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

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6.1 INTRODUCTION

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

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

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

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

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

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

6.2 INFLUENCE OF THE PARTICLE SIZE

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

6.2.1 On Cytotoxicity

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

TABLE 6.1
Photon Correlation Spectroscopy Diameters of Solid Lipid Nanoparticles

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

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

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

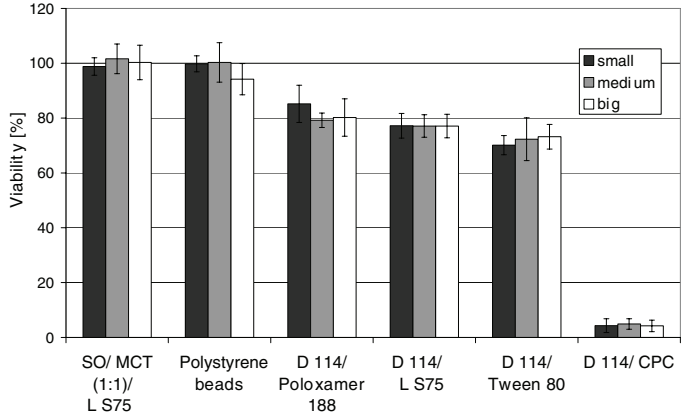

FIGURE 6.1 Viability (MTT assay) of mouse peritoneal macrophages after 20 h of incubation with SLN, polystyrene particles, or control emulsion (1:1 mixture of soybean oil and MCT stabilized with Lipoid S75) of different sizes at a concentration of 0.1% (means ± standard deviation, n = 3). The viability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

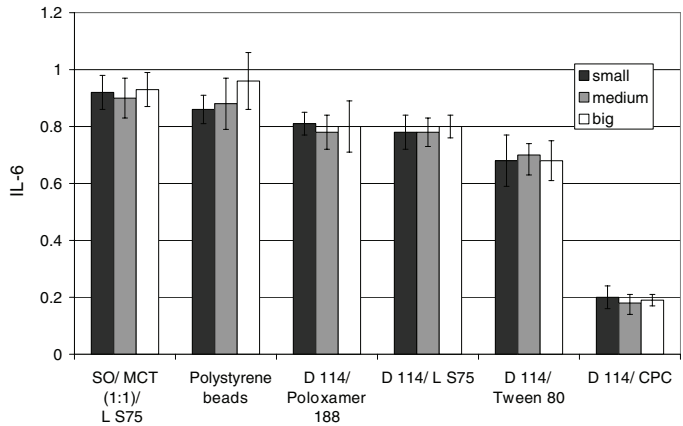

FIGURE 6.2 IL-6 secretion of mouse peritoneal macrophages after 20 h of incubation with SLN, polystyrene particles, or control emulsion (1:1 mixture of soybean oil and MCT stabilized with Lipoid S75) of different sizes at a concentration of 0.1% (means ± standard deviation, n = 3). The viability of untreated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

6.2.2 On Biodegradation

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

TABLE 6.2
Photon Correlation Spectroscopy Diameters of Solid Lipid Nanoparticles

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

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

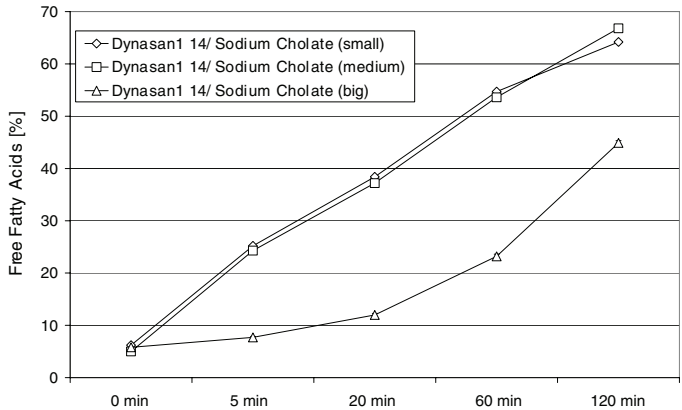

FIGURE 6.3 Degradation profile of sodium cholate–stabilized Dynasan 114 SLN of different sizes. The percentage of free fatty acids after complete hydrolysis is 66%.

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

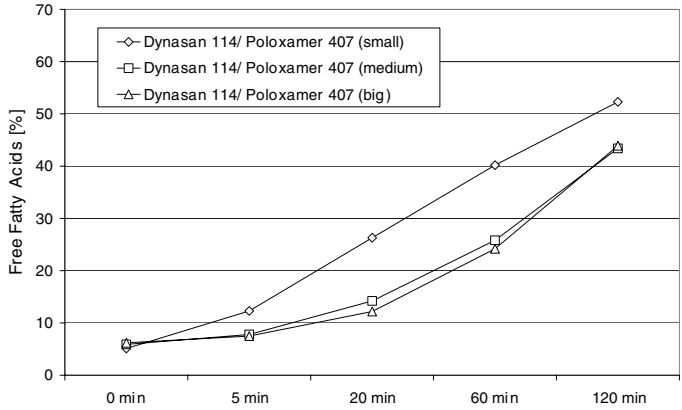

FIGURE 6.4 Degradation profile of poloxamer 407–stabilized Dynasan 114 SLN of different sizes. The percentage of free fatty acids after complete hydrolysis is 66%.

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

6.3 INFLUENCE OF THE MATRIX LIPID

6.3.1 On Cytotoxicity

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

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

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

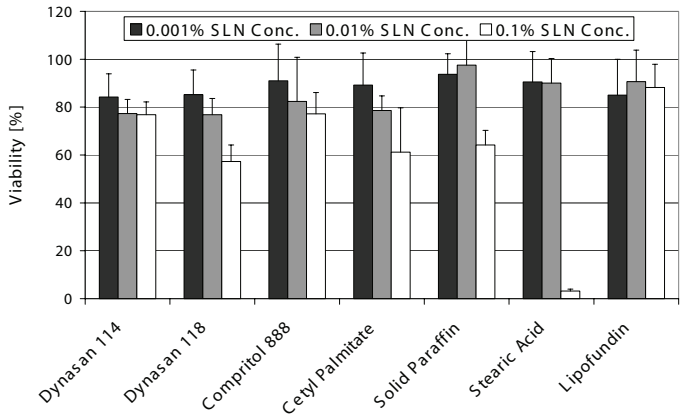

FIGURE 6.5 Viability of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different lipid matrices and poloxamer 188) or Lipofundin MCT in different concentrations (means ± standard deviation, n = 3, untreated cells have a viability of 100%).

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

6.3.2 On Biodegradation

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

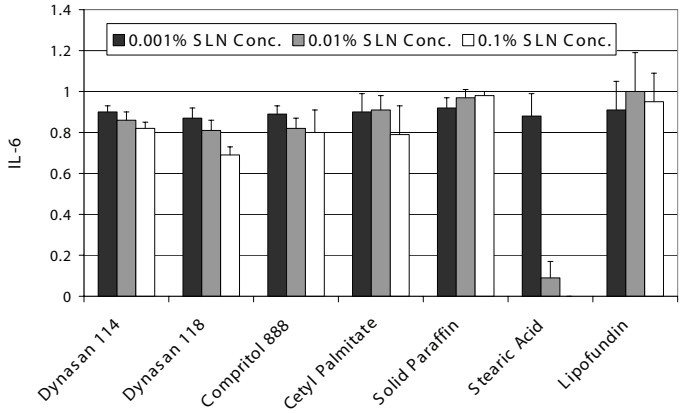

FIGURE 6.6 IL-6 production of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different lipid matrices and poloxamer 188) or Lipofundin MCT in different concentrations (means ± standard deviation, n = 3, untreated cells have a IL-6 secretion of 1).

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

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

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

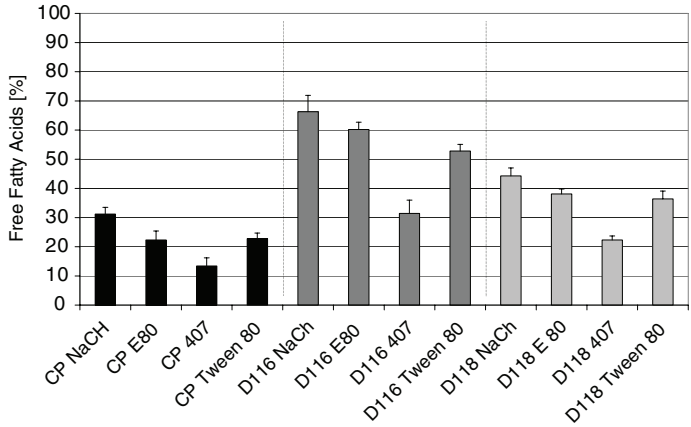

FIGURE 6.7 Lipase/colipase degradation of SLN with different matrix lipids: CP, cetyl palmitate; D116, Dynasan 116; D118, Dynasan 118; NaCh, sodium cholate; E80, Lipoid E80; 407, poloxamer 407; Tween 80, polysorbate 80. The lipid concentration was 5% and the surfactant concentration 0.5%. The values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

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

6.3.3 On *In Vitro* Transfection Efficacy

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

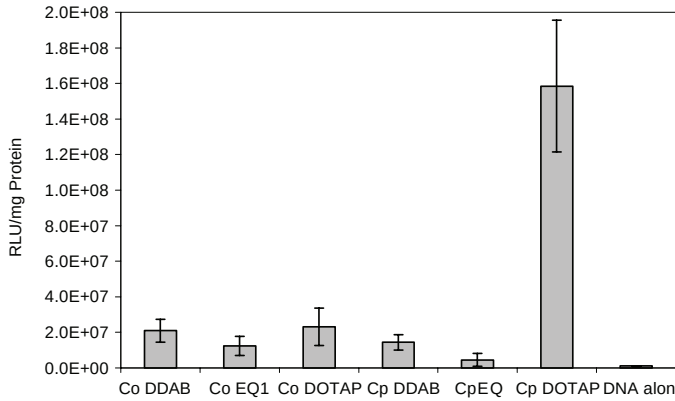

FIGURE 6.8 Transfection efficiency (quantified as "relative light units" [RLU]/mg protein) of six different SLN formulations (Co DDAB: 4% Compritol ATO 888, 1% dimethyldioctadecylammonium bromide = DDAB; Co EQ: 4% Compritol, 1% N,N-di-(β-stearoylethyl)-N,N-dimethyl-ammonium chloride = EQ; Co DOTAP: 4%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

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

6.4 INFLUENCE OF THE SURFACTANT

6.4.1 On Cytotoxicity

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

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

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

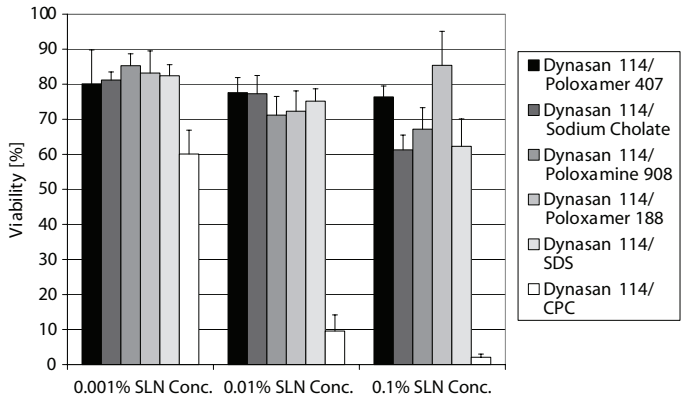

FIGURE 6.9 Viability of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different surfactants and Dynasan 114) in different concentrations (means ± standard deviation, n = 3, untreated cells have a viability of 100%).

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

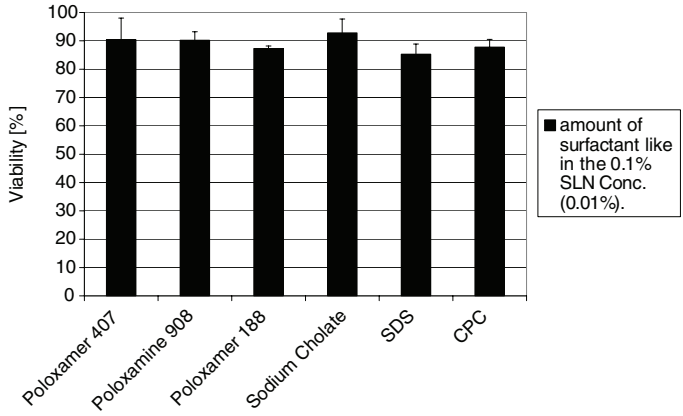

FIGURE 6.10 Viability of peritoneal macrophages of the mouse after 20 h of incubation with different surfactant solutions (0.01%). This corresponds to the surfactants present in the 0.1% formulation (means ± standard deviation, n = 3, untreated cells have a viability of 100%).

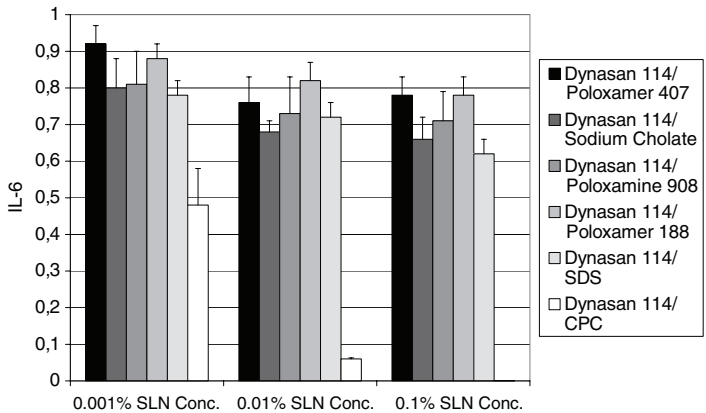

FIGURE 6.11 IL-6 production of peritoneal macrophages of the mouse after 20 h of incubation with SLN (different surfactants and Dynasan 114) in different concentrations (means ± standard deviation, n = 3, untreated cells have an IL-6 secretion of 1).

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

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

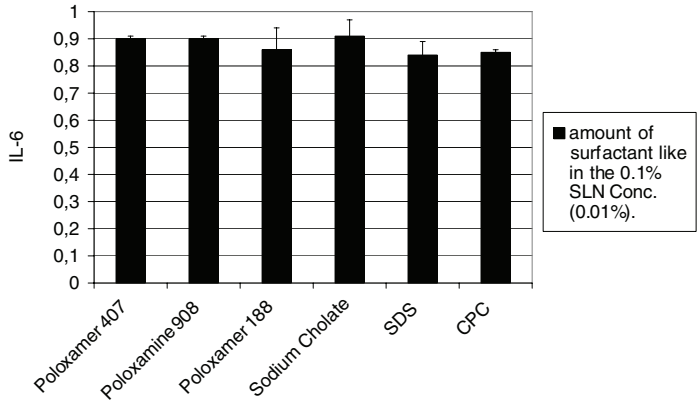

FIGURE 6.12 IL-6 production of peritoneal macrophages of the mouse after 20 h of incubation with different surfactant solutions (0.01%). This corresponds to the surfactants present in the 0.1% formulation (means ± standard deviation, n = 3, untreated cells have an IL-6 secretion of 1).

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

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

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

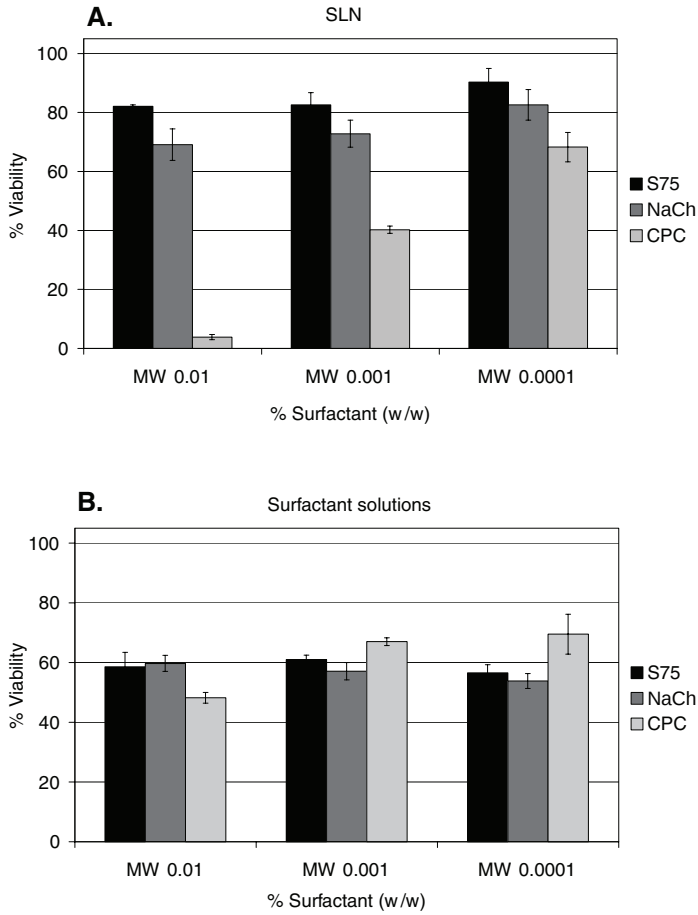

FIGURE 6.13 **(A)** Viability of RAW 264.7 macrophages treated with different SLN formulations compared with cells treated with pure medium (100% viability). Viability is related to the total surfactant concentration in the cell culture medium and was quantified by an MTT assay. All SLN contained 10%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

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

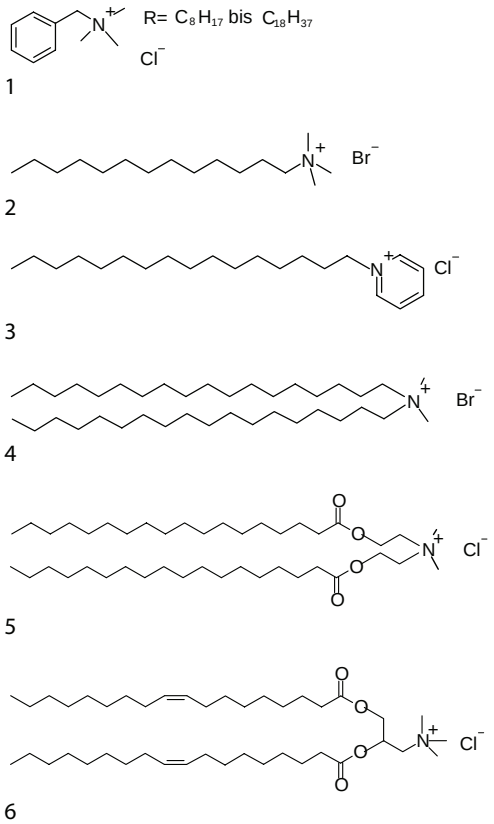

FIGURE 6.14 Organic formulas of the following cationic lipids: **(1)** benzalkonium chloride (alkyldimethylbenzylammonium chloride, BA); **(2)** cetrimide (tetradecyltrimethylammonium bromide, CTAB); **(3)** cetylpyridinium chloride (hexadecylpyridinium chloride, CPC); **(4)** dimethyldioctadecylammonium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

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

6.4.2 On Biodegradation

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

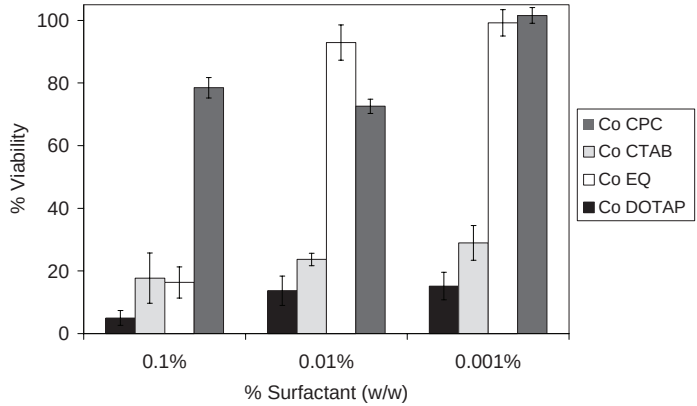

FIGURE 6.15 Viability of Cos-1 cells (African green monkey kidney) after incubation with different SLN formulations. All were made from the matrix lipid Compritol ATO 888 (4% w/w), the surfactants Tween 80/Span 85 (7:3, 2% w/w), and 1% of different cationic lipids. CPC, cetylpyridinium chloride; CTAB,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

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

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

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

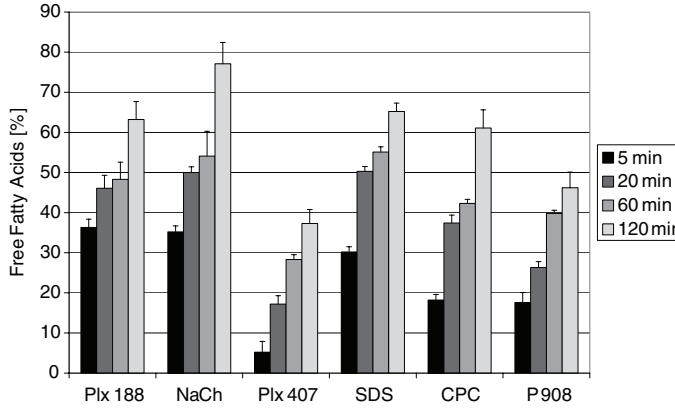

FIGURE 6.16 Degradation of different Dynasan 114 formulations to assess the influence of different surfactants. Plx 188, poloxamer 188; NaCh, sodium cholate; Plx 407, poloxamer 407; SDS, sodium dodecyl sulfate; CPC, cetylpyridinium chloride; P 908, poloxamine 908.

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

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

6.4.3 On *In Vitro* Transfection Efficacy

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

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

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

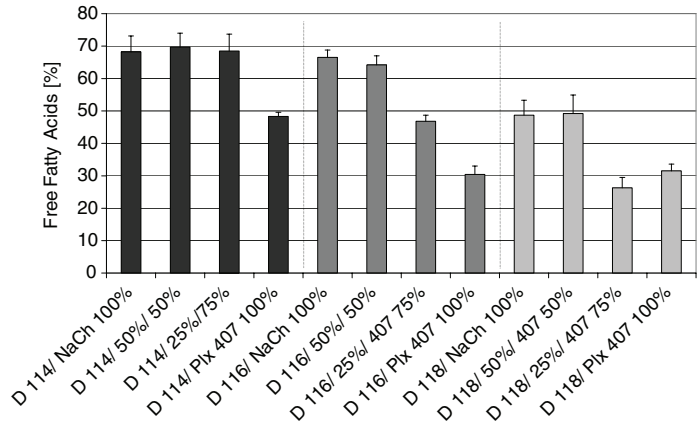

FIGURE 6.17 Degradation of different lipids with mixtures of sodium cholate (NaCh) and poloxamer 407 (Plx 407). D 114, Dynasan 114; D 116, Dynasan 116; D 118, Dynasan 118.

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

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

6.5 INFLUENCE OF PRESERVATIVES ON CYTOTOXICITY

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

6.6 MECHANISM OF UPTAKE AND FATE IN THE CELL

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

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

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

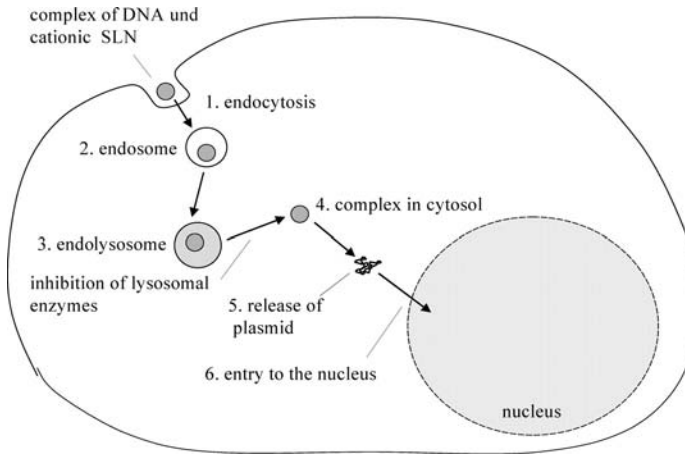

FIGURE 6.18 Proposed cell uptake mechanism for cationic solid lipid nanoparticles. **(1)** The slightly cationic complex of SLN and DNA interacts electrostatically with the anionic cell surface and is internalized by endocytosis; **(2)** the complex is localized in the endosome; **(3)** after fusion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

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

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

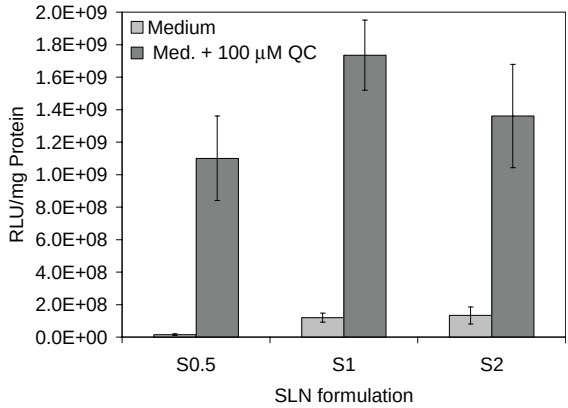

FIGURE 6.19 Transfection efficiency (quantified as "relative light units" [RLU]/mg protein) of three different SLN formulations (all made from 4% cetyl palmitate, 2% Tween 80/Span 85 [7:3], and 0.5% [S0.5], and 1.0% [S1] or 2.0% [S2] of the cationic lipid DOTAP) without medium (Medium) and with medium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

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

6.7 CONCLUSION

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

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