



Review

Solid lipid nanoparticles as nucleic acid delivery system: Properties and molecular mechanisms



Marcelo B. de Jesus^{a,b,*}, Inge S. Zuhorn^a

^a Department of Cell Biology, University of Groningen, University Medical Center Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

^b Biochemistry and Tissue Biology, Institute of Biology, University of Campinas – UNICAMP, Campinas, SP, Brazil

ARTICLE INFO

Article history:

Received 2 October 2014

Received in revised form 6 January 2015

Accepted 7 January 2015

Available online 9 January 2015

Keywords:

Solid lipid nanoparticles

Gene delivery

ABSTRACT

Solid lipid nanoparticles (SLNs) have been proposed in the 1990s 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 is 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, that have proven useful in the assessment of structure–function relationships. Finally, we will give suggestions for improving SLN-based gene delivery systems, by pointing to alternative methods for SLNplex assembly, focusing on the realization of a sustained nucleic acid release.

© 2015 Elsevier B.V. All rights reserved.

Contents

1. Introduction	1
1.1. Composition and production of solid lipid nanoparticles	2
1.2. Physico-chemical properties of complexes consisting of SLNs and nucleic acids	5
1.2.1. SLN structure and morphology	5
1.2.2. Assembly of SLN–DNA lipoplexes	5
1.2.3. Transfection mechanisms	6
2. Storage stability & lyophilization	9
3. Concluding remarks	10
Acknowledgments	10
References	10

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 pharmaceuticals 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, 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

* Corresponding author at: Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas, Rua Monteiro Lobato n° 255, CEP 13083-862 Campinas, SP, Brazil.

E-mail address: dejesusmb@gmail.com (M.B. de Jesus).

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]. Some viral-based systems, such as Glybera® (alipogene tiparvec), have reached the market. However, serious set-backs 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, because of cell-mediated immunity [5]. The potential side effects of viral vectors (e.g. induction of a host inflammatory and immune response) are of continuous concern. Additional challenges involve issues of controlling viral mutagenesis and the ability to make the production of viral vectors at a large scale economically feasible. Although important advances have been made for the targeted integration of transgenes at specific sites within the human genome, the safety of the introduction of transgenes at these target sites, preventing cell malfunctioning or oncogenesis, remains to be guaranteed [7]. 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 [8]. Some have been tested *in vivo*, proving the potential efficiency of these vectors as delivery vehicle for nucleic acids [9–11]. Moreover, the efficiency of some of these formulations in conjunction with a negligible toxicity has 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, 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 homogenization [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 [34–40]. 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 [41]. In fact, these nanoparticles have also been used in combined delivery protocols of drugs and genes, trapped in the same carrier [42,43]. Moreover, they are readily amenable to surface-coupling of specific ligands, thus allowing the formation of decorated particles for specific targeting of specific cells, tissues or organs, including the brain [36,44]. 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. [41] 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.

1.1. 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 [39,45,46].

A variety of lipid(ic) compounds (i.e., lipids or appropriate solid fatty acids; Fig. 1) have been used as waxy solid lipid matrix in the preparation of SLNs, including Compritol 888 ATO (glyceryl behenate), behenic acid, cetylpalmitate, Precirol ATO 5, stearic acid, Imwitor 900PTM (IMW, 40–50% glyceryl monostearate), tricaprins, 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 cetylpalmitate as the lipid matrix, when compared to Compritol (a mixture of mono-, di- and triglycerides of behenic acid) [47]. 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 the case of solid lipid nanoparticles, or high oil concentrations in the preparation of nanostructured lipid carriers (NLCs) [34,48]. Although the highly ordered crystal packing of lipids in the SLN matrix will ensure a superior physical stability [49], as will the preclusion of distinct lipid (phase) transitions [50], an appropriate balance between stability and drug expulsion is pivotal 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 acid (see Fig. 2). 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 [51]. Interestingly, even hydrophilic surfactants can alter polymorphic transitions in the core of the nanoparticles. Most importantly,

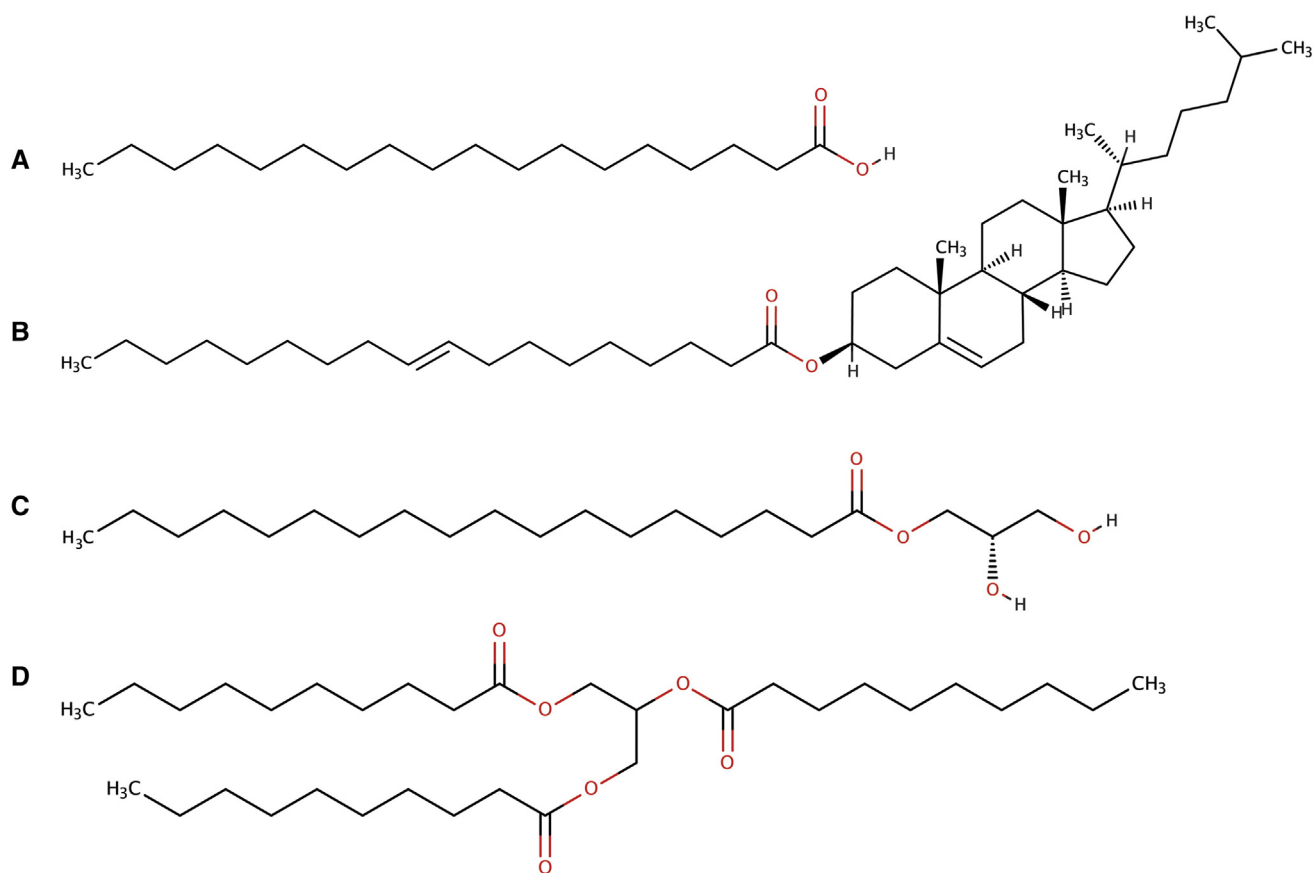


Fig. 1. Representative lipid(ic) structures, i.e., lipids or appropriate solid fatty acids, used as waxy solid lipid matrix in the preparation of cationic SLNs: a) stearic acid, b) cholesteryl oleate, c) glyceryl monostearate, and d) tricaprin.

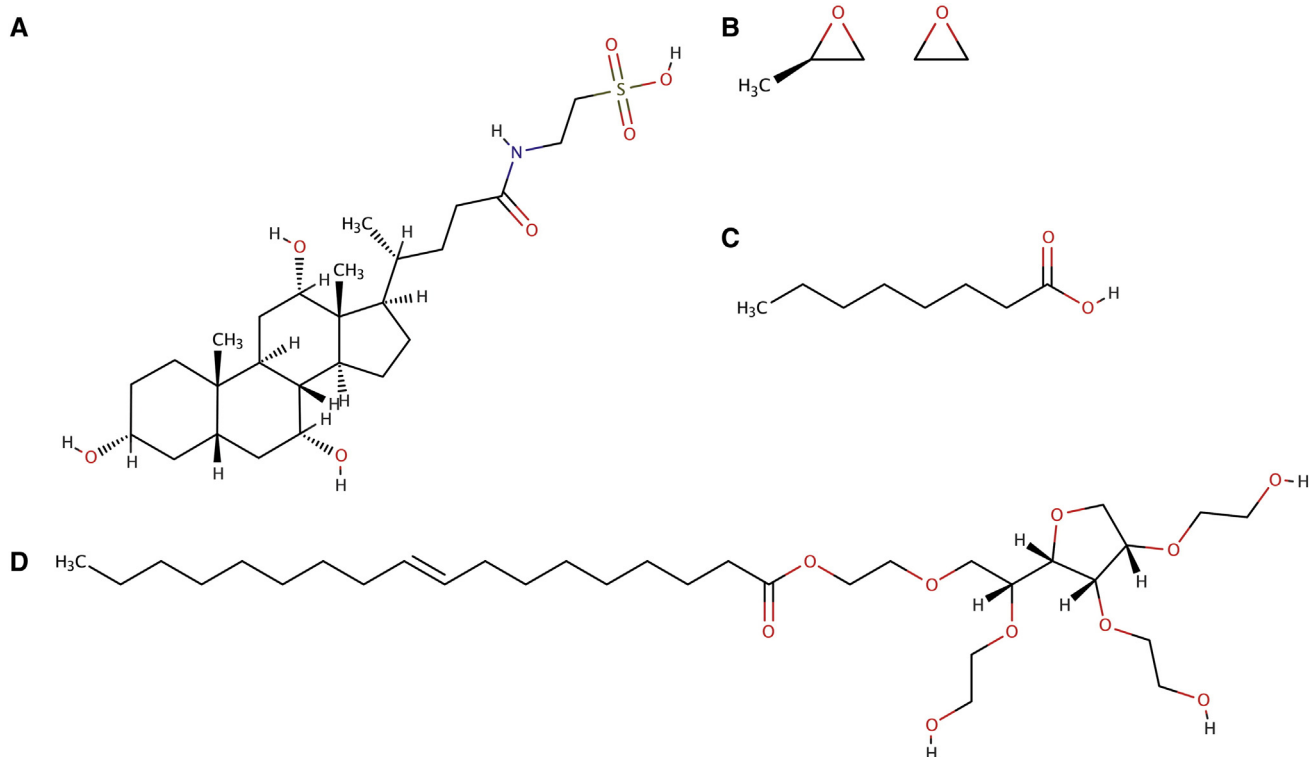


Fig. 2. Representative surfactant structures used as interface stabilizers in cationic SLN formulations: a) taurocholate, b) Pluronic F68 (polyoxyethylene–polyoxypropylene block copolymer), c) octanoic acid, and d) Tween 80.

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 [51,52].

To accommodate the negatively charged nucleic acids, cationic SLNs are prepared by including a positively charged co-surfactant in their formulation (cf. Fig. 3), for example N,N-di-(b-stearoyl)ethyl)-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 [38,53]. Regarding SLN-mediated delivery of nucleic acids, the choice of an appropriate combination of cationic and matrix lipids seems imperative as it may exert a significant impact on the transfection efficiency [47].

A variety of procedures have been described for preparing SLNs [53–57]. 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 [58]. However, the choice of the appropriate method to prepare SLNs should also take into account issues such as 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 [59], (ii) the hot high pressure homogenization method [60], and (iii) the solvent emulsification method [52].

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 [55,61]. 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 [52]. 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 to meet conditions of therapeutic efficacy [62]. 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 (see below) [42].

The high pressure homogenization technique (ii, HPH) is considered a standard procedure in the preparation of SLNs [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 [57,63]. 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 production of cationic SLNs in this manner does not depend on the presence of organic solvents [64]. However, the application of this method is obviously restricted to formulations that resist degradation at the desired temperature and high pressure. Furthermore, as in the 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 [52]. 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 [38,52]. On the other hand, this procedure may raise concerns about the effectivity of the removal of the organic solvent, which may cause undesired toxic effects of the formulation upon its use in vitro and in vivo.

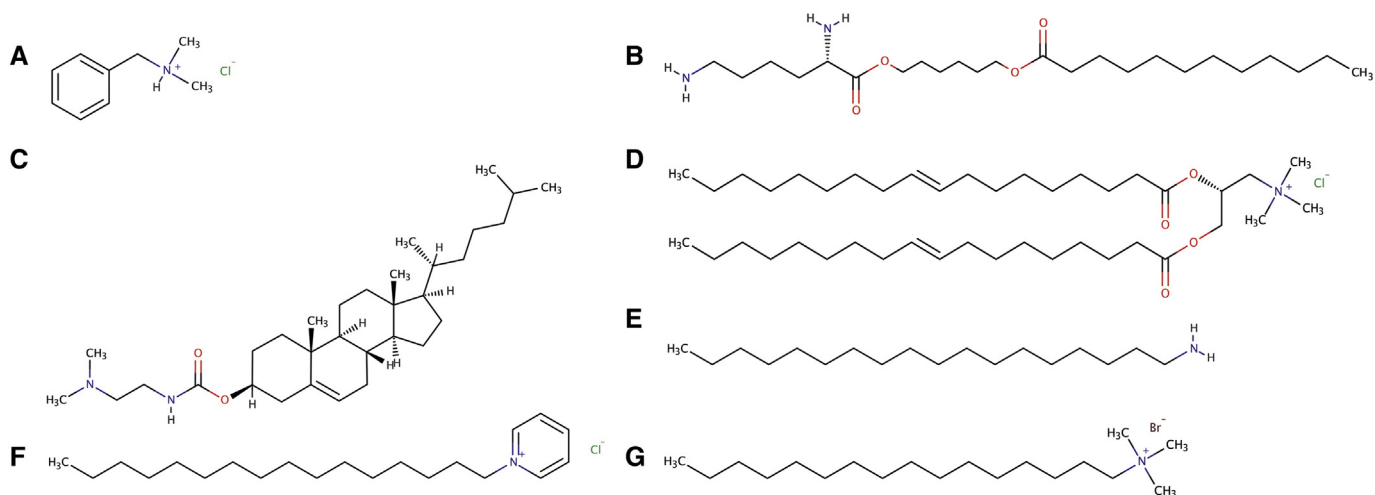


Fig. 3. Representative cationic lipid(ic) structures used in cationic SLN formulations: a) benzalkonium chloride, b) 6-lauroxyhexyl lysinate, c) DC-Chol, d) DOTAP, e) stearylamine, f) cetylpyridinium chloride, and g) cetrimide.

Although the three procedures discussed in the foregoing represent the most commonly applied ones, several other procedures are also in use [56], while 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 [65]. 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 [66]. 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 [67]. Following a different approach, Lobovkina and coworkers [67] 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-containing 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 effectivity 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 the assembly of these types 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.

1.2. 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, including the determination of the coexistence of other colloidal structures, such as liposomes, supercooled melts, and micelles. Sucrose gradient centrifugation of SLN can be used to determine particle size as well as composition, based on the different buoyant densities of particles with different lipid/DNA contents, addressing the question of the presence of multiple particle structures [10].

Indeed, it has been reported that the choice of the lipid matrix in conjunction with that of the cationic lipid appears as the major determinants in the outcome of the efficiency of SLN gene delivery [63]. 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.

1.2.1. 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 the addition of nucleic acids, 'lipoplexes' with sizes up to approx. 500 nm are obtained. Although such diameters are not desirable 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 [68]. 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 [5,69]. 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 observed for cationic liposomes [6,70]. 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 [71–75]. The SLNs (empty particles) are commonly spherical in shape [76], although this may vary and depend on the formulation, some of which may lead to imperfect spheres [73].

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 SLN 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 [77]. 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 [78] and, in cases, a clear lamellar-like structure with a dense core has been observed [73]. As expected, SLN formulations for the delivery of lipid-soluble drugs, showed incorporation of the drugs within the matrix [48,79,80], although also systems have been reported where the drugs were located at the surface of the SLN. Accordingly, the latter formulations did not show sustained release properties [72,81].

1.2.2. 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 [14,28,33,82,83]. 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 [84–91]. 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 [88–90,92,93]. 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) [86,88,89,94]. This interaction reaches the highest ΔS at isoelectricity, which has important implications for the lipoplex geometry, as further discussed below [86,90].

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 the case of the use of lipid vesicles and promoting polymorphic (lipid) transitions, both of which accompany the process of lipoplex assembly [95]. Indeed, the proximity of molecules may allow hydrophobic interactions to take place and contribute to complex formation, driven by a significant entropy increase [96]. 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 [28,87,91,94,97,98]. 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 [99].

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 [88,92,97,100–104]. As result of these reorganizations, lipoplexes can adopt distinct tridimensional ordered multilayered structures (known as finger print structures) with DNA intercalated between the lipid layers [54,104–106]. Three different organizations of the lipids have been described for these interactions, i.e., (i) the lamellar structure ($LC\alpha$), characterized by DNA rods inserted between lipid bilayers, (ii) the intercalated hexagonal structure (H_I), characterized by DNA rods surrounded by three cylindrical lipid micelles arranged on a hexagonal lattice, and (iii) the inverted hexagonal structure (H_{II}), characterized by DNA rods covered with lipid monolayers organized on a hexagonal matrix [54,98,101,107]. Adoption of the latter structure is particularly promoted when dioleoylphosphatidylethanolamine ($C_{18:1}$) is included in the formulation [88,108–110], and strongly facilitates cellular delivery of nucleic acids and their subsequent functional expression [14] (for reviews see [83]). However, also formulations containing cationic lipids capable of adopting the H_I 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. 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 [111]. 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 [111].

These findings suggest that for the 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. 4 [41,73,75,112]. 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 [73]. 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 [112]. However, nuclease treatment revealed that the treated particles still express transfection efficiency, implying that part of the DNA must have been protected from degradation [73]. 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 intimately bound versus loosely bound pools of DNA in bringing about transfection (see Fig. 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 [113].

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 [47]. 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 [69]. 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 lipids mix completely [47,114]. Interestingly, the interaction between SLNs and nucleic acids does not appear to be significantly affected by surfactants [114,115]. 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 [78].

1.2.3. 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 many important physicochemical properties of lipoplexes that bear relevance to their transfection

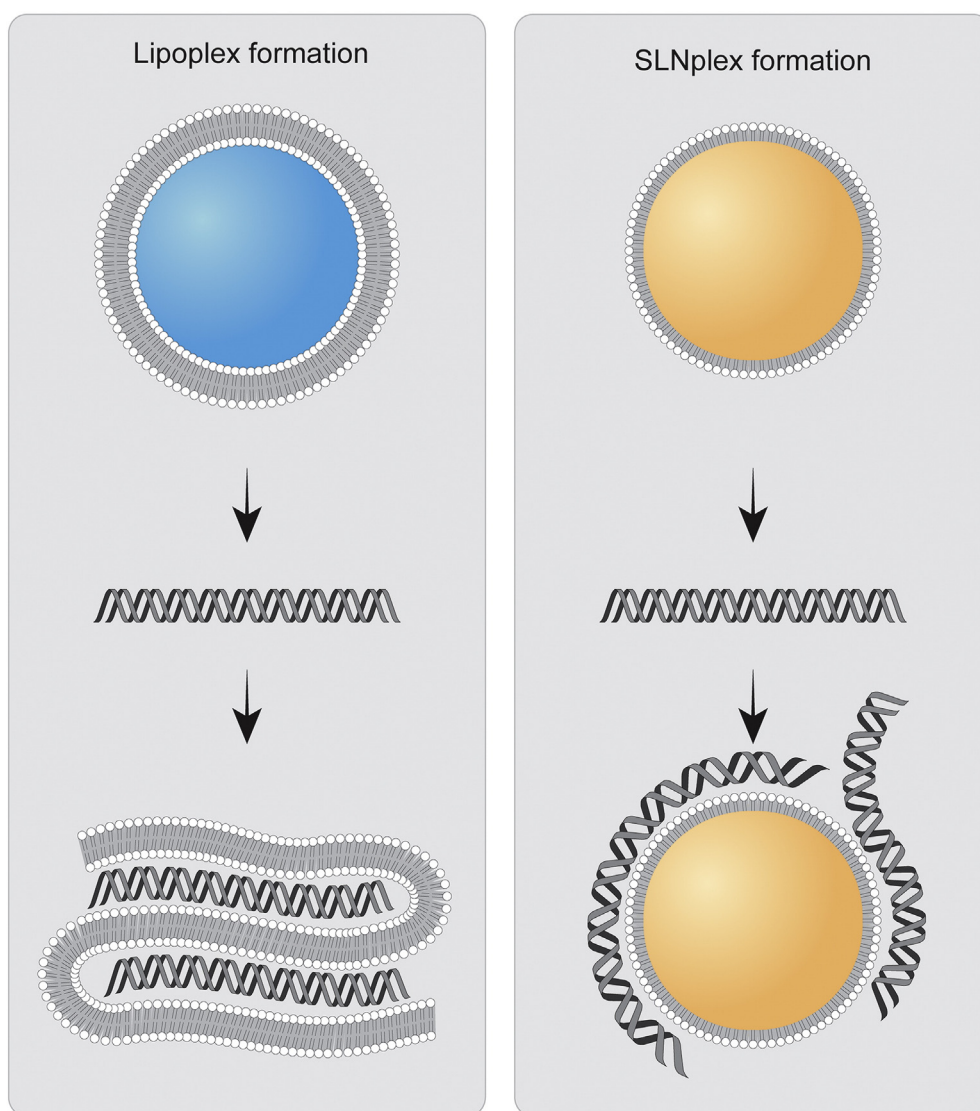


Fig. 4. Schematic representation of the formation of a Lipoplex and an SLNplex. Assembly of lipoplexes is an endothermic process, which is driven by the release of counterions into the bulk solution followed by the binding of the two macro-ions. Initially, the DNA adsorbs at the cationic liposomes surface, with further addition of DNA the liposome structure is entirely reorganized, resulting in fusion and aggregation, which lead 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 limit the internal mixing of DNA into SLN. Presumably, in the 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.

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 [116], 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 [117–119]. 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 [118,120]. For polymers, it has been advocated that the high buffering capacity (i.e. within a pK_a range between 5–6) of some polymers can absorb a significant amount of protons in acidifying endosomes, which drives an inflow of counterions 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 [121]. More recently, direct experimental evidence, favoring endosomal membrane rupture rather than endosomal lysis [14,120], while excluding an effect on the lysosomal pH [122], 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 non-bilayer structures (Fig. 5, 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 the case of applying (ionizable) cationic lipids or, if needed, by inclusion of a helper lipid such as DOPE that may promote and facilitate such a transition [123–128]. Consistently, the inclusion of bilayer-stabilizing components (such as phosphatidylcholine or PEGylated lipids) effectively blocks delivery of cargo and hence transfection efficiency [108,129–131]. Evidently, the non-bilayer configurations referred to also mediate a rapid lipid mixing, which is often taken to suggest a role of membrane fusion. However,

fusion-abrogating non-bilayer phases, such as the H_I phase, do promote delivery, implying that fusion is unlikely the driving force in cationic lipid-mediated delivery [14,83,128].

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 non-ionic surfactant that is capable of enhancing membrane permeability, thereby causing an improvement of transfection efficiency [33,132]. 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 H_{II} -adopting, cationic lipid-based systems while inhibiting that of H_I phase adopting systems, is without effect on transfection when included in SLN formulations [73,115]. 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 endosomolytic molecules such as chloroquine [133], 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 [134]. 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 have been proposed as a mechanism of drug release from these nanoparticles [135]. However, although cargo might be released in this manner from the SLNs, their required escape across the lysosomal membrane remains a mystery, particularly because released nucleic acids are thought to be rapidly degraded in the lysosomal lumen. Currently, it can't be excluded that matrix degradation and ensuing cargo release occur within the cytosol, i.e., after the endosomal escape of SLN (Fig. 5).

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 observations 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 (our unpublished observations), 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 [136]. These results could thus suggest that SLNs, compared to liposomal formulations, could display a higher intracellular stability because 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 approximately 7 days has been observed [137]. 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.

While the internal structure of SLN seems most important in determining the loading and delivery efficiency for lipophilic compounds, it is likely that the surface composition of cationic SLN is the more determinant factor for nucleic acid binding and delivery. This is in the first place a result from the current method of SLN–nucleic acid complex preparation. Because SLN–nucleic acid complexes are generally made by the addition of the nucleic acids to preformed SLN, and SLNs are –unlike liposomes– not likely to restructure upon nucleic acid addition, it is highly unlikely that the nucleic acids end up within the matrix. Instead the nucleic acids are expected to adsorb onto the SLN surface (Fig. 4). Tabatt et al. showed that the nature of the cationic lipid determined DNA binding by SLN [47]. It will be relevant to investigate the effects of the chain length and (un)saturation of the cationic lipid on DNA binding and delivery with SLN, which will also be influenced by the relative fluidity as well as the biodegradability of the matrix lipid.

When lipid nanoparticles were prepared by microfluidic mixing of lipids (DLinkC2-DMA/DSPC/cholesterol/PEG-c-DMA) in ethanol buffer with nucleic acids in aqueous buffer, the resulting nanoparticles showed a nanostructured core with aqueous cavities that contained nucleic acids [138]. Likewise, it would be of interest to investigate the encapsulation of nucleic acids during SLN formation, and evaluate its effect on SLN structure and function. In fact, Lobovkina et al. followed such an approach. By the preformation of electrically neutral siRNA–cationic lipid complexes, the lipophilicity of the siRNA was increased, allowing its encapsulation within the SLN matrix. Interestingly, intradermal injection of the resulting SLN formulation into mice showed prolonged siRNA release over a period of 10 days [10]. Such sustained release of siRNA is indicative of the presence of siRNA within the SLN matrix. Xue et al. further improved the loading efficiency of siRNA into SLN by preparing siRNA–cationic polymer complexes in oil prior to the mixing with solid lipids, generating siRNA-loaded NLC. The NLC formulation showed high encapsulation efficiency of the siRNA, while the release kinetics could be manipulated by varying the oil content and – to a minor extent – the solid lipid composition [136]. This points to the importance of the NLC internal structure in determining its degradation. While NLC appears promising for generating sustained siRNA release, its suitability for the (sustained) delivery of other types of nucleic acids, including plasmid DNA, remains to be investigated. Nevertheless, the enclosure of liquid lipids that is aimed for the formation of NLCs, is challenging. Whether proper mixing will occur is dependent on the type of solid lipids that are used as well as the mixing ratio of liquid and solid lipids. Several reports point to the presence of liquid lipid films or droplets at the surface of the NLC, instead of within the NLC matrix [72,139].

Examination of the position of the cationic component in SLN (/NLC) may shed light on SLN structure and function. To determine the amount of external cationic lipid in lipid nanoparticles a FRET-based lipid mixing assay between the cationic particles and negatively charged DOPS liposomes has been successfully employed [138]. In addition, quenching of cationic lipid fluorescence by means of a non-membrane permeable quencher molecule can be used to determine the amount of cationic lipid within the nanoparticle interior [140]. Furthermore, ^{31}P NMR can be used to measure the mobility of nucleic acids after their complexation with nanoparticles. However, when phospholipid surfactants are used for stabilization of the SLN, their phosphate groups may interfere with the signal from the nucleic acid-based phosphates. This can be prevented by using backbone-modified nucleic acids such as phosphorothioate DNA and RNA, as was shown by Leung et al. [138]. Coarse-grained molecular dynamics simulation has been employed to reveal

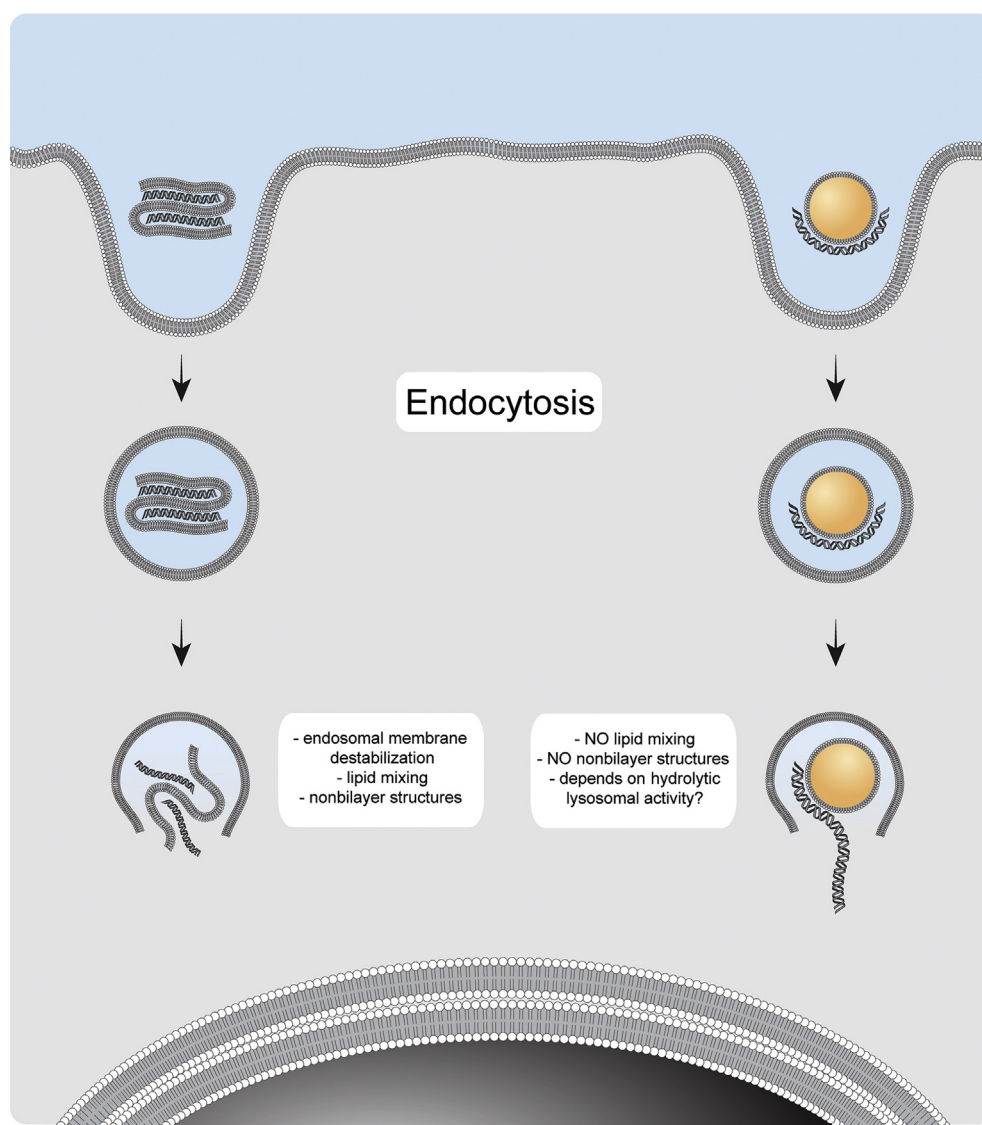


Fig. 5. Schematic representation of internalization and intracellular delivery of nucleic acids from lipoplexes and SLNplexes. Both lipoplexes and SLNplexes are taken up by cells via endocytosis. For lipoplexes, the endosomal membrane destabilization is driven by a process that likely involves a (local) endosomal membrane destabilization, mediated by the ability of the lipid-based nanoparticles to adopt non-bilayer 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).

the overall structure of lipid nanoparticles [138]. Finally, fluorescent labeling of both the carrier and the nucleic acid will allow for intracellular tracking of the complexes, and provide information on the time and place of complex dissociation. Live cell imaging using fluorescent complexes, while visualizing the endosomal membranes, may provide an answer to the question if matrix degradation and payload release occur prior or after endosomal escape. All of the above-mentioned techniques have been successfully used to determine the structure–function relationships of lipid–DNA particles [138], including lipoplexes and genospheres [140], but have yet to find their application in SLN research. Together with data on gene delivery efficiency this information will be valuable to tailor SLN for time-controlled nucleic acid delivery.

2. 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 [50,52]. 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 [52]. Cationic SLN formulations may maintain their activity for over 2 years [73]. 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 [141,142], and an appropriate match between the hydrophilic–lipophilic balance value of the surfactant and the solid lipid is necessary [141,143]. 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 [50,144].

Electrostatic repulsion created by the co-surfactants, generally cationic 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 [145,146]. However, recently it has been shown that the zeta potential of SLNs may not accurately predict SLN stability. Rheological analysis can serve as an

alternative tool in predicting the storage stability of SLNs [147]. Next to the zeta potential, the co-surfactant also seems to affect the size of SLN formulations, the absence of cationic components leading to a larger mean diameter and a higher PDI value compared to SLN formulations containing cationic lipids [148]. These findings confirm that co-surfactants may play a role at the interface of cationic SLN formulations [31,32,78,148]. 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) [27,47].

Lyophilization is an alternative for improving the shelf-stability of SLNs [144,149]. 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 [148, 149]. However, both the freezing and drying processes should be carefully controlled as it may perturb the physical integrity of the particles and eventually lead to their aggregation [38,150]. Moreover, lyophilization can damage plasmid DNA or lead to conformational changes, resulting into loss of activity [151]. However, this can be prevented by the addition of lyoprotectants to the formulation [148,149,152].

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 [38,53,63,150]. 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 [148]. 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 [36,44,73,75]. 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 [144]. The concentrations of cryoprotectants and nanoparticles should be considered for optimization of the process. Thus a nanoparticle concentration around 1% showed better results, while cryoprotectant 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 [45,71,145,150], as previously described for non-cationic SLN formulations [144,149]. Remarkably, in several cases the transfection efficiency was preserved when comparing the functionality of SLN formulations before and after freeze-drying [63,73,132,150,153].

3. Concluding remarks

Clearly, although very compelling, the concept of a magic bullet for targeted drug delivery proves to be a utopic challenge rather than a realistic concept. This also holds in the development of an ideal SLN-based nanocarrier device. While 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. In particular, it is rather unclear how SLNs accommodate their nucleic acid 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, freeze fracture electron microscopy (FFEM), nuclear magnetic resonance (NMR) and electron spin resonance (ESR) spectroscopy. In addition, (FRET-based) lipid mixing assays and molecular

dynamics simulation, in combination with fluorescent particle tracking, could be a useful approach to obtain detailed insight into the interaction of SLNplexes with target membranes. Such studies will be of help in properly evaluating and appreciating the validity of SLNs as suitable nanocarriers for a variety of nucleic acid-based cargos.

Acknowledgments

We thank C.E.P.S. Filho for helping with the illustrations. This research has benefited from the financial support of State of São Paulo Research Foundation (FAPESP, Proc. 2012/01038-9) and the Groningen University Institute for Drug Exploration (GUIDE).

References

- [1] C. Sheridan, Gene therapy finds its niche, *Nat. Biotechnol.* 29 (2011) 121–128. <http://dx.doi.org/10.1038/nbt.1769>.
- [2] C.M. Wiethoff, C.R. Middaugh, Barriers to nonviral gene delivery, *J. Pharm. Sci.* 92 (2003) 203–217. <http://dx.doi.org/10.1002/jps.10286>.
- [3] I.S. Zuhorn, J.B.F.N. Engberts, D. Hoekstra, Gene delivery by cationic lipid vectors: overcoming cellular barriers, *Eur. Biophys. J.* 36 (2007) 349–362. <http://dx.doi.org/10.1007/s00249-006-0092-4>.
- [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 (2011) 1843–1858. <http://dx.doi.org/10.1007/s11095-010-0364-7>.
- [5] C.S. Manno, G.F. Pierce, V.R. Arruda, B. Glader, M. Ragni, J.J. Rasko, 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. <http://dx.doi.org/10.1038/nm1358>.
- [6] F. Mingozzi, J.J. Meulenberg, D.J. Hui, E. Basner-Tschakarjan, N.C. 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. <http://dx.doi.org/10.1182/blood-2008-07-167510>.
- [7] M. Sadelain, E.P. Papapetrou, F.D. Bushman, Safe harbours for the integration of new DNA in the human genome, *Nat. Rev. Cancer* 12 (2012) 51–58. <http://dx.doi.org/10.1038/nrc3179>.
- [8] A. Pathak, S. Patnaik, K.C. Gupta, Recent trends in non-viral vector-mediated gene delivery, *Biotechnol. J.* 4 (2009) 1559–1572. <http://dx.doi.org/10.1002/biot.200900161>.
- [9] J. Wei, J. Jones, J. Kang, A. Card, M. Krimm, P. Hancock, et al., RNA-induced silencing complex-bound small interfering RNA is a determinant of RNA interference-mediated gene silencing in mice, *Mol. Pharmacol.* 79 (2011) 953–963. <http://dx.doi.org/10.1124/mol.110.070409>.
- [10] T.T. Lobovkina, G.B.G. Jacobson, E.E. Gonzalez-Gonzalez, R.P.R. Hickerson, D.D. Leake, R.L.R. Kaspar, et al., In vivo sustained release of siRNA from solid lipid nanoparticles, *ACS Nano* 5 (2011) 9977–9983. <http://dx.doi.org/10.1021/nn203745n>.
- [11] R. Goyal, S.K. Tripathi, S. Tyagi, K. Ravi Ram, K.M. Ansari, Y. Shukla, et al., Gellan gum blended PEI nanocomposites as gene delivery agents: evidences from in vitro and in vivo studies, *Eur. J. Pharm. Biopharm.* 79 (2011) 3–14. <http://dx.doi.org/10.1016/j.ejpb.2011.01.009>.
- [12] G. McLachlan, H. Davidson, E. Holder, L.A. Davies, I.A. Pringle, S.G. Sumner-Jones, et al., Pre-clinical evaluation of three non-viral gene transfer agents for cystic fibrosis after aerosol delivery to the ovine lung, *Gene Ther.* 18 (2011) 996–1005. <http://dx.doi.org/10.1038/gt.2011.55>.
- [13] S.L. Ginn, I.E. Alexander, M.L. Edelstein, M.R. Abedi, J. Wixon, Gene therapy clinical trials worldwide to 2012 – an update, *J. Gene Med.* 15 (2013) 65–77. <http://dx.doi.org/10.1002/jgm.2698>.
- [14] Z.U. Rehman, I.S. Zuhorn, D. Hoekstra, How cationic lipids transfer nucleic acids into cells and across cellular membranes: recent advances, *J. Control. Release* 166 (2013) 46–56. <http://dx.doi.org/10.1016/j.jconrel.2012.12.014>.
- [15] M.J. Hope, Enhancing siRNA delivery by employing lipid nanoparticles, *Ther. Deliv.* 5 (2014) 663–673. <http://dx.doi.org/10.4155/tde.14.37>.
- [16] J.R. Viola, S. El Andaloussi, I.I. Oprea, C.I.E. Smith, Non-viral nanovectors for gene delivery: factors that govern successful therapeutics, *Expert Opin. Drug Deliv.* 7 (2010) 721–735. <http://dx.doi.org/10.1517/17425241003716810>.
- [17] X. Guo, L. Huang, Recent advances in nonviral vectors for gene delivery, *Acc. Chem. Res.* 45 (2012) 971–979. <http://dx.doi.org/10.1021/ar200151m>.
- [18] H. Cui, Y. Feng, W. Ren, T. Zeng, H. Lv, Y. Pan, Strategies of large scale synthesis of monodisperse nanoparticles, *Recent Pat. Nanotechnol.* 3 (2009) 32–41.
- [19] J. Kim, I. Hwang, D. Britain, T.D. Chung, Y. Sun, D.-H. Kim, Microfluidic approaches for gene delivery and gene therapy, *Lab Chip* 11 (2011) 3941–3948. <http://dx.doi.org/10.1039/c1lc20766k>.
- [20] J. Clement, K. Kiefer, A. Kimpfler, P. Garidel, R. Peschka-Süss, Large-scale production of lipoplexes with long shelf-life, *Eur. J. Pharm. Biopharm.* 59 (2005) 35–43. <http://dx.doi.org/10.1016/j.ejpb.2004.06.001>.
- [21] T.A. Balbino, A.R. Azzoni, L.G. de La Torre, Microfluidic devices for continuous production of pDNA/cationic liposome complexes for gene delivery and vaccine therapy, *Colloids Surf., B* 111C (2013) 203–210. <http://dx.doi.org/10.1016/j.colsurfb.2013.04.003>.
- [22] N. Saranya, A. Moorthi, S. Saravanan, M.P. Devi, N. Selvamurugan, Chitosan and its derivatives for gene delivery, *Int. J. Biol. Macromol.* 48 (2011) 234–238. <http://dx.doi.org/10.1016/j.ijbiomac.2010.11.013>.

- [23] W. Khan, H. Hosseinkhani, D. Ickowicz, P.-D. Hong, D.-S. Yu, A.J. Domb, Polysaccharide gene transfection agents, *Acta Biomater.* 8 (2012) 4224–4232. <http://dx.doi.org/10.1016/j.actbio.2012.09.022>.
- [24] V. Sokolova, M. Epple, Inorganic nanoparticles as carriers of nucleic acids into cells, *Angew. Chem. Int. Ed. Engl.* 47 (2008) 1382–1395. <http://dx.doi.org/10.1002/anie.200703039>.
- [25] K. Chaturvedi, K. Ganguly, A.R. Kulkarni, V.H. Kulkarni, M.N. Nadagouda, W.E. Rudzinski, et al., Cyclodextrin-based siRNA delivery nanocarriers: a state-of-the-art review, *Expert Opin. Drug Deliv.* 8 (2011) 1455–1468. <http://dx.doi.org/10.1517/17425247.2011.610790>.
- [26] C. Ortiz Mellet, J.M. García Fernández, J.M. Benito, Cyclodextrin-based gene delivery systems, *Chem. Soc. Rev.* 40 (2011) 1586–1608. <http://dx.doi.org/10.1039/c0cs00019a>.
- [27] R. Laga, R. Carlisle, M. Tangney, K. Ulbrich, L.W. Seymour, Polymer coatings for delivery of nucleic acid therapeutics, *J. Control. Release* 161 (2012) 537–553. <http://dx.doi.org/10.1016/j.jconrel.2012.02.013>.
- [28] A. Elouahabi, J. Ruyschaert, Formation and intracellular trafficking of lipoplexes and polyplexes, *Mol. Ther.* 11 (2005) 336–347. <http://dx.doi.org/10.1016/j.jymthe.2004.12.006>.
- [29] V.P. Torchilin, Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery, *Biopolymers* 90 (2008) 604–610. <http://dx.doi.org/10.1002/bip.20989>.
- [30] A. Bolhassani, Potential efficacy of cell-penetrating peptides for nucleic acid and drug delivery in cancer, *Biochim. Biophys. Acta* 1816 (2011) 232–246. <http://dx.doi.org/10.1016/j.bbcan.2011.07.006>.
- [31] B. Ruozi, F. Forni, R. Battini, M.A. Vandelli, Cationic liposomes for gene transfection, *J. Drug Target.* 11 (2003) 407–414. <http://dx.doi.org/10.1080/10611860310001655600>.
- [32] H.-S. Zhou, D.-P. Liu, C.-C. Liang, Challenges and strategies: the immune responses in gene therapy, *Med. Res. Rev.* 24 (2004) 748–761. [http://dx.doi.org/10.1002/\(ISSN\)1098-1128](http://dx.doi.org/10.1002/(ISSN)1098-1128).
- [33] M.L. Bondi, E.F. Craparo, Solid lipid nanoparticles for applications in gene therapy: a review of the state of the art, *Expert Opin. Drug Deliv.* 7 (2010) 7–18. <http://dx.doi.org/10.1517/17425240903362410>.
- [34] H. Bunjes, Lipid nanoparticles for the delivery of poorly water-soluble drugs, *J. Pharm. Pharmacol.* 62 (2010) 1637–1645. <http://dx.doi.org/10.1111/j.2042-7158.2010.01024.x>.
- [35] S.A. Wissing, O. Kayser, R.H. Müller, Solid lipid nanoparticles for parenteral drug delivery, *Adv. Drug Deliv. Rev.* 56 (2004) 1257–1272. <http://dx.doi.org/10.1016/j.addr.2003.12.002>.
- [36] P.P. Blasi, S.S. Giovagnoli, A.A. Schoubben, M.M. Ricci, C.C. Rossi, Solid lipid nanoparticles for targeted brain drug delivery, *Adv. Drug Deliv. Rev.* 59 (2007) 454–477. <http://dx.doi.org/10.1016/j.addr.2007.04.011>.
- [37] M. Muchow, P. Maincent, R.H. Müller, Lipid nanoparticles with a solid matrix (SLN, NLC, LDC) for oral drug delivery, *Drug Dev. Ind. Pharm.* 34 (2008) 1394–1405. <http://dx.doi.org/10.1080/03639040802130061>.
- [38] K. Manjunath, J.S. Reddy, V. Venkateswarlu, Solid lipid nanoparticles as drug delivery systems, *Methods Find. Exp. Clin. Pharmacol.* 27 (2005) 127–144. <http://dx.doi.org/10.1358/mf.2005.27.2.876286>.
- [39] R.H. Müller, K. Mäder, S. Gohla, Solid lipid nanoparticles (SLN) for controlled drug delivery – a review of the state of the art, *Eur. J. Pharm. Biopharm.* 50 (2000) 161–177.
- [40] L.H. du Plessis, E.B. Marais, F. Mohammed, A.F. Kotze, Applications of lipid based formulation technologies in the delivery of biotechnology-based therapeutics, *Curr. Pharm. Biotechnol.* 15 (2014) 659–672.
- [41] C. Olbrich, U. Bakowsky, C.M. Lehr, R.H. Müller, C. Kneuer, Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA, *J. Control. Release* 77 (2001) 345–355.
- [42] M.B. de Jesus, A. Radaic, I.S. Zuhorn, E. Paula, Microemulsion extrusion technique: a new method to produce lipid nanoparticles, *J. Nanopart. Res.* 15 (2013) 1–15.
- [43] Y.H. Yu, E. Kim, D.E. Park, G. Shim, S. Lee, Y.B. Kim, et al., Cationic solid lipid nanoparticles for co-delivery of paclitaxel and siRNA, *Eur. J. Pharm. Biopharm.* 80 (2012) 268–273. <http://dx.doi.org/10.1016/j.ejpb.2011.11.002>.
- [44] S. Patel, S. Chavhan, H. Soni, A.K. Babbar, R. Mathur, A.K. Mishra, et al., Brain targeting of risperidone-loaded solid lipid nanoparticles by intranasal route, *J. Drug Target.* (2010). <http://dx.doi.org/10.3109/1061186X.2010.523787>.
- [45] W. Yu, C. Liu, J. Ye, W. Zou, N. Zhang, W. Xu, Novel cationic SLN containing a synthesized single-tailed lipid as a modifier for gene delivery, *Nanotechnology* 20 (2009) 215102. <http://dx.doi.org/10.1088/0957-4484/20/21/215102>.
- [46] A. Garud, D. Singh, N. Garud, Solid lipid nanoparticles (SLN): method, characterization and applications, *Int. Curr. Pharm. J.* 1 (2012) 384–393.
- [47] K. Tabatt, M. Sameti, C. Olbrich, R.H. Müller, C.-M. Lehr, Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer, *Eur. J. Pharm. Biopharm.* 57 (2004) 155–162. <http://dx.doi.org/10.1016/j.ejpb.2003.10.015>.
- [48] V. Jenning, A.F. Thünemann, S.H. Gohla, Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids, *Int. J. Pharm.* 199 (2000) 167–177.
- [49] V. Jenning, S. Gohla, Comparison of wax and glyceride solid lipid nanoparticles (SLN), *Int. J. Pharm.* 196 (2000) 219–222.
- [50] C. Freitas, R.H. Müller, Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase, *Eur. J. Pharm. Biopharm.* 47 (1999) 125–132. [http://dx.doi.org/10.1016/S0939-6411\(98\)00074-5](http://dx.doi.org/10.1016/S0939-6411(98)00074-5).
- [51] H. Bunjes, M.H.J. Koch, K. Westesen, Influence of emulsifiers on the crystallization of solid lipid nanoparticles, *J. Pharm. Sci.* 92 (2003) 1509–1520. <http://dx.doi.org/10.1002/jps.10413>.
- [52] W. Mehnert, K. Mäder, Solid lipid nanoparticles: production, characterization and applications, *Adv. Drug Deliv. Rev.* 47 (2001) 165–196.
- [53] A.A. Attama, SLN, NLC, LDC: state of the art in drug and active delivery, *Recent Pat. Drug Deliv. Formul.* 5 (2011) 178–187.
- [54] C. Tros de Ilarduya, Y. Sun, N. Düzgüneş, Gene delivery by lipoplexes and polyplexes, *Eur. J. Pharm. Sci.* 40 (2010) 159–170. <http://dx.doi.org/10.1016/j.ejps.2010.03.019>.
- [55] R. Parhi, P. Suresh, Preparation and characterization of solid lipid nanoparticles—a review, *Curr. Drug Discov. Technol.* 9 (2012) 2–16.
- [56] F. Corrias, F. Lai, New methods for lipid nanoparticles preparation, *Recent Pat. Drug Deliv. Formul.* 5 (2011) 201–213.
- [57] E.B. Souto, P. Severino, M.H.A. Santana, S.C. Pinho, Solid lipid nanoparticles: classical methods of lab production, *Quim. Nova* 34 (2011) 1762–1769.
- [58] A. Radomska-Soukharev, Stability of lipid excipients in solid lipid nanoparticles, *Adv. Drug Deliv. Rev.* 59 (2007) 411–418. <http://dx.doi.org/10.1016/j.addr.2007.04.004>.
- [59] M.R. Gasco, Method for producing solid lipid microspheres having a narrow size distribution, 5250236, 1993.
- [60] R. Muller, J. Lucks, Arzneistoffträger Aus Festen Lipid Teilchen – Feste Lipid Nanosphären (SLN), EP 0605497, 1996.
- [61] Z. Jiang, C. Sun, Z. Yin, F. Zhou, L. Ge, X. Liu, et al., Comparison of two kinds of nanomedicine for targeted gene therapy: premodified or postmodified gene delivery systems, *Int. J. Nanomedicine* 7 (2012) 2019–2031. <http://dx.doi.org/10.2147/IJN.S30928>.
- [62] R.H. Müller, R. Shegokar, C.M. Keck, 20 years of lipid nanoparticles (SLN and NLC): present state of development and industrial applications, *Curr. Drug Discov. Technol.* 8 (2011) 207–227.
- [63] K. Tabatt, C. Kneuer, M. Sameti, C. Olbrich, R.H. Müller, C.-M. Lehr, et al., Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes, *J. Control. Release* 97 (2004) 321–332. <http://dx.doi.org/10.1016/j.jconrel.2004.02.029>.
- [64] A. Dingler, S. Gohla, Production of solid lipid nanoparticles (SLN): scaling up feasibility, *J. Microencapsul.* 19 (2002) 11–16. <http://dx.doi.org/10.1080/02652040010018056>.
- [65] L.S. Battaglia, R. Cavalli, M. Trotta, Method for the preparation of solid lipid micro and nanoparticles, WO2008149215A2, 2008.
- [66] Zhang, Yun, Shen, Chen, Yao, Chen, Formation of solid lipid nanoparticles in a microchannel system with a cross-shaped junction, *Chem. Eng. Sci.* 63 (2008) 5600–5605. <http://dx.doi.org/10.1016/j.ces.2008.08.005>.
- [67] T.A. Balbino, N.T. Aoki, A.A.M. Gasperini, C.L.P. Oliveira, A.R. Azzoni, L.P. Cavalcanti, et al., Continuous flow production of cationic liposomes at high lipid concentration in microfluidic devices for gene delivery applications, *Chem. Eng. J.* 226 (2013) 423–433. <http://dx.doi.org/10.1016/j.cej.2013.04.053>.
- [68] Y.H. Bae, K. Park, Targeted drug delivery to tumors: myths, reality and possibility, *J. Control. Release* 153 (2011) 198–205. <http://dx.doi.org/10.1016/j.jconrel.2011.06.001>.
- [69] A. del Pozo-Rodríguez, D. Delgado, M.A. Solinís, A.R. Gascón, J.L. Pedraz, Solid lipid nanoparticles: formulation factors affecting cell transfection capacity, *Int. J. Pharm.* 339 (2007) 261–268. <http://dx.doi.org/10.1016/j.ijpharm.2007.03.015>.
- [70] H.R. Kim, I.K. Kim, K.H. Bae, S.H. Lee, Y. Lee, T.G. Park, Cationic solid lipid nanoparticles reconstituted from low density lipoprotein components for delivery of siRNA, *Mol. Pharm.* 5 (2008) 622–631. <http://dx.doi.org/10.1021/mp8000233>.
- [71] C. Carrillo, N. Sánchez-Hernández, E. García-Montoya, P. Pérez-Lozano, J.M. Suñé-Negre, J.R. Tico, et al., DNA delivery via cationic solid lipid nanoparticles (SLNs), *Eur. J. Pharm. Sci.* 49 (2013) 157–165. <http://dx.doi.org/10.1016/j.ejps.2013.02.011>.
- [72] K. Jores, W. Mehnert, M. Drechsler, H. Bunjes, C. Johann, K. Mäder, Investigations on the structure of solid lipid nanoparticles (SLN) and oil-loaded solid lipid nanoparticles by photon correlation spectroscopy, field-flow fractionation and transmission electron microscopy, *J. Control. Release* 95 (2004) 217–227. <http://dx.doi.org/10.1016/j.jconrel.2003.11.012>.
- [73] M.B. de Jesus, A. Radaic, W.L.J. Hinrichs, C.V. Ferreira, E. de Paula, D. Hoekstra, et al., Inclusion of the helper lipid dioleoyl-phosphatidylethanolamine in solid lipid nanoparticles inhibits their transfection efficiency, *J. Biomed. Nanotechnol.* 10 (2014) 355–365. <http://dx.doi.org/10.1166/jbnn.2014.1804>.
- [74] W. Yu, C. Liu, Y. Liu, N. Zhang, W. Xu, Mannan-modified solid lipid nanoparticles for targeted gene delivery to alveolar macrophages, *Pharm. Res.* 27 (2010) 1584–1596. <http://dx.doi.org/10.1007/s11095-010-0149-z>.
- [75] E. Vighi, B. Ruozi, M. Montanari, R. Battini, E. Leo, pDNA condensation capacity and in vitro gene delivery properties of cationic solid lipid nanoparticles, *Int. J. Pharm.* 389 (2010) 254–261. <http://dx.doi.org/10.1016/j.ijpharm.2010.01.030>.
- [76] G. Montana, M.L. Bondi, R. Carotta, P. Picone, E.F. Craparo, P.L. San Biagio, et al., Employment of cationic solid-lipid nanoparticles as RNA carriers, *Bioconjug. Chem.* 18 (2007) 302–308. <http://dx.doi.org/10.1021/bc0601166>.
- [77] E.B. Souto, A.J. Almeida, R.H. Müller, Lipid nanoparticles (SLN, NLC) for cutaneous drug delivery: structure, protection and skin effects, *J. Biomed. Nanotechnol.* 3 (2007) 317–331. <http://dx.doi.org/10.1166/jbnn.2007.049>.
- [78] E.E. Vighi, E.E. Leot, M.M. Montanari, A.A. Mucci, M.M. Hanuskuva, V.V. Iannucelli, Structural investigation and intracellular trafficking of a novel multicomposite cationic solid lipid nanoparticle platform as a pDNA carrier, *Ther. Deliv.* 2 (2011) 1419–1435. <http://dx.doi.org/10.4155/tde.11.118>.
- [79] V. Jenning, K. Mäder, S.H. Gohla, Solid lipid nanoparticles (SLN) based on binary mixtures of liquid and solid lipids: a ¹H-NMR study, *Int. J. Pharm.* 205 (2000) 15–21.
- [80] M. García-Fuentes, M.J. Alonso, D. Torres, Design and characterization of a new drug nanocarrier made from solid-liquid lipid mixtures, *J. Colloid Interface Sci.* 285 (2005) 590–598. <http://dx.doi.org/10.1016/j.jcis.2004.10.012>.
- [81] J. Pietkiewicz, M. Sznitowska, M. Placzek, The expulsion of lipophilic drugs from the cores of solid lipid microspheres in diluted suspensions and in concentrates, *Int. J. Pharm.* 310 (2006) 64–71. <http://dx.doi.org/10.1016/j.ijpharm.2005.11.038>.

- [82] P.P. Karmali, A. Chaudhuri, Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises, *Med. Res. Rev.* 27 (2007) 696–722. <http://dx.doi.org/10.1002/med.20090>.
- [83] L. Wasungu, D. Hoekstra, Cationic lipids, lipoplexes and intracellular delivery of genes, *J. Control. Release* 116 (2006) 255–264. <http://dx.doi.org/10.1016/j.jconrel.2006.06.024>.
- [84] C.H. Spink, J.B. Chaires, Thermodynamics of the binding of a cationic lipid to DNA, *J. Am. Chem. Soc.* 119 (1997) 10920–10928.
- [85] R. Bruinsma, Electrostatics of DNA–cationic lipid complexes: isoelectric instability, *Eur. Phys. J. E Soft Matter* 4 (1998) 75–88.
- [86] D. Harries, S. May, W.M. Gelbart, A. Ben-Shaul, Structure, stability, and thermodynamics of lamellar DNA–lipid complexes, *Biophys. J.* 75 (1998) 159–173. [http://dx.doi.org/10.1016/S0006-3495\(98\)77503-4](http://dx.doi.org/10.1016/S0006-3495(98)77503-4).
- [87] N.J. Zuidam, D. Hirsch-Lerner, S. Margulies, Y. Barenholz, Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency, *Biochim. Biophys. Acta* 1419 (1999) 207–220.
- [88] M.T. Kennedy, E.V. Pozharski, V.A. Rakhmanova, R.C. Macdonald, Factors governing the assembly of cationic phospholipid–DNA complexes, *Biophys. J.* 78 (2000) 1620–1633. [http://dx.doi.org/10.1016/S0006-3495\(00\)76714-2](http://dx.doi.org/10.1016/S0006-3495(00)76714-2).
- [89] P.C.A. Barreleiro, G. Olofsson, P. Alexandridis, Interaction of DNA with cationic vesicles: a calorimetric study, *J. Phys. Chem. B* 104 (2000) 7795–7802. <http://dx.doi.org/10.1021/jp000636c>.
- [90] K. Wagner, D. Harries, S. May, V. Kahl, J.O. Rädler, A. Ben-Shaul, Direct evidence for counterion release upon cationic lipid–DNA condensation, *Langmuir* 16 (2000) 303–306. [http://dx.doi.org/10.1016/S0006-3495\(00\)76720-8](http://dx.doi.org/10.1016/S0006-3495(00)76720-8).
- [91] D. Matulis, I. Rouzina, V.A. Bloomfield, Thermodynamics of cationic lipid binding to DNA and DNA condensation: roles of electrostatics and hydrophobicity, *J. Am. Chem. Soc.* 124 (2002) 7331–7342.
- [92] S.J. Eastman, C. Siegel, J. Tournant, A.E. Smith, S.H. Cheng, R.K. Scheule, Biophysical characterization of cationic lipid: DNA complexes, *Biochim. Biophys. Acta* 1325 (1997) 41–62. [http://dx.doi.org/10.1016/S0005-2736\(96\)00242-8](http://dx.doi.org/10.1016/S0005-2736(96)00242-8).
- [93] E. Pozharski, R.C. Macdonald, Lipoplex thermodynamics: determination of DNA–cationic lipid interaction energies, *Biophys. J.* 85 (2003) 3969–3978. [http://dx.doi.org/10.1016/S0006-3495\(03\)74811-5](http://dx.doi.org/10.1016/S0006-3495(03)74811-5).
- [94] K. Shirahama, K. Takashima, N. Takisawa, Interaction between dodecyltrimethylammonium chloride and DNA, *Bull. Chem. Soc. Jpn* 60 (1987) 43–47.
- [95] D. Hirsch-Lerner, Y. Barenholz, Hydration of lipoplexes commonly used in gene delivery: follow-up by laurdan fluorescence changes and quantification by differential scanning calorimetry, *Biochim. Biophys. Acta* 1461 (1999) 47–57.
- [96] T. Lazaridis, *Hydrophobic Effect*, John Wiley & Sons, Ltd., 2013. <http://dx.doi.org/10.1021/ar010058y>.
- [97] V. Oberle, U. Bakowsky, I.S. Zuhorn, D. Hoekstra, Lipoplex formation under equilibrium conditions reveals a three-step mechanism, *Biophys. J.* 79 (2000) 1447–1454. [http://dx.doi.org/10.1016/S0006-3495\(00\)76396-X](http://dx.doi.org/10.1016/S0006-3495(00)76396-X).
- [98] B. Ma, S. Zhang, H. Jiang, B. Zhao, H. Lv, Lipoplex morphologies and their influences on transfection efficiency in gene delivery, *J. Control. Release* 123 (2007) 184–194. <http://dx.doi.org/10.1016/j.jconrel.2007.08.022>.
- [99] C.S. Braum, G.S. Jas, S. Choosakoonkriang, G.S. Koe, The structure of DNA within cationic lipid/DNA complexes, *Biophys. J.* 84 (2003) 1114–1123.
- [100] I.S. Kikuchi, A.M. Carmona-Ribeiro, Interactions between DNA and synthetic cationic liposomes, *J. Phys. Chem. B* 104 (2000) 2829–2835.
- [101] V. Pector, J. Backmann, D. Maes, M. Vandenbranden, J.M. Ruysschaert, Biophysical and structural properties of DNA_{diC(14)}-amidine complexes. Influence of the DNA/lipid ratio, *J. Biol. Chem.* 275 (2000) 29533–29538. <http://dx.doi.org/10.1074/jbc.M909996199>.
- [102] H. Gershon, R. Ghirlando, S.B. Guttman, A. Minsky, Mode of formation and structural features of DNA–cationic liposome complexes used for transfection, *Biochemistry* 32 (1993) 7143–7151.
- [103] B. Sternberg, F.L. Sorgi, L. Huang, New structures in complex formation between DNA and cationic liposomes visualized by freeze–fracture electron microscopy, *FEBS Lett.* 356 (1994) 361–366.
- [104] D.D. Lasic, H. Strey, M.C.A. Stuart, R. Podgornik, P.M. Frederik, The structure of DNA–liposome complexes, *J. Am. Chem. Soc.* 119 (1997) 832–833. <http://dx.doi.org/10.1021/ja962713g>.
- [105] J. Smisterová, A. Wagenaar, M.C. Stuart, E. Polushkin, G. ten Brinke, R. Hulst, et al., Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleoylphosphatidylethanolamine–DNA complexes and the efficiency of gene delivery, *J. Biol. Chem.* 276 (2001) 47615–47622. <http://dx.doi.org/10.1074/jbc.M106199200>.
- [106] J.O. Rädler, I. Koltover, T. Salditt, C.R. Safinya, Structure of DNA–cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, *Science* 275 (1997) 810–814.
- [107] P.C.A. Barreleiro, R.P. May, B.R. Lindman, Mechanism of formation of DNA–cationic vesicle complexes, *Faraday Discuss.* 122 (2002) 191–201. <http://dx.doi.org/10.1039/b200796g>.
- [108] I. Koltover, T. Salditt, J.O. Rädler, C.R. Safinya, An inverted hexagonal phase of cationic liposome–DNA complexes related to DNA release and delivery, *Science* 281 (1998) 78–81.
- [109] V.A. Rakhmanova, T.J. McIntosh, R.C. Macdonald, Effects of dioleoylphosphatidylethanolamine on the activity and structure of O-alkyl phosphatidylcholine–DNA transfection complexes, *Cell. Mol. Biol. Lett.* 5 (2000) 51–66.
- [110] C.R. Safinya, Structures of lipid–DNA complexes: supramolecular assembly and gene delivery, *Curr. Opin. Struct. Biol.* 11 (2001) 440–448. [http://dx.doi.org/10.1016/S0959-440X\(00\)00230-X](http://dx.doi.org/10.1016/S0959-440X(00)00230-X).
- [111] V. Rullaud, M. Siragusa, A. Cumbo, D. Gygax, P. Shahgaldian, DNA surface coating of calixarene-based nanoparticles: a sequence-dependent binding mechanism, *Chem. Commun.* 48 (2012) 12186–12188. <http://dx.doi.org/10.1039/c2cc36859e>.
- [112] K. Helltunen, P. Shahgaldian, Self-assembly of amphiphilic calixarenes and resorcinarenes in water, *New J. Chem.* 34 (2010) 2704–2714. <http://dx.doi.org/10.1039/c0nj00123f>.
- [113] J. Ye, A. Wang, C. Liu, Z. Chen, N. Zhang, Anionic solid lipid nanoparticles supported on protamine/DNA complexes, *Nanotechnology* 19 (2008) 285708. <http://dx.doi.org/10.1088/0957-4484/19/28/285708>.
- [114] S. Doktorovova, R. Shegokar, E. Rakovsky, E. Gonzalez-Mira, C.M. Lopes, A.M. Silva, et al., Cationic solid lipid nanoparticles (cSLN): structure, stability and DNA binding capacity correlation studies, *Int. J. Pharm.* 420 (2011) 341–349. <http://dx.doi.org/10.1016/j.ijpharm.2011.08.042>.
- [115] R. Asatjarit, S.-I. Lorenzen, S. Sirivichayakul, K. Ruxrungham, U. Ruktanonchai, G.C. Ritthidej, Effect of solid lipid nanoparticles formulation compositions on their size, zeta potential and potential for in vitro pHIS-HIV-hugag transfection, *Pharm. Res.* 24 (2007) 1098–1107. <http://dx.doi.org/10.1007/s11095-007-9234-3>.
- [116] B. Moghaddam, S.E. McNeil, Q. Zheng, A.R. Mohammed, Y. Perrie, Exploring the correlation between lipid packaging in lipoplexes and their transfection efficacy, *Pharmaceutics* 3 (2011) 848–864. <http://dx.doi.org/10.3390/pharmaceutics3040848>.
- [117] R.F. Minchin, S. Yang, Endosomal disruptors in non-viral gene delivery, *Expert Opin. Drug Deliv.* 7 (2010) 331–339. <http://dx.doi.org/10.1517/17425240903512931>.
- [118] A.K. Varkouhi, M. Scholte, G. Storm, H.J. Haisma, Endosomal escape pathways for delivery of biologicals, *J. Control. Release* 151 (2011) 220–228. <http://dx.doi.org/10.1016/j.jconrel.2010.11.004>.
- [119] W. Liang, J.K.W. Lam, Endosomal escape pathways for non-viral nucleic acid delivery systems, *Intechopen.com*, 10.5772/46006InTech, 2012.
- [120] Z.U. Rehman, D. Hoekstra, I.S. Zuhorn, On the mechanism of polyplex- and lipoplex-mediated delivery of nucleic acids: real-time visualization of transient membrane destabilization without endosomal lysis, *ACS Nano* (2013). <http://dx.doi.org/10.1021/nn3049494>.
- [121] J.-P. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, *Chimia* 51 (1997) 1–2.
- [122] R.V. Benjaminsen, M.A. Matthebjerg, J.R. Henriksen, S.M. Moghimi, T.L. Andresen, The possible “proton sponge” effect of polyethylenimine (PEI) does not include change in lysosomal pH, *Mol. Ther.* 21 (2013) 149–157. <http://dx.doi.org/10.1038/mt.2012.185>.
- [123] P. Pinnaduwage, L. Schmitt, L. Huang, Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells, *Biochim. Biophys. Acta* 985 (1989) 33–37.
- [124] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer, *Biochim. Biophys. Acta* 1235 (1995) 289–295.
- [125] O. Zelfhati, F. Szoka, Mechanism of oligonucleotide release from cationic liposomes, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 11493–11498.
- [126] E.R. Lee, J. Marshall, C.S. Siegel, C. Jiang, N.S. Yew, M.R. Nichols, et al., Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung, *Hum. Gene Ther.* 7 (1996) 1701–1717. <http://dx.doi.org/10.1089/hum.1996.7.14-1701>.
- [127] K.W. Mok, P.R. Cullis, Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA, *Biophys. J.* 73 (1997) 2534–2545. [http://dx.doi.org/10.1016/S0006-3495\(97\)78282-1](http://dx.doi.org/10.1016/S0006-3495(97)78282-1).
- [128] I.S. Zuhorn, U. Bakowsky, E. Polushkin, W.H. Visser, M.C.A. Stuart, J.B.F.N. Engberts, et al., Nonbilayer phase of lipoplex–membrane mixture determines endosomal escape of genetic cargo and transfection efficiency, *Mol. Ther.* 11 (2005) 801–810. <http://dx.doi.org/10.1016/j.ythm.2004.12.018>.
- [129] I. Wrobel, D. Collins, Fusion of cationic liposomes with mammalian cells occurs after endocytosis, *Biochim. Biophys. Acta* 1235 (1995) 296–304.
- [130] I.M. Hafez, P.R. Cullis, Roles of lipid polymorphism in intracellular delivery, *Adv. Drug Deliv. Rev.* 47 (2001) 139–148.
- [131] F. Shi, L. Wasungu, A. Nomden, M.C.A. Stuart, E. Polushkin, J.B.F.N. Engberts, et al., Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions, *Biochem. J.* 366 (2002) 333–341. <http://dx.doi.org/10.1042/BJ20020590>.
- [132] S.H. Choi, S.-E. Jin, M.-K. Lee, S.-J. Lim, J.-S. Park, B.-G. Kim, et al., Novel cationic solid lipid nanoparticles enhanced p53 gene transfer to lung cancer cells, *Eur. J. Pharm. Biopharm.* 68 (2008) 545–554. <http://dx.doi.org/10.1016/j.ejpb.2007.07.011>.
- [133] C. Rudolph, U. Schillinger, A. Ortiz, K. Tabatt, C. Plank, R.H. Müller, et al., Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo, *Pharm. Res.* 21 (2004) 1662–1669.
- [134] D. Delgado, A. del Pozo-Rodríguez, M.Á. Solinís, A. Rodríguez-Gascón, Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: the importance of the entry pathway, *Eur. J. Pharm. Biopharm.* 79 (2011) 495–502. <http://dx.doi.org/10.1016/j.ejpb.2011.06.005>.
- [135] C.C. Olbrich, R.H.R. Müller, Enzymatic degradation of SLN-effect of surfactant and surfactant mixtures, *Int. J. Pharm.* 180 (1999) 31–39. [http://dx.doi.org/10.1016/S0378-5173\(98\)00404-9](http://dx.doi.org/10.1016/S0378-5173(98)00404-9).
- [136] H.-Y. Xue, H.-L. Wong, Tailoring nanostructured solid-lipid carriers for time-controlled intracellular siRNA kinetics to sustain RNAi-mediated chemosensitization, *Biomaterials* 32 (2011) 2662–2672. <http://dx.doi.org/10.1016/j.biomaterials.2010.12.029>.
- [137] A. del Pozo-Rodríguez, D. Delgado, M.Á. Solinís, J.L. Pedraz, E. Echevarría, J.M. Rodríguez, et al., Solid lipid nanoparticles as potential tools for gene therapy: in vivo protein expression after intravenous administration, *Int. J. Pharm.* 385 (2010) 157–162. <http://dx.doi.org/10.1016/j.ijpharm.2009.10.020>.

- [138] A.K.K. Leung, I.M. Hafez, S. Baoukina, N.M. Belliveau, I.V. Zhigaltsev, E. Afshinmanesh, et al., Lipid nanoparticles containing siRNA synthesized by microfluidic mixing exhibit an electron-dense nanostructured core, *J. Phys. Chem. C Nanomater. Interfaces* 116 (2012) 18440–18450. <http://dx.doi.org/10.1021/jp303267y>.
- [139] H. Bunjes, M. Drechsler, M.H. Koch, K. Westesen, Incorporation of the model drug ubidecarenone into solid lipid nanoparticles, *Pharm. Res.* 18 (2001) 287–293.
- [140] M.E. Hayes, D.C. Drummond, K. Hong, J.W. Park, J.D. Marks, D.B. Kirpotin, Assembly of nucleic acid-lipid nanoparticles from aqueous-organic monophasic, *Biochim. Biophys. Acta* 1758 (2006) 429–442. <http://dx.doi.org/10.1016/j.bbame.2006.03.020>.
- [141] P. Severino, S.C. Pinho, E.B. Souto, M.H.A. Santana, Polymorphism, crystallinity and hydrophilic-lipophilic balance of stearic acid and stearic acid-capric/caprylic triglyceride matrices for production of stable nanoparticles, *Colloids Surf., B* 86 (2011) 125–130. <http://dx.doi.org/10.1016/j.colsurfb.2011.03.029>.
- [142] Y.-C. Kuo, C.-C. Wang, Electrophoresis of human brain microvascular endothelial cells with uptake of cationic solid lipid nanoparticles: effect of surfactant composition, *Colloids Surf., B* 76 (2010) 286–291. <http://dx.doi.org/10.1016/j.colsurfb.2009.11.006>.
- [143] W.C. Griffin, Classification of surface-active agents by "HLB", *J. Soc. Cosmet. Chem.* 1 (1949) 311–326.
- [144] B. Heurtault, P. Saulnier, B. Pech, J.-E. Proust, J.-P. Benoit, Physico-chemical stability of colloidal lipid particles, *Biomaterials* 24 (2003) 4283–4300. [http://dx.doi.org/10.1016/S0142-9612\(03\)00331-4](http://dx.doi.org/10.1016/S0142-9612(03)00331-4).
- [145] M.R. Gasco, Lipid nanoparticles: perspectives and challenges, *Adv. Drug Deliv. Rev.* 59 (2007) 377–378. <http://dx.doi.org/10.1016/j.addr.2007.05.004>.
- [146] Z. Liu, Z. Zhong, G. Peng, S. Wang, X. Du, D. Yan, et al., Folate receptor mediated intracellular gene delivery using the charge changing solid lipid nanoparticles, *Drug Deliv.* 16 (2009) 341–347. <http://dx.doi.org/10.1080/10717540903047387>.
- [147] N. Seetapan, P. Bejrapha, W. Srinuanchai, U.R. Ruktanonchai, Rheological and morphological characterizations on physical stability of gamma-oryzanol-loaded solid lipid nanoparticles (SLNs), *Micron* 41 (2010) 51–58. <http://dx.doi.org/10.1016/j.micron.2009.08.003>.
- [148] E. Vighi, B. Ruozi, M. Montanari, R. Battini, E. Leo, Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid, *Eur. J. Pharm. Biopharm.* 67 (2007) 320–328. <http://dx.doi.org/10.1016/j.ejpb.2007.02.006>.
- [149] W. Abdelwahed, G. Degobert, S. Stainmesse, H. Fessi, Freeze-drying of nanoparticles: formulation, process and storage considerations, *Adv. Drug Deliv. Rev.* 58 (2006) 1688–1713. <http://dx.doi.org/10.1016/j.addr.2006.09.017>.
- [150] A. del Pozo-Rodríguez, M.A. Solinís, A.R. Gascón, J.L. Pedraz, Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy, *Eur. J. Pharm. Biopharm.* 71 (2009) 181–189. <http://dx.doi.org/10.1016/j.ejpb.2008.09.015>.
- [151] S.W. Poxon, J.A. Hughes, The effect of lyophilization on plasmid DNA activity, *Pharm. Dev. Technol.* 5 (2000) 115–122. <http://dx.doi.org/10.1081/PDT-100100526>.
- [152] H. Heiati, R. Tawashi, N.C. Phillips, Drug retention and stability of solid lipid nanoparticles containing azidothymidine palmitate after autoclaving, storage and lyophilization, *J. Microencapsul.* 15 (1998) 173–184. <http://dx.doi.org/10.3109/02652049809006847>.
- [153] P. Chollet, M.C. Favrot, A. Hurbin, J.-L. Coll, Side-effects of a systemic injection of linear polyethylenimine-DNA complexes, *J. Gene Med.* 4 (2002) 84–91. <http://dx.doi.org/10.1002/jgm.237>.