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Full Length Article

Transfection of plasmid DNA by nanocarriers containing a gemini cationic lipid with an aromatic spacer or its monomeric counterpart

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ABSTRACT

This study performed a biophysical characterization (electrochemistry, structure and morphology) and assessment of the biological activity and cell biocompatibility of GCL/DOPE-pDNA lipoplexes comprised of plasmid DNA and a mixed lipid formed by a DOPE zwitterionic lipid and a gemini cationic lipid N-N′-(1,3-phenylene bis (methylene)) bis (N,N-dimethyl-N-(1-dodecyl) ammonium dibromide (12PH12) containing an aromatic spacer or its monomeric counterpart surfactant, N-benzyl-N,N-dimethyl-N-(1-dodecyl) ammonium bromide (12PH). Electrochemical results reveal that i) the gemini cationic lipid (12PH12) and the plasmid pDNA yield effective charges less than their nominal charges (+2 and −2/bp, respectively) and that ii) both vectors (12PH12/DOPE and 12PH/DOPE) could compact pDNA and protect it from DNase I degradation. SAXS and cryo-TEM experiments indicate the presence of a lamellar lyotropic liquid crystal phase represented as alternating layers of mixed lipid and plasmid. Transfection efficiency (by FACS and luminometry) and cell viability assay in COS-7 cells, performed with two plasmid DNAs (pEGFP-C3 and pCMV-Luc VR1216), confirm the goodness of the proposed formulations (12PH12/DOPE and 12PH/DOPE) to transport genetic material, with efficiencies and biocompatibilities comparable to or better than those exhibited by the control Lipofectamine 2000*. In conclusion, although major attention has been paid to gemini cationic lipids in the literature, due to the large variety of modifications that their structures may support to improve the biological activity of the resulting lipoplexes, it is remarkable that the monomeric counterpart surfactant with an aromatic group analyzed in the present work also exhibits good biological activity. The *in vitro* results reported here indicate that the optimum formulations of the gene vectors studied in this work efficiently transfect plasmid DNA with very low toxicity levels and, thus, may be used in forthcoming *in vivo* experiments.

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1. Introduction

The goal of gene therapy is to accomplish the treatment of diseases that are caused by genetic disorders [1,2]. Among the different approaches, introduction of a nucleic acid in living cells to substitute for a certain damaged gene has been widely explored in recent decades [3–5]. Insertion and expression of exogenous DNA into cells require the use of carriers that must be able to condense the nucleic acid, protect it from degradation, promote its cellular uptake, and release it into the cytoplasm [6,7]. Firstly, viral vectors were used to compact DNA; however, they had several drawbacks, such us immune responses, oncogenicity, and limitations on the size of the therapeutic gene [1,8]. Compared to viral vectors, cationic lipids are safe (there is no immune response), cost-effective and easier to fabricate, and for that, they have been explored as nanocarriers of nucleic acids in the last two decades, opening a wide range of possibilities [3,5,9,10]. Several factors on the rational design of the cationic lipid structure, *i.e.*, nature of the cationic head, spacer group and tail length, are important for DNA delivery [11,12]. Among the wide variety of cationic lipids (CLs), gemini lipids (GCLs) are known to show better performance due to their greater ability to compact nucleic acids [5,13–15]. The GCLs, constituted by two equivalent cationic surfactants (one head-one tail) linked with a molecular spacer through their cationic head

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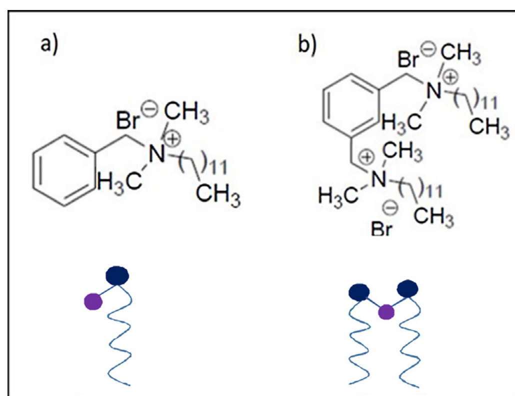

Scheme 1. Structure of a) monomeric (12PH) and b) gemini (12PH12) cationic lipids.

groups, offer a wide variety of potential structural modifications to improve their biological activity [3]. In this regard, different types of spacer groups (*i.e.*, hydroxyethyl, oxyethylene, alkyl and hydroxyethyl moieties) and cationic heads (*i.e.*, quaternary ammonium or imidazole groups) have been studied [5,16,17]. Previous studies reveal that determination of the optimum transfection efficiency is strongly influenced by the length and type of the spacer moiety. Thus, short groups as oligo-oxyethylene and hydrophobic rigid aromatic spacers have shown better performance in gene delivery [18–20]. With respect to the type and role of the positive charge on the surfactant head, better transfection efficiency and viability results were found with imidazolium groups in which the charge is known to be delocalized [17,21]. On the other hand, the length of the hydrophobic tail is another key parameter that affects crossing through the cellular membrane; for instance, GCLs with 12-carbon tails seem to show significantly better results than those with 14 carbons [22], although both the cationic head and hydrophobic tail are known to play an important role [23]. In any case, better transfection efficiencies are obtained when CLs are used together with a neutral or zwitterionic lipid, such as 1,2-dioleoyl-sn-*glycero*-3-phosphatidylethanolamine (DOPE) or mono-oleoylglycerol (MOG), which, working as helper lipids, decrease the gel-transition temperature; *i.e.*, the mixed lipid bilayer becomes, in turn, more fluid, and its fusion with the cell membrane is enhanced [24–27]. All these previous considerations indicate that the success of the transfection of nucleic acids strongly depends on the final structure and charge distribution in the nanocarrier formed by the GCL mixed with a neutral or zwitterionic lipid [3,5].

Consequently, in the present work, we report the rational design, physicochemical characterization, and transfection performances of a cationic gemini (two tails-two heads) lipid based on two 12-carbon hydrophobic tails and two quaternary dimethyl ammonium cationic heads linked *via* an aromatic ring (12PH12, see Scheme 1). Bearing in mind the high variety of parameters that influence the transfection efficiency, we have established a structure activity relationship (SAR) by comparing a gemini-type lipoplex (GCL/DOPE-pDNA) with its cationic monovalent (12PH, see Scheme 1) counterpart-type lipoplex (CL/DOPE-pDNA). Firstly, lipoplexes have been analyzed through zeta potential, agarose gel electrophoresis, small angle X-ray scattering (SAXS) and cryo-TEM to establish a relationship between the charge and the structure, as well as lipid vector-plasmid interaction. Secondly, to have a better comprehension of their behavior in *in vitro* biochemical studies, we have performed transfection experiments and determined the cell viability of those lipoplexes in COS-7 (African green monkey kidney) cells. The *in vitro* results of the present work provide interesting information to design gene lipid-type nanocarriers with efficient transfection capabilities and adequate levels of cell viability that may be used in *in vivo* applications.

2. Materials and methods

2.1. Materials

A zwitterionic lipid (1,2-dioleoyl-sn-*glycero*-3-phosphatidylethanolamine, DOPE) with the best purity was purchased from Avanti Polar Lipids, Inc., Alabaster, USA. The synthesis of the GCL, N-N′-(1,3-phenylene bis (methylene)) bis (N,N-dimethyl-N-(1-dodeyl) ammonium dibromide (12PH12), and its monomeric counterpart surfactant, N-benzyl-N,N-dimethyl-N-(1-dodecyl) ammonium bromide (12PH), was recently fully described [28]. Sodium salt of calf thymus DNA (ctDNA), provided by Sigma-Aldrich (St. Louis, USA) was used as linear DNA to determine the effective charge of the gemini cationic vector (12PH12). pEGFP-C3 plasmid DNA (4700 bp), used on biophysical and biological experiments, was extracted from competent *Escherichia coli* bacteria previously transformed with pEGFP-C3. The extraction was carried out using a GenElute HP Select Plasmid Gigaprep Kit (Sigma Aldrich). The plasmid pCMV-Luc VR1216 (6934 bp) encoding luciferase (Clontech, Palo Alto, USA), used for biological experiments, was amplified in *E. coli* and isolated and purified using a Qiagen Plasmid Giga Kit (Qiagen GMBH, Hilden, Germany). All the reagents and solvents, of the highest grade commercially available, were used without further purification.

2.2. Preparation of lipoplexes

Appropriate amounts of cationic lipids, 12PH12 or 12PH, and helper lipid, DOPE, were dissolved in chloroform to obtain the desired CL molar fraction (α) on the lipid mixtures. After briefly vortexing this solution, chloroform was removed to yield a dry lipid film. The resulting dry lipid films were then hydrated with 40 mM HEPES, pH 7.4, and homogenized by means of a combination of vortexing and sonication. The resulting multilamellar liposomes were transformed into the desired unilamellar liposomes by a sequential extrusion procedure that is widely explained elsewhere [16,19]. To prepare the lipoplex, appropriate amounts of a pDNA stock solution, prepared one day before, were added to lipid suspensions. pDNA concentrations in HEPES solution were chosen to fit the optimum conditions for each experimental technique as follows: 1 mg/mL for zeta potential, 1 mg/mL for cryo-TEM, 200 μg/capillar ($\approx$5 mg/mL) for SAXS, and 1 μg/well (2 μg/mL) for biological studies.

2.3. Zeta potential and particle size

A phase analysis light scattering technique (Zeta PALS, Brookhaven Instruments Corp., Holtsville, USA) was used to measure electrophoretic mobility, which was used to obtain the zeta potential (ζ) of the nanoaggregates [19,29]. Particle size was determined by a dynamic light scattering (DLS) method using a particle analyzer (Zeta Nano Series; Malvern Instruments, Barcelona, Spain). In both studies, samples were prepared with buffer 40 mM HEPES, pH 7.4. Experimental conditions were as follows: 25 °C, dispersant refractive index of 1.33 (water), viscosity of 0.9 cP, and dispersant dielectric constant of 78.5. Each zeta potential and particle size data point was taken as an average over 50 and 30 independent measurements, respectively. Measurements were carried out for the two studied lipoplexes as a function of the lipid/DNA mass ratio, $(m_{L+} + m_L^0)/m_{DNA}$ ($m_{L+,}$ m_L^0 and m_{DNA} being the masses of the cationic gemini lipid, of the zwitterionic helper lipid and of

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