Drug Delivery

ISSN: 1071-7544 (Print) 1521-0464 (Online) Journal homepage: http://www.tandfonline.com/loi/idrd20

The role of protamine amount in the transfection performance of cationic SLN designed as a gene nanocarrier

Eleonora Vighi, Monica Montanari, Barbara Ruozi, Valentina Iannuccelli & Eliana Leo

To cite this article: Eleonora Vighi, Monica Montanari, Barbara Ruozi, Valentina Iannuccelli & Eliana Leo (2012) The role of protamine amount in the transfection performance of cationic SLN designed as a gene nanocarrier, Drug Delivery, 19:1, 1-10, DOI: 10.3109/10717544.2011.621989

To link to this article: http://dx.doi.org/10.3109/10717544.2011.621989

Published online: 09 Nov 2011.

Drug Delivery, 2012; 19(1): 1–10

ISSN 1071-7544 print/ISSN 1521-0464 online
DOI: 10.3109/10717544.2011.621989

informa
healthcare

ORIGINAL ARTICLE

The role of protamine amount in the transfection performance of cationic SLN designed as a gene nanocarrier

Eleonora Vighi[1], Monica Montanari[2], Barbara Ruozi[1], Valentina Iannuccelli[1], and Eliana Leo[1]

[1]Department of Pharmaceutical Sciences and [2]Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via Campi, 41100 Modena, Italy

Abstract

Cationic solid lipid nanoparticles (SLN) have been recently proposed as non-viral vectors in systemic gene therapy. The aim of this study was to evaluate the effect of the protamine amount used as the transfection promoter in SLN-mediated gene delivery. Three protamine-SLN samples (Pro25, Pro100, and Pro200) prepared by adding increasing amounts of protamine were characterized for their size, zeta potential, and protamine loading level. The samples were evaluated for pDNA complexation ability by gel-electrophoresis analysis and for cytotoxicity and transfection efficiency by using different cell lines (COS-I, HepG2, and Na1300). The size of SLN was ~230 nm and only Pro200 showed few particle aggregates. Unlike the Pro25 sample with the lowest protamine loading level, the others SLN samples (Pro100 and Pro200) exhibited a good ability in complexing pDNA. A cell-line dependent cytotoxicity lower than that of the positive control PEI (polyethilenimmine) was observed for all the SLN. Among these, only Pro100, having an intermediate amount of protamine, appeared able to promote pDNA cell transfer, especially in a neuronal cell line (Na1300). In conclusion, the amount of protamine as the transfection promoter in SLN affects not only the gene delivery ability of SLN but also their capacity to transfer genes efficiently to specific cell types.

Keywords: Gene delivery, solid lipid nanoparticles, protamine, transfection promoter, pEGFP

Introduction

Gene therapy requires the introduction of foreign DNA encoding a therapeutic gene into the target cells, using viral or non-viral delivery systems. Non-viral vectors, even if not so efficacious as viral ones, are safer and may be used in the in vivo approach, allowing the DNA administration by a systemic route such as intravenous infusion (Huang & Viroonchatapan, 1999; Morille et al., 2008). Solid Lipid Nanoparticles (SLN) represent one of the most studied non-viral lipid vectors (Olbrich et al., 2001; Rudolph et al., 2004; Tabatt et al., 2004a, b; Pedersen et al., 2006; del Pozo-Rodriguez et al., 2009). However, it is known that the transfection efficiency of non-viral vectors, including SLN, is limited by several steps, such as cell internalization, disruption of the endosome membrane, and translocation of the genetic material towards the cell nucleus (Canine et al., 2008). In order to promote the transfection, cationic non-viral vectors are described to facilitate an electrostatic interaction of the nanocarrier with both the negative backbone of DNA and the negative cell surface inducing unspecific endocytosis. For this reason, cationic lipids such as Esterquat 1 (EQ1) (N,N-di-(β-stearoylethyl)-N,N-dimethylammonium chloride) or DOTAP (N-(1-(2,3-dioleoyloxy) propyl)-N,N,N-trimethylammoniumchloride) have been used in the development of cationic gene delivery devices (Olbrich et al., 2001; del Pozo-Rodriguez et al., 2007). Moreover, the addition of cationic protein/peptide transfection promoters, such as protamine, has been shown to enhance lipid-mediated gene transfection (Vangasseri et al., 2005). Protamine is a cationic nuclear protein enriched in arginine, FDA approved for the parenteral administration, and widely studied in the gene delivery (Tsuchiya et al., 2006) owing to its ability to improve DNA packaging in sperm cells (Braun, 2001), to protect

Address for Correspondence: Professor Eliana Leo, Department of Pharmaceutical Sciences, University of Modena and Reggio Emilia, Via Campi 183, 41100 Modena, Italy. Tel: + 39 59 205 5148. Fax: + 39 59 205 5131. Email: eliana.leo@unimore.it

(Received 06 April 2011; accepted 06 September 2011)

DNA from degradation and to promote DNA delivery into the nucleus by means of Nuclear Localization Signal sequence (Tsuchiya et al., 2006).

In previous studies, we referred that the transfection by SLN-Protamine:pDNA was more effective compared with the Protamine:pDNA polyplexes while no transfection capacity was observed for SLN-EQ1:pDNA complexes due to their inability to be internalized caused by their larger dimension (Vighi et al., 2010a). Red Nile-SLN-Protamine were localized in endocytic-like vesicles inside the nuclear membrane, suggesting that the inclusion of protamine in nano-lipophilic systems may reduce the complex size and enhance the nuclear pDNA translocation as well as the pDNA release into the cells (Vighi et al., 2010a, b).

Since the protamine concentration added to the SLN composition could represent a crucial parameter affecting the transfection efficiency, the present work aims to investigate the effect of the amount of protamine employed in the vector preparation on the in vitro performance of cationic SLN. These vectors were formulated using stearic acid as the solid lipid matrix, Pluronic F68 as the surfactant, and stearylamine as the cationic component. Protamine was included in three different percentages, i.e., 0.25, 1, and 2% w/v, obtaining three different SLN samples. SLN physical properties (morphology, size, and zeta potential), pDNA complexation, as well as in vitro cytotoxicity and gene transfection ability were examined in order to establish the optimum protamine amount useful to enhance SLN transfection efficiency and expression level in a perspective of a gene therapy.

Materials and methods

Materials

Stearic acid was purchased from Carlo Erba Reagenti (Milan, Italy). Stearylamine was provided from Fluka (Deisenhofen, Germany). Protamine sulfate and polyethilenimmine (PEI, Mw 25,000) were provided from Sigma-Aldrich (St. Louis, MO). Agarose GellyPhor, Dulbecco's modified eagle medium (DMEM), Dulbecco's Phosphate Buffer (PBS), and other culture reagents were purchased from Euroclone Celbio (Milan, Italy). All other chemical reagents were obtained commercially as reagent-grade products. The cell lines COS-I, HepG2, and Na1300 were provided from Zoo-prophylactic Institute of Emilia Romagna (Bologna, Italy).

SLN preparation

Cationic SLN were produced by a modification of the oil-in-water microemulsion method (Heydenrich et al., 2003). In practice, the solid lipid component (250 mg stearic acid) was melted at ~68°C, and an aqueous phase (10 mL) containing Pluronic F68 (2% w/v), stearylamine (3% w/v), and protamine (0.25, 1, or 2% w/v) was heated at the same temperature. Then, the melted lipid phase 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 obtain a hot O/W emulsion. The emulsion was then cooled at 4°C by an ice bath under mechanical stirring at 20,500 rpm for 10 min, i.e., until the formation of solid lipid nanoparticles (SLN) suspension. This suspension was purified and concentrated until 10 mL volume by vacuum ultra-filtration using a polycarbonate holder (Sartorius, Goettingen, Germany) equipped with a polyethersulfone (PESU) filter (cut-off 100 nm, Sartorius) and freeze-dried (Heto-Holten A/S, Allerod, Denmark) for 48 h at–55°C and at a pressure of 10^{-2} Torr. The freeze-dried SLN powder was collected and stored at 4°C. The samples obtained by using increasing concentrations of protamine were named Pro25, Pro100, and Pro200 throughout the text.

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

Determination of protamine loading

In order to evaluate the total protamine in the SLN samples, an accurately weighed amount of freeze-dried SLN (~20 mg) was dissolved in 1 mL of dichloromethane. Then, 2 mL of deionized water was added and stirred until the complete evaporation of the organic solvent. The sediment was separated by centrifugation (Spectrafuge 24D, Labnet International Inc., Edison, NJ) at 12,000 rpm for 25 min. The protamine total amount was determined on the clear supernatant by Bio-Rad DC Protein Assay (Bio-Rad laboratories, Milan, Italy), measuring the absorbance spectrophotometrically (UV/VIS spectrometer V-530, Jasco, Tokyo, Japan) at 750 nm. The calibration curve for the protamine quantification was linear in the range of 0.1–1 mg/mL with a correlation coefficient of $R^2 = 0.9995$. Each sample was analyzed in triplicate. The total amount of protamine in each sample was expressed as a percentage (% w/w) according to the following formula:

$$\text{Total protamine (\%, w/w)} = \frac{\text{determined protamine (mg)}}{\text{weighed SLN (mg)}} \times 100$$

In order to quantify the protamine fraction non-associated to the particles (free fraction) an accurately weighed amount of freeze-dried SLN (~ 20 mg) was re-constituted in deionized water (2 mL) as described above and centrifuged at 10,000 rpm for 15 min in a Microcon device (Microcon 10K, Millipore Corporation, Bedford, MA). The recovered solution was analyzed for protamine concentration (%, w/w) by Bio-Rad DC Protein Assay, as referred above. Each sample was analyzed in triplicate. The percentage of free protamine was calculated using the following formula:

$$\text{Free protamine (\%, w/w)} = \frac{\text{determined free protamine (mg)}}{\text{weighed SLN (mg)}} \times 100$$

The protamine fraction associated to the nanocarrier was calculated from the difference between total and free protamine amounts. The data are averaged on three determinations for each sample.

Plasmid DNA production

The plasmid vector expressing Enhanced Green Fluorescent Protein pEGFP-C3 (pDNA) was transformed into *Escherichia coli* (XL_1 Blue MR). A Maxiprep from 500 mL of the overnight culture was performed by Invitrogen Kit according to the manufacturer's instruction (PureLink™ Hipure Plasmid Maxiprep Kit, Invitrogen, Carlbad, CA).

Preparation of SLN:pDNA complexes

pDNA was mixed with freeze-dried SLN (Pro25, Pro100, Pro200) re-dispersed in water (5 mg/mL) at the following SLN:pDNA w/w ratios: 20:1, 40:1, 60:1, and 80:1. The mixtures were kept at room temperature (25 ± 0.5°C) for 45 min to allow the formation of the complexes. The water suspensions containing the complexes, named Pro25:pDNA, Pro100:pDNA, and Pro200:pDNA throughout the text, were used immediately after their preparation.

Physical characterization of SLN and SLN:pDNA complexes

Z-average diameter, size distribution, and polydispersity index (PDI) measurements were performed using dynamic light scattering (DLS) by a Nanosizer NanoZS (Malvern Instruments, Worcs, UK) equipped with a 4 mW He-Ne laser (633 nm) and a DTS software (Version 5.0). The value of *Z*-average diameter is referred to as the mean hydrodynamic diameter weighted according to light scattering intensity. The size distribution, i.e., the plot of the relative intensity of light scattered by particles in various size classes, was reported as the peak of the most represented dimensional class of each particle population.

The zeta potential values were determined by Laser Doppler Anemometry by a folded capillary electrophoresis cell of the Zetasizer NanoZS at 25°C using deionized water with a refraction index of 1.33 and a viscosity of 0.8872 cP.

All the data were expressed as the mean ± standard deviation (SD) calculated on three determinations.

Atomic force microscopy

The morphology of SLN and the structure of SLN:pDNA complexes were examined by Atomic Force Microscopy (AFM) (Park Autoprobe Atomic Force Microscope, Park Instruments, Sunnyvale, CA). The experiments were carried out in water at room temperature (20 ± 1°C) under atmospheric pressure operating in non-contact mode. Triangular silicon tips were used for this analysis. The resonant frequency of this cantilever was found to be ~120 KHz.

Agarose gel electrophoresis

The SLN:pDNA complexes were prepared by using 1 μL of a pDNA solution at a concentration of 0.5 mg/mL diluted in serum-free DMEM medium (1:5, v/v), following the same procedure outlined above. 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.

Cell culture

COS-I (SV-40 transformed African Green Monkey kidney cell line) and HepG2 (Human hepatocellular liver carcinoma cell line) cells were cultured in DMEM medium with high glucose, supplemented with 10% (v/v) of fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C under 95% relative humidity. Na1300 cells (murine neuroblastoma cell line) were cultured in the same conditions, but using FBS at 20% (v/v).

Cytotoxicity

SLN cytotoxicity, expressed as a percentage of dead cells, was assessed by the PI (propidium iodide) test using flow cytometry. The cells were seeded at 500,000 cells/dish in 6 cm Petri dishes and then cultured for 24 h in complete medium at 37°C. The experiments were conducted at ~80% confluence. The medium was aspirated from each dish and replaced with 2 mL of serum-free DMEM. Then, 40, 80, 120, or 160 μL/well of SLN suspension at a concentration of 5 mg/mL (corresponding to SLN amounts of 0.2, 0.4, 0.6, and 0.8 mg/well, respectively) were added followed by 12 h cell incubation. The cytotoxicity of polyethilenimmine (PEI), used as a positive control, was determined by incubating the cells with 15 μg of PEI for 2 h in complete medium according to the literature procedure (Pintel & Tullis, 2006).

Then, all the treated cells were washed with PBS, added with 5 mL of DMEM containing FBS (10% v/v), and incubated at 37°C for 24 h, in order to simulate the transfection procedure, before the flow cytometry analysis.

Cell transfection

In order to study the transfection efficiency of the vectors, cells were grown in 6 cm Petri dishes, seeding the cells at 500,000 cells/dish. After plating, the cells were incubated at 37°C for 24 h and transfection experiments were conducted at ~ 80% confluence. The culture medium was aspirated from each dish, replaced with 2 mL of serum-free medium, and added with SLN:pDNA complexes at w/w ratios ranging from 20:1–60:1. The complexes were prepared as described above by using 10 μL of 1 mg/mL pDNA solution and appropriate volumes of 5 mg/mL SLN suspensions. The cells were incubated in a 5% CO_2 incubator at 37°C for 12 h (incubation time). Then, the transfected cells were washed with PBS and incubated at 37°C in 5 mL DMEM containing FBS for 24 h (expression

time) in order to allow the expression of the protein, and then analysed by flow cytometry.

Cells transfected by 10 μg of naked pDNA were used as a negative control and cells transfected by PEI:pDNA poliplexes were adopted as a positive control. In the last case the procedure described in the literature has been followed (Pintel & Tullis, 2006; Huh et al., 2007); in practice, 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. The PEI:pDNA polyplexes were incubated with the cells for 2 h (first incubation time) in complete medium (DMEM+10% v/v FBS). Then, the medium was replaced with 5 mL of fresh complete medium and the cells were incubated for 12 h (second incubation time) at 37°C. Before the flow cytometry analysis, another medium replacement was carried out and the cells were incubated again for 24 h (expression time), likewise for SLN:pDNA complexes.

Flow cytometry analysis

Cytotoxicity expressed as a percentage of dead cells as well as quantitative transfection efficiency was evaluated by flow cytometry. The incubated cells were washed with 500 μL of PBS and detached with 500 μL of 0.25% trypsin added with EDTA (1% v/v).

With regard to cytotoxicity, 800 μl of cells re-suspended in 4 mL of the removed medium were centrifuged at 1000 rpm for 5 min. The supernatant was removed and the cells were suspended in 500 μL of PI solution, prepared according to Nicoletti staining (50 mg/mL PI, 0.1% w/v sodium citrate, 0.1% w/v Triton X-100), and incubated in the dark at 4°C for 10 min.

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

The percentage of dead cells and the transfection efficiency were determined at 540 nm and 488 nm, respectively, by using a Coulter Epics XL cytofluorimeter (Coulter Electronics Inc., Hialeah, FL) equipped with a 488 nm argon laser. At least 10,000 events were collected for each sample.

Results and discussion

Protamine loading

In all SLN samples both the total and the free protamine content increased with the increase of the initial amount of protamine (Table 1). On the contrary, the protamine fraction associated to the nanocarriers, calculated as the difference between the total and the free content, increased significantly from 4.5% to 13% w/w by increasing the initial amount of protamine in Pro25 and Pro100, respectively, and then it remained constant by further protamine increase (Pro200), reaching a plateau value. The lack of correlation between the increase of initial protamine amount and that of the associated protamine loading could be explained by considering that protamine is localized on the surface of the particles by an ionic interaction between its negatively charged amino acid tails and the positive amino group of stearylamine (Vighi et al., 2010a). Therefore, a saturation of these interaction sites in the Pro100 sample could be reasonably supposed.

SLN physical properties

SLN physical properties such as morphology, size, polydispersity index (PDI), and surface charge, known to play a critical role in cell transfection efficiency (Rejman et al., 2004), were investigated.

SLN morphology analysis examined by AFM revealed spherical shaped particles in the nanometric size for

Table 1. Protamine content in SLN samples. Mean values ± SD.

SLN samples	Initial protamine amount in the SLN preparation (mg)	Total protamine (%, w/w)	Free protamine (%, w/w)	Associated protamine (%, w/w)
Pro25	25	4.5 ± 0.5	0.00 ± 0.01	4.5 ± 0.5
Pro100	100	23.0 ± 1.5	10.0 ± 0.7	13.0 ± 0.5
Pro200	200	33.4 ± 1.0	20.1 ± 1.0	13.3 ± 0.5

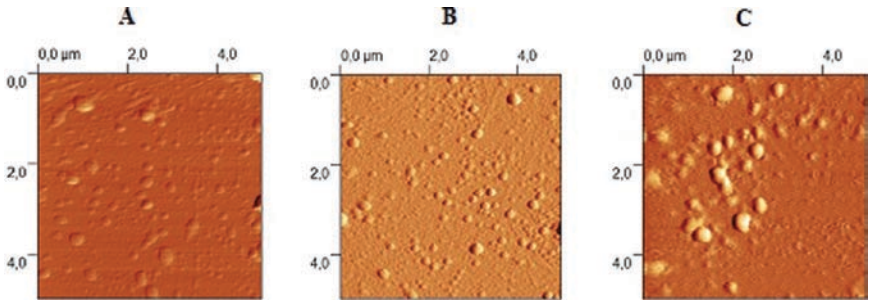

Figure 1. Atomic force microscopy (AFM) images of SLN (A: Pro25; B: Pro100; C: Pro200).

Pro25 and Pro100, and small particles combined with several aggregates for Pro200 (Figure 1). *Z*-average diameters, size distributions by intensity, and zeta potential values of the three SLN samples are reported in Table 2. According to AFM images, Pro25 and Pro100 showed no significant differences in *Z*-average diameters (~230 nm), PDI values of ~0.25, and monomodal distributions of the size. Conversely, Pro200 exhibited a *Z*-average diameter of 430 nm, with a higher PDI value (~0.5) and a bimodal size distribution characterized by two distinct populations being the first one, with the main dimensional class at 228 nm (73% intensity), attributable to the nanoparticles, and the second one, with the main dimensional class at 2446 nm (27% intensity), attributable to particle aggregates. This finding confirmed the AFM analysis and suggested that the highest initial amount of protamine in the preparation (2% w/v) induced particle aggregation, decreasing the size homogeneity.

By comparing AFM image with DLS results, it is evident that plurimodal systems, unlike monomodal ones, are not correctly described by the *Z*-average value. Therefore, in such cases the analysis of the size distribution instead of *Z*-average has to be considered.

As far as zeta potential is concerned (Table 2), all SLN samples showed a positively charged surface owing to the presence of the cationic lipid, being the value +19 mV, +17 mV, and +9 mV for Pro25, Pro100, and Pro200, respectively. The decreased surface charge of Pro200 could be attributed to the higher free protamine amount accumulated on the nanoparticle surface, inducing a less positive charge than that offered by the cationic lipid (Asasutjarit et al., 2007; Jain et al., 2009). The reduced zeta potential would give an explanation of the nanoparticle aggregation observed for this sample.

Agarose gel electrophoresis

To evaluate the ability of the SLN to bind pDNA, an essential pre-requisite for transfection, gel mobility experiments were carried out on the SLN:pDNA complexes obtained at different SLN:pDNA ratios (Figure 2). Spots attributable to the plasmid migration were provided only by the sample Pro25 at SLN:pDNA ratios from 20:1–60:1, indicating that pDNA was not stably complexed with the SLN. Otherwise, no pDNA migration was observed for the sample Pro25 at a SLN:pDNA ratio of 80:1 (Figure 2A), as well as for the samples Pro100 and Pro200, regardless of the SLN:pDNA ratio (Figures 2B and C), giving evidence of a complete pDNA complexation. Therefore, the minimum amount of protamine able to provide a complete plasmid complexation was found to be ranging from 2.7–3.6 μg/μg pDNA, corresponding to the amount of protamine in Pro25 at 80:1 and in Pro100 at 20:1 SLN:pDNA ratio, respectively.

SLN:pDNA complex physical properties

The results of DLS analysis performed on the SLN:pDNA complexes in terms of *Z*-average diameter, PDI, size distribution, and zeta potential value are reported in Table 3 along with the size and the zeta potential of naked pDNA.

With regard to Pro25:pDNA complexes, until 60:1 SLN:pDNA w/w ratio, a bimodal size distribution was observed with PDI values > 0.4. Therefore, the size of each population (size distribution by intensity) instead of the *Z*-average value was taken into account, as highlighted above. The first population, with the main dimensional class at ~210 nm, could be attributable to SLN, whereas the second one, at ~580 nm, could be due to the naked pDNA. Otherwise, at a 80:1 SLN:pDNA ratio a monomodal size distribution with a *Z*-average diameter of 400 nm combined with a low PDI value (0.3) was observed, suggesting the formation of Pro25:pDNA complexes. The negative surface charge of pDNA (–27 mV) determined an overall negative surface charge for Pro25:pDNA at low ratios, owing to the lack of interaction between the plasmid and the cationic nanoparticles, with a tendency to shift towards positive values as the increase in SLN:pDNA ratio, as evidence of the occurred pDNA complexation.

Pro100:pDNA complexes exhibited a monomodal size distribution with a *Z*-average diameter ranging between 310–400 nm and PDI values of ~0.4, regardless of the SLN:pDNA ratio, suggesting the formation of SLN:pDNA complexes. The surface charge was still positive and without significant differences according to SLN:pDNA ratio, confirming the stable complexation of pDNA.

As far as Pro200:pDNA complexes are concerned, a high PDI value (>0.5) and three different particle populations were observed for all SLN:pDNA ratios. The largest particles of ~2200 nm could be ascribed to the SLN aggregates, whereas the peak at 350 nm may be attributable to the complexes with the plasmid. Moreover, the smallest population (90 nm) having a high frequency by intensity (~60%) can be presumably provided by protamine/pDNA polyplexes according to the high free protamine amount found in this sample. This hypothesis is confirmed by the positive zeta potential values observed, regardless of the SLN:pDNA ratio.

To give further support to the observed SLN:pDNA complex physical properties, AFM topographical images

Table 2. *Z*-average, PDI, particle size distribution by intensity, and Zeta-potential of Pro25, Pro100, and Pro200. Mean values ± SD.

		Particle size distribution by intensity		
SLN samples	*Z*-average (nm) (PDI)	Peak 1 (nm) (%)	Peak 2 (nm) (%)	Zeta potential (mV)
Pro25	230 ± 30 (0.220 ± 0.002)	202 ± 25 (100 ± 2%)	—	+17 ± 5
Pro100	235 ± 20 (0.280 ± 0.001)	204 ± 22 (100 ± 3%)	—	+19 ± 3
Pro200	428 ± 80 (0.502 ± 0.004)	228 ± 18 (73 ± 2%)	2446 ± 158 (27 ± 3%)	+9 ± 2

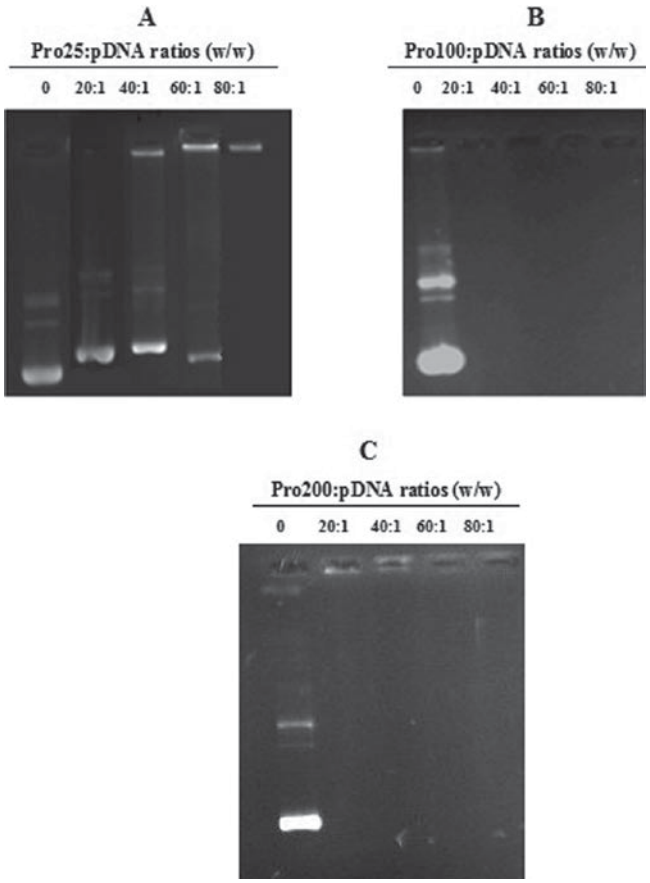

Figure 2. (A) Agarose gel electrophoresis of pDNA incubated with Pro25 and diluted in DMEM. Lane from left: naked plasmid DNA (0); Pro25:pDNA mixtures at 20:1, 40:1, 60:1, and 80:1 w/w ratios, respectively. (B) Agarose gel electrophoresis of pDNA incubated with Pro100 and diluted in DMEM. Lane from left: naked plasmid DNA (0); Pro100:pDNA mixtures at 20:1, 40:1, 60:1, and 80:1 w/w ratios, respectively. (C) Agarose gel electrophoresis of pDNA incubated with Pro200 and diluted in DMEM. Lane from left: naked plasmid DNA (0); Pro200:pDNA mixtures at 20:1, 40:1, 60:1, and 80:1 w/w ratios, respectively.

were acquired for SLN:pDNA complexes at a 60:1 ratio (Figure 3). Pro25:pDNA image revealed the presence of a pDNA coil, indicating the incomplete pDNA complexation in accordance with electrophoresis and DLS data. Pro100:pDNA exhibited homogeneous and globular structures, suggesting a wrapping of the pDNA around the particles. On the contrary, Pro200:pDNA appeared as a non-homogenous system with globular particles combined with small and irregular shaped structures, whereas no free pDNA was observed in agreement to DLS and gel electrophoresis results.

Therefore, Pro25 at 80:1 SLN:pDNA ratio, Pro100 and Pro200 at all the SLN:pDNA ratios were able to condense pDNA forming complexes 300–400 nm in size. Furthermore, Pro200 sample, having a higher free protamine content, led to the coexistence of smaller protamine/pDNA polyplexes along with large aggregates of SLN. Because of their pDNA immobilization ability as well as positive surface charge allowing the interaction with negatively charged cell membranes, these SLN:pDNA complexes were selected to be further studied for transfection efficiency.

Cytotoxicity

Before the transfection studies, cytotoxicity of SLN was evaluated on three different cell lines, i.e., COS-I, HepG2, and Na1300, by measuring the percentage of dead cells using PI test. The experiments were performed using SLN amounts ranging between 0.2–0.8 mg/well for Pro100 and Pro200 and 0.8 mg/well for Pro25. Cells incubated in the presence of PEI were used as the positive control; untreated cells and cells incubated with naked pDNA as the negative ones.

As far as COS-I cells are concerned, Pro25 (0.8 mg/well) demonstrated the highest cytotoxicity, whereas Pro100 and Pro200 induced a cell death which essentially increased with an increase of the SLN:pDNA ratio, reaching a value comparable to that of positive control (PEI) (Figure 4A). By considering HepG2 and Na1300, no relevant differences were observed between the samples which showed a cytotoxicity lower than that of the positive control. Moreover, the degree of cytotoxicity on HepG2 and Na1300 cells was found negligible and 5-fold lower compared with COS-I cell line, respectively (Figures 4B and C).

Therefore, SLN exhibited toxic effect on cell lines decreasing in the following order: COS-I > Na1300 >

Table 3. *Z*-average, PDI, particle size distribution by intensity, and Zeta-potential SLN:pDNA complexes at w/w ratios from 20:1 to 80:1, in comparison with naked pDNA. Mean values ± SD.

			Particle size distribution by intensity			Zeta potential
	SLN:pDNA (w/w)	*Z*-average (nm) (PDI)	Peak 1 (nm) (%)	Peak 2 (nm) (%)	Peak 3 (nm) (%)	(mV)
Naked pDNA	—	530 ± 15 (0.477 ± 0.05)	580 ± 40 (100 ± 2%)	—	—	−27 ± 3
Pro25:pDNA	20:1	320 ± 25 (0.483 ± 0.03)	210 ± 15 (40 ± 4%)	570 ± 32 (60 ± 6%)	—	−12 ± 2
	40:1	330 ± 30 (0.545 ± 0.04)	212 ± 21 (50 ± 3%)	581 ± 36 (50 ± 4%)	—	−8 ± 3
	60:1	410 ± 24 (0.504 ± 0.06)	208 ± 19 (65 ± 4%)	575 ± 39 (35 ± 5%)	—	+7 ± 1
	80:1	406 ± 19 (0.302 ± 0.08)	350 ± 45 (100 ± 2%)	—	—	+16 ± 2
Pro100:pDNA	20:1	330 ± 15 (0.352 ± 0.02)	325 ± 15 (100 ± 2%)	—	—	+21 ± 3
	40:1	310 ± 33 (0.427 ± 0.01)	333 ± 23 (100 ± 3%)	—	—	+18 ± 2
	60:1	405 ± 28 (0.323 ± 0.03)	350 ± 33 (100 ± 3%)	—	—	+17 ± 4
	80:1	400 ± 42 (0.401 ± 0.05)	348 ± 35 (100 ± 2%)	—	—	+18 ± 3
Pro200:pDNA	20:1	268 ± 33 (0.544 ± 0.02)	90 ± 11 (20 ± 3%)	330 ± 22 (50 ± 3%)	2022 ± 152 (30 ± 3%)	+14 ± 3
	40:1	261 ± 51 (0.619 ± 0.05)	85 ± 15 (33 ± 2%)	351 ± 18 (57 ± 5%)	2050 ± 159 (10 ± 4%)	+15 ± 4
	60:1	267 ± 43 (0.702 ± 0.08)	88 ± 14 (35 ± 4%)	325 ± 23 (62 ± 2%)	2111 ± 148 (30 ± 3%)	+16 ± 5
	80:1	275 ± 48 (0.502 ± 0.07)	93 ± 8 (32 ± 3%)	340 ± 25 (65 ± 4%)	2502 ± 169 (30 ± 2%)	+13 ± 3

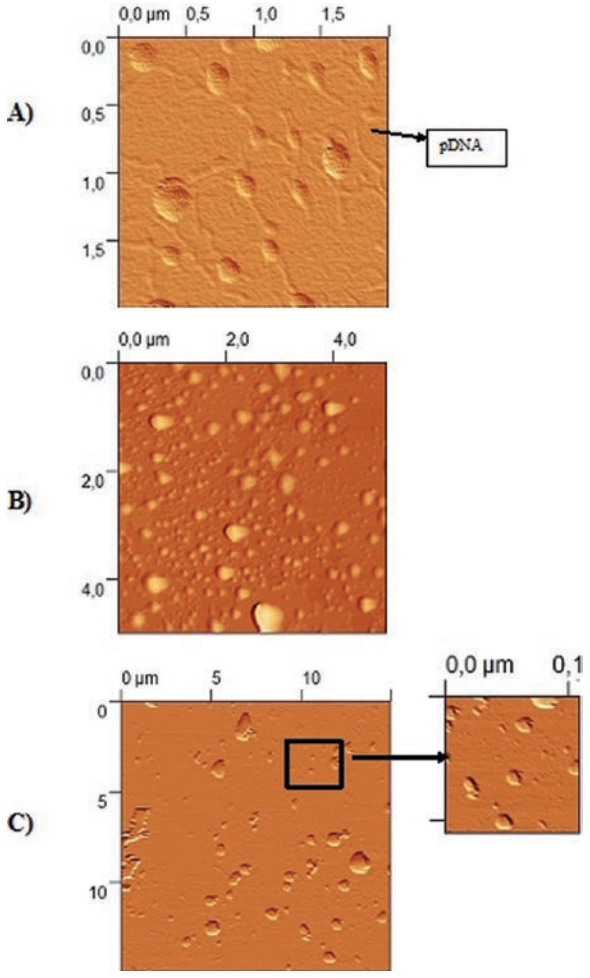

Figure 3. Atomic force microscopy (AFM) images at 60:1 SLN:pDNA ratio of Pro25:pDNA (A), Pro100:pDNA (B), and Pro200:pDNA (C) complexes.

HepG2 according to the increasing cell lipid metabolism rate COS-I < Na1300 < HepG2 (Gröne, 1994; Karten et al., 2005; Guo et al., 2006). The faster lipid metabolism rate is provided by cells the faster the degradation of the up-taken particles and, consequently, the stronger the cell toxicity prevention will result.

Otherwise, PEI demonstrated the same cytotoxicity on all the three tested cell lines because of its strongly positive charge and because its degradation pathway was unaffected by the cellular metabolism (Moghimi et al., 2005).

Transfection experiments

Tranfection experiments were carried out by flow cytometry using the same SLN amounts used in the cytotoxicity experiments; naked pDNA and PEI were used as negative and positive control, respectively. Sample Pro25, having the lowest protamine level which was unable to reduce the toxicity of the lipid matrix and to protect COS-I cells against apoptosis, was excluded from the transfection assay on such a cell line.

Compared with the gene transfection efficiency of the plasmid, Pro25:pDNA and Pro200:pDNA complexes were unable to improve transfection efficiently, regardless of the cell line and the SLN:pDNA ratio (Figure 5). On the contrary, Pro100:pDNA complexes at the SLN:pDNA ratio providing the best transfection efficiency (40:1) improved the transfection capacity of the naked pDNA 5-fold on COS-I cells, 7-fold on HepG2 cells, and 12-fold on Na1300 cells. By considering the level of the transfection mediated by PEI, the efficiency of Pro100:pDNA at the same SLN:pDNA ratio was lower on both COS-I and HepG2 cell line (4-fold and 7-fold, respectively), but 2-fold higher on Na1300 cells.

Therefore, protamine amount plays an important role in determining the transfection performance of SLN. The insufficient protamine content could reasonably justify the failure in cell transfection of Pro25:pDNA complexes; on the other hand, the presence of an excess of protamine inducing the formation of protamine:pDNA polyplexes, incapable to transfect the cells efficiently owing to either their inability to overcome the cell membrane (Liu et al., 2009) or their strong pDNA binding (Mayer et al., 2005), could explain the poor transfection capacity of

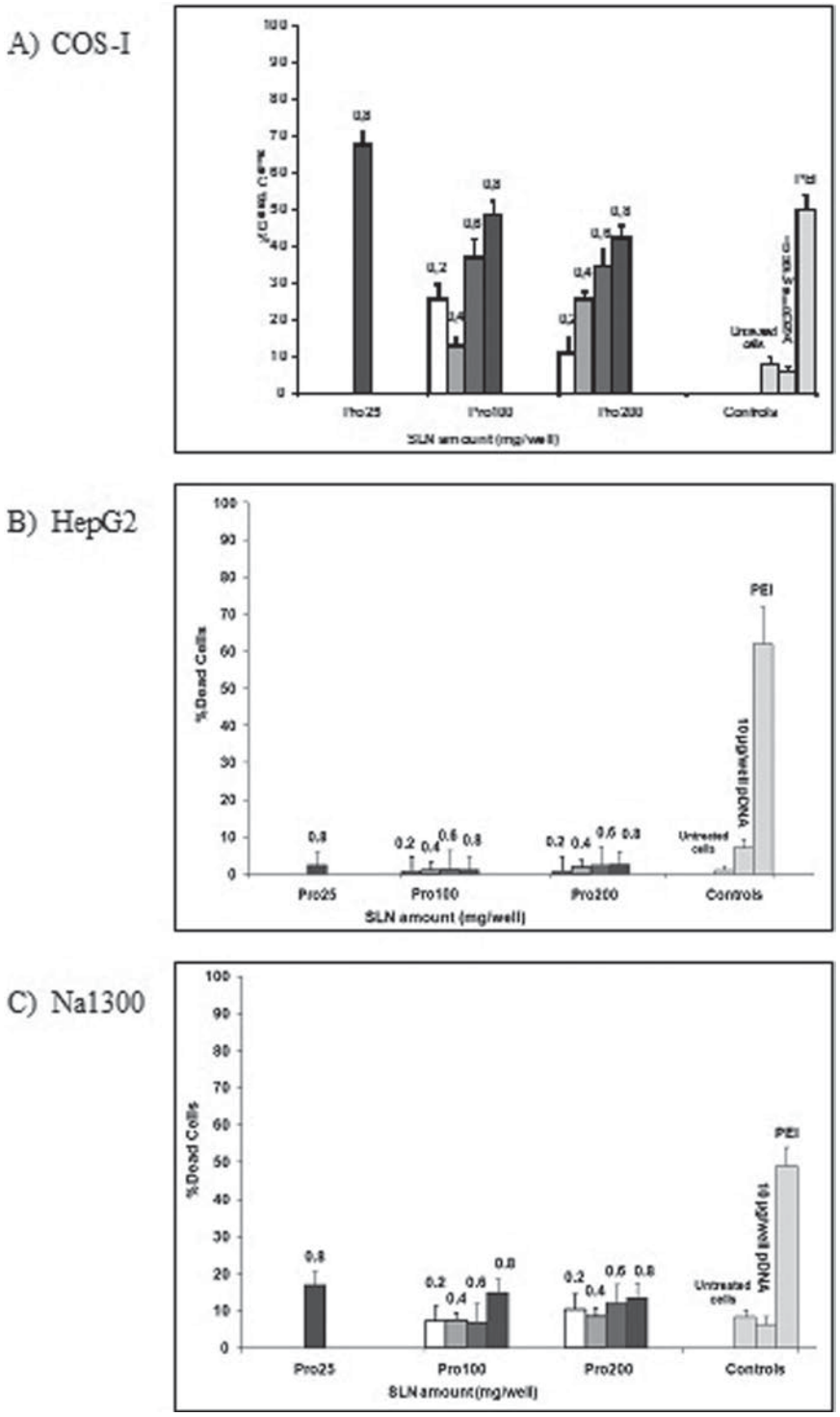

Figure 4. Cytotoxicity expressed as a percentage of dead cells of Pro25, Pro100, and Pro200 on COS-I (A), HepG2 (B), and Na1300 (C) cell lines, as provided by PI assay from flow cytometry analysis.

Pro200:pDNA. Contrary to this, Pro100:pDNA seems to contain the optimal protamine amount able to promote the transfection capacity of the carrier. Moreover, since protamine is an arginine rich peptide like TAT of HIV, and since HIV is able to provide a brain reservoir owing to the presence of TAT (Zink et al., 1999; Van der Meer et al., 2000), protamine may play a role in the active internalization process by neuronal Na1300 cells.

Conclusions

The amount of protamine, used as a pDNA condenser and cell transfer enhancer in the development of lipid nanoparticles, strongly affects the plasmid condensation and the gene transfection efficiency. Only protamine amount used in Pro100 preparation allowed us to obtain nanoparticles able to stabilize SLN:pDNA complexes promoting the plasmid wrapping around the particles and achieving

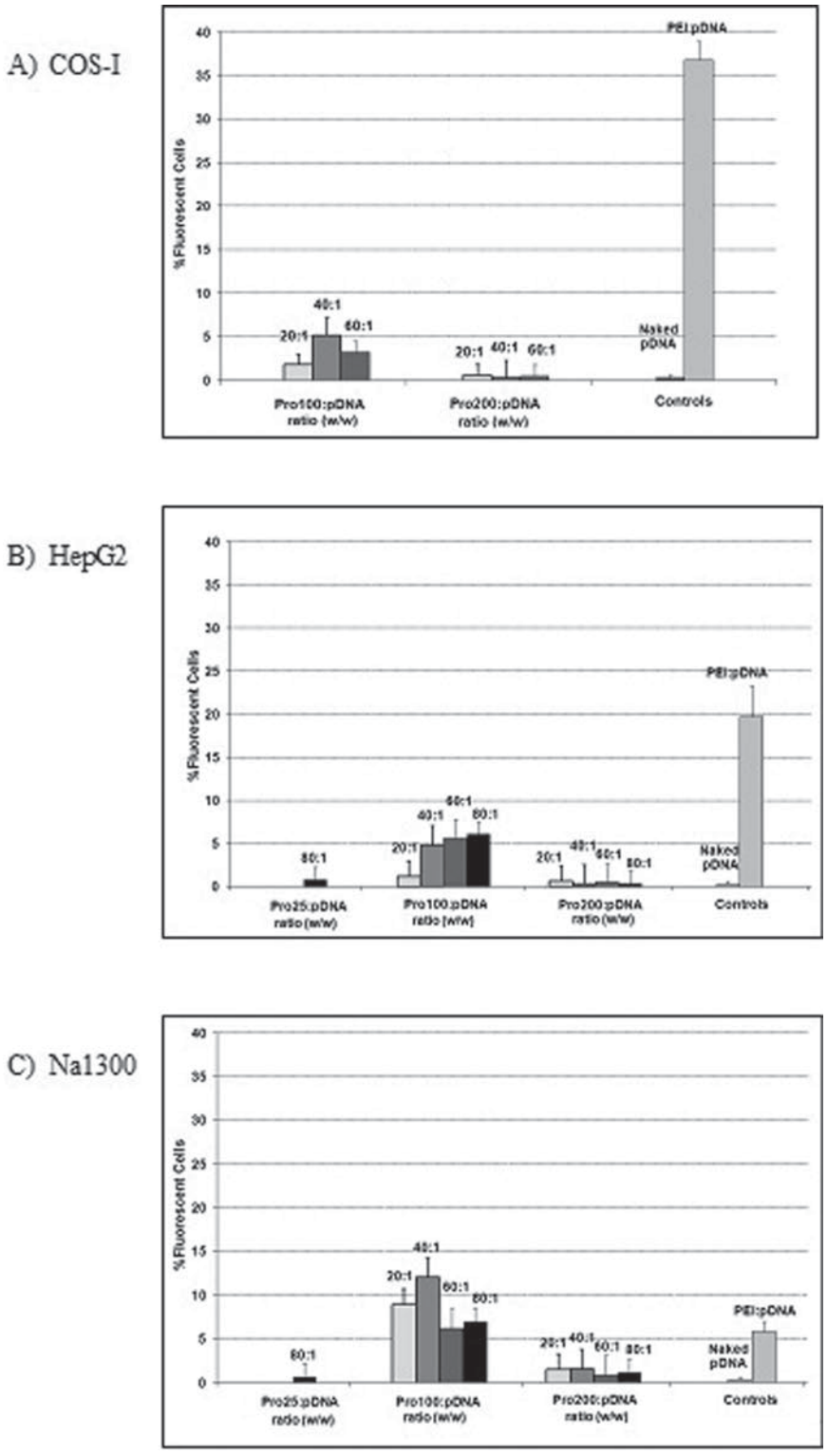

Figure 5. Transfection efficiency of Pro25:pDNA, Pro100:pDNA, and Pro200:pDNA complexes on COS-I (A), HepG2 (B), and Na1300 (C) cell lines, as assessed by flow cytometry.

a successful tropism for a neuronal cell line, known to be poorly transfectable. Since the objective of gene therapy is to obtain targeted vectors transferring genes efficiently to specific cell types, the proposed nanocarrier could be considered in a perspective of DNA-based pharmaceuticals for the management of brain-associated disorders.

Acknowledgments

This work was financially supported by *Fondazione Cassa di Risparmio di Modena*. Thanks are due to Dr M. Tonelli for the technical advice in the AFM analysis and to Professor G. Coppi for the critical reading of the manuscript.

Declaration of interest

The authors report no conflicts of interest.

References

Asasutjarit, R., Lorenzen, S.I., Sirivichayakul, S., Ruxrungtham, K., Ruktanonchai, U., Ritthidej, G.C. (2007). Effect of solid lipid nanoparticles formulation composition on their size, zeta potential and potential for in vitro pHIS-HIV-Hugag transfection. Pharm Res. 24:1098–7.

Braun, R.E. (2001). Packaging paternal chromosomes with protamina. Nat Genet. 28:10–12.

Canine, B.F., Wang, Y., Hatefi, A. (2008). Evaluation of the effect of vector architecture on DNA condensation and gene transfer efficiency. J Contr Rel. 129:117–23.

Del Pozo-Rodríguez, A., Delgado, D., Solinís, M.A., Gascon, A.R., Pedraz, J.L. (2007). Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity. Int J Pharm. 339: 261–8.

Del Pozo-Rodríguez, A., Solinís, M.A., Gascón, A.R., Pedraz, J.L.(2009). Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. Eur J Pharm Biopharm. 71:181–9.

Gröne, E.F., Walli, A.K., Gröne, H.J., Miller, B., Seidel, D. (1994). The role of lipids in nephrosclerosis and glomerulosclerosis. Atherosclerosis. 107:1–13.

Guo, W., Huang, N., Cai, J., Xie, W., Hamilton, J.A. (2006). Fatty acid transport and metabolism in HepG2 cells. J Physiol Gastrointest Liver Physiol. 290:528–34.

Heydenrich, A.V., Westmeier, R., Pedersen, N., Polsen, H.S., Kristensen, H.G. (2003). Preparation and purification of cationic solid lipid nanospheres-effect on particle size, physical stability and cell toxicit. Int J Pharm. 254:355–64.

Huang, L., Viroonchatapan, E. (1999). Introduction. In: Huang, L., Wagner, M., ed. Nonviral vectors for gene therapy. New York: Academic Press, 39–68.

Huh, S.H., Do, H.J., Lim, H.Y., Kim, D.K., Choi, S.J., Song, H., Kim, N.H., Park, J.K., Chang, W.K., Chung, H.M., Kim, J.H. (2007). Optimazation of 25 KDa linear polyethylenimine for efficient gene delivery. Biologicals. 35:165–71.

Jain, V., Prasad, V., Jadhav, P., Mishra, P.R. (2009). Preparation and performance evaluation of saquinavir laden cationic submicron emulsions. Drug Deliv. 16:37–44.

Karten, B., Hayashi, H., Campenot, R.B., Vance, D.E., Vance, J.E. (2005). Neuronal models for studying lipid metabolism and transport. Methods. 36:117–28.

Liu, J., Guo, S., Li, Z., Liu, L., Gu, J. (2009). Synthesis and characterization of stearyl protamine and investigation of their complexes with DNA for gene delivery. Coll Surf B: Bioint. 73:36–41.

Mayer, G., Vogel, V., Weyermann, J., Lochmann, D., van den Broek, J.A., Tziatzios, C., Haase, W., Wouters, D., Schubert, U.S., Zimmer, A., Kreuter, J., Schubert, D. (2005). Oligonucleotide–protamine-albumin nanoparticles: Protamine sulfate causes drastic size reduction. J Contr Rel. 106:181–7.

Moghimi, S.M., Symonds, P., Murray, J.C., Hunter, A.C., Debska, G., Szewczyk, A. (2005). A two-stage poly(ethylenimine)-mediated cytotoxicity: Implications for gene transfer/therapy. Mol Ther. 11:990–5.

Morille, M., Passirani, C., Vonarbourg, A., Clavreul, A., Benoit, J.P. (2008). Progress in developing cationic vectors for non viral systemic gene therapy against cancer. Biomaterials. 29:3477–96.

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 Contr Rel. 77:345–55.

Pedersen, N., Hansen, S., Heydenreich, A.V., Kristensen, H.G., Poulsen, H.S. (2006). Solid lipid nanoparticles can effectively bind DNA, streptavidin and biotinylated ligands. Eur J Pharm Biopharm. 62:155–62.

Pintel, D.J., Tullis, G.E. (2006). Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. J Virol Methods. 138:85–98.

Rejman, J., Oberie, V., Zuhon, I.S., Hoekstra, D. (2004). Size-dependent internalization of particles via pathways of clathrin and cavoelae-mediated endocytosis. Biochem J. 377:159–69.

Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., Müller, R.H., Rosenecker, J. (2004). Application of novel solid lipid nanoparticles (SLN)-gene vector formulations based on a dimeric HIV-1 Tat-peptide in vitro and in vivo. Pharm Res. 21:1662–9.

Tabatt, K., Kneuer, C., Sameti, M., Olbrich, C., Muller, R.H., Lehr, C.M., Bakowsky, U. (2004a). Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes. J Contr Rel. 97:321–32.

Tabatt, K., Sameti, M., Olbrich, C., Muller, R.H., Lehr, C.M. (2004b). Effect of cationic lipid and matrix lipid composition on solid lipid nanoparticle-mediated gene transfer. Eur J Pharm Biopharm. 57:155–62.

Tsuchiya, Y., Ishti, T., Okahata, Y., Sato, T. (2006). Characterization of Protamine as a tranfection accelerator for gene delivery. J Bioact Comp Polym. 21:519–37.

Van der Meer, P., Ulrich, A.M., Gonzalez-Scarano, F., Lavi, E. (2000). Neurotoxicity of HIV-1 proteins gp120 and Tat in the rat striatum. Exp Mol Pathol. 69:233–41.

Vangasseri, D.P., Han, S.J., Huang, L. (2005). Lipid-protamine-DNA-mediated antigen delivery. Curr. Drug Deliv. 2:401–6.

Vighi, E., Montanari, M., Ruozi, B., Tosi, G., Magli, A., Leo, E. (2010b). Nuclear localization of cationic solid lipid nanoparticles containing Protamine as transfection promoter. Eur J Pharm Biopharm. 76:384–93.

Vighi, E., Ruozi, B., Montanari, M., Battini, R., Leo, E. (2010a). pDNA condensation capacity and in vitro gene delivery properties of cationic solid lipid nanoparticles. Int J Pharm. 389:254–61.

Zink, W.E., Zheng, J., Persidsky, Y., Poluektova, L., Gendelman, H.E. (1999). The neuropathogenenesis of HIV-1 infection. FEMS Immunol Med Mic. 26:233–41.