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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.

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

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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 [22–24]. 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 5′-CGT TTC CTT TGT TCT GGA-3′) 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 5′-CTG TCT GAC GTT CTT TGT-3′) 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 Dulbecco's Modified Eagle's 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 °C for 20 min. The dispersion was vortexed and sonicated for 3 min 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 min). 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 min and finally incubated at 37 °C for 30 min.

2.3. Morphology of lipoplexes

The morphology of the resulted lipoplexes was assessed by transmission electron microscopy (TEM). Briefly, 5 μL 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 s. Samples were observed under the microscope, TECNAI G2 20 TWIN (FEI, Eindhoven, The Netherlands), operating at an accelerating voltage of 200 keV in