CHAPTER 1

SOLID LIPID NANOPARTICLES: PROPERTIES AND THEIR APPLICATION IN NUCLEIC ACID DELIVERY

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Abstract. Solid lipid nanoparticles (SLNs) have been proposed in the 1990’s as appropriate drug delivery systems, and ever since they have been applied in a wide variety of cosmetic and pharmaceutical applications. In addition, SLNs are considered suitable alternatives as carriers in gene delivery. Although important advances have been made in this particular field, fundamental knowledge of the underlying mechanisms of SLN-mediated gene delivery are conspicuously lacking, an imperative requirement in efforts aimed at further improving their efficiency. Here, we address recent advances in the use of SLNs as platform for delivery of nucleic acids as therapeutic agents. In addition, we will discuss available technology for conveniently producing SLNs. In particular, we will focus on underlying molecular mechanisms by which SLNs and nucleic acids assemble into complexes, and how the nucleic acid cargo may be released intracellularly. In discussing underlying mechanisms, we will, when appropriate, refer to analogous studies carried out with systems based on cationic lipids and polymers. Work carried out so far emphasizes the need to obtain further insight into fundamental issues of SLN/DNA complex assembly and underlying mechanisms of delivery in order to appreciate the potency of SLNs as nanocarriers.
1.1 Introduction

Since early proposals on the potential of applying nucleic acids (e.g. plasmids, antisense oligonucleotides, and siRNA) as therapeutic agents, great expectations were raised as to the impact of this technology on pharmaceutics and human healthcare. Specifically, therapeutic approaches for several currently untreatable diseases have been and still are anticipated [1]. To achieve their therapeutic effects, nucleic acids need to cross several biological barriers, including membranes, in order to successfully gain access to their intracellular targets. Another potential ‘barrier’ to take into account in the delivery of nucleic acids is that of avoiding an encounter with nucleases, present in biological fluids (e.g. blood) and intracellular compartments (e.g. lysosomes and cytosol) [2-4]. To tackle these hurdles, several gene delivery devices have been developed that effectively protect nucleic acids from being degraded while circulating in the bloodstream and also facilitate their efficient translocation across membrane barriers, resulting in intracellular (cytosolic) delivery.

Typically, gene delivery devices are divided into viral and non-viral delivery systems, each system displaying its specific advantages and limitations. Viral delivery systems are extensively used for gene delivery and promising results in vivo have been presented [5,6], although serious setbacks because of medical complications have also been reported. For example, by employing recombinant adeno-associated viral vectors, successful transduction of coagulation factor (Factor IX) in hepatocytes at therapeutically relevant levels in vivo has been accomplished, although maintenance of expression at a therapeutically effective level, was limited [5]. However, potential side effects of viral vectors (e.g. induction of a host inflammatory and immune response) are of continuous concern. Moreover, additional challenges involve issues of controlling viral mutagenesis and the ability to make the production of viral vectors at a large scale economically feasible. For those particular reasons, major efforts remain focused on the development of non-viral delivery devices as a potentially less-hazardous and convenient alternative.

A variety of non-viral delivery systems for nucleic acids has been developed, including nanoparticles, assembled from lipidic, polymeric and inorganic materials [7]. Some have been tested in vivo, proving the potential efficiency of these vectors in a composition-dependent manner [8] as delivery vehicle for nucleic acids [9-11]. Moreover, the efficiency of some of these formulations in conjunction with a negligible toxicity have led to their application as gene delivery vehicles in clinical trials [12,13]. Clearly, over the years viral systems have proven to be more potent in nucleic acid delivery than non-viral systems. Yet numerous studies in which non-viral systems were carefully modified to define parameters for optimizing delivery are now paying off, highlighting the impressive advances in delivery
efficiency of such (non-viral) systems [for reviews see 14,15] raising expectations for further successful applications in the near future.

Non-viral delivery systems can be rationally developed because of the relative ease in bringing about chemical modifications in the compounds, employed in their formulations, in order to obtain optimal delivery efficiency and, if needed, specific targeting to tissues and cells. Furthermore, the use of appropriate non-immunogenic, biodegradable and biocompatible materials enables to prepare delivery devices of relatively low toxicity [16,17]. In addition, large scale production can be readily accomplished by exploiting sophisticated industrial technologies, such as spray drying and high pressure homogenizers [18]. For example, for the production of lipoplexes, i.e., nanoparticles consisting of cationic lipids and nucleic acids, devices have been developed allowing the continuous on-line complexation of DNA and cationic liposomes, a most fertile approach for the large-scale preparation of such lipoplexes [19-21].

As noted, non-viral delivery systems usually rely on the use of (ionizable) polycations, which provide an efficient binding platform for negatively charged nucleic acids via electrostatic interactions. Guided by this principle, a great variety of vectors have been designed for nucleic acid delivery, including cationic polysaccharide [22,23], metallic nanoparticles [24], cyclodextrins [25,26], polymers [27,28], cell penetrating peptides [29,30], liposomes [28,31,32], cationic lipids [14,15] and solid lipid nanoparticles [33].

Although relatively less attention has been paid so far towards the application of solid lipid nanoparticles (SLNs), they have shown promising prospects in drug delivery. [34-39]. Their ability to trap drugs within the solid lipidic matrix enables not only to protect against chemical degradation, but also causes a modulation of the drug release profile, i.e., giving rise to a so-called sustained release profile [34]. In addition, SLNs have attracted widespread academic and industrial interest because of pharmacological advantages. Specifically, SLNs can be produced at a large scale without the use of organic solvent and with a long-term physical stability (generally over a year). They also can accommodate a high drug payload, and several formulations have been assembled that are perfectly stable following steam sterilization or lyophilization. Since early 2000, cationic SLNs have been developed and are applied as gene delivery systems [40]. In fact, these nanoparticles have also been used in combined delivery protocols of drugs and genes, trapped in the same carrier [41,42]. Moreover, they are also readily amenable to surface-coupling of specific ligands, thus allowing formation of decorated particles for specific targeting of specific cells, tissues or organs, including the brain [36,43]. However, so far, very little is known about underlying mechanism(s) in SLN-mediated delivery, knowledge that is imperative for further optimizing their delivery capacity, similarly as reported in the development of cationic lipids and polymers as
nanocarriers.

Here, we will address some recent advances in the application of SLNs as vehicle for the delivery of nucleic acids as therapeutic agents. In particular we will discuss underlying molecular mechanisms by which SLNs and nucleic acids assemble into complexes, and how the nucleic acid cargo may be released intracellularly. Since Olbrich et al. (2001) introduced SLNs as gene delivery systems; such issues have been barely addressed and/or compared to closely related systems such as cationic lipids and polymers.

1.2 Composition and production of solid lipid nanoparticles

SLNs are aqueous colloidal dispersions, produced in solution using (in part) solid lipidic material, which comprises the so-called lipid matrix, and surfactants(s) and co-surfactants(s), which confer stability and ligand properties to the formulation, respectively. Depending on the procedure of preparation the particles obtained are usually in the submicron range (10 – 1000 nm) [38]. In general, the compounds used to produce SLNs are well tolerated and show little toxicity. Indeed, several SLN formulations have been approved and applied for pharmaceutical applications in humans [39,44,45].

A variety of lipid(ic) compounds (i.e., lipids or appropriate solid fatty acids; Fig. 1.1) have been used as waxy solid lipid matrix in the preparation of SLNs, including compritol 888 ATO (glyceryl behenate), behenic acid, cetyl palmitate, precirol ATO 5, stearic acid, imwitor 900PTM (IMW, 40–50% glyceryl monostearate), tricaprin, cholesteryl oleate, glyceryl trioleate, cholesterol, soya lecithin, and glyceryl monostearate. The choice of the lipid modulates the gene delivery properties of the SLN formulation. For example, better transfection efficiencies were achieved when using the wax cetyl palmitate as the lipid matrix, when compared to Compritol (a mixture of mono-, di- and triglycerides of behenic acid) [46]. Furthermore, the choice of the lipid matrix will also determine the physicochemical stability of the SLNs. Thus long term physical stability can be achieved when using formulations that consist of oxidation-resistant saturated lipids/fatty acids in case of solid lipid nanoparticles, or high oil concentrations in the preparation of nanostructured lipid carriers [34,47]. Although the highly ordered crystal packing of lipids in the SLN matrix will ensure a superior physical stability [48], as will the preclusion of distinct lipid (phase) transitions [49], an appropriate balance between stability and drug expulsion is pivotal in order to accomplish the desired therapeutic effect.

Surfactants and co-surfactants are part of the SLN formulation, and the most frequently applied surfactants are pluronic F68, tween 80 as such and in mixtures with span 85, taurocholate, glycocholate, and octanoic
Surfactants do not affect the solid nature of the lipid matrix but they may modulate the rate of structural, so-called polymorphic transitions of the lipid core, and thereby SLN assembly [50]. Interestingly, even hydrophilic surfactants can alter polymorphic transitions in the core of the nanoparticles. Most importantly, the net effect of surfactants on polymorphic transitions and thus the lipid matrix organization as such usually results in a more efficient stabilization of SLNs [50,51].

To accommodate the negatively charged nucleic acids, cationic SLNs are prepared by including a positively charged co-surfactant in their formulation (cf. Fig. 1.3), for example N,N-di-(b-stearoylethyl)-N,N-dimethyl-ammonium chloride (Esterquat 1, EQ 1), benzalkonium chloride (BA), cetylpyridinium chloride (CPC), cetrimide (CTAB), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP), dimethyldioctadecylammonium bromide (DDAB), stearylamine, 3beta-[N-(N0, N0-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol), or 6-lauroxyhexyl lysinate [38,52]. Regarding SLN-mediated delivery of nucleic acids, the choice of an appropriate combination of cationic and matrix lipid seems imperative as it may exert a significant impact on the transfection
efficiency [46].

Figure 1.2. Representative surfactant structures used as interface stabilizers in cationic SLN formulations: a) taurocholate, b) Pluronic F68 (polyoxyethylene-polyoxypropylene block copolymer), c) octanoic acid, d) Tween 80.

A variety of procedures have been described for preparing SLNs [52-56]. To optimize the various production methods, individual steps in SLN production have been evaluated. It has thus been found that the physicochemical properties (e.g., lipid matrix organization) were not greatly influenced by the method applied [57]. However, the choice of the appropriate method to prepare SLNs should also take into account issues like size, and stability of drugs during the production process of the nanocarrier, which may also be affected by composition. In the following, we will therefore critically discuss the methods used for the production of cationic SLNs, and highlight several advantages and limitations. In particular we will focus on three different procedures that have been frequently applied for preparing cationic SLNs, i.e., (i) the warm oil-in-water (o/w) microemulsion procedure [58], (ii) the hot high pressure homogenization method [59], and (iii) the solvent emulsification method [51].

In the microemulsion technique (i), the solid core lipid(s) is(are) heated above its (their) melting point. Separately, a mixed dispersion of surfactant, co-surfactant and cationic lipid(s) is preheated at the same temperature, and subsequently added to the melted lipid under stirring [54,60]. Next, the hot microemulsion is dispersed in cold water under stirring, the rapid recrystallization leading to the formation of SLNs. Importantly, the dilution
in cold water as such suffices to produce submicron particles, irrespective of the stirring rate. Of particular interest is the observation that the solvent used affects particle size, water-miscible solvents leading to smaller SLNs, while the use of water-immiscible solvents leads to larger SLNs \cite{51}. Although this method can produce cationic SLNs and avoids the use of organic solvents, the final dilution step in water, which occurs in a relatively large volume (dilutions range from 1:25 up to 1:200 and frequently resulting in less than 1% of particles in the final formulation), is an obvious disadvantage when relatively expensive drugs are employed, while the final SLN concentration will be such that relatively large volumes have to be administered in order to meet conditions of therapeutic efficacy \cite{61}. However, some adaptations of this method can overcome these limitations. Recently, we have developed a microemulsion extrusion method for SLN production, which is an attractive alternative as it relies on the use of small volumes \cite{41}.

The high pressure homogenization technique (ii, HPH) is considered a standard procedure in the preparation of SLNs \cite{39}. Similarly as for the microemulsion technique, the melted solid lipid is initially co-dispersed in a hot aqueous solution, containing the surfactant, co-surfactant and cationic lipid(s). Next, the hot microemulsion is submitted through a high pressure homogenizer at the desired temperature, and the thus generated high pressure (100-2000 bar) and high shear stress produce SLNs at a sub-micron scale \cite{56,62}. In this procedure the temperature is of particular relevance, i.e., the lower the temperature the larger the size of the SLNs, a parameter of relevance for in vivo application, where relatively smaller particles (below 120 nm) are preferred. As for the microemulsion technique (i), the produc-

Figure 1.3. Representative cationic lipid(ic) structures used in cationic SLN formulations: a) benzalkonium chloride, b) 6-lauroxyhexyl lysinate, c) DC-Chol, d) DOTAP, e) stearylamine, f) cetylpyridinium chloride, g) cetrimide.
tion of cationic SLNs in this manner does not depend on the presence of organic solvents [63]. However, the application of this method is obviously restricted to formulations that resist degradation at the desired temperature and high pressure. Furthermore, as in case of the microemulsion procedure, the use of large volumes precludes the preparation of formulations aimed at delivering relatively expensive agents. Yet, the HPH procedure enables the production of cationic SLNs at a large-scale and, given the ability to avoid the use of organic solvents, may thus be considered as a suitable commercial application for the production of cationic SLNs.

Finally, in the solvent emulsification-evaporation technique (iii), the solid lipids are dissolved in water-immiscible solvents and are then emulsified in an aqueous phase containing the surfactant, co-surfactant and cationic lipid(s). Generally, the emulsion is obtained by sonication and mechanical stirring under vacuum subsequently evaporates the solvent. Upon solvent evaporation, the SLNs are assembled by precipitation of the solid lipid in the aqueous phase [51]. The particles thus produced can be concentrated and washed by centrifugation. Unlike the previously described methods, cationic SLNs can thus be prepared at conditions that circumvent thermal stress, thereby allowing the use of thermolabile compounds [38,51]. On the other hand, this procedure may raise concerns about the effectivity of the removal of the organic solvent, which may give rise to undesired toxic effects of the formulation upon its use in vitro and in vivo.

Although the three procedures discussed in the foregoing represent the most commonly applied ones, several other procedures are also in use [55], although less intensely, but some comments are warranted. One of these is known as the coacervation method, which avoids the use of organic solvents and requires relatively mild temperatures (40 °C to 50 °C), which thus allows the use of thermolabile drugs. In addition, the required equipment does not involve significant capital costs, thus making this procedure affordable for laboratory and industrial application [64]. Another example to produce SLNs relies on the use of microchannels, a relative simple procedure which results in the preparation of particles at the nano-scale with a narrow size distribution [65]. Moreover, this method can be conveniently used for the complexation of nanoparticles and nucleic acids, and relative to other procedures, offers a more consistent and more reproducible alternative [21]. Additionally, this method has been shown to allow for complexation between cationic lipids and DNA as a continuous process, thus being relatively labor-insensitive as it reduces the number of commonly required steps [66]. Following a different approach, Lobovkina and coworkers [10] used a hydrophobic ion pairing technique, in which they produced a complex between siRNA and DOTAP prior to the assembly of the SLNs as such. Interestingly, this approach increases the lipophilicity of the siRNA, allowing a more efficient incorporation of the nucleic acid into the lipid core of the SLNs (see below). Of particular interest, siRNA con-
taining SLNs, prepared in this manner, showed a sustained release profile, implying a long-term (up to 13 days) release of siRNA in vivo [10], a beneficial procedure that promotes the effectiveness of SLN-mediated delivery of siRNA. This observation supports a potential for the use of SLNs in mediating delivery of nucleic acids. However, little insight is currently available on the mechanism underlying assembly of these kind of ‘lipoplexes’ or the mechanism of internalization by cells and subsequent release of the nucleic acid cargo. Evidently, this knowledge will be of relevance in further improving the development of SLNs as gene delivery vehicle in the future applications, as will be discussed next.

1.3 Physico-chemical properties of complexes consisting of SLNs and nucleic acids

To control SLN production in terms of loading efficiency, the effect of varying the lipid composition that will affect electrostatic interactions with nucleic acids, is an important parameter. In addition, transfection efficiency is known to be further controlled by physicochemical properties such as size, surface chemistry, morphology, and aggregation, as revealed by studies of other nanocarrier systems. Thus these parameters critically determine the interactions between nanoparticles and biological systems in terms of activity and, importantly, toxicity. Consequently, an investigation of the application of new SLN formulations will benefit from a detailed physicochemical characterization. Indeed, it has been reported that the choice of the lipid matrix in conjunction with that of the cationic lipid appear major determinants in the outcome of the efficiency of SLN gene delivery [62]. Accordingly, we will discuss next some important SLN physicochemical properties, and how such properties influence the interaction of SLNs with the cellular environment, and determine the consequences for nucleic acid delivery.

1.3.1 SLN Structure and Morphology

The size of nanoparticles is one of the more important physicochemical features for in vivo applications. Irrespective of the preparation method, cargo-free SLNs are often in the nanoscale range (50 – 200 nm). Following addition of nucleic acids, ‘lipoplexes’ with sizes up to approx. 500 nm are obtained. Although such diameters are not undesirable for in vivo use [14], for some applications such as delivery of therapeutics to solid tumors, such particles may nevertheless be of interest. In fact an enhanced permeation and retention effect with an upper limit of 500 nm for accumulation of particles in solid tumors has been reported, while particles larger than 1 µm can still be internalized by Peyer’s patches and may be subsequently directed to mesenteric lymph nodes, although such large particles could be
quite hazardous and lead to embolization [67]. Therefore, sizes of SLNs should be strictly controlled upon their preparation and should not exceed 500 nm in diameter, while for a more general use in vivo a size of approx. 120 nm or less is preferred [14,15].

An additional parameter that strongly determines the interaction efficiency of SLNs with cell surfaces is the charge of the nanocarrier. Although the zeta potential gives an indirect measurement of the charge at the particle surface, it provides an estimate of the level of interaction between SLNs and nucleic acids. Common formulations for SLNs usually show a high positive value, i.e., higher than +30 mV, due to the cationic lipids that are located at the particle interface, which decreases upon addition of increasing concentrations of DNA [5,68]. As anticipated, the charge ratio (+/-) of the SLN lipoplexes strongly influences the biological outcome in terms of delivery and transfection efficiency. At a relatively high (+/-) charge ratio, an enhancement in SLN-mediated delivery is observed, while their stability in the presence of serum (proteins) is improved. In this regard, the effect appears similar to that seen for cationic liposomes [6,69]. Therefore, for the optimization of SLN as a platform for nucleic acid delivery it is important to take into account in this context the pronounced effect of the charge (+/-) ratio.

Several techniques have been employed to elucidate the morphology and ultrastructure of SLNs. These include transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM and AFM have been mainly used to investigate morphology, size and surface properties of SLNs, with or without the loading of drugs or nucleic acids [70-74]. The SLNs (empty particles) are commonly spherical in shape [75], although this may vary and depend on the formulation, some of which may lead to imperfect spheres [72].

TEM can provide a more detailed insight into the SLNs’ ultrastructure. The monolithic matrix of SLNs can be identified, and at some experimental conditions a detailed ultrastructure can be observed. The solid lipid used in the formulation, stabilized by the co-surfactant at the water interface, in essence forms the SLNs matrix. Different polymorphic features of the lipid matrix have been described. For triacylglycerols hexagonal (α), orthorhombic perpendicular (β’) and triclinic parallel (β) forms have been claimed, and the role of such phases in expressing various properties of SLNs, such as the efficiency of drug incorporation and SLN stability, has been discussed [76]. However, so far little insight is available as to how complexation of SLNs with nucleic acids affects the organization of the lipid matrix and how this may affect release and expression. Depending on the composition, co-surfactant can be found in the matrix [77] and, in cases, a clear lamellar-like structure with a dense core has been observed [72].
1.3.2 Assembly of SLN-DNA lipoplexes

Usually, complexes between nucleic acids and cationic lipids (e.g. in cationic liposomes, nanoemulsions or SLNs) are prepared by simply adding DNA to preformed, cationic lipid containing nanoparticles, suspended in aqueous solution [14,28,33,78,79]. For cationic liposomes, lipoplex formation is a spontaneous process ($\Delta G < 0$). The enthalpy contribution for this process has been evaluated by different methodologies (ITC, DSC and theoretical studies), and the endothermic nature of the assembly process has been well established [80-87]. Although an increase in ionic strength causes a decrease in the binding enthalpy, a net positive enthalpy gain is maintained, regardless of the ionic strength or the order of DNA and lipid addition [84-86,88,89]. Thus, an increase in entropy must be the thermodynamic driving force for the spontaneous formation of lipoplexes. In fact, this entropy increase stems from the release of counterions into the bulk solution, following the interaction of the two macro-ions (i.e. DNA and cationic lipid). [82,84,85,90]. This interaction reaches the highest $\Delta S$ at isoelectricity, which has important implications for the lipoplex geometry, as further discussed below [82,86].

Besides counterion release and macro-ion interactions, it has also been shown that water is released from the system, thereby increasing the degree of freedom, allowing close intermembrane interactions in case of the use of lipid vesicles and promoting polymorphic (lipid) transitions, both of which accompany the process of lipoplex assembly [91]. Indeed, the proximity of molecules may allow hydrophobic interactions to take place and contribute to complex formation, driven by a significant entropy increase [92]. This has led investigators to evaluate the hydrophobic contribution in lipoplex formation. It has been found that besides the release of counterions and water, hydrophobic interactions between the hydrocarbon chains also contribute to lipoplex stabilization, involving hydrophobic (fatty acyl chain) interactions among the aligned lipids [28,83,87,90,93,94]. Further investigations using FTIR and Raman spectroscopy revealed that structural changes also occur in the DNA itself, such as rearrangements in hydrogen bonds between the base pairs [95].

Thus, lipoplex formation has a considerable impact on the structure of both the cationic liposomes and DNA. The vesicular structures, formed initially, are completely reorganized due to nucleic acid-induced aggregation and fusion, which leads to lipid mixing and rupture of the bilayer structure [84,88,93,96-100]. As result of these reorganizations, lipoplexes can adopt distinct tridimensional ordered multilayered structures (known as fingerprint structures) with DNA intercalated between the lipid layers [53,100-102]. Three different organizations of the lipids have been described for these interactions, i.e., (i) the lamellar structure (L$\alpha$), characterized by DNA rods inserted between lipid bilayers, (ii) the intercalated hexagonal
structure (HI), characterized by DNA rods surrounded by three cylindrical lipid micelles arranged on a hexagonal lattice, and (iii) the inverted hexagonal structure (HII), characterized by DNA rods covered with lipid monolayers organized on a hexagonal matrix \[53,94,97,103\]. Adoption of the latter structure is particularly promoted when dioleoylphosphatidylethanolamine (C18:1) is included in the formulation \[84,104-106\], and strongly facilitates cellular delivery of nucleic acids and their subsequent functional expression \[14, for review see 79\]. However, also formulations containing cationic lipids capable of adopting the HI structure promote nucleic acid delivery and transfection, suggesting that adoption of non-bilayer structures of the lipoplex as such is instrumental in nucleic acid delivery. Importantly, in spite of their non-bilayered nature, these structures nevertheless provide a compactness that effectively prevents exogenous accessibility of agents such as intercalating dyes or nucleases to the nucleic acids (Fig. 1.4).

Complexes formed between SLNs and nucleic acids, which will be further referred to as ‘SLNplexes’, display several features that are similar to those observed for lipoplex formation, i.e., between cationic lipids and nucleic acid, although the final structural organization may be different. Similarly to lipoplex formation, SLNplex formation is a spontaneous process (\(\Delta G < 0\)) and ITC measurements showed a positive enthalpy \[107\]. Next to electrostatic interactions, hydrophobic interactions also appear to play a role in assembly and stabilization of the SLNplexes. However, increasing the ionic strength showed little effect on SLN/DNA affinity. Details of this interaction, as obtained by circular dichroism, revealed rearrangements of the DNA helices, giving rise to the presence of a mixture of B- and A-DNA structures after complex assembly \[107\]. These findings suggest that for formation of SLNplexes may rely on the same driving forces (i.e., culminating from the release of counterions and macro-ion interactions) as in lipoplex formation.

Due to the presence of the solid lipid, the core of SLNs is relatively dehydrated, with few if any counterions inside. Therefore, a significant entropic driving force that contributes to further mixing of DNA into the SLN core is virtually negligible. It has been suggested that upon assembly of SLNplexes the interaction between nucleic acids and SLNs is mainly through adsorption of the nucleic acid to the particle surface, as schematically represented in Fig. 1.4 \[40,72,74,108\]. Among others, in support of this hypothesis are observations by AFM, showing that DNA bound to the surface of cationic SLNs is readily removed after exposure to nucleases \[72\]. Clearly, the localization of DNA molecules at the surface of the SLNs thus makes the system susceptible to nuclease attack, thereby compromising the therapeutic application \[108\]. However, nuclease treatment revealed that the treated particles still express transfection efficiency, implying that part of the DNA must have been protected from degradation.
Indeed, it has also been demonstrated that SLNs can deliver genes in vivo, showing that at these conditions potential problems due the natural presence of nucleases in serum can be overcome and do not frustrate the application of SLNs as gene delivery system. On the other hand, it is unclear to what extent surface bound DNA is effectively removed by nucleases, which would require a precise quantitation of the localization of the different DNA pools, for example, by applying fluorescence DNA binding assays. Application of quantitative assays could thus shed further light on the relevance of internally trapped pool of DNA within the nanoparticle versus loosely bound pools of DNA in bringing about transfection (see Fig. 1.4). Nevertheless, of interest in this regard, the nucleic acid surface binding capacity of SLNs has been explored as a strategy in DNA delivery. Thus, by starting out with anionic SLNs, their surfaces have been modified by the addition of cationic ligands, such as protamine, which in turn effectively accommodates DNA, thus allowing the system to be applied as gene delivery system [109].

In efforts to further clarify important factors in SLNplex assembly, it has been noted that the amount of cationic lipid in SLN formulations, required for a complete recruitment of nucleic acid, may differ substantially, thereby significantly affecting the charge ratio of the various formulations. Thus, it has been reported that depending on the SLN core lipid composition, the amount of cationic lipid required to accommodate the same amount of DNA, may differ as much as an order of magnitude between formulations [46]. This reinforces the importance of the cationic lipid in the formulation, but also draws attention to the fact that the molecular mechanisms underlying these observations are still unclear. Likewise, at a relatively high cationic lipid ratio in the SLN/nucleic acid formulation, excessive condensation of DNA may occur and result in a poor transfection efficiency, due to a reduced release of the nucleic acid from the complex [68]. In addition to cationic lipids, the solid lipid used in the SLN core seems to play a role in this interaction. SLN formulations prepared from solid lipids that mix with the cationic lipid but still preserve the capacity of crystallization, are thought to achieve a better stability than the ones where the solid and cationic lipid mix completely [46,110]. Interestingly, the interaction between SLNs and nucleic acids does not appear to be significantly affected by surfactants [110,111]. Recently, it has been demonstrated that the interaction between surfactants and the SLN nanoparticles might be of a dynamic nature, revealing the surfactant’s partitioning between the lipid core of the nanoparticle and the surrounding aqueous phase. Thus, although the surfactant may play an important role in SLN stability, it does not seem to interfere with the interaction between DNA and the cationic SLNs [77].
Figure 1.4. Schematic representation of formation of a Lipoplex and an SLNplex. Assembly of lipoplexes is an endothermic process, which is driven by the release of counterions into the bulk solution followed by the binding of the two macro-ions. Initially, the DNA adsorbs at the cationic liposomes surface, with further addition of DNA the liposome structure is entirely reorganized, resulting in fusion and aggregation, which leads to a compact structure in which the DNA helix is sandwiched between lipid layers (for details see text). SLNplex formation is also an endothermic process, which proceeds very similar as for lipoplexes. The solid, relatively dehydrated core and the virtual absence of counterions within the SLNs, limits the internal mixing of DNA into SLN. Presumably, in case of the SLNplex, the DNA largely adsorbs to the surface of the SLNs, resulting in a fraction intimately bound to the SLN (left) and one that is more exposed (right), and susceptible towards nucleases.
1.3.3 Transfection mechanisms

It is well known that transfection relies on a multifactorial process and some factors are directly related to the physicochemical properties of the gene delivery system [14]. Although a number of important physicochemical properties of lipoplexes that bear relevance to their transfection efficiency (lipid polymorphic properties and particle size, see e.g. [14], for a review) have been identified, it is still a challenge to predict in vitro transfection efficiency [112], not in the least because of the variety in cell-type dependent processing of the nanoparticles. Particularly in the research field that focuses on the internalization of lipoplexes and polyplexes, considerable efforts have been made to elucidate the underlying mechanisms of intracellular gene delivery. These studies have clearly revealed the importance of endocytosis in nanoparticle internalization and the subsequent need of endosomal escape of its cargo, which appears a major hurdle in the actual cytosolic deposition of drugs and genes in cells [113-115]. However, although direct evidence is still lacking, insight into likely mechanisms by which cationic lipid-based carriers and polymers mediate the transfer of their cargo across these intracellular membrane barriers, is gradually emerging [114,116]. For polymers, it has been advocated that the high buffering capacity (i.e. within a pKₐ range between 5-6) of some polymers can absorb a significant amount of protons in acidifying endosomes, which drives an inflow of counter ions and water into these compartments to maintain the electric and osmotic balance, respectively. As a result, endosomes will swell, which subsequently causes a destabilization of their membrane, thereby allowing contents to be released into the cytosol [117]. More recently, direct experimental evidence, favoring endosomal membrane rupture rather than endosomal lysis [14,116], while excluding an effect on the lysosomal pH [118], has been provided.

For cationic liposomes, the endosomal membrane destabilization is claimed to be driven by a process that likely involves a (local) endosomal membrane destabilization, mediated by the ability of the lipid-based nanoparticles to adopt nonbilayer structures (reviewed in [14]). Both hexagonal and inverted hexagonal phases suffice to bring about such a destabilization, which may be triggered by a mild acidic pH, as occurs in endosomes, in case of applying (ionizable) cationic lipids or, if needed, by inclusion of a helper lipid like DOPE that may promote and facilitate such a transition [119-124]. Consistently, inclusion of bilayer-stabilizing components (such as phosphatidylcholine or PEGylated lipids) effectively block delivery of cargo and hence transfection efficiency [104,125-127]. Evidently, the nonbilayer configurations referred to also mediate a rapid lipid mixing, which is often taken to suggest a role of membrane fusion. However, fusion abrogating nonbilayer phases, like the HI phase, do promote delivery, implying, together with other evidence discussed elsewhere,
that fusion is unlikely the driving force in cationic lipid-mediated delivery [14,79,124].

Although cationic lipids are an inherent part of SLN formulations, it is unclear to which extent their presence bears relevance to the mechanism by which SLNs deliver their cargo. Evidently, the cationic charge provides a binding platform for the negatively charged nucleic acids. Whether their polymorphic properties play a role in destabilizing cellular membranes, as discussed above for cationic lipid-based nanocarriers, remains to be determined. However, SLNplexes consisting of stearic acid (7 mM), Pluronic F68 (1 mM), and DOTAP (2.5 mM) as cationic lipid, neither adopt inverted hexagonal structures nor show any capacity of lipid mixing (our unpublished observations). Accordingly, although SLNplexes, like lipoplexes, are primarily internalized by endocytosis, their mechanism of endosomal escape is unclear. Nevertheless some observations may shed further light on the mechanism by which SLNplexes release nucleic acids inside the cells. Among others, it is possible that (some) surfactants can play a role as reported for Tween 80, a nonionic surfactant that is capable of enhancing membrane permeability, thereby causing an improvement of transfection efficiency [33,128]. Furthermore, differences in the final organization of SLNplexes and lipoplexes revealed a key idiosyncratic difference in transfection mechanism of either type of nanoparticle. Thus, inclusion of helper lipids (e.g., cholesterol and DOPE) that often promote the transfection capacity of HII-adopting, cationic lipid-based systems while inhibiting that of HI phase adopting systems, is without effect on transfection when included in SLN formulations [72,111]. This suggests that a non-bilayer based mechanism is likely not critically involved in SLN-mediated transfection. It has been shown, however, that SLN formulations may take advantage of the presence of endosomolitic molecules such as chloroquine [129], showing an enhancement of transfection at such conditions. While this implies that low intraluminal pH of late endosome and lysosomes could hinder transfection efficiency, others have suggested the opposite, namely that DNA release from SLNs requires lysosomal activity [130]. Although the reason underlying this discrepancy is still rather unclear, the perturbation and/or degradation of SLNs, likely by hydrolytic lysosomal activity, and the ensuing disorganization of the lipid matrix, has been proposed as a mechanism of drug release from these nanoparticles [131]. However, although cargo might be released in this manner from the SLNs, their required escape across the lysosomal membrane remains a mystery, particularly since released nucleic acids are thought to be rapidly degraded in the lysosomal lumen.

Evidently, further work will be needed to elucidate the mechanism by which SLNs deliver their cargo into the cytoplasm. This is also particularly relevant in the frame of observations on the likelihood of a prolonged intracellular stability of these nanocarriers. This might be inferred from ob-
servations on the release of different cargos, including pDNA, siRNA, and ODNs, in both in vitro and in vivo experiments. For example, in in vitro experiments using prostate cancer cells [132], it has been shown that ODN release from SLN formulations was considerably slower than that observed from lipoplexes consisting of Lipofectamine. For siRNA, it has been shown that lipofectamine or liposomal formulations led to knockdown of survivin over a time period that lasted for three days, while the application of SLN formulations extended this period up to five to nine days, depending on the formulation [133]. These results could thus suggest that SLNs, compared to liposomal formulations, could display a higher intracellular stability as a consequence of which intracellular delivery may be relatively delayed, thereby giving rise to sustained release of its contents. However, as noted above, the intracellular site of release remains enigmatic.

In this context, similar observations of an enhanced stability of SLNs have also been reported for in vivo experiments. Intradermal injection of SLN nanoparticles carrying siRNA gave rise to a sustained release for a period of up to 10-13 days [10]. Analogously, by employing SLN-based vectors, a prolonged expression of foreign proteins in the spleen and the liver for about 7 days has been observed [134]. Accordingly, also these data raise intriguing questions as to the underlying mechanism of SLN-mediated release of cargo, and inherently to the mechanism by which these nanoparticles are processed by cells.

1.4 Storage Stability & Lyophilization

Given their long storage stability and options for large-scale production, SLNs display features that may provide an added value regarding their future application in healthcare. Optimized SLN formulations maintain their physical integrity for over at least 3 years [49,51]. As discussed above, SLN stability is determined by several factors, including chemical features, such as the nature of solid lipids and surfactants, and their physical features, such as size, zeta potential and degree of crystallinity of the solid lipid [51]. Cationic SLN formulations may maintain their activity for over 2 years [72]. Concerning their stability two factors have been highlighted: the surfactant and co-surfactant. Thus, choosing the correct surfactant is critical for long-term stability of SLNs [135,136], and an appropriate match between the hydrophilic-lipophilic balance value of the surfactant and the solid lipid is necessary [135,137]. Adjustments in surfactant concentrations may be necessary during the optimization phase of the formulation to avoid the gelling phenomena, described as the transformation of the SLN formulation into a viscous gel after a certain period of time [49,138].

Electrostatic repulsion created by the co-surfactants, generally cationic
Figure 1.5. Schematic representation of internalization and intracellular delivery of nucleic acids from Lipoplexes and SLNplexes. Both Lipoplexes and SLNplexes are taken up by cells via endocytosis. For Lipoplexes, the endosomal membrane destabilization is driven by a process that likely involves a (local) endosomal membrane destabilization, mediated by the ability of the lipid-based nanoparticles to adopt nonbilayer structures, which, following lipid mixing, will destabilize intracellular (endosomal) target membranes and allow cargo release. For SLNplexes, it has been suggested that these carriers neither adopt inverted hexagonal structures nor show any capacity of lipid mixing. It has been proposed that destabilization of SLNplexes in lysosomes, following endocytic internalization, is of relevance for SLN-mediated delivery (see text for details).

lipids, can also contribute to the colloidal stability of SLNs. Actually, it has been previously established that zeta potential values greater than 30 mV significantly increase nanoparticle stability in solution [139,140]. The co-
surfactant also seems to affect the size of SLNs formulation, the absence of cationic components leading to a larger mean diameter and a higher PDI value compared to SLN formulations containing cationic lipids [141]. These findings confirm that co-surfactants may play a role at the interface of cationic SLN formulations [31,32,77,141]. However, a careful selection of the co-surfactant should be made in this regard as they can also compromise the stability of SLNs. For example, SLN formulations containing DOTAP are sensitive to oxidation, requiring storage at low temperature (4-8 °C [27,46].

Lyophilization is an alternative for improving the shelf-stability of SLNs [138,142]. In addition, it brings several pharmaceutical advantages. The removal of water makes the formulation lighter for storage and transportation, which can be done at room temperature [141,142]. However, both the freezing and drying process should be carefully controlled as it may perturb the physical integrity of the particles and eventually lead to their aggregation [38,143]. Moreover, lyophilization can damage plasmid DNA or lead to conformational changes, resulting into loss of activity [144]. However, this can be prevented by the addition of lyoprotectants to the formulation [141,142,145].

Yet, the effect of lyophilization on the physicochemical properties of cationic SLNs remains of concern. Upon rehydration, it has been shown to cause an increase in particle size, generally up to 100 nm and a slight decrease of zeta potential, which depends on the SLN formulation [38,52,62,143]. This decrease of the zeta potential can be attributed to rearrangements in the SLN structure and requires a compensation in charge for proper binding of the nucleic acid; therefore, an increase in the N/P ratio is required to accommodate a certain amount of nucleic acid [141]. On the other hand, for other formulations the process appears to be harmless, leading to no important alterations in the size or zeta potential of re-suspended lyophilized powder [36,43,72,74]. The extent of sample dilution seems to play an important role in maintaining the physicochemical integrity upon rehydration after lyophilization, as aggregation can be precluded with increasing dilution.

Many types of cryoprotectants, mainly carbohydrates (e.g. glucose, mannitol, lactose, mannose, trehalose), have been used to prevent damage caused by lyophilization [138]. The concentrations of cryoprotectants and nanoparticles should be considered for optimization of the process. Thus a nanoparticle concentration around 1% showed better results, while cryoprotectants concentrations from 10-20% seem to suffice to stabilize the formulations. Among available cryoprotectants, trehalose has proven to be the more effective lyoprotectant for cationic formulations [44,70,139,143], as previously described for non-cationic SLN formulations [138,142]. Remarkably, in several cases the transfection efficiency was preserved when comparing the functionality of SLN formulations before and after freeze-drying [62,72,128,143,146].
1.5 Concluding Remarks

Clearly, although very compelling, the concept of a magic bullet proves to be a utopic challenge rather than a realistic concept. This also holds in the development of an ideal SLN-based nanocarrier device. Whilst there is a considerable body of work on formulations of cationic SLNs as transfection agents, there is no clear-cut correlation between SLN composition or structure, and its potency in delivery or subsequent efficacy of drug release. Accordingly, further successful development of solid lipid nanoparticles as gene delivery vehicles will greatly depend on the improvement of knowledge on structure-function relationships of SLNplexes and, consequently, the underlying mechanisms of intracellular processing.

It is expected that these issues will be tackled in the near future and that this insight may help in further improving the development of SLNs as gene delivery system. So far, amazingly little insight has been obtained into the structure-function relationship of SLNplexes. In particular, it is rather unclear how SLNs accommodate their cargo, in particular nucleic acids. This would be important in clarifying underlying mechanisms that might be of relevance for SLN-mediated delivery of its cargo. In that context, further insight is needed in the structure of SLNs and SLNplexes, as might be obtained by applying, for example, small-angle X-ray scattering, electron microscopy, and nuclear magnetic resonance (NMR) and electron spin resonance (ESR) spectroscopy. In addition, molecular dynamics simulation could be a useful approach to obtain detailed insight into the interaction of SLNplexes with target membranes [147]. Such studies may be of help in properly evaluating and appreciating the validity of SLNs as suitable nanocarriers for a variety of nucleic acid-based cargos.

1.6 Scope of this thesis

The applications of nanomaterials on medicine (nanomedicine) can achieve disease diagnostics and therapeutic approaches that cannot be reached by conventional therapeutic strategies. Therefore, the developments on this field allow us to dream about a future where nanomaterials are versatile therapeutic weapons used to fight the most overpowering diseases, such as cancer. The initial step was made, the first generation nanomaterials are being approved for human use. Although, some fundamental knowledge of the underlying mechanisms about how nanomaterials interact with human cells are still unclear and they are imperative requirement for further improving the safety and efficiency of this applications. Hence, the aim of this thesis is to develop and investigate lipid nanoparticles as resourceful tools for delivery of therapeutics (drugs and/or genes) into cancer cells. Initially, we develop a new method to produce lipid
nanoparticles at laboratory scale; to overcome some problems imposed by
the high-pressure homogenization, the standard technique that is adopted
nowadays for lipid nanoparticles production. In addition, we evaluated
the capacity of lipid nanoparticles as drug and gene delivery system (Chapter
2). Next, we decided to explore strategies to improve transfection ef-
ficiency of solid lipid nanoparticles into prostate cancer cells. Consider-
ing the beneficial effect of helper lipids on the transfection efficiency with
cationic liposomes, we decided to evaluate the effect of the helper lipid 1,2-
dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) on transfection with
cationic lipid-containing solid lipid nanoparticles in PC3 prostate cancer
cells (Chapter 3). Having determined the effect of DOPE in SLN formula-
tions, we decided to compare the transfection efficiency of SLNs in normal
prostate and cancer prostate cells. After we observe the transfection was
significantly higher in cancer cells compared with normal cells, we decide
to obtain further insight into the underlying mechanism responsible for this
effect, we subsequently investigated the internalization and subsequently
intracellular trafficking in both cell lines (Chapter 4). Finally, we investi-
gated an alternative tool to defeat cancer cells; the effects of the natural
compound ferruginol were evaluated in prostate cell survival (Chapter 5).

1.7 References

121-128. doi:10.1038/nbt.1769.
[4] F.C. Pérez-Martínez, J. Guerra, I. Posadas, V. Ceña, Barriers to non-viral
vector-mediated gene delivery in the nervous system, Pharm Res. 28
et al., Successful transduction of liver in hemophilia by AAV-Factor IX and
limitations imposed by the host immune response, Nat Med. 12 (2006)
342-347. doi:10.1038/nm1358.
Hasbrouck, S.A. Edmonson, et al., AAV-1-mediated gene transfer to skeletal
muscle in humans results in dose-dependent activation of capsid-specific
T cells, Blood. 114 (2009) 2077-2086. doi:10.1182/blood-2008-07-
167510.


[21] T.A. Balbino, A.R. Azzoni, L.G. de La Torre, Microfluidic devices for


[35] S.A. Wissing, O. Kayser, R.H. Müller, Solid lipid nanoparticles for


[49] C. Freitas, R.H. Müller, Correlation between long-term stability of solid
[65] Zhang, Yun, Shen, Chen, Yao, Chen, et al., Formation of solid lipid nanoparticles in a microchannel system with a cross-shaped junction,


V.V. Iannuccelli, Structural investigation and intracellular trafficking of a novel multicomposite cationic solid lipid nanoparticle platform as a pDNA carrier, Ther Deliv. 2 (2011) 1419-1435. doi:10.4155/tde.11.118.


[131] C.C. Olbrich, R.H.R. Müller, Enzymatic degradation of SLN-effect


[143] A. del Pozo-Rodríguez, M.A. Solinís, A.R. Gascón, J.L. Pedraz, Short- and long-term stability study of lyophilized solid lipid nanopar-


