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Research paper

Nuclear localization of cationic solid lipid nanoparticles containing Protamine as transfection promoter

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ABSTRACT

Protamine has attracted much attention as DNA condenser and nuclear transfer enhancer although the excess of hydrophilicity and the strong DNA pack restrain its potentialities. In order to overcome this limitation, we added Protamine in the composition of solid lipid nanoparticles (SLN-Protamine) and we compared this carrier with the same kind of SLN containing Esterquat 1 instead of Protamine (SLN-EQ1). Carriers cytotoxicity was assessed on COS-I cells evaluating the cell cycle by propidium iodide test, while the transfection efficiency was studied using pEGFP as plasmid model. The cell penetrating activity of Protamine inside the lipid vectors was evaluated studying cell internalization by confocal microscopy using Red Nile-labeled carriers. SLN-Protamine:pDNA showed a mean diameter five-times smaller than the size of SLN-EQ1:pDNA and a remarkably lesser cytotoxicity. Transfection by SLN-Protamine:pDNA was seven-times 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 owing to their larger dimension. Red Nile-SLN-Protamine were localized in endocytic-like vesicles into the nuclear membrane suggesting the inclusion of Protamine in nano-lipophilic systems may enhance the reduction in the complex dimensions, the nuclear pDNA translocation and the pDNA release in the cells.

1. Introduction

The effective cure of genetic or acquired human diseases can be achieved by the genetic approaches pursuing the controlled introduction of therapeutic nucleic acids into target cells. In the in vivo gene therapy, viral vectors, even if engineered to minimize their adverse biological effects, may induce severe side effects involving immune response [1], secondary oncogenesis [2] and transfection of untargeted cells. These aspects along with safety concerns and a negative public perception of viral vectors [3] have addressed researchers to develop biologically inactive non-viral strategies for the in vivo gene delivery [4]. Non-viral vectors are safer to use for their low immunogenic response and easier to produce also in large scale manufacture than viral ones; in addition, the advantages associated with these vectors include the capacity to carry large inserts (52 kb) [5]. Non-viral vectors are mainly of a cationic nature: cationic polymers and cationic lipids that interact with negatively charged DNA through electrostatic interactions leading to polyplexes and lipoplexes, respectively.

Among cationic non-viral systems, solid lipid nanoparticles (SLN) have emerged in the last years as an effective alternative to liposomes and PEI (poly-ethylene-imine) principally due to their better stability profile owing to their possibility to be freeze-dried, ease of scalability and commercialization and relative cost efficacy [6].

However, the main limitations of cationic SLN, as well as of non-viral systems in general are the low and only transient expression levels owing to their inability to support the amplification, the cell-to-cell transmission [7] and the toxicity at high doses [8]. Moreover, it is known that the transfection efficiency of non-viral vectors is conditioned by many barriers like cell internalization, the disruption of the endosome membrane and the translocation of the genetic materials towards the cell nuclei [9].

Therefore, recently, a great attention has been focused on the improvement of the therapeutic potential of the cargo nucleic acids [6,10–13].

The approach proposed in this study is based on the addition of a transfection promoter as Protamine in the lipid nanocarrier structure. Protamine is a cationic small protein with high arginine content that is FDA approved for the parenteral administration [14]. Since Protamine is a nuclear protein that helps DNA packaging in sperm cells [15], it is also used as transfection accelerator in the gene delivery [14]. However, Protamine/DNA polyplexes demonstrated relatively low transfection efficiency [16]. One possible reason of this finding may lie in the strong hydrophilicity of Protamine, which makes it difficult to cross cellular membrane

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[16]. Another reason is the difficult release of DNA from the polyplexes given that Protamine can pack strongly DNA into small particles with highly compact structure [17]. In order to overcome these obstacles, the most common strategies included the addition of Protamine to the transfection media [18,19] or the preparation of ternary systems nanoparticles/protamine/DNA [17,20–22].

In this study, we added Protamine in the matrix of SLN based on stearic acid and stearylamine, and we compared this vector, in terms of cytotoxicity, transfection capacity and cell internalization, with a vector prepared by adding another transfection promoter, i.e. Esterquat 1 (EQ1). EQ1 ((N,N-di-(β-steaorylethyl)-N,N-dimethylammonium chloride) is a cationic detergent used to enhance the transfection efficacy of SLN with a good tolerability and biocompatibility [6].

Stearic acid is one of the most used lipids in the SLN formulation owing to its low toxicity, high biocompatibility and ease of manipulation [24,25]. On the other hand, stearylamine was chosen as cationic lipid for its affinity to the stearic acid structure and for its strong surfactant activity which allows the final dimension of the nanoparticles to be reduced [26].

In previous works, we have characterized these two cationic SLN formulations (SLN-EQ1 and SLN-Protamine), and it was demonstrated that both the samples were able to complex pDNA encoding for the Enhanced Green Fluorescent Protein (pEGFP) in the presence of cell culture medium [23,27].

In this paper, the cytotoxicity and the transfection efficiency of the freeze-dried SLN containing Protamine or EQ1 were assessed by flow cytometry on COS-I cells, while the intracellular distribution of SLN:pDNA complexes was analyzed by confocal microscopy.

2. Materials and methods

2.1. Materials

Stearic acid was purchased from Carlo Erba Reagenti (Milan, Italy). Stearylamine (ST) and Red Nile were 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 and chloroquine were provided from Sigma Aldrich (St. Louis, MO, USA). DAPI (4′,6-diamidino-2-phenylindole) was purchased from Vysis (Abbott Laboratories, Illinois, USA). Dulbecco's modified Eagle medium (DMEM), Dulbecco's Phosphate Buffer Solution (PBS) and other culture reagents were purchased from Euroclone Celbio (Milan, Italy). The cell line COS-I was purchased from Zoo-prophylactic Institute of Emilia Romagna (Bologna, Italy).

All other chemical reagents were obtained commercially as reagents-grade products.

2.2. SLN formulation

The cationic SLN were produced according to the method previously described [23]. Briefly, the solid component (250 mg of stearic acid) was melted at around 68 °C and at 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. 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 which was cooled at 4 °C by an ice bath, maintaining the mechanical stirring at 20,500 rpm for 10 min. The same procedure was employed to prepare blank SLN (bSLN) without adding any transfection promoter in the formulation. The formed solid lipid nanoparticles (SLN) suspension was washed as previously described [23] and freeze-dried (Heto-Holten A/S, AllerØd, Denmark) during 48 h at −55 °C at a pressure of 10^{-2} Torr. The freeze-dried SLN powder was collected and stored at 4 °C.

In order to prepare fluorescent samples, 200 μL of a methanol solution of Red Nile (2 mg/mL) was added to the stearic acid before melting.

The re-dispersion of the freeze-dried SLN (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 pEGFP production

The plasmid vector expressing Enhanced Green Fluorescent Protein 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) [A]. The pDNA was precipitated in 70% ethanol and reconstituted in 10 mM Tris–HCl, pH 8.5. The DNA concentration was determined using Hoechst 33258 dye. The $OD_{260/280}$ ratio was always >1.8.

2.4. Formation of SLN:pDNA complexes

SLN:pDNA complexes were prepared by mixing the SLN-EQ1 or SLN-Protamine (5 mg/mL) with 0.5 or 10 μg of plasmid DNA, for Photon Correlation Spectroscopy (PCS) analysis or transfection experiments, respectively. The mixture was kept at room temperature (25 ± 0.5 °C) for 45 min to allow the complexes to be formed. After the incubation, the SLN:pDNA complexes were diluted (1:5) in serum free medium (DMEM). Different SLN:pDNA (w/w) ratios were tested, i.e. 10:1, 20:1, 30:1 and 40:1.

2.5. Particle size, zeta potential and stability measurement

The size of both naked SLN and SLN:pDNA complexes was measured by a Zetasizer Nano ZS (Malvern, Worcs, UK) equipped with a 4 mW He–Ne laser (633 nm) [23].

Similarly, the zeta potential was measured using the same equipment by a combination of laser Doppler velocimetry and a phase analysis light scattering (PALS) [23].

2.6. Cell culture

COS-I cell line (SV-40 transformed African Green Monkey kidney cells) was cultured in DMEM medium with high glucose (6 mL), supplemented with 10% v/v (0.6 mL) of Fetal Bovine Serum (FBS), 100 units/mL penicillin and100 μg/mL streptomycin in 5% CO_2 incubator at 37 °C under 95% humidity.

2.7. Propidium iodide test (PI test)

Cytotoxicity of the SLN was determined by using the PI test (propidium iodide test [28]) evaluating their effect on the cell cycle distribution in comparison with an untreated cell population. In brief, COS-I cells were seeded at 500,000 cells/dish in 6 cm Petri's dishes, then cultured for 24 h as describe above. Immediately prior to the addition of various amounts of SLN-EQ1 or SLN-Protamine vectors, the medium was aspirated from each dish and replaced with 1 mL of DMEM serum free. The cells were incubated for 4 h or overnight (12 h). Then, the treated cells were washed with PBS, added with 5 mL of DMEM with FBS and incubated at 37 °C for 24 h. 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 (1% w/v). Detached cells were resuspended in the removed

medium, and 800 μL of cell suspension were centrifuged at 200*g* 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; sodium citrate 0.1% w/v, Triton X-100 0.1% w/v), and incubated in the dark at 4 °C for 10 min before the flow cytometry analysis.

2.8. Transfection studies

In order to study the transfection efficiency of the vectors, COS-I cells were grown in 6 cm Petri's dishes seeding the cells at 500,000 cells/dish and cultured for 24 h as described above; the transfection experiments were conducted at approximately 80% of confluence. Immediately prior to the addition of SLN:pDNA (10 μg pDNA/well) at various [carrier]/[pDNA] ratios, the culture medium was aspirated from each dish and replaced with 1 mL of serum free medium. The cells were incubated in 5% CO_2 incubator at 37 °C for different time periods (4 or 12 h) and then were washed with PBS and incubated at 37 °C for 24 h (expression time) in 5 mL DMEM with FBS.

Different SLN:pDNA w/w ratios were tested (10:1, 20:1, 30:1, 40:1) corresponding, respectively, to N/P ratios of 0.2:1, 0.4:1, 0.6:1, 0.8:1 for SLN-EQ1:pDNA and of 0.4:1, 0.9:1, 1.3:1, 1.8:1 for SLN-Protamine:pDNA, calculated in both cases using the amount of Nitrogen determined by a previous performed Electron Spectroscopy for Chemical Analysis (ESCA) [27]. In the case of SLN-EQ1:pDNA, the incubation was also conducted in the presence of chloroquine (50 μg/well) added in the cell medium before the transfection.

Cells transfected with naked pDNA at same concentration than in the SLN:pDNA (i.e. 10 μg/well) were used as control following an identical procedure. Protamine:pDNA and EQ1:pDNA polyplexes (N/P ratio of 2.3:1 and 1.8:1, respectively), obtained by incubating 10 μg of pDNA with the same amount of transfection promoter (EQ1 or Protamine, 110 μg) present in the 40:1 w/w ratio of SLN:pDNA, were used as additional control. Finally, further control was represented by the transfection performed using blank SLN (bSLN) formulated without any transfection promoters. bSLN:pDNA at 40:1 w/w ratio (corresponding to N/P ratio of 0.4:1) was used in presence or not of the transfection promoters (EQ1 or Protamine, 110 μg/well) in the cell culture medium.

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 (1% w/v). Detached cells were resuspended in 5 mL of DMEM with serum and 500 μL were analyzed by flow cytometry in order to assay the expression of GFP.

2.9. Flow cytometry

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

2.10. Confocal microscopy

Before to perform confocal microscopy analysis, the in vitro release of the SLN marker (Red Nile) was evaluated until 48 h. Labeled SLN-EQ1 or SLN-Protamine (40 mg) was incubated in 40 mL of phosphate buffer (20 mM, pH 7.4) or in 40 mL of complete medium (DMEM + serum) at 37 °C under magnetic stirring. One milliliter of SLN suspension was withdrawn from the system at time intervals of 30 min. and replaced with 1 mL of fresh solvent to maintain constant volume. The sample was centrifuged (10,000 rpm for 15 min) with Microcon 10 K (Millipore), and Red Nile content was determined in the filtrate by vis-spectroscopy at 525 nm.

To perform the internalization studies, COS-I cells were incubated 4 h or overnight (12 h) with Red Nile-SLN-EQ1:pDNA complexes and overnight (12 h) with Red Nile-SLN-Protamine:pDNA complexes. SLN:pDNA ratio was 40:1 (w/w) in all the experiments.

In order to visualize the expressed GFP in the cells treated by Red Nile-SLN-Protamine:pDNA complexes, an incubation time of 12 + 24 h was employed, according to the transfection procedure. After the incubation, the cells were fixed at room temperature with 50:50 (v/v) acetone/methanol solution, treated with DAPI, which gives blue fluorescence to the nuclei [29], and observed by a confocal microscope.

The confocal laser scanning microscopy (CLSM) of fixed cells was performed with a Leica DM IRE2 microscope (Mannheim, Germany) and a Leica Confocal System equipped with a scanner multiband 3-channel Leica TCS SP2 with AOBS, laser diode blu COH (405 nm/25 mW), laser Ar (458 nm/5 mW) (476 nm/5 mW) (488 nm/20 mW) (496 nm/5 mW) (514 nm/20 mW), laser HeNe (543 nm/1.2 mW), laser HeNe (594 nm) (orange) and laser HeNe (633 nm/102 mW).

3. Results and discussion

3.1. Particle size and zeta potential of SLN and SLN:pDNA complexes

The preparation of the SLN was performed by a modified oil-in-water microemulsion method which allows small and homogeneous nanoparticles around 230 nm to be obtained, as reported in the Table 1. Owing to the instability of the fresh prepared SLN suspensions [30], the reconstituted freeze-dried SLN powder was employed in all the experiments.

Even if the amount of two transfection promoters used in the SLN formulation was the same, the different structure of the two promoters leads to different numbers of N on the particle surface. However, after the freeze-drying process, the same positive charge (about +20 mV) was observed for the two free SLN samples because not all the N present on SLN-Protamine surface displayed a positive charge [27].

The PCS analysis of the SLN:pDNA complexes was performed at different weight ratios ranging from 10:1 to 40:1 corresponding to different N/P values for each sample (Table 1).

SLN-Protamine:pDNA complexes showed smaller sizes in comparison with the naked pDNA (530 nm) in all the SLN:pDNA ratios tested. Moreover, SLN-Protamine:pDNA complexes showed mean diameters less than 400 nm by far below the size of SLN-EQ1:pDNA (larger than 2 μm). Considering that EQ1 presents two hydrophobic chains, it is probably that its incorporation into the lipid matrix was higher than the Protamine [27]. Consequently, EQ1 may confer a rigid positive surface of the SLN, which would allow for the DNA adsorption by means of the formation of bridges between the particles and causes an increase in the mean diameter of the complexes. The increase of the PDI value increasing the SLN:pDNA ratios may be due to the co-presence of free SLN. Indeed, the size distribution by intensity obtained by PCS analysis showed that the percentage of free SLN (of around 230 nm) for SLN-Protamine:pDNA complexes increased from 2% (at the ratio 10:1 w/w) to 14% (at the ratio 40:1 w/w) and for SLN-EQ1:pDNA complexes from 0.5% to 5% at the same ratios.

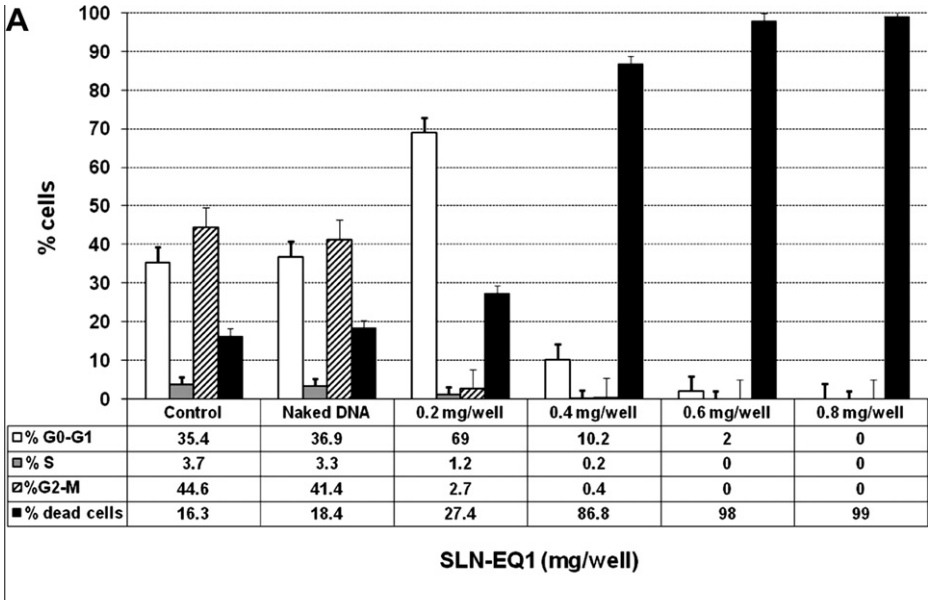
Relating to zeta potential value, SLN:pDNA complexes showed a positive surface: this value was about +20 mV for SLN-Protamine:pDNA and +25 mV for SLN-EQ1:pDNA complexes, regardless the N/P value. The positive *z*-potential at low N/P ratios provides evidence that at these N/P values, the negative charges of pDNA were completely redressed. Indeed, at a lower N/P value, not considered in this work (i.e. 5:1 w/w ratio), a negative charge of

Table 1
Size and zeta potential values of water resuspended freeze-dried naked SLN, SLN-EQ1:pDNA and SLN-Protamine:pDNA.

	SLN:pDNA ratio (w/w)	SLN:pDNA ratio (N/P)	Mean diameter (nm) ± SD (PDI)	Zeta potential (mV) ± SD
SLN-EQ1	–	–	230 ± 40 (0.280)	+23 ± 3
SLN-Protamine	–	–	235 ± 20 (0.291)	+19 ± 3
Naked pDNA	–	–	530 ± 30 (0.230)	−27 ± 3
SLN-EQ1:pDNA	10:1	0.2:1	2047 ± 110 (0.564)	+27 ± 2
	20:1	0.4:1	2070 ± 230 (0.710)	+28 ± 6
	30:1	0.6:1	2513 ± 320 (0.850)	+23 ± 5
	40.1	0.8:1	3584 ± 285 (0.800)	+16 ± 7
SLN-Protamine:pDNA	10:1	0.4:1	268 ± 15 (0.230)	+21 ± 3
	20:1	0.9:1	356 ± 30 (0.352)	+21 ± 2
	30:1	1.3:1	330 ± 23 (0.401)	+20 ± 4
	40:1	1.8:1	358 ± 29 (0.427)	+18 ± 6

the complexes (−19 mV) was obtained indicating a partial pDNA complexation. The slight difference in the *z*-potential between SLN-Protamine:pDNA and SLN-EQ1:pDNA complexes can be ascribed to the initial difference in the *z*-potential of the two kinds of free particles. A positive zeta potential of non-viral vectors is advantageous because it facilitates the interaction with the negative charged cell surface and the cell entry [13,31], notwithstanding a too high positive charge can induce severe cytotoxic effects [32].

In conclusion, SLN-Protamine demonstrated excellent property of pDNA condensation, being able to form small and positive



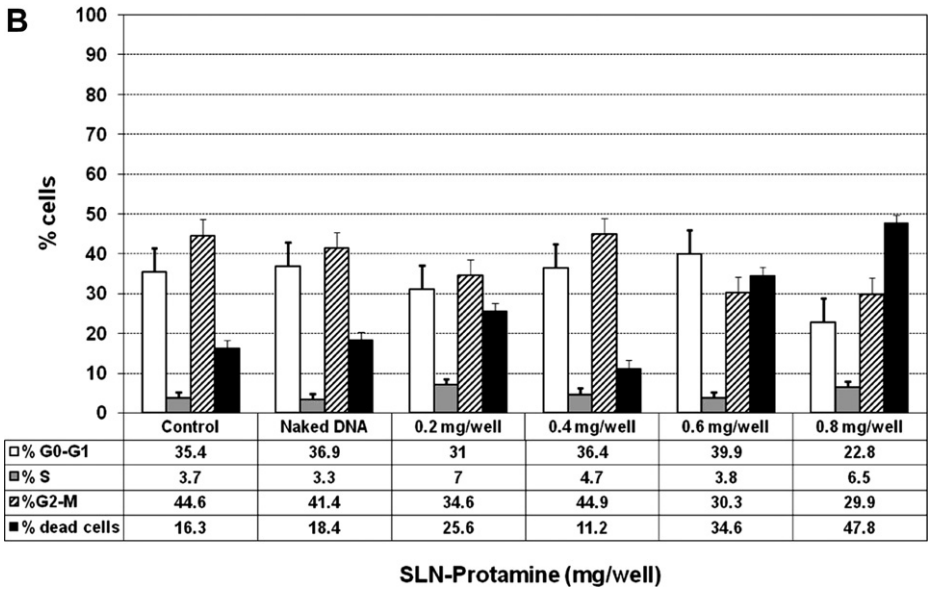


Fig. 1. Viability of COS-I cells exposed overnight (12 h) to various SLN-EQ1(A) and SLN-Protamine (B) concentrations, as assessed by PI assay. Untreated cells were used as control.

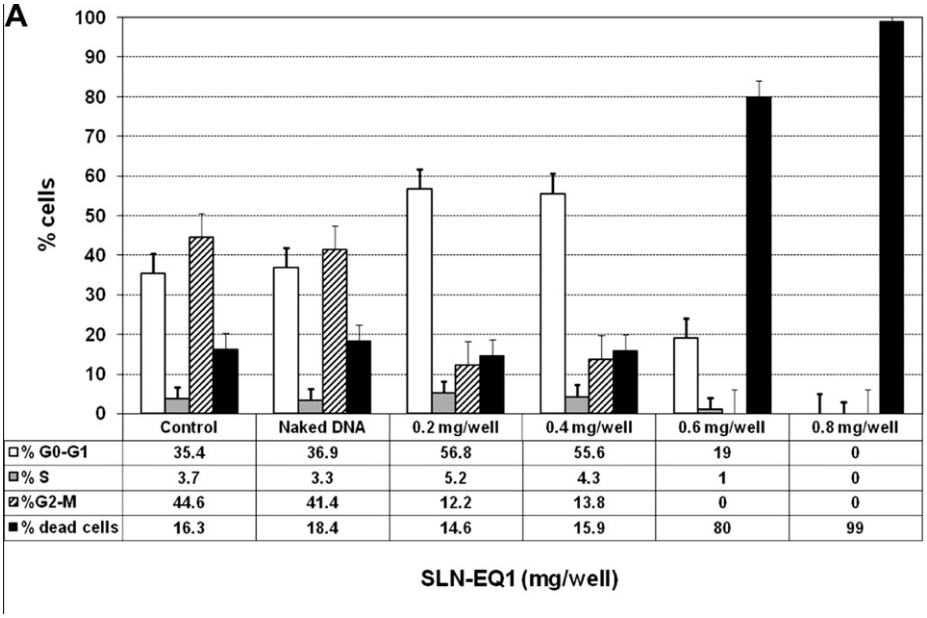
complexes, while SLN-EQ1 formed with pDNA very large aggregates. Cell viability and transfection efficiency were evaluated in order to understand how the different dimension of the complexes and different pDNA condensation capacity of the two samples influenced their in vitro behavior.

Concerning Protamine:pDNA and EQ1:pDNA polyplexes, the size (*z*-average) measured by PCS was of 320 ± 15 and 450 ± 22 nm, respectively, and the PDI value was about 0.2 in both the cases; finally, *z*-potential of Protamine:pDNA was around +20 mV, while EQ:1:pDNA complex presented a surface charge around +35 mV. As dimension and *z*-potential values of these polyplexes can be considered suitable for the DNA delivery, these complexes were applied as control in the transfection experiments.

3.2. Cell viability assay

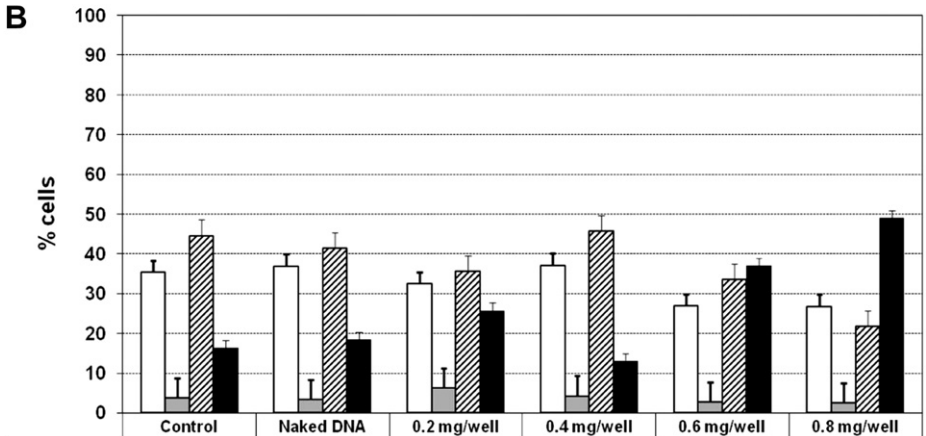
The cytotoxicity of free SLN was assessed on the COS-I cells by the PI test. Naked SLN, instead of SLN:pDNA complexes, were used in this assay in order to simulate the worst case scenario and obtain larger sensitive results, since, in general, toxicity is reduced when polyplexes with DNA are formed [33]. The PI test is an alternative to the most used MTT test and offers the advantage to highlight the distribution of the cells in the complete cell cycle after the incubation with the gene carriers [28]. The experiments were performed using a range between 0.2 and 0.8 mg/well of particles and employing two incubation times, i.e. 12 h (overnight) or 4 h.

In Fig. 1, the results obtained after the overnight incubation by SLN-EQ1 and SLN-Protamine were reported: the inhibition of the cell viability by SLN was clearly observed in a dose-dependent manner for the two samples. In details, cells treated with SLN-EQ1 (Fig. 1A) showed a G2/M arrest and an increase of the dead cells percentage even when incubated with only 0.2 mg/well of SLN; using 0.6 mg/well, almost the complete apoptosis of the cells occurred. On the other hand, cells treated with SLN-Protamine (Fig. 1B) showed a cell cycle distribution very similar to the control until the use of 0.4 mg/well; afterward, a slight G2/M reduction



	Control	Naked DNA	0.2 mg/well	0.4 mg/well	0.6 mg/well	0.8 mg/well
% G0-G1	35.4	36.9	56.8	55.6	19	0
% S	3.7	3.3	5.2	4.3	1	0
%G2-M	44.6	41.4	12.2	13.8	0	0
% dead cells	16.3	18.4	14.6	15.9	80	99

SLN-EQ1 (mg/well)



	Control	Naked DNA	0.2 mg/well	0.4 mg/well	0.6 mg/well	0.8 mg/well
% G0-G1	35.4	36.9	32.5	37.1	26.9	26.8
% S	3.7	3.3	6.2	4.3	2.7	2.6
%G2-M	44.6	41.4	35.6	45.7	33.5	21.7
% dead cells	16.3	18.4	25.7	12.9	36.9	48.9

SLN-Protamine (mg/well)

Fig. 2. Viability of COS-I cells exposed for 4 h to various SLN-EQ1 (A) and SLN-Protamine (B) concentration, as assessed by PI assay. Untreated cells were used as control.

and an increase of the percentage of dead cells were observed when 0.6 mg/well of SLN were employed. Summarizing, this incubation time (12 h) was considered inappropriate for the SLN-EQ1 but suitable for SLN-Protamine (0.4 mg/well maximum).

In Fig. 2, the complete cell cycle after an incubation of COS-I cells with SLN-EQ1 and SLN-Protamine during 4 h is reported. After this incubation time, a decrease of the cytotoxicity of SLN-EQ1 (Fig. 2A) in comparison with the results obtained after the overnight incubation was observed, suggesting that for this sample cytotoxicity was a time-dependant event. In fact, the effect on cell cycle of 0.4 mg/well of SLN-EQ1 was in this case very similar to the one observed for the untreated cells (controls) and only when 0.6 mg/well was employed a noticeable toxicity was observed (i.e. significant increase of cell death and G2-/M arrest). On the contrary, the effect of the SLN-Protamine on the cell cycle after 4 h (Fig. 2B) was very similar to the one observed after the overnight incubation suggesting that in the case of the SLN-Protamine, the cytotoxicity on the COS-I cells was not influenced by the incubation time.

It is evident that SLN-EQ1 exerts a higher cytotoxic activity on COS-I cells than SLN-Protamine. However, this different behavior should not be related to the positive charge of the particles, since both the samples showed a very similar charge (Table 1) and the value of this charge was too low to induce important cytotoxic events [34]. This finding may be due to the higher toxicity of cationic lipid with quaternary amine head-group, such as EQ1, in comparison with the biodegradable cationic polymers like proteins or polysaccharides [35]. This toxic effect should be attributable to a cell membrane damage mechanism or to the inhibition of critical enzymes, such as PKC, both events induced by the quaternary amine head-group of cationic lipid [35].

On the other hand, SLN-Protamine exhibited a low cytotoxicity, dose-dependent but not time-dependent. This property can be very important for in vivo gene therapy application, a strategy that requires a prolonged contact time between vectors and living cells [5].

The results obtained here were considered in determining not only the amount of SLN applicable per well (0.4 mg/well maxi-

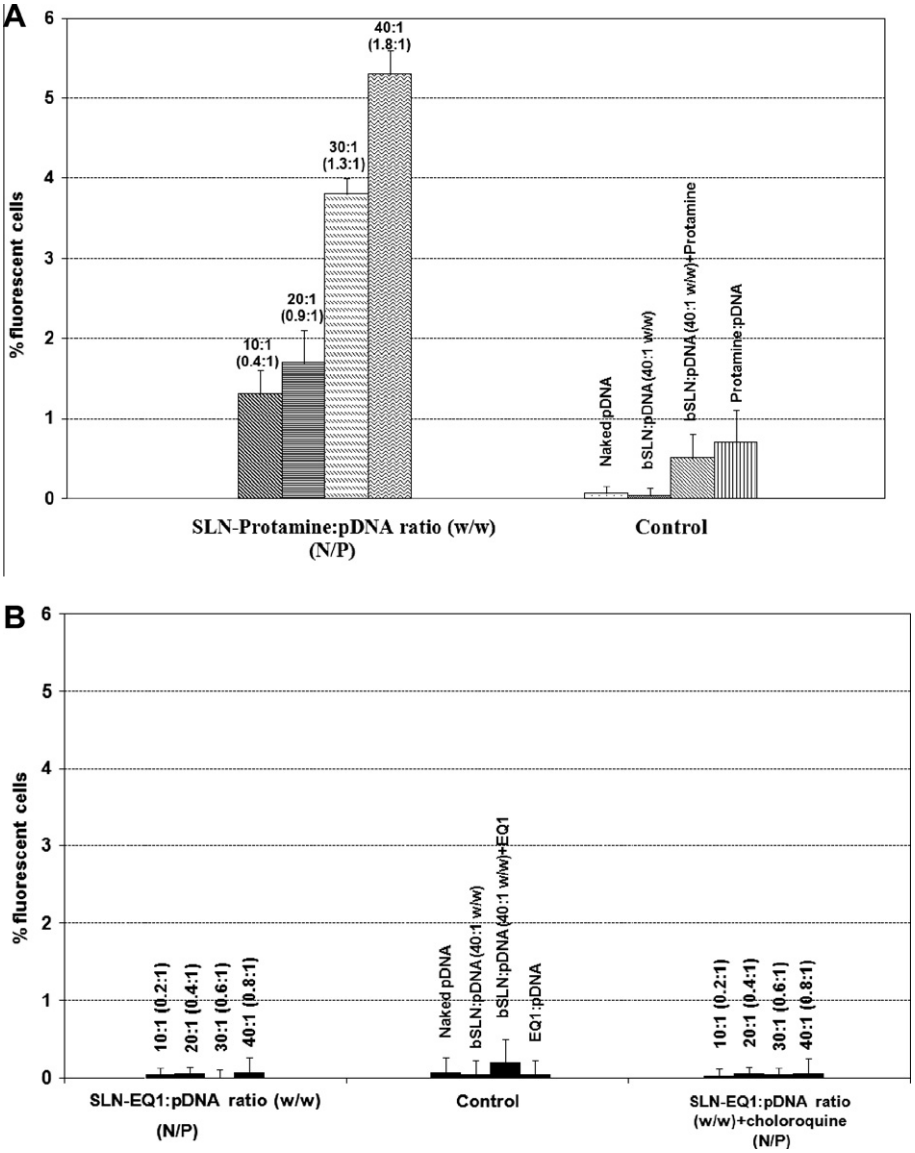


Fig. 3. (A) Transfection efficiency on COS-I cells of SLN-Protamine:pDNA at various SLN:pDNA ratios (w/w), bSLN:pDNA at 40:1 (w/w) in presence or not of Protamine in the cell culture medium (110 μg/well) and Protamine:pDNA polyplexes, incubated overnight (12 h) + 24 h, as assessed by flow cytometry. (B) Transfection efficiency on COS-I cells of SLN-EQ1:pDNA at various SLN:pDNA ratios (w/w), bSLN:pDNA at 40:1 (w/w) in presence or not of EQ1 in the cell culture medium (110 μg/well), EQ1:pDNA lipoplexes and SLN-EQ1:pDNA at various SLN:pDNA ratios (w/w) in presence of chloroquine in the cell culture medium (50 μg/well), incubated 4 h + 24 h, as assessed by flow cytometry.

mum) but also the incubation time reachable for each sample in the transfection studies (4 h for the SLN-EQ1 and 12 h for the SLN-Protamine).

3.3. Gene transfection experiment

The expression level of GFP was evaluated by flow cytometry assay after the incubation of the cells with the SLN:pEGFP complexes during 4 h for the SLN-EQ1 and 12 h (overnight) for the SLN-Protamine; in the same way, the amount of nanoparticles used in this experiment was ranged from 0.1 to 0.4 mg/well employing the following SLN:pEGFP ratios (w/w): 10:1, 20:1, 30:1, 40:1.

In Fig. 3A, the transfection efficiency of SLN-Protamine:pDNA is showed along with the control experiments. Increasing the SLN:pDNA ratio, the efficiency increased too and the maximum transfection efficiency was observed at a Protamine-SLN:pDNA ratio of 40:1 obtaining a value of 5.3 ± 0.2%. This fairly good transfection level is probably due to the transfection promoter activity of the Protamine [14] incorporated in the SLN. In order to understand if Protamine was able to promote in the same way the pDNA transfection if added in the cell medium or simply complexed with pDNA, several experiments as control were performed (Fig. 3A). SLN without transfection promoters (bSLN), having a positive charge thank to the presence of stearylamine, were used to transfect pDNA adding or not Protamine in the cell culture medium. bSLN demonstrated to be unable to transfer pDNA into the cells (0.06%) even if a slight enhancement was observed in the presence of Protamine (0.5%). In the same way, Protamine:pDNA polyplex showed unsatisfactory transfection level (0.7%). This finding demonstrated that the inclusion of Protamine in the SLN structure has determined a 7-folds enhancement in the transfection efficiency in comparison with the Protamine:pDNA polyplex (from 0.7% to 5.4%). These data are in agreement with the data reported in literature regarding the activity of Protamine as promoter transfection and provide evidence that the inclusion of Protamine in lipophilic systems like SLN may permit a sensible improvement of its transfection capacity.

In Fig. 3B, the results of the transfection experiments carried out with SLN-EQ1:pDNA were reported. The efficiency observed was very poor regardless of the SLN:pDNA ratio, and the same unsatisfying results were obtained either adding EQ1 in the culture medium of cells treated by bSLN:pDNA or using EQ1:pDNA polyplex. As in the transfection condition adopted (incubation time and SLN concentration) the cytotoxicity effect is slight, the failed transfec-

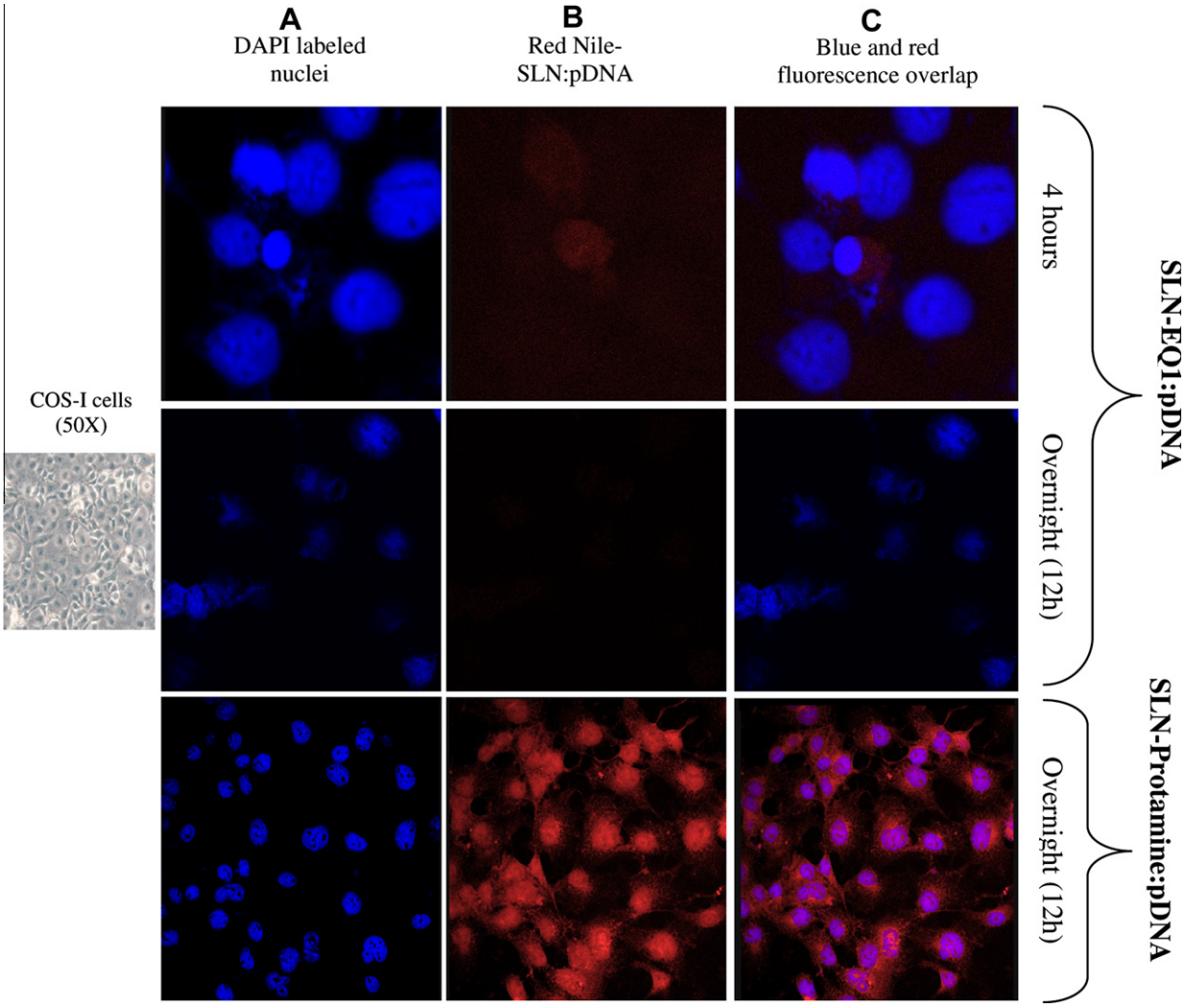


Fig. 4. Confocal images of COS-I cells after the incubation with Red Nile-SLN:pDNA complexes at 40:1 ratio (w/w). The cell nuclei were stained with DAPI (blue florescence, column A), and SLN were labeled with Red Nile (red fluorescence, column B). The column C reported blue and red fluorescence overlapped. COS-I cells were incubated with Red Nile-SLN-EQ1:pDNA complexes 4 h (first line) or overnight (second line), while with Red Nile-SLN-Protamine:pDNA complexes, the cells were incubated only overnight (third line). The images were taken at the same *z*-thickness. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tion can be ascribable to the big dimensions of the SLN-EQ1:pDNA complexes (Table 1). On the other hand, it is possible that the SLN-EQ1:pDNA complexes could be internalized notwithstanding their dimensions since transfections with microparticles are reported [36,37], but they were unable to escape from endosome after endocytosis. To further elucidate this possibility, chloroquine, a lysosomolytic agent [6,11,38], was added. The results indicated that this addition did not determine any increase in the SLN-EQ1:pDNA transfection efficiency (Fig. 3B). Thus, the low transfection level achieved by SLN-EQ1:pDNA cannot be ascribable to the failed endosome escape.

As discussed in previous papers [23,27], both the SLN samples exhibited in the agarose gel electrophoresis analysis a good pDNA binding ability at the same w/w ratios used for transfections. However, in the physicochemical characterization of the complexes (Table 1), a significant difference in the dimension of the two kinds of complexes was highlighted suggesting a different capacity in the pDNA condensation. Therefore, the noticeable difference in the transfection efficiency could be attributable to the different internalization capability probably related to the complexes dimensions. In order to elucidate this point, the intracellular fate of the SLN-Protamine in comparison with SLN-EQ1 was studied by confocal fluorescence microscopy (CLSM) using Red Nile-labeled SLN.

3.4. Confocal fluorescence microscopy

Before the CLSM analysis, the stability of the marker (Red Nile) in the SLN was evaluated incubating the labeled samples at 37 °C in PBS or in complete medium for 48 h, according to the procedure described in Section 2. During this time period, the Red Nile was not released at all from both of the samples meaning that the marker was stably associated to the carriers (data not shown).

The cellular uptake by COS-I cell line of SLN:pDNA complexes was visualized by confocal microscopy after an incubation of the cells with Red Nile-SLN:pDNA complexes at a ratio of 40:1 (w/w), according to the transfection procedure.

The major advantage of CLSM analysis is that the cell can be optically sectioned and the distribution of the fluorescent probe in the cell can be visualized by images parallel to the surface of the cell. The intracellular distributions of the Red Nile-SLN were observed in a single plane in the same z-thickness value, using appropriate excitation and emission filters and three fluorescence probes (i.e.: DAPI for nuclei (blue), Red Nile for the SLN (red) and the GFP as the protein expressed (green)).

As showed in Fig. 4, no red spots were detected in the cells transfected by Red Nile-SLN-EQ1:pEGFP complexes after 4 h or after 12 h of incubation (first and second line, respectively), while in the case of cell transfected by Red Nile-SLN-Protamine:pEGFP complexes, the red spots were observed after overnight incubation not only in the cytosol but also in the nucleus of cells (Fig. 4, third line). Interestingly, in this case, as reported in Fig. 5, grainy white spots indicating the site of interaction between red fluorescent and the blue fluorescence were localized in endocytic-like vesicles into the nuclear membrane. The interaction is well recognized also in the spots included into the yellow ellipsis (Fig. 5A) because of their same position in the thickness of the sample. The use of confocal microscopy, owing to the 3D possibility of investigation, demonstrated in unequivocal manner the position of the red fluorescence (due to the labeled SLN) relating to the nucleus (blue fluorescence). However, the red spots could be due to either the labeled free SLN or the Red Nile-SLN-Protamine:pDNA complexes seeing that the complexes were formulated in excess of free particles.

In order to evaluate the real internalization of the complexes, the GFP expression in COS-I cells was evaluated by the confocal micros-

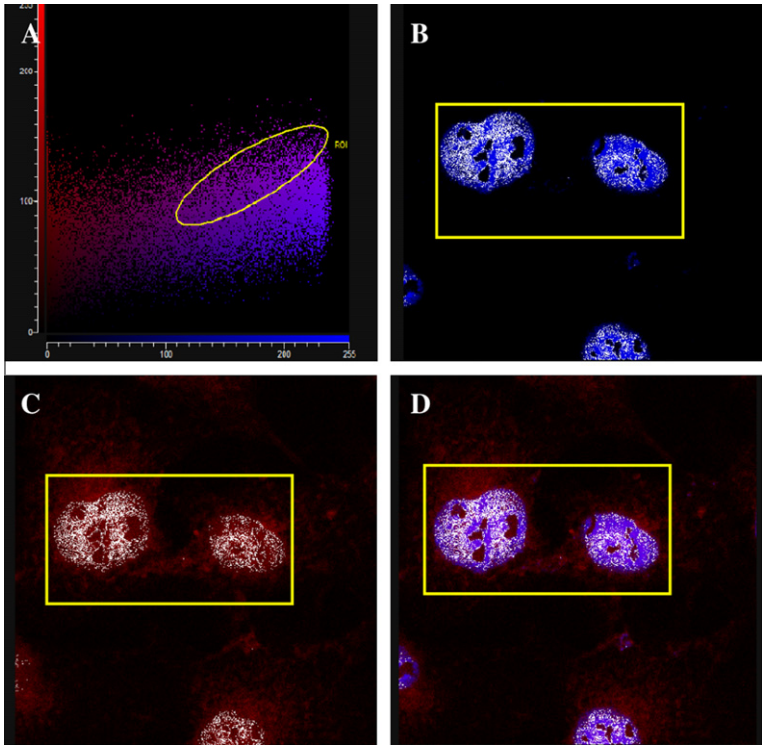


Fig. 5. Confocal study performed in a single plane in z-thickness on COS-I cells. Image A is referred to the intensity of red and blue coloration coming, respectively, from fluorescent SLN-Protamine and DAPI. The spots included into the yellow ellipsis are considered as the points of interaction between fluorescent SLN and nuclei because of their same position in the thickness of the sample. These sites of interaction are also well recognizable as the white spots enclosed in the yellow squares in (B) (DAPI labeled nuclei), (C) (Red Nile-labeled SLN-Protamine:pDNA) and D (blue and red fluorescence overlap). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

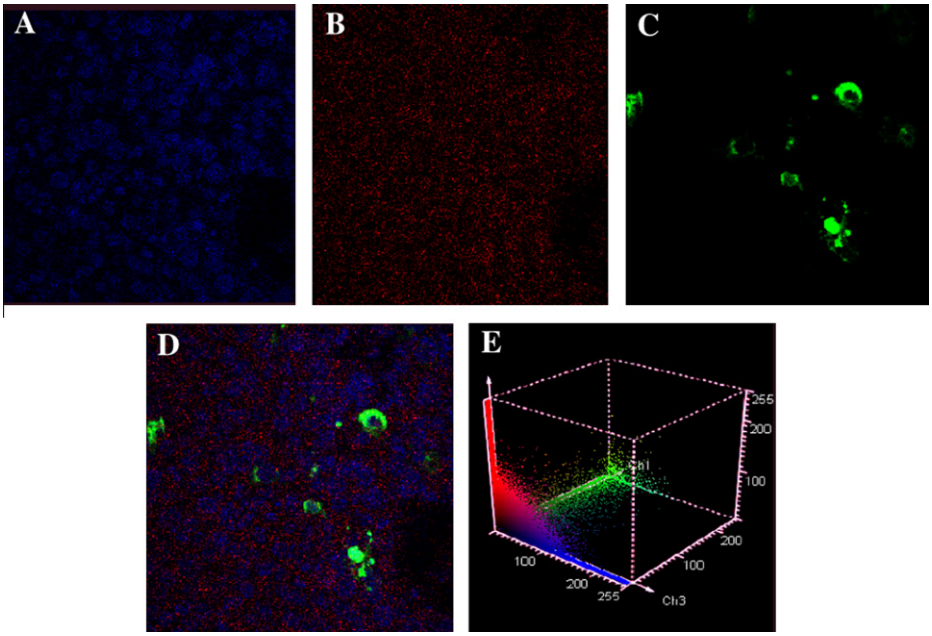


Fig. 6. Confocal images of COS-I cells incubated with Red Nile-SLN-Protamine:pDNA at 40:1 ratio (w/w). The blue (cell nuclei stained with DAPI, A), red (Red Nile-SLN:pDNA complexes internalized into the cells, B) and green (expressed GFP, C) fluorescence were detected after overnight incubation (12 h, incubation time) followed by another 24 h (protein expression time), as in the transfection procedure. Image D reports the overlap of blue, red and green fluorescence. Image E represents the blue, red and green fluorescence intensity and their intensity overlap. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

copy after the time necessary for the protein expression (24 h) (Fig. 6). In this case, the presence of the green spots (Fig. 6; C and D images) was evident in some cells confirming the internalization of the labeled complexes. The green fluorescence was localized in the cytoplasm, while red fluorescence was co-localized with the blue fluorescence of nucleus (Fig. 6; E image). The intensity of the red fluorescence (Fig. 6; B images) after the expression time (24 h) appeared weak and diffused in the cells, probably due to the beginning of the intracellular particle metabolization.

The basic conclusion from the CLMS analysis is that EQ1 is not an efficient transfection promoter for solid lipid nanoparticles probably owing to its inability to promote the pDNA condensation and internalization. Nevertheless, SLN-EQ1 demonstrated a considerable cell toxicity which cannot be attributable to a critical enzyme inhibition, because this event requires the cell internalization of the particles. Moreover, on the basis of the CLMS analysis, no red signal in the cells attributable either to the complexes or to the particles was detected after 4 h or overnight incubation. Thus, the cytotoxicity of SLN-EQ1 can probably be due to cell membrane damage, caused by the quaternary amine head-group. In fact, the cell membrane damage mechanism is often mediated by ionic interactions between anionic domains on the cell surface and cationic moieties present on the vector [39].

The better cell internalization capacity of the SLN-Protamine:pDNA can be due not only to their small dimensions (Table 1) but also to the action of Protamine. Indeed, as underlined before, the red fluorescence visible in the cells transfected by SLN-Protamine complexes (Figs. 4 and 6) could be due not only to the complexes but also to the free particles, since not all the red cells were able to express GFP. Thus, as free SLN-Protamine and free SLN-EQ1 showed the same dimensions (Table 1), the cell internalization ability of the free SLN-Protamine may be attributable to the Protamine transfection ability. Moreover, the nuclear localization of the Red Nile-SLN-Protamine as either free particles or pDNA complexed may be attributable to the NLS (nuclear localization signal) of Protamine [40]. In fact, particles over 30 nm in diameter required active transport through the nuclear pore complex (NPC) to reach the nucleus [41]. Such nuclear transport can be accomplished by incorporating appropriate NLS in the vehicle. Since Protamine is a nuclear protein that helps DNA packaging in sperm cells [15], it presents a NLS that can help both the naked SLN-Protamine and SLN-Protamine:pDNA complexes to enter the cell nucleus.

4. Conclusions

The results here highlight that the inclusion of EQ1 into the SLN lipid matrix leads to particles forming with pDNA large aggregates unable to enter the cell and to induce the expression of the encoded protein. On the other hand, the incorporation of Protamine in the solid lipid nanostructured carriers not only does not affect the ability of this protein to promote DNA condensation and nuclear translocation of exogenous DNA but also allows the use of Protamine in the in vivo gene therapy. Indeed, notwithstanding the use of temperature of about 68 °C during the SLN formulation, no modification of the protein propriety to bind and delivery the plasmid DNA to the nuclei occurred. In conclusion, since Protamine/DNA polyplexes are not efficient gene vectors [14], the inclusion of Protamine in lipophilic systems like SLN may enhance on one hand the pDNA release in the cells thank to the less strong packaging of the pDNA [14] in comparison with the Protamine:pDNA polyplex, on the other hand it does not modify the pDNA nuclear transport ability of Protamine.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.07.012.

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