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pDNA condensation capacity and in vitro gene delivery properties of cationic solid lipid nanoparticles

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ABSTRACT

Cationic solid lipid nanoparticles (SLN) are promising nonviral gene delivery carriers suitable for systemic administration. The objective of this study was to investigate the relationship between the composition of cationic SLN and their ability to condense plasmid DNA (pDNA) and to transfer it in neuroblastoma cells. The SLN were prepared by using stearic acid and stearylamine as lipid core along with Esterquart 1 (EQ1) or Protamine obtaining two samples (SLN-EQ1 and SLN-Protamine, respectively). The cationic SLN were freeze-dried after preparation and their physical–chemical properties, including the surface composition and the transfection efficiency were investigated. The results showed that the two samples had similar size, zeta potential and pDNA binding properties but SLN-Protamine were able to condense pDNA more efficaciously than SLN-EQ1 forming smaller and less positive complexes. SLN-Protamine:pDNA complexes demonstrated to be less cytotoxic and more efficient in the transfection of Na1300 cell line than SLN-EQ1:pDNA. These findings were attributed to the different surface composition of the two samples and in particular to the localization of the Protamine on the surface of the particle while EQ1 in the lipid core. In conclusion the results here suggest that not only the z-potential but also the surface composition may affect the pDNA condensation proprieties and thus the transfection efficiency of nonviral gene nanocarriers.

1. Introduction

Many human gene products developed by means of biotechnology are being investigated for their potential use as commercial drugs employed for hereditary disorder improvement, life threatening diseases treatment such as cancer, infectious and vaccination in healthy people. To date, the production of effective gene delivery vectors is the limitation of the success of gene-based drug in clinical trials. Nonviral vectors have great potential as a strategy for gene therapy and can be used to treat genetic diseases which require systemic administration (Alonso, 2004).

Although cationic polymers and liposome are promising (Li and Huang, 2007; Li and Szoka, 2007), solid lipid nanoparticles (SLN) have been recently proved to be a useful vehicle for gene therapy (del Pozo-Rodríguez et al., 2009). SLN are basically composed of high melting point lipids that act as a solid core, covered by surfactants. The use of materials that are generally recognized as safe (i.e. triglycerides, partial glycerides, fatty acids, steroids) (Mehnert and Mader, 2001) leads to an advantageous toxicity profile (Olbrich et al., 2001) when compared with many highly efficient cationic polymers vectors such as poly-L-lysine (Jeong and Park, 2002) or polyethylenimine (PEI) that can lead to liver necrosis and animal death after intravenous application (Chollet et al., 2002). Additionally SLN have advantages under the technological point of view over the highly efficient cationic polymers, including good storage stability, the possibility of steam sterilization and lyophilization (Schwarz et al., 1994; Muller et al., 2000; Vighi et al., 2007). The increasing attention toward such as vectors is highlighted in the recent reviews about the potentialities of the SLN (Kaur et al., 2008; Joshi and Müller, 2009; Tseng et al., 2009).

The positively charged SLN would bind to polyanionic DNA via electrostatic force leading to SLN:DNA complex that will protect DNA from interaction with small molecules in environment and will be taken into cell by an endocytosis process (Asasutjarit et al., 2007). Recently great attention has been applied on the development of several strategies aimed to improve the transfection efficiency of the cationic SLN, including the use of endosomolytic agents as clorochine (Tabatt et al., 2004), or of synthetic cationic lipids used normally in the preparation of liposomes, such as DOTAP (N-(1-(2,3-dioleoyloxy) propyl)-N,N,N-trimethylammoniumchloride) (del Pozo-Rodríguez et al., 2007) and the application of cell penetrating peptides (del Pozo-Rodríguez et al., 2009). Additionally, SLN made of tricaprin, DOPE (dioleoylphosphatidyl ethanolamine) and Tween 80 were found

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able to transfect human non-small cell lung cancer cells (Choi et al., 2008) and the capacity of SLN–DNA vectors to induce the expression of a foreign protein after intravenous administration has been demonstrated (del Pozo-Rodríguez et al., 2010).

Poor attention has been paid on the determination of the effect of the SLN composition on the ability of the SLN to condense plasmid DNA and thus on their transfection ability.

This paper aims at evaluating the plasmid DNA condensation by means of SLN consisting of stearic acid as lipid matrix, stearylamine as a cationic agent, and Protamine or Esterquart 1 (EQ1) as transfection promoters. EQ1 (N,N-di-(β-stearoylethyl)-N,N-dimethylammonium chloride) is a cationic detergent with a good tolerability and biocompatibility used to enhance the transfection efficacy of SLN (del Pozo-Rodríguez et al., 2007), while Protamine is a cationic small peptide with high arginine content that is FDA-approved for the parenteral administration and is used as transfection accelerator for gene delivery (Tsuchiya et al., 2006).

We investigated here the effect of the composition of the two samples, prepared in the same experimental condition, on their ability to condense the pEGFP (plasmid encoding for enhanced green fluorescent protein) and to transfer it in a murine neuroblastoma cell line (Na1300). Using this approach we compared the dimension and the morphology of the two SLN:pDNA complexes along with their transfection efficiency.

2. Materials and methods

2.1. Materials

Stearic acid was purchased from Carlo Erba Reagenti (Milan, Italy). Stearylamine (ST) was provided from Fluka (Deisenhofen, Germany). Esterquat 1 (EQ1) (N,N-di-(β-stearoylethyl)-N,N-dimethyl-ammonium chloride) was gift from Gerbu Biotechnik (Gaiberg, Germany). Protamine sulfate was provided from Sigma–Aldrich (St. Louis, MO, USA). DAPI (4′,6-diamidino-2-phenylindole) was purchased from Vysis (Abbott Laboratories, IL, USA).

Polyethilenimmine (PEI, MW 25,000) was provided from Sigma–Aldrich (St. Louis, MO, USA). Agarose GellyPhor, Dulbecco's modified eagle medium (DMEM), Dulbecco's Phosphate Buffer (PBS) and other culture reagent were purchased from Euroclone Celbio (Milan, Italy). All other chemical reagents were obtained commercially as reagents-grade products. The cell line Na1300 was provided from Zoo-prophylactic Institute of Emilia Romagna (Bologne, Italy).

2.2. SLN formulation

The cationic SLN were produced by a modification of the oil-in-water microemulsion methods (Heydenreich et al., 2003). Briefly, the solid component (250 mg of stearic acid) was melted at around 68 °C (i.e. 10 °C above its melting point). In the same time, an aqueous phase (10 mL) containing Pluronic F68 (2%, w/v), stearylamine (3%, w/v) and EQ1 or Protamine (1%, w/v) was heated at the same temperature of the melted mass. Then, the melted mass of stearic acid was dispersed in the aqueous phase using a high-speed stirrer (Ultra-Turrax T25, IkaWerk, Staufen, Germany) at 20,500 rpm for 5 min to form a hot O/W emulsion. Then, the hot emulsion was cooled at 4 °C by an ice bath, maintaining the mechanical stirring at 20,500 rpm for 10 min, i.e. until the formation of the solid lipid nanoparticle (SLN) suspension. This suspension was washed using 100 mL of deionised water and concentrated until 10 mL by vacuum ultra-filtration using a polycarbonate holder (Sartorius, Goettingen, Germany) equipped with a polypropylene filter (cut-off 0.2 μm; Pall Corporation, Ann Arbor, MI, USA). Then the SLN suspension was freeze-dried (Heto-Holten A/S, Allerød, Denmark) during 48 h at −55 °C at a pressure of 10^{-4} and the freeze-dried SLN powder was collected and stored at 4 °C.

The re-dispersion of the freeze-dried samples (10 mg) was performed in deionised water (2 mL) by three cycles of vortex (30 s) (Zx^3, Velp Scientifica, Milan, Italy) followed by a treatment in an ultrasound bath (Sonorex™, Bandelin, Mörfelden, Wan, Germany) (30 s).

2.3. Plasmid DNA production

The plasmid vector expressing Enhanced Green Fluorescent Protein (EGFP) pEGFP-C3 (pDNA) was purchased from Invitrogen (Carlsbad, California, USA) and transformed into *Escherichia coli* (XL_1 Blue MR). A Maxiprep from 500 mL of overnight culture was performed with Qiagen Kit according to the manufacture's instruction (Endofree Maxi Prep, QIAGEN, Hilden, Germany).

2.4. Formation of SLN:pDNA complexes

SLN:pDNA complexes were prepared using 0.5 or 10 μg of plasmid, for the gel electrophoresis or transfection experiments, respectively. pDNA was mixed with the re-suspended freeze-dried SLN (5 mg/mL) at different SLN:pDNA w/w ratios (20:1, 40:1, 60:1, 80:1) corresponding to N/P ratios, respectively, of 0.4:1, 0.8:1, 1.2:1, 1.6:1 for SLN-EQ1:pDNA and of 0.9:1, 1.8:1, 2.7:1, 3.6:1 for SLN-Protamine:pDNA. The mixture was kept at room temperature (25 ± 0.5 °C) for 45 min to allow the formation of the complexes. After the incubation, the SLN:pDNA complexes were diluted in water (1:5) or in serum free medium (DMEM).

2.5. PCS (photon correlation spectroscopy) experiments

The PCS experiments were carried out on the fresh prepared SLN samples, the re-dispersed freeze-dried samples and the SLN:pDNA complexes. A Zetasizer Nano ZS (Malvern, Worcs, UK) equipped with a 4 mW He–Ne laser (633 nm) was used for the size determination. Each experiment was carried out at 25 °C using deionised water with a refraction index of 1.33, a viscosity of 0.8872 cP and repeated three times for each samples. The data are expressed as mean value ± standard deviation.

Similarly, the z-potential was measured using the same equipment described previously with a combination of laser Doppler velocimetry and a phase analysis light scattering (PALS).

2.6. Atomic force microscopy (AFM)

AFM observation of SLN:pDNA complexes was performed by a Park Autoprobe Atomic Force Microscope (Park Instruments, Sunnyvale, CA, USA). The experiments were conducted in water at room temperature (20 °C) and at atmospheric pressure (760 mmHg) operating in non-contact mode (NC-AFM). Triangular silicon tips were used for this analysis. The resonant frequencies of this cantilever were found to be about 120 kHz.

2.7. Electron spectroscopy for chemical analysis (ESCA) studies

In order to determine the distribution of the atoms in the surface of the particles, the electron spectroscopy for chemical analysis (ESCA) was performed on the SLN-Protamine sample either as fresh prepared and as re-dispersed freeze-dried powder. The spectra were recorded on an analysis system 04-153 X-ray source (PHI, Uvalca-PHI, Tokyo, Japan) and an hemispherical electron analyser EA11 (Leybold Optics, Germany) by MgK1,2 radiations (E = 1253.6 eV). The analysis was carried out in FAT (fixed retardation ratio) mode with 190 eV pass energy. The pressure in the

sample analysis chamber was ca. 10^{-9} mbar. Data acquisition and processing were performed using the RBD AugerScan 2 program.

2.8. Agarose gel electrophoresis

The SLN:pDNA complexes were formed as reported in Section 2.4 and the analysis by agarose gel electrophoresis was performed at 50 V for 4 h in 0.8% (w/v) agarose gel in TBE buffer (0.045 M Tris-borate pH 8.3 and 0.001 M EDTA) after adding to each sample the loading buffer (40% (v/v) glycerol and 0.25% (w/v) bromophenol blue in TBE buffer). The results of pDNA migration were visualized under UV light, after staining with ethidium bromide. The total amount of pDNA in each lane was 0.5 μg for all the samples.

2.9. Cell culture

Na1300 cells (murine neuroblastoma) were cultured at 37 °C with 5% CO_2. The culture medium was DMEM with high glucose, supplemented with 20% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and 1 mM glutamine.

2.10. Cytotoxicity

SLNs cytotoxicity was assessed by the PI (propidium iodide) test (Wrobel et al., 1996) as percentage of death cells by flow cytometry analysis. In brief, Na1300 cells were seeded at 500,000 cells/dish in 6 cm Petri's dishes, then cultured for 24 h in complete medium at 37 °C and experiments were conducted at approximately 80% confluence. Immediately prior to the addition of various amounts of SLN-EQ1 and SLN-Protamine vectors, the medium was aspirated from each dish and replaced with 2 mL of DMEM serum free and the cells were incubated 4 h or 12 h (overnight). The PEI cytotoxicity was determined as positive control using 15 μg of PEI for 2 h on cells in complete medium. Then the all the treated cells were washed with PBS, added with 5 mL of DMEM with FBS and incubated at 37 °C for 24 h.

2.11. Transfection experiments

In order to study the transfection efficiency of the vectors, Na1300 cells were growth in 6 cm Petri's dishes seeding the cells at 500,000 cells/dish and cultured for 24 h as described above. Immediately prior to the addition of SLN:pDNA (10 μg pDNA) complexes at various (carrier)/(pDNA) (w/w) ratios (20:1, 40:1 and 60:1), corresponding, respectively, to N/P ratios of 0.4:1, 0.8:1, 1.6:1 for SLN-EQ1:pDNA and of 0.9:1, 1.8.1, 2.7:1 for SLN-Protamine:pDNA, the culture medium was aspirated from each dish and replaced with 2 mL of serum free medium. The cells were incubated in 5% CO_2 incubator at 37 °C for 4 h or overnight, for SLN-EQ1 or SLN-Protamine respectively. After the incubation time, the transfected cells were washed with PBS and incubated at 37 °C for 24 h in 5 mL DMEM with FBS in order to allow the EGFP expression.

Cells transfected by 10 μg of naked pDNA were used as negative control and cells transfected by PEI-pDNA polyplex was adopted as positive control. In this case 2 μg of plasmid DNA was incubated with 15 μg of PEI for 8 min at room temperature, in order to obtain stable polyplexes with a w/w ratio of 7.5:1 (corresponding to an N/P ratio of 15:1). This PEI:pDNA polyplex was incubated with Na1300 cells for 2 h in DMEM with serum (complete medium). After the incubation time, the medium was replaced with 5 mL of fresh complete medium and the cells were incubated overnight at 37 °C. Then, another medium replacement was carried out and the cells were incubated again for 24 h. Then the procedure was identical to that used for SLN:pDNA complexes.

2.12. Flow cytometry

Percentage of death cells and quantitative transfection evaluation were carried out by flow cytometry. Briefly, at the end of incubation, cells were washed with 500 μL of PBS and were detached with 500 μL of 0.25% trypsin added with EDTA.

In the PI assay cells were re-suspended in 4 mL of removed medium and 800 μL of cell suspension were centrifugated at 200 × *g* for 5 min. The supernatant was removed and the cells were suspended in 500 μL of PI solution, according to Nicoletti staining (50 mg/mL PI; sodium citrate 0.1% (w/v), Triton X-100 0.1% (w/v)), and incubated in the dark at 4 °C for 10 min.

In the transfection experiments, detached cells were re-suspended in 5 mL of DMEM with serum and 500 μL were analysed in order to assay the expression of EGFP.

The percentage of death cells and the transfection efficiency was determined using a Coulter Epics XL cytofluorimetry, equipped with 488 nm argon laser (Coulter Electronics Inc., Hialeah, FL, USA) respectively at 540 nm and 488 nm. For each sample 10,000 events were collected.

3. Results and discussion

3.1. Nanoparticle characterization

The SLN prepared by means of the modified oil-in-water microemulsion method were freeze-dried after preparation. Before the formation of the SLN:pDNA complexes, the ability of the particles to be re-suspended after liophylization was investigated by the detection of both their size and z-potential before and after the freeze-dried process (Table 1).

The SLN obtained in presence of Protamine (SLN-Protamine) or in presence of EQ1 (SLN-EQ1) showed a diameter in a range between 210 nm and 250 nm and an acceptable homogeneity in the size distribution, as indicating by the polidispersity indices (PDI < 0.3). From the data reported in Table 1, it appears that no remarkable differences in the size occurred between fresh prepared samples and the re-suspended lyophilized powder.

Concerning the z-potential value of the fresh prepared sample the use of Protamine in the place of EQ1 involve a decrease of the z-potential from +40 mV to +20 mV. Moreover, after the freeze-drying process a reduction of the z-potential of SLN-EQ1 was observed, while no modification in the case of SLN-Protamine occurred. In other words,, even if SLN-Protamine showed a lower z-potential value respect of the SLN-EQ1, a higher stability of the particle after the freeze-dried process was observed probably due to the presence of Protamine in the place of EQ1. In fact the reduction of the z-potential of SLN-EQ1 after the freeze-drying process has been attributed to a rearrangement of the lipid core due to the liophilization and to the consequent loss of the cationic lipid EQ1 from the particle surface, demonstrated by the ESCA analysis (Vighi et al., 2007).

In order to understand the role of Protamine in the determination of the z-potential of the particles before and after the liophilization, the atomic composition of the SLN-Protamine nanoparticle surface was analysed by the X-ray photoelectron spectroscopy (ESCA). The analysis was performed on both the fresh prepared and the freeze-dried particles obtained in presence of Protamine (SLN-Protamine) or in its absence (SLN-St). ESCA is a surface-sensitive technique that gives information on the elementary composition of the particle surface using the photoelectric effect. In this technique, X-rays are directed at the sample, causing the electrons of surface atoms to be emitted from their orbitals. Only those electrons that leave the surface without energy loss (i.e.

Table 1
Size and zeta potential value of fresh prepared and in water re-suspended freeze-dried SLN-Protamine and SLN-EQ1 sample.

	Fresh sample		Re-suspended samples	
	Particle size (nm) ± S.D. (PDI)	Zeta potential (mV)	Particle size (nm) ± S.D. (PDI)	Zeta potential (mV)
SLN-Protamine	246 ± 50 (0.254)	+20 ± 2	235 ± 20 (0.291)	+19 ± 3
SLN-EQ1	209 ± 23 (0.219)	+40 ± 10	230 ± 40 (0.280)	+23 ± 3

Table 2
Actual percentage (from quantitative analysis of the ESCA spectra) of carbon, oxygen and nitrogen on the surface of SLN prepared in presence (SLN-Protamine) or in absence (SLN-St) of Protamine, both as fresh prepared sample and as in water re-suspended powder.

Formulation	%C	%O	%N	%S
Fresh prepared SLN-St	84.4	13.2	2.4	0
Re-suspended SLN-St	84.5	12.9	2.6	0
Fresh prepared SLN-Protamine	83.2	10.6	4.5	1.7
Re-suspended SLN-Protamine	83.4	10.4	4.6	1.6

10 nm distance) will contribute to the peak signifying that element (Sherwood, 1990).

The survey scan of the SLN gave quantitative data of the surface atomic composition of carbon, oxygen, nitrogen and sulfate (the last one only in the SLN-Protamine sample) (Table 2).

From the data reported in Table 2, no difference in the chemical composition (C, O, S and in particular in the N-percentage) where found between the fresh prepared and the re-suspended SLN-Protamine sample, according to the unchanging of the z-potential value observed after the freeze-drying process. It is relevant to notice that the percentage of nitrogen found in the re-suspended SLN-Protamine sample (4.6%) was almost double respect of that observed in the re-suspended SLN-St samples (2.4%), notwithstanding the z-potential of SLN-Protamine (+19 mV) was less than half of that of SLN-St (+42 mV). The high value of nitrogen in the SLN-St sample indicates that the hydrophilic amino groups of stearylamine are situated on the surface of particles while, presumably, its carboxylic chain was located in the lipid core of the particles. The increase in the nitrogen percentage observed in the SLN-Protamine may be explained by the prevalent presence of Protamine on the surface of particles, given that Protamine displays much more nitrogen atoms respect of stearylamine, due to the amino groups of arginine. In other words, we can hypothesize that Protamine may be localized on the surface of the particles by an ionic interaction between its negative charge (carboxylic terminal group) and the positive amino group of stearylamine, inducing a reduction of the *z*-potential. This model (Fig. 1A) may explain how

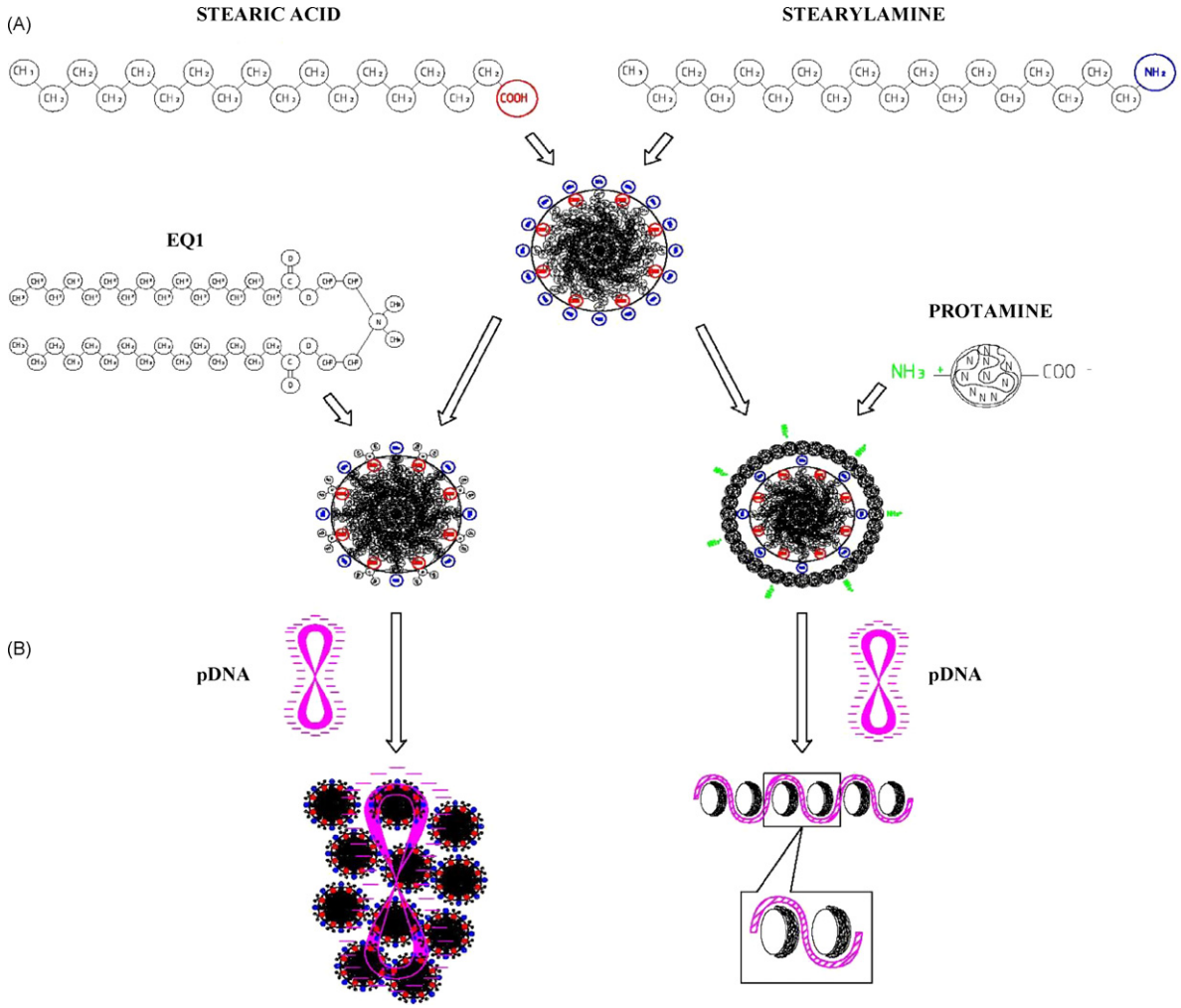

Fig. 1. Schematic illustration of SLN-Protamine and SLN-EQ1 particles, their surface topology (A) and the respective complexes with pDNA (B).

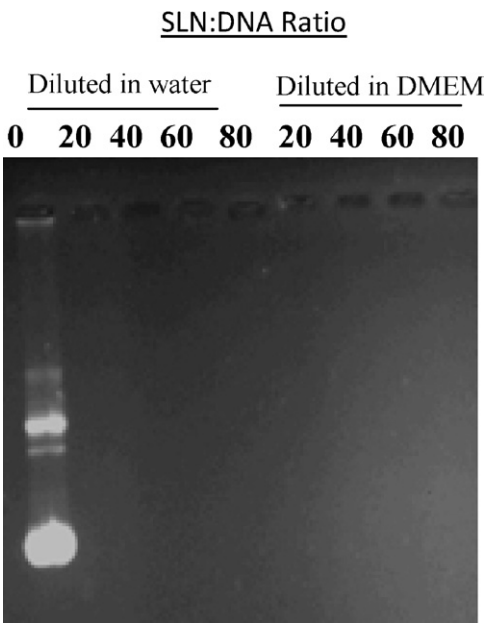

Fig. 2. Agarose gel electrophoresis retardation of pDNA. Lanes from left: (1) naked plasmid DNA; (2–5) ratio (w/w) of SLN:pDNA mixtures diluted in deionised water; (6–9) ratio (w/w) of SLN:pDNA mixtures diluted in DMEM without serum.

SLN-Protamine possesses a strong signal relative to the presence of N and a low z-potential.

Besides, the presence of a relevant S-percentage revealed by the ESCA analysis on the surface of SLN-Protamine, due probably to cistein and tioglicin can support this hypothesis. The S-percentage remains unchanged after the liophilization process indicating that no rearrangement of the localization of Protamine occurred in the re-suspended powder. On the contrary, by the ESCA analysis on the SLN-EQ1 sample it was previously demonstrated that a rearrangement of the surface composition occurred after the liophilization determining a partial expulsion of the EQ1 from the lipid matrix of the sample and thus a reduction of the z-potential, as mentioned above.

Finally, considering that no change in the surface composition occurred for the SLN-Protamine, the re-constituted freeze-dried powder was utilized for all the following characterizations.

3.2. Gel electrophoresis analysis

The binding of the cationic SLN-Protamine to the polyanionic plasmid DNA (pEGFP) was studied using the electrophoretic mobility of the pDNA within an agarose gel. The complexes were formed in deionised water by mixing fixed amount of pDNA (500 ng) with increasing amounts of cationic SLN in such way to obtain cationic SLN:pDNA weight ratios ranging from 20:1 to 80:1, corresponding to the N/P ratios indicated in Section 2. The efficiency of the pDNA complexation by cationic SLN-Protamine after 45 min of incubation was evaluated by the amount of cationic SLN required to retard or to hinder the migration of the pDNA toward the cathode during agarose gel electrophoresis.

Efficient complexation of pEGFP by cationic SLN-Protamine leads to the pDNA immobilization. As can be seen in Fig. 2, in all the ratios SLN:pDNA assayed, stable complexes were formed and their stability were not perturbed by the addition of DMEM. Most of the plasmid is supercoiled (the fast moving band). The absence of DNA in all the lines from 2 to 9 proves that DNA itself is completely immobilized inside complexes.

The transfection of cells in culture generally are performed in serum free medium (i.e. DMEM) thus it was important to assure

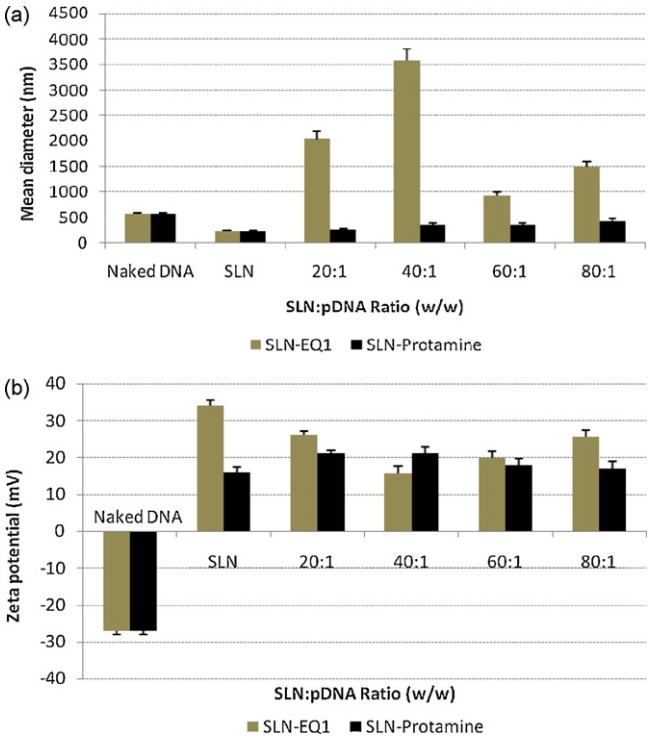

Fig. 3. Size (A) and z-potential (B) of SLN:pDNA complexes, formed with SLN-EQ1 and SLN-Protamine samples.

that SLN:pDNA complexes were stable in this environment. Similar results have been previously obtained in the gel electrophoresis of the SLN-EQ1:pEGFP complex (Vighi et al., 2007) where the re-suspended SLN-EQ1 sample was analysed. Therefore SLN-Protamine and SLN-EQ1, as re-suspended powder, showed a similar capacity to complex pDNA.

In conclusion of this part of characterization, re-suspended SLN-Protamine results to possess size and z-potential values similar to that of SLN-EQ1 and the same capacity to complex pDNA. The two samples (in both cases in their freeze-dried form) were characterized regarding their capacity to condense pDNA and to transfect NA1300 cells.

3.3. Physicochemical characterization of the cationic SLN:pDNA complexes

In living cells, DNA is usually found associated with cationic proteins, such as Protamine and histones, or with cationic domains of viral capsid proteins, as thermodynamically stable, tight complexes. In the gene therapy landscape, the condensed state that is promoted by such interaction is essential for nuclease protection and stabilization in the extracellular media and for efficient translocation to the cell nucleus and further transgene expression (Tagawa et al., 2002).

The pDNA condensation by SLN-Protamine and SLN-EQ1 was evaluated by the PCS and the AFM analysis. The size of the SLN:pDNA complexes were compared with both those of the simple particles (SLN-Protamine or SLN-EQ1) and the naked pDNA (Fig. 3A).

The size of the pDNA was around 550 nm, indicating that pDNA assumes the OC (open circular) topology or wormlike chains as reported by several authors (Smith et al., 1998; Manickama et al., 2005). The weight ratios of the SLN:pDNA complex was ranged from 20:1 to 80:1, corresponding to N/P ratios ranging from 0.4:1 to 1.6:1 for SLN-EQ1:pDNA and from 0.9:1 to 3.6:1 for SLN-Protamine:pDNA, as well as in the agarose gel electorphore-

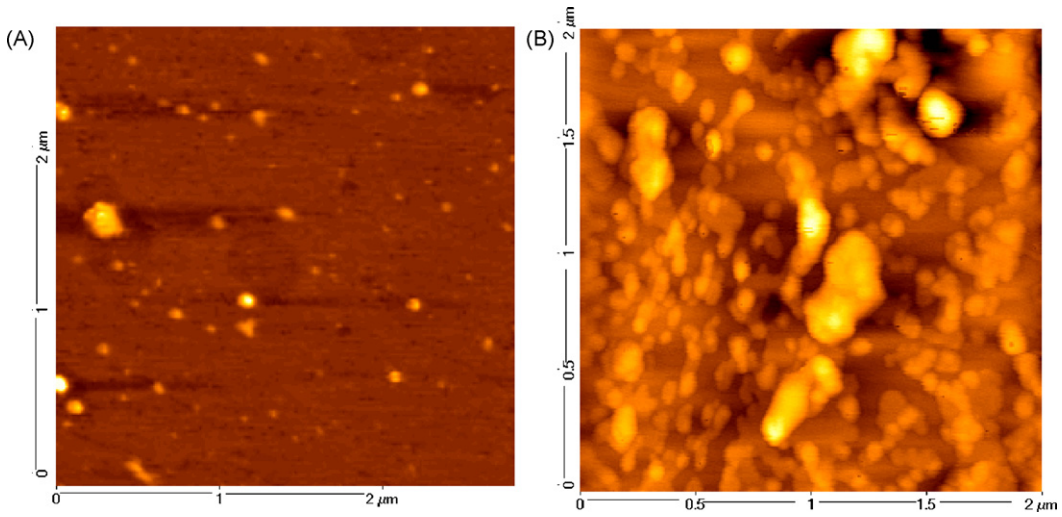

Fig. 4. Atomic force microscopy images of SLN:pDNA complexes at a weight ratio of 40:1: SLN-Protamine:pDNA (A) and SLN-EQ1:pDNA (B).

sis analysis. The size of the SLN-Protamine:pDNA complexes were varied from 268 nm to 430 nm according to the increase of the SLN:pDNA ratios, displaying an increase respect of the initial size of the particles (235 nm) and a reduction respect of the size of the naked plasmid (576 nm). The PDI value also increased with from 0.23 to 0.47 according to the increase of the SLN:pDNA weigh ratios, probably owing to the co-presence of the free SLN. The z-potential value of the complexes was positive (from +21 mV to +17 mV) decreasing with the SLN:pDNA ratio probably owing to the progressive condensation effect of the pDNA. In conclusion SLN-Protamine is able to condense pDNA forming small and positive SLN:pDNA complexes and promoting, presumably, the turn of the pDNA from OC topology to the SC (super coiled) structure around the particles (Fig. 1B). The AFM images of the complexes (Fig. 4A) revealed a very compact and quit globular morphology in agreement with the results of the PCS analysis.

On the other hand, concerning the SLN-EQ1:pDNA complexes a bigger size (from 935 nm to 3584 nm) than the initial naked DNA was obtained for all the ratios studied, suggesting that the condensation capacity of this carrier was weak. The z-potential was positive for all the SLN-EQ1:pDNA ratios increasing from +16 mV to +26 mV (Fig. 3B). The increase of both the size and the z-potential value with the increase of the SLN:pDNA ratio may be explained by the progressive growing of the clusters formed by the particles around the pDNA. The AMF analysis of the SLN-EQ1:pDNA complexes confirmed a cluster-like morphology and a very irregular structure of the complexes (Fig. 4B) where the single particles were visible aggregated around the pDNA presumably in its OC topology.

The different behaviour of the two kinds of SLN may be attributable only to the different tranfection promoters used in their formulation (i.e. EQ1 or Protamine). EQ1, a cationic lipid very similar to the N-(1-(2,3-dioleoyloxy) propyl)-N,N,N-trimethylammonium chloride (DOTAP) or to the dimethyldioctadecyl ammonium bromide (DDAB) used in other formulations (Asasutjarit et al., 2007; del Pozo-Rodríguez et al., 2007), is involved in the formation of the core of SLN (Fig. 1) and assured a stable complexation of pDNA, as discussed in a previous paper (Vighi et al., 2007). However this cationic lipid results less efficient than DOTAP or DDAB in the condensation capacity of pDNA hindering the turn from the OC conformation in the SC ones. On the contrary, Protamine even if give a less positive charge to the particles remaining adsorbed onto their surface, allow the formation of small complexes with pDNA stabilizing the SC pDNA topology.

3.4. In vitro experiments

In gene therapy, gene vectors should not induce cytotoxic effects (Conwell and Huang, 2005) so the low cytotoxicity of SLN is very important. Here, the cytotoxicity of SLN-EQ1 and SLN-Protamine samples was evaluated in Na1300 cells by PI (Propidium iodide) assay considering 2 time periods of incubation, i.e. 4 h and overnight. Cells treated overnight with SLN-EQ1 showed a very high percentage of death (from 37% for the lowest amounts to 82% for the highest one) but a significant decrease in toxicity was observed for 4 h of incubation time (Fig. 5), suggesting that, in this case, cytotoxicity was a time-dependant event.

On the contrary, the cytotoxicity of the SLN-Protamine after 4 h was similar to that observed after the overnight incubation (Fig. 5) suggesting that the toxicity of this sample on the Na1300 cells was not influenced by the incubation time. Summarizing the effect on

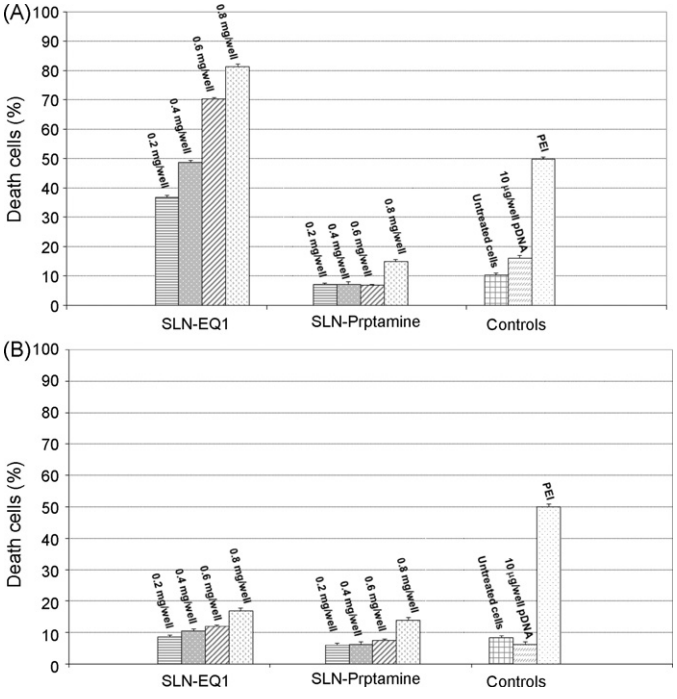

Fig. 5. Citotoxicity of SLN-EQ1 and SLN-Protamine samples on Na1300 cells, incubated overnight (A) or 4 h (B), as evaluated by PI assay.

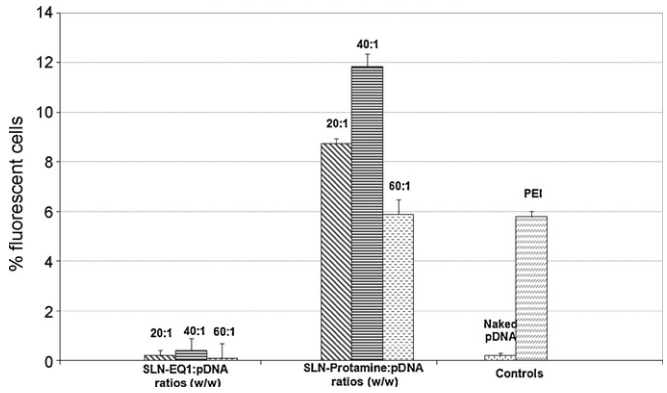

Fig. 6. Transfection efficiency on Na1300 cell line of SLN-EQ1:pDNA and SLN-Protamine:pDNA complexes, as assessed by flow cytometry.

cell viability of SLN-EQ1 after 4 h was very similar to that observed after an overnight incubation for SLN-Protamine, suggesting the lower cytotoxicity of the SLN-Protamine respect of the SLN-EQ1 sample.

The high and time dependent cytotoxicity of SLN-EQ1 may be attributable to the effect of the EQ1 quaternary ammine headgroup that should exert cell membrane damage according to both the concentration and the contact time. On the contrary, in the case of SLN-Protamine, the higher cell viability observed is probably due to the better cell-compatibility of Protamine, a small and naturally occurring protein. As a consequence, SLN-Protamine particles maintain the same low level of cytotoxicity even increasing the particles concentration (until 600 μg/well) and the cell contact time.

The cytotoxicity results obtained here were considered in the study of the transfection efficiency of the two samples not only to establish the maximum working amount of SLN (i.e. 600 μg/well) but also the more appropriate incubation time period for each sample: i.e. 4 h in the case of SLN-EQ1 and overnight for SLN-Protamine. In fact in this experimental condition both the samples showed a cytotoxicity lower than PEI, as reported in Fig. 5.

Thus, the transfection efficiency of SLN:pDNA complexes was evaluated at weight ratios ranging from 20:1 to 60:1, corresponding to N/P ratios from 0.4:1 to 1.2:1 for SLN-EQ1:pDNA and from 0.9:1 to 2.7:1 for SLN-Protamine:pDNA, using naked pDNA and 25 kDa PEI as controls.

As shown in Fig. 6, the transfection efficiency of naked pDNA was very low (0.1%), according to the expectative. In fact, as known, most internalized free DNA is rapidly destroyed by endosome or cytoplasmatic DNAses, before entering the nucleus (Lv et al., 2006). Transfection efficiency of SLN-EQ1:pDNA complexes resulted dramatically low for all the SLN:pDNA ratios analysed, while SLN-Protamine:pDNA complexes showed higher transfection efficiency than PEI for all SLN:pDNA ratios tested, notwithstanding the lower citotoxicity demonstrated. In details, SLN-Protamine:pDNA complexes at 40:1 SLN:pDNA w/w ratio had a transfection efficiency around 12% that can be considered a good level for this poor transfectable cell line.

From transfection data it is evident that SLN-EQ1 sample is unable to transfect Na1300 cells even if employed in low cytotoxicity conditions (incubation time and concentration). As in such as conditions the cell membrane damage effect is slight, the failed transfection can be ascribable to the big dimensions of the SLN-EQ1:DNA complexes. On the other hand, the encouraging transfection data obtained by SLN-Protamine:pDNA complexes were probably due to the pDNA condensation ability of the cationic peptide Protamine, since pDNA condensation is a crucial factor determining the size of the complexes and thus the transfection capacity of nonviral vectors. Indeed the pDNA condensation influences not only the size and z-potential of the complexes but also their cell entry, their gene protection from DNAses, the pDNA delivery from nanoparticles and finally the pDNA transcription.

Generally, the small particle size of the complexes would facilitate the cellular uptake and enhance transfection efficiency (Lv et al., 2006). In our case, the transfection data fit well with the complex size determination for both the samples: the smaller was the size the higher was the transfection efficiency. Moreover the positive charge of the nonviral systems is necessary for the interaction with the negative charged cell surface and the cell entry facilitating the invagination of the cell plasma membrane (Rejman et al., 2005). However when the complexes display a too much high z-potential, a low capacity to release the pDNA may be observed (Moore et al., 2009) owing to the low delivery of the pDNA. Thus, in the case of SLN-Protamine:pDNA complexes, the moderate z-potential (about +20 mV) may promote the release of pDNA from endosome to the cytoplasm. Finally, the good transfection efficiency achieved by SLN-Protamine may be also probably due to the nuclear transfer of complexes as a result of the nuclear localization signal of Protamine (Noguchi et al., 2002; Masuda et al., 2005) that promotes penetration of pDNA into the nucleus and transcription of exogenous pDNA.

4. Conclusion

The addition of a cationic lipid such as EQ1 or a cationic protein such as Protamine in the SLN formulations provided a multivalent positive surface charge that could interact via electrostatic with the negative charge of the pDNA phosphate backbone. The results here suggest that the ability of SLN to condense the pDNA depends not only on the value of their charge but also on the surface composition of the carrier. In fact SLN-Protamine even if displayed a similar z-potential value and a similar capacity to complex DNA respect to SLN-EQ1 demonstrated a higher pDNA condensation capacity, better transfection proprieties and lower citotoxicity. Further studies of transfection efficiency will be carried out in other cell lines and studies of cell localization will be performed in order to evaluate the capacity of the two complexes to be internalized in the cells and to clarify how the different composition affects the cell entry capacity of the complexes.

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References

Alonso, M.J., 2004. Nanomedicine for overcoming biological barriers. Biomed. Pharmacother. 58, 168–172.

Asasutjarit, R., Lorenzen, S.I., Sirivichayakul, S., Ruxrungtham, K., Ruktanonchai, U., Ritthidej, G.C., 2007. Effect of Solid Lipid Nanoparticles formulation compositions on their size, zeta potential and potential for in vitro pHIS-HIV-Hugag transfection. Pharm. Res. 24, 1098–1107.

Choi, S.H., Jin, S.-E., Lee, M.-K., Lim, S.-J., Park, J.-S., Kim, B.-G., Ahn, W.S., Kim, C.-K., 2008. Novel cationic solid lipid nanoparticles enhanced p53 gene transfer to lung cancer cells. Eur. J. Pharm. Biopharm. 68, 545–554.

Chollet, P., Favrot, M.C., Hurbin, A., Coll, J.L., 2002. Side-effects of a systemic injection of linear polyethylenimine–DNA complexes. J. Gene Med. 4, 84–91.

Conwell, C.C., Huang, L., 2005. Recent advances in non-viral gene delivery. Adv. Genet. 53, 1–18.

del Pozo-Rodríguez, A., Delgado, D., Solinıs, M.A., Gascon, A.R., Pedraz, J.L., 2007. Solid lipid nanoparticles: formulation factors affecting cell transfection capacity. Int. J. Pharm. 339, 261–268.

del Pozo-Rodríguez, A., Delgado, D., Solinís, M.Á., Pedraza, J.L., Echevarríac, E., Rodríguezc, J.M., Gascóna, A.R., 2010. Solid lipid nanoparticles as potential tools

for gene therapy: in vivo protein expression after intravenous administration. Int. J. Pharm. 385, 157–162.
del Pozo-Rodríguez, A., Pujals, S., Delgado, D., Solinís, M.A., Gascón, A.R., Giralt, E., Pedraz, J.L., 2009. Proline-rich peptide improves cell transfection of solid lipid nanoparticle-based non-viral vectors. J. Control. Release 133, 52–59.
Heydenreich, A.V., Westmeier, R., Pedersen, N., Poulsen, H.S., Kristensen, H.G., 2003. Preparation and purification of cationic solid lipid nanospheres-effect on particle size, physical stability and cell toxicity. Int. J. Pharm. 254, 83–87.
Jeong, J.H., Park, T.G., 2002. Poly(L-lysine)-γ-poly(D,L-lactic-co-glycolic acid) micelles for low cytotoxic biodegradable gene delivery carriers. J. Control. Release 82, 159–166.
Joshi, M.D., Müller, R.H., 2009. Lipid nanoparticles for parenteral delivery of actives. Eur. J. Pharm. Biopharm. 71, 161–172.
Kaur, I.P., Bhandari, R., Bhandari, S., Kakkar, V., 2008. Potential of solid lipid nanoparticles in brain targeting. J. Control. Release 127, 97–109.
Li, W., Szoka Jr., F.C., 2007. Lipid-based nanoparticles for nucleic acid delivery. Pharm. Res. 24, 438–449.
Li, S.D., Huang, L., 2007. Non-viral is superior to viral gene delivery. J. Control. Release 123, 181–183.
Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. J. Control. Release 114, 100–109.
Manickama, D.S., Bishta, H.S., Wanb, L., Maob, G., Oupickya, D., 2005. Influence of TAT-peptide polymerization on properties and transfection activity of TAT/DNA polyplexes. J. Control. Release 102, 293–306.
Masuda, T., Akita, H., Harashima, H., 2005. Evaluation of nuclear transfer and transcription of plasmid DNA condensed with Protamine. Mol. Ther. 11, S289–S290.
Mehnert, W., Mader, K., 2001. Solid lipid nanoparticles: production, characterization and applications. Adv. Drug Deliv. Rev. 47, 165–196.
Moore, N.M., Sheppard, C.C., Sakiyama-Elbert, S.E., 2009. Characterization of a multifunctional PEG-based gene delivery system containing nuclear localization signals and endosomal escape peptide. Acta Biomater. 5, 854–864.
Muller, R.H., Mader, K., Gohla, S., 2000. Solid lipid nanoparticles (SLN) for controlled drug delivery—a review of the state of the art. Eur. J. Pharm. Biopharm. 50, 161–177.
Noguchi, A., Hirashima, N., Nakanishi, M., 2002. Cationic cholesterol promotes gene transfection using the nuclear localization signal in Protamine. Pharm. Res. 19, 933–938.
Olbrich, C., Bakowsky, U., Lehr, C.M., Muller, R.H., Kneuer, C., 2001. Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid DNA. J. Control. Release 77, 345–355.
Rejman, J., Bragonzi, A., Conese, M., 2005. Role of clathrin- and caveolae mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol. Ther. 12, 468–474.
Schwarz, C., Mehnert, W., Lucks, J.S., Muller, R.H., 1994. Solid Lipid Nanoparticles (SLN) for controlled drug delivery. 1. Production, characterization and sterilization. J. Control. Release 30, 83–96.
Sherwood, M.A., 1990. Data analysis in X-ray Photoelectron Spectroscopy, Practical Surface Analysis, 2nd ed. Wiley, New York.
Smith, L.C., Duguid, J., Wadhwa, M.S., Logan, M.J., Tung, C.H., Edwards, V., Sparrow, J.T., 1998. Synthetic peptide-based DNA complexes for nonviral gene delivery. Adv. Drug Deliv. Rev. 30, 115–131.
Tabatt, K., Sameti, M., Olbrich, C., Muller, R.H., Lehr, C.M., 2004. Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer. Eur. J. Pharm. Biopharm. 57, 155–162.
Tagawa, T., Manvell, M., Brown, N., Keller, M., Perouzel, E., Murray, K.D., Harbottle, R.P., Tecle, M., Booy, F., Brahimi-Horn, M.C., Coutelle, C., Lemoine, N.R., Alton, E.W.F.W., Miller, A.D., 2002. Characterization of LMD virus-like nanoparticles self-assembled from cationic liposomes, adenovirus core peptide mu and plasmid DNA. Gene Ther. 9, 564–576.
Tseng, Y.-C., Mozumdar, S., Huang, L., 2009. Lipid-based systemic delivery of siRNA. Adv. Drug Del. Rev. 61, 721–731.
Tsuchiya, Y., Ishti, T., Okahata, Y., Sato, T., 2006. Characterization of Protamine as a tranfection accelerator for gene delivery. J. Bioact. Compat. Pol. 21, 519–537.
Vighi, E., Ruozi, B., Montanari, M., Battini, R., Leo, E., 2007. Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid. Eur. J. Pharm. Biopharm. 67, 320–328.
Wrobel, K., Claudio, E., Segade, F., Ramos, S., Laze, P.S., 1996. Measurement of cytotoxicity by propidium iodide staining of target cell DNA. Application to the quantification of murine TNF-α. J. Immunol. Methods 189, 243–249.