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Research paper

pH and reduction dual-responsive dipeptide cationic lipids with α–tocopherol hydrophobic tail for efficient gene delivery

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ABSTRACT

A series of tocopherol-based cationic lipid **3a-3f** bearing a pH-sensitive imidazole moiety in the dipeptide headgroup and a reduction-responsive disulfide linkage were designed and synthesized. Acid-base titration of these lipids showed good buffering capacities. The liposomes formed from **3** and co-lipid 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) could efficiently bind and condense DNA into nanoparticles. Gel binding and HPLC assays confirmed the encapsulated DNA could release from lipoplexes **3** upon addition of 10 mM glutathione (GSH). MTT assays in HEK 293 cells demonstrated that lipoplexes **3** had low cytotoxicity. The *in vitro* gene transfection studies showed cationic dipeptide headgroups clearly affected the transfection efficiency (TE), and arginine-histidine based dipeptide lipid **3f** give the best TE, which was 30.4 times higher than Lipofectamine 3000 in the presence of 10% serum. Cell-uptake assays indicated that basic amino acid containing dipeptide cationic lipids exhibited more efficient cell uptake than serine and aromatic amino acids based dipeptide lipids. Confocal laser scanning microscopy (CLSM) studies corroborated that **3** could efficiently deliver and release DNA into the nuclei of HeLa cells. These results suggest that tocopherol-based dipeptide cationic lipids with pH and reduction dual-sensitive characteristics might be promising non-viral gene delivery vectors.

1. Introduction

Gene therapy involves the use of genes for therapeutic purposes is a very promising method for the treatment of various diseases [1,2]. The success of gene therapy critically depends on the development of safe and efficient gene vectors [3]. Traditionally, gene delivery systems are based on either viral or synthetic non-viral mediated vectors. Viral vectors are more efficient than non-viral vectors, however, their clinic use were impeded due to their immunogenicity and toxicity [4,5]. Therefore, the use of non-viral vectors, such as cationic lipids-based vectors that offer advantage over viral vectors in these aspects, has attracted broad attention. The basic architecture of a cationic lipid consists of three functional units: a positively charged hydrophilic head group, a hydrophobic steroid or alkyl backbone, and a linker between above two parts [6,7]. All these domains can significantly affect the TE and toxicity of the cationic lipids.

Gene transfection with cationic lipids needs to overcome multiple delivery barriers, including the formation of lipid/DNA complexes (lipoplexes) and their initial binding to the cell surface, endocytosis, endosomal escape, nuclear entry, and final expression [8]. Efficiently condense and protect DNA under extracellular conditions is a prerequisite for efficient gene delivery [9]. Multivalent cationic lipids are expected to form liposomes with a greater surface charge density than monocationic lipids [10], which thus led to better DNA condensation and easier interaction with the negative cell membrane [11]. Synthetic peptides are perspective substances for the construction of the multivalent polar domains [11–14], besides they also show many advantages over other non-viral vectors, such as good biodegradability, excellent biocompatibility and potential application in improving the delivery of gene therapeutics [15,16]. In addition to good DNA condensation, efficient endosomal escape and cytosolic delivery would also largely affects the gene transfection efficacy [17]. The development pH-sensitive lipids which could escape from endosomes due to membrane disturbance is an efficient strategy to overcome the endosome barrier. As histidine imidazole group with an appropriate pKa (~6) which could increase the buffering capacity in endosomes and lysosomes [18]. Thus, introducing histidine into cationic lipid might benefit the endosome escape, leading to better gene transfection

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[7,19]. Besides, the introduction of another stimulus-responsive or biocompatible functional group into the structure of cationic lipid may not only further enhance the TE, but also decrease the cytotoxicity of the vectors. Disulfide bond is a characteristic linkage which can be degraded in the cytoplasm specifically in response to redox potential through thiol-disulfide exchange reactions due to the elevated levels of the reducing tripeptide glutathione (GSH), which are 100–1000 times higher than that in extracellular media [20]. In recent years, many disulfide linker bearing cationic lipids with the merit of low cytotoxicity and high transfection efficiency were synthesized [21–27]. The design and synthesis of new lipid systems with alternative structural types are also crucial for the development of potent synthetic vectors for gene delivery. As a biocompatible hydrophobic moiety, we [28,29] and other groups [30–35] recently demonstrated the potential of novel tocopherol-based cationic lipids for use in liposomal gene delivery.

With these inspirations in mind, herein we describe a series of tocopherol-based cationic lipids with a reduction-responsive disulfide linkage and various pH sensitive dipeptide headgroups (Scheme 1). Their interaction with plasmid DNA was studied, and the structure-activity relationship in the gene delivery mediated by these lipids was discussed.

2. Results and discussions

2.1. Syntheses of new di-peptide cationic lipids

The title lipids **3** were synthesized according to the route shown in Scheme 1. Compound **2** was synthesized by coupling **1** with Boc-protected (Boc = *tert*-butyloxycarbonyl) amino acids (*N*-Boc-L-serine, *N*-Boc-L-phenylalanine, *N*-Boc-L-tyrosine, *N*-Boc-L-tryptophan, *N*,*N*-di-Boc-L-lysine or N^{α},N^{ω},$N'^{\omega\prime}$-*tri*-Boc-L-arginine) in the presence of 1-hydroxybenzotrizole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl). Finally, target lipids were obtained by removing the Boc groups with trifluoroacetic acid in anhydrous CH_2Cl_2. All the structures of lipidic compounds were characterized by using NMR spectroscopy and HRMS.

2.2. Buffering capability

It is well known that polyethyleneimine (PEI) has been considered as the “golden standard” for polymeric gene vectors because of its relatively high TE, which might come from its excellent pH buffering capacity and the consequent “proton-sponge effect” [36]. The introduction of imidazole which has a pKa of ~ 6 may significantly contribute to its endosomal pH buffering capacity [7,18,19]. Two lipids (**3e** and **3f**) were chosen to examine their buffering capacities by acid–base titration experiments, and branched PEI (average molecular weight 25 kDa, bPEI-25K) was used as the control. The results showed that the histidine imidazole-attached molecules gave a relatively slow pH increase with the addition of NaOH as well as PEI, indicating their good buffering capacities (Fig. 1).

2.3. Interaction with DNA and characterization of liposome/DNA complexes (lipoplexes)

Our previous study showed that cationic liposomes had higher transfection efficiency with an equivalent molar ratio of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) [28], so the liposomes are formed with the dipeptide lipids with DOPC as co-lipid at molar ratio of 1:1. Agarose-gel retardant and ethidium bromide (EB) dye displacement assays were used to evaluate their plasmid DNA-binding abilities [21]. Fig. 2A shows **3a-3f** could effectively bind to DNA and retard its electrophoresis. Complete DNA retardation could be achieved at N/P ratio of 2 for the liposomes formed from all lipids except **3a** (N/P = 4). Most dipeptide-functionalized cationic lipids showed higher pDNA-binding abilities than histidine-containing lipid **1** [28]. In addition, ethidium bromide dye displacement was also used to evaluate their pDNA-binding abilities (Fig. 2B). It was shown that the fluorescent intensities were significantly decreased with the rise of N/P ratio (from 0 to 5), indicating that EB was replaced by the act of cationic lipid binding. It is noteworthy that lipid **3f** which utilized arginine-histidine di-peptide as cationic headgroup exhibited a stronger fluorescent quenching effect, and this may infer an important headgroup structure dependence of pDNA binding affinity for the di-peptide lipids. Hence, the agarose-gel retardant and EB dye displacement assays consistently showed strong interaction between new prepared dipeptide cationic lipids and pDNA.

2.4. Reduction-triggered active unpacking of lipoplex

To gain an insight into whether the disulfide linker of the newly dipeptide based cationic lipids are reducible by reducing molecules such as glutathione (GSH) in living cells, gel retardation assay in the presence of 10 mM GSH was first investigated using **3**-based lipoplexes from at N/P ratio of 2 for **3b-f**, and 4 for **3a**, respectively. Interestingly, DNA was fully released from lipoplexes of all lipids when incubated for 60 min with 10 mM GSH (Fig. 3A). This demonstrated GSH-responsive disulfide cleavages of dipeptide based delivery carriers, which thus led to the release of the encapsulated pDNA. Besides, according to this experimental setup, the use of DTT (10 mM), another reducing agents, was also able to reduce lipids **3** resulting in the release of free pDNA (Fig. S1). The same assay was also conducted in serum containing medium, and similar results were obtained. (Figs. S2 and S3). Further, we used HPLC to monitor the time dependent degradation of liposomes **3f** by both GSH and DTT. Incubation with GSH or DTT resulted in cleavage of the disulfide linker of the newly dipeptide-based cationic lipids (Fig. 3B and Fig. S4). This results correlated well with the gel retardation assay (Fig. 3A and Fig. S1). Besides, arginine-histidine dipeptide containing lipid **3f** exhibited a faster degradation than histidine-containing lipid **1** (Fig. S5). Although both GSH and DTT could reduce the disulfide bond, the reaction rates were substantially different. This result is in accordance with our previous study [28]. The reaction rate of DTT was much higher than that of GSH (Fig. 3C). These rate differences may be due to their unequivalent chemical structures. GSH and DTT possess one and two thiol functional groups, respectively; furthermore, as glutathione is an anionic molecule at physiological pH (7.0), it may interact with the head group of cationic lipids; thus the probability of the interaction between this reducing agent and **3** disulfide bonds could be diminished [27]. These results show that reducible di-peptide cationic lipids might elegantly resolve the DNA condensation and release dilemma of non-viral gene carriers, i.e. on one hand improving DNA condensation ability and lipoplex vesicle stability and on the other hand actively releasing DNA under intracellular mimicking reductive environments.

2.5. Size and zeta-potential of the formed lipoplexes

Proper size and zeta potential are important factors for liposome/DNA complexes (lipoplexes) used as gene vectors. The particle sizes of different lipoplexes were measured by dynamic light scattering (DLS) assays. The result showed that liposomes formed from **3** could condense plasmid DNA into nanoparticles around