Expert Opinion

Solid lipid nanoparticles for applications in gene therapy: a review of the state of the art

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Importance of the field: Gene therapy represents a new paradigm in the prevention and treatment of many inherited and acquired diseases, including genetic disorders, such as cystic fibrosis, haemophilia and many somatic diseases, such as tumours, neurodegenerative diseases and viral infections, such as AIDS.

Areas covered in this review: Among a large array of non-viral transfection agents used for in-vitro applications, cationic SLNs are the topic of this review, being recently proposed as an alternative carrier for DNA delivery, due to many technological advantages such as large-scale production from substances generally recognized as safe, good storage stability and possibility of steam sterilization and lyophilisation.

What the reader will gain: The authors give some information on the knowledge of intracellular trafficking and SLNs-DNA complex chemical-physical properties reported until now in the literature.

Take home message: The future success of cationic SLNs for administration of genetic material will depend on their ability to efficiently cross the physiological barriers, selectively targeting a specific cell type in vivo and expressing therapeutic genes.

Keywords: cationic solid lipid nanoparticles, gene delivery, gene therapy, non-viral vectors

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1. Introduction

Gene therapy is a rapidly advancing field with great potential for the treatment of inherited and acquired diseases, including genetic disorders, such as cystic fibrosis, haemophilia and many somatic diseases, such as tumours, neurodegenerative diseases and viral infections, for example AIDS [1-3]. Despite the considerable interest generated in gene therapy, delivery of genes to the target cells remains the utmost challenge.

Polynucleotide molecules (e.g., DNA or RNA) are large, hydrophilic macromolecules with a negative charge. Unlike other drugs, they are very labile in the biological environment and do not cross biological membranes effectively. Both the macromolecular genes and biological cell surfaces are negatively charged, therefore spontaneous entry of naked genes inside cells is unlikely to be an efficient process. Naked nucleic acids can be locally injected into specific organs such as muscle or liver, producing high gene expression [4,5]. Of course, this strategy is limited to tissues that are easily accessible by direct injection, such as skin, whereas it is not applicable to systemic gene delivery or is unrealistic for a commercial gene therapy. So, the clinical success of gene therapy is critically dependent on the development of efficient and safe gene delivery systems, popularly known as transfection vectors.

In general, gene delivery vectors can be categorised into viral and non-viral vectors [6,7]. Viral vectors include the use of genetically engineered retroviruses,

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adenoviruses, adeno-associated viruses and other viruses that have been used for gene transfer procedures, the viruses having evolved exquisite mechanisms through the course of evolution to deliver their genetic material into host cells. Although the viral vectors are remarkably efficient in transfecting the body cells, the myriads of potentially adverse immunogenic aftermaths associated with their use, such as their potential to generate replication-competent virus through various recombination events with the host genome, to induce inflammatory and adverse immunogenic responses, and to produce insertional mutagenesis, call for reassessing their use with regard to their safety in human gene therapy [8-11].

Thus, the current consensus is to develop suitable vectors systems that are minimally invasive and highly efficient for gene therapy in humans. Non-viral vectors include cationic liposomes and complexes prepared either using cationic lipids, such as octadecylamidoglycylspermine (DOGS), or cationic polymers, such as poly(L-lysine) (PLL) [6,7,12-14]. These systems are generally preferred over viruses because they are non-immunogenic, relatively easy to assemble, form stable complexes with plasmid, provide protection of plasmid DNA from nucleases, and are amenable to scale-up for industrial production [15,16]. Unlike viruses, they have no restrictions on the size of DNA to be delivered, and they can deliver nucleic acids of essentially unlimited size ranging up to large mammalian artificial chromosomes. The specific characteristics concerning non-viral vectors include small size and stability against aggregation in blood, serum and extracellular fluids. Moreover, they must show efficiency to be easily internalised by the target cells or to disassemble and release the payload into the cell nucleus once internalised, as well as self-regulate drug release without clinical side effects [17].

1.1 Solid lipid nanoparticles

In the past few years, solid lipid nanoparticles (SLNs) have been developed as potential carriers for several drugs [18-22]. However, there are only few reports on the use of SLNs for gene delivery [23-37]. SLNs have been shown to condense DNA onto nanometer colloidal particles and to transfect mammalian cells *in vitro* [25]. Compared with standard DNA or RNA carriers, such as cationic lipids or cationic polymers, SLNs offer several technological advantages. They are relatively easy to produce without the use of organic solvents, and large-scale production is possible with qualified production lines [18-22]. They show good stability during long-term storage and are amenable to both lyophilisation and steam sterilisation [22,38,39]. However, the physical stability during steam sterilisation cannot be assured in a general manner, as it depends strongly on the composition of the SLNs' formulation [22]. It was found that lecithin is a suitable surfactant for autoclaving SLNs because only a minor increase in particle size and number of microparticles was observed, whereas autoclaving at 121°C cannot be performed by using Poloxamer series because of dehydration of the ethylene glycol chains, which leads to a decrease of the thickness of the SLNs' protecting layer [22].

Moreover, SLNs consist of physiologically well tolerated ingredients often already approved for pharmaceutical application in humans and generally recognised as safe, so they are less toxic than cationic polymers such as polyethylenimine (PEI) [40,41]. What is more, the possibility to modulate the SLNs' chemical composition, that is, the particle surface charge, thus allowing binding of oppositely charged molecules by means of electrostatic interactions, is another important feature of these systems. However, in most of cases, although the surface charge can easily be increased, in the absence of endosomolytic agents such as chloroquine, gene transfer efficiency mediated by SLN-derived gene vectors (even when the lipid composition is optimised) remains lower than that observed with standard transfection agents [25,28].

2. Biological obstacles for systemic gene delivery

Investigation of the systemic and cellular itinerary of plasmid DNA, complexed by a vector, has provided insight into the nature of potential barriers to gene transfer. Systemic delivery of drugs targeted at the nuclear compartment is challenged primarily by the hostile extracellular environment, which may present several barriers, such as extreme pH, proteases and nucleases, and the immune defence and scavenger systems [42].

After intravenous administration, cationic SLN–DNA complexes with diameter < 150 nm, characterised by neutral surface charge and dimensionally stable *in vivo*, could have an enhanced permeation and retention effect (EPR) in inflammation sites and in solid tumours owing to the leakage in vasculature and endothelial junctions of such tissues [43-45], which allows small particles to extravasate from the bloodstream into the disease site.

Nucleic acids are unable by themselves to permeate the mammalian cell membrane because they are highly anionic, hydrophilic macromolecules, so the complexation with cationic SLNs could permit an increase in their intracellular delivery.

The endocytosis route is generally seen as the main pathway for the uptake of particles into cells [46-48]. Cells have several pathways useful for the cellular uptake of macromolecules or particles, which could be exploited from non-viral vectors in order to be internalised and processed by cells to achieve transgenic expression. Unless a specific targeting ligand is incorporated into the system, the binding of SLN–DNA complexes to the cell surface is the result of a nonspecific ionic interaction between the positive charge of the complexes and the negative charge of the cell surface. However, the internalisation mechanism of SLN–DNA complexes is not yet well known. It is now believed that most of the uptake for transfection occurs through the endocytosis pathway, although a wide variety of factors may affect this mechanism [30,49,50]. For example, interference of the endocytic pathway of liposomes with lysosomotropic

reagents such as chloroquine was found to enhance the gene expression [51].

After internalisation by means of endocytosis, the internalised particles exist in endosomes that either fuse with lysosomes for degradation or recycle their contents back to the cell surface. Therefore, escape from endosomes is essential for efficient transfection. Only DNA or the complex between DNA and SLNs as a whole is likely to be released to the cytosol after fusion. If SLN–DNA complexes reach the cytosol, the dissociation of DNA must occur in the cytosol or even at the nuclear membrane to achieve transfection.

The nuclear envelope is a significant barrier against transfection, containing nuclear pores with a passive transport limit of 70 kDa molecular mass or ~ 10 nm diameter, which is much smaller than the size of DNA, even when condensed on SLNs [52]. In fact, although DNA width is ~ 2.2 – 2.6 nm, plasmid size ranges between 0.1 and 10 kb (1 kb corresponds to ~ 650 kDa), and some of these used recombinant DNA molecules are circular [53]. Microinjection of plasmid DNA encoding β-galactosidase into the nucleus produced a much higher gene expression than when the same plasmid was microinjected into the cytosol [54]. Cell division is without doubt an important factor in the nuclear translocation of transgenes [55].

3. SLN-based gene delivery systems

Since SLNs were introduced as non-viral transfection systems, few reports of their use for gene delivery have been published [23-37]. To obtain systems with positive surface charge and for cationic SLNs to be efficacious as gene therapy agents, the use of cationic lipids is extremely important, and could allow further interaction between these systems and negative charged DNA or RNA to form SLN–DNA or RNA complexes [29]. **Table 1** shows the most common cationic lipids used to prepare cationic SLNs, and the most common plasmids, reported in the literature, to evaluate the potential of SLNs such as gene therapy agents.

Leaving an excess of positive charges on the SLN–DNA complex surface, that is, modulating the SLN/DNA weight ratios, could also help the complexes bind to negatively charged mammalian cell membranes and induce the uptake of the associated nucleic acid into cells. Nevertheless, some cationic lipids could be toxic on repeated use and can induce inflammatory reactions '*in vivo*' [56].

SLN–DNA complexes, however, are multicomponent systems whose properties and functions, such as effective intracellular delivery, depend on how components are assembled. In particular, a nucleic acid-containing particle should have a diameter < 150 nm to be suitable for systemic gene therapy. Moreover, this particle should be stable and resistant to nonspecific uptake in the circulation, but be quickly destabilised to release DNA once it is taken up into the target cells. In fact, the release of DNA from the complexes is one of the most crucial steps in determining the optimal ratio for cationic lipid system-mediated transfection [57].

3.1 Preparation of cationic SLNs

SLNs can be produced on the nanoscale size (< 200 nm), where the particles are sufficiently small to traverse the microvascular system, prevent macrophage uptake and be systemically delivered [23]. Other physicochemical and structural properties play a key role in their fate, such as surface charge, morphology and the composition of the nanoparticle matrix [40]. Several methods could be followed to prepare cationic SLNs that are useful as delivery systems for genetic material [18-21,58]. Such methods have already been developed to obtain SLNs with an overall positive, neutral or negative surface charge [59]. These preparation methods have largely been reported in the literature [18,19,21,58].

To obtain cationic SLNs for gene delivery, it is necessary to add one or more cationic lipid to the composition. In particular, cationic SLNs can be formulated by using the following techniques: warm oil-in-water (o/w) microemulsion (see **Figure 1A**); solvent emulsification/evaporation technique (see **Figure 1B**); and cold and hot high-pressure homogenisation (see **Figure 1C**). A schematic representation of these techniques is given in **Figure 1**.

The warm o/w microemulsion technique is favourable when substances are unstable to the high mechanical stress produced by high-pressure homogenisation [59]. Briefly, this technique consists of two steps: the formulation of an o/w microemulsion, first, and the preparation of the cationic SLNs by dispersing the warm microemulsion into cold water (see **Figure 1A**) [59]. The lipids (fatty acids and/or glycerides) are melted; a mixture of water, co-surfactant(s) and the cationic lipid(s) is heated to the same temperature as the lipids and added under mild stirring to the melted lipids. A transparent, thermodynamically stable system is formed when the compounds are mixed in the correct ratio for microemulsion formation. This microemulsion is then dispersed in a cold aqueous medium (2 – 3°C) under mild mechanical mixing, thus leading to the achievement of fine particles, owing to the solidification of the lipid phase [24,59-61]. However, using this method, huge amounts of surfactants and co-surfactants have to be used [60].

The solvent emulsification/evaporation is another method to produce cationic SLNs (see **Figure 1B**) [22,62,63]. Lipids must be dissolved in the organic solvent immiscible with water, and then emulsified in an aqueous phase containing the cationic lipid(s) and surfactant(s). Then, a finest emulsion could be obtained by sonication; the organic solvent is subsequently removed by evaporation using a magnetic stirring or a rotavapor under reduced pressure, and a cationic SLNs dispersion is formed by precipitation of the SLNs in the aqueous medium.

Either the hot or the cold homogenisation techniques have in common an initial step 40. R.H. Müller, C. Schwarz, W. Mehnert and J.S. Lucks, Production of solid lipid nanoparticles (SLN) for controlled drug delivery. *Proc. Int. Symp. Control. Release Bioact. Mater.* 20 (1993), pp. 480-481. View Record in Scopus | Cited By in Scopus

Table 1 Lipid composition and plasmids commonly used for obtaining SLN–DNA complexes for gene delivery.

Cationic lipid	Lipid	Plasmid	Ref.
DOTAP	Cetylpalmitate	pCMVβ	[25]
DOTAP	Precirol 5 ATO	pCMS-EGFP	[23,40,48,68]
Cetylpyridinium chloride (CPC) Cetrimide (CTAB) Benzalkonium chloride (BA) dimethyldioctadecylammonium bromide (DDAB) *N*,*N*-di-(*b*-stearoylethyl)-*N*,*N*-dimethyl-ammonium chloride (Esterquat 1, EQ 1); *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,Ntrimethylammonium chloride (DOTAP)	Compritol 888 ATO Cetyl palmitate	pCMVβ	[26]
Dimethyldioctadecyl ammonium bromide (DDAB)	Cetylpalmitate	pHIS-HIV-hugag	[71]
3b-[*N*-(*N*′, *N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) Dioleoylphosphatidylethanolamine (DOPE)	Tricaprin (TC)	pp53-EGFP	[69]
6-lauroxyhexyl lysinate (LHLN)	Glyceril monostearate	p-EGFP-N1	[72]
Esterquat 1	Cetylpalmitate	pCMV-Luc	[27]
Esterquat 1 or CPC	Paraffin or Compritol 888 ATO	pCMVβ	[28]
Esterquat 1	Cetylpalmitate, Compritol 888 ATO, stearic acid	p-EGFP-C3	[68]
Protamine	Stearic acid	p-EGFP-N_1	[64]
Stearylamine	Compritol 888 ATO	p-EGFP-N_1	[51]
DDAB	Compritol 888 ATO	pCMVβ-gal	[55]
DDAB	Compritol 888 ATO	*bep3*RNA	[24]

(2), consisting of the lipid melting at ~ 5 – 10°C above the mixture melting point [58]. For the hot homogenisation technique, the melted lipids are dispersed under stirring in a hot aqueous surfactant solution at the same temperature. Then, the obtained pre-emulsion is broken down into nanodroplets by using a homogeniser at high values of temperature and pressure for several cycles; the hot o/w nanoemulsion produced is cooled down to room temperature, and the lipid recrystallises and leads to SLNs [23,25,28]. For the cold homogenisation technique, the melted lipids are cooled, the solid lipids ground to lipid microparticles (~ 50 – 100 µm), and these lipid microparticles are dispersed in a cold surfactant solution yielding a pre-suspension. Then this pre-suspension is homogenised at or below room temperature, and the cavitation forces are strong enough to break the lipid microparticles directly to SLNs. In general, compared with the hot homogenisation, larger particles sizes and a broader size distribution are observed in the cold homogenised sample [22].

3.2 Formation of cationic SLN–DNA complexes

On mixing with DNA, electrostatic interactions occur between the cationic surface charge of the particle and the negative charge of the DNA, resulting in the formation of SLN–DNA complexes. Complexes are generally formed with a slight excess of positive charge to allow them to interact with the negatively charged cell surface; these complexes could have diameters that range from as small as 200 nm to structures as large as 2 µm, which are still suitable for systemic administration [24,61]. In fact, the size of intravenously administered particles must be < 5 µm to avoid blocking of fine capillaries leading to embolism [40].

The formation of these complexes is generally difficult to control, and different structures and aggregates are obtained in the same batch preparation. The main proposed model to describe the interaction between cationic nanoparticles and DNA or RNA involves two or three nanoparticles for each DNA or RNA molecule (see **Figure 2**) [24,29].

Usually, positively charged SLNs lead to the compaction and neutralisation of DNA onto the nanoparticle surface, and also provide a protective role against extra- and intracellular nucleases [24]. To a greater extent, the complex size and charge depend on the weight ratio between the particle and DNA. Nanoparticle surface can be easily modified with targeting ligands or PEG to escape the recognition by opsonins as well as macrophages after systemic administration [64]. To obtain surface pegylated SLNs, pegylated lipid(s) such as Compritol HD5 ATO can be used as component(s).

3.3 Characterisation of cationic SLN–DNA complexes

For successful gene delivery to the cell nucleus, an ideal cationic SLN should have the following attributes. A good loading capacity (i.e., the final formulation should have a high DNA loading efficiency for the maximum delivery of payload

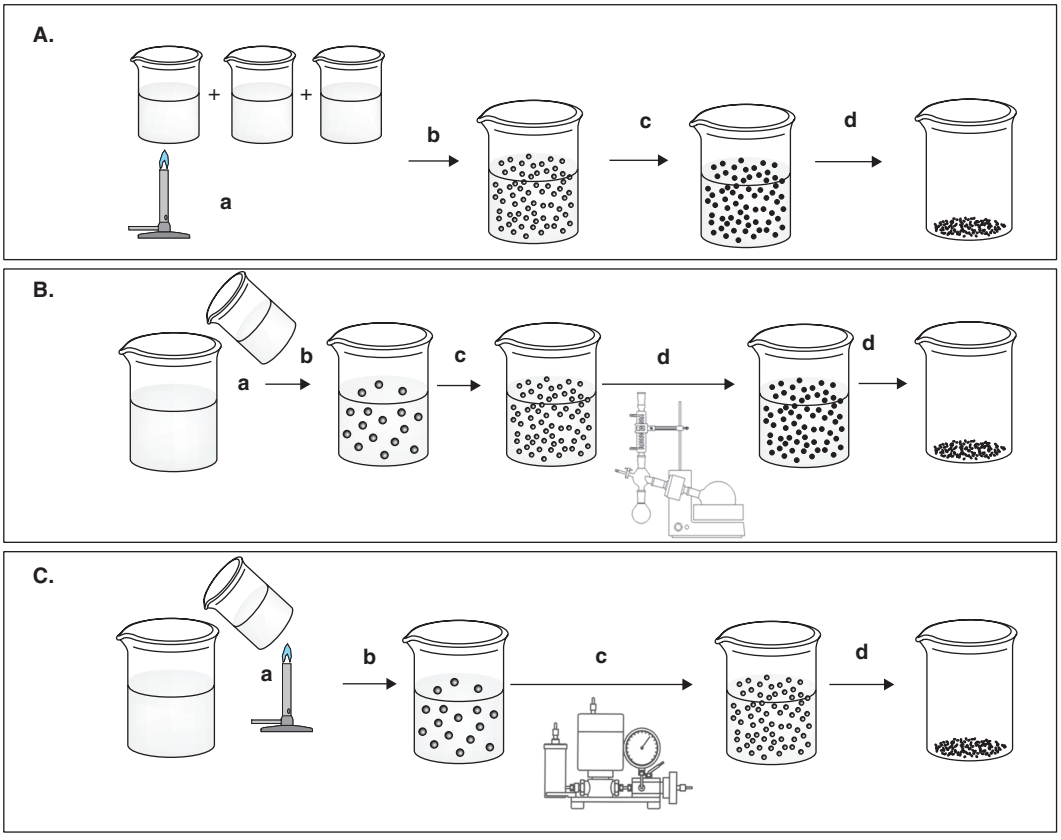

Figure 1. Schematic diagrams for the preparation of cationic SLNs. A. Oil-in-water microemulsion technique: (a) heating the lipid to ~ 10°C of its melting point; (b) addition of the cationic lipid and surfactant aqueous solutions to the melted lipid; (c) dispersion of the warm microemulsion in cold water (2 – 3°C); and (d) purification and freeze-drying procedure. **B.** Solvent emulsification/evaporation technique: (a) dissolution of the lipid in the organic solvent immiscible in water; (b) addition of the organic solution in an aqueous phase containing the cationic lipid(s) and the surfactant(s); (c) sonication of the obtained emulsion; (d) evaporation by rotavapor to remove organic solvent; and (e) purification and freeze-drying procedure. **C.** Hot high-pressure homogenisation technique: (a) heating the lipid to ~ 10°C of its melting point; (b) addition of the melted lipid in a hot aqueous phase containing the cationic lipid(s) and the surfactant(s) and formation of the primary emulsion by vigorous stirring with a high-speed stirrer; (c) homogenisation in a heated homogeniser for several homogenisation cycles; and (d) purification and freeze-drying procedure.

to the cells); the resulting SLN–DNA complex size should be in the nanometer range for optimal cellular internalisation, unless used for gene delivery to phagocytic cells. In this case, it can be in the micrometer range; the SLN–DNA complexes should posses an optimum net positive surface charge for interaction with the negatively charged cell membrane. Moreover, the complex formed between DNA and SLNs should be able to protect the payload from pH and enzymatic degradation. That is why, after formulating, SLN–DNA complexes are usually characterised according to the above-mentioned parameters.

Either direct or indirect methods can be used for determination of the DNA loading efficiency of formulations. For systems that physically complex DNA, a direct approach might be used that involves extracting and quantifying the loaded DNA, to obtain the amount incorporated per mass of delivery system. The indirect method determines the amount of DNA material that remains free after complexation; it can be subtracted from the total amount of DNA initially added to the mixture. Fluorochromes such as ethidium bromide and Hoechst 33258, which bind strongly with double-stranded DNA and fluoresce, are also used for quantitative analysis [65-67].

SLN–DNA complex size is the most important parameter to be assessed and it is often measured thanks to instruments working on the light scattering principle that can measure small particles up to 20 µm [68]. Other instruments are also available. They use a laser beam, work on the principle of laser diffraction and can measure particle sizes from 0.6 nm to 6.0 µm. The Zetasizer Nano series particle sizers from Malvern Instruments use laser beams for particle size measurement and can accurately measure particles in the nanometer range.

In addition to SLN–DNA complex size, the surface charge of formulation also has to be determined. The surface charge on particles can be determined with commercially available instruments measuring the electrophoretic mobility of particles

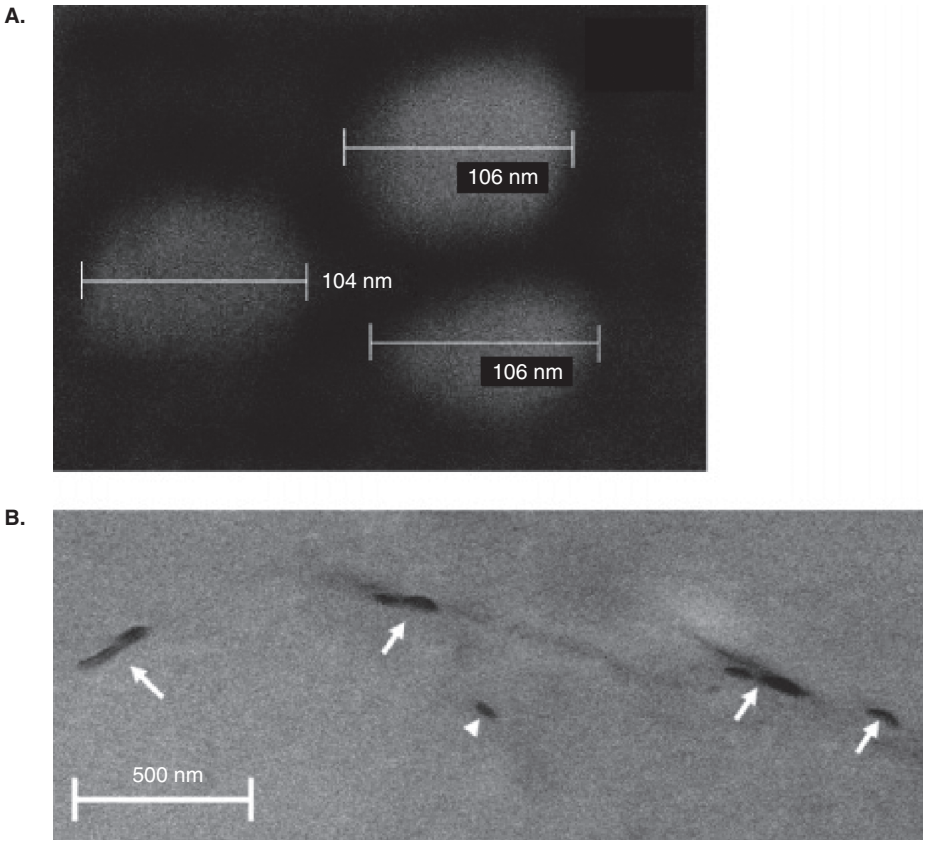

Figure 2. Scanning electron microscopy images of (A) cationic SLNs and (B) a group of representative cationic SLN–RNA complexes obtained at a w/w ratio of 200:1. The arrows indicate the complexes; the arrowhead indicates the free nanoparticles [24].

under the influence of an electric field. Often, the complex sizing and zeta-potential measurements can be done on the same instrument (e.g., Zetasizer Nano series, Malvern Instruments, Malvern, UK; and 90Plus® nanoparticle size analyser, Brookhaven Instruments Corp., Worcestershire, UK) [69].

Size and shape of SLNs and SLN–DNA complexes can also be characterised by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (see **Figure 2**) [24,33]. Moreover, the shape of cationic SLNs and their complexes with genetic material can be observed by the use of an atomic force microscope (AFM), which is one of the most powerful tools for determining the surface topography [25,29].

Most of the above-mentioned methods for preparation of SLN–DNA complexes do not involve steps that would put significant physical strain on the DNA molecule, but assessment of the physical integrity of the encapsulated payload is necessary, as it can affect the final outcome of gene therapy.

The stability of plasmid DNA can be determined by agarose gel electrophoresis, by running an appropriate standard along with the sample in the same gel, and then by comparing the bands between the sample and standards. The ability of the formulation to protect the payload from endogenous nucleases, such as DNase I, also has to be determined by agarose gel electrophoresis. For a more precise result, the DNA sample can be sequenced to determine its physical integrity, but this is a more expensive and time-consuming process. Agarose gel electrophoresis also permits determination of the changes on DNA topology when it is incubated in the presence of DNase. DNA can feature three forms: supercoiled (SC), open circular (OC) and linear (L). SC-DNA has been reported in the literature to be the most bioactive form and incubation in the presence of DNase could give changes in DNA topology [70-72].

Finally, DNA condensation is a crucial factor, which determines the transfection capacity of SLNs, because it influences the superficial charge of the complexes and thus cell entry, DNA delivery from nanoparticles, gene protection from DNases and DNA topology. An optimal DNA condensation must be achieved when designing cationic SLNs as non-viral vectors. Complexes must have enough DNA condensation capacity to create equilibrium between the gene protection degree, the binding forces of DNA to SLNs, and the DNA topology. This equilibrium is determined by cationic lipid/DNA ratio and it must be optimised with every new formulation [29].

Some authors reported the preparation and chemical-physical characterisation of several cationic SLNs batches obtained by using EQ1 and CPC as cationic lipids and the

hot homogenisation technique [28]. In particular, it was found that average sizes ranged between 101 and 105 nm with low polydispersity indices. Surface charge of all particle batches in aqueous medium was found to be in the range between +40.2 and 44.8 mV, whereas particles obtained without any cationic surfactant showed a negative zeta-potential value (-23.1 mV). However, only the cationic SLNs based on Compritol 888 ATO and EQ1 resulted in a shift of DNA motility, by immobilising a detectable amount of DNA at low w/w ratios, and typical complexes were 300 – 800 nm in size, determined by AFM [28].

To investigate the influence of cationic lipid and matrix lipid composition on transfection efficiency, some authors have carried out the preparation and characterisation of 12 batches of cationic SLNs by the use of CTAB, CPC, BA, DDAB, EQ1 and DOTAP as cationic lipids, and Compritol 888 ATO and CP as lipid matrix [26]. Sizes ranged between 113 and 327 nm, with surface charge being between +34.1 and +44.8 mV, that is, suitable values for complexing DNA, although the toxicity seemed to be strictly related to the chemical cationic lipid structure. Compritol 888 ATO and stearylamine were used for the production of cationic SLNs with a size of ~ 100 nm and a zeta-potential of + 15 mV, which were able to condense DNA [23].

Cationic SLNs were successfully prepared by the o/w microemulsion method [73]. In this case, stearylamine was used as cationic lipid and different lipids as matrix, such as CP, stearic acid and Compritol 888 ATO, obtaining particles with an average size ranging between 160 and 209 nm and zeta-potential values ranging between +34.8 and +39.9 mV. AFM investigation was used for determining the redispersion of SLNs and the sample obtained by the use of stearic acid resulted in the best candidate for binding DNA, although it displayed a sensible reduction of the zeta-potential (from +39.2 to +23.3 mV).

4. Gene therapy using SLNs

Knowledge of intracellular trafficking and SLN–DNA complex physicochemical properties is very useful for designing more efficient vectors, taking into account the characteristics of the specific cell line to be transfected.

Del Pozo-Rodriguez and co-workers have investigated DNA delivery systems for the treatment of diseases related to problems in the retina by using human retinal pigment epithelial (ARPE-19) cells [30]. In particular, plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was used for obtaining SLN–DNA complexes. Cells were incubated with the obtained complexes for 4 h, left to grow for a further 72 h, and then EGFP fluorescence was collected by flow cytometry. The transfection capacity of SLNs based on Precirol® (glyceril palmitostearate (Gatefossè, Madrid, Spain)) 5 ATO and DOTAP in these cells was evaluated, although there was a lower transfection level in ARPE-19 cells than in human embryonic kidney cell line (HEK293). Trafficking studies were carried out by using either SLNs labelled with Nile red or plasmid pCMS-EGFP with ethidium monoazide, which revealed a delay in cell uptake of the vectors in ARPE-19 cells. Differences in the internalisation process into the two cell lines studied explain, in part, the difference in the gene expression.

To improve the transfection efficacy of SLNs into ARPE-19 cells, the authors have added to the SLN–DNA complexes the Sweet Arrow Peptide (SAP) [32]. First, SAP–DNA complexes at ratios of at least 50:1 were prepared, and then they were incorporated into SLNs. All formulations were able to protect DNA, and the peptide favoured the most bioactive form (supercoiled) of open circular DNA turns. *In vitro* transfection studies of the vectors containing the pCMS-EGFP plasmid in HEK293 and ARPE-19 cell lines revealed that incorporation of SAP led to greater transfection in both cell lines, although by means of different mechanisms. In ARPE-19 cells, SAP induced a change in the dominant entrance mechanism, from clathrin endocytosis to caveolae/raft-dependent endocytosis, thereby decreasing use of the lysosomal pathway and consequently reducing vector degradation.

Choi and co-workers evaluated the *in vitro* and *in vivo* transfection efficiency of cationic SLNs into non-small human lung cancer cells (H1299), obtained by using tricaprin (TC) as a core and 3b-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl] cholesterol (DC-Chol) as cationic surfactant, and compared it with Lipofectin [34]. As model DNA, the pp53-EGFP plasmid DNA encoding the EGFP and p53 was used and transfection was determined by either flow cytometry or confocal laser scanning microscopy. The authors concluded that cationic SLN-mediated p53 gene delivery may have potential for clinical application as a non-viral vector-mediated lung cancer therapy owing to its effective induction of apoptosis and tumour growth inhibition.

Bondì and co-workers evaluated the suitability of new cationic SLNs based on Compritol 888 ATO as matrix lipid, dimethyldioctadecylammonium bromide (DDAB) as charge carrier and Pluronic F68 as surfactant, as non-viral transfection agents for gene delivery on human hepatoma (HuH-6) cell line [35]. Plasmid DNA encoding the β-galactosidase gene (plasmid pCMV-β-gal) under the control of the human cytomegalovirus (CMV) was used in this study as the reporter gene, and the expression gene coding for β-galactosidase was estimated by measuring the corresponding activated substrate. The authors demonstrated that cationic SLN–DNA complexes could promote transfection of liver cancer cells. To test the applicability of these SLNs on a living organism, Montana and co-workers [24] used the sea urchin as a model system. With a microinjection experiment, it was demonstrated that SLNs do not inference with the viability of the paracentrotus lividus embryo and the complex nanoparticles-bep3 permits movement of the RNA during its localisation in the egg, suggesting that it could be a suitable system for gene delivery.

Del Pozo-Rodriguez and co-workers have focused on several cationic SLN formulations in order to evaluate the influence of the formulation components on the '*in vitro*' transfection capacity [29]. In particular, SLNs were composed of Precirol ATO 5, the cationic lipid DOTAP and the surfactant Tween 80, and SLN–DNA complexes prepared by using pCMS-EGFP plasmid, at different DOTAP/DNA ratios. Transfection activity in HEK293 cultured cells was found to be dependent on the DOTAP/DNA ratio because it influences the DNA condensation into the SLNs. These authors have also investigated short- and long-term stability of lyophilised SLNs for gene therapy [31], being physically and chemically stable vectors necessary to achieve large easily shipped and stored batches. After lyophilisation, an increase in particle size was found, which did not imply a reduction of '*in vitro*' transfection capacity.

Asasutjarit and co-workers reported the effect of SLNs' formulation compositions on their characteristics and potential for *in vitro* pHIS-HIV-Hugag transfection [36]. SLNs were prepared using cetylpalmitate as lipid matrix, Tween 80 and Span 85 mixture, DDAB and cholesterol. The ability of SLNs to form a complex with pHIS-HIV-hugag and transfection capability was demonstrated to be affected by formulation compositions.

The transfection activity of SLNs, as well as the toxicity, seem to be strongly governed by the cationic lipid composition [26,37]. In particular, cationic lipid composition is very important for *in vitro* transfection performance in comparison with the kind of colloidal structure it is arranged in.

An interesting investigation is based on the enhancement of *in vitro* transfection activity by optimising cationic lipid and matrix lipid composition of SLNs [26]. For this purpose, SLNs were formulated by using two different matrix lipids and six different cationic detergents; the formulations obtained were tested for their main physical parameters. *In vitro* cytotoxicity measurements on COS-1 cells revealed that cytotoxicity is strongly dependent on the cationic lipid used, SLNs made from one-tailed cationic detergents being highly cytotoxic, whereas the two-tailed cationic lipids are well tolerated. Transfection activity seems to be determined by both the cationic lipid and the matrix lipid used. Here, the combination of cetylpalmitate and DOTAP led to significantly higher transfection efficiency than in all other tested combinations.

Cos-1 cells were also used by other authors to demonstrate that SLNs systems can efficiently bind and transfect plasmid DNA [28]. SLNs were produced by hot homogenisation using either Compritol 888 ATO or paraffin as matrix lipid, and either *N*,*N*-di-(*b*-stearoylethyl)-*N*,*N*-dimethyl-ammonium chloride (EQ1) or cetylpyridinium chloride (CPC) as charge carriers. Cationic SLNs produced by modification with EQ1 were well tolerated, and SLN–DNA complexes efficiently transfected the galactosidase expression plasmid pCMVβ in the absence and presence of the endosomolytic agent chloroquine.

Yu and co-workers prepared cationic SLNs for gene therapy by using a new single-tailed cationic lipid, 6-lauroxyhexyl lysinate (LHLN), as a modifier to obtain stable SLN–DNA complexes by a nanoprecipitation method [37]. LHLN-modified SLNs had a higher gene transfection efficiency and lower cytotoxicity than the naked DNA and Cetrimide (CTAB)-modified SLN–DNA formulation and Lipofectamine–DNA complexes.

Some SLNs were easily obtained by a simple method with a great potential as targeted systems to specific surface receptors [23]. In particular, to prepare SLNs able to condense DNA in very stable complexes under physiological conditions and with low cytotoxicity, substantial amounts of streptavidin were bound directly by means of electrostatic interactions. The SLN:DNA:streptavidin complexes are stable and can bind biotinylated ligands, so interact with surface receptors.

In other systems, the incorporation of a dimeric HIV-1 TAT peptide (TAT2) into SLN gene vectors was demonstrated to optimise gene transfer on bronchial epithelial cells *in vitro* because it induced an up to 100-fold sequence-dependent increase of gene expression as compared with the mutant TAT2-M1 and was 4 to 8 times higher as compared with PEI *in vitro* [27].

Ye and co-workers developed new anionic ternary nanoparticles for gene delivery, equipped with protamine/DNA binary complexes (150 – 200 nm) as the support, and the anionic formation was achieved by absorption of anionic SLNs (≤ 20 nm) onto the surface of the binary complexes in water by means of electrostatic interactions [33]. These particles were prepared starting from stearic acid as lipid matrix and lecithin as surfactant and by using a modified film dispersion-modification method [33]. The authors demonstrated that the protamine/DNA complexes had been coated by small SLNs, and that the anionic ternary nanoparticles formed did not disturb the formation of the binary complexes. SYBR Green I analysis suggested that the ternary nanoparticles could protect the DNA from nuclease degradation. Moreover, cell viability assay results showed that they have lower cytotoxicity and higher transfection efficiency to A549 cells compared with the binary complexes.

5. Conclusions

Gene therapy represents a new paradigm in the prevention and treatment of many inherited and acquired diseases. Among a large array of non-viral transfection agents used for *in vitro* applications, cationic SLNs have been proposed recently as an alternative carrier for DNA delivery, owing to many technological advantages over other existing non-viral vectors. They include large-scale production from substances generally recognised as safe, good storage stability and the possibility of steam sterilisation and lyophilisation. However, although cationic lipid-based SLNs possess several advantages over other forms of nucleic acid transfer agents in cell culture, cytotoxicity remains a problem, especially *in vivo*.

6. Expert opinion

As genetic alterations are responsible for several pathologies, the next generation of therapeutics is expected to be based on charged molecules (e.g., plasmid DNA, siRNA, oligonucleotides and proteins) that have poor stability and low diffusion properties in the biological environments. In gene therapy, the foreign nucleic acids must be introduced into the target cells by the use of gene vectors to facilitate the cellular uptake and the intracellular processing of the exogenous nucleic acid. Viral vectors are still the most efficient systems for gene transfer, but the limitations associated with their use, in terms of safety, immunogenicity and costs, have encouraged researchers to focus on alternative systems, such as lipoplexes or polyplexes. However, these systems have not demonstrated high efficiency and specificity. Cationic SLNs have recently been suggested for non-viral gene delivery as a promising alternative to the other synthetic vectors. Cationic SLNs, as charged molecules carriers, may offer several technological advantages, such as excellent storage stability, a relatively easy production without the use of any organic solvent, the possibility of steam sterilisation and lyophilisation, and large-scale production. Moreover, cationic SLNs are made by using physiologically well-tolerated ingredients already approved for pharmaceutical applications in humans. Cationic SLNs can be produced in nanoscale size and can condense nucleic acids onto the surface by means of electrostatic interactions. The cationic SLN/nucleic acid complexes can protect nucleic acid from enzymatic degradation and deliver the genetic material into cells by interacting with the negatively charged cell membrane. These systems are also suitable for enhancing target-specific delivery and sustained release mechanisms, and for promoting cellular stability of the plasmid for nuclear uptake in order to optimise transfection efficiency.

Although present nanotechnologies are focused on the efficient delivery of a therapeutic to the target site, a future goal may be to enhance the functionality by introducing both drug and gene combination and to deliver them at a desired time point. The future success of cationic SLNs for administration of genetic material will depend on their ability to cross efficiently the physiological barriers, selectively targeting a specific cell type *in vivo* and expressing therapeutic genes. It is clear that the success of gene therapy needs to be evaluated in higher organism disease models before it can be moved up to human trials. However, despite many obstacles, the development of topical and/or systemic gene therapy has great clinical promise and must continue to move forward developments in innovative delivery strategies.

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Declaration of interest

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