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Self-assembling complexes between binary mixtures of lipids with different linkers and nucleic acids promote universal mRNA, DNA and siRNA delivery

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ABSTRACT

Protein expression and RNA interference require efficient delivery of DNA or mRNA and small double stranded RNA into cells, respectively. Although cationic lipids are the most commonly used synthetic delivery vectors, a clear need still exists for a better delivery of various types of nucleic acids molecules to improve their biological activity. To optimize the transfection efficiency, a molecular approach consisting in modifying the chemical structure of a given cationic lipid is usually performed, but an alternative strategy could rely on modulating the supramolecular assembly of lipidic lamellar phases sandwiching the nucleic acids molecules. To validate this new concept, we synthesized on one hand two paromomycin-based cationic lipids, with either an amide or a phosphoramide linker, and on the other hand two imidazole-based neutral lipids, having as well either an amide or a phosphoramide function as linker. Combinations of cationic and helper lipids containing the same amide or phosphoramide linkers led to the formation of homogeneous lamellar phases, while hybrid lamellar phases were obtained when the linkers on the cationic and helper lipids were different. Cryo-transmission electron microscopy and fluorescence experiments showed that liposomes/nucleic acids complexes resulting from the association of nucleic acids with hybrid lamellar phases led to complexes that were more stable in the extracellular compartment compared to those obtained with homogeneous systems. In addition, we observed that the most active supramolecular assemblies for the delivery of DNA, mRNA and siRNA were obtained when the cationic and helper lipids possess linkers of different natures. The results clearly show that this supramolecular strategy modulating the property of the lipidic lamellar phase constitutes a new approach for increasing the delivery of various types of nucleic acid molecules.

1. Introduction

Synthetic delivery systems represent today an attractive and well-documented approach for the intracellular delivery of recombinant informative macromolecular drugs including proteins and nucleic acids [1,2]. Among synthetic vectors used to deliver nucleic acids inside the cells, cationic lipids are recognized as a classical and versatile family of nanocarriers [3].

The usual approach applied until now to optimize the transfection efficiency consisted in chemical modifications on the cationic lipid structure only [4,5]. Indeed, performing structure-activity relationship (SAR) is the strategy of choice in the field of medicinal chemistry for the "hit-to-lead" approach and has proven its efficacy in various applications such as enzyme inhibition, taking advantage of the fact that the drug of interest interacts directly with its target [6,7]. Regarding the design of cationic lipids used as delivery systems, a ceiling effect may have been reached with this SAR strategy. Indeed, almost 30 years after the pioneer work on the synthesis of the first cationic lipids [8] and even if great improvements have been achieved compared to the original molecules, the ultimate goal of identifying a unique and universal

Abbreviations: C2C12, mouse myoblast cell line; CR, charge ratios; CLN, cationic lipid with N-based linker; CLP, cationic lipid with P-based linker; Cryo-TEM, cryogenic transmission electron microscopy; DMEM, Dulbecco's Modified Eagle's Medium; DNA, deoxyribonucleic acid; EtBr, ethidium bromide; eGFP, enhanced green fluorescent protein; HeLa, human cervical cancer cells; HLN, helper lipid with N-based linker; HLP, helper lipid with P-based linker; HPRT, hypoxanthine-guanine phosphoribosyltransferase; FACS, fluorescence activated cell sorting; Luc, luciferase; MEF, mouse embryonic fibroblasts; MEM, minimum essential medium; mRNA, messenger Ribonucleic acid; qRT-PCR, quantitative Real-Time PCR; RNAi, RNA interference; siRNA, small interfering RNA.

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delivery system able to efficiently transfect different classes of nucleic acids (DNA, mRNA, siRNA) into different cell lines or primary cells has not been reached yet. This situation probably results from the fact that cationic lipids do no act by themselves as "medical drugs" but represent only one piece of a complex process involving numerous steps that all need to be overcome to reach good levels of transfection.

The mechanism of gene delivery is known to proceed via i) formation of the lipoplex (complex nucleic acid + cationic lipid), ii) cellular uptake by endocytosis, iii) endosomal escape, iv) release of the nucleic acid from the complex and v) protein expression in the case of mRNA, protein inhibition in the case of siRNA in the cytosol, or nuclear uptake then protein expression in the case of DNA [9–14]. In addition, another difficulty for the discovery of an "universal" vector probably relies on identifying molecular features adapted to different structures of nucleic acids such as single or double stranded RNA and double stranded DNA that moreover possess different molecular weights.

The structure of cationic lipids used in the field of nucleic acid delivery is typically divided into three distinct parts i) a positively charged headgroup, able to electrostatically interact with nucleic acids, ii) an hydrophobic tail, to allow interactions with the cell membranes and iii) a linker (spacer) that links the head and the tail moieties together. Our team has developed a specific class of cationic lipids, using aminoglycoside-based headgroup. Aminoglycosides represent a family of natural products with a precise 3D-organization carrying several positively charged primary amines and having the property of naturally binding to nucleic acids [15–20]. We assessed different aminoglycosides (including paromomycin, neomycin, kanamycin and tobramycin) in previous studies and prepared various combinations of aminoglycoside/linker/hydrophobic part conjugates. The resulting amphiphilic molecules proved to be efficient vectors for the delivery of DNA, siRNA and more recently mRNA [21–24].

A well-known strategy to enhance transfection efficiency of a given cationic lipid relies in the incorporation of a neutral helper lipid in the nucleic acid formulation, forming cationic liposomes with binary mixtures of lipids in the liposomal membrane [25]. The helper lipids are known to facilitate the endosomal escape and display structural features comparable to cationic lipids (headgroup/linker/hydrophobic domain), except that the headgroup does not carry any positive charge. In the present study, we used helper lipids with an imidazole headgroup, based on encouraging results from the literature [26].

We hypothesized that the various steps of the transfection mechanism could be affected by the self-assembling properties of the complexes formed between cationic lipids (or liposomes) and nucleic acids, rather than the structure of the cationic lipids (or liposomes) themselves [9]. In these supramolecular assemblies, the nucleic acid molecules are sandwiched between lipid bilayers forming a lamellar organization of various periodicities [27–29]. The transfection pathway is governed by "membrane interactions", as the membranes of the lipoplexes first interact with the cytoplasmic membranes for internalization, and then with the endosomal membrane to trigger the release of the transported molecules [10,12]. We believe that the gene delivery process could thus be optimized by taking advantage of a new strategy consisting in finely modulating the lamellar phases of the lipoplex membrane, in order to confer enhanced nucleic acids complexation and decomplexation properties, outside and inside the cells, respectively.

In a previous study on DNA transfection, we compared two different formulation systems made with the same cationic lipid whose structure consisted of a paromomycin headgroup, a dioleyl hydrophobic backbone and a nitrogen-based linker (amide bond) [30]. Two imidazole-based helper lipids with either a nitrogen or a phosphorous-based linker were used to prepare liposomes. We discovered that better stability and higher DNA transfection were obtained when the hybrid liposomes (meaning with N-based linker on the cationic lipid and P-based linker on the helper lipid) were used compared to homogeneous liposomes (with N-based linkers both on the cationic and helper lipids).

These interesting preliminary observations prompted us to further investigate on these types of supramolecular assemblies. In the present study we assessed the systems formed by the combination of two paromomycin-based cationic lipids and two neutral imidazole-based helper lipids with three types of nucleic acids, DNA, mRNA and siRNA. It is known that binary mixtures of lipids with different hydrophobic backbone (nature, chain length and/or insaturations) can give rise to spatial separation of phase domains in the membranes; we therefore decided to maintain the same hydrophobic moiety for the cationic and the helper lipids and selected a dioleyl backbone for this study. Regarding the linker, two bio-inspired functions consisting of either an amide (nitrogen-based) or a phosphoramide (phosphorous-based) bond were used to link the polar and the lipophilic parts together.

The combinations polar headgroup/linker/hydrophobic tail provided four molecules that were evaluated; two cationic lipids: **CLN** (cationic lipid with nitrogen-based linker) and **CLP** (cationic lipid with phosphorous-based linker), and two helper lipids: **HLN** (helper lipid with nitrogen-based linker) and **HLP** (helper lipid with phosphorous-based linker). With these four molecules, supramolecular assemblies consisting of associations of the two cationic lipids with the two neutral lipids led to either homogeneous lamellar phases (when the two lipids have the same linker, **CLN**:**HLN** and **CLP**:**HLP**) or hybrid ones (when the linkers on the two lipids are different, **CLN**:**HLP** and **CLP**:**HLN**).

The major objective of the work was to understand how the physicochemical properties of the lipoplexes can affect the stability and the transfection efficiency of nucleic acids and to decipher the potential "universal" transfection properties of these types of liposomal formulations.

2. Materials and methods

pGWIZ-Luc (Genlantis, San Diego, CA) is a plasmid encoding the luciferase reporter gene under the control of the human cytomegalovirus immediate-early gene promoter. pGWIZ-eGFP (Clontech, Mountain View, CA) is a plasmid encoding green fluorescent protein reporter gene, under the control of the human cytomegalovirus immediate-early gene promoter. Plasmids were purified from recombinant *Escherichia coli* by means of Endofree plasmid purification columns (Qiagen, Venlo, Netherlands).

mRNA encoding the luciferase and the green fluorescent protein were purchased from Trilink Biotechnologies (San Diego, CA).

Human anti-laminA/C siRNA (sc-35,776) was purchased in Santa Cruz Biotechnology (Dallas, Texas) and murine anti-laminA/C siRNA (sense sequence: GGA-CCU-CGA-GGC-UCU-UCU-C) was obtained from Eurogentec (Seraing, Belgium).

3. Lipids synthesis

All reagents were purchased from Acros Organics or Sigma-Aldrich and were used without further purification. Reactions requiring anhydrous conditions were performed under nitrogen. NMR experiments were recorded on a Bruker Advance 400 MHz spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C and 162 MHz for [31] P). Spectra were acquired at 300 K, using deuterated methanol (MeOD) as solvent. Chemical shifts are reported in parts per million (ppm); coupling constant are reported in units of Hertz [Hz]. The following abbreviations were used: s = singlet, d = doublet, t = triplet, m = multiplet. High-resolution mass spectra (HRMS) were recorded with a qTOF Synaptg2 HDMS (Waters Corporation) apparatus; electrospray in positive mode was used. All the products were purified by flash chromatography (GRACE REVELERIS Flash Chromatography System) equipped with UV and DLS detectors. The purity of **CLP** was controlled by HPLC using UPLC H-Class from Waters using a gradient of H_2O + 0.1% Formic acid and CH_3CN.