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Long-chain cationic derivatives of PTA (1,3,5-triaza-7-phosphaadamantane) as new components of potential non-viral vectors

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ABSTRACT

The purpose of this study was to investigate the potential of new positively charged solid lipid nanoparticles (SLN) to convey nucleic acids. The cationic character of SLN was obtained by adding as cationic molecules two different long-chain cationic phosphines (CP), namely hexadecyl-PTA iodide (CP16) and octadecyl-PTA iodide (CP18). The obtained CP-SLN are characterized by a positive charge on the surface and reproducible dimensions around 220 nm. These nanosystems are able to efficiently bind nucleic acid molecules and to protect DNA from the activity of serum nucleases up to 120 min. Lastly, *in vitro* experiments demonstrated that CP-SLN exhibit a quite pronounced antiproliferative effect on cultured human K562 erythroleukemic cells and a limited effect as transfecting adjuvant.

These data, and particularly the ability of CP-SLN to protect DNA from degradation, encourages further studies aimed at proposing these nanosystems as a potential approach to deliver nucleic acid to cells in living organisms.

1. Introduction

Gene transfection protocols typify important experimental therapeutics for tumor, infectious and genetic diseases (Deng et al., 2010; Edelstein et al., 2007; Brenner and Okur, 2009). The replacement of defect genes by introducing normal exogenous genes into target cells is an intriguing therapeutic system to restore the normal cell function (Patil et al., 2005). To replace or arrest the expression of specific genes, it is necessary that administered nucleic acid molecules maintain their stability within the extra- and intracellular environment for a sufficient period of time to exert a pharmacological effect (Patil et al., 2005; Pedroso de Lima et al., 2001; Merdan et al., 2002). In order to obtain this effect an efficient delivery system for nucleic acid molecules is required. There are two kinds of gene delivery vectors, viral and non-viral vectors. Viral vectors have shown high and stable gene expression *in vitro*. However, the immunogenicity and potential mutagenicity limit their applications *in vivo* (Chirmule et al., 1999; Manno et al., 2006). Therefore, more attention has been paid to non-viral vectors. Indeed, the use of non-viral vectors such as cationic systems (*i.e.*, liposomes, nanoparticles, microparticles) (Patil et al., 2005) appears suitable since these carriers are known to be able to carry large inserts, to be the safer to use and the easier to produce in large scale with respect to viral vectors (Morille et al., 2008). However, these non-viral systems show low and transient expression levels owing to their inability to support the amplification, the cell-to-cell transmission (Ferrer-Miralles et al., 2008) and the toxicity at high doses (Somia et al., 2000). Among cationic non-viral systems, cationic solid lipid nanoparticles (SLN) have recently emerged as an alternative to liposomes due to their better stability profile and ease of industrial scalability (Tabatt et al., 2004).

Cationic systems bind DNA molecules by ionic interactions on their positively charged surface due to the presence of cationic detergents on carrier composition. Particularly, in the present paper, as cationic detergent two long-chain cationic phosphines (CP) have been considered.

CP are derivatives of PTA (1,3,5-triaza-7-phosphaadamantane), an hydrophilic phosphine firstly prepared in the early seventies (Daigle et al., 1974). CP have been designed as a new class of aminophosphine bearing a large lipophilic portion beside the hydrophilic positively charged PTA cage and they have been obtained introducing long aliphatic chains on PTA nitrogen. CP structure, recalling cationic species with amphiphilic surfactant properties, makes them good candidates for the formation of positively charged SLN.

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The purpose of this study was to investigate the potential of new positively charged SLN to convey nucleic acids. The cationic character of SLN was obtained by the addition of two long-chain CP, namely hexadecyl-PTA iodide (CP16) and octadecyl-PTA iodide (CP18). Particularly, this report describes: (a) the preparation and characterization of CP-SLN; (b) the ability of CP-SLN to bind DNA; (c) the effect of the obtained CP-SLN on cell proliferation of *in vitro* cultured human K562 erythroleukemic cells; (d) the effect on the stability of DNA molecules exposed to exo- and endo-nucleases after complexation to CP-SLN; and (e) the ability of CP-SLN to transfect DNA into BHK-21 (Syrian hamster kidney fibroblast, PHLS) cultured cells.

2. Materials and methods

2.1. Materials

Cationic derivatives of PTA (1,3,5-triaza-7-phosphaadamantane), namely CP16 and CP18, were synthesized by Dr. Paola Bergamini at the Department of Chemistry of our University as below reported. The reagents C16H33I and C18H37I were purchased and used without further purification. The phosphine PTA was prepared as described in the literature (Daigle, 1998).

Lutrol F 68, oxirane, methyl-, polymer with oxirane (75:30) (poloxamer 188) was obtained from BASF (Ludwigshafen, Germany). Miglyol 812, caprylic/capric triglyceride (tricaprin) was purchased from Eigenmann & Veronelli (Rho, Italy). Agarose and tristearin (stearic triglyceride) were purchased from Fluka (Buchs, Switzerland). The expression vectors for the red fluorescent protein (pHcRed1-N1) and the firefly luciferase (pGL3) were available in the laboratory. All other materials and solvents were from Sigma–Aldrich S.r.l. (Milan, Italy).

2.2. CP16 and CP18 synthesis and characterization

CP16 and CP18 have been prepared as iodides by treating in acetone PTA with $C_{16}H_{33}I$ and $C_{18}H_{37}I$, respectively. In the case of CP16, PTA (0.40 g, 2.58 mmol) was dissolved in 25 ml of degassed acetone and 1-iodohexadecane $C_{16}H_{33}I$ (1.62 ml, 5.14 mmol) was added to the solution, which was stirred at room temperature under argon for 20 h. $(PTAC_{16}H_{33})I$, precipitated as a white solid, was filtered and washed with n-hexane (0.95 g, 1.87 mmol, 72%).

Concerning the synthesis of CP18, PTA (0.20 g, 1.29 mmol) and $C_{18}H_{37}I$ (0.98 g, 2.57 mmol) reacted in the same conditions as above to give $(PTAC_{18}H_{37})I$ as a white solid (0.57 g yield 83%).

Elemental analyses were carried out using a Carlo Erba instrument model EA1110. The ESI mass spectra were acquired with a Micromass LCQDuo Finningan. NMR spectra were recorded by a Varian Gemini 300 MHz spectrometer (^1H at 300 MHz, ^{13}C at 75.43 MHz, ^{31}P at 121.50 MHz). The ^{13}C and ^{31}P spectra were run with proton decoupling and ^{31}P spectra are reported in ppm relative to an external 85% H_3PO_4 standard, with positive shifts downfield. ^{13}C NMR spectra are reported in ppm relative to external tetramethylsilane (TMS), with positive shifts downfield.

2.3. CP-SLN preparation

CP-SLN were prepared by stirring, homogenization and ultrasonication (Esposito et al., 2008). Briefly, 1 g of lipid was melted at 80 °C. The lipid mixture was constituted of tristearine and CP in 200:1 by weight. The fused lipid phase was dispersed in 19 ml of an aqueous solution of poloxamer 188 (2.5%, w/w). The obtained emulsion was subjected to ultrasonication (Microson™, Ultrasonic cell Disruptor) at 6.75 kHz for 15 min and then cooled down to room temperature by placing it in a water bath at 22 °C. CP-SLN dispersions were stored at room temperature.

2.4. Characterization of CP-SLN: size, ζ potential and morphology

Submicron particle size analysis was performed using a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for injections. Measurements were made at 25 °C at an angle of 90°. Samples were diluted with MilliQ water to an adequate scattering intensity prior the measurement. The results are presented as intensity weighted average (*z*-ave) value obtained from three measurements (10 runs each) with corresponding standard deviation. Each experimental value results from three independent experiments performed in triplicate.

The electrophoretic mobility of CP-SLN/pDNA complexes was measured at room temperature by mean of a Zetasizer 3000 PCS (Malvern Instr., Malvern, England) in 1 mM NaCl solution to avoid the fluctuation in the ζ potential due to variations in the conductivity of purified water. Samples were injected in a glass capillary cell and analyzed under a constant voltage after focusing with a 5 mW helium neon laser. The ζ potential, in mV, was automatically calculated from the electrophoretic mobility based on the Helmholtz–Smolukowski equation. Each sample was measured three times then mean value and standard deviation (SD) are presented.

Morphological characterization of CP-SLN was performed by Cryo-TEM. Samples were vitrified as described by Esposito et al. (2008). The vitrified specimen was transferred to a Zeiss EM922 transmission electron microscope for imaging using a cryoholder (CT3500, Gatan). The temperature of the sample was kept below −175 °C throughout the examination. Specimens were examined with doses of about 1000–2000 e/nm^2 at 200 kV. Images were recorded digitally by a CCD camera (Ultrascan 1000, Gatan) using a image processing system (GMS 1.4 software, Gatan). A drop of dispersion prepared for TEM measurements was placed on a bare copper grid and plunged frozen in liquid ethane at approximately 100 K. The sample was transferred into a cryo electron microscope (CEM902a, Zeiss, D-Oberkochen, Philips CM120, NL-Eindhoven) operated at 80 kV respectively 120 kV. Samples were viewed under low dose conditions at a constant temperature around 77–100 K. Images were acquired by a Dage SIT low intensity TV camera system and processed by a Kontron IBAS image processing system in the case of the Zeiss CEM902A and a Tietz Fastscan CCD camera system for the Philips CM120.

2.5. Analysis of the electrophoretic mobility of complexes between CP-SLN and DNA

CP-SLN were mixed with pHcRed1-N1 vector in different +/− molar ratios (*i.e.* from 0.5:1 to 32:1) and incubated at 37 °C for 5 min, then each sample was subjected to electrophoresis. Electrophoresis was carried on in 0.8% agarose gel at constant voltage (100 mV) for 2 h. The relative band migration was determined, after staining the gels with ethidium bromide.

2.6. DNA stability studies

The stability of CP-SLN/pDNA complexes towards fetal calf serum (FCS) contained nucleases was studied following the above protocol. 0.3 μg of pHcRed1-N1 vector were complexed to different amount of SLN resulting in final +/− molar charge ratios SLN/pDNA of 8:1 and 16:1, for CP16-SLN and CP18-SLN, respectively. The complexes were then incubated at 37 °C in a thermostatic bath.

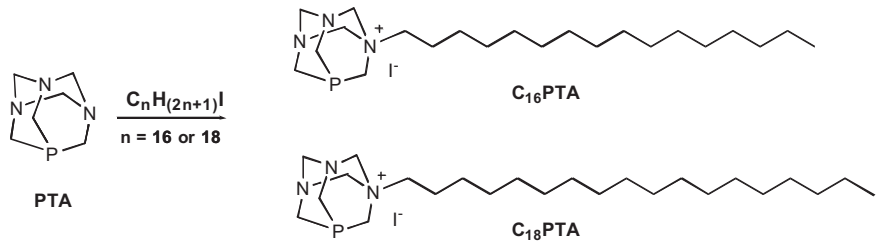

Fig. 1. Synthetic scheme and chemical structure of the cationic phosphines hexadecyl-PTA iodide (CP16) and octadecyl-PTA iodide (CP18).

At different time intervals, between 0 and 240 min, samples were withdrawn and stored at −20 °C until electrophoretic analysis was performed. Electrophoresis was performed on 0.8% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide for 2 h at 25 mV constant current. After electrophoresis, the bands corresponding to the pHcRed1-N1 vector were visualized by UV shadowing (Maniatis et al., 1982).

2.7. Effect of CP-SLN on cell proliferation

The effect of CP-SLN on cell proliferation was determined on cultured human leukemic K562 cells (Lozzio and Lozzio, 1975). Standard conditions for cell growth were α-medium (Gibco, Grand Island, NY), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) in 5% CO_2 at 90% humidity.

Human leukemic K562(S) cells were treated with different concentrations (10, 5, 2.5, 1.25 or 0.62 μg/ml) of cationic CP-SLN. After 5 days of culture, the cells were counted by a cell counter Fuchs (Tosenthal, Preciss, France) and the number of cells/ml was compared with the value obtained in untreated cell cultures in order to determine the percentage of surviving cells. As control the activity of SLN constituted of sole tristearine without CP, named "blank SLN" were also assayed under the same conditions. Assays were carried out in triplicate and usually counts differed by <7%. Cells were counted with a Model ZF Coulter Counter (Coulter Electronics Inc., Hielah, FL) and the cell growth rate was computed. IC_{50} values, namely the compound concentration inhibiting the 50% of the cell growth, were calculated using the free ED50plus v1.0 software.

2.8. Transfection studies

BHK-2 cells were cultured as previously reported (Pinotti et al., 1998). For fluorescence microscopy, cells were cultured on 24-mm glass coverslips.

As control, BHK cells were transfected with 2 μg of plasmid DNA (pDNA) using the TransIT®-2020 (Mirus, Madison, WI, USA), in accordance to the manufacturer's protocol.

CP-SLN/pDNA complexes were prepared by mixing the C16-SLN (8:1 +/− molar charge ratio) or C18-SLN (16:1 +/− molar charge ratio) with 2 μg of plasmid DNA for transfection experiments. The mixture was kept at room temperature (25 ± 0.5 °C) for 20 min to allow the complexes to be formed. After the incubation, the SLN/pDNA complexes were used following the previously mentioned transfection protocol.

48 h post-transfection cells were analyzed for red fluorescence (NIKON Eclipse 50i) or luciferase activity, as previously described (Bertolucci et al., 2008).

3. Results and discussion

3.1. Synthesis and characterization of CP16 and CP18

N-alkyl PTA derivatives CP16 and CP18 have been prepared in high yields by reacting 1,3,5-triaza-7-phosphaadamantane (PTA) with the appropriate alkyl iodide. Each product precipitated from solution as an air-stable solid that needed little purification (see Fig. 1). The obtained products were firstly characterized by mass spectroscopy. Concerning CP16, Anal. calcd. for $C_{22}H_{45}IN_3P$ (509) C, 51.84; H, 8.91; N, 8.25. Found C, 51.84; H, 8.97; N, 8.19. Electrospray

Table 1
NMR data for CP16 and CP18.

	^1H δ (ppm), J (Hz)	$^{13}C\{^1H\}$ δ (ppm), J (Hz)	$^{31}P\{^1H\}$ δ (ppm), J (Hz)
CP16	0.85, bt, 3H, CH_3 1.23, s, 26H, CCH_2C 1.62, m, 2H, $CH_2CH_2N^+$ 2.80, m, 2H, CH_2CH_2N 3.90, m, 4H, PCH_2N 4.35, bs, 2H, PCH_2N^+ 4.35, d, $^2J_{HH}$ 14, 1H, NCH_2N, 4.55, d, $^2J_{HH}$ 14, 1H, NCH_2N 4.80, d, $^2J_{HH}$ 11, 2H, NCH_2N^+ 4.92, d, $^2J_{HH}$ 11, 2H, NCH_2N^+	13.9, s, CH_3 18.9, s, CH_2 22.1, 26.1, 28.4, 28.7, 28.8, s, CH_2 29.0, bs, $7CH_2$ 31.3, s, CH_2 45.4, d, $^1J_{PC}$ 20.6, PCH_2N 51.7, d, $^1J_{PC}$ 31.2, PCH_2N^+ 61.4, s, RCH_2N^+ 69.3, s, NCH_2N 78.6, s, NCH_2N^+	−84.27, s
CP18	0.92, t, 3H, CH_3 1.24, s, 30H, CCH_2C 1.62, m, 2H, $CH_2CH_2N^+$ 2.80, m, 2H, CH_2CH_2N 3.90, m, 4H, PCH_2N 4.33, d, 2H, PCH_2N^+, 4.35, d, 1H, NCH_2N, 4.53, d, $^2J_{HH}$ 14, 1H, NCH_2N 4.77, d, $^2J_{HH}$ 11, 2H, NCH_2N^+ 4.95, d, $^2J_{HH}$ 11, 2H, NCH_2N^+	13.9, s, CH_3 18.9, s, CH_2 22.1, 26.1, 28.4, 28.7, 28.8, s, CH_2 29.0, bs, 9 CH_2 31.3, s, CH_2 45.4, d, $^1J_{PC}$ 20.6, PCH_2N 51.7, d, $^1J_{PC}$ 32.2, PCH_2N^+ 61.4, s, RCH_2N^+ 69.3, s, NCH_2N 78.6, s, NCH_2N^+	−84.26, s

The spectra were recorded at 25 °C in d_6-dmso, at 300 MHz (^1H), 100.58 MHz ($^{13}C\{^1H\}$) and 121.50 MHz ($^{31}P\{^1H\}$) respectively.

Table 2
Some physicochemical characteristics of long-chain cationic phosphines.

Cationic phosphine	Molecular weight	λ_{max} [a] (nm)	log P[b]
CP16	509	219 octanol 227 water	0.53
CP18	537	219 octanol 227 water	0.70

[a] λ_{max}, lambda max. It is the wavelength yielding the highest absorbance value.
[b] log P, the logarithm of the partition coefficient (P) of a compound, being P the ratio of the concentration of the compound in octanol to the concentration of the same compound in water.

MS (in H_2O): observed m/z 382, calcd. 382 for $C_{22}H_{45}N_3P$ $(M-I)^+$. In the case of CP18, Anal. calcd. for $C_{24}H_{49}IN_3P$ (537): C, 53.60; H, 9.19; N, 7.82. Found C, 53.56; H, 9.35; N, 7.77. Electrospray MS (in H_2O): observed m/z 410.3, calcd. 410 for $C_{24}H_{49}N_3P$ $(M-I)^+$.

Secondly, CP16 and CP18 were characterized by ^1H, ^{13}C and ^{31}P NMR whose data are summarized in Table 1. As clearly evident, ^{31}P NMR is diagnostic of the conversion of PTA into N-alkylated products, being the peak of PTA at −100 ppm shifted downfield of about 20 ppm in $[(PTAC_nH_{2n+1})I]$ derivatives (δ −84.27 and −84.26 ppm for n = 16, 18 respectively).

The octanol–water partition coefficient of the in $[(PTAC_nH_{2n+1})I]$ derivatives was obtained by a slow-stirring method providing accurate log P results over a wide range of concentration values (Hajji et al., 2011). The obtained results are reported in Table 2 together with other physicochemical characteristics of CP16 and CP18.

3.2. Production and characterization of CP-SLN

The use of pure tristearin for producing CP-SLN allows the obtaining of stable and homogenous dispersions, free from aggregates (Esposito et al., 2008).

Table 3 summarizes the results concerning size, ζ potential and effect on K562 cell growth of the produced CP-SLN. From the analysis of these data it can be achieved that mean size is almost the same for both colloidal systems, being 216.8 ± 5.1 nm (P.I. 0.29) for CP16-SLN and 228.9 ± 6.3 nm (P.I. 0.22) for CP18-SLN. SLN dispersions maintained their dimensions almost unchanged for more than 6 months (data not shown).

Concerning ζ potential both systems show a slight cationic charge being +28.0 ± 0.63 and +26.9 ± 1.21, for CP16-SLN and CP18-SLN respectively.

Cryo-transmission electron microscopy (Cryo-TEM) analyses have been conducted in order to shed light on the structure of the dispersed particles in both CP-SLN dispersions. Fig. 2 reports cryo-TEM images of CP containing colloidal dispersions.

The electron microscopic analysis demonstrates that CP16-SLN are mainly characterized by the presence of three-dimensional particles projected in a two dimensional way. In panel A elongated circular platelet-like crystalline particles and dark, “needle” like structures edge-on viewed can be observed. The calculated thickness of nanoparticles was 10 nm.

On the other hand CP18-SLN (reported in panel B) showed a more inhomogeneous population of particles. Beside the “normal” SLN there were also many smaller and larger spherical particles characterized by a lamellar ultrastructure. In fact, together with the characteristics platelet-like structure of SLN, it is possible to note vesicular-like shapes with an internal multilamellar structure.

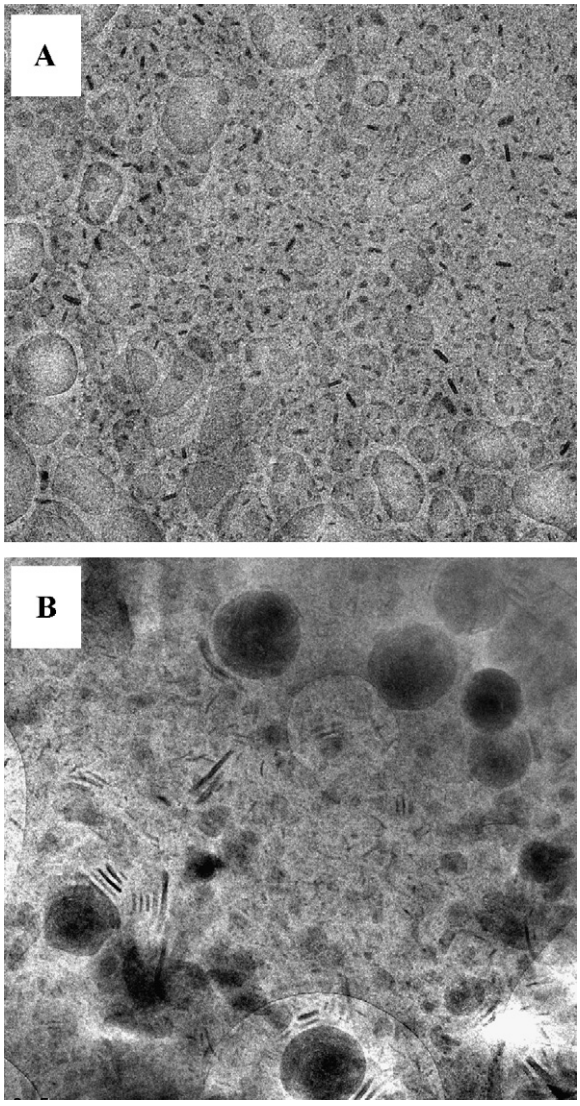

Fig. 2. Cryo-TEM photographs of CP-SLN studied in the present paper. Panel A: CP16-SLN; Panel B: CP18-SLN. Bar represents 0.2 μm.

Moreover, it has to be underlined that due to the presence of phosphorus, the samples were very radiation sensitive on the surface therefore the images resulted a bit noisy.

To obtain information about the potential cytotoxic activity of CP-SLN, some *in vitro* assay were performed cultivating human erythroleukemic K562 cells in the presence of CP-SLN. Particularly, CP-SLN containing 0.5 or 2% of CP in their composition, corresponding to 0.6–10 μM of CP, were added to the cells. The results, reported in Fig. 3 and Table 2, indicate that both types of CP-SLN containing 2% of CP showed a rather pronounced cytotoxicity with an IC_{50} of 0.63 μM and IC_{50} of 0.61 μM for CP16-SLN and CP18-SLN, respectively (where IC_{50} is the compound concentration inhibiting the 50% of the cell growth). On the other hand when the content of CP was 0.5% of the total composition, the antiproliferative effect was obviously lower, being IC_{50} = 7.83 μM for CP16-SLN and IC_{50} = 6.86 μM for CP18-SLN.

Table 3
Average diameter, ζ potential and effect on human erythroleukemic K562 cell growth of the produced carrier systems.

Carrier system	Mean diameter (nm)	Polydispersity	ζ potential (mV)	CP 0.5% IC_{50} (μM)	CP 2% IC_{50} (μM)
CP16-SLN	216.8 ± 5.1	0.29 ± 0.07	+28.0 ± 0.63	7.83 ± 0.12	0.63 ± 0.04
CP18-SLN	228.9 ± 6.3	0.22 ± 0.04	+26.9 ± 1.21	6.86 ± 0.09	0.61 ± 0.07

Data represent the mean of three independent experiments ± SD.

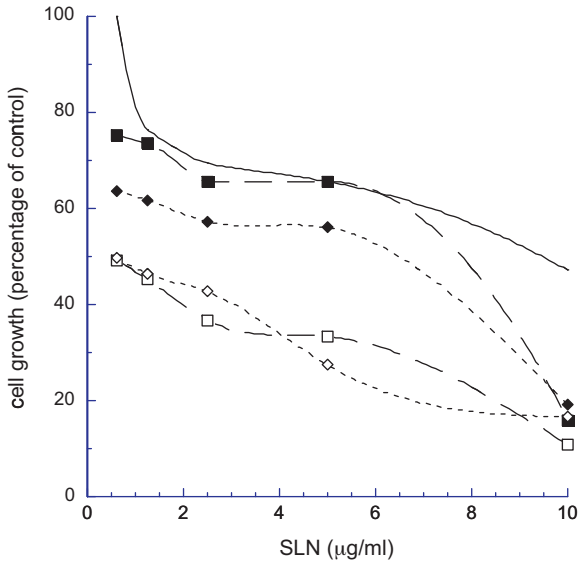

Fig. 3. *In vitro* antiproliferative effect of different cationic SLN containing CP16 (squares) or CP18 (diamonds) and blank SLN (line) on human erythroleukemic K562 cells. The concentration of CP was 0.5% (black symbols) or 2% (white symbols). Determinations were performed after 5 days of cell culture. Data represent the % of cell number/ml compared to untreated control K562 cells. The reported results are the average of 3 independent experiments, SD ≤ 7%.

It is well known that in general uncharged SLN do not exhibit any cytotoxic effects *in vitro* up to concentrations of 2.5% lipid (Schubert and Muller-Goymann, 2005), while lipid concentrations higher than 10% have been shown a viability of 80% in culture of human granulocytes (Muller et al., 1996). Moreover, the use of biocompatible fatty acids leads to no toxic effect from SLN degradation products. Hence, SLN cytotoxicity can be attributable to the presence of emulsifiers, surfactants, cationic lipids and preservatives that are used in the production of these systems (Heydenreich et al., 2003; Tabatt et al., 2004). Cationic lipids are needed to prepare SLN used on gene therapy due to their surfactant activity and their positive charge. The surfactant activity is necessary to obtain the initial emulsion while the positive charge is needed to provide the superficial charge to SLN for their further interaction with negative charged DNA to form SLN–DNA complexes. However, cationic lipids can be toxic on repeated use (Han et al., 2000). One of the most commonly used cationic lipids in gene therapy is DOTAP. In particular, in the present study one-tailed cationic phosphines are used for the preparation on SLN. Many studies in literature reported that for these SLN formulations the presence of one-tailed cationic lipids is more critical with respect to cell toxicity than the usage of two tailed lipids (Tabatt et al., 2004; Olbrich et al., 2001), thus a higher toxicity is normally observed. Due to the risk of toxicity of cationic surfactant its concentration in the formulation has to be as lower as possible. Accordingly with the obtained results, the lower the concentration of CP used, the lower is expected to be the cytotoxic effect.

3.3. Binding migration studies of CP-SLN

In order to evaluate the strength of the interaction occurring between DNA and CP-SLN, CP-SLN were incubated with the pHcRed1-N1 plasmid DNA to different final charge +/− molar ratios SLN/DNA (see legend to Fig. 4) and subjected to electrophoresis. The results reported in Fig. 4 indicate that DNA migration is retarded by the presence of CP-SLN, due to the formation of high-molecular-weight complexes with DNA molecules. These complexes were attributed to inter-nanoparticle bridges formed by DNA molecules (Sternberg et al., 1994). This hypothesis was

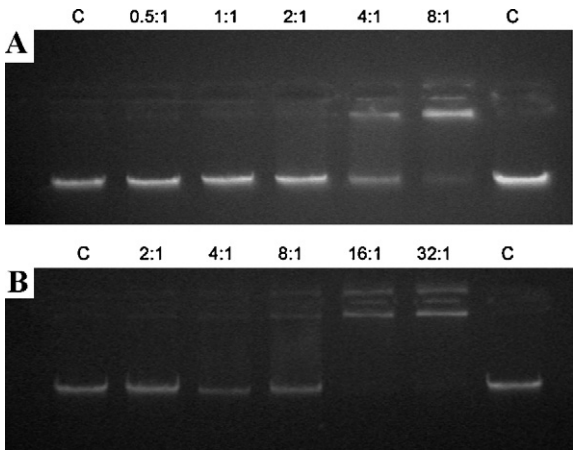

Fig. 4. Effect of cationic complexation on electrophoretic migration of pHcRed1-N1 plasmid. For each type of CP-SLN the used +/− molar charge ratio between CP-SLN and DNA was indicated. C = free DNA. Panel A: CP16-SLN. Panel B: CP18-SLN.

supported by the presence of non-migrating bands in the agarose gels. From the analysis of Fig. 4 it emerges that the strength of complex formation is higher in the case of CP16 with respect to CP18, being the charge +/− molar ratio between SLN and DNA 8:1 and 16:1, respectively.

Moreover we would like to mention that, as expected, when lower concentrations of cationic phosphines are used (*i.e.*, 0.5% *vs* 2%), DNA migration is only slightly retarded by CP-SLN (data not shown).

3.4. Stability studies

An important advantage of non-viral systems in gene therapy is their capacity to protect DNA from components of the medium, and fundamentally from DNases digestion. The protective effect of SLN was studied on degradation of pHcRed1-N1 plasmid catalyzed by nucleases. It is to be underlined that we considered CP18-SLN as model CP-SLN and fetal calf serum (FCS) as source for nucleases. FCS was used since it is routinely employed in cell culture experiments. CP16-SLN/DNA and CP18-SLN/DNA complexes were incubated at 37 °C from 0 to 120 min in the presence of 10% FCS, samples were then loaded on agarose gel and subjected to electrophoresis. As control, the same amount of pHcRed1-N1 plasmid was incubated in the absence of CP-SLN. The result of this experiment indicates that the complete degradation of free pHcRed1-N1 plasmid occurs within 90 min (see Fig. 5A), while the complexation of DNA to CP-SLN protects pHcRed1-N1 plasmid from degradation (Fig. 5B and C).

As above reported, the formation of CP-SLN/pHcRed1-N1 complex leads to high-molecular-weight aggregates that give rise to a non-migrating band on agarose gel. However, we cannot formally exclude that DNA complexed to CP-SLN would be still integer after exposure to FCS nucleases. In order to answer this question, we performed a further experiment. Both CP16-SLN/pHcRed1-N1 and CP18-SLN/pHcRed1-N1 complexes were exposed to FCS nucleases, then phenol extraction of DNA was performed and the extracted DNA was analyzed by gel-electrophoresis. As reported in Fig. 6, the extracted DNA shows a single band with a molecular weight superimposable to that of undigested DNA. This result confirms the maintenance of DNA integrity in the presence of CP-SLN, while in their absence the DNA completely degrades within 60 min.

3.5. Gene transfection experiments

Once the formulations possessed the technologically suitable characteristics for the desired application, the next step was to test their transfection activity "*in vitro*." For transfection assays we

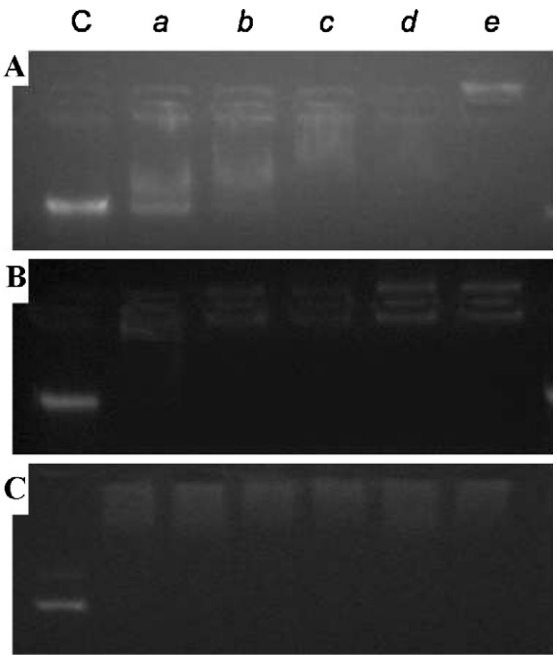

Fig. 5. Agarose gel electrophoresis patterns of pHcRed1-N1 plasmid (0.3 μg) incubated in the presence of 10% FCS at 37 °C for different length of time, namely 0 min (lane a), 30 min (lane b), 60 min (lane c), 90 min (lane d), and 120 min (lane e), untreated DNA (C). Panel A: uncomplexed DNA. Panel B: CP16-SLN at a final +/− molar charge ratio of 8:1. Panel C: CP18-SLN at a final +/− molar charge ratio 16:1.

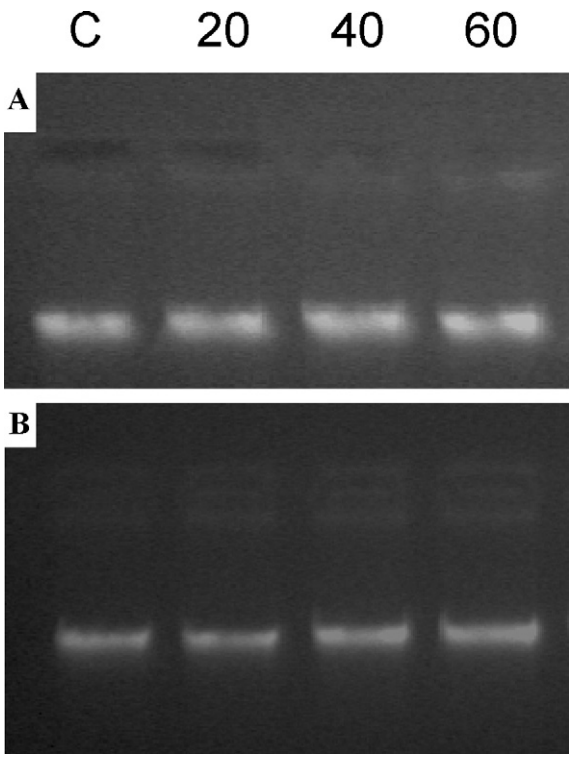

Fig. 6. Electrophoretic analysis of pHcRed1-N1 plasmid DNA phenol extracted from CP-SLN. pHcRed1-N1 plasmid (0.3 μg) was incubated at 37 °C in the presence of CP-SLN for 20, 40 and 60 min, then DNA was extracted with a standard protocol and analyzed by agarose gel electrophoresis. Panel A: CP16-SLN. Panel B: CP18-SLN.

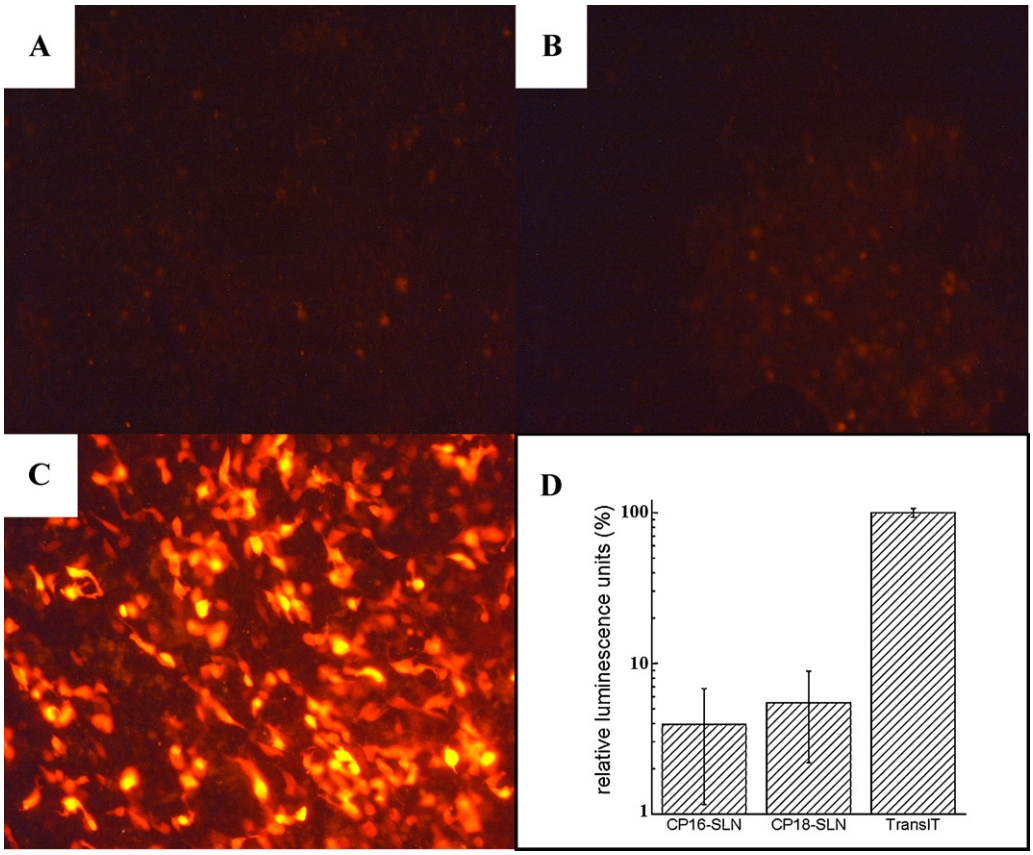

Fig. 7. Transfection efficiency of CP-SLN in BHK-21 cells. (A–C) Representative examples of fluorescence microscopy images of cells transfected with the pHcRed1-N1 and the CP16-SLN (A), CP18-SLN (B) or TransIT® (C). (D) Luciferase activity levels in cells transfected with the pGL3 and TransIT®, CP16-SLN or CP18-SLN. The histograms report the mean ± standard deviation from three independent experiments.

worked with those +/− molar ratios that bound all DNA, provided high positive surface charge and protected DNA from enzyme degradation.

The ability of CP-SLN to transfect DNA was tested on BHK cells by exploiting the expression (i) of the red fluorescent protein (RFP) through fluorescence microscopy (Fig. 7A–C) and (ii) of the firefly luciferase through luciferase activity assays (Fig. 7D), which allows a better quantitative evaluation. Particularly the expression levels of RFP and firefly luciferase was evaluated after incubating BHK cells with the corresponding vectors complexed either to C16-SLN or C18-SLN, or TransIT® as control. As clearly reported in Fig. 7, notwithstanding the ability of CP-SLN to efficiently bind DNA, their transfection efficiency appeared very limited as compared to the commercial product TransIT®. While the red fluorescence in cell transfected with the TransIT® reagent and pHcRed1-N1 was very intense (Fig. 7C), that in cells transfected with C16-SLN and C18-SLN was barely appreciable (Fig. 7A and B).

Moreover, the luciferase activity levels measured upon transfection of cells with the pGL3 vector and the C16-SLN or C18-SLN was 3.9% and 5.5% of that detected with the TransIT® reagent (Fig. 7D). These low transfection efficiencies might be attributable either to the cytotoxic of the CP-SLN or to their ability to deliver the DNA into cells.

In fact, many findings on this topic indicated that the low transfection activity could be attributed to the excessive condensation at high +/− molar charge ratio, which make difficult the dissociation of DNA from cationic SLN (Faneca et al., 2002; del Pozo-Rodríguez et al., 2007). Indeed, DNA condensation is a crucial factor that determines the transfection capacity of SLN, because it influences the superficial charge of the complexes and thus cell entry, DNA delivery from nanoparticles, gene protection from DNAses and hence DNA topology. An optimal DNA condensation must be achieved when designing non-viral vectors. Complexes must have enough DNA condensation capacity to create equilibrium between those factors to obtain good transfection levels.

4. Conclusions

Taking into account these results, we demonstrated that both hexadecyl-PTA iodide (CP16) and octadecyl-PTA iodide (CP18) cationic phosphines can be exploited to produce CP-SLN. The obtained CP-SLN are characterized by a net positive charge on the surface and a reproducible size. Moreover, these nanosystems can bind nucleic acid molecules allowing the formation of stable complexes that are able to protect DNA from the activity of exo- and endo-nucleases present in serum. Lastly, *in vitro* experiments demonstrated that CP-SLN exhibit a quite pronounced antiproliferative effect on cultured human K562 erythroleukemic cells and a limited effect as transfecting adjuvant. The "*in vitro*" transfection levels provided by the formulations developed could be mainly conditioned by their DNA condensation capacity. There must be equilibrium between the gene protection degree, the binding forces of DNA to SLN and the DNA topology. This equilibrium is determined by cationic lipid/DNA ratio and it must be optimized with every new formulation.

Notwithstanding the scarce ability of CP-SLN to transfect RFP into mammalian cells, our results indicated the capacity of CP-SLN to protect DNA from degradation and encourage further studies aimed at proposing this method as an innovative potential approach to deliver nucleic acid to cells in living organisms.

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