts of liquid lipids. This model is called the imperfect type.

If higher amounts of oil are mixed with the solid lipid, a different type of nanostructure is present. Here, the solubility of the oil molecules in the solid lipid is exceeded; this leads to phase separation and the formation of oily nanocompartments within the solid lipid matrix (Jenning, Mader et al 2000; Jennings, Thunemann et al 2000; Jennings and Gohala 2001). Many drugs show a higher solubility in oils than in solid lipids so that they can be dissolved in the oil and still be protected from degradation by the surrounding solid lipids. This type of NLC is called the multiple type, and can be regarded as an analogue to w/o/w emulsions since it is an oil-in-solid lipid-in-water dispersion.

Since drug expulsion is caused by ongoing crystallization or transformation of the solid lipid, this can be minimized by the formation of a third type, the amorphous type. Here, the particles are solid but crystallization upon cooling is avoided by mixing special lipids (eg, hydroxyoctacosanylhydroxystearate and isopropylmyristate) (Jenning, Mader et al 2000; Jennings, Thunemann et al 2000; Wissing et al 2004).

The methods used to produce NLC for peptide and protein are the above described w/o/w double emulsion, and the hot HPH technique. Examples of bioactives associated to NLC for oral delivery are the calcitonin and cyclosporine A. NLC for the delivery of calcitonin have been produced by w/o/w double emulsion, resulting an association efficiency higher than 90% (Garcia-Fuentes, Prego et al 2005; Garcia-Fuentes, Torres et al 2005). NLC with cyclosporine A have been produced by hot HPH (Muller et al 2002a).

## Toxicological concerns

One can anticipate that lipid-based colloidal carriers are well tolerated in living systems because they are made of physiological compounds and therefore, metabolic pathways exist decreasing the risk of acute and chronic toxicity. Of course, the toxicity of the emulsifiers has to be considered (Mehnert and Mader 2001).

Lipid carriers prepared with several lipids and emulsifying agents did not exhibit any cytotoxic effects *in vitro* up to concentrations of 2.5% lipid (Muller et al 2005). In fact, it has been shown that even concentrations higher than 10% of lipid phase led to a viability of 80% with human granulocytes in culture (Muller et al 1996). For comparison, PLA nanoparticles showed complete cell death at 0.5%. The low toxicity of lipid nanoparticles has already been shown in human promyelotic cells (HL60) (Garcia-Fuentes, Prego et al 2005; Muller et al 2005).

It can be assumed that the cytotoxicity of the SLN can be mainly attributed to components of the aqueous phase, especially to non-ionic emulsifiers and preservatives that have eventually been used (Schubert and Muller-Goymann 2005).

Considering a future application of these systems for peptide or protein delivery, for most biomacromolecules a parenteral application is inevitable. For that reason, the cytotoxicity of liposomes and lipid nanoparticles is an essential product parameter with regard to a prospective *in vivo* tolerance evaluation in humans and/or animals. Most studies have been conducted with acylglycerols composed of fatty acids and lecithins, which are mostly accepted as safe. Good tolerability depends in the first line on the used surfactant and secondarily on the lipid. To formulate parental lipid-based carriers, surfactant of GRAS status must be employed, eg, lecithin, Tween 80, Poloxamer 188, Span 85, and sodium glycocholate. When performing bolus injections into mice good tolerability was found for most of these surfactants coating SLN (Wissing et al 2004). As described, for cetyl palmitate SLN with different surfactants no acute toxicity and no increase in liver and spleen weight was observed. After autopsy and histopathology no significant evidence was documented that SLN were acute toxic to tested animals (Wissing et al 2004).

No problems should be observed for peroral or transdermal administration and intramuscular or subcutaneous injection if appropriate surfactants are used. The particle size is not a very critical issue for these administration routes, because a low content of microparticles might decrease the performance of the colloidal system, but will not cause toxic events. Size is relevant if such formulations are intended for intravenous administration. In this case toxicological problems may arise if size is not controlled. Injections of highly polydispersed particle dispersions can cause embolism due to the risk of particle aggregation during injection. When particle dispersions are intended for intravenous administration production of monodispersed formulations

must be guaranteed. Moreover, colloidal carriers can be coated with hydrophilic polymers or albumin to actively target the incorporated drugs to specific cells, tumors and/or organs. This strategy can be successfully applied to concentrate drugs in such target tissues minimizing systemic side effects and therefore toxicity. The absence of pyrogens must be checked for parenteral administration. Problems may arise, because colloidal carriers may interfere with the pyrogens causing gelation of the suspension, which could result in embolism.

## Conclusions

In the future we can expect an increasing number of therapeutic molecules such as proteins, oligonucleotides and DNA as vaccine or as drug for gene therapy. The employment of lipid-based carriers has been found to be suitable for the administration of peptides and proteins through different routes.

Liposomes have been used successfully in the field of biology, biochemistry and medicine since its origin (Bangham et al 1965). It exhibits a number of advantages in terms of amphiphilic character. Biocompatibility, and ease of surface modification rendering it a suitable candidate delivery system for peptides and proteins molecules.

Once lipid nanoparticles are a new and innovative therapeutic delivery system, we can expect in the future an increasing number of contributions describing delivery of recombinant proteins (Wissing et al 2004). Lipid nanoparticles, particularly, NLC are a promising approach for the formulation of peptides and proteins with poor aqueous solubility. Since nearly 40% of the new drug compounds are hydrophobic, it appears that more drug products will be formulated as NLC for the pharmaceutical market in the very near future.

## References

- Almeida AJ, Runge S, Muller RH. 1997. Peptide loaded solid lipid nanoparticles (SLN): influence of production parameters. *Int J Pharm*, 149:255–65.
- Anselem S, Domb AJ, Alving CR. 1992. Lipospheres as a vaccine carrier system: effects of size, charge, and phospholipid composition. *Vaccine*, 1:383–95.
- Anne SU. 2002. Biophysical Aspects of Using Liposomes as Delivery Vehicles. *Bioscience Reports*, 22:129–50.
- Arifin DR, Palmer AF. 2003. Determination of size distribution and encapsulation efficiency of liposome-encapsulated hemoglobin blood substitutes using asymmetric flow field-flow fractionation coupled with multi-angle static light scattering. *Biotechnol Prog*, 19:1798–811.
- Arulsudar N, Subramanian N, Mishra P, et al. 2004. Preparation, characterization, and biodistribution study of technetium-99m-labeled leuprolide acetate-loaded liposomes in Ehrlich ascites tumor-bearing mice. *AAPS PharmSci*, 6 (1) Article 5.
- Attama AA, Nzekwe IT, Nnamani PO, et al. 2003. The use of solid self-emulsifying systems in the delivery of diclofenac. *Int J Pharm*, 262:23–8.
- Aungustan BJ, Nguyen N, Rogers NJ, et al. 1994. Improved oral bioavailability of an HIV protease inhibitor using Gelucire 44/14 and Labrasol vehicles. *B T Gattefosse*, 87:49–54.
- Badiee A, Jaafari MR, Khamesipour A. 2007. Leishmania major: Immune response in BALB/c mice immunized with stress-inducible protein 1 encapsulated in liposomes. *Exp Parasitol*, 115:127–34.
- Bangham AD. 1993. Liposomes: the Babraham connection. *Chem Phys Lipids*, 64:275–85.
- Bangham AD, Standish MM, Watkins JC. 1965. The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J Mol Biol*, 13:253–9.
- Boltri L, Coceani N, De-Curto D, et al. 1997. Enhanced and modification of etoposide release from crospovidone particles loaded with oil-surfactant blends. *Pharm Dev Technol*, 2:373–81.
- Bouchemal K, Briancon S, Perrier E, et al. 2004. Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. *Int J Pharm*, 280:241–51.
- Brgles M, Miroslavljevic K, Noethig-Laslo V, et al. Spin-labelling study of interactions of ovalbumin with multilamellar liposomes and specific anti-ovalbumin antibodies. *Int J Biol Macromol*, In Press.
- Carafa M, Marianucci C, Annibaldi V, et al. 2006. Novel O-palmitoylscleroglucan-coated liposomes as drug carriers: Development, characterization and interaction with leuprolide. *Int J Pharm*, 325:155–62.
- Carsten R, Ulrike S, Aurora O, et al. 2004. Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo. *Pharm Res*, 21:1662–9.
- Cavalli R, Bocca C, Miglietta A, et al. 1999. Albumin adsorption on stealth and non-stealth solid lipid nanoparticles. *STP Pharma Sci*, 9:183–9.
- Cilek A, Celebi N, Tirnaksiz F. 2006. Lecithin-based microemulsion of a peptide for oral administration: preparation, characterization, and physical stability of the formulation. *Drug Deliv*, 13:19–24.
- Cilek A, Celebi N, Tirnaksiz F, et al. 2005. A lecithin-based microemulsion of rh-insulin with aprotinin for oral administration: Investigation of hypoglycemic effects in non-diabetic and STZ-induced diabetic rats. *Int J Pharm*, 298:176–85.
- Constantinides P. 1995. Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. *Pharm Res*, 12:1561–72.
- Couvreur P, Dubernet C, Puisieux F. 1995. Controlled drug delivery with nanoparticles: current possibilities and future trends. *Eur J Pharm Biopharm*, 41:2–13.
- Couvreur P, Kante B, Roland M, et al. 1979. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. *J Pharm Pharmacol*, 31:331–2.
- Dai C, Wang B, Zhao H, et al. 2005. Factors affecting protein release from microcapsule prepared by liposome in alginate. *Colloid Surface B*, 42:253–8.
- Dai C, Wang B, Zhao H, et al. 2006. Preparation and characterization of liposomes-in-alginate (LIA) for protein delivery system. *Colloid Surface B*, 47:205–10.
- Duncan MR, Lee JM, Warchol MP. 1995. Influence of surfactants upon protein/peptide adsorption to glass and polypropylene. *Int J Pharm*, 120:179–8.
- Flanagan J, Singh H. 2006. Microemulsions: A potential delivery system for bioactives in food. *Crit Rev Food Sci*, 46:221–37.
- Frkanec R, Noethig-Laslo V, Vranesic B, et al. 2003. A spin labeling study of immunomodulating peptidoglycan monomer and adamantyltripeptides entrapped into liposomes. *Biochimica et Biophysica Acta (BBA) – Biomembranes*, 1611:187–96.
- Fundaro A, Cavalli R, Bargoni A, et al. 2000. Non-stealth and stealth solid lipid nanoparticles (SLN) carrying doxorubicin: pharmacokinetics and tissue distribution after i.v. administration to rats. *Pharmacol Res*, 42:337–43.

- Gabizon A, Catane R, Uziely B, et al. 1994. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res*, 54:987–92.
- Garcia-Fuentes M, Prego C, Torres D, et al. 2005. A comparative study of the potential of solid triglyceride nanostructures coated with chitosan or poly(ethylene glycol) as carriers for oral calcitonin delivery. *Eur J Pharm Sci*, 25:133–43.
- Garcia-Fuentes M, Torres D, Alonso MJ. 2005. New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin. *Int J Pharm*, 296:122–32.
- García-Fuentes M, Torres D, Alonso MJ. 2002. Design of lipid nanoparticles for the oral delivery of hydrophilic macromolecules. *Colloid Surface B*, 27:159–68.
- García-Santana MA, Duconge J, Sarmiento ME, et al. 2006. Biodistribution of liposome-entrapped human gamma-globulin. *Biopharm Drug Dispos*, 27:275–83.
- Ge W, Sui Y-F, Wu D-C, et al. 2006. MAGE-1/Heat shock protein 70/MAGE-3 fusion protein vaccine in nanoemulsion enhances cellular and humoral immune responses to MAGE-1 or MAGE-3 in vivo. *Cancer Immunol Immunother*, 55:841–9.
- Gerhardt NI, Dungan SR. 2002. Time-dependent solubilization of IgG in AOT-brine-isooctane microemulsions: Role of clusters formation. *Biotechnol Bioeng*, 78:60–72.
- Goppert TM, Muller RH. 2003. Plasma protein adsorption of tween 80- and poloxamer 188-stabilized solid lipid nanoparticles. *J Drug Targ*, 11:225–31.
- Göppert TM, Müller RH. 2004. Alternative sample preparation prior to two-dimensional electrophoresis protein analysis on solid lipid nanoparticles. *Electrophoresis*, 25:134–40.
- Gorodetsky R, Levdansky L, Vexler A, et al. 2004. Liposome transduction into cells enhanced by haptotactic peptides (Haptides) homologous to fibrinogen C-termini. *J Control Release*, 95:477–88.
- Goto T, Morishita M, Nishimura K, et al. 2006. Novel mucosal insulin delivery systems based on fusogenic liposomes. *Pharm Res*, 23:384–91.
- Gregoriades G, Florence AT, Patel HM. 1993. Liposomes in drug delivery. Amsterdam: Harwood Academic Publishers.
- Gualbert J, Shahgaldian P, Coleman AW. 2003. Interactions of amphiphilic calix[4]arene-based solid lipid nanoparticles with bovine serum albumin. *Int J Pharm*, 257:69–73.
- Guo J, Ping Q, Jiang G, et al. 2003. Chitosan-coated liposomes: characterization and interaction with leuprolide. *Int J Pharm*, 260:167–73.
- Gursoy RN, Benita S. 2004. Self-emulsifying drug delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. *Biomed Pharmacother*, 58:173–82.
- Hossain MA, Maesaki S, Razzaque MS, et al. 2000. Attenuation of nephrotoxicity by a novel lipid nanosphere (NS-718) incorporating amphotericin B. *J Antimicrob Chemother*, 46:263–8.
- Hu FQ, Hong Y, Yuan H. 2004. Preparation and characterization of solid lipid nanoparticles containing peptide. *Int J Pharm*, 273:29–35.
- Hu FQ, Yuan H, Zhang HH, et al. 2002. Preparation of solid lipid nanoparticles with clobetasol propionate by a novel solvent diffusion method in aqueous system and physicochemical characterization. *Int J Pharm*, 239:121–8.
- Igartua M, Saulnier P, Heurtault B, et al. 2002. Development and characterization of solid lipid nanoparticles loaded with magnetite. *Int J Pharm*, 233:149–57.
- Janknecht R, Marie SD, Bakker-Woudenberg IAJM, et al. 1992. Liposomal and lipid formulations of amphotericin B. *Clin Pharmacokinetic*, 23:279–91.
- Jenning V, Gohala S. 2001. Encapsulation of retinoids in solid lipid nanoparticles (SLN). *J Microencapsul*, 18:149–58.
- Jenning V, Mader K, Gohla SH. 2000. Solid lipid nanoparticles (SLN) based on binary mixtures of liquid and solid lipids: a <sup>1</sup>H-NMR study. *Int J Pharm*, 205:15–21.
- Jenning V, Thunemann AF, Gohla SH. 2000. Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids. *Int J Pharm*, 199:167–77.
- Jizomoto H, Kanoaka E, Sugita H, et al. 1993. Gelatin-acacia microcapsules for trapping micro oil droplets containing lipophilic drugs and ready disintegration in the gastrointestinal tract. *Pharm Res*, 10:1115–22.
- Jumaa M, Muller BW. 2000. Lipid emulsions as a novel system to reduce the hemolytic activity of lytic agents: mechanism of the protective effect. *Eur J Pharm Sci*, 9:285–90.
- Kommuru TR, Gurley B, Khan MA, et al. 2001. Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q10: formulation development and bioavailability assessment. *Int J Pharm*, 212:233–46.
- Kreuter J. 1978. Nanoparticles and nanocapsules – new dosage forms in the nanometer size range. *Pharm Acta Helv*, 53:33–9.
- Kullberg E, Wei Q, Capala J, et al. 2005. EGF-receptor targeted liposomes with boronated acridine: Growth inhibition of cultured glioma cells after neutron irradiation. *Int J Radiat Biol*, 81:621–9.
- Langston MV, Ramprasad MP, Kararli TT, et al. 2003. Modulation of the sustained delivery of myelopietin (Leridistim) encapsulated in multivesicular liposomes (DepoFoam). *J Control Release*, 89:87–99.
- Lindmark T, Nikkila T, Artursson P. 1995. Mechanisms of absorption enhancement by medium chain fatty acids in intestinal epithelial Caco-2 monolayers. *J Pharmacol Exp Ther*, 275:958–64.
- Luck M, Pistel KF, Li YX, et al. 1998. Plasma protein adsorption on biodegradable microspheres consisting of poly(-lactide-co-glycolide), poly(-lactide) or ABA triblock copolymers containing poly(oxyethylene) Influence of production method and polymer composition. *J Control Release*, 55:107–20.
- Luo X, Belcastro R, Cabacungan J, et al. 2003. Transfection of lung cells in vitro and in vivo: effect of antioxidants and intraliposomal bFGF. *Am J Physiol – Lung C*, 284:L817–L25.
- Martin F, Papahadjopoulos D. 1982. Irreversible coupling of immunoglobulin fragments to performed vesicles. *J Biol Chem*, 257:286–8.
- Marty JJ, Oppenheim RC, Speiser P. 1978. Nanoparticles – a new colloidal drug delivery system. *Pharm Acta Helv*, 53:17–23.
- Mathiowitz E, Jacob JS, Jong YS, et al. 1997. Biologically erodable microspheres as potential oral drug delivery systems. *Nature*, 386:410–4.
- Mehnert W, Mader K. 2001. Solid lipid nanoparticles: Production, characterization and applications. *Adv Drug Deliv Rev*, 47:165–96.
- Miglietta A, Cavalli R, Bocca C, et al. 2000. Cellular uptake and cytotoxicity of solid lipid nanospheres (SLN) incorporating doxorubicin or paclitaxel. *Int J Pharm*, 210:61–7.
- Morel S, Ugazio E, Cavalli R, et al. 1996. Thymopentin in solid lipid nanoparticles. *Int J Pharm*, 132:259–61.
- Muller RH, Dingler A. 1998. The next generation after the liposomes: solid lipid nanoparticles (SLN<sup>TM</sup>) as dermal carrier in cosmetics. *Eurocosmetics*, 7/8:19–26.
- Muller RH, Dingler A, Weyhers H, et al. 1997. Solid lipid nanoparticles – ein neuartiger Wirkstoff-carrier für kosmetika und Pharmazeutika. 3. Mitteilung: Langzeitstabilität, Gerfrier- und Toxizität, Anwendung in Kosmetika und Pharmazeutika. *Pharm Ind*, 59:614–9.
- Muller RH, Heinemann S. 1994a. Fat emulsions for parenteral nutrition. III. Lipofundin MCT regimens for total parenteral nutrition (TPN) with high electrolyte load. *Int J Pharm*, 101:175–89.
- Muller RH, Heinemann S. 1994b. Fat emulsions for parenteral nutrition. IV. Lipofundin MCT regimens for total parenteral nutrition (TPN) with high electrolyte load. *Int J Pharm*, 107:121–32.
- Muller RH, Keck CM. 2004. Challenges and solutions for the delivery of biotech drugs – a review of drug nanocrystal technology and lipid nanoparticles. *J Biotechnol*, 113:151–70.
- Muller RH, Lippacher A, Gohala S. 2000. Solid lipid nanoparticles (SLN) as carrier system for the controlled release of drugs. Handbook of pharmaceutical controlled release technology. Marcel Dekker. p 377–91
- Muller RH, Maaen S, Weyhers H, et al. 1996. Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles. *Int J Pharm*, 138:85–94.

- Muller RH, Mader K, Gohla S. 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art. *Eur J Pharm Biopharm*, 50:161–77.
- Muller RH, Radtke M, Wissing SA. 2002a. Nanostructured lipid matrices for improved microencapsulation of drugs. *Int J Pharm*, 242:121–8.
- Muller RH, Radtke M, Wissing SA. 2002b. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv Drug Deliv Rev*, 54:S131–55.
- Muller RH, Radtke M, Wissing SA. 2004. Solid lipid nanoparticles and nanostructured lipid carriers. *Encyclopedia of nanoscience and nanotechnology*. American Scientific Publishers. p 43–56
- Muller RH, Runge S, Ravelli V, et al. 2006. Oral bioavailability of cyclosporine: Solid lipid nanoparticles (SLN(R)) versus drug nanocrystals. *Int J Pharm*, 317:82–9.
- Muller RH, Schwarz C, Mehnert W, et al. 1993. Production of solid lipid nanoparticles (SLN) for controlled drug delivery. *Proc Int Symp Control Rel Bioact Mater*, 20:480–1.
- Muller RH, Souto EB, Goppert T, et al. 2005. Production of biofunctionalized solid lipid nanoparticles for site-specific drug delivery. In: *Nanotechnologies for the life sciences biological and pharmaceutical nanomaterials*. p 291–307
- Muller RH, Weyhers H, Muhlen AZ, et al. 1997. Solid lipid nanoparticles (SLN)-ein neuartiger Wirkstoff-Carrier fur Kosmetika und Pharmazeutika: I. Systemeigenschaften, Herstellung and Scaling up. *Pharm Ind*, 59:423–7.
- Muller RH, Wissing SA. 2003. Lipopearls for topical delivery of active compounds and controlled release. Modified-release drug delivery systems. New York: Marcel Dekker Inc.
- Murakami S, Ono T, Sakai S, et al. 2006. Effect of Diglucosamine on the Entrapment of Protein into Liposomes. *J Lipos Res*, 16:103–12.
- Newton M, Peterson J, Podzcek F, et al. 2001. The influence of formulation variables on the properties of pellets containing a self-emulsifying mixture. *J Pharm Sci*, 90:987–95.
- Olbrich C, Bakowsky U, Lehr C-M, et al. 2001. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. *J Control Release*, 77:345–55.
- Olbrich C, Muller RM, Tabatt K, et al. 2002. Stable biocompatible adjuvants – a new type of adjuvant based on solid lipid nanoparticles: study on cytotoxicity, compatibility and efficacy in chicken. *Alter Lab Anim*, 30:443–58.
- Patton JN, Palmer AF. 2005. Photopolymerization of bovine hemoglobin entrapped nanoscale hydrogel particles within liposomal reactors for use as an artificial blood substitute. *Biomacromolecules*, 6:414–24.
- Pedersen N, Hansen S, Heydenreich AV, et al. 2006. Solid lipid nanoparticles can effectively bind DNA, streptavidin and biotinylated ligands. *Eur J Pharm Biopharm*, 62:155–62.
- Plum SM, Holaday JW, Ruiz A, et al. 2000. Administration of a liposomal FGF-2 peptide vaccine leads to abrogation of FGF-2-mediated angiogenesis and tumor development. *Vaccine*, 19:1294–303.
- Ramprasad MP, Amini A, Kararli T, et al. 2003. The sustained granulopoietic effect of progenipoietin encapsulated in multivesicular liposomes. *Int J Pharm*, 261:93–103.
- Robert GS. 2004. Solubilizing excipients in oral and injectable formulations. *Pharm Res*, 21:201–30.
- Rohloff CM, Shimek JW, Dungan SR. 2003. Effect of added alpha-lactalbumin protein on the phase behavior of AOT-brine-isooctane systems. *J Colloid Interface Sci*, 261:514–23.
- Ruzica GR, Karmela B, Zeljka P, et al. 2002. High efficiency entrapment of superoxide dismutase into mucoadhesive chitosan-coated liposomes. *Eur J Pharm Sci*, 15:441–8.
- Schubert MA, Muller-Goymann CC. 2005. Characterisation of surface-modified solid lipid nanoparticles (SLN): Influence of lecithin and nonionic emulsifier. *Eur J Pharm Biopharm*, 61:77–86.
- Schwarz C, Mehnert W. 1999. Solid lipid nanoparticles (SLN) for controlled drug delivery. II. Drug incorporation and physicochemical characterization. *J Microencapsul*, 16:205–13.
- Segota S, Tezak Du-i. 2006. Spontaneous formation of vesicles. *Adv Colloid Interfac*, 121:51–75.
- Senior JH. 1987. A review of controlling factors. *Crit Rev Ther Drug Carrier Syst*, 3:123–93.
- Shahiwal A, Misra A. 2005. A preliminary pharmacokinetic study of liposomal leuprolide dry powder inhaler: a technical note. *AAPS PharmSciTech*, 6:E482–6.
- Souto EB. 2005. SLN and NLC for topical delivery of antifungals. PhD Thesis. Freie Universitat Berlin, Berlin.
- Souto EB, Muller RH. 2006. Lipid nanoparticles (SLN and NLC) for drug delivery. nanoparticles for pharmaceutical applications. Berlin: American Scientific Publishers. p 103–22
- Souto EB, Wissing SA, Barbosa CM, et al. 2004. Development of a controlled release formulation based on SLN and NLC for topical clotrimazole delivery. *Int J Pharm*, 278:71–7.
- Souto EB, Wissing SA, Barbosa CM, et al. 2004. Evaluation of the physical stability of SLN and NLC before and after incorporation into hydrogel formulations. *Eur J Pharm Biopharm*, 58:83–90.
- Speiser P. 1973. New drug formulations as the result of recent technological development efforts. *Act Pharm Suec*, 10:381–400.
- Swenson ES, Curatolo WJ. 1992. Means to enhance penetration. *Adv Drug Deliv Rev*, 8:39–42.
- Tabatt K, Kneuer C, Sameti M, et al. 2004. Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes. *J Control Release*, 97:321–32.
- Takeuchi H, Matsui Y, Sugihara H, et al. 2005. Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs. *Int J Pharm*, 303:160–70.
- Takeuchi H, Matsui Y, Yamamoto H, et al. 2003. Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. *J Control Release*, 86:235–42.
- Teiji O, Tetsuhiro K, Yoshitaka O, et al. 2005. Hemodilution with liposome-encapsulated low-oxygen-affinity hemoglobin does not attenuate hypothermic cerebral ischemia in rats. *Journal of Artificial Organs*, 8:263–9.
- Thongborisute J, Tsuruta A, Kawabata Y, et al. 2006. The effect of particle structure of chitosan-coated liposomes and type of chitosan on oral delivery of calcitonin. *J Drug Targ*, 14:147–54.
- Tobio M, Gref R, Sánchez A, et al. 1998. Stealth PLA-PEG Nanoparticles as Protein Carriers for Nasal Administration. *Pharm Res*, 15:270–5.
- Torchilin VP, Rammohan R, Weissig V, et al. 2001. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *PNAS*, 98:8786–91.
- Trotta M, Cavalli R, Gallarate M, et al. 2006. Microencapsulation of insulin into lipid carriers. Proc 5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology.
- Tsuji Y, Kakegawa H, Myataka H, et al. 1996. Pharmaceutical properties of freeze-dried formulations of egg albumin, several drugs and olive oil. *Biol Pharm Bull*, 19:636–40.
- Ugazio E, Cavalli R, Gasco MR. 2002. Incorporation of cyclosporin A in solid lipid nanoparticles (SLN). *Int J Pharm*, 241:341–4.
- Visser CC, Stevanovic S, Voorwinden LH, et al. 2005. Targeting liposomes with protein drugs to the blood-brain barrier in vitro. *Eur J Pharm Sci*, 25:299–305.
- Vondercher J, Meizner A. 1994. Rationale for the development of Sandimmune oral. *Transplant Proc*, 26:2925–7.
- Walde P, Ichikawa S. 2001. Enzymes inside lipid vesicles: preparation, reactivity and applications. *Biomol Eng*, 18:143–77.
- Watnasirichaikul S, Davies NM, Rades T, et al. 2000. Preparation of biodegradable insulin nanocapsules from biocompatible microemulsions. *Pharm Res*, 17:6:684–9.
- Watnasirichaikul S, Rades T, Tucker IG, et al. 2002. Effects of formulation variables on characteristics of poly (ethylcyanoacrylate) nanocapsules prepared from w/o microemulsions. *Int J Pharm*, 235:237–46.

- Wissing SA, Kayser O, Muller RH. 2004. Solid lipid nanoparticles for parenteral drug delivery. *Adv Drug Deliv Rev*, 56:1257–72.
- Xi J, Guo R. . . Interactions between flavonoids and hemoglobin in lecithin liposomes. *Int J Biol Macromol*, In Press.
- Xie Y, Ye L, Zhang X, et al. 2005. Transport of nerve growth factor encapsulated into liposomes across the blood-brain barrier: In vitro and in vivo studies. *J Control Release*, 105:106–19.
- Yang S, Zhu J, Lu Y, et al. 1999. Body Distribution of camptothecin solid lipid nanoparticles after oral administration. *Pharm Res*, 16:751–7.
- Ye Q, Asherman J, Stevenson M, et al. 2000. DepoFoam(TM) technology: a vehicle for controlled delivery of protein and peptide drugs. *J Control Release*, 64:155–66.
- Yu L, Bridgers A, Polli J, et al. 1999. Vitamin E-TPGS increases absorption flux of an HIV protease inhibitor by enhancing its solubility and permeability. *Pharm Res*, 16:1812–17.
- Zara GP, Cavalli R, Fundaro A, et al. 1999. Pharmacokinetics of doxorubicin incorporated in solid lipid nanospheres (SLN). *Pharmacol Res*, 40:281–6.
- Zhang N, Ping Q, Huang G, et al. 2006. Lectin-modified solid lipid nanoparticles as carriers for oral administration of insulin. *Int J Pharm*, 327:153–9.
- Zhang N, Ping QN, Huang GH, et al. 2005. Investigation of lectin-modified insulin liposomes as carriers for oral administration. *Int J Pharm*, 294:247–59.
- Zheng JY, Fulu M-Y. 2006. Decrease of genital organ weights and plasma testosterone levels in rats following oral administration of leuprolide microemulsion. *Int J Pharm*, 307:209–15.

