Colloids and Surfaces B: Biointerfaces 109 (2013) 197–203

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Synthesis and characterization of N-ethyl-N'-(3-dimethylaminopropyl)-guanidinyl-polyethylenimine polymers and investigation of their capability to deliver DNA and siRNA in mammalian cells

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ARTICLE INFO

Article history:
Received 22 October 2012
Received in revised form 9 January 2013
Accepted 26 March 2013
Available online 9 April 2013

Keywords:
Polyethylenimine
Transfection
Cytotoxicity
siRNA
Buffering capacity

ABSTRACT

Recent advancements in polymeric gene delivery have raised the potential of gene therapy as treatment for various acquired and inherited diseases. Here, we report on the synthesis and characterization of N-ethyl-N'-(3-dimethylaminopropyl)-guanidinyl-polyethylenimine (sGP) polymers and investigation of their capability to carry DNA and siRNA in vitro. Zinc triflate-mediated activation of primary amines of branched polyethylenimine (bPEI) followed by reaction with varying amounts of N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDAC) resulted in the generation of a small series of trisubstituted guanidinyl-modified polyethylenimine polymers. Determination of primary amines on modified polymers by TNBS assay revealed 62–84% of the attempted conjugation of EDAC onto bPEI. These modified polymers were shown to condense plasmid DNA and retard its mobility on 0.8% agarose gel. Further, these polymers were evaluated for their capability to carry pDNA into the cells by performing transfection assay on various mammalian cells. All the modified polymer/pDNA complexes exhibited significantly higher levels of gene expression with one of the complexes, sGP3/pDNA complex, displayed ~1.45 to 3.0 orders of magnitude higher transfection efficiency than that observed in the native bPEI and the commercial transfection reagent, Lipofectamine™. The efficacy of sGP3 polymer was further assessed by siRNA delivery, which resulted in ~81% suppression of the target gene. In conclusion, these studies demonstrate the potential of these substituted guanidinyl-modified PEIs as efficient gene delivery vectors.

1. Introduction

Non-viral vectors have received increasing interest as safe and efficient carriers of nucleic acids intracellularly [1–4]. These vectors employ synthetic chemical materials, such as cationic lipids, polymers, dendrimers, peptides, etc., to deliver the genes of interest to the interiors of the target cell [5–9]. These materials interact with negatively charged nucleic acids to form compact nanoparticles and provide protection to them from degradation by nucleases. These particles are efficiently taken up by the cells and resulted in the higher level of gene expression [7,10,11]. Among these vectors, cationic polymers have shown promising results in nucleic acid delivery. Of these, branched polyethylenimine (bPEI) has been the most efficient and widely used polymer for such applications. The prominent features of bPEI are its intrinsic proton sponge property and high charge density comprising of 1°, 2° and 3° amines in 1:2:1 ratio, which enable it to condense nucleic acids into small-sized polyplexes that are effectively endocytosed by the cells. The proton sponge property facilitates the release of the polyplexes into the cytosol, which are further transported into the nucleus. High molecular weight bPEI exhibits high transfection efficiency, however, associated with high cytotoxicity due to high charge density (in particular, high density of primary amines) and has a tendency to interact non-specifically with the blood components, which have hampered its clinical applications [12,13]. In order to address these concerns, various modifications have been suggested. Hydrophilic modifications reduce the toxicity by lowering the charge density but also affect the transfection efficiency due to diminished interactions with the cell membranes that cause lower uptake and internalization [14]. Alternatively, hydrophobic modifications have also been shown to significantly improve the cell viability and gene delivery efficiency by enhancing pDNA condensation through cooperative binding as well as promoting interactions with the lipophilic cell membranes that facilitate pDNA release for transgene expression [15]. Therefore, a large number of ligands have been conjugated with cationic polymers [16–22]. In such studies, a relatively higher amount of modified cationic polymers are required to

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http://dx.doi.org/10.1016/j.colsurfb.2013.03.052

deliver a fixed amount of pDNA due to partial blockage of charge. Taking a clue from these investigations, we report the synthesis and evaluation of trisubstituted guanidinyl-modified PEI polymers as efficient and safe carriers of nucleic acids in vitro. As literature records, multisubstituted guanidines constitute an important class of compounds, which serve as antimicrobial compounds, thrombin inhibitors, transporter for the delivery of anticancer agents, antifungal agents building blocks for many biologically relevant therapeutics [23–30].

In this study, we have synthesized N-ethyl-N'-(3-dimethylaminopropyl)-guanidinyl-modified polyethylenimine polymers, characterized by spectroscopic techniques and eventually evaluated their capability to carry nucleic acids into the cells. Further, these modified polymers have been assessed for their buffering capacity and cell viability. One of the modified polymers in the series, sGP3, has been examined for its ability to bind pDNA and provide protection against nucleases. Versatility of sGP3 was examined by delivering GFP-specific siRNA to knockdown the target gene expression.

2. Materials and methods

2.1. Synthesis of sGP polymers

In a solution of bPEI (110 mg) in N,N-dimethylformamide (5 ml), $Zn(OTf)_2$ (22.687 mg, 0.062 mmol, for 10% grafting) was added and stirred for 3 h at 80 °C in an argon atmosphere [31]. EDAC (11.98 mg, 0.062 mmol, for 10% grafting) was then added to the resulting PEI-zinc complex and stirred the reaction mixture for 12 h at ambient temperature. After removal of the solvent, the residue was taken up in water (10 ml) and subjected to dialysis (cut off 12 kDa) against deionised water for three days with intermittent change of water. Similarly, reactions were carried out for 30, 50, 70, 100% substitution on bPEI with varying amount of $Zn(OTf)_2$ and EDAC. The dialyzed solutions were lyophilized to obtain sGP polymers as white solids in ~78–85% yield. These polymers were characterized by ^{1}H-NMR and the percent grafting of EDAC on bPEI was determined by 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay.

2.1.1. sGP3 Polymer

^{1}H-NMR (D_2O) δ (ppm): 2.5–3.3 (PEI protons), 2.3 (s, $-N(CH_3)_2$, 6H), 2.0–2.1 (m, $3x(-N-CH_2)$, 6H), 1.18 (m, $-CH_2$, 2H), 1.05 (t, $-CH_3$, 3H).

2.2. In vitro transfection assay

HEK293 cells were seeded in 96-well plates at a density of 10,000 cells per well and cultured with medium containing 10% FBS for 16 h. Then cells were washed with 1× PBS (2 × 200 μl) and pDNA complexes of modified polymers prepared at w/w ratio of 1.6, 2.6, 3.3, 4.0, 5.0, bPEI at w/w ratio of 1.6 and Lipofectamine™ (according to manufacturer's protocol) were diluted with serum free as well as serum containing DMEM. After incubation for 30 min, these were gently added onto the cells. The plates were incubated for 36 h at 37 °C under humidified 5% CO_2 atmosphere. Similarly, assay was performed on Hela and CHO cells. Post 36 h of incubation, the plates were visualized under Nikon Eclipse TE 2000-S inverted microscope (Kanagwa, Japan) fitted with C-F1 epifluorescence filter (Excitation, 488 nm; Emission 505 nm; barrier filter BA 520) and the images of the cells expressed GFP were captured using Nikon Digital Imaging System. The transfection assay was carried out thrice to generate statistical data. Mock treated cells were used as blank.

2.3. siRNA delivery

To demonstrate the capability of modified polymer to carry siRNA into the cells, GFP-specific siRNA was delivered and the knockdown efficiency of the target gene expression was determined. Cells were treated first with complex of sGP3/pDNA for 3 h, washed with 1x PBS and followed by treatment with complex of sGP3/siRNA (2 μl, 2.5 μM). Transfection with sGP3/pDNA alone was used as control. Similarly, pDNA and GFP-specific siRNA were also delivered by Lipofectamine™ for comparative study and the GFP expression was quantified after 36 h.

3. Results and discussion

In order to address the issue of charge-associated toxicity in high molecular weight PEIs, several modifications, such as pegylation, acylation, conjugation of polysaccharides, etc., have been studied [1–5]. Forrest et al. [32] and Gabrielson and Pack [33] investigated the effect of partial acetylation of bPEI (25 kDa) on the transfection efficiency and cytotoxicity of the resulting polymers. The results of these studies revealed that capping of primary amines, enhanced lipophilicity and weakening of polymer/pDNA complexes significantly improved the transfection efficiency and reduced the cytotoxicity. Similarly, Nouri et al. [34] conjugated N,N-dimethylaminoethylmethacrylate (DMAEMA) to bPEI (25 kDa) and investigated toxicity and transfection capability of the resulting tertiary amine bearing polymers. The grafting of DMAEMA was found to be beneficial and on increasing the percent grafting i.e. incorporation of tertiary amines, the polymer/pDNA interaction energy decreased, which resulted in the easy release of pDNA from the vector. It was observed that bPEI with 88% conjugation of DMAEMA exhibited the highest transfection efficiency and cell viability. These studies established that blocking of primary amines and weakening of polymer/pDNA complex significantly reduced the cytotoxicity and transfection ability of the vector. Here, the goal of the present study was also to improve the cell viability of bPEI without hampering the overall charge. The rationale to undertake the present investigation was to address following issues, viz., toxicity, transfection efficiency and surface charge of the modified polymers. It was speculated that grafting of EDAC onto bPEI would (i) decrease the density of the primary amines, which are responsible for toxicity in cationic polymers [32–36], (ii) improve the interactions of the polymers with cell membranes, as alkyl chains in EDAC would impart hydrophobicity to the polymers, (iii) facilitate the uptake and internalization of the pDNA complexes, and (iv) weaken the vector/pDNA complex, which would result in the easy disassembly of the complexes leading to marked improvement in the transfection efficiency. Keeping these points into consideration, we conjugated N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide onto bPEI to yield trisubstituted guanidinyl-modified PEI polymers, which were then evaluated for their transfection efficiency, cell viability and buffering capacity.

The synthesis of trisubstituted guanidinyl-modified PEI polymers was carried out following a published protocol with some modifications [31]. Branched PEI was activated by varying amounts of zinc triflate to form amine-coordinated zinc complex, which acted as an excellent precursor for the addition of N—H bonds to varying amounts of carbodiimide (EDAC) under an inert atmosphere. The reaction resulted in the formation of trisubstituted guanidinyl-modified PEI polymers (sGP1-sGP5) (Scheme 1) in high yields, which were characterized by ^{1}H-NMR. In a typical spectrum of sGP3, appearance of a singlet peak at δ 2.3 due to dimethyl groups of modified guanidine moiety confirmed the formation of the desired products. The extent of conjugation of EDAC onto bPEI was determined by TNBS assay [37], which was found to be 10