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Diaminododecane-based cationic bolaamphiphile as a non-viral gene delivery carrier

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ABSTRACT

The advancement in gene therapy relies upon the discovery of safe and efficient delivery agents and methods. In this study, we report the design and synthesis of a cationic bolaamphiphile as a non-viral gene delivery agent. The bolaamphiphile is composed of 1,12-diaminododecane as the central hydrophobic unit linked to the hydrophilic pentaethylenehexamine *via* thioether-based glycidyl units. This bolaamphiphile condensed DNA efficiently into nanoparticles of sizes around 150–200 nm with positive zeta potential of 30–35 mV. *In vitro* luciferase expression levels and percentage of GFP expressing cells induced by the bolaamphiphile/DNA complexes were higher than those mediated by the often used "golden" standard of non-viral systems, polyethyleneimine (PEI, branched, 25 kDa) at its optimal N/P ratio in HEK293, HepG2, NIH3T3, HeLa and 4T1 cells. *In vitro* cytotoxicity testing revealed that the DNA complexes fabricated from this cationic bolaamphiphile displayed marginal toxicity towards all the cell lines tested. In addition, *in vivo* transfection studies carried out in a 4T1 mouse breast cancer model showed that the cationic bolaamphiphile delivered DNA more efficiently than PEI. This cationic bolaamphiphile may make a promising gene delivery vector for future gene therapy.

1. Introduction

The ultimate goal of non-viral gene delivery research is to develop biomaterials that are capable of delivering genes in an efficient and safe manner. As a result, most researchers have been focusing on designing and synthesizing biomaterials that can be manufactured with ease and also allow multifunctional end usage such as having targeting ability as well as possessing low *in vitro* and *in vivo* cytotoxicity. Unfortunately, synthetic non-viral gene delivery systems still continue to suffer from low transfection efficiency and to some extent from certain toxicity issues when compared to their viral counterparts. Although employing viral vectors as delivery agents is known to induce high gene transfer efficiency, concerns have been raised over their general safety issues such as immunogenicity, difficult to manufacture on a large scale and low gene loading capacity. Consequently, alternative delivery vectors have been proposed based on synthetic materials such as cationic lipids [1–6], polymers [7–19] and peptides [20–24].

Amongst the non-viral delivery systems reported in the literature, the most studied class of biomaterials that have shown great potential in terms of delivering genes *in vitro* as well as *in vivo* so far has been cationic lipids [25–29]. A majority of the earlier studies that utilized cationic lipids for gene delivery contained a quaternary ammonium group for gene binding. However, increasing amount of evidence gathered from studies in which cationic polymers are used for gene delivery have demonstrated that the presence of multiple amines and various types of amines such as primary, secondary and tertiary is an important aspect in the design of gene delivery vectors, since these enhance DNA condensation and endosomal buffering capacity [30,31]. Therefore, in more recent studies, cationic bolaamphiphiles containing different types of amines have been proposed as non-viral gene delivery agents. Bolaamphiphiles are simply surfactants that contain one or two hydrophobic chains linked to a hydrophilic portion on both ends of the hydrophobic chain. One of the earliest studies that utilized cationic bolaamphiphiles as non-viral gene delivery agents was carried out by Vierling et al. [32,33]. In both cases non-symmetrical bolaamphiphiles were employed, which had sugar molecules such as galactose attached to the bolaamphiphiles to aid in targeting signal for delivering genes to a specific type of cells. More recent studies have shown that it is possible to obtain efficient non-viral gene delivery vectors from cationic bolaamphiphiles containing primary and secondary amines in the hydrophilic portions [34,35]. In particular, Brunelle et al. [34] studied the structure-activity

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relationship of non-symmetrical hemiflourinated bolaamphiphiles containing lysine and histidine in the polar head groups, and found that these bolaamphiphiles gave rise to good transfection efficiency.

Apart from these examples, there is scarcity of studies on bolaamphiphile-based non-viral gene delivery vectors as compared to the huge number of reports that detail the usage of traditional lipids. Herein we designed and synthesized a symmetrical cationic bolaamphiphile that is composed of 1,12-diaminododecane as the hydrophobic chain linked via a thioether-based glycidyl unit to hydrophilic cationic pentaethylenehexamine (containing primary and secondary amines) on two ends. DNA binding by the bolaamphiphile was evaluated using gel electrophoresis and the bolaamphiphile/DNA complexes were characterized via particle size and zeta potential. Luciferase and GFP expression efficiency induced by the bolaamphiphile/DNA complexes was investigated in various cell lines including HEK293 (human embryonic kidney), HepG2 (human hepatocellular liver carcinoma), 4T1 (mouse breast cancer), and HeLa (human cervical cancer), and NIH3T3 (mouse embryonic fibroblast) cell lines in comparison with PEI (branched, 25 kDa). In addition, the cytotoxicity of bolaamphiphile/DNA complexes was evaluated against these cell lines. Moreover, the *in vivo* luciferase expression efficiency of the bolaamphiphile/DNA complexes was studied in a 4T1 mouse breast cancer model.

2. Materials and methods

2.1. Materials

PEI (branched, 25 kDa), pentaethylenehexamine, 1,12-diaminododecane, tris(2-aminoethyl) amine, epichlorohydrin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich and used as received. RPMI1640 medium, Dulbecco's modified eagle medium (DMEM), fetal bovine serum, penicillin and streptomycin were obtained from Invitrogen Corporation (U.S.A.). Dimethyl sulfoxide (DMSO), methanol and HPLC grade water were bought from Fisher Scientific (U.S.A.). Tris–boric acid EDTA (TBE) buffer was purchased from 1st BASE (Malaysia). Plasmid DNA encoding 6.4 kb firefly luciferase (pCMV-luciferase VR1255C) driven by cytomegalovirus (CMV) promoter was kindly provided by Car Wheeler, Vical (U.S.A.), which was amplified in *E. coli* DH5α and purified with endotoxin-free plasmid purification kit supplied by Qiagen (Dutch). HEK293 human embryonic kidney, HepG2 human hepatocellular liver carcinoma, HeLa human cervical cancer, NIH3T3 mouse embryonic fibroblast and 4T1 mouse breast carcinoma cell lines were obtained from ATCC (U.S.A.).

2.2. Synthesis of diaminododecane-based bolaamphiphile precursor (precursor molecule, Scheme 1)

Into a flask, was placed 50 ml of methanol, 2 g of diaminododecane (0.01 mol), 2.42 ml of methyl-3-mercaptopropoinate (0.022 mol) and 10 ml of triethylamine (0.07 mol). The flask was left to stir at 80 °C until all the diaminododecane had dissolved. Next, 100 ml of DMSO was added to the stirring reaction mixture, and the reaction was carried out under nitrogen with stirring at 80 °C for 24 h. The contents of the flask were cooled down to 30 °C and the crude product was precipitated into excess tetrahydrofuran several times to give rise to a pale yellow solid. The precursor molecule was characterized by IR (PerkinElmer spectrum 100 FT–IR spectrometer, U.S.A.), ^{1}H NMR and ^{13}C NMR spectroscopy (Bruker AVANCE 400, Germany) at 400 and 100 MHz respectively, using the indicated deuterated solvent. CS ChemNMR Pro version 6.0 (Upstream Solutions GbmH Scientific Software Engineering CH-6052 Hergiswil, Switzerland) was employed to analyze various protons and carbons. Bolaamphiphile precursor molecule: υ_{max}/cm^{-1} 2950 medium (sharp) [υ(C–H)]; 2890 medium (sharp) [υ(C–H)]; 1650 strong (sharp) [υ(C=O)]. δ_H (400 MHz, CD_3OD) 3.00–2.20 (2H, t, HS–C***H***$_2$–C***H***$_2$–C(=O)–NH–C***H***$_2$–CH$_2$–CH$_2$–); 1.60–1.00 (m, HS–CH$_2$–CH$_2$–C(=O)–NH–CH$_2$–(C***H***$_2$)$_{10}$–CH$_2$–NH–C(=O)–CH$_2$–CH$_2$–SH, signal A). δ_C (100 MHz, CD_3OD) 174, 39, 35, 29.6, 29, 28 and 26.

2.3. Synthesis of diaminododecane-based bolaamphiphile (Scheme 1)

Into a flask, was placed 1.0 g of the precursor molecule (0.0027 mol) in conjunction with 50 ml of methanol, and the mixture was left to stir at 80 °C until all of the compound had dissolved. Then 5 ml of triethylamine (0.035 mol) in conjunction with 100 ml of DMSO was added to the stirring mixture. The contents of the flask were cooled down to 40 °C and 0.67 ml of epichlorohydrin (0.0081 mol) was added to the mixture, which was then left to stir under nitrogen at 40 °C for 24 h. Pentaethylenehexamine (60 ml, 0.26 mol) was added to the reaction mixture and the reaction was conducted under nitrogen at 90 °C with stirring for 24 h. The contents of the flask were cooled down to 30 °C and the crude product was precipitated into excess tetrahydrofuran. Then a small amount of water was added to dissolve the precipitate. The pH of the solution was adjusted to 4–6 using HCl (aq), and dialyzed against water for 24 h with a continuous flow by a membrane dialysis method using dialysis tubing with a molecular weight cut-off (MWCO) of 1 kDa (Spectrum Laboratories, U.S.A.). Sodium hydroxide solution was then added to the dialyzed contents until a pH of 9–10 was achieved, and using fresh tubing the solution was further dialyzed against water for 24 h. Diaminododecane-based bolaamphiphile was harvested by freeze-drying, and characterized by IR, ^{1}H NMR and ^{13}C NMR spectroscopy at 400 and 100 MHz respectively using the indicated deuterated solvent. Bolaamphiphile: υ_{max}/cm^{-1} 3360 strong (sharp) [υ(N–H)]; 3310 strong (sharp) [υ(N–H)]; 2960 medium (sharp) [υ(C–H)]; 2830 medium (sharp) [υ(C–H)]; 1660 strong (sharp) [υ(C=O)]; 1120 weak (sharp) [υ(C–OH)]. δ_H (400 MHz, D_2O) 3.70 (1H, m, –CH$_2$–C***H***(OH)CH$_2$–, signal C); 3.30–2.20 (2H, t, NH$_2$–C***H***$_2$–C***H***$_2$–NH–, –CH$_2$–CH$_2$–C***H***$_2$–NH–C(=O)–C***H***$_2$–C***H***$_2$–S–, –C***H***$_2$–CH(OH)C***H***$_2$–, signal B) and 1.60–1.00 ppm (–(O=)C–NH–CH$_2$–(C***H***$_2$)$_{10}$–CH$_2$–NH–C(=O)–, signal A). δ_C (100 MHz, D_2O) 174, 67, 55, 54, 53, 52, 50, 48, 47, 46, 45, 44, 39, 38, 37, 36, 29, 28 and 27.

2.4. Gel permeation chromatography (GPC)

Molecular weight (M_n) of bolaamphiphile was analyzed by GPC (Waters 2690, MA, USA) with a differential refractometer detector (Waters 410, MA, U.S.A.). The mobile phase consisted of HPLC water with a flow rate of 1 ml/min. A Shodex OHpak SB-803 HQ (8.0 mm × 300 mm) column was used. Number and weight average molecular weights (M_n and M_w) as well as polydispersity index were calculated from a calibration curve using a series of PEG standards (PPS Polymer Standards Service, U.S.A., with molecular weight ranging from 600 to 20,600).

2.5. Infra-red spectroscpy (IR)

IR spectra of bolaamphiphile precursor and bolaamphiphile were recorded on a PerkinElmer spectrum 100 FT–IR spectrometer in conjunction with a Pike MIRacle single reflection ATR and Germanium crystal.

2.6. Preparation of bolaamphiphile/DNA complexes

Bolaamphiphile/DNA complexes were fabricated in aqueous solution using HPLC grade water by addition of DNA solution to bolaamphiphile solution at various N/P ratios. N/P ratio refers to the molar ratio of nitrogen atom in the polymer to phosphorus atom in DNA. In brief, for preparation of 50 μl of complexes, 25 μl of DNA solution containing 2.5 μg DNA was added to 25 μl of bolaamphiphile solution. The solution was vortexed gently for 3 s, and then allowed to equilibrate at room temperature for 30 min.

2.7. Particle size and zeta potential analyses

The particle size and zeta potential of the bolaamphiphile//DNA complexes were measured by dynamic light scattering (Brookhaven Instrument Corp., Holtsville, NY, U.S.A.) and Zetasizer (Malvern Instrument Ltd., Worchestershire, UK), respectively. In brief, bolaamphiphile/DNA complexes were formed in ultra pure water (pH 7.0) at room temperature at various N/P ratios (i.e. 2, 4, 6, 8, 10 and 12). All the particle size measurements were performed with a He–Ne laser beam at 658 nm and a scattering angle of 90°. The particle size and zeta potential measurements were repeated for 5 runs for each sample, and the data were reported as the average of 5 readings.

2.8. Gel retardation assay

The DNA binding ability of cationic bolaamphiphile and PEI at various N/P ratios was investigated by examining electrophoretic mobility of DNA in the DNA complexes at 1% agarose gel containing 4 μl of ethidium bromide (10 μg/μl) per 50 ml of gel solution. Briefly, 8 μL of the DNA complex solution containing 0.4 μg of DNA and corresponding polymer at their respective N/P ratios were mixed with 2 μl of 5 × DNA loading dye and loaded to each well. The same amount of naked DNA was used as the control. The gel was run at 80 mV for 70 min in TBE buffer. Following completion of assay, the gel was imaged under UV irradiation (Vilber Lourmat, France) and the picture was taken.

2.9. In vitro gene transfection

The *in vitro* gene transfection of bolaamphiphile/DNA complexes was performed in 5 cell lines namely HEK293, HepG2, NIH3T3, HeLa and 4T1. The cells were seeded onto 24-well plates at a density of 8×10^4 cells/well for luciferase transfection, and onto 12-well plates at a density of 2×10^5 cells/well for GFP transfection, and cultivated in 0.5 ml and 1.0 ml of the respective growth medium. After 24 h, the culture medium was replaced with fresh medium supplemented with serum, and the complex solution (50 μl) containing 2.5 μg luciferase reporter gene or 3.5 μg GFP reporter gene was added to each well. After 4 h of incubation, the culture media were replaced with the fresh media. For luciferase transfection, the cells were