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Multifunctional cationic polyurethanes designed for non-viral cancer gene therapy

Jian Cheng [1], Xin Tang [1], Jie Zhao, Ting Shi, Peng Zhao, Chao Lin *

Shanghai East Hospital, Institute for Biomedical Engineering and Nanoscience, Tongji University School of Medicine, Tongji University, 200092 Shanghai, PR China

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ABSTRACT

Nano-polyplexes from bioreducible cationic polymers have a massive promise for cancer gene therapy. However, the feasibility of cationic polyurethanes for non-viral gene therapy is so far not well studied. In this work, a linear cationic polyurethane containing disulfide bonds, urethane linkages and protonable tertiary amino groups was successfully generated by stepwise polycondensation reaction between 2,2′-dithiodiethanol bis(p-nitrophenyl carbonate) and 1,4-bis(3-aminopropyl)piperazine (BAP). We confirmed that the cationic polyurethane (denoted as PUBAP) displayed superior gene delivery properties to its cationic polyamide analogue, thus causing higher *in vitro* transfection efficiency in MCF-7 and SKOV-3 cells. Besides, further folate-PEGylation and hydrophobic deoxycholic acid (DCA) conjugation to amino-containing PUBAP can be conducted to afford multifunctional polyurethane gene delivery system. After optimization, folate-decorated nano-polyplexes from the PUBAP conjugated with 8 folate-PEG chains and 12 DCA residues exhibited superb colloidal stability under physiological conditions, and performed rapid uptake via folate receptor-mediated endocytosis, efficient intracellular gene release and nucleus translocation into SKOV-3 cells *in vitro* and in vivo. Importantly, PUBAP based polyplexes possess low cytotoxicity as a result of PUBAP biodegradability. Therefore, marked growth inhibition of SKOV-3 tumor xenografted in Balb/c nude mice was achieved with negligible side effects on the mouse health after intravenous administration of PUBAP based polyplexes with a therapeutic plasmid encoding for TNF-related apoptosis-inducing ligand. This work provides a new insight into biomedical application of bio-responsive polyurethanes for cancer therapy.

Statement of significance

In this study, we have confirmed that disulfide-based cationic polyurethane presents a new non-viral vector for gene transfer and cancer gene therapy. The significance of this work includes: (1) design and synthesis of a group of novel disulfide-based cationic polyurethane by non-isocyanate chemistry; (2) comparative study of transfection activity between cationic polyurethanes and cationic polyamides; (3) feasibility of bioreducible cationic polyurethanes for in vivo cancer gene therapy.

1. Introduction

The essential concept of cancer gene therapy involves the utilization of therapeutic genes instead of chemotherapeutic drugs for killing cancer cells [1]. However, for successful cancer gene therapy, how to safely and efficiently deliver therapeutic gene into targeted cells remains a challenge. This delivery problem is addressed by the development of safe and robust gene delivery vehicles, both viral and non-viral vectors. Although viral vectors such as adeno-associated virus have been approved and applied for clinical trials, viral vector-induced side effects such as systemic toxicity and inflammatory response critically impede their clinical translations [2]. As an alternative to viral vectors, non-viral vectors based on cationic polymers and cationic lipids hold much safer features and gain additional advantages including flexible synthesis and chemical conjugation, high gene-carrying ability and easy large-scale production at low cost [3]. Over the past two decades, biodegradable cationic polymers having biodegradable linkages for non-viral gene transfer have received rapid growing interest since these polymers have lower toxicity when compared to their

* Corresponding author.
E-mail address: chaolin@tongji.edu.cn (C. Lin).
[1] The two authors contribute equally as co-first author.

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non-degradable analogues like polyethylenimine (PEI) [4,5]. Besides, disulfide-based (bioreducible) polymers as non-viral gene vectors have received much attention in the past few years [6]. Disulfide bond is known as a bio-responsive linkage due to unique bio-property, that is, cleavable in an intracellular reducing environment but inert in an extracellular environment [7]. This bio-responsive cleavaging of disulfide bond in bioreducible polyplexes can trigger adequate dissociation and then gene release from the polyplexes, thereby promoting transfection efficacy [8]. Thus, bioreducible cationic polymer systems have been regarded to be highly potential for safe and efficient gene therapy [9].

Most of bioreducible polymer systems reported to date have either amide or ester linkage. For example, a great deal of bioreducible poly(amido amine)s were prepared by Michael-type addition and investigated as non-viral vectors for gene delivery *in vitro* [10]. Recently, Green et al. designed bioreducible poly(amino ester)s for nucleic acid transfection [11]. However, the design of cationic polymers with urethane linkage for gene delivery has not received much attention. Although a lot of nanoscale polyurethanes have been applied widely in biomedical applications such as drug delivery and tissue engineering [12], cationic polyurethanes as gene delivery vectors have only been rarely reported. For example, Yang et al. prepared cationic polyurethanes via polyaddition reaction between diisocyanate and N-methyldiethanolamine and they found moderate *in vitro* transfection ability of these polyurethanes [13]. Nevertheless, no report has appeared on the synthesis and evaluation of bioreducible cationic polyurethanes as non-viral gene delivery vectors. Thus, there is a fundamental need to elucidate the potential of bioreducible cationic polyurethanes for non-viral gene delivery.

Despite of high *in vitro* transfection efficacy of bioreducible polymer systems based on polyamides and PEI, their further applications for in vivo gene therapy are relatively limited. Currently, in vivo studies with bioreducible polymer systems largely focus on local gene administration for cancer therapy and cardiovascular disease therapy [14–17]. Moreover, for systemic (e.g. intravenous) gene administration of cationic polyplexes, non-directed targeting ability and low colloidal stability in physiological conditions are major problems. To tackle these, Kim et al. reported on RGD peptide-directed polyplexes of bioreducible poly(disulfide amine)s for gene delivery targeting MCF-7 cells [18]. We recently showed that a bioreducible cationic dextran system was efficient for prolonged gene delivery targeting SKOV-3 tumor [19]. However, further study on anti-tumor activity of bioreducible polymer systems is still needed by using therapeutic gene plasmids.

The aim of this work is to design bioreducible cationic polyurethanes and evaluate their potential as non-viral vectors for both *in vitro* and in vivo gene delivery targeting cancer cells. To this end, an aminolysis chemistry of *p*-nitrophenyl ester compounds was used in this study. By this method, a bioreducible cationic polyurethane containing multiple disulfide and 1,4-bis(3-amnio propyl)piperazine (BAP) residue (denoted as PUBAP) was designed and prepared. For comparison, a bioreducible cationic polyamide having disulfide bonds and BAP residues (denoted as PABAP) was also prepared as PUBAP analogue. Initially, a comparative study between PUBAP and PABAP was performed in terms of gene delivery properties and transfection ability. Next, chemical modification and conjugation of amino-containing PUBAP with folate-PEG and deoxycholic acid were conducted and optimized to achieve multifunctional PUBAP system for gene delivery targeting SKOV-3 cells over-expressing folate receptor [20]. Moreover, anti-tumor efficacy of SKOV-3 tumor bearing in a Balb/c nude mouse was investigated by intravenous injection of PUBAP-based formulation containing a therapeutic plasmid encoding for the tumor necrosis factor related apoptosis inducing ligand (TRAIL). Preliminary biocompatibility study of PUBAP system was also carried out by routine blood assay and immunohistochemistry.

2. Materials and methods

2.1. Materials

All chemicals were purchased in the highest purity and used directly without further purification. Tris(2-aminoethyl) amine (TAA), dicyclohexyl carbodiimide (DCC), N-hydroxysuccinimide (NHS), 1,4-bis(3-aminopropyl)piperazine (BAP), dithiothreitol (DTT), *p*-nitrophenyl chloroformate (PNC), di-tert-butyl dicarbonate (Boc_2O), anhydrous pyridine, trifluoroacetic acid (TFA), Rhodamine B isocyanate (Rh), deoxycholic acid (DCA), branched polyethylenimine (BPEI, 25 kDa), heparin, and 2,2′-dithiodiethanol (DTDE) were purchased from Sigma–Aldrich. The plasmids, pCMV-GFP and pCMV-Luc, contained a CMV promoter and encoded for the green fluorescent protein (GFP) and luciferase (Luc), respectively, were ordered from Plasmid Factory (Germany). The plasmid pUNO1-TRAILa, contained an EF-1α and HTLV hybrid promoter, was purchased from InvivoGen Co. and amplified in *Escherichia coli*. DH5α culture. Bis(2-aminoethyl) 2-(tert-butoxycarbonylamino) ethyl amine (Boc-TAA) was obtained by the method as reported previously [21]. Bioreducible cationic polyamide comprising BAP residue (PABAP) was prepared according to our previous method [22]. Poly (ethylene glycol) (PEG) derivative, α-folate, ω-carboxylic acid PEG (denoted as folate-PEG-COOH) was prepared by coupling folate to α-amine, ω-carboxylic acid PEG (M_w = 3350) via DCC/NHS activation.

2.2. Monomer and polymer synthesis

2.2.1. Synthesis of 2,2′-dithiodiethanol bis(p-nitrophenyl carbonate) (DTDE-PNC)

DTDE-PNC was synthesized by esterification reaction of OH group in DTDE with PNC for overnight (Scheme S1). As a typical example, PNC (10.3 g, 51.0 mmol), DTDE (4 g, 46.7 mmol OH) and pyridine (7.4 g, 93.4 mmol) in DCM (100 mL) solvent were stirred at 0 °C under nitrogen protection over 2 h. Next, the reaction was continued for overnight at room temperature. After the removal of DCM solvent under vacuum, the residue was purified on a silica column with DCM as a dilute (R_f = 0.89). DTDE-PNC was finally obtained as a waxy solid (yield: 5.6 g, 40%). ^{1}H NMR ($CDCl_3$, ppm): δ 8.26 (aromatic protons, 4H), 7.37 (aromatic protons, 4H), 4.57 (2 × $SSCH_2C\underline{H}_2$, 4H), and 3.08 (2 × $SSC\underline{H}_2CH_2$, 4H) (Fig. S1).

2.2.2. Synthesis of bioreducible cationic polyurethane containing BAP residue (PUBAP)

Bioreducible cationic polyurethane derived from BAP was prepared by polycondensation reaction of DTDE-PNC and BAP for 3 days (Scheme 1a). In a typical procedure, DTDE-PNC (1.2 g, 2.5 mmol) and BAP (0.5 g, 2.5 mmol) were mixed in anhydrous DMF (15 mL) as a solvent in a bottom flask. The reaction was preceded for 3 days in the dark at 40 °C under nitrogen protection. Next, an excess amount of BAP (0.05 g) was added into reaction system to consume any unreacted *p*-nitrophenyl residues for 2 days. The resulting mixture was then dissolved with deionzed water at pH ~ 4 and purified by ultrafiltration process (1000 MWCO) with deionzed water at pH ~ 4. PUBAP was obtained as a white powder after freeze-drying (yield: 0.55 g, 76%).^{1}H NMR (D_2O, ppm): δ4.30 (2 × $SSCH_2C\underline{H}_2$, 4H), 3.4–4.0 (2 × $NC\underline{H}_2C\underline{H}_2N$, 8H), 3.31 (2 × $OCONHC\underline{H}_2$, 4H), 3.21 (2 × $OCONHCH_2C\underline{H}_2$, 4H), 2.95 (2 × $SSC\underline{H}_2CH_2$, 4H), and 1.95 (2 × $CH_2C\underline{H}_2CH_2$, 4H).

2.2.3. Synthesis of amine-containing bioreducible cationic polyurethane (pBT)

Bioreducible cationic polyurethane with primary amine side chain (denoted as pBT) was prepared by polycondensation reaction