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Cationic vesicles based on non-ionic surfactant and synthetic aminolipids mediate delivery of antisense oligonucleotides into mammalian cells

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Dedicated to the Memory of Dr. Francisco Sánchez-Baeza and Dr. Nuria Azemar

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Abstract

A formulation based on a synthetic aminolipid containing a double-tailed with two saturated alkyl chains along with a non-ionic surfactant polysorbate-80 has been used to form lipoplexes with an antisense oligonucleotide capable of inhibiting the expression of *Renilla* luciferase mRNA. The resultant lipoplexes were characterized in terms of morphology, zeta potential, average size, stability and electrophoretic shift assay. The lipoplexes did not show any cytotoxicity in cell culture up to 150 mM concentration. The gene inhibition studies demonstrated that synthetic cationic vesicles based on non-ionic surfactant and the appropriate aminolipid play an important role in enhancing cellular uptake of antisense oligonucleotides obtaining promising results and efficiencies comparable to commercially available cationic lipids in cultured mammalian cells. Based on these results, this amino lipid moiety could be considered as starting point for the synthesis of novel cationic lipids to obtain potential non-viral carriers for antisense and RNA interference therapies.

Keywords

Cationic lipids, cationic vesicles, non-ionic surfactant vesicles, antisense oligonucleotide, antisense therapy, gene delivery

1. Introduction

The discovery of antisense technology and more recently RNA interference has allowed new strategies in the search of novel therapeutics by controlling gene expression [1]. These approaches incorporate different action mechanisms than those used in conventional therapies. For this reason the use of nucleic acids may provide enormous benefits for therapy since they inhibit target proteins drugs with high specificity and also become potential units in the treatment of genetic disordered diseases or even in cancer [2].

However, there are many obstacles in developing nucleic acids into therapeutics since they are polyanionic macromolecules. Fortunately, chemical modifications to nucleic acid backbones and/or sugars have accelerated the discovery process of new compounds in addition to improving the properties of nucleic acids in terms of stability against nucleases [3] and decreasing off-targets effects [4] without losing their initial biological activities. Nevertheless, delivery problems continue to be the major bottleneck in the development of nucleic acids as drugs.

Although viral vectors like retroviruses or adenoviruses have shown high transfection efficiencies and have been used in some clinical trials [5] there are concerns about the immunogenicity or the recombination of oncogenes that have still not been solved. Alternatively, non-viral vectors such as lipids [6], cell-penetrating peptides [7], polymers [8] or gold nanoparticles [9] have emerged as promising alternatives to safely delivering nucleic acids.

There are two strategies used for transfecting nucleic acids with non-viral vectors. The first one is the use of formulations [10] which are the simplest and the fastest way to bind non-viral vectors to nucleic acids by taking advantage of the electrostatic interactions between them. The second strategy is the use of covalent approaches in which non-viral carriers and nucleic acids are covalently linked obtaining stable nucleic acid conjugates which improve their biological properties in both *in vitro* and *in vivo* [11, 12].

There have been great advances made in the last few years in the search for both nucleic acid conjugates as well as formulations for generating active complexes. In addition, there has been renewed interest in the development of new, more efficient and less toxic formulations for nucleic acid delivery.

Since the first transfection experiments carried out by Felgner [13] demonstrated an efficient lipid mediated DNA-transfection by using DOTMA as a cationic lipid, a variety of synthetic cationic lipids have been widely used in formulation in order to deliver therapeutic biomolecules which are becoming promising non-viral tools for nucleic acid delivery [14]. One of the factors that must be considered when using cationic lipids is the tendency of positively charged particles to interact with plasma proteins which induce aggregation and consequently produce low transfection efficiencies in gene delivery [15]. The reduction of the net cationic charge of cationic lipids in formulation, the presence of either serum-resistant cationic lipids [16], an increase of lipid/DNA charge ratios [17], PEGylation [18] and finally the addition of helper lipids into formulations [19] are essential modifications which minimize undesirable effects like cell toxicity in cells and avoiding rapid plasma clearance. Despite efforts made in the development of new formulations or designing novel cationic lipids, obtaining effective non-viral carriers remains crucial for optimal gene transfection.

The use of surfactant agents in colloidal carrier systems might mask or reduce the undesirable effects of cationic lipids. In addition, surfactants may also play an important role in gene delivery [20, 21] because the resulting complexes show a high stability. Moreover, their synthesis is readily scalable and the structure is comparable to liposomes. However, there are few studies that have analyzed the effects of such surfactants agents in gene delivery processes [22, 23, 24]. In an effort to develop new formulations based on non-ionic surfactant vesicles, we have recently reported the use of a novel formulation which is composed of a mixture of non-ionic surfactant polysorbate-80 and a synthetic aminolipid containing a double-tailed hydrocarbonated alkyl chain. This demonstrated the ability to efficiently deliver plasmid DNA

into the retina with good efficiency and low toxicity [25]. In addition, the use of cationic niosome formulations based on the same cationic aminolipid moiety, polysorbate-80 and squalene were also condensed using plasmid DNA which obtained the corresponding complexes and mediated delivery in several cell lines with high efficiencies [26]. These results prompted us to further investigate the versatility of the aforementioned surfactant formulations in order to encapsulate oligonucleotides and evaluate their effectiveness as a drug delivery system in antisense therapy.

There are few reports in the literature describing the encapsulation of oligonucleotides with non-ionic surfactant vesicles [27] which use (in the majority of formulations) a mixture of commercially available cationic lipids which deliver oligonucleotides into cells. Herein, we describe a formulation based on of non-ionic synthetic surfactant vesicles which contain a modified synthetic cationic lipid and an antisense oligonucleotide (ASO) which is designed to knockdown the expression of a *Renilla* Luciferase gene.

The resulting lipoplexes were fully characterized in terms of zeta potential, average size, stability and electrophoretic shift assay. Finally, the best compositions were used to study the potential toxicity and transfection processes of antisense oligonucleotides mediated by surfactant vesicles. These results were compared to transfections that were carried out using commercially available cationic lipids.

2. Materials and Methods

2.1 Materials

All reagents employed in this work were used as received, having an analytical grade and used without further purification. Polysorbate-80 (Tween-80) and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) were purchased from Sigma-Aldrich. Antisense

phosphorothioate oligonucleotide (sequence 5ô-CGT TTC CTT TGT TCT GGA-3ô) complementary to the mRNA of the *Renilla* luciferase gene targeted to a predominant accessible site between 20 and 40 nt was purchased from Proligo (Sigma-Aldrich). A 18-mer scrambled antisense oligonucleotide sequence (sequence 5ô- CTG TCT GAC GTT CTT TGT-3ô) was synthesized in-house and purified according to well-established methods (DMTon-based protocols). All the standard phosphoramidites and ancillary reagents used for oligonucleotide synthesis were purchased from Applied Biosystems or Link Technologies. The synthetic aminolipid, 2,3-di(tetradecyloxy)propan-1-amine was obtained as described in the literature [28]. Lipofectamine 2000 was purchased from Invitrogen. PBS buffer and Dulbeccoôs Modified Eagleôs Medium (DMEM) which was supplemented with a 10% heat-inactive fetal serum bovine (FBS) along with distilled water (DNAse/RNAse free) were purchased from Gibco. Additional nuclease-free water was also prepared by using 0.1% of diethylpyrocarbonate (DEPC) to ensure the removal of RNase contamination, autoclaved and filtered before using. Luciferase assay kits were purchased from Promega. Qiagen Giga plasmid purification kit was purchased from Qiagen.

2.2 Preparation of synthetic non-ionic surfactant vesicles with an antisense oligonucleotide (ASO) containing lipoplexes

Non-ionic synthetic surfactant vesicles were prepared using a hydration method with equimolecular amounts of both the synthetic aminolipid and the non-ionic surfactant polysorbate-80. Specifically, equimolecular amounts containing the cationic lipid and polysorbate-80 (6.40 µmol) were dissolved in 1 mL of chloroform. The solvent was evaporated and the resulting crude was kept under vacuum overnight at room temperature. The dried lipid film was hydrated with 1 mL of sterile HEPES (20 nM; pH 7.4) buffer, filtered previous to use through a 0.2 µm membrane filter, and heated to 60 ºC for 20 minutes. The dispersion was vortexed and sonicated for 3 minutes before being used.

The dispersion was resuspended in HEPES buffer at stock concentrations of 0.484 mM or 828 µM vortexed and was sonicated (3 minutes). Cationic surfactant vesicle/antisense oligonucleotide complexes (lipoplexes) were then formed by adding the required amount of cationic lipid dispersion to aliquots containing fixed amounts of either antisense oligonucleotide targeting the *Renilla* luciferase mRNA expression or a scrambled oligonucleotide at 14:1 or 16:1 charge ratios ($[\text{cationic amino groups}]_{\text{cationic lipid}}/[\text{anionic phosphate groups}]_{\text{nucleic acid}}$, respectively. The resultant lipoplexes were vortexed and sonicated for 2 minutes and finally incubated at 37 ºC for 30 minutes.

2.3 Morphology of lipoplexes

The morphology of the resulted lipoplexes was assessed by Transmission Electron Microscopy (TEM). Briefly, 5 εl of the sample were adhered onto glow discharged carbon coated grids for 60 s. The remaining liquid was removed by blotting on a paper filter and stained with 2% uranyl acetate for 60 seconds. Samples were observed under the microscope, TECNAI G2 20 TWIN (FEI, Eindhoven, The Netherlands), operating at an accelerating voltage of 200 KeV in a bright-field image mode. Digital images were acquired with an Olympus SIS Morada digital camera.

2.4 Electrophoretic mobility shift assay

Antisense oligonucleotide (0.5 µM) was mixed with increasing concentrations of the cationic surfactant dispersion giving rise to cationic vesicle/antisense oligonucleotide molar ratios ranging from 2 to 20. Lipoplexes were analyzed using electrophoresis on a 20% polyacrylamide gel at 150 V for 5 hours in TBE buffer 1X. Pictures were taken using Fujifilm LAS-1000 Intelligent Dark Box II using IR LAS-1000 Lite v1.2

2.5 Zeta potential

Zeta potential values were obtained by laser doppler velocimetry by using a Zetasizer Nano ZS (Malvern Instruments) equipped with a He-Ne red light laser (λ = 633 nm). All measurement parameters and the sample preparation (0.5 µM of antisense oligonucleotide in a final volume of 50 µL) were carried out according to the experimental procedure described before. Studies in the presence of fetal serum bovine (FBS) were carried out by adding 5 µL of FBS to the lipoplex solutions (50 µL) followed by 5 minutes of incubation. In both cases, the resulting lipoplex solutions were diluted with mili-Q water to a final volume of 1 mL. Data is shown as the average value of three runs.

2.6 Size measurement and physical stability of vesicles

The particle size of cationic vesicles and cationic vesicle/antisense oligonucleotide lipoplexes were determined by using a dynamic light scattering (DLS) spectrometer (LS Instruments, 3D cross correlation multiple-scattering) equipped with a He-Ne laser (632.8 nm) with variable intensity. Cationic surfactant vesicles were stored at 4 ºC and stability studies of cationic surfactant vesicle/antisense oligonucleotide complexes were carried out by analyzing the average size of the particles at room temperature by dynamic light scattering for one month. In both cases, cationic vesicles and lipoplexes were previously sonicated and measurements were taken at a scattering angle of 90º, in triplicates, without diluting samples and at a constant temperature of 25ºC. The particle radius was calculated by fitting of the first cumulant parameter.

2.7 Cell culture

HeLa cells were cultured at 37ºC, 5% CO_2 in DMEM partially supplemented with 10% fetal bovine serum, 100 mg/mL penicillin and 100 µg/mL streptomycin. Cells were regularly passaged in order to maintain exponential growth. Twenty-four hours before transfection at 40 to 80% confluency, cells were trypsinized and diluted 1:5 with a fresh medium without

antibiotics (about 1-3 x 10^5 cells/mL) and transferred to a 24-well plate (500 µL per well). Two luciferase plasmids, *Renilla* luciferase (pRL-TK) and Firefly luciferase (pGL3) from Promega, were used as a reporter and as a control, respectively. *Renilla* and Firefly luciferase vectors (0.1 µg and 1.0 µg per well, respectively) were transfected into the cells using Lipofectamine 2000. Cells were incubated with the plasmids for 5 hours. The medium was discarded and cells were washed with PBS twice (500 µL). Then, 500 µL of fresh DMEM without antibiotics were added to each well. Two transfection experiments were carried out using either DMEM without FBS or DMEM supplemented with 10% of FBS. Then antisense oligonucleotides were prepared at concentrations of 20, 60, 100 and 150 nM, respectively. Lipoplexes (300 µL) (which were prepared according to the procedure described before), were previously incubated for 30 min at 37°C using HEPES buffer (20mM, pH 7.4). Later, 100 εL of antisense oligonucleotide and scrambled lipoplexes were added to each well. Cell lysates were prepared and analyzed twenty-four hours after transfection using the Dual-Luciferase Reporter Assay System according to the manufacturerôs protocol. Lumminiscence was measured using SpectraMax M5 spectrophotometer.

2.8 Analysis of cell viability by the MTT colorimetric assay

HeLa cells viability in the presence of both cationic vesicles and lipoplexes were tested at different concentrations (20, 60, 100 and 150 nM, respectively) using the MTT colorimetric assay. For each assay, cells were seeded (about 6 x 10^3 cells per well) on a 96-well plate in 200 µL of DMEM and cultured for 24h. Later, the culture medium was discarded and both vesicles and lipoplexes were added at gradually larger concentrations between 20 and 150 nM. Cells were incubated for 4h at 37ºC under 5% CO_2 atmosphere. The media was discarded and additional DMEM (200 µL) was added. Cells were incubated for 15 hours at 37°C. The MTT reagent was added at a final concentration of 0.5 mg/mL per (25 µL) and incubated for two additional hours at 37°C. Finally, the medium was absorbed, DMSO (200 µL) and was added to

dissolve formazan crystals (15 minutes of stirring at room temperature) and absorbance (λ = 570 nm) was measured. Absorbance was measured using SpectraMax M5 spectrophotometer.

3. Results

3.1 Morphology of the lipoplexes

TEM pictures showed that lipoplexes at 14/1 N/P ratio exhibited a discrete spherical morphology and did not aggregate under our experimental conditions (Fig. 1). Particle size of resulting lipoplexes was around 200 nm.

3.2 Zeta potential measurements and oligonucleotide binding capacity measurements

Cationic surfactant vesicle/antisense oligonucleotide lipoplexes were characterized in terms of Zeta potential and oligonucleotide binding capacity. Non-ionic surfactant synthetic vesicles composed of a modified amino lipid with a double-tailed hydrocarbonated alkyl chain and polysorbate-80 were used at several ratios ranging from 1 to 20 and using a fixed amount of antisense oligonucleotide (0.5 μM) in order to determine the optimal charge ratio to form lipoplexes in serum-free conditions. As depicted in Fig. 2A, the Zeta potential displayed negative values at the lowest N/P charge ratios (-20 mV for a N/P charge ratio of 2) due to the presence of the negatively charged antisense oligonucleotide. Clearly, as the concentration of cationic vesicles increased, the tendency of Zeta potential changed by decreasing this negative character in the system and thereby obtained positive values in a range of +12 mV to +20 mV indicating an electrostatic stabilization level. The effect of fetal bovine serum (FBS) in the cationic vesicles was also evaluated (Fig. 2A). As expected, all charge ratios that were tested displayed similar behaviors in the presence of FBS. They obtained negative Zeta potential values close to -18 mV, except for charge ratios of 14 and 16, in which there was a slight increase that reached values of the Zeta potential between -14 and -12 mV, respectively.

These results clearly indicate that all charge ratios were serum-dependent in which negatively charged particles of serum were absorbed onto the surface of lipoplexes. Moreover, these results may suggest that the exact influence of serum might be dependent on the charge ratio of the cationic vesicles. Alternatively, a native electrophoretic gel assay of lipoplexes was also carried out in order to confirm at which N/P ratio oligonucleotides are complexed with our cationic surfactant vesicles. As illustrated in Fig. 2B, free DNA and complexes can be separated by native polyacrylamide gel electrophoresis. Free ASO oligonucleotide is observed in N/P charge ratios from 0 to 8. When ASO oligonucleotide is complexed with the cationic vesicles, the resulting complexes do not enter the gel because of the size restriction. The ASO oligonucleotide was retained in wells from N/P charge ratios 10 to 18 indicating complete complexation.

3.3 Particle size and physical stability of cationic vesicles

Besides the characterization of lipoplexes by superficial charge and the evaluation of their binding capacity to antisense oligonucleotides, dynamic light scattering (DLS) was also used to measure the average particle size of cationic surfactant vesicle/antisense oligonucleotide complexes at charge N/P ratios of 14 and 16 along with the stability evaluation of the cationic surfactant complexes stored at 4ºC for one month. Physical characterization of cationic surfactant vesicle/antisense oligonucleotide complexes in terms of Zeta potential and average size is displayed in Table 1. The average particle size of both N/P ratios was almost identical (ranging from 324 and 334 nm for charge ratios of 14 and 16, respectively). As illustrated in Fig. 3, the average size of the complex remained practically constant for one month, according to DLS measurements.

3.4 Cell culture

3.4.1 Transfection activity in serum-free conditions

We first evaluated the transfection efficiencies at several N/P ratios (12, 14, 16, 18 and 20-fold, respectively) of our cationic surfactant vesicle/antisense oligonucleotide which formed complexes in serum-free medium conditions in order to determine which charge ratio would be more effective at carrying out gene transfection experiments, as shown in Fig. 4. All selected N/P ratios were able to silence gene expression in a range of 50 to 70% of inhibition at 60 nM. In particular, we observed good efficiencies for charge ratios of 12, 14 16 and 18 obtaining similar silencing activities (63, 70, 69 and 68% respectively) whereas lower transfection efficiencies were observed at a charge ratio of 20 which reached only 51% of luciferase inhibition. Therefore, we chose selected charge ratios of 14 and 16 to evaluate the effect of both cationic surfactant vesicles and antisense oligonucleotide forming complexes on cell viability and gene transfection, respectively.

3.4.2 Cytotoxicity assay

Cell viability and cytotoxicity studies were carried out (previous to cell transfection experiments) by incubating HeLa cells with cationic surfactant vesicle dispersion and lipoplexes at both charge ratios of 14 and 16, respectively. They contained different concentrations of antisense oligonucleotide (20, 60, 100 and 150 nM, respectively) in the presence of DMEM supplemented with serum (10%) by using a tetrazolium-based colorimetric assay [29]. As depicted in Fig. 5, no significant toxicity was observed in cells compared with control samples in the presence of either cationic surfactant vesicles or lipoplexes formed with a charge ratio of 14, which obtained viabilities around 90% in all tested concentrations. HeLa cells in the presence of lipoplexes formed at a N/P charge ratio of 16 displayed a concentration-dependent toxicity which did not show any cytotoxicity at low concentrations (around 90 and 80% of cell viability at 20 and 60 nM, respectively) whereas the aforementioned lipoplexes were detrimental at high concentrations (100 and 150 nM) to cell viability (40%) probably due to an increase of polysorbate-80 content in the composition of the surfactant dispersion. Cell viability was also compared to positive controls by using Lipofectamine (Figure S2; Supplementary

Part). Lipoplexes at a charge ratio of 14 along with cells in the presence of Lipofectamine did not show any cytotoxicity at the tested concentrations. Consequently, all further studies in cell culture were therefore performed at a charge ratio of 14.

3.4.3 In vitro transfection: Evaluation of the antisense activity

The efficiency in the transfection of cationic surfactant vesicle/antisense oligonucleotide complexes at the optimal charge ratio of 14 was carried out at several concentrations (20, 60, 100 and 150 nM, respectively) targeting the *Renilla* luciferase mRNA expression in the absence of a commercially available cationic lipids. We observed that complexes were able to mediate cellular uptake and hence exhibited high efficiencies in gene transfection at both 100 and 150 nM (83% and 87% for 100 and 150 nM, respectively). Lipoplexes were also able to silence luciferase expression at 20 and 60 nM (4% and 60% for 20 and 60 nM, respectively). A phosphorothioate scrambled sequence forming complexes with cationic vesicles at the same charge ratio of 14 had no effect on luciferase expression at the same tested concentrations thereby indicating the specificity of the gene knockdown experiment (Fig. 6).

The transfection efficiencies mediated by our cationic surfactant vesicles dispersion at their optimal N/P ratio were compared to commercially available cationic lipids like Lipofectamine, as a positive control, at concentrations of 20, 60, 100 and 150 nM. As illustrated in Fig. 7, gene transfections carried out in the presence of Lipofectamine were more efficient only at 60 nM (84% *versus* 60% for Lipofectamine and cationic surfactant vesicles, respectively). However, both systems reached a *plateau* at higher concentrations (100 and 150 nM), and hence obtained similar gene knockdown activities (approximately 85% of luciferase inhibition).

3.4.4 Effect of the fetal bovine serum (FBS) on transfection experiments

In order to evaluate the effect of serum on cellular uptake, we studied the efficiency of cationic vesicle/antisense oligonucleotide complexes at a charge ratio of 14 to mediate cellular uptake at several FBS concentrations (0, 10, 40 and 60%, respectively) at 60 nM. The results depicted in Fig. 8 showed a clear reduction in the transfection efficiency at high FBS concentrations although cellular uptake was not totally inhibited (23% for FBS at 60%). In contrast, while the use of lower FBS concentrations (10%) promoted similar transfection efficiencies than in the absence of serum (from 70% to 60% of gene knockdown activity), cellular uptake experiments carried out with FBS at 40% caused a 2-fold decrease in luciferase inhibition activity (36% reduction) in comparison with the maximal efficiency achieved in serum-free conditions. These results demonstrated the dependency of cationic lipoplexes at their optimal N/P ratio in the presence of high serum concentration and their efficiencies on cellular transfection.

4. Discussion

The emergence of new therapies such as antisense technology or more recently RNA interference has allowed therapeutic oligonucleotides to be seen as promising units for fighting against several diseases because of their high specificity in the inhibition of target proteins. However, it is also well-known that delivery of nucleic acids is the major challenge because they have to cross several cellular barriers including plasma membrane. Low cellular uptake is the main bottleneck for the development of nucleic acids as therapeutics.

Consequently, the development of new drug delivery systems that may facilitate cellular uptake of nucleic acids is becoming necessary. Herein, we described a novel non-viral carrier based on the use of a formulation made up of a synthetic aminolipid and non-ionic surfactant polysorbate-80 which were able to form vesicles and consequently were able to form complexes with antisense oligonucleotides and mediate delivery in cell culture.

It is well-reported in literature that encapsulating oligonucleotides in lipid vesicles may turn out to be a limiting step in the development of an effective formulation. Although there are several techniques to improve the efficiency in encapsulation of oligonucleotides [30] herein, we used hydration of lipid films as the main approach for forming stable cationic vesicle/oligonucleotide complexes.

To get a direct evidence of the lipoplexes formation, we examined such lipoplexes at 14/1 N/P ratio under TEM at different magnifications Fig. 1. Adding oligonucleotides to our formulation resulted in lipoplexes which did not aggregate to our experimental conditions (Fig 1-A). These lipoplexes adopted spherical morphology, the most favorable structure conformation from an energetic point of view. Lipoplexes size was around 200 nm, slightly smaller than the size reported by dynamic light scattering in Table 1 (320 nm). At high magnification (Fig 1B), small cavities were displayed in the lipoplexes. The heterogeneous distribution of the oligonucleotides during the spontaneously and self-assembly process might cause such cavities.

We characterized which N/P ratio between cationic vesicles and antisense oligonucleotides was the optimal way to form complexes with a range of 0 to 20-fold. The antisense oligonucleotide was partly condensed by the synthetic aminolipid unit after adding 6-fold of cationic dispersion, according to Zeta potential and electrophoretic mobility shift assay experiments. Increasing N/P ratios from 12 to 20, more amino groups were introduced to bind to antisense oligonucleotides and thereby lead to the formation of the expected cationic surfactant vesicle/antisense oligonucleotide complexes with an increase of surface charge reaching a *plateau* at a charge ratio of 20.

One of the existing milestones in gene transfection is the effect induced by negatively charged plasma proteins on the surface of cationic lipoplexes which are prone to aggregation and lead to the failure of cell transfection. Antisense oligonucleotide forming complexes in a selected range of charge ratios from 12 to 20 were evaluated to promote delivery in the absence of fetal bovine

serum in order to know the best N/P ratio for carrying out cell transfection. All tested N/P ratios were able to impart cellular uptake, being charge ratios of 14 and 16 those which obtained the best transfection efficiencies and silencing activities than the rest of the selected N/P ratios.

In order to correctly explain our cellular uptake results, we first evaluated whether the viability of HeLa cells were not affected with the use of our cationic surfactant vesicles as well as lipoplexes using the MTT colorimetric assay. Although some surfactants are seldom used in cell culture due to their toxicity [31], cationic vesicle dispersions along with lipoplexes at the charge ratio of 14 did not induce any cytotoxicity in HeLa cells at the selected concentrations. However, in the case of using lipoplexes at the N/P ratio of 16, lipoplexes did not display any cytoxicity effect at lower concentrations (20 and 60 nM) whereas toxicity of the aforementioned lipoplexes was detrimental at higher concentrations (100 and 150 nM) which had a significant negative effect on cell growth at the same charge ratio of 16. This was probably due to the toxic effect induced by our cationic vesicle formulation and surfactant agent proportions used [31] to form lipoplexes with antisense oligonucleotides.

Cellular uptake experiments mediated by cationic surfactant vesicle/antisense oligonucleotide complexes at their optimal N/P ratio of 14 triggered in a dose-response manner the best gene knockdown activities at 100 and 150 nM. These silencing activities were comparable when commercially available cationic lipids were also used at the same tested concentrations. These results suggest that this transfection properties mediated by our surfactant lipoplexes might be due to structural changes caused in either the morphology or phase evolution of lipoplexes and their possible ability to undergo a lamellar to non-lamellar phase transition [32].

Another factor that can limit the effectiveness of cellular uptake is the presence of negatively charged serum proteins in medium due to the tendency of these particles to interact with the cationic charged surface of vesicles that induce aggregation in the system and deactivate their internalization into cells. This serum-dependence of cationic lipoplexes causes a serious

limitation for *in vivo* applications and hence reduces the efficacy of the non-viral vector in gene therapy. In order to overcome this unwanted effect, authors have described some strategies like the use of serum-resistance amino acid-based cationic lipids [16], the use of PEGylated synthetic surfactant vesicles [22], the addition of helper lipids [19] or PEGylated lipids [33]. All these modifications have shown an improvement in the potency and stability of the formulation and an increase in the cellular uptake carried out by DNA complexes. Transfection experiments mediated by our surfactant cationic vesicle/antisense oligonucleotide complexes (as expected) were dependent as FBS concentration increased. In our hands, the efficacy in the transfection process of cationic particles at the optimal N/P ratio diminished obtaining only 20% of luciferase inhibition when a high concentration of FBS (60%) was used. In the case of using low serum levels (10 or 40%, respectively) better transfection results were achieved, as was expected. These results are in accordance to the literature in which the use of non-ionic surfactant molecules such as sorbitan monoesters (Spans) or polysorbate-80 (Tween-80) to stabilize cationic formulations normally have shown poor efficiency in the presence of a high concentration of serum [34].

There is a general consensus in which endocytosis mechanism has been proposed as the major mode of action of lipoplex-mediated delivery antisense oligonucleotide [35, 36] although other kinds of mechanisms like fusion have been suggested as well [37]. It is also well-accepted that the type of endocytosis can also depend on the particle size [38] and the net positive charged surfaces of non-viral carriers [39] which may interact with the negative charges at the cell surface and consequently release the genetic cargo from endosomes. However, despite all efforts and studies carried out to understand how this cellular entry pathway works for nucleic acid forming complexes, knowledge of their uptake mechanism is still limited [40]. According to our results, the average size of cationic surfactant vesicle/antisense oligonucleotide complexes at their optimal charge ratios of 14 and 16 gave similar sizes of around 320 nm. This result may suggest a receptor-mediated endocytosis through the clarthrin-mediated pathway [41] as the main entering pathway for the aforementioned cationic surfactant lipoplexes by ensuing

membrane destabilization and leading to the release of antisense oligonucleotides and therefore efficiently inhibit gene expression [34].

5. Conclusions

The urge to obtain new vehicles capable of improving the delivery properties of nucleic acids is essential for the development of oligonucleotide-based therapeutics. Cationic liposomes composed of helper lipids like DOPE or cholesterol are normally used for stabilizing complexes with nucleic acids and consequently often increase their transfection potencies. In this article, we used a formulation based on a modified aminolipid containing two saturated alkyl chains and a non-ionic surfactant polysorbate-80 in order to form stable modified cationic vesicles with antisense oligonucleotides. Lipoplexes were characterized in terms of Zeta potential and dynamic light scattering. Furthermore, the ability of these surfactant cationic vesicle/antisense oligonucleotide complexes to impart cellular uptake in cell culture was also evaluated. The results confirmed that lipoplexes at their optimal N/P ratio of 14 did not show any sign of cytotoxicity in cell culture except for lipoplexes with a charge ratio of 16 in which cell viability was compromised. Lipoplexes showed a clear serum-dependence at high serum concentrations however, the use of low serum conditions (10%) on our modified cationic surfactant vesicles promoted comparable results in cellular uptake to those obtained with commercially available cationic lipids. We believe that the presence of the modified amino containing a double-tailed hydrocarbonated lipid chain may be considered as a starting point in order to design novel cationic lipids by modifying some elements such as headgroup or a glycerol backbone and lead to the study the subsequent structure-activity relationship for the development of new gene delivery systems.

Supporting Information

Average size measurements by Dinamic Light Scattering at a charge ratio of 16 and normalized cell viability by MTT assay.

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Fig. 1 TEM images of lipoplexes at 14/1 N/P ratio, original magnification 7.100x (A) and 88.000x (B).

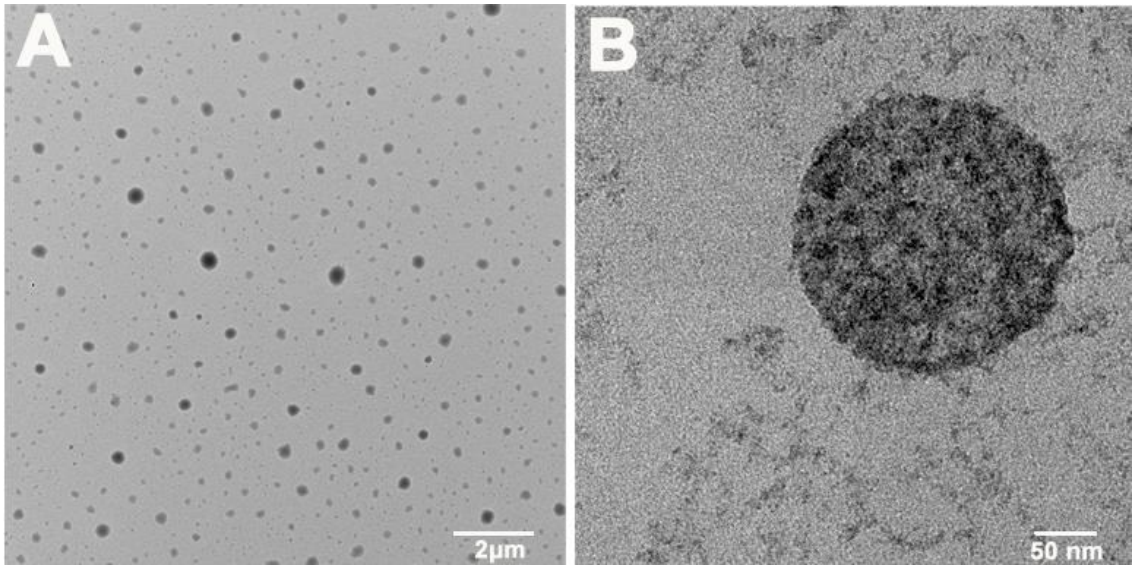
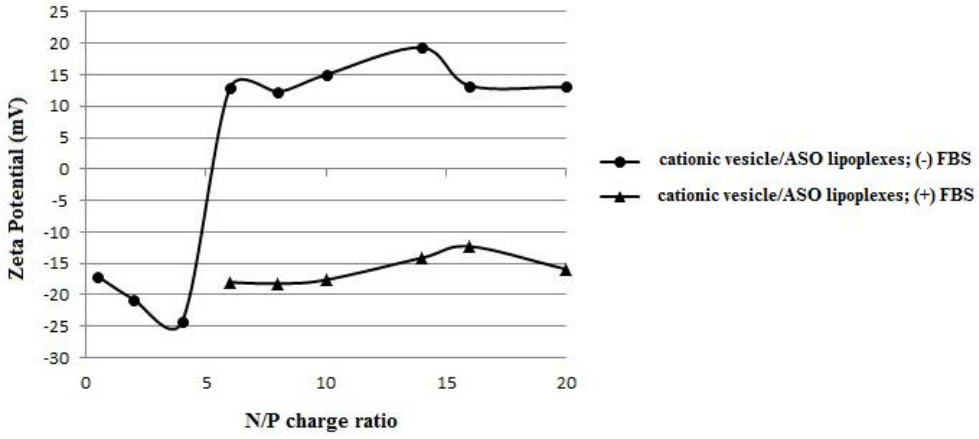


Fig. 2. Zeta potential of cationic vesicle/antisense oligonucleotide (ASO) lipoplexes at several N/P charge ratios in the absence (circles) and the presence (triangles) of fetal bovine serum (FBS) (A) and the characterization of lipoplexes by electrophoretic mobility shift assay (B).

A



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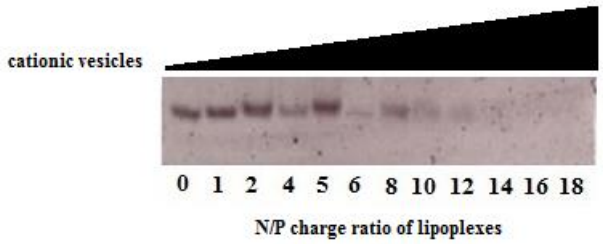

Table 1

Physical characterization (size and Zeta potential) of cationic vesicle lipoplexes containing an antisense oligonucleotide at N/P charge ratios of 14 and 16. Results are means ±S.D. for three independent experiments.

Cationic surfactant vesicle / antisense oligonucleotide complexes		
N/P charge ratio	14	16
size (nm)	324 ±32.0	332±3.0
ɛ potential (mV)	19.3±0.61	13.2±2.0

Fig. 3. Physical stability measurements of cationic vesicle/antisense oligonucleotide (ASO) lipoplexes at a N/P charge ratio of 14 by dynamic light scattering for one month.

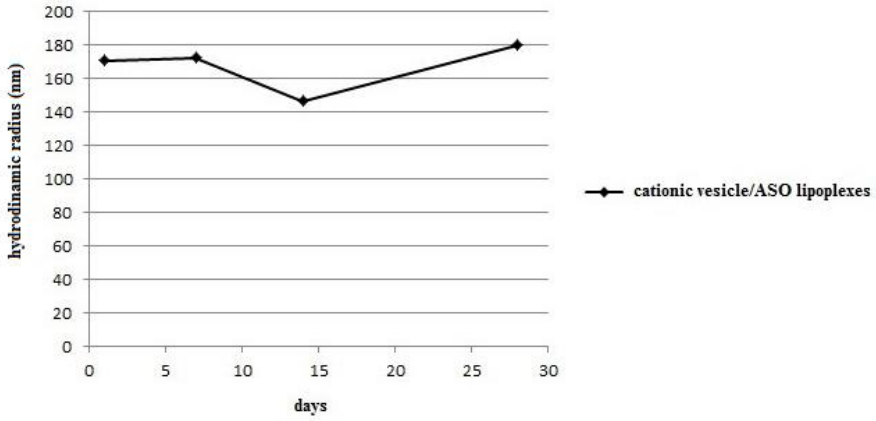

Fig. 4. Normalized transfection efficiencies mediated by cationic vesicle/antisense oligonucleotide lipoplexes targeting *Renilla* luciferase mRNA in serum-free conditions at several charge ratios at 60 nM. Antisense oligonucleotide (ASO) in the absence of non-viral carrier was evaluated as a control. Results are means ±S.D. for three independent experiments.

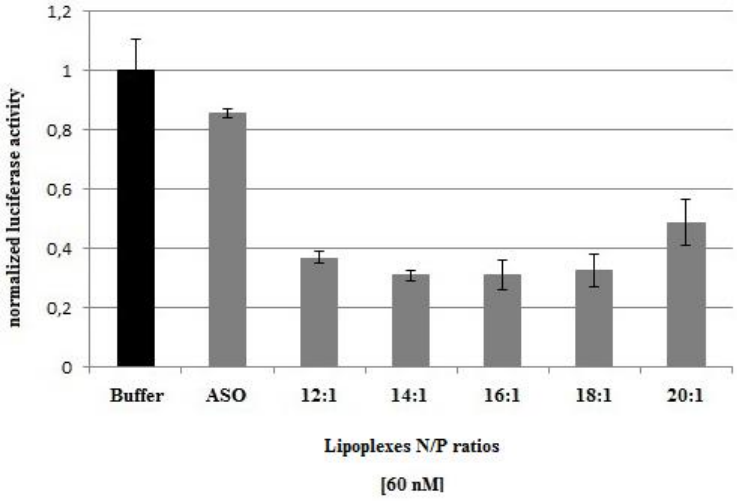

Fig. 5. Normalized cell viability of both cationic vesicle dispersions and antisense oligonucleotide forming lipoplexes at charge ratios of 14 and 16. Results are means ±S.D. for nine independent experiments.

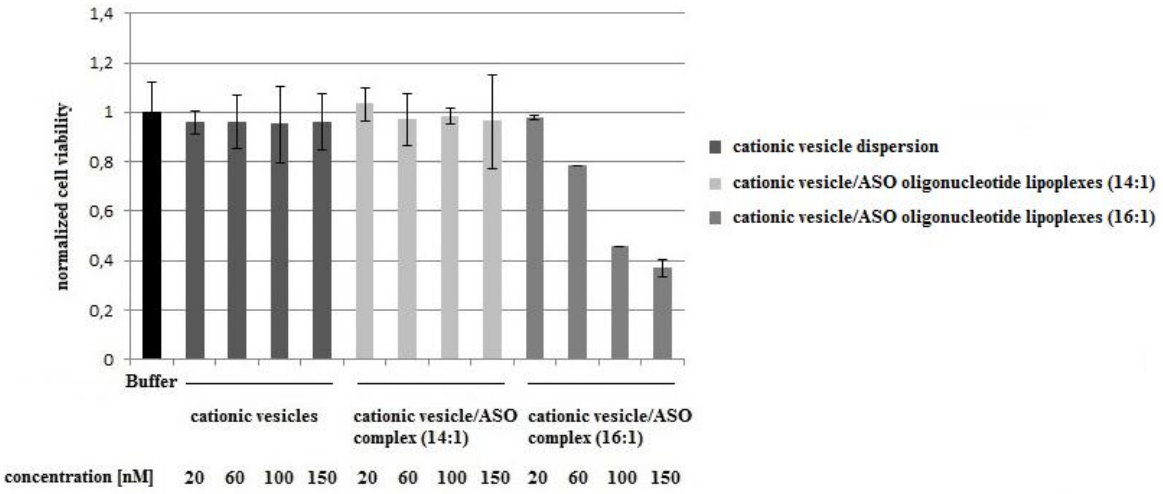

Fig. 6. Normalized gene-specific silencing activities targeting *Renilla* luciferase mRNA for cationic vesicle/antisense oligonucleotide (ASO) lipoplexes at a charge ratio of 14 at several concentrations (20, 60, 100 and 150 nM, respectively). Antisense oligonucleotide at the same tested concentrations in the absence of a non-viral carrier along with a scramble sequence (Scr) forming lipoplexes were used as controls. Results are means ±S.D. for three independent experiments.

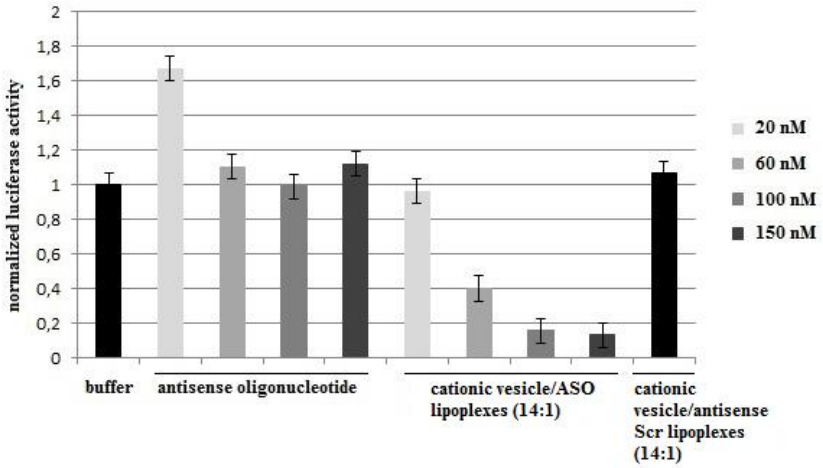

Fig. 7. Normalized gene-specific silencing activities targeting *Renilla* luciferase mRNA for cationic vesicles containing unmodified antisense oligonucleotide (ASO) at a N/P charge ratio of 14. Comercially available lipofectamine was used as a positive control at several concentrations (20, 60, 100 and 150 nM, respectively). Results are means ±S.D. for three independent experiments.

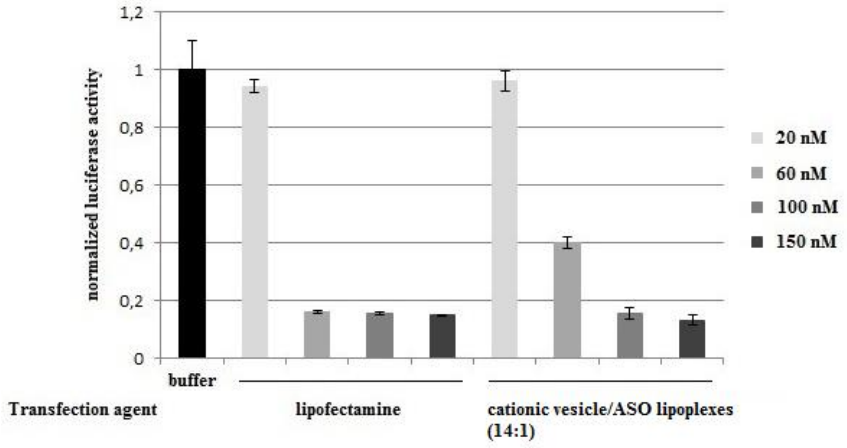

Fig. 8. Effect of fetal bovine serum (0 to 60%) on cell transfection mediated by cationic vesicle/antisense oligonucleotide (ASO) lipoplexes at a N/P charge ratio of 14. Antisense oligonucleotide in the absence of non-viral carrier was used as a control. Results are means ±S.D. for three independent experiments.