Biomaterials 34 (2013) 5391–5401

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Linear polycations by ring-opening polymerization as non-viral gene delivery vectors

Qin-Fang Zhang [a], Wen-Jing Yi [a], Bing Wang [a], Ji Zhang [a,**], Laifeng Ren [b], Qian-Ming Chen [c], Liandi Guo [b,**], Xiao-Qi Yu [a,*]

[a] Key Laboratory of Green Chemistry and Technology (Ministry of Education), College of Chemistry, Sichuan University, Chengdu 610064, PR China
[b] Laboratory of Genome Stability, Development and Stem Cell Institute, The West China Second Hospital, Sichuan University, Chengdu 610041, PR China
[c] State Key Laboratory of Oral Diseases, Sichuan University, Chengdu 610041, PR China

ARTICLE INFO

Article history:
Received 6 February 2013
Accepted 27 March 2013
Available online 10 April 2013

Keywords:
Gene delivery
Cationic polymer
Non-viral vector
Ring-opening polymerization
Structure–activity relationships

ABSTRACT

For a clinically effective non-viral gene delivery system, a non-toxic and highly efficient vector is of great importance. A series of linear cationic polymers were synthesized by the ring-opening polymerization between diglycidyl ethers and diamines. Their structure–activity relationships as gene delivery vectors were systematically studied. Besides the amino groups with various densities, these polymers have uniform distribution of hydroxyl groups, which were formed in the polymerization and may benefit their biocompatibility and serum-tolerance. These polymers have good DNA binding ability and could condense DNA into nanoparticles with proper sizes and zeta-potentials. MTT assay revealed that polyplexes formed from title polymers have lower cytotoxicity than that derived from PEI. Most of the polymers have higher transfection efficiency than 25 kDa PEI in the *in vitro* transfection experiments. Polymers prepared from diglycidyl ethers with less or no N atom (**2a** and **2b**) gave dramatically decreased TE, indicating that secondary amine on the backbone is highly required for efficient gene transfection, and compound **2** may be a good building block in the design of cationic polymers for gene delivery. More importantly, these polymers showed much better serum tolerance. Unlike PEI, the transfection mediated by **P5** was seldom affected by the presence of 10% serum. Cellular uptake and intracellular distribution studies also confirmed the good performance of **P5** in the transfection process with serum.

1. Introduction

In the past decades, cationic polymers as safer alternatives to viral vectors have received enormous attention for gene delivery [1,2]. Among the polymers explored, linear and branched polyethylenimine (PEI) are certainly the most studied vectors. The advantages of PEI include high condensation capability toward DNA, strong buffering capacity in the pH range of 7.4–5.1, and relatively high transfection efficiency (TE) [3,4]. These characteristics made PEI to be the golden standard for newly designed non-viral vectors, especially cationic polymers. However, the high charge density of PEI contributes significantly to its evident cytotoxicity due to the interaction with negatively charged cellular membranes. Besides, the fact that PEI failed to show similarly high TE when applied *in vivo* or in simulated physiological serum-contained environment also limited its application. This limitation is believed to have strong association with the non-specific interaction between DNA/polycation polyplexes and negatively charged protein [5,6]. Therefore, the design of new vectors to overcome the problem of cytotoxicity and increase the TE has become significant to improve clinically efficient gene therapy.

So far, a number of polycation vectors have been successfully fabricated with delicate designs [7–9]. Most of those polycation vectors were aimed at reduced cells cytotoxicity and improved TE *in vitro*, but intensive investigation for improving the stability and TE in the presence of serum was lack [10,11]. The introduction of polyethylene glycol (PEG) chains or other hydrophilic neutral polymers has previously been demonstrated to reduce surface charge and improve serum-tolerance by protecting the polyplexes from undesired interactions with serum albumin [12,13]. However, conjugation of PEG to the surface of cationic polymer particles might decrease their TE, and the surface-conjugated PEG hindered

* Corresponding author. Fax: +86 28 85415886.
** Corresponding authors.
E-mail addresses: ji_zhang@163.com (J. Zhang), lcmicro@yahoo.com (L. Guo), xqyu@scu.edu.cn (X.-Q. Yu).

0142-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.03.083

the interaction between the functional groups on polymer and receptors on cell surface [14,15]. Polysaccharides bearing plenty of hydroxyl groups were renewable, non-toxic, and biodegradable materials [16]. Reineke detailed the effect of hydroxyl groups on the process of gene delivery based on the research of a new library of poly(glycoamidoamine)s, and the results showed that the biological properties, particularly the delivery efficiency and serum-tolerance, of these polymers were affected by the hydroxyl groups [17–20]. More recently, many research groups have reported that the inclusion of hydroxyl groups in polycations would help to reduce cytotoxicity and improved gene delivery efficiency. Zhuo and co-workers found that through the reaction between a hydroxyl-containing cyclic carbonate of 5-ethyl-5-hydroxymethyl-1, 3-dioxan-2-oxo (EHDO) and PEI, the amine groups on PEI surface were replaced by two folds amount of hydroxyl groups, leading to enhance TE due to the good serum-tolerance and the shielding effect of the hydroxyl groups [7]. However, they also found that the introduction of hydroxyl groups into poly(aminoethyl methacrylate) (PAEMA) might decrease its TE toward 293T cells [21]. Therefore, a better understanding of such structure–activity relationships (SAR) is required.

We recently prepared a series of cationic polymers through the epoxide ring-opening polymerization, in which nonionic hydrophilic hydroxyl groups were introduced [22,23]. These compounds were expected to improve the cytotoxicity and serum-tolerance ability. Differing from the oxygen coating polymers, these materials have a uniform distribution of hydroxyls groups. Thus the disadvantageous effect on the transfection activity caused by the steric covering might be minimized, avoiding the aforementioned PEG dilemma. In this report, we further studied this type of cationic polymers with broader structural diversity. Their SAR in buffering and DNA binding ability, cytotoxicity and TE were systematically investigated.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals and reagents were obtained commercially and used without further purification. Absolute chloroform ($CHCl_3$) and dichloromethane (CH_2Cl_2) were distilled after being dried with calcium hydride (CaH_2). Anhydrous acetonitrile (CH_3CN) was distilled after being dried with P_2O_5. Column chromatography was performed using 200–300 mesh silica gel or 200–300 mesh Al_2O_3. All aqueous solutions were prepared from deionized or distilled water. 2-[1,4,7] Triazonan-1-yl-ethanol was prepared according to reported procedures [24]. The ^1H NMR and ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (^1H, 0.00 ppm). HR-MS spectral data was recorded on a Bruker Daltonics Bio TOF mass spectrometer. MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). Luciferase assay kit was purchased from Promega (Madison, WI, USA). Endotoxin-free plasmid purification kit was purchased from TIANGEN (Beijing, China). 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. Cy5™ was obtained from Molecular Probe (Mirus, Madison, WI, USA). 293T human embryonic kidney cell lines, A549 lung cancer cell lines and U-2OS human osteosarcoma cancer cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

2.2. Synthesis of title polymers

2.2.1. Preparation of N, N′-2Boc-N, N′-Bis (2-hydroxyethyl) ethylenediamine (compound **1**)

Di-tert-butyl dicarbonate (8.72 g, 40 mmol) in anhydrous dichloromethane (100 mL) was added dropwise to a stirred solution of *N*, *N*′-Bis (2-hydroxyethyl) ethylenediamine (2.96 g, 20 mmol) and triethylamine (6.7 mL, 4.84 g, 48 mmol) in anhydrous dichloromethane (50 mL) under the ice bath. The resulting reaction mixture was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure to give the product as pale yellow solid. Then 150 mL ethyl acetate was added to the solid and stirred for 0.5 h, filtered and gave the product as colorless solid, which was further purified by chromatography over silica (v/v 30:1, CH_2Cl_2-MeOH) as colorless solid. Yield: 50%. ^1H NMR (400 MHz, DMSO, TMS): $\delta = 1.39$ (s, 18H, −Boc), 3.18 (m, $J = 4$ Hz, 4H, $-CH_2CH_2OH$), 3.29 (s, 4H, $-NCH_2CH_2N-$), 3.45 (m, $J = 4$ Hz, 4H, $-CH_2CH_2OH$), 4.66 (s, 2H, $-OH$). ^{13}C NMR (DMSO, 100 MHz): δ 28.02, 48.93, 49.60, 55.99, 58.95, 59.35, 78.38, 154.55. HR-MS (ESI): Calcd for: $C_{16}H_{32}N_2O_6$: 371.2158 $[M + Na]^+$; Found: 371.2156 $[M + Na]^+$.

2.2.2. Preparation of N, N′-2Boc-N, N′-Bis (2-(oxiran-2-ylmethoxy)ethyl) ethylenediamine (compound **2**)

A mixture of epichlorohydrin (3.5 g, 37.9 mmol), sodium hydroxide pellets (1.5 g, 37.5 mmol), water (0.18 mL, 10 mmol), tetrabutylammonium bromide (96 mg, 0.3 mmol) and compound **1** (2.2 g, 6.3 mmol) were stirred for 4 h at 40 °C. The reaction mixture was filtered off and the solid was washed with dichloromethane. The combined organic layer was dried with anhydrous magnesium sulfate. The solvent and excess epichlorohydrins were distilled off under reduced pressure and the residue was purified by silica gel column chromatography (v/v 4: 1, PE–EA) to give compound **2** as colorless oil. Yield: 80%. ^1H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 1.45$ (s, 18H, −Boc), 2.60 (s, 2H, Epoxyresin $-CH_2-$), 2.78 (t, $J = 4$ Hz, 2H, Epoxyresin $-CH_2-$), 3.12 (m, $J = 4$ Hz, 2H, Epoxyresin $-CH-$), 3.38 (m, 10H, $-CH_2OCH_2CH_2N-$ and $-NCH_2CH_2N-$), 3.61 (m, 4H, $-OCH_2CH_2N-$), 3.72 (m, 4H, $-CH_2OCH_2CH_2N-$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 28.40, 44.11, 47.20, 50.71, 66.36, 69.85, 71.62, 79.63, 155.33. HR-MS (ESI): Calcd for: $C_{22}H_{40}N_2O_8$: 483.2682 $[M + Na]^+$; Found: 483.2675 $[M + Na]^+$.

2.2.3. Preparation of compound ***R5***

O-Boc–phenol [25] was firstly prepared. PhOH (6.8 g, 72 mmol) and DMAP (0.88 g, 7.2 mmol) were dissolved in anhydrous CH_2Cl_2 (100 mL). Under ice bath, a solution of $(Boc)_2O$ (16 g, 72 mmol) in CH_2Cl_2 was added dropwise to the above solution. After stirring 2 h at room temperature, the reaction mixture was condensed under reduced pressure. The residue was purified by alumina gel column chromatography (v/v 20: 1, PE–EA) to give *O*-Boc–phenol as colorless oil. Yield: 82%.

O-Boc–phenol (9.5 g, 49 mmol) in anhydrous ethanol (100 mL) was added dropwise to a stirred solution of triethylenetetramine (3.6 g, 25 mmol) in anhydrous ethanol (50 mL) under the ice bath. And then refluxed for 12 h, the reaction mixture was condensed under reduced pressure. The residue was purified by alumina gel column chromatography (v/v 10: 1, CH_2Cl_2–MeOH) to give compound **R5** as colorless oil. Yield: 30%. ^1H NMR (400 MHz, $CDCl_3$, TMS): $\delta = 1.45$ (s, 18H, −Boc), 2.74 (t, $J = 4$ Hz, 8H, $BocNHCH_2CH_2NHCH_2-$), 3.22 (d, 4H, $BocNHCH_2CH_2NHCH_2-$). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 28.40, 40.24, 48.75, 49.04, 79.13, 156.20. HR-MS (ESI):Calcd for: $C_{16}H_{34}N_4O_4$: 347.2658 $[M + H]^+$; Found: 347.2659 $[M + H]^+$.

2.2.4. Preparation of polymers (***P1*** ∼ ***P7***)

Amino compound (**R1** ∼ **R7**) (0.658 mmol) was dissolved in C_2H_5OH, respectively. Then compound **2** (303 mg, 0.658 mmol) was added to the solution. Under the protection of N_2, the reaction mixture was stirring at 80 °C for 72 h. The solvent was removed under reduced pressure. Then polymers were dissolved in CH_2Cl_2, and MeOH/HCl solution was added. The reaction mixture was stirring overnight at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in a small amount of water. Then 5 N NaOH aqueous solution was added to adjust pH to 12 and dialyzed (MWCO 3500 kDa) against deionized water for 3 days. The product was obtained as white or pale-yellow solid after lyophilization. Yield: 22%–56%.

2.2.5. Preparation of polymers ***P5a*** and ***P6a***

Amino compound (**R5 or R6**) (0.5 mmol) was dissolved in C_2H_5OH, then compound **2a** [22] (159 mg, 0.5 mmol) was added to the solution. Under the protection of N_2, the reaction mixture was stirring at 80 °C for 72 h. The solvent was removed under reduced pressure. Then polymers were dissolved in CH_2Cl_2, and MeOH/HCl solution was added. The reaction mixture was stirring overnight at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in a small amount of water. Then 5 N NaOH aqueous solution was added to adjust pH to 12 and dialyzed (MWCO 3500 kDa) against deionized water for 3 days. The product was obtained as white or pale-yellow solid after lyophilization. Yield: 30% ∼ 50%.

2.2.6. Preparation of polymers ***P5b*** and ***P6b***

Amino compound (**R5 or R6**) (0.5 mmol) was dissolved in C_2H_5OH, then compound **2b** [23] (131 mg, 0.5 mmol) was added to the solution. Under the protection of N_2, the reaction mixture was stirring at 80 °C for 72 h. The solvent was removed under reduced pressure, and the residue was dissolved in a small amount of water. Then 5 N NaOH aqueous solution was added to adjust pH to 12 and dialyzed (MWCO 3500 kDa) against deionized water for 3 days. The product was obtained as white or pale-yellow solid after lyophilization. Yield: 45% ∼ 70%.

2.2.7. Polymer characterization

^1H NMR spectra in D_2O were obtained on a Bruker AV 400-MHz instrument at 25 °C. The molecular weights (M_w) and polydispersity (PDI, M_w/M_n) of prepared cationic polymers were determined by a gel permeation chromatography (GPC) system, which consisted of a Waters 515 pump, a Linear 7.8 × 300 mm column (Waters Corp, Milford, MA, USA), an 18-angle laser scattering instrument (Wyatt