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Low molecular weight PEI-based polycationic gene vectors via Michael addition polymerization with improved serum-tolerance

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ABSTRACT

A series of polycationic gene delivery vectors were synthesized via Michael addition from low molecular weight PEI and linking compounds with various heteroatom compositions. Agarose gel electrophoresis results reveal that these polymers can well condense plasmid DNA and can protect DNA from degradation by nuclease. The formed polyplexes, which are stable toward serum, have uniform spherical nanoparticles with appropriate sizes around 200–350 nm and zeta-potentials about +40 mV. *In vitro* experiments show that these polymers have lower cytotoxicity and higher transfection efficiency than 25 KDa PEI. Furthermore, the title materials exhibit excellent serum tolerance. With the present of 10% serum, up to 19 times higher transfection efficiency than PEI was obtained, and no obvious decrease of TE was observed even the serum concentration was raised to >40%. Flow cytometry and confocal microscopy studies also demonstrate the good serum tolerance of the materials.

1. Introduction

Gene therapy represents a promising option for the treatment of various diseases such as viral infections, inherited disorders and cancers [1–3]. The safe and effective delivery of genes with various types of vectors promises exceptional advancements in clinical disease treatment, next-generation vaccines, and tissue engineering [4–6]. Current gene delivery vectors can be divided into viral and non-viral. The use of non-viral vectors mitigates some issues generally associated with viral gene therapy, such as limited gene insertion size, immune response, mutagenesis and large scale production limitations [7–9]. These non-viral vectors consist of a diverse set of materials which are typically cationic, including natural and synthetic polymers, lipids, peptides, dendrimers and combinations of these structures [10,11]. Cationic polymers, which can efficiently complex with negatively charged DNA, thereby increasing DNA stability, were frequently studied. Among the polycations, poly(ethyleneimine) (PEI), a commercially available material, has been used as a gene delivery vector since 1995 and become one of the most promising and widely studied gene carriers [12,13] due in large part to efficient escape from the endocytic pathway through the proton-sponge mechanism. However, the gene transfection efficiency (TE) and cytotoxicity of PEI are heavily correlated with their chain length and topology [14,15]. High molecular weight (HMW) PEIs are effective in condensing nucleic acids but exhibit pronounced cytotoxicity and induce membrane damage in the initial stages of treatment [16] and mitochondrial-mediated apoptosis in the later stages. Meanwhile, low molecular weight (LMW) PEIs, bearing buffering capacity equivalent to their longer chain counterparts, are almost non-toxic but display poor TE owing to inefficient pDNA condensation and low cellular uptake through diminished charge-mediated interactions [17].

To achieve high TE as well as low toxicity, PEI might be modified by many strategies such as covalent grafting [18], cross linking [19] and electrostatic coating [20]. Crosslinked LMW PEIs have been examined over the past decade, and various types of hydrolytically or reductively degradable PEI polymers and networks have been designed for *in vitro* transfection. Zhong et al. reported the reversibly hydrophobilized 10 KDa PEIs based on rapidly acid-degradable acetal-containing hydrophobe for nontoxic and marked enhanced non-viral gene transfection [21]. Zhuo and co-

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workers described the disulfide cross-linked PEI via click reaction [22]. It was proved that these materials can maintain the TE of 25 KDa PEI with much less cytotoxicity.

For potential clinical applications, the interaction between electropositive polycation/pDNA complexes (polyplexes) and the negatively charged blood components cannot be ignored [23]. After intravenous injection, some unwanted effects would arise including the rapid clearance by the RES (Reticuloe Endothelin System) upon polyplexes aggregation, and structure destabilization as well as the premature DNA release and degradation [24]. Thereby it is highly indispensable to make models about the serum-conditioned transfection for the forecast evaluation on the *in vivo* TE of polycationic vectors. For example, zwitterionic betaine species were used for the functionalization of polymeric materials to enhance their serum-tolerance [25,26]. Zhuo et al. also put forward that branched PEI could be modified via the catalyst-free aminolysis reaction with 5-ethyl-5-(hydroxymethyl)-1,3-dioxan-2-oxo (EHDO) to promote the serum-tolerant capability [27]. Poly(ethylene glycol) (PEG) was also used to enhance the biocompatibility of the polymer vectors, and these modified PEIs maintained low cytotoxicity and showed enhanced transfection activity [28]. Meanwhile, they protected the polyplexes from undesired interactions with the negatively charged components in the bloodstream.

In the present study, we developed a series of polycations (**MP1**–**MP6**) via Michael addition from LMW PEI 600 Da and linking compounds (**LC1**–**LC6**). These materials showed good pH buffering capacity and DNA binding ability. Improved TEs were achieved compared to 25 KDa PEI, especially in the presence of serum.

2. Experimental details

2.1. Materials and methods

All chemicals and reagents were obtained commercially and were used as received. Anhydrous methanol and anhydrous chloroform were dried and purified under nitrogen by using standard methods and were distilled immediately before use. LMW PEI (branched, average molecular weight 600 Da, 99%) was purchased from Aladdin (Shanghai, China). 25 KDa PEI (branched, average molecular weight 25 KDa) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and nucleic acid labeling kit Label IT® Cy5™ was obtained from Mirus Bio Corporation (USA). The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA, coding for luciferase DNA) and pEGFP-N1 (Clontech, Palo Alto, CA, USA, coding for EGFP DNA). The Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen Corp. The MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). The luciferase assay kit was purchased from Promega (Madison, WI, USA). The endotoxin free plasmid purification kit was purchased from TIANGEN (Beijing, China).

^{1}H NMR spectra were obtained on a Bruker AV400 spectrometer. $CDCl_3$ or D_2O was used as the solvent and TMS as the internal reference. The molecular weight of polymers were determined by gel permeation chromatography (GPC) (Waters 515 pump, Waters 2410 Refractive Index Detector (25 °C, incorporating Shodex columns OHPAK KB-803). A filtered mixture of 0.5 mol L^{-1} HAc/NaAc buffer was used as the mobile phase with a flow rate of 0.5 mL min^{-1}. Molecular weights were calculated against poly(-ethylene glycol) standards of number average molecular weights ranging from 900 to 80,000 Da.

2.2. Synthesis and characterization linker *LC1*–*LC6*

Diols or diamine (0.048 mol) and triethylamine (4.81 g, 0.057 mol) were dissolved in anhydrous CH_2Cl_2 (50 mL). $(Boc)_2O$ (12.45 g, 0.057 mol) in anhydrous CH_2Cl_2 solution was added dropwise to the above stirred solution under the ice bath. The mixture was stirred overnight at room temperature, followed by evaporation of the organic solvents. The residue was purified with silica gel column chromatography (dichloromethane/methyl alcohol = 30: 1). The synthesis of naked two primary amine compound according to the literature [29]. Then, acryloyl chloride (6.95 g, 0.077 mol) in anhydrous dichloromethane (50 mL) was added dropwise to a stirred solution of diol or diamine (0.038 mol) and triethylamine (7.77 g, 0.077 mol) in anhydrous dichloromethane (50 mL) under the ice bath. The mixture was stirred overnight at room temperature and then filtered off generated salt, followed by evaporation of the volatile solvent. The residue was purified with silica gel column chromatography (PE: EA = 3: 1, v/v) to give **LC1**–**LC6**.

LC1 (Yield 41.2%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.44 (s, 9H, $(CH_3)_3CO$), 4.19–4.31 (m, 4H, $OCH_2CH(NH)CH_2O$), 4.84 (m, 1H, $OCH_2CH(NH)CH_2O$), 5.85–5.88 (d, *J* = 12 Hz, 2H, CH_2CHCO), 6.09–6.15 (t, *J* = 8 Hz, 2H, CH_2CHCO), 6.40–6.45 (d, *J* = 20 Hz, 2H, CH_2CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.92, 155.24, 131.80, 127.88, 80.24, 63.45, 48.61, 28.43. MALDI-HRMS: *m*/*z* 322.1265 ($[M+Na]^+$), $C_{14}H_{21}NO_6Na^+$, calc. 322.1267.

LC2 (Yield 55.3%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.42–1.71 (m, 6H, $CH_2(CH_2)_3CH_2$), 4.12–4.15 (m, 4H, $OCH_2(CH_2)_3CH_2O$), 5.77–5.80 (m, 2H, $(CH_2CHCO)_2$), 6.05–6.12 (m, 2H, $(CH_2CHCO)_2$), 6.34–6.39 (m, 2H, $(CH_2CHCO)_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.27, 130.67, 128.54, 64.32, 28.28, 22.52. MALDI-HRMS: *m*/*z* 235.0947 ($[M+Na]^+$), $C_{14}H_{21}NO_6Na^+$, calc. 235.0946.

LC3 (Yield 52.6%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.71–3.73 (t, *J* = 4 Hz, 4H, $CH_2CH_2OCH_2CH_2$), 4.28–4.30 (t, *J* = 4 Hz, 4H, $CH_2CH_2OCH_2CH_2$), 5.80–5.83 (m, 2H, $(CH_2CHCO)_2$), 6.08–6.15 (m, 2H, $(CH_2CHCO)_2$), 6.37–6.42 (m, 2H, $(CH_2CHCO)_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.13, 131.18, 128.22, 69.08, 63.58. MALDI-HRMS: *m*/*z* 237.0742 ($[M+Na]^+$), $C_{14}H_{21}NO_6Na^+$, calc. 237.0739.

LC4 (Yield 45.4%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.43 (s, 9H, $(CH_3)_3CO$), 3.50–3.54 (t, *J* = 16 Hz, 4H, $CH_2CH_2N(CO)CH_2CH_2$), 4.24–4.26 (t, *J* = 8 Hz, 4H, $OCH_2CH_2N(CO)CH_2CH_2O$), 5.83 (m, 2H, CH_2CHCO), 6.06–6.13 (t, *J* = 8 Hz, 2H, CH_2CHCO), 6.37–6.41 (d, *J* = 16 Hz, 2H, CH_2CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.02, 155.26, 131.36, 131.18, 128.26, 128.21, 80.43, 62.81, 62.68, 47.16, 46.93, 28.41. MALDI-HRMS: *m*/*z* 336.1426 ($[M+Na]^+$), $C_{15}H_{23}NO_6Na^+$, calc. 336.1423.

LC5 (Yield 33.2%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.44 (s, 18H, $\{(CH_3)_3CO\}_2$), 3.32–3.49 (t, *J* = 20 Hz, 8H, $CH_2CH_2N(CO)(CH_2)_2N(CO)CH_2CH_2$), 4.23–4.26 (t, *J* = 4 Hz, 4H, $OCH_2CH_2N(CO)(CH_2)_2N(CO)CH_2CH_2O$), 5.83–5.85 (d, *J* = 8 Hz, 2H, CH_2CHCO), 6.08–6.15 (t, *J* = 12 Hz, 2H, CH_2CHCO), 6.39–6.43 (d, *J* = 16 Hz, 2H, CH_2CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.04, 155.42, 155.21, 131.46, 131.26, 131.09, 128.35, 80.34, 80.14, 62.75, 62.61, 46.71, 46.19, 45.50, 28.46. MALDI-HRMS: *m*/*z* 479.2370 ($[M+Na]^+$), $C_{22}H_{36}N_2O_8Na^+$, calc. 479.2369.

LC6 (Yield 19.5%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.40 (s, 9H, $(CH_3)_3CO$), 3.43–3.53 (m, 8H, $NCH_2CH_2N(CO)CH_2CH_2N$), 5.63–5.66 (d, *J* = 12 Hz, 2H, CH_2CHCO), 6.07–6.34 (m, 4H, $\{CH_2CHCO\}_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.04, 157.12, 132.11, 131.06, 128.41, 126.61, 80.81, 49.59, 47.96, 40.04, 39.03, 28.41. MALDI-HRMS: *m*/*z* 334.1740 ($[M+Na]^+$), $C_{15}H_{25}N_3O_4Na^+$, calc. 334.1743.

2.3. Synthesis and characterization of target cationic polymers *MP1*–*MP6*

Polymers were successfully synthesized following modified Michael addition reaction as reported previously. Briefly, PEI 600 (1.26 mmol) and linker **LC1**–**LC6** (1.26 mmol) were separately dissolved in 1.5 mL of anhydrous methanol and 1.5 mL anhydrous