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trans-2-Aminocyclohexanol-based amphiphiles as highly efficient helper lipids for gene delivery by lipoplexes

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ABSTRACT

Lipidic amphiphiles equipped with the *trans*-2-aminocyclohexanol (TACH) moiety are promising pH-sensitive conformational switches ("flipids") that can trigger a lipid bilayer perturbation in response to increased acidity. Because pH-sensitivity was shown to improve the efficiency of several gene delivery systems, we expected that such flipids could significantly enhance the gene transfection by lipoplexes. Thus a series of novel lipids with various TACH-based head groups and hydrocarbon tails were designed, prepared and incorporated into lipoplexes that contain the cationic lipid 1,2-dioleoyl-3-trimethylammonio-propane (DOTAP) and plasmid DNA encoding a luciferase gene. B16F1 and HeLa cells were transfected with such lipoplexes in both serum-free and serum-containing media. The lipoplexes consisting of TACH-lipids exhibited up to two orders of magnitude better transfection efficiency and yet similar toxicity compared to the ones with the conventional helper lipids 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or cholesterol. Thus, the TACH-lipids can be used as novel helper lipids for efficient gene transfection with low cytotoxicity.

1. Introduction

The development of gene delivery systems of high efficiency and low toxicity continues to draw much attention because of their critical applications in modern medicine, including gene therapy in the clinic and basic research using transgenic cell lines and animals. Since their inception in 1987 [1], the cationic liposome/DNA complexes (lipoplexes) have become one of the most commonly used non-viral gene delivery systems. Cationic lipoplexes have several advantages over viral gene delivery systems, including convenience in preparation, high capacity, virtually unlimited cargo gene size and lower toxicity. Compared to polyplexes, which are another major non-viral gene delivery system, the lipoplexes are less immunogenic. However, the main drawback of lipoplexes is their relatively low efficiency compared to viral gene vectors [2].

Extensive investigation over the past three decades has established that lipoplexes deliver genes into cells by a multi-step mechanism. After administration, cationic lipoplexes first adsorb onto the negatively charged cell surface by electrostatic interaction with anionic heparin sulfate, which is part of the extracellular matrix [3,4]. The adsorption causes endocytosis [5] that transfers the lipoplexes first into the endosomes, and then into the lysosomal compartment. While processed in the endosomal/lysosomal pathway, the cationic lipoplexes exchange lipids with the negatively charged endosomal/lysosomal membranes to destabilize the membranes and to release a small portion of the cargo DNA from the endosomal/lysosomal compartments into the cytosol [6,7]. Some of the released DNA eventually partition from the cytosol into the nucleus for the transgene expression [8]. However, the majority of the cargo DNA are degraded in the lysosomal compartment, which represents a major barrier that limits the efficiency of gene delivery by lipoplexes [9].

One important feature of the endosomal/lysosomal pathway is the decrease of pH inside the endosomal and lysosomal compartments [10]. Such feature is exploited by many viruses to enhance their gene transfection inside the host cells. For example, a glycoprotein of the influenza virus, hemagglutinin fuses the viral membrane with the endosome membrane in response to the pH drop to transfer the viral genome from the endosome to the cytosol [11]. pH-sensitivity has also been exploited to enhance gene transfection by lipoplexes, such as the incorporation of pH-sensitive peptides/polymers [12], and the development of pH-sensitive cationic lipids [13].

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's Modification of Eagle's Medium; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonio-propane; FBS, fetal bovine serum; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid; HRMS, high resolution mass spectrometry; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PEG-ceramide, mPEG2000-ceramide; TACH, *trans*-2-aminocyclohexanol.

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Thus far the major strategy to enhance gene delivery by cationic lipoplexes has been to develop cationic lipids of optimized structure. In this regard, numerous cationic lipids carrying diverse headgroups, lipid tails, as well as linker groups have been reported, some of which successfully commercialized as gene transfection agents in biological and pharmaceutical research [14]. Nevertheless, neutrally charged "helper lipids" also participate in the formation of lipoplexes and play an essential role in transformation of lipoplexes to non-bilayer (inverted hexagonal) phases that more efficiently destabilizes the endosomal/lysosomal membranes and consequently increases the amount of DNA that can escape the endosomal/lysosomal pathway [15,16]. Therefore the discovery of novel and effective helper lipids can be another approach to enhance the gene delivery efficiency. To date, the vast majority of reported gene transfections by lipoplexes used either 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) or cholesterol as the helper lipid and only a few other helper lipids have been studied, including partially fluorinated DOPE analogs [17], a phosphatidylcholine bearing dodecanedioic acid monobenzyl ester chains [18], N-lauroylsarcosine [19], and more recently, 1-alkyl-2-acyl phosphatidylcholines [20]. Furthermore, no pH-sensitive helper lipids for the lipoplexes have been reported to date.

We recently designed a novel type of amphiphiles based on *trans*-2-aminocyclohexanol (TACH) and introduced these TACH-lipids as a pH-sensitive conformational switch of liposome bilayer [21–24] (Scheme 1). In the original conformational equilibrium **A** ⇆ **B** (Scheme 1), the amine and the hydroxy groups of TACH-lipid are predominantly in axial positions while both lipidic *trans*-ester groups adopt equatorial positions (**A**). The protonation of the amine nitrogen upon addition of acid results in a strong intramolecular hydrogen bond between the amine and the hydroxy groups, forcing both groups to adopt equatorial positions. This impulse is mechanically transmitted via the conformational flip of the cyclohexane ring to force both ester groups into axial positions (**B**H^+ on Scheme 1), thereby drastically increasing the spatial separation of the lipid tails and perturbing the bilayer structure. After incorporation into liposome membranes, TACH-lipids significantly enhanced the release from both the traditional liposomes based on phospholipids and the sterically hindered liposomes containing mPEG2000-ceramide (PEG-ceramide) upon exposure to lowered pH [21–24]. To highlight the key role of the conformational flip in the pH-triggered liposome leakage, we introduced the terms "*flipids*" for the amphiphiles containing a pH-sensitive conformational switch and "*fliposomes*" for the liposomes comprising these amphiphiles [22–24].

Because pH-sensitivity was shown to improve the efficiency of a number of gene delivery systems, including viral vectors, polyplexes and lipoplexes [25–27], we postulated that such TACH-derived, pH-sensitive conformational switches could also serve as helper lipids to significantly enhance the gene transfection by lipoplexes. To explore this possibility, we prepared a series of novel TACH-lipids with different amino groups and lipid tails (**1**–**8** in Fig. 1). The analogous amphiphiles **9** [21,22] and **10** (Fig. 1) were also synthesized as controls that do not perform pH-triggered conformational switch. The commonly used helper lipids DOPE and cholesterol were used as pH-insensitive lipids for comparison. The pH-driven conformational flip of compounds **1**–**8** (Scheme 1) was studied by ^{1}H NMR titration. All the amphiphiles were incorporated into lipoplexes containing a well-studied cationic lipid 1,2-dioleoyl-3-trimethylammonio-propane (DOTAP) [28,29] and plasmid DNA encoding luciferase to mediate gene transfection into B16F1 and HeLa cancer cells. To the best of our knowledge, this is the first report on pH-sensitive amphiphiles as helper lipids in lipoplexes for gene delivery.

2. Materials and methods

2.1. Materials

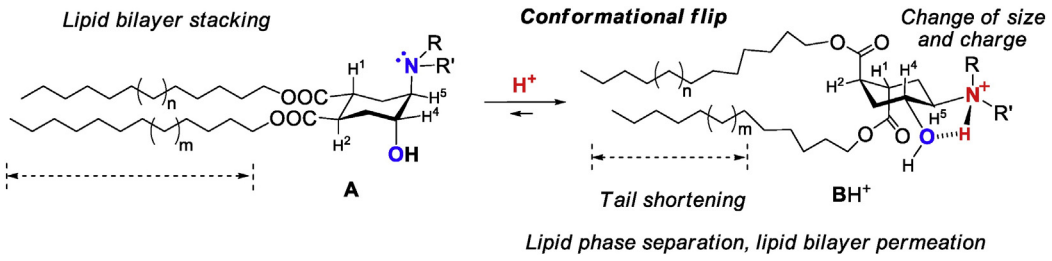
The lipids 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and used without further purification. All other chemicals including 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid (HEPES) and sodium chloride (NaCl) were purchased from Sigma-Aldrich or Fisher Scientific. All solvents were purified by conventional techniques prior to use. Dulbecco's Modification of Eagle's Medium (DMEM), penicillin–streptomycin solution, L-glutamine, trypsin, and phosphate-buffered saline (PBS) were purchased from Corning Cellgro (Manassas, VA, USA). Fetal bovine serum (FBS) was purchased from Tissue Culture Biologicals (Tulare, CA, USA). HeLa cells and B16F1 cells were purchased from American Type Culture Collection (ATCC) (Rockville, MA, USA). pCMV-GLuc Control Plasmid and BioLux® *Gaussia* Luciferase Assay Kit were purchased from New England Biolabs (Hitchin, Hertfordshire, UK). M-PER® Mammalian protein extraction reagent, Pre-diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set and Micro BCA Protein Assay Kit were purchased from Thermo Scientific Pierce Protein Biology Products (Rockford, IL, USA). The 96-well plates were purchased from TPP (Switzerland).

2.2. Synthesis

The amphiphiles **1**–**3**, **6**, and **9**, and the corresponding intermediates were prepared as described earlier [21–24]. Other TACH-lipids (Fig. 1) were synthesized using similar procedures (Scheme 2). In order to render "kinks" in the lipid tails of amphiphiles **4** and **8**, a cyclopropyl moiety was introduced into the hydrocarbon chains of the starting unsaturated alcohol by a diethylzinc-based Simmons–Smith reaction [30] (Scheme 3). ^{1}H NMR and ^{13}C NMR spectra were acquired on a JEOL ECA-600 NMR-spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C). High resolution mass spectra (HRMS) were obtained on a JEOL AccuTOF time-of-flight mass spectrometer (Peabody, MA) coupled with an Ionsense DART open-air ionization source (Saugus, MA).

2.2.1. Dihexadecyl fumarate (*11b*)

Fumaryl chloride (3.22 g, 20 mmol) was refluxed for 12 h with 1-hexadecanol (10.2 g, 42 mmol) in anhydr. $CHCl_3$ (30 mL). The reaction mixture was diluted with $CHCl_3$ (60 mL), washed with 5% aq. NaOH (2 × 20 mL), 5% aqueous HCl (2 × 20 mL), and brine (10 mL),



Scheme 1. Protonation-induced spatial separation and efficient shortening of the TACH-lipid tails, and change of the charge, effective size and shape of the polar head cause a quick perturbation of the lipid bilayer.