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Research paper

Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: The importance of the entry pathway

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ABSTRACT

The aim of our study was to evaluate the effect of protamine on the transfection capacity of solid lipid nanoparticles (SLNs) by correlating it to the internalization mechanisms and intracellular trafficking of the vectors. Vectors were prepared with SLN, DNA, and protamine. ARPE-19 and HEK-293 cells were used for the evaluation of the formulations. Protamine induced a 6-fold increase in the transfection of SLNs in retinal cells due to the presence of nuclear localization signals (NLS), its protection capacity, and a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis. However, protamine produced an almost complete inhibition of transfection in HEK-293 cells. In spite of the high DNA condensation capacity of protamine and its content in NLS, this does not always lead to an improvement in cell transfection since it may impair some of the limiting steps of the transfection processes.

1. Introduction

Gene therapy, which involves systems for the targeted delivery of DNA into cells, has a great potential for treating a large number of diseases [1–4]. Non-viral vectors are safer, cheaper, and more reproducible than viral vectors and have no limitation in the size of DNA they can transport, but their transfection efficacy is still low [5]. Therefore, the enhancement of non-viral vectors is a priority in the field of gene therapy, in order to manage effective and safe vectors.

Non-viral vectors based on cationic lipids are being extensively studied [6–9], and among lipidic systems, solid lipid nanoparticles (SLNs) have become a promising strategy as gene delivery systems. In previous studies, we showed the capacity of SLNs for transfection "in vitro" [10,5] and "in vivo" [11], and from the point of view of application, SLNs have good stability and are subject to be lyophilized [12], which facilities the industrial production.

Transfection efficacy is conditioned by the entry and posterior intracellular trafficking of gene delivery vectors. These processes are cell line dependent, but there are steps common to all cells [13]: binding to cell surface by electrostatic interactions between the positively charged systems and the negative charges of the cell membrane, entry into the cell by following a particular pathway [14,15], release of DNA into the cytoplasm and crossing the nuclear envelope to reach the cellular machinery for protein synthesis. The entry into the nucleus is, in general, quite difficult, as the nuclear membrane is a selective barrier to molecules longer than 40 kDa, and plasmids surpass that size. There are two mechanisms those molecules can use to overcome that barrier: the disruption of the nuclear membrane during mitosis, which is conditioned by the division rate of the targeted cells, or the import through the nuclear pore complex (NPC). This latter mechanism requires nuclear localization signals (NLS), which can be used to improve transfection by non-viral vectors [16].

NLS peptides have sequences rich in basic amino acids (lysine or arginine) that promote active nuclear transport through the NPC, mediated by importin α–importin β interactions [17]. Peptides with arginine residues in their sequences have shown translocation activity, and those containing six or eight consecutive arginine residues exhibited the maximum internalization and accumulation in the nucleus [18,19]. Among these peptides, protamine sulfate is an USP (United States Pharmacopeia) compound isolated from the sperm of mature fish. It condenses DNA and presents sequences of six consecutive arginine residues [20], which make this peptide able to translocate molecules such as DNA from the cytoplasm to the nucleus of living cells. This feature would solve the passage of DNA from the cytoplasm into the nucleus. Moreover, protamine improves intra-nuclear transcription [21].

The present study considers the effect of protamine on the transfection capacity of SLNs composed by the core lipid Precirol® ATO 5, and the surfactants DOTAP and Tween 80 as surface components. Protamine–DNA–SLN complexes were characterized in terms of size, superficial charge, and DNA protection capacity. The mechanism of internalization and the intracellular trafficking

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of the vectors were also studied into cell lines with different division rates and related to the transfection capacity of the vectors.

2. Materials and methods

2.1. Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). 1,2-Dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) was acquired from Avanti Polar Lipids, Inc. Deoxyribonuclease I (DNase I), lauryl sulfate sodium (SDS), Nile Red, and protamine sulfate were purchased from Sigma–Aldrich (Madrid, Spain). Tween 80 was provided by Vencaser (Bilbao, Spain), and dichloromethane by Panreac (Barcelona, Spain).

The plasmid pCMS-EGFP, which encodes the enhanced green fluorescent protein (EGFP), was purchased from BD Biosciences Clontech (Palo Alto, California, US) and amplified by Dro Biosystems S.L. (San Sebastian, Spain). The labeling of the plasmid pCMS-EGFP with ethidium monoazide (EMA) was carried out by Dro Byosystems S.L. (San Sebastian, Spain).

The gel electrophoresis materials were acquired from Bio-Rad (Madrid, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). Antibiotic Normocin™ was acquired from InvivoGen (San Diego, California, US). The BD Viaprobe kit was provided by BD Biosciences (Belgium).

Hoechst 33258, AlexaFluor488-Cholera toxin, and AlexaFluor488-Transferrin were provided by Molecular Probes (Barcelona, Spain), and Fluoromount-G by SouthernBiotech (Coultek, España).

2.2. Production of solid lipid nanoparticles (SLNs)

The SLNs were produced by a solvent emulsification–evaporation technique previously described by del Pozo-Rodríguez et al. [22]. Briefly, Precirol® ATO 5 was dissolved in dichloromethane (5% w/v) and then emulsified by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W in an aqueous phase containing DOTAP (0.4% w/v) and Tween 80 (0.1% w/v). The organic solvent was removed by evaporation, and SLN suspension was formed upon solidification of the Precirol® ATO 5. The SLNs were washed by centrifugation (3000 rpm, 20 min, 3×) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

2.3. Binding of protamine to DNA

pCMS-EGFP plasmid was added to an aqueous solution of protamine at protamine to DNA ratios (w/w) of 0.25:1, 0.5:1, 1:1, 2:1, and 5:1. The resulting complexes were diluted in Milli-Q™ water up to a final concentration of 0.03 μg DNA/μl and then subjected to electrophoresis on a 1% agarose gel (containing ethidium bromide for visualization) for 30 min at 120 V. The bands were observed with an Uvidoc D-55-LCD-20M Auto transilluminator (Uvitec).

2.4. Preparation of vectors

DNA–SLN vectors were obtained by mixing the pCMS-EGFP plasmid with an aqueous suspension of SLNs. The SLN to DNA ratio, expressed as the ratio of DNA to DOTAP (w/w), was fixed at 1:5.

Protamine–DNA–SLN vectors were prepared by first binding protamine to DNA at the ratios (w:w:w) 0.5:1:5, 1:1:5, 2:1:5, 3:1:5, and 5:1:5 under agitation for 30 min. Then protamine–DNA complexes were put in contact for 15 min with a suspension of previously prepared SLNs, and electrostatic interactions between complexes and SLNs led to the formation of vectors. These vectors have protamine–DNA complexes adsorbed on the nanoparticle surface.

2.5. Size and zeta potential measurements

Sizes of SLNs, DNA–SLN (1:5), and protamine–DNA–SLN (2:1:5) vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials were measured by laser Doppler velocimetry (LDV). Both measurements were taken on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl (aq.).

2.6. DNase I protection and SDS-induced release of DNA

DNase I was added to DNA–SLN and protamine–DNA–SLN vectors to a final concentration of 1 U DNase I/2.5 μg DNA. The mixtures were then incubated at 37 °C for 30 min. Afterward 1% SDS solution was added to release the DNA from the SLNs. Additionally, the vectors were treated only with SDS to study the ability of DNA to be released from the vectors. The samples were then analyzed by electrophoresis on agarose gel (described above), and the integrity of the DNA in each sample was compared to a control of untreated DNA.

2.7. Cell culture and transfection protocol

In vitro assays were performed with two different cell lines: a human embryonic kidney (HEK-293) cell line and a human retinal pigmented epithelial (ARPE-19) cell line, obtained from the American Type Culture Collection (ATCC).

HEK-293 cells were 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% Normocin™. Cells were incubated at 37 °C with 5% CO_2 in air and subcultured every 2–3 days using trypsin/EDTA. For transfection, HEK-293 cells were seeded on 24-well plates at density of 150,000 per well and allowed to adhere overnight.

ARPE-19 cells were maintained in Dulbecco's modified Eagle's medium-Ham's nutrient mixture F-12 (1:1) medium (D-MEM/F-12) supplemented with 10% heat-inactivated fetal calf serum and 1% Normocin™ antibiotic solution. Cells were incubated at 37 °C under 5% CO_2 atmosphere and subcultured every 2–3 days using trypsin–EDTA. For transfection, ARPE-19 cells were seeded on 12-well plates at a density of 30,000 cells per well and allowed to adhere overnight.

The formulations were diluted in HBS and added to the cell cultures. In all cases, 2.5 μg of DNA was added. The cells were incubated with the vectors at 37 °C, and after 4 h, the medium containing the complexes in the wells was refreshed with 1 ml of complete medium. The cells were then allowed to grow for another 72 h. Transfection efficacy was quantified at 12 h, 24 h, 48 h, 72 h, and 1 week.

As control, a DNA–SLN vector without protamine whose transfection ability was evaluated in previous works [10,22] was also assayed.

2.8. Flow cytometry mediated analysis of transfection efficacy and cell viability

At the end of the incubation period, the cells were washed once with 400 μl of PBS and then detached with 400 μl of 0.05% trypsin–EDTA. The cells were then centrifuged at 1500*g* and then resuspended with PBS and directly introduced into a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, US). For each sample, 10,000 events were collected.

Transfection efficacy was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). For cell viability measurements, the BD Via-Probe kit was employed. The reagent (5 μl) was added to each sample, and after 10 min of incubation, the fluorescence corresponding to dead cells was measured at 650 nm (FL3).

2.9. Cellular uptake of DNA–SLN and protamine–DNA–SLN vectors

Entry of vectors into the cells was studied quantitatively by flow cytometry. For this purpose, pCMS-EGFP was labeled with ethidium monoazide (DNA–EMA), as previously described [10].

Twenty-four hours after the addition of vectors, cells were washed three times with PBS and detached from plates. Cells incorporating either DNA–EMA–SLN or protamine–DNA–EMA–SLN vectors were quantified by flow cytometry at 650 nm (FL3). For each sample, 10,000 events were collected.

2.10. Internalization mechanism

First, endocytosis mechanisms were evaluated in both cell lines by flow cytometry, and the entry of AlexaFluor488-Cholera Toxin and AlexaFluor488-Transferrin, which are markers of caveolae raft-mediated endocytosis and clathrin-mediated endocytosis, respectively, was quantified (FL1).

The endocytic processes involved in the internalization of the non-viral systems were analyzed qualitatively by colocalization studies with both markers. SLNs were labeled with the fluorescent dye Nile Red (λ = 590 nm) according to a previously reported method [23]. Briefly, Nile Red was incorporated into the dichloromethane employed to prepare SLNs by the emulsification–evaporation technique described above. Cells were seeded in coverslips and co-incubated for 2 h with Nile Red-labeled vectors and either AlexaFluor488-Cholera toxin (10 μg/ml) or AlexaFluor488-Transferrin (50 μg/ml). Next, medium was removed, and cells were washed with PBS and fixed with paraformaldehyde 4%, which did not interact with the fluorescence of Nile Red or AlexaFluor488. Preparations were mounted on Fluoromount G, and after air-drying, images were obtained with an Olympus Fluoview FV500 confocal microscope using sequential acquisition to avoid overlapping of fluorescent emission spectra. Confocal laser scanning microscopy study (CLSM) images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV-EHU).

Colocalization results were estimated by means of Manders overlap coefficient (R), where $0.6 \leqslant R \leqslant 1.0$ indicates colocalization (overlap of the signals) [24]. The value of every coefficient was obtained from 15 images.

2.11. Detection of intracellular EMA-labeled DNA by fluorescence microscopy

In order to follow the DNA into the cytoplasm, cells were seeded in culture plates and treated with vectors containing DNA–EMA. Prior to the observation of the samples through the microscope, nuclei were labeled with Hoechst 33258. Images were analyzed with an inverted fluorescent microscopy (model EclipseTE2000-S, Nikon).

2.12. Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. The different formulations were compared with the student's t test, whereby differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Binding of protamine to DNA

The gel in Fig. 1A illustrates that the capacity of protamine to condensate DNA depends on the protamine to DNA ratio. In lanes 1–3 (protamine to DNA ratios from 0.25:1 to 1:1), the intensity of the bands indicates that most DNA was free. However, in lines 4 and 5 (protamine to DNA ratios 2:1 and 5:1, respectively), no band is present in the gel, indicating that all DNA was completely bound to protamine.

3.2. Characterization

3.2.1. Particle size and zeta potential of the formulations

When protamine–DNA (ratio 2:1) complexes were bound to SLNs, protamine did not induce any significant change in surface charge or particle size of SLNs, being the measures in the region of +35 mV (SD = 2.40) and 250 nm (SD = 43.20), with normal distribution and polydispersion index lower than 0.4. The labeling of vectors with Nile Red or EMA did not induce changes in the particle size or zeta potential ($p > 0.05$).

3.2.2. DNase I protection and SDS-induced release of DNA

DNA formulated in DNA–SLN or protamine–DNA–SLN was protected from nuclease action (gel electrophoresis in Fig. 1B). The addition of protamine to the formulations increased the DNA protection capacity of the DNA–SLN formulation. Fig. 1C shows that after treatment with SDS, DNA release is more difficult as protamine to DNA ratio increases.

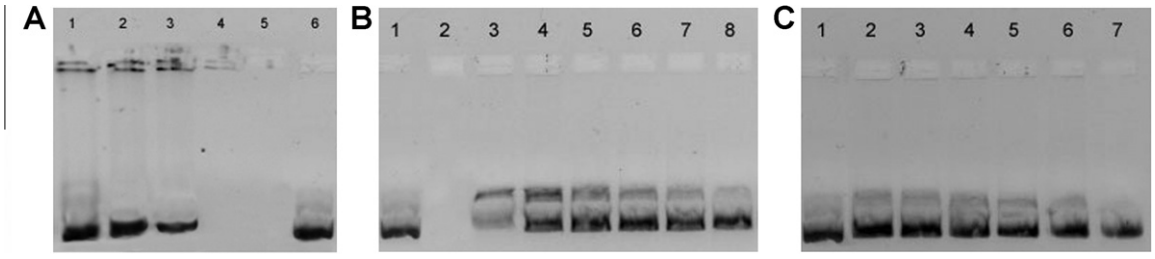

Fig. 1. (A) Binding efficiency of DNA with protamine at different protamine–DNA ratios (w/w) studied by agarose gel electrophoresis. Lane 1 = 0.25:1; lane 2 = 0.5:1; lane 3 = 1:1; lane 4 = 2:1; lane 5 = 5:1; and lane 6 = free DNA. (B) Protection of DNA by DNA–SLN and protamine–DNA–SLN vectors from DNase digestion at different protamine–DNA–SLN ratios (w/w/w) visualized by agarose gel electrophoresis. Vectors were treated with DNase I. Lane 1 = non-treated free DNA; lane 2 = DNase-treated free DNA; lane 3 = DNA–SLN vectors; lane 4 = 0.5:1:5; lane 5 = 1:1:5; lane 6 = 2:1:5; lane 7 = 3:1:5; lane 8 = 5:1:5. (C). Release of DNA by DNA–SLN and protamine–DNA–SLN at different protamine–DNA–SLN ratios (w/w/w) visualized by agarose gel electrophoresis. Vectors were treated with SDS. Lane 1 = free DNA; lane 2 = SLN–DNA vectors; lane 3 = 0.5:1:5; lane 4 = 1:1:5; lane 5 = 2:1:5; lane 6 = 3:1:5; lane 7 = 5:1:5.

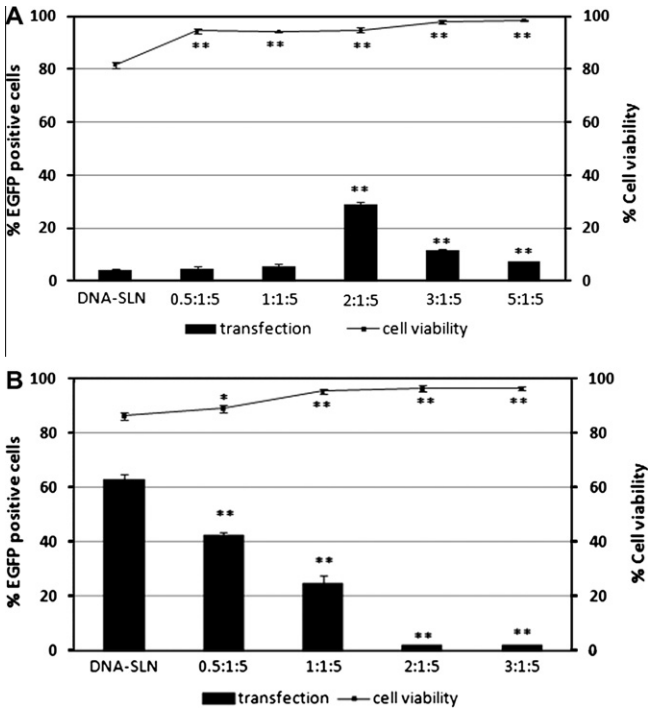

Fig. 2. Transfection (bars) and cell viability (line) for each formulation assayed in ARPE-19 cells (A) and HEK-293 cells (B) 72 h after the addition of vectors; the SLN to DNA ratio (w/w) was 5:1 in all cases, and the protamine to DNA ratio varied from 0.5:1 to 5:1. Error bars represent SD ($n = 3$). $^{*}p < 0.05$ respect to SLN–DNA formulation. $^{**}p < 0.01$ respect to DNA–SLN formulation.

3.3. Transfection and cell viability studies in ARPE-19 cells and HEK-293 cells

Prior to the evaluation of the efficacy of protamine-containing vectors, we showed that protamine–DNA complexes were not able to transfect cells. As observed in Fig. 2A (bars), transfection levels in ARPE-19 cells depend on the protamine content, reaching the highest level (29% EGFP positive cells) with the protamine–DNA–SLN formulation prepared at 2:1:5 ratio ($p < 0.01$). However, vectors without protamine (DNA–SLN) produced only 5% transfected cells. Cell viability (lines in Fig. 2A) was maintained around 95% with all the formulations bearing protamine, with a significantly higher viability ($p < 0.01$) than that in cells treated with DNA–SLN vectors (80% viability).

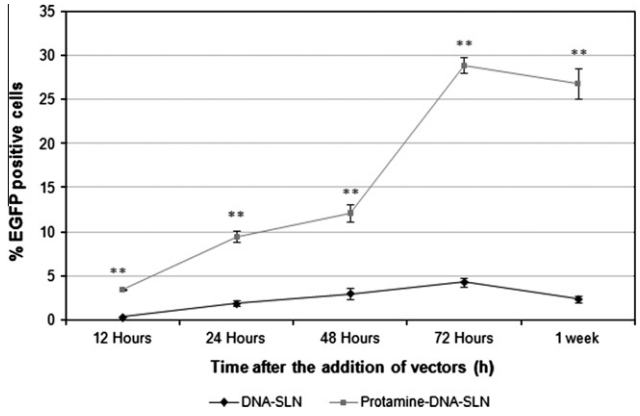

Fig. 3. Transfection of non-viral vectors in ARPE-19 cells over time. The SLN to DNA ratio (w/w) was 5:1 and the protamine to DNA ratio 2:1. Error bars represent SD ($n = 3$). $^{**}p < 0.01$ respect to DNA–SLN formulation.

Contrary to ARPE-19 cells, the highest transfection level in HEK-293 cells was achieved with the formulation prepared without protamine (Fig. 2B). The incorporation of this peptide into the vectors caused a significant decrease ($p < 0.01$) in the transfection levels, although cell viability (line in Fig. 2B) was higher with the formulations containing protamine ($p < 0.01$).

Fig. 3 shows transfection of vectors over time in ARPE-19 cells. Transfection levels were significantly higher ($p < 0.01$) with the formulation containing protamine. Seven days after the addition of vectors, transfection did not increase in HEK-293 cells with any of the formulations (data not shown).

3.4. Cellular uptake of non-viral vectors

The presence of protamine at ratio protamine:DNA 2:1 in the vectors decreased the entry into HEK-293 cells from 75% to 45% DNA–EMA positive cells; however, protamine did not modify the cellular uptake of vectors in ARPE-19 cells (77% vs. 72% DNA–EMA positive cells, with DNA–SLN 1:5 and protamine–DNA–SLN 2:1:5, respectively).

3.5. Internalization mechanism

Fig. 4 features the flow cytometry results and confocal images of ARPE-19 cell after the treatment with the endocytosis markers and with the vectors (DNA–SLN and protamine–DNA–SLN at a ratio of 2:1:5).

Colocalization with both transferrin and cholera toxin was observed with the two vectors (with and without protamine), indicating that both clathrin and caveolae were involved in the internalization process. However, transferrin colocalization obtained when the cells are treated with the formulation containing protamine ($R = 0.641 \pm 0.039$) is higher than colocalization achieved by the formulation without protamine ($R = 0.424 \pm 0.124$).

Fig. 5 features the entry of endocytosis markers and the colocalization study in HEK-293 cells. The cholera toxin showed a significant colocalization with the formulation elaborated without protamine ($R = 0.618 \pm 0.035$); when protamine was incorporated, colocalization with the cholera toxin decreased ($R = 0.491 \pm 0.046$). The low levels of transferrin compared to cholera toxin levels indicate that this cell line uses barely endocytosis via clathrin.

3.6. Intracellular distribution of EMA-labeled DNA in culture cells

Fig. 6 presents images captured by fluorescence microscopy from 4 h to 72 h after the addition of the vectors. When ARPE-19 cells were treated with DNA–SLN vectors, red fluorescence appeared homogeneously distributed in the cytoplasm, but DNA was much more condensed and closer to the nucleus with protamine–DNA–SLN vectors. However, in HEK-293 cells, DNA appeared condensed and close to the nucleus with both vectors.

4. Discussion

The incorporation of NLS into non-viral vectors has been studied by several authors, and it has shown improvement in transfection due to an effect on the nuclear membrane [25–27]. Protamine is a peptide widely used to improve lipofection due to its content in NLS. The enhancement of transfection efficacy with protamine is attributed to nuclear localization and stabilization against DNase degradation [28]. However, there is very little information describing its role in lipofection [29]. It is well known that transfection efficiency of nanoparticular vectors is conditioned by cell

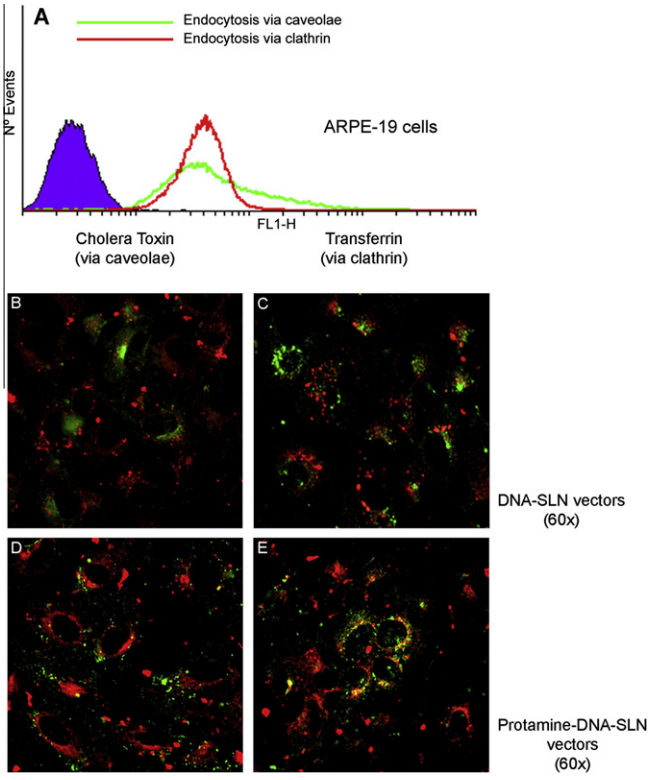

Fig. 4. Presence of AlexaFluor 488-Cholera toxin and AlexaFluor 488-Transferrin quantified by flow cytometry (A) and CLSM images of Nile Red-labeled DNA–SLN vectors (B–C) and protamine–DNA–SLN vectors (D and E) in ARPE-19 cells, with AlexaFluor 488-Cholera toxin (green) at 10 μg/ml, (B and D); or with AlexaFluor 488-Transferrin (green) at 50 μg/ml, (C and E). SLN to DNA ratio (w/w) was 5:1, and protamine to DNA ratio was 2:1.

line-dependent factors such as division rate, internalization pathway, and intracellular trafficking [30,31]. Thus, in this work, we evaluated the transfection efficiency of protamine–DNA–SLN vectors and its correlation with those factors by using two different cell lines: ARPE-19 and HEK-293 cells.

Our results indicated that entry through clathrin-mediated endocytosis seems to be necessary in order to achieve transfection when protamine–DNA complexes are incorporated into the SLNs. In spite of the high DNA condensation capacity of protamine and its content in NLS, this does not always lead to an improvement in cell transfection; although NLS may have a positive effect in the entry to the nucleus, they may impair the previous steps due to the multistage nature of the transfection process. Therefore, the influence of any new component involved on each step of transfection process should always be studied.

As expected, the capacity of protamine to condensate DNA depends on the protamine to DNA ratio. Fig. 1A shows that a ratio of at least 2:1 is needed to bind all DNA. Gel electrophoresis (Fig. 1B) also shows that the presence of protamine increased the DNA protection capacity of the DNA–SLN complexes. Another important issue to be considered is the ability of the nanoparticles to deliver the DNA. Fig. 1C shows that the higher the amount of protamine in the vectors, the more difficult the release of plasmid is. The formulation prepared at a protamine:DNA:SLN ratio of 2:1:5 possesses the suitable characteristics to transfect because this formulation increases the protection capacity of the DNA–SLN vector without hampering the plasmid release. Furthermore, the inclusion of protamine modified neither particle size of the vectors nor their zeta potential, and all formulations prepared had a particle size around 250 nm (SD = 43.20) and a zeta potential about +35 mV (SD = 2.40). Modification of particle size depends on the balance between the ability of the peptide to precondense DNA, which would imply a reduction in size, while demanding greater space itself, which would cause an increase in size. Thus, a change in particle size does not always happen. In a previous study we carried out with SLN containing other cationic peptide [5], the peptide did not modify the surface charge of the nanoparticles. This could be due the limitations associated to the photon correlation to measured zeta potential [32]. Moreover, changes in the amount of charges may not always imply modification in the disposition of charges at the surface of the nanoparticles.

Once the vectors were characterized, the transfection capacity and cell viability in ARPE-19 and HEK-293 cells were studied. ARPE-19 cells divide slowly and are a good "in vitro" model to evaluate transfection systems targeted to the treatment of retinal diseases. On the contrary, HEK-293 cells divide rapidly and are usually employed for "in vitro" transfection studies. In a previous work, we have shown different transfection capacity of SLNs to transfect ARPE-19 and HEK-293 cells [10], achieving significant higher transfection levels in HEK-293 cultures. In a later work [5], we suggested that the passage to the nucleus may be the main limitation for transfection of retinal pigment epithelial cells.

The highest ARPE-19 positive level (Fig. 2A) was reached with a 2:1 protamine to DNA ratio (29% cells transfected), according to the condensation, protection against DNase, and delivery studies. On the one hand, at lower protamine to DNA ratios, all the basic amino acids of protamine may be consumed in the condensation of DNA, which results in the limitation of the recognition of NLS [21]. On the other hand, if the protamine to DNA ratio is too high, DNA may be too condensed, and its release is more difficult. It should be noted that the increase in the transfection level in

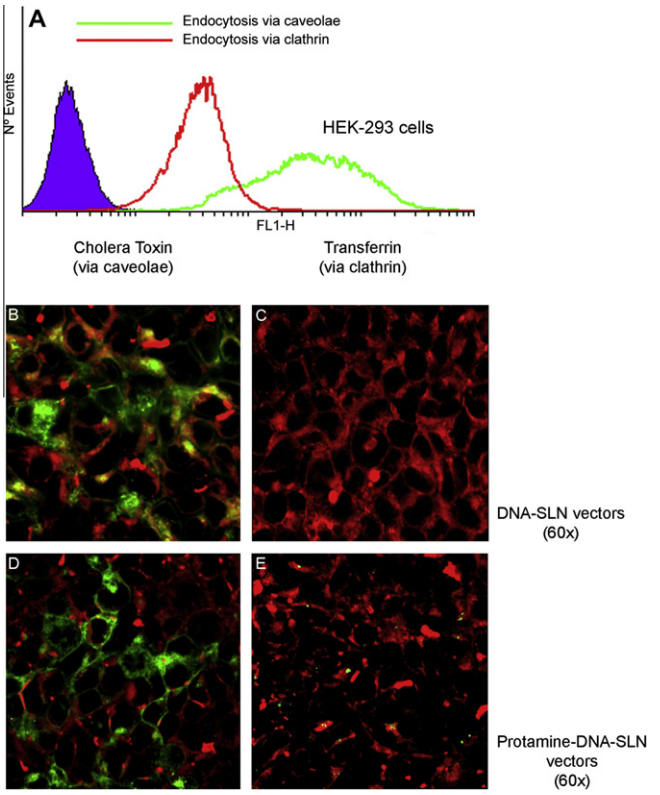

Fig. 5. Presence of AlexaFluor 488-Cholera toxin and AlexaFluor 488-Transferrin quantified by flow cytometry (A) and CLSM images of Nile Red-labeled DNA–SLN vectors (B and C) and protamine–DNA–SLN vectors (D and E) in HEK-293 cells, with AlexaFluor 488-Cholera toxin (green) at 10 μg/ml (B and D); or with AlexaFluor 488-Transferrin (green) at 50 μg/ml (C and E). SLN to DNA ratio (w/w) was 5:1, and protamine to DNA ratio was 2:1.

ARPE-19 due to protamine (from 5% to 29% transfected cells) must be considered a relevant achievement, since difficulties for transfecting this cell line are well known [33]. Until now, no other paper in the literature has shown a similar transfection level in ARPE-19 cells with non-viral vectors. These results confirm the importance of optimizing the proportion of components in the design of formulations.

Unexpectedly, the incorporation of protamine into our vectors decreased transfection in HEK-293 cells (from 60% to 2% transfected cells) in a protamine concentration manner (Fig. 2B). Taking into account that this cell line shows a high division rate, nuclear entry should not be a limiting step in transfection. That is why we expected that the inclusion of protamine in the vectors might not affect or increase transfection in these cells. Our results suggest that protamine may also affect other critical limiting steps in the transfection process. Therefore, the whole process involved in transfection should be considered, in order to properly explain the mechanism of protamine action, and not only the nuclear effect.

Other authors have also used protamine with lipid formulations at different protamine:DNA ratios, and as in our study, protamine not always produced an increase in transfection. In these studies, the effect of protamine was not related to the internalization mechanism and intracellular trafficking of the vectors [34,35].

In ARPE-19 cultures, the percentage of cells that captured vectors did not change ($p > 0.05$) with the presence of protamine in the formulations (77% for DNA–SLN vs. 72% for protamine–DNA–SLN at ratio w/w/w 2:1:5); this lack of correlation between cell internalization and transfection in cell lines with low division rate has been described by other authors [36]. In HEK-293 cells, protamine brought about a decrease in cell uptake from 75% to 45% when the formulation prepared at protamine to DNA ratio of 2:1 was studied. As in ARPE-19 cells, no correlation between uptake and transfection was found, since a decrease of 30% in cell uptake caused an almost complete inhibition of transfection.

Endocytosis has been postulated as the main entry mechanism for non-viral systems. Various endocytosis mechanisms have been described to date: phagocytosis, pinocytosis, clathrin-mediated and caveolae/raft-mediated. Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes, which degrade their content, whereas caveolae/raft-mediated endocytosis avoids the lysosomal pathway and its consequent vector degradation.

In ARPE-19 cells, clathrin-mediated endocytosis and caveolae-mediated endocytosis are present in a similar extent. Both mechanisms are involved in the internalization process of our vectors. However, the protamine-containing vectors presented a higher colocalization with transferrin ($R = 0.641 \pm 0.039$ for protamine–DNA–SLN vectors vs. $R = 0.424 \pm 0.124$ for DNA–SLN vectors), which indicates that protamine induces a shift in the internalization mechanism from caveolae/raft to clathrin endocytosis. Since the internalization pathway conditions later steps, we investigated the intracellular disposition of vectors prepared with EMA-labeled DNA. We observed that DNA was highly condensed and near the nucleus after the treatment of both cell lines with the formulation containing protamine (Fig. 6). However, when the cells were treated with DNA–SLN (without protamine), the plasmid was broadly distributed in the cytoplasm. In ARPE-19 cells, which show a slow division rate, an active transport of DNA through the nuclear pores is necessary; otherwise the DNA stays longer in the cytoplasm. Protamine is an excellent DNA condenser that reduces the exposition to degradation by different cytoplasmic agents such as DNases and helps the transport of DNA to the nucleus. This justified the high level of transfection with the formulation containing

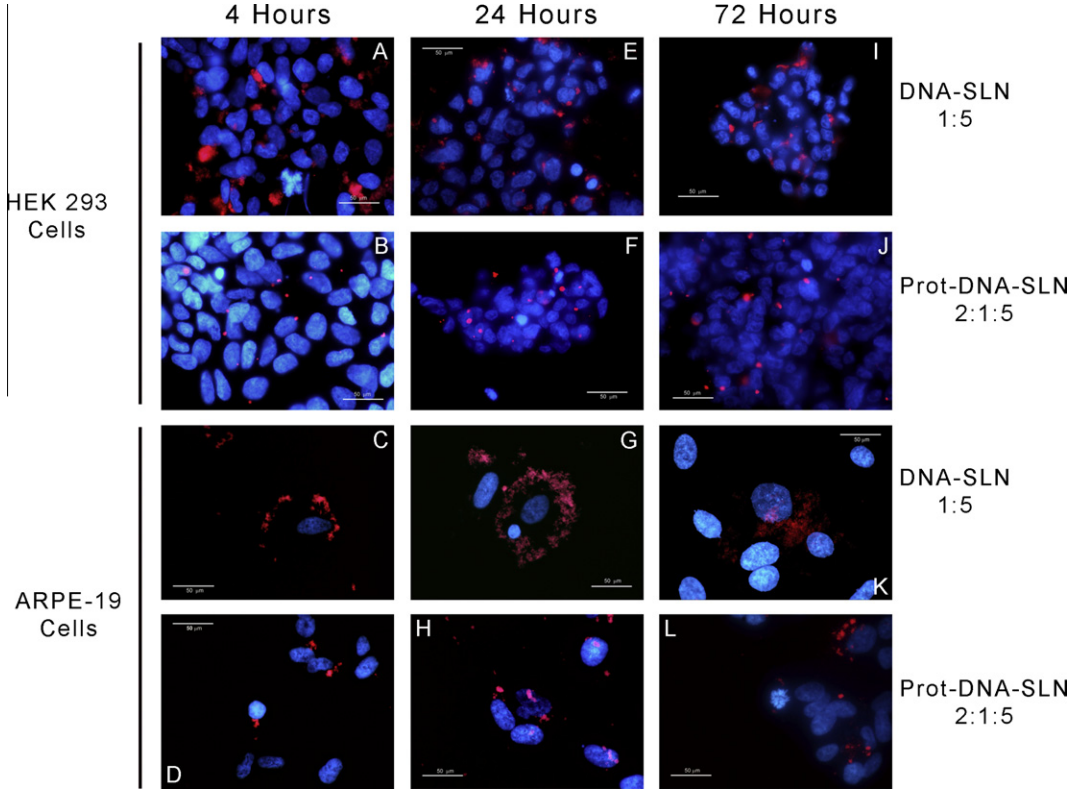

Fig. 6. Fluorescence microscopy images of HEK-293 cells and ARPE-19 cells at different times after the addition of DNA–SLN vectors or protamine–DNA–SLN vectors. Cells were treated with Hoechst 33258 in order to detect the nucleus (blue) and vectors containing EMA-labeled pCMS-EGFP plasmid (red). The SLN to DNA ratio (w/w) was 5:1, and the protamine to DNA ratio 2:1.

protamine. Additionally, it has been shown that DNA condensed by protamine is more accessible to intra-nuclear transcription [21].

In HEK-293 cells, the evaluation of endocytosis markers by flow cytometry indicated that endocytosis via caveolae is much more active than via clathrin (Fig. 5). The images obtained by confocal microscopy also showed that the DNA–SLN vectors mainly enter by caveolae/raft-dependent endocytosis. The vectors containing protamine also used caveolae/raft-dependent endocytosis but to a lesser extent as suggest the colocalization coefficients ($R = 0.491 \pm 0.046$ for protamine–DNA–SLN vectors vs. $R = 0.618 \pm 0.035$ for DNA–SLN vectors). When vectors enter the cells by caveolae/raft-dependent endocytosis, there is a lack of lysosomal activity and the vectors are localized around the nucleus. Fig. 6 shows that DNA presents the same localization and the same condensation degree with both vectors. The lack of lysosomal activity makes difficult the release of DNA from the solid lipid nanoparticles.

Since lipid particles are physiologically degraded, we followed transfection during 7 days to discard that delivery of the DNA in the cytoplasm might be dependent on time. Seven days after the addition of vectors, transfection levels did not increase in any of the two cell lines.

In a previous study carried out by our group [5], we improved cell transfection of ARPE-19 by using a cell penetration peptide (SAP) with solid lipid nanoparticles. This peptide presents lower capacity of condensation and protection of DNA than protamine, thus via caveolae is its optimum endocytic mechanism since it localizes the vectors around the nucleus and lysosomal activity is reduced. With protamine, endocytosis via clathrin is necessary for transfection because of the lysosomal activity that facilitates the release of the complex protamine–DNA to reach the nucleus. Depending on the composition of the vector, the most appropriate internalization mechanism may be modulated. Therefore, the knowledge of the cellular entry of gene delivery systems is a crucial step in the field of gene therapy. Nevertheless, developing studies to understand the process of endocytosis and intracellular trafficking of gene carriers "in vivo" is still the real challenge for the next years of gene delivery [37].

5. Conclusions

In summary, protamine induced a 6-fold increase in the transfection capacity of SLNs in retinal cells due to a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis, which promotes the release of the protamine–DNA complexes from the SLNs; afterward the transport of the complexes into the nucleus is favoured by the NLS of the protamine. On the contrary, protamine diminished transfection in HEK-293 cells, due to the decrease in the cell uptake of the vectors and to the difficulty of the protamine–DNA complexes to be released from the nanoparticles in the absence of lysosomal activity, which is characteristic of via caveolae. This study reinforces the importance of the knowledge of the internalization mechanism and intracellular trafficking processes, which can be modulated by optimizing the formulations to better design more efficient non-viral vectors.

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