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A family of bioreducible poly(disulfide amine)s for gene delivery

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ABSTRACT

A family of bioreducible poly(disulfide amine)s, which differ in the length of polymethylene spacer [–$(CH_2)_n$–] in the main chain and the side chain, has been synthesized. These bioreducible poly(disulfide amine)s exhibit local environment specific degradability and are associated with lower cytotoxicity than branched poly(ethylenimine) (bPEI, 25 kDa). These cationic polymers also show higher buffering capacity and protonation degree than bPEI, facilitating the endosomal escape of carried genetic materials. The transfection efficiency of these agents is oligomethylene length dependent. Poly(cystaminebisacrylamide-spermine) [poly(CBA-SP)], poly(cystaminebisacrylamide-bis(3-aminopropyl)-1,3-propanediamine) [poly-(CBA-APPD)], and poly(cyxtaminebisacrylamide-bis(3-aminopropyl)-ethylenediamine) [ploy(CBA-APED)] with longer propylene [–$(CH_2)_3$–] side spacer, demonstrate higher transfection efficacy than the counterpart poly(cystaminebisacrylamide-bis(2-aminoethyl)-1,3-propanediamine) [poly(CBA-AEPD)] and poly(cyst-aminebisacrylamide-triethylenetetramine) [poly(CBA-TETA)], which have shorter ethylene [–$(CH_2)_2$–] side spacer. The poly(CBA-SP), poly(CBA-APPD), poly(CBA-APED) with the main chain spacer of –$(CH_2)_4$–, –$(CH_2)_3$–, –$(CH_2)_2$– demonstrate similar transfection efficiency, indicating the length of polymer main chain spacer has less influence on transfection efficiency. However, with the same short ethylene [–$(CH_2)_2$–] side spacer, poly(CBA-AEPD), with the longer main chain oligomethylene units [–$(CH_2)_3$–], showed relatively higher transfection efficiency than poly(CBA-TETA), having shorter main chain oligomethylene units [–$(CH_2)_2$–]. Of these polymeric carriers, poly(CBA-SP) demonstrated the highest transfection in the C2C12 cell line, while poly(CBA-APED) showed the highest transfection in the HeLa cell line. All of these agents showed greater transfection activity than commercialized bPEI 25 kDa. The poly(disulfide amine)s are promising safe and efficient non-viral vectors for gene delivery.

1. Introduction

The development of safe and efficient vectors is a major challenge in the development of gene therapy to treat human diseases [1,2]. Gene delivery vectors are classified into viral and non-viral vectors, whose advantages and disadvantages have been well documented [3,4]. Cationic polymers are one of the main categories of non-viral vectors, having received greater attention recently because of their inherent advantages, including non-immunogenicity, stability, capacity to carry large nucleic acid loads, and ease of manufacturing [5,6]. By condensing nucleic acids into nanoparticles through electrostatic interactions, polymeric carriers can protect nucleic acids from nuclease degradation, facilitate cellular uptake of polymer/DNA complexes (polyplexes) via endocytosis, and induce efficient gene expression [5,7]. Over the last two decades, many cationic polymers have been synthesized for gene delivery, such as poly(ethylenimine) (PEI), poly(L-lysine) (PLL) and polyamidoamine dendrimers [8,9]. The main drawback for these cationic polymers, however, is their high level of cytotoxicity, mostly due to their non-degradability and accumulation [10]. Therefore, the design of new vectors to overcome the problem of cytotoxicity and increase the efficiency of gene transfection has become mandatory to advance the development of clinically efficacious gene therapy.

To successfully deliver DNA into nucleus, polymeric vectors must have multiple functional groups to overcome a series of extra- and intracellular barriers. The first barrier is that polymers need to condense DNA and form stable and positively-charged polyplexes [1,9,10]. Previously, poly(amido amine)s (PAAs) with protonated amines bind DNA more strongly than their counterpart polymers lacking protonated amine groups [11]. The modification of poly-(β-amino ester)s end groups from alcohol to primary amine groups also increases the polymers' DNA binding affinity [12,13].

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Protonatable primary amine groups in the pendant side chains allow for strong DNA binding ability and charge density and therefore the ability to condense DNA into nanosized particles extracellularly.

The second barrier to gene delivery is the ability of the polymer to escape from the endosomal–lysosomal pathway [3,10]. Behr and others introduced the hypothesis of the "proton sponge effect" and defined polymers having good buffering capacity (as the percentage of amine groups becoming protonated between pH 7.4 and 5.1) as facilitating endosomal rupture via osmolysis and thereby inducing efficient gene expression [9,14]. The combination of titratable amine groups with basic p*Ka* values enables good buffering capacity of a polymer and enhances DNA binding capability. As a result, gene transfection efficiency increases by incorporating different amine groups into polymer structures. Our previously synthesized poly(amido ethylenimine)s containing different amine groups have demonstrated significant higher transfection efficiencies than bPEI 25 kDa in a variety of cell lines [15,16]. Poly(amido amine)s (PAAs) containing different amine groups showed high buffering capacities and similar or higher transfection efficiency than bPEI 25 kDa in COS-7 cells [17].

To efficiently induce gene expression with low cytotoxicity, polymeric vectors should not only have superior DNA binding capability and buffering capacity, but also be able to be degraded into small and non-toxic molecules [4]. To achieve this goal of degradability, the use of disulfide bonds has received more attention in the design of "bioreducible" polymers [18,19]. Polymers with disulfide bonds can form stable complexes with DNA in the extracellular oxidative environment, while readily release DNA intracellularly via the cleavage of the disulfide bonds by glutathione and other small redox molecules with free thiol groups (5–10 mM) through the efficient disulfide-thiol exchange reaction [20,21]. As a result, gene expression is enhanced and cytotoxicity is significantly reduced. Previously, improved gene transfection efficiency and decreased cytotoxicity have been observed when using low molecular weight PEI with disulfide bonds [22,23]. Recently, PAAs containing disulfide bonds have been demonstrated to have higher transfection efficiency and lower cytotoxicity than the counterpart PAAs without disulfide linkages [24].

To explore the studies of (1) side chain length and (2) main chain spacer on polymeric DNA binding capability, buffering capacity, protonation degree and basicity, biodegradability and gene transfection, we synthesized a family of biodegradable poly(disulfide amine)s as polymeric gene vectors. These poly(disulfide amine)s are synthesized with defined structures: two pendant primary amine groups at the side chains, two tertiary amine groups and one disulfide bond in the backbond in each repeating unit and different lengths of oligomethylene spacers [$-(CH_2)_n-$, $n = 2$–4] in the main and side chains. The characterization of the polymers includes the measurement of buffering capacity and particle size, DNA condensation and releasing ability, *in vitro* transfection efficiency, cytotoxicity and fluorescence-labeled cellular uptake. We hypothesized that chemical structures in polymer design may play important roles in gene transfection efficiency and cytotoxicity.

2. Materials and methods

2.1. Materials

All chemicals: spermine (SP, Sigma, St. Louis, MO), *N,N′*-bis(3-aminopropyl)-1,3-propanediamine (APPD, Sigma–Aldrich, St. Louis, MO), *N,N′*-bis(3-aminopropyl)-ethylenediamine (APED, Acros Organics, Fair Lawn, NJ), *N,N′*-bis(2-aminoethyl)-1,3-propanediamine (AEPD, Sigma–Aldrich, St. Louis, MO), triethylenetetramine (TETA, Sigma–Fluka, St. Louis, MO), *N,N′*-cystaminebisacrylamide (CBA, PolySciences, Warrington, PA), branched polyethylenimine (bPEI, $M_w = 25$ kDa, Sigma, St. Louis, MO), ethylenediamine (EDA, Sigma–Aldrich, St. Louis, MO), 2-acetyldimedone (Dde-OH, EMD Chemicals, Inc. Gibbstown, NJ), hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, Sigma–Aldrich, St. Louis, MO), imidazole (Sigma–Aldrich, St. Louis, MO), *N*-methyl-2-pyrrolidinone (NMP, Sigma–Aldrich, St. Louis, MO), *N,N*-dimethylformamide (DMF, Sigma–Aldrich, St. Louis, MO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St. Louis, MO), dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO), dithiothreitol (DTT, Sigma–Aldrich, St. Louis, MO), and SYBR® Safe DNA gel stain (10,000× concentrate in DMSO, Invitrogen, Carlsbad, CA) were purchased in the highest purity and used without further purification. The plasmid pCMV-Luc, containing a firefly luciferase reporter gene, was amplified in *E. coli* DH5α and purified by standard Maxiprep kit (Invitrogen, Carlsbad, CA). Dulbecco's Modified Eagle's medium (DMEM), penicillin–streptomycin (P/S), fetal bovine serum (FBS), trypsin-like enzyme (TrypLE Express) and Dulbecco's phosphate buffered saline (PBS) were all purchased from Invitrogen-Gibco (Carlsbad, CA). Luciferase assay system with reporter lysis buffer was purchased from Promega (Madison, WI). The BCA™ protein assay system was purchased from Thermo Scientific (Rockford, IL). YOYO-1 iodide (1 mM solution in DMSO) was purchased from Molecular Probes (Eugene, OR). HeLa cells (human cervical cancer cell line) and C2C12 (mouse myoblast cell line) were purchased from the American Type Culture Collection (ATCC) and cultured according to recommended protocols.

2.2. Polymer synthesis

The synthesis of five novel poly(disulfide amine)s was shown in Scheme 1. The synthetic route of poly(cystaminebisacrylamide-spermine) [poly(CBA-SP)] is described here as the representative procedure. Briefly, SP (0.202 g, 1 mmol) and Dde-OH (0.419 g, 2.3 mmol) were dissolved in 1 mL MeOH, stirring at room temperature for 24 h to let Dde group specifically protect primary amine groups in spermine. The next day, CBA (0.260 g, 1 mmol) was added into above system in 1.5 mL of MeOH/diH_2O (9/1 v/v). Polymerization was conducted at 60 °C in the dark under nitrogen atmosphere for 3–4 days. Then, 10% mol excess EDA was added to consume any unreacted acrylamide groups, stirring at 60 °C for at least additional 2 h. The product was precipitated in 40 mL anhydrous diethyl ether to get the intermediate polymer poly(CBA-SP-Dde). The Dde groups were removed in the deprotection mixture of $NH_2OH{\cdot}HCl$/Imidazole/NMP/DMF, stirring at room temperature for 4 h. The deprotection mixture was prepared as follows: 1.25 g (1.80 mmol) of $NH_2OH{\cdot}HCl$ and 0.918 g (1.35 mmol) of imidazole were suspended in 5 mL of NMP. The mixture was sonicated until complete dissolution. Just before reaction, 5 volume of this solution was diluted with 1 volume of DMF to make the deprotection mixture [25]. After deprotection, the crude product was purified by dialysis (MWCO = 1000) against MilliQ water for 24 h, followed by lypholization to obtain poly(CBA-SP) as solid gel. Poly(cystaminebisacrylamide-bis(3-aminopropyl)-1,3-propanediamine) [poly(CBA-APPD)], poly(cyxtaminebisacrylamide-bis(3-aminopropyl)-ethylenediamine) [poly-(CBA-APED)], poly(cystaminebisacrylamide-bis(2-aminoethyl)-1,3-propanediamine) [poly(CBA-AEPD)] and poly(cystaminebisacrylamide-triethylenetetramine) [poly-(CBA-TETA)] were synthesized in the same way. These poly(disulfide amine)s were then analyzed by ^{1}H NMR (400 MHz, D_2O, δ, ppm) and the data were listed as below:

Poly(CBA-SP): $\delta = 3.37$ ($CONHCH_2CH_2SS$, 4H), 2.87 ($CONHCH_2CH_2SS$, 4H), 2.77 ($NCH_2CH_2CH_2NH_2$, 4H), 2.70 ($NHCOCH_2CH_2N$, 4H), 2.53 ($NCH_2CH_2CH_2NH_2$, 4H), 2.47 ($NCH_2CH_2CH_2CH_2N$, 4H), 2.34 ($NHCOCH_2CH_2N$, 4H), 1.75 ($NCH_2CH_2CH_2NH_2$, 4H), 1.35 ($NCH_2CH_2CH_2CH_2N$, 4H).

Poly(CBA-APPD): $\delta = 3.36$ ($CONHCH_2CH_2SS$, 4H), 2.86 ($CONHCH_2CH_2SS$, 4H), 2.71 ($NHCOCH_2CH_2N$, 4H; $NCH_2CH_2CH_2NH_2$, 4H), 2.46 ($NCH_2CH_2CH_2CH_2N$, 4H), 2.36 ($NCH_2CH_2CH_2NH_2$, 4H), 2.30 ($NHCOCH_2CH_2N$, 4H), 1.70 ($NCH_2CH_2CH_2NH_2$, 4H), 1.53 ($NCH_2CH_2CH_2N$, 2H).

Poly(CBA-APED): $\delta = 3.38$ ($CONHCH_2CH_2SS$, 4H), 2.86 ($CONHCH_2CH_2SS$, 4H), 2.70 ($NHCOCH_2CH_2N$, 4H; $NCH_2CH_2CH_2NH_2$, 4H), 2.49 ($NCH_2CH_2CH_2NH_2$, 4H; NCH_2CH_2N, 4H), 2.30 ($NHCOCH_2CH_2N$, 2H), 1.50 ($NCH_2CH_2CH_2NH_2$, 4H).

Poly(CBA-AEPD): $\delta = 3.37$ ($CONHCH_2CH_2SS$, 4H), 2.94 ($CONHCH_2CH_2SS$, 4H), 2.82 ($NCH_2CH_2NH_2$, 4H), 2.70 ($NHCOCH_2CH_2N$, 4H), 2.60 ($NCH_2CH_2NH_2$, 4H), 2.44 ($NCH_2CH_2CH_2N$, 4H), 2.30 ($NHCOCH_2CH_2N$, 4H), 1.48 ($NCH_2CH_2CH_2N$, 2H).

Poly(CBA-TETA): $\delta = 3.38$ ($CONHCH_2CH_2SS$, 4H), 2.93 ($CONHCH_2CH_2SS$, 4H), 2.83 ($NCH_2CH_2NH_2$, 4H), 2.69 ($NHCOCH_2CH_2N$, 4H), 2.62 ($NCH_2CH_2NH_2$, 4H), 2.49 (NCH_2CH_2N, 4H), 2.30 ($NHCOCH_2CH_2N$, 4H). The ^{1}H NMR spectra of all poly(disulfide amine)s are given in the Supporting Information.

2.3. Polymer characterization

The molecular weights of five poly(disulfide amine)s were determined by size exclusion chromatography (SEC) on an AKTA FPLC system (Amersham Biosciences, Piscataway, NJ) equipped with a superose 12 column, and UV and refractive index detectors, eluted with Tris buffer (20 mM, pH 7.4) at a rate of 0.5 mL/min. The molecular weights and polydispersity index ($PDI = M_w/M_n$) were calibrated with standard poly[N-(2-hydroxyproyl)methacrylamide] (pHPMA). The FPLC spectra data of polymers are given in the Supporting Information.

2.4. Acid–base titration

The buffering capacity of poly(disulfide amine)s was determined by acid–base titration. An amount equal to 5 mmol of amine groups of poly(disulfide amine)s was