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Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity

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Abstract

Since solid lipid nanoparticles (SLNs) were introduced as non-viral transfection systems, very few reports of their use for gene delivery have been published. In this work different formulations based on SLN–DNA complexes were formulated in order to evaluate the influence of the formulation components on the "in vitro" transfection capacity. SLNs composed by the solid lipid Precirol® ATO 5, the cationic lipid DOTAP and the surfactant Tween 80, and SLN–DNA complexes prepared at different DOTAP/DNA ratios were characterized by studying their size, surface charge, DNA protection capacity, transfection and cell viability in HEK293 cultured cells. The incorporation of Tween 80 allowed for the reduction of the cationic lipid concentration. The formulations prepared at DOTAP/DNA ratios 7/1, 5/1 and 4/1 provided almost the same transfection levels (around 15% transfected cells), without significant differences between them ($p > 0.05$). Other assayed formulations presented lower transfection. Transfection activity was dependent on the DOTAP/DNA ratio since it influences the DNA condensation into the SLNs. DNA condensation is a crucial factor which conditions the transfection capacity of SLNs, because it influences DNA delivery from nanoparticles, gene protection from external agents and DNA topology.

Keywords: Solid lipid nanoparticles; Non-viral vectors; Gene therapy; "In vitro" transfection

1. Introduction

Gene therapy is a rapidly advancing field with great potential for the treatment of genetic and acquired systemic diseases. This therapy requires the introduction of foreign DNA into the target cells and a gene delivery system must be used to facilitate the cellular uptake and the intracellular processing of the exogenous DNA. Gene delivery systems include viral vectors and non-viral vectors. Viral vectors are the most effective, but their application is limited by their immunogenicity, oncogenicity and the small size of the DNA they can transport. Non-viral vectors, however, are safer, lowered cost, and more reproducible and do not present DNA size limit. The main problem of non-viral systems is their low transfection efficiency.

Non-viral transfection systems may be composed by cationic peptides, cationic polymers or cationic lipids, and the combination of some of those components is also possible (Tokunaga et al., 2004; Hyndman et al., 2004).

Although there is a large number of publications about cationic liposomes and cationic lipid emulsions for gene therapy, only a few reports about the use of solid lipid nanoparticles (SLNs) for delivery of genes (Tabatt et al., 2004a,b; Rudolph et al., 2004; Pedersen et al., 2006) have been published since Olbrich et al. (2001) introduced these particles as a non-viral transfection system. From the point of view of application, SLNs have good stability (Freitas and Müller, 1999), which facilitates the industrial elaboration and the manipulation for different processes such as lyophilization.

In most cases the elaboration of SLNs in addition to the matrix lipid and the cationic lipid requires additional surfactants. Tween 80 is one of the most employed surfactants in pharmaceutical industry, and it has some interesting characteristics to be used in formulations for gene therapy, because of the presence of poly(ethyleneglycol) (PEG) chains in its structure. Different research groups have observed that the presence of PEG in cationic lipid emulsions (Liu et al., 1996a,b) and in liposomes

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(Meyer et al., 1998) improves their transfection capacity. Liu et al. (1996a) showed that Tween 80 was the most effective non-ionic surfactant to avoid the formation of aggregates. In their opinion, when complexes are formed each molecule of DNA may bind more than one emulsion particle such that large aggregates are formed. However, Tween 80 may prevent sterically each DNA molecule from binding to more than one particle and the formation of such large aggregates does not occur. Besides, Tween 80 has another important characteristic for the transfection of these systems "in vivo". It creates a steric barrier (Harvie et al., 2006) which neutralizes the excess of positive charges of the systems and reduces the interaction with blood components, such as serum proteins, which could limit the arrival of the gene therapy system to the cell surface.

In order to form *lipoplexes* the positive superficial charge of the systems is necessary to electrostatically bind the DNA, which has negative charge. When DNA binds with these systems it is condensed, and that condensation increases as the charges ratio (+/−) increases (Faneca et al., 2002). Condensation is necessary to facilitate the mobility of DNA molecules, which is limited by their large size, and to protect the DNA from agents present inter and intracellulary. Condensation reduces the exposure of the DNA to those agents and improves its protection. However, Faneca et al. (2002) also indicated that DNA condensation may limit the transfection efficiency of non-viral systems because the larger the condensation the more difficult the release of the DNA from the complexes.

The objective of this study was to evaluate the influence of the composition of SLN–DNA complexes for gene therapy on their transfection capacity of culture cells. These non-viral vectors have not been studied as extensively as liposomes for gene therapy, and the aim of this work was to evaluate some relevant formulation factors which may be important to improve their application in gene therapy. The influence of composition of SLNs and DOTAP/DNA ratio was studied.

2. Materials and methods

2.1. Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). *N*-[1-(2,3-Dioleoyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), deoxyribonuclease I (DNase I), lauryl sulfate sodium (SDS), antibiotic/antimycotic and DNA from salmon sperm were purchased from Sigma–Aldrich (Madrid, Spain). Tween 80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain).

Plasmid pCMS-EGFP encoding the green fluorescent protein (GFP) was purchased from BD Biosciences Clontech (Palo Alto, USA) and amplified by Dro S.L. (San Sebastián, Spain).

The materials employed for the electrophoresis on agarose gel were acquired from Bio-Rad (Madrid, Spain). PicoGreen® dsDNA quantitation reagent (PicoGreen®) was provided by Molecular Probes (Oregon, USA).

The cell culture reagents were purchased from LGC Promochem (Barcelona, Spain).

BD Viaprobe kit was provided by BD Biosciences (Belgium).

2.2. SLN production

The SLNs were produced by a solvent emulsification/evaporation technique (Mehnert and Mader, 2001). The lipid Precirol® ATO 5 was dissolved in the organic solvent dichloromethane (5%, w/v), and then emulsified in an aqueous phase that contained the cationic lipid DOTAP and the surfactant Tween 80. Different concentrations (w/v) of DOTAP and Tween 80 were used—formulation 1: DOTAP 0.4% and Tween 80 0.1%; formulation 2: DOTAP 0.3% and Tween 80 0.2%; formulation 3: DOTAP 0.3% and Tween 80 0.1%. The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) during 30 s at 50 W. The organic solvent then was removed from the emulsion by evaporation using a magnetic agitator for 45 min followed by vacuum conditions for 15 min. Upon dichloromethane evaporation SLNs suspension was formed by precipitation of the Precirol® ATO 5 in the aqueous medium. Finally, SLNs were washed by centrifugation (3000 rpm, 20 min, three times) using the Amicon® Ultra centrifugal filters (Millipore, Madrid, Spain).

2.3. Preparation of SLN–DNA complexes

Twenty-five microliters of pCMS-EGFP plasmid DNA solution at 2 μg/μL concentration was mixed with different amounts of SLNs suspensions in Mili-Q™ water. The amounts of SLNs required for complete DNA binding were determined individually by agarose gel electrophoresis and expressed as w/w ratio of DOTAP/DNA. DOTAP/DNA ratios assayed ranged from 15/1 to 1/1. SLN–DNA complexes were prepared by mixing DNA solution and SLNs suspension during 30 min at 25 °C.

2.4. Size and zeta potential measurements

The sizes of SLNs and SLN–DNA complexes were determined by photon correlation spectroscopy (PCS). Zeta potential was measured by laser doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in NaCl 0.1 mM.

2.5. Atomic force microscopy

SLNs and SLN–DNA complexes were observed by atomic force microscopy (AFM) using the Multimode™ model from Digital Instruments. The images were captured in Tapping Mode™ using a cantilever of silicon rotated tapping etched silicon probe type (RTESP) with a resonance frequency of about 300 kHz.

2.6. Agarose gel electrophoresis

SLN–DNA complexes were diluted in water Mili-Q™ to a final concentration of 0.1 μg DNA/μL and subjected to electrophoresis on an agarose gel (1% ethidium bromide included for visualization) for 30 min at 120 V. The bands were observed with a model TFX-20M transilluminator (Vilber-Lourmat). Images

were captured using a digital camera from BioRad, DigiDoc model.

2.7. Quantitation of DNA binding: PicoGreen® assay

SLN–DNA complexes were washed by centrifugation using the PVDF centrifugal filters Ultrafree® MC GV 0.22 μm (Millipore, Madrid, Spain). The filtered phase during the centrifugation was recovered and mixed with the PicoGreen® reagent. The fluorescence of the samples was measured by a SFM 25 fluorometer (excitation 480 nm, emission 520 nm). From the measured fluorescence we calculated the amount of DNA which was not bound to the SLNs.

2.8. DNase I protection study

DNase I was added to SLN–DNA complexes to a final concentration of 1 U DNase I/2.5 μg DNA, and the mixtures were incubated at 37 °C for 30 min. Afterwards 2% SDS solution was added to the samples to a final concentration of 1% to release DNA from SLNs. Samples were then analysed by electrophoresis on agarose gel and the integrity of the DNA in each sample was compared with untreated DNA as control.

2.9. Cell culture and transfection protocol

The Human Embrionic Kidney (HEK293) cell line was obtained from the American Type Culture Cell (ATCC) and maintained in Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic/antimycotic. Cells were incubated at 37 °C with 5% CO_2 in air and subcultured every 2–3 days using trypsin/EDTA.

For transfection HEK293 cells were seeded on 24-well plates at a density of 150,000 cells per well and allowed to adhere overnight. Seventy-five microliters of the complexes solution (2.5 μg DNA) was added, and cells were incubated with the complexes for 4 h at 37 °C. The medium containing the complexes in the wells was diluted with 1 mL of complete medium and cells were allowed to grow for further 72 h.

As a blank we used SLN–DNA complexes formulated in the same conditions but with DNA from salmon sperm instead of pCMS-EGFP plasmid.

2.10. Qualitative analysis of transfection efficacy: fluorescent microscopy

Detection of expression of GFP was carried out at 72 h post-transfection using an inverted microscope equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon). Observations and image captures were performed using a 20× objective.

2.11. Quantitative analysis of transfection efficacy and cytotoxicity: flow cytometry

At the end of the incubation, cells were washed once with 300 μL of PBS and were detached with 300 μL of trypsin/EDTA. Then the cells were centrifuged at 1500 × *g* and the supernatant was discarded. The cells were resuspended with PBS and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA). For each sample 10,000 events were collected.

For transfection efficacy quantitation fluorescence of GFP was collected at 525 nm (FL1). For cytotoxicity measurements BD Via-Probe kit was employed. This reagent was used for dead cell exclusion. Five microliters of the kit was added to each sample and after 10 min of incubation fluorescence correspondent to dead cells was measured at 650 nm (FL3).

2.12. Statistical analysis

Results are reported as means (S.D. = standard deviation). Statistical analysis was made with SPSS 14.0 for Windows® (SPSS®, Chicago, USA). Normal distribution of samples was assessed by the Shapiro–Wilk's test, and homogeneity of the variance by the Levene's test. The statistical analysis between different groups was determined with an ANOVA test. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Characterization of SLNs

Table 1 summarizes particle size, zeta potential and polydispersity indexes of SLNs composed by different amounts of DOTAP and Tween 80. The particle size ranged from 340 to 373 nm and the zeta potential was positive, around +45 mV, for all formulations. Polydispersity indexes were 0.4, 0.2 and 0.3 for SLNs composed by DOTAP 0.4% Tween 80 0.1%, DOTAP 0.3% Tween 80 0.2% and DOTAP 0.3% Tween 80 0.1%, respectively. No statistically significant differences ($p > 0.05$) were observed between the formulations.

The atomic force microscopy (AFM) images of SLNs indicated that nanoparticles were spherical. The image in Fig. 1A corresponds to the visualization of a batch of SLNs.

3.2. Characterization of SLN–DNA complexes

SLN–DNA complexes for an extensive characterization were prepared with the SLNs composed by DOTAP 0.4% and Tween 80 0.1%.

As can be seen in Table 2, the size of the complexes obtained at the DOTAP/DNA ratios from 15/1 to 4/1 decreased when comparing with their corresponding SLNs (Table 1), whereas

Table 1
Composition and physicochemical characterization of SLN formulations

Cationic lipid	Tween 80 (%)	Size (nm)	Zeta potential (mV)	Polydispersity index
DOTAP 0.4%	0.1	339.1 (12.4)	+42.9 (1.3)	0.4 (0.1)
DOTAP 0.3%	0.2	373.2 (42.9)	+48.1 (5.6)	0.2 (0.2)
DOTAP 0.3%	0.1	367.3 (129.6)	+49.8 (5.9)	0.3 (0.1)

Mean (S.D. = standard deviation) ($n = 3$). The matrix lipid was Precirol® ATO 5 at 5% in all formulations.

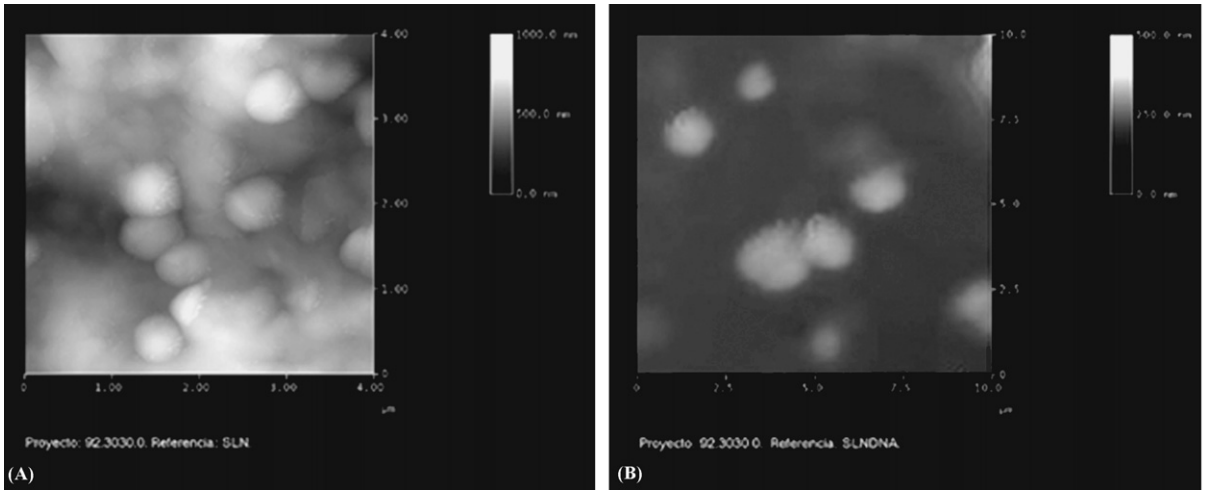

Fig. 1. Image of SLNs (A) and SLN–DNA complexes (B) taken by atomic force microscopy. SLNs were composed by DOTAP 0.4% and Tween 80 0.1%, and SLN–DNA complexes were prepared at DOTAP/DNA ratio 5/1 (w/w).

a size increase took place with the ratio 3/1 or smaller (bigger than 1 μm). No significant differences between the sizes of the complexes at ratios 15/1 to 4/1 were detected ($p > 0.05$). Polydispersity indexes were higher than those obtained with SLNs.

The zeta potential (Table 2) was highly positive for DOTAP/DNA ratios 15/1 to 4/1, ranging from +30 to +40 mV. However, when DOTAP/DNA ratio was 3/1 or lower zeta potential became negative.

Fig. 2 shows the gel electrophoresis resulting from the binding efficiency assay carried out with the different DOTAP/DNA ratios used in the elaboration of the complexes. Lane 1 corresponds to free DNA. On lanes 2–7 (ratios from 15/1 to 4/1) there was no band corresponding to free DNA, which means that all DNA was bound to the SLNs. Ratios 3/1, 2.5/1 and 1/1 (lanes 8, 9 and 10, respectively) revealed bands indicating that SLNs were not capable to bind all DNA. These results are in agreement with those obtained with the PicoGreen® assay, which showed that DOTAP/DNA ratios equal or higher than 4/1 presented a binding efficiency of 100%, whereas when this ratio was 3/1 or smaller the binding efficiency decreased.

Table 2
Physicochemical characterization of SLN–DNA complexes

DOTAP/DNA ratio (w/w)	Size (nm)	Zeta potential (mV)	Polydispersity index
15/1	258.5 (23.1)	+40.6 (5.6)	0.5 (0.3)
10/1	216.7 (44.2)	+46.6 (4.4)	0.7 (0.3)
7/1	260.1 (18.7)	+38.9 (7.1)	0.7 (0.2)
6/1	255.9 (61.7)	+35.3 (2.6)	0.7 (0.2)
5/1	249.2 (25.3)	+36.5 (2.8)	0.4 (0.3)
4/1	240.4 (10.9)	+32.4 (0.9)	0.7 (0.1)
3/1	>1 μm	−11.9 (5.2)	–
2.5/1	>1 μm	−23.7 (2.9)	–
1/1	>1 μm	−37.1 (1.9)	–

SLNs were composed by DOTAP 0.4% and Tween 80 0.1%. The size of the corresponding SLNs was 339.1 (12.4). Mean (S.D. = standard deviation) ($n = 3$).

Fig. 2. Binding efficiency of DNA with SLNs at different DOTAP/DNA ratios (w/w) studied by agarose gel electrophoresis. Lane 1: free DNA; lane 2: 15/1; lane 3: 10/1; lane 4: 7/1; lane 5: 6/1; lane 6: 5/1; lane 7: 4/1; lane 8: 3/1; lane 9: 2.5/1; lane 10: 1/1.

3.3. "In vitro" resistance against DNase I

Fig. 3 features the gel electrophoresis with the results of resistance of DNA bound to SLNs against the attack of DNase I. Lanes 1 and 2 correspond to non-treated free DNA and DNase I treated free DNA, respectively. No band on lane 2 is observed because free DNA was totally digested by the enzyme. The

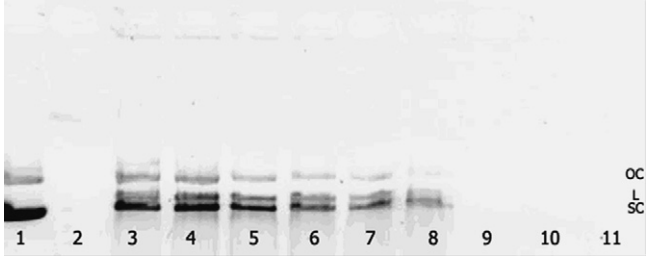

Fig. 3. Protection of DNA by SLNs from DNase digestion at different DOTAP/DNA ratios (w/w) visualized by agarose gel electrophoresis. SLN–DNA complexes were treated with DNase I. Lane 1: not treated free DNA; lane 2: DNase-treated free DNA; lane 3: 15/1; lane 4: 10/1; lane 5: 7/1; lane 6: 6/1; lane 7: 5/1; lane 8: 4/1; lane 9: 3/1; lane 10: 2.5/1; lane 11: 1/1; OC: open circular form; L: lineal form; SC: supercoiled form.

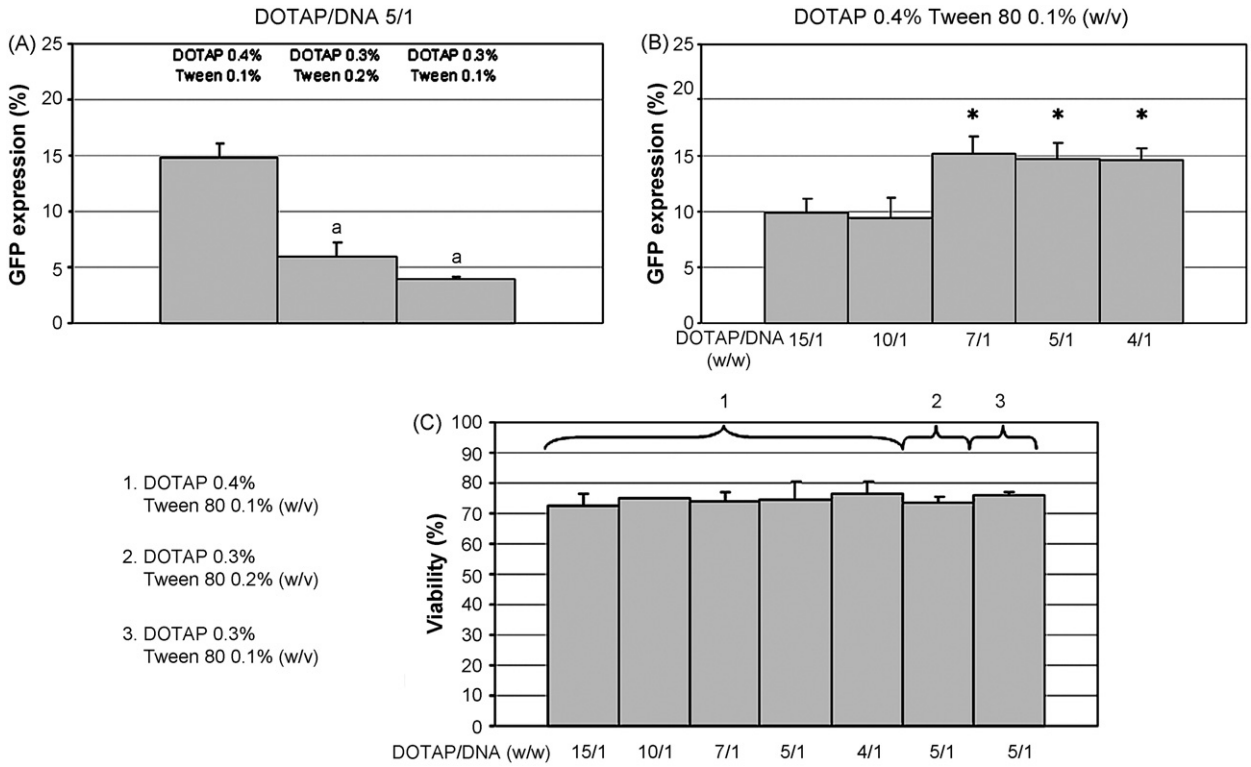

Fig. 4. Transfection activity and cell viability of the different formulations assayed: (A) influence of SLNs composition on transfection activity; (B) influence of DOTAP/DNA ratio on transfection activity; (C) percentage of alive cells after the treatment with the different formulations. [a]$p < 0.05$ against to DOTAP 0.4%, Tween 80 0.1%. *$p < 0.05$ against to DOTAP/DNA ratios 15/1 and 10/1.

presence of bands on lanes 3–8 demonstrated that SLN–DNA complexes prepared at DOTAP/DNA ratios from 15/1 to 4/1 were able to protect DNA. DOTAP/DNA ratios equal or below 3/1 failed to prevent nuclease digestion of the DNA (lanes 9–11).

The agarose gel electrophoresis also allowed us to detect the presence of different DNA topology forms after the treatment with DNase I. Non-treated free DNA (lane 1), which was not in contact with the DNase, presented only two bands. The lower band corresponds to the supercoiled form (SC) and the upper band to the open circular form (OC). When the SLN–DNA complexes were treated with DNase I another intermediate band appeared, which corresponds to the linear form (L).

3.4. Transfection "in vitro"

The highest transfection activity (Fig. 4A) was obtained with SLNs composed by DOTAP 0.4% and Tween 80 0.1%: 14.8% cells produced green fluorescent protein (GFP). This transfection level was statistically higher ($p < 0.05$) than the percentage of transfected cells obtained with the SLNs composed by DOTAP 0.3% and Tween 80 0.2% or DOTAP 0.3% and Tween 80 0.1% (5.9% and 4.0% transfected cells, respectively). No statistically significant differences appeared between these two formulations ($p > 0.05$). Fig. 5A shows green fluorescence due to the transfection of cells with SLN–DNA complexes composed by DOTAP 0.4% and Tween 80 0.1%, at DOTAP/DNA ratio 5/1, and Fig. 5B the correspondent phase contrast image.

In order to study the influence of the ratio SLN/DNA on the transfection ability we chose the formulation composed by DOTAP 0.4% and Tween 80 0.1% because it was the formulation with the highest transfection capacity. DOTAP/DNA ratios employed were 15/1, 10/1, 7/1, 5/1 and 4/1.

As can be seen in Fig. 4B, the complexes prepared at the highest ratios (15/1 and 10/1) provided smaller transfection levels ($p < 0.05$), around 9.5% GFP-positive cells, than the complexes prepared using ratios 7/1, 5/1 and 4/1, which transfected about 15% of the cells in culture. No significant differences were detected between these three formulations ($p > 0.05$).

3.5. Cytotoxicity of the different formulations on HEK293 cell culture

In order to evaluate the effect of the formulations on cell viability, the percentage of dead cells was determined by flow cytometry. Fig. 4C presents these results. The viability was near 75% for both the cells treated with transfection systems and the non-treated cells ($p > 0.05$).

After 1-month storage of the SLNs at 4 °C the transfection levels and the cell viability obtained with these systems were maintained (data not shown).

4. Discussion

In spite of the advantages of SLNs over liposomes, mainly in relation to their stability and manufacture processes, very few

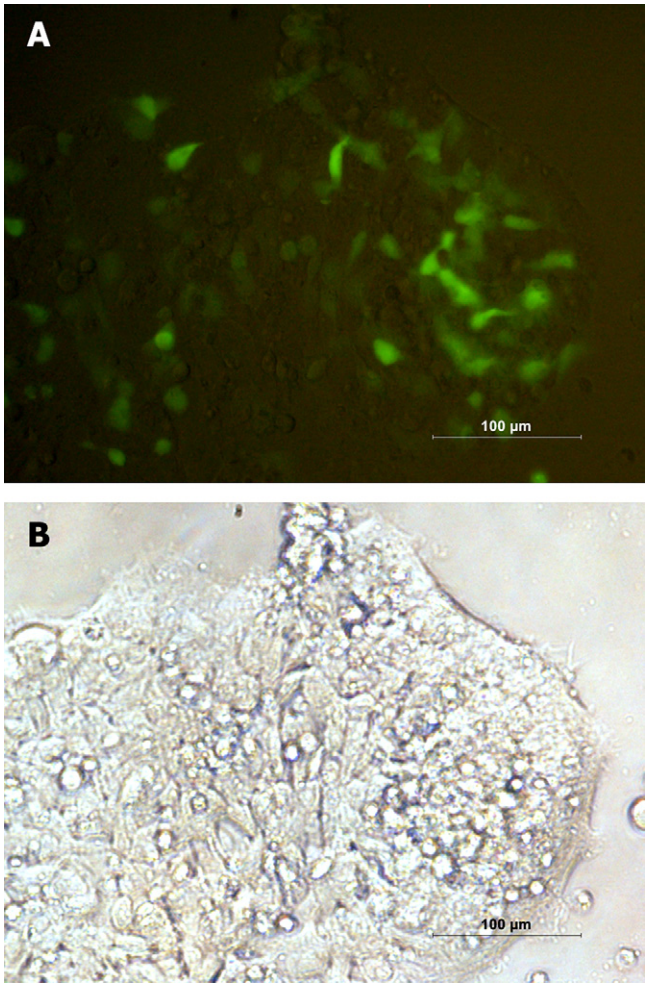

Fig. 5. GFP-positive cells obtained with SLN–DNA complexes composed by DOTAP 0.4% and Tween 80 0.1%, at DOTAP/DNA ratio 5/1 (w/w) observed by inverted fluorescent microscopy (20× objective): (A) fluorescence image; (B) phase contrast image.

reports about the use of SLNs in gene delivery (Olbrich et al., 2001; Tabatt et al., 2004a,b; Rudolph et al., 2004; Pedersen et al., 2006) have been published.

We have prepared nanoparticles with Precirol® ATO 5, DOTAP and Tween 80, and we evaluated their transfection capacity. Furthermore, we evaluated the influence of the DOTAP/DNA ratio on the transfection activity of the SLN–DNA complexes.

Cationic lipids are needed for the preparation of SLNs used on gene therapy because of their surfactant activity and their positive charge. The surfactant activity is necessary to obtain the initial emulsion, and the positive charge to provide the superficial charge to the SLNs. The positive superficial charge of the SLNs is needed for their further interaction with negative charged DNA to form SLN–DNA complexes. However, cationic lipids can be toxic on repeated use and can induce inflammatory reactions "in vivo" (Han et al., 2000). We prepared SLNs with one of the most commonly used cationic lipids in gene therapy, DOTAP. The minimum proportion necessary to form a stable emulsion was 1%. Due to the risk of toxicity of cationic tensioactives we decreased the proportion of DOTAP in the formulations thanks to the addition of Tween 80. As we mentioned above this tensioactive has flattering properties for gene therapy in terms of formulation and transfection activity.

Once the SLNs were synthesized, we proceeded to prepare the SLN–DNA complexes. As can be seen in Fig. 1 the spherical shape was maintained. However, the size of the complexes (Table 2) was smaller than the size of the correspondent SLNs when DOTAP/DNA ratios from 15/1 to 4/1 were used (339 nm versus approximately 250 nm). The reduction in size is due to the DNA compactation. At the minor ratios (3/1, 2.5/1 and 1/1) the size increased because the SLNs lost the DNA binding and condensation capacity. We have observed that the degree of DNA condensation increases when increasing the cationic lipid/DNA ratio. Similar results have been obtained by Mahato et al. (1995), Ferrari et al. (1998) and Faneca et al. (2002).

The zeta potential (Table 2) was highly positive, between +30 and +40 mV, for the highest DOTAP/DNA ratios (from 15/1 to 4/1), but for the minor ratios (3/1, 2.5/1 and 1/1) the zeta potential became negative. The smaller the DOTAP/DNA ratio is, the lower DNA condensation is provided by the complexes, which allows for the exposure of the DNA-negative charges. For gene therapy the positive charge of the non-viral systems is advantageous because it facilitates the interaction with the negative charged cell surface and the cell entry. Elouahabi and Ruysschaert (2005) have postulated that this positive charge could also improve the entry in the cell facilitating the invagination of the cell plasma membrane and inducing the early steps of the endocytosis process. Clathrin-mediated endocytosis is the main entry process for the cationic lipid formulations (Rejman et al., 2005), but fusion is also possible. We have prepared formulations with positive superficial charge, which improves endocytic pathways because, when the systems enter by fusion, part of the DNA can be released to the medium and not to the cytoplasm (Elouahabi et al., 2003).

The binding efficiency assay showed that DOTAP/DNA ratios 4/1 or higher are necessary to bind DNA completely. When DOTAP/DNA ratios from 3/1 to 1/1 were employed the binding efficiency decreased and it was reduced to almost half at DOTAP/DNA ratio 1/1.

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. In order to study the protection capacity of the SLNs, we put SLN–DNA complexes prepared at different DOTAP/DNA ratios in contact with DNase I during 30 min at 37 °C, and we analyzed the integrity of the DNA by agarose gel electrophoresis (Fig. 3). Results showed that SLNs were able to protect DNA only at DOTAP/DNA ratios 4/1 or higher. However, the intensity of the bands decreased as the DOTAP/DNA ratio decreased, which indicates that protection of the genes from DNAses depends on condensation degree. When the condensation decreases DNA exposition to components in medium increases, and the digestion by enzymes is easier.

Once the formulations possessed the technologically suitable characteristics for the desired application, the next step was their evaluation "in vitro".

On the basis of the results mentioned above, for transfection assays we worked with DOTAP/DNA ratios from 15/1 to 4/1: those which bound all DNA provided high positive surface charge and protected DNA from enzyme degradation.

Regarding the transfection activity of the different SLN–DNA formulations, those composed by the highest proportion of DOTAP, 0.4% (w/v), provided the highest transfection levels (Fig. 4A), whereas the transfection obtained from those containing a lower proportion of DOTAP, 0.3% (w/v), were statistically smaller, independently of the amount of Tween 80. Although the transfection activity "in vitro" was influenced by the cationic lipid proportion, and not by the Tween 80 concentration, this non-ionic surfactant determined the size and morphology of SLNs because it was necessary to form a stable and homogeneous emulsion employing a lower DOTAP amount, and thus, reduce the formulation toxicity.

The transfection efficiency of the SLNs was also influenced by the DOTAP/DNA ratio. At ratios 15/1 and 10/1 the transfection levels were significantly smaller than those obtained with the ratios 7/1, 5/1 and 4/1 (Fig. 4B). These differences could be explained by the condensation degree of the DNA. Condensation is necessary to facilitate the mobility of DNA molecules, which is limited by their large size. Furthermore, it is interesting that formulations are able to bind and condensate DNA, providing positive charged complexes which protect DNA from external agents. However, if condensation is excessive, the DNA release from the complexes is more difficult and transfection could be limited. In fact, the release of DNA from the complexes may be one of the most crucial steps determining the optimal ratio for cationic lipid system-mediated transfection (Sakurai et al., 2000). In order to have an indication about the release of DNA from our SLNs based complexes, we quantified free DNA in the culture medium of cells after 4 h in contact with the complexes. For ratios 15/1, 10/1 and 7/1 about 16% of the DNA incorporated into the complexes and added to the culture cell was free in the medium, compared to 37% detected at the minor ratios (5/1 and 4/1). These results fit in with those observed in Fig. 3, where the different bands intensity could suggest that DNA condensation is higher at ratios 15/1 to 7/1 than at 5/1 and 4/1.

Faneca et al. (2002) studied the transfection capacity of cationic liposomes at different charge ratios (+/−) and obtained lower gene expression levels with complexes prepared at charges ratio 8/1 (+/−) as compared to 4/1 (+/−) complexes. They attributed that difference to the excessive condensation at charge ratio 8/1 (+/−), which made the dissociation of DNA from the liposomes difficult. Our results coincide with those reported in their work.

Another important aspect to be considered for the design of this kind of formulations is DNA topology. DNA can feature three forms: supercoiled (SC), open circular (OC) and linear (L). SC-DNA has been reported in the literature to be the most bioactive form (Middaugh et al., 1998; Remaut et al., 2006). Fig. 3, obtained from the DNase I protection study, shows the presence of the L-form after the treatment of the complexes with that enzyme. DNase I turns the SC-DNA, which is the DNA topology with the most transfection capacity, into OC by cutting one of the DNA double strands, and in a successive cut, it breaks the OC- to the L-DNA (Sanders et al., 2006). Thus, as the formulations with higher DOTAP/DNA ratio are the ones that better protect the DNA from DNases, they might be the most convenient for transfection from a point of view of DNA topology.

Therefore, DNA condensation is a crucial factor which determines the transfection capacity of SLNs, because it influences the superficial charge of the complexes and thus cell entry, DNA delivery from nanoparticles, gene protection from DNAses and hence DNA topology. An optimal DNA condensation must be achieved when designing non-viral vectors. Complexes must have enough DNA condensation capacity to create an equilibrium between those three factors to obtain good transfection levels.

We have also shown that SLN-complexes composed by Precirol® ATO 5, DOTAP and Tween 80 do not decrease cell viability and present good stability properties after the storage of the SLNs 1 month at 4 °C (data not shown).

In conclusion, this study shows the potential of solid lipid nanoparticles (SLNs) as non-viral vectors for gene therapy and the main factors which can determine the efficacy of these systems. The "in vitro" transfection levels provided by the formulations developed are conditioned mainly by their DNA condensation capacity. There must be an equilibrium between the gene protection degree, the binding forces of DNA to SLNs, and the DNA topology. This equilibrium is determined by cationic lipid/DNA ratio and it must be optimized with every new formulation.

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References

Elouahabi, A., Thiry, M., Pector, V., Ruysschaert, J.M., Vandenbranden, M., 2003. Calorimetry of cationic liposome–DNA complex and intracellular visualization of the complexes. Meth. Enzymol. 373, 312–332.

Elouahabi, A., Ruysschaert, J.M., 2005. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol. Ther. 11, 336–347.

Faneca, H., Simoes, S., de Lima, M.C.P., 2002. Evaluation of lipid-based reagents to mediate intracellular gene delivery. Biochim. Biophys. Acta 1567, 23–33.

Ferrari, M.E., Nguyen, C.M., Zelphati, O., Tsai, Y.L., Felgner, P.L., 1998. Analytical methods for the characterization of cationic lipid nucleic acid complexes. Hum. Gene Ther. 9, 341–351.

Freitas, C., Müller, R.H., 1999. Correlation between long-term stability of solid lipid nanoparticles (SLN™) and crystallinity of the lipid phase. Eur. J. Pharm. Biopharm. 47, 125–132.

Han, S.O., Mahato, R.I., Sung, Y.K., Kim, S.W., 2000. Development of biomaterials for gene therapy. Mol. Ther. 2, 302–317.

Harvie, P., Wong Frances, M.P., Bally Marcel, B., 2006. Use of poly(ethylene glycol)–lipid conjugates to regulate the surface attributes and transfection activity of lipid–DNA particles. J. Pharm. Sci. 89, 652–663.

Hyndman, L., Lemoine, J.L., Huang, L., Porteous, D.J., Boyd, A.C., Nan, X.S., 2004. HIV-1 Tat protein transduction domain peptide facilitates gene transfer in combination with cationic liposomes. J. Control. Rel. 99, 435–444.

Liu, F., Yang, J., Huang, L., Liu, D., 1996a. Effect of non-ionic surfactants on the formation of DNA/emulsion complexes and emulsion-mediated gene transfer. Pharm. Res. 13, 1642–1646.

Liu, F., Yang, J., Huang, L., Liu, D., 1996b. New cationic lipid formulations for gene transfer. Pharm. Res. 13, 1856–1860.

Mahato, R.I., Kawabata, K., Nomura, T., Takakura, Y., Hashida, M., 1995. Physicochemical and pharmacokinetic characteristics of plasmid DNA cationic liposome complexes. J. Pharm. Sci. 84, 1267–1271.

Mehnert, W., Mader, K., 2001. Solid lipid nanoparticles—production, characterization and applications. Adv. Drug Deliv. Rev. 47, 165–196.

Meyer, O., Kirpotin, D., Hong, K.L., Sternberg, B., Park, J.W., Woodle, M.C., Papahadjopoulos, D., 1998. Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. J. Biol. Chem. 273, 15621–15627.

Middaugh, C.R., Evans, R.K., Montgomery, D.L., Casimiro, D.R., 1998. Analysis of plasmid DNA from a pharmaceutical perspective. J. Pharm. Sci. 87, 130–146.

Olbrich, C., Bakowsky, U., Lehr, C.M., Muller, R.H., Kneuer, C., 2001. Cationic solid–lipid nanoparticles can efficiently bind and transfect plasmid DNA. J. Control. Rel. 77, 345–355.

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, 155–162.

Rejman, J., Bragonzi, A., Conese, M., 2005. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol. Ther. 12, 468–474.

Remaut, K., Sanders, N.N., Fayazpour, F., Demeester, J., De Smedt, S.C., 2006. Influence of plasmid DNA topology on the transfection properties of dotap/dope lipoplexes. J. Control. Rel. 115, 335–343.

Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., Müller, R., 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, 1662–1669.

Sakurai, F., Inoue, R., Nishino, Y., Okuda, A., Matsumoto, O., Taga, T., Yamashita, F., Takakura, Y., Hashida, M., 2000. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. J. Control. Rel. 66, 255–269.

Sanders, N.N., De Smedt, S.C., Demeester, J., 2006. In: McGrath, B.M., Walsh, G. (Eds.), Therapeutic Enzymes. Taylor & Francis Group, Boca Raton, pp. 97–116.

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. Control. Rel. 97, 321–332.

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, 155–162.

Tokunaga, M., Hazemoto, N., Yotsuyanagi, T., 2004. Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes. Int. J. Pharm. 269, 71–80.