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Research paper

Rigid aromatic linking moiety in cationic lipids for enhanced gene transfection efficiency

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ABSTRACT

Although numerous cationic lipids have been developed as non-viral gene vectors, the structure-activity relationship (SAR) of these materials remains unclear and needs further investigation. In this work, a series of lysine-derived cationic lipids containing linkages with different rigidity were designed and synthesized. SAR studies showed that lipids with rigid aromatic linkage could promote the formation of tight liposomes and enhance DNA condensation, which is essential for the gene delivery process. These lipids could give much higher transfection efficiency than those containing more flexible aliphatic linkage in various cell lines. Moreover, the rigid aromatic linkage also affords the material higher serum tolerance ability. Flow cytometry assay revealed that the target lipids have good cellular uptake, while confocal microscopy observation showed weaker endosome escape than Lipofectamine 2000. To solve such problem and further increase the transfection efficiency, some lysosomotropic reagents were used to improve the endosome escape of lipoplex. As expected, higher transfection efficiency than Lipofectamine 2000 could be obtained via this strategy. Cytotoxicity assay showed that these lipids have lower toxicity in various cell lines than Lipofectamine 2000, suggesting their potential for further application. This work demonstrates that a rigid aromatic linkage might distinctly improve the gene transfection abilities of cationic lipids and affords information to construct safe and efficient gene vector towards practical application.

1. Introduction

Gene therapy is a promising treatment method for genetic disorders and acquired diseases, as well as an alternative means of cancer treatment to traditional chemotherapy [1–4]. However, the intrinsic physicochemical properties of nucleic acid make itself grossly unsatisfactory as an independent therapeutic pharmaceutical, and a series of barriers exist in their transfection process [5]. Generally, free nucleic acids will be rapidly degraded by nucleases and/or esterases in the fluid circulatory system. On the other hand, naked genes cannot easily pass through the negatively charged cell membranes because of electrostatic repulsion [6,7]. Therefore, a great amount of research works have concentrated on the development and utilization of gene vectors with the ability to condense and protect gene cargos from degradation during the extra- and intra-cellular delivery [8]. The early research efforts focused on using viral gene vectors such as retrovirus and adenovirus since these vectors revealed excellent transfection efficiency (TE) in numerous cell lines [9]. However, many issues such as toxicity, immunogenic, inflammatory, and mutagenic effects may lead to safety risks and limit their application, prompting the extensive studies of non-viral vectors.

In the past decades, many kinds of non-viral vectors, such as lipids, organic polymers or inorganic materials, have been designed for safer gene delivery [10–13]. Among these materials, cationic lipids, a family of amphipathic small molecules usually containing a hydrophilic cationic headgroup, hydrophobic tails and a linking moiety, have been realized as promising gene delivery platforms because of their exact structure, good biocompatibility, high efficiency, and potential for clinical application [14,15]. Since the pioneer work from Felgner and coworkers utilizing DOTMA as cationic gene delivery vector [16], a wide range of lipids were investigated with the hope to overcome *in vitro* and *in vivo* transfection barriers [17]. In order to obtain ideal lipid materials with

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high efficiency and biocompatibility, many works have focused on the clarification of the structure-activity relationship (SAR) [18–20]. Various kinds of cationic groups, such as amines, amino-acids, guanidiniums and peptides, were used as polar head for nucleic acid condensation and further balancing the TE and cyto-toxicity [21]. Meanwhile, as another one of the important structural domain of the lipids, the hydrophobic tails were also considered to play significant roles in the liposome formation and gene delivery. Efforts were carried out on discussing the influence of the number, length, unsaturation, branching and asymmetry of hydrophobic chains on the TE [22]. Compared to the headgroup and hydrophobic tails, the studies on linking moiety were rare. It is known that biodegradable linkage (such as disulfide, ester, acetal, etc.) may benefit the lipoplex dissociation and thus to improve TE [23]. Recently, it was found that the integration of rigid elements might benefit the gene vectors with enhanced self-assembly, biocom-patibility and efficiency [24–27].

To obtain instructional information about the SAR of linking moiety of cationic lipids, we herein designed and synthesized a series of lipids containing the linking groups with different rigidity. Besides the linking moiety, these lipids contain a bio-essential lysine group as cationic headgroup, a hydrophobic tail formed by C18 oleyl group, which has been used in many efficient lipidic gene vectors [21,28]. Transfection experiment results revealed that lipids with rigid aromatic linkage could promote the formation of tight liposomes and enhance DNA condensation, which is essential for the gene delivery process. Moreover, these lipids exhibited higher serum tolerance and TE than those containing more flexible aliphatic linkage. Mechanism studies revealed that the target lipids have good cellular uptake but weak endosome escape, which is the main reason for their lower TE than Lipofectamine 2000. Thus, with the help of lysosomotropic reagents, the TE of the lipids could be effectively promoted as expected.

2. Results and discussion

2.1. Synthesis of target lipids

Cationic lipids designed for gene therapy usually utilize biocompatible amino acids as functional headgroup for nucleic acid binding while using oleyl chain as hydrophobic tail for lipid bilayer formation and cell/endosome membrane fusion. Lysine, which has two primary amines in the structure, was chosen as headgroup of the target lipids, and several linking structures were constructed to study their SAR in gene delivery. The synthetic route of the lipids was shown in Scheme 1. The target lipids were named according to their chemical structures, e.g. **LBO** represents the lysine-butylidene-oleyl architecture, while **LPO** represents lysine-phenyl-oleyl structure. Two types of rigid aromatic ring (phenyl in **LPO** and naphthyl in **LNO**) were introduced into the structure of cationic lipids as linking moiety. For comparison, aliphatic linkage with similar carbon atoms (cyclohexyl in **LHO** and butyl in **LBO**) were also designed and prepared. Further, a lipid with only an amide bond as linking group (**LO**) was also prepared as control lipid. For the preparation of **LBO** and **LHO** containing aliphatic linkage, the linkage was first reacted to the oleyl tail, and then coupled with lysine headgroup. However, the aromatic ring-contained lipids **LPO** and **LNO** could not be obtained via similar method. In their preparation, the aromatic linkage was first reacted to the lysine headgroup in the presence of IBCF, a stronger condensation reagent, and then coupled with oleyl amine. The structures of all target lipids were confirmed by ^{1}H NMR, ^{13}C NMR and HRMS.

2.2. The properties of lipids, liposomes and lipoplexes

It is widely recognized that amphiphilic lipids can self-assemble in aqueous solution when the lipid concentration surpasses a critical value, which is defined as the critical aggregation concentration (CAC) value [29]. Herein, two representative lipids without (**LBO**) or with (**LPO**) rigid aromatic group were chosen to measure the CAC. The results in Fig. S1 show that **LPO** holds a distinctly lower CAC value (0.04 mg/mL) than **LBO** (0.18 mg/mL), demonstrating that the aromatic ring in the linkage would promote the aggregation. The five lipids were then mixed with DOPE (1: 2, mol/mol) to form relative liposomes, of which the particle sizes and zeta potentials were measured via dynamic light scattering (DLS) assay. It was found that with the increase of rigidity, the particle size decreased gradually from **LO** to **LNO** (Fig. S2A). This also indicates that rigid structure might facilitate the formation of tight aggregates. On the other hand, the zeta potentials of the five liposomes were similar around +55 mV (Fig. S2B), indicating their remarkable cationic property. Subsequently, agarose gel electrophoresis assay was performed to evaluate the DNA binding ability of the liposomes at different N/P ratios. As shown in Fig. 1, complete DNA retardation could be observed at N/P ratio of 4 in the case involving **LO**. The aliphatic linkage seemed not to have any effect on the binding, meanwhile, the aromatic linkage might increase the binding, and complete retardation was found at lower N/P ratio (N/P = 2). Such results suggest that the rigid aromatic structure in the lipid may not only promote the formation of tight liposomes, but also enhance the DNA condensation, which is essential for the gene delivery process [30,31].

The physical properties including particle size and zeta potential of the liposome/DNA complexes (lipoplexes) were also studied for their significance in cellular uptake, cytotoxicity and release of gene cargo. The DLS results shown in Fig. 2A reveal that at N/P ratio of 1, the liposomes and DNA formed particles with the sizes larger than 500 nm, indicating an incomplete DNA condensation. With the increase of N/P ratio, the sizes of all the lipoplexes reduced gradually and finally became steady at 100–150 nm, suggesting the full condensation. On the other hand, the zeta potentials of the lipoplexes were negative at the N/P of 1, and charge reverse occurred from N/P of 2 and remained stable at 30–45 mV (Fig. 2B). The positive surface charge may facilitate their interaction with negatively charged cell membrane, leading to better cellular uptake. In addition, a visual image of **LPO**/DNA complexes at N/P of 4 by TEM (Fig. S3) revealed an irregular spherical morphology features with diameter of ~50 nm, which was smaller than that measured by DLS. This might be due to the different measure conditions of the two assays [20].

2.3. In vitro gene transfection

The gene transfection activity of the lipoplexes was first assessed by using enhanced green fluorescent protein (eGFP) as reporter gene in HeLa cells for direct visualization and preliminary investigation. Fig. 3 shows the eGFP gene expression mediated by lipoplexes at various N/P ratios, and Lipofectamine 2000 (Lipo) was used as control. Compared to naked DNA transfection, all the complexes induced large area of green florescent dots and the florescence density reached the maximum at N/P of 2. It was observed that by comparison with **LO**, the aromatic ring-contained lipids **LPO** and **LNO** (especially the phenyl-derived **LPO**) gave higher transfection efficiency (TE), while alkyl linkage-contained **LBO** and **LHO** did not lead to enhanced TE.

To quantitatively study the TE of the lipids and to confirm the superiority of **LPO**, luciferase reporter gene was also applied for the transfection. As shown in Fig. 4A, in HeLa cells, the five lipids gave