Colloids and Surfaces B: Biointerfaces 99 (2012) 27–37

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Bioreducible polyether-based pDNA ternary polyplexes: Balancing particle stability and transfection efficiency

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ARTICLE INFO

Article history:
Available online 22 September 2011

Keywords:
pDNA
Non-viral gene delivery
Polyplex
Ternary complex

ABSTRACT

Polyplex particles formed with plasmid DNA (pDNA) and Pluronic P85-*block*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (P85-*b*-P[Asp(DET)]) demonstrated highly effective transfection ability compared to PEG-based block cationomer, PEG-*b*-P[Asp(DET)]. Ternary polyplexes comprising PEG-*b*-P[Asp(DET)], poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide)-*b*-P[Asp(DET)] (P(EPE)-*b*-P[Asp(DET)]) used as an analog of P85-*b*-P[Asp(DET)], and pDNA were prepared in this work aiming at maintaining adequate transfection efficiency while solving the stability issues of the P85-*b*-P[Asp(DET)] polyplexes. Furthermore, a bioreducible P(EPE)-SS-P[Asp(DET)] possessing a redox potential-sensitive disulfide linkage between the P(EPE) polymer and the cationic block was used as a substitute for P(EPE)-*b*-P[Asp(DET)] during ternary complex formation to investigate whether the transfection ability of the ternary polyplex system could be enhanced by triggered release of P(EPE) polymers from the polyplexes. The ternary complexes showed significant improvement in terms of stability against salt-induced aggregation compared to binary complexes, although the gene delivery ability dropped with the amount of PEG-*b*-P[Asp(DET)] used for complexation. By manipulating the difference in redox potential between the extracellular and intracellular environments, the reducible ternary complexes achieved higher transfection compared to the non-reducible polyplexes; moreover, the reducible polyplexes exhibited comparable stability to the non-reducible ones. These results suggest that reducible ternary complexes could provide satisfactory transfection efficiency without comprising the colloidal stability of the particles.

1. Introduction

Development of non-viral plasmid DNA (pDNA) delivery vehicles, for example, polyplexes [1–3], polymersomes [4], and liposomes [5,6], has been an attractive area of research, aiming for alternatives to virus-based gene therapy. Non-viral gene carriers are usually regarded as a safer gene delivery tool for therapeutic purposes compared to viruses [7]; however, the low level of target gene expression provided by non-viral vectors still prohibits them from practical use [8]. A major cause for this unsatisfactory transfection ability is the colloidal instability of the gene delivering particles at physiological conditions [9].

One of the promising methods for producing stable particles with pDNA is using cationic polymers covalently linked with hydrophilic poly(ethylene glycol) (PEG) [10–12]. Upon electrostatic interaction between anionic pDNA and cationic polymer molecules, a PEG layer will be formed around the complexed pDNA, and then polyplex particles with highly hydrated PEG surface will be result. Among the cationic polymer candidates for pDNA complexation, PEG-based diblock copolymer, poly(ethylene glycol)-*block*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-*b*-P[Asp(DET)]) has achieved high transfection efficiency in both *in vitro* and *in vivo* studies [13–15]. Nonetheless, high N/P ratios are usually crucial to obtain adequate transfection level, and implying that a large excess amount of free polymers should be present with polyplexes. This imposes a barrier for this system to be used in systemic administration as free polymers may not be able to concentrate at the target sites along with the pDNA polyplex particles.

Recently we reported that amphiphilic Pluronic could be used as a substitute for hydrophilic PEG for decorating P[Asp(DET)] polyplex particles to improve transfection ability especially at low N/P ratios. However, it was also demonstrated that the Pluronic P85-based nanoparticles suffered from salt-induced aggregation at physiological salt concentration [16]. Kabanov and coworkers improved the colloidal stability of the

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doi:10.1016/j.colsurfb.2011.09.026

Pluronic-based polyplex particles by including free Pluronic polymers during the polyplex formation. The utilization of free Pluronic in those studies also improved the overall transfection efficiency of their systems [17–19]. Unfortunately, preliminary results revealed that free Pluronic could not improve neither the stability nor the transfection level of the P[Asp(DET)]-based polyplexes. Therefore, in order to balance the stability and transfection efficiency of the polyplexes, ternary polyplexes consisting of PEG-*b*-P[Asp(DET)], poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide)-*b*-P[Asp(DET)] (P(EPE)-*b*-P[Asp(DET)]), and pDNA were prepared and investigated whether the colloidal stability of the ternary complexes could be improved by incorporating PEG-based polymers for complexation while maintaining satisfactory level of transfection.

Another aim of this study was to explore if the transfection ability of the present ternary complex system could be further improved by replacing the P(EPE)-*b*-P[Asp(DET)] used in ternary complexation with a bioreducible P(EPE)-SS-P[Asp(DET)] polymer, which has a redox potential-responsive disulfide linkage located between the P(EPE) polymer and the cationic block. It has been demonstrated that the reduction of disulfide bond could occur intracellularly in the reduced glutathione-rich cytoplasm [20] or extracellularly due to the presence of free thiols secreted by cells [21] or protein disulfide-isomerase on cellular membrane [22,23]. Moreover, several reviews suggested that amphiphilic polymers and polyelectrolytes (cationic/anionic polymers) interact with lipid bilayer using different mechanisms [24,25] and thus the co-presence of those two species might lead to a more effective membrane perturbation. If the cleavage of disulfide linkage occurs at proximity of cell membrane surface or along the endocytotic pathway after particle internalization, the detachment of P(EPE) polymers from the polyplex particles might allow higher cellular uptake or more efficient endo/lysosomal escape of the particles through the combined membrane-disturbing effects of the amphiphilic P(EPE) and cationic polymers.

Here we described the syntheses of two P(EPE)-based block copolymers, P(EPE)-*b*-P[Asp(DET)] and bioreducible P(EPE)-SS-P[Asp(DET)], and showed that both of them could form ternary polyplexes with additions of PEG-*b*-P[Asp(DET)] and pDNA. Characterizations on particle stability at physiological conditions, sensitivity to reducing environment, and transfection ability of the ternary complexes were performed. The ternary complexes revealed higher stability against salt-induced aggregation compared to the P(EPE)-based binary polyplex particles. Moreover, the ternary polyplexes with P(EPE)-detachable property demonstrated improved transfection efficiency compared to the non-bioreducible counterparts.

2. Materials and methods

2.1. Materials

α-Methoxy-ω-amino poly(ethylene glycol) (PEG-NH_2, M_n = 12,000 g/mol, M_w/M_n = 1.03) was obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Amphiphilic triblock copolymers, α-methoxy-ω-amino poly(ethylene oxide)$_{27}$-*block*-poly(propylene oxide)$_{40}$-*block*-poly(ethylene oxide)$_{27}$ (P(EPE)-NH_2, M_n = 4800 g/mol, M_w/M_n = 1.10) and α-methoxy-ω-hydroxy poly(ethylene oxide)$_{27}$-*block*-poly(propylene oxide)$_{40}$-*block*-poly(ethylene oxide)$_{27}$ (P(EPE)-OH, M_n = 4800 g/mol, M_w/M_n = 1.07), were synthesized by Advanced Polymer Materials Inc. (Montreal, Canada). L-Aspartic acid β-benzyl ester, triphosgene, 4-nitrophenyl chloroformate (*p*-NPC), branched polyethylenimine (bPEI, M_w = 25,000 g/mol), cysteamine, diethylenetriamine (DET), benzene, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), hexane, *N*-methyl-2-pyrrolidone (NMP), and tetrahydrofuran (THF) were purchased from Sigma-Aldrich (St. Louis, MO). DET was distilled by conventional method before use. DCM, DMF, hexane, NMP, and THF were purchased as anhydrous grade and used without further purification. Monomer β-benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was synthesized from L-aspartic acid β-benzyl ester by the Fuchs-Farthing method using triphosgene and purified through repetitive crystallizations [26]. Dialysis tubings (MWCO's 1000 and 6–8000) were purchased from Spectra/Por (Rancho Dominguez, CA). The pDNA coding for luciferase in pGL4 vector with a CMV promoter (Promega, Madison, WI) was amplified in DH5α *Escherichia coli* cells and purified using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). Luciferase Assay System Kit, and CellTiter-Blue® Cell Viability Assay were purchased from Promega (Madison, WI). *RC DC* Protein Assay Kit was purchased from Bio-Rad (Hercules, CA). MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line were obtained from ATCC.

2.2. ^{1}H NMR and gel permeation chromatography (GPC) analyses

The ^{1}H NMR spectrum of each polymer was obtained with Varian Unity-Inova 400 MHz NMR spectrometer (Palo Alto, CA) with temperature regulated at designated temperature. Chemical shift were reported in ppm relative to the residual protonated solvent resonance. Polymer molecular weight distributions were monitored using Agilent 1100 series equipped with TOSOH TSK-gel G3000PWXL and G4000PWXL columns with temperature regulated at 40 °C and an internal refractive index (RI) detector. DMF with 10 mM LiCl was used as the eluent at a flow rate of 1 ml/min. PEG standards were used for calibration.

2.3. Synthesis of P(EPE)-SS-NH_2

Disulfide containing P(EPE) monoamine(P(EPE)-SS-NH_2) was prepared from P(EPE)-OH as previously described with modifications [27]. Fifty equivalent of *p*-NPC (660 mg, 3.14 mmol) was dissolved in benzene and then added dropwise to P(EPE)-OH (301 mg, 0.0627 mmol) dissolved in benzene. The reaction proceeded for 24 h at room temperature under an argon atmosphere. Benzene was then evaporated under reduced pressure and the crude product was dissolved in methanol, precipitated in dry ice-chilled diethylether, and then subjected to centrifugation. The precipitation and centrifugation procedure was repeated 3 times to ensure the complete removal of unreacted *p*-NPC. The white solid obtained was dissolved in benzene and lyophilized. The degree of activation was determined to be about 100% from the peak intensity ratio of the aryl protons of the nitrophenyl groups (NO_2–C_6H_4–, δ = 7.5 and 8.25 ppm) to the methyl protons of P(EPE) (–CH_3, δ = 1.0 ppm) in ^{1}H NMR spectrum taken in DMSO-d_6 at 25 °C. The activated P(EPE) was subsequently reacted with cysteamine to obtain P(EPE)-SS-NH_2. P(EPE)-*p*-NPC (0.17 g, 0.0342 mmol) and excess amount of cysteamine (0.54 g, 7 mmol) were dissolved in methanol and mixed. The reaction was allowed to proceed for 48 h at room temperature. GPC result showed that there was no dimer present after 48 h reaction and the reaction mixture was transferred to a dialysis tubing (MWCO:1000) and dialyzed extensively against methanol. The product was then lyophilized from benzene. The degree of amine functionality was confirmed to be about 80% from the peak intensity ratio of the methylene protons of the cysteamine moieties (–CH_2–, δ = 2.7–2.9 ppm) to the methyl protons of P(EPE) (–CH_3, δ = 1.0 ppm) in ^{1}H NMR spectrum obtained in DMSO-d_6 at 80 °C. GPC was used again to monitor the molecular weight distribution after dialysis and it showed that P(EPE)-SS-P(EPE) dimers were formed during dialysis; however,