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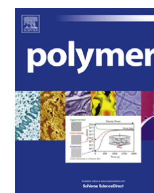

Guanidinated multi-arm star polyornithines with a polyethylenimine core for gene delivery

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ABSTRACT

Multi-arm star polyornithines PEI-P(Orn)$_n$ are prepared by grafting polyornithine arms onto branched polyethylenimine (PEI) with an M_w of 600 via the ring-opening polymerization of *N*-carboxyanhydride of benzyloxycarbonyl ornithine. To enhance gene delivery efficiency and reduce cytotoxicity, the amino side groups on the polyornithine arms are partially guanidinated that transforms the ornithine units to arginine units. Thus, the guanidinated products G-PEI-P(Orn)$_n$ contain multiple poly(ornithine-co-arginine) arms. PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_{71}$ mediate the transfection of pGL3 plasmid to 293T cells almost as efficient as 25 kDa PEI in serum-free medium. Notably, in contrast to the dramatically lowered efficiency of 25 kDa PEI in the presence of serum, the efficiency of G-PEI-P(Orn)$_{71}$ can be retained or even enhanced in the medium containing 10% serum. The improved serum-compatibility and high efficiency of the guanidinium-modified star polyornithines make them promising for gene delivery.

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1. Introduction

Gene therapy is a very promising way for treating congenital diseases and acquired diseases [1–6]. One of the major factors for effective gene therapy is an efficient and safe vector that delivers genes into cells. Viral vectors are usually highly efficient but with difficulty in large scale preparation and with safety issues of immunogenicity and mutagenesis. The disadvantages of viral vectors have stimulated the investigation of synthetic vectors. Commonly used non-viral vectors include linear polymers such as polylysine [7–10], branched polymers such as polyethylenimine (PEI) [11–14], and dendritic polymers such as poly amidoamine dendrimers [15–19].

Dendritic and hyperbranched polymers have attracted intensive attentions in gene delivery because of their three-dimensional architectures with abundant terminal functional groups and inner cavities. Dendritic polyamidoamine polymers consist of primary amines on the surface and tertiary amines in the interior. This structure has high buffer capacity that helps the escape of DNA from endosome [20–23]. Among these polyamidoamines, the partially degraded are especially noticeable. Due to the random solvolysis of amide bonds between polyamidoamine units, these polymers can collapse into a compact form when complexed with DNA and swell in endosome when released from DNA. The partially degraded dendrimers with a flexible structure mediate higher level transfection than intact dendrimers [24–25]. Redox-responsive hyperbranched polyamidoamines with a thiol-responsive core and aminoethylpiperazine terminal groups exhibited high transfection efficiency and low cytotoxicity, showing that both the degradation of disulfide bonds in the core and the type of the terminal groups play an important role [26]. Investigation on the influence of molecular weight and architecture of polylysine in gene transfection efficiency showed that hyperbranched polylysine has higher transfection efficiency than linear and dendritic polylysine [27]. Hyperbranched PEI-methyl acrylate-PEI conjugate prepared with low-molecular weight PEIs exhibited lower cytotoxicity and a rather high gene transfection efficiency than 25 kDa PEI in various cell lines [28].

Basic peptides such as arginine-rich peptides have been reported to have a membrane permeability and a carrier function for intracellular protein delivery [29–34]. These functions are related to the guanidinium groups. Guanidinium groups displayed from numerous non-proteinogenic scaffolds can facilitate internalization [35]. Primary guanidinium groups bear a positive charge under physiological conditions and have the potential to donate up to five hydrogen bonds to electron-rich functional groups [36] such as the carboxyl, phosphoryl, and sulfuryl groups of cell-surface carbohydrates and phospholipids which are essential for efficient cellular uptake [37]. Arginine-rich peptides or poly(amino acid)s were reported to be effective gene vectors [38–40]. The synthesis of

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arginine-containing poly(amino acid)s, however, is comparatively more complex because additional protection of the guanidine side groups is required [41–43].

In this work, we design multi-arm star polyornithines consisting of a hyperbranched PEI (M_w = 600) core and multiple positively charged polyornithine arms. The multi-arm structure endows the polymer with a three-dimensional architecture while the arms are yet not as crowded as the branches in typical dendrimers or hyperbranched polymers. The star polyornithines are transformed to ornithine-arginine copolymers by guanidination of the amino side groups using *O*-methylisourea hemisulfate. Compared to the original polyornithines, the guanidinated polymers show reduced cytotoxicity and increased gene transfection efficiency.

2. Experimental section

2.1. Materials

PEI with an M_w of 600 Da was purchased from Alfa Aesar, and PEI with an M_w of 25 kDa was purchased from Aldrich. ε-Benzyloxycarbonyl ornithine (ZOrn) and 33 wt% solution of HBr in HOAc were supplied by Chengdu Chengnuo New-Tech Co., Ltd. Tetrahydrofuran (THF) was distilled over Na–K alloy in the presence of benzophenone before use. Dimethyl formamide (DMF) was dried over CaH_2 and distilled under vacuum before use. Plasmid pGL3 under the control of SV40 promoter and with enhancer sequences encoding luciferase was obtained from Promega, Madison, WI, USA. Plasmids were propagated in *Escherichia coli* in Luria–Bertani (LB) medium containing 60 μg mL^{-1} ampicillin respectively at 37 °C and purified using E. Z. N. A. Fastfilter Endofree Plasmid Midi kits (Omega) according to the manufacturer's instruction. The purity of DNA was assessed spectrophotometrically by measuring absorbance at wavelengths of 260 and 280 nm (OD_{260}/OD_{280} 1.8 or greater) and confirmed using 0.7% agarose gel electrophoresis containing GelRed. The DNA concentration was determined by measuring the UV absorbance at 260 nm. DNA aliquots of pGL3 were stored at −20 °C prior to use. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen Corp. RPMI 1640 medium was purchased from Biological Industries. Phosphate buffered saline (PBS) and Trypsin-EDTA, Penicillin-streptomycin, fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Bioind. Other commercially available reagents were used as received.

2.2. Synthesis of PEI-polyornithine (PEI-P(Orn)$_n$)

ε-Benzyloxycarbonyl ornithine *N*-Carboxyanhydride (ZOrn-NCA) was synthesized by following a literature procedure [44]. Briefly, ZOrn (5.00 g, 18.7 mmol) and triphosgene (3.50 g, 11.8 mmol) was suspended in 100 mL of dry THF under argon. The mixture was stirred in a 55 °C oil bath until the cloudy solution turned clear. The solution was precipitated by addition of excess petroleum ether. The precipitate was collected by filtration and purified by recrystallization from ethyl acetate and petroleum ether. ^1H NMR (300 MHz, $CDCl_3$, δ): 7.35 (br s, C_6H_5), 5.09 (s, $C_6H_5CH_2$), 4.32 (s, COCHNHCOO), 3.23 (s, $CH_2CH_2CH_2NHCOO$), 1.95–1.61 (m, $CHCH_2CH_2CH_2NH$).

A representative procedure for the preparation of PEI-poly(ε-benzyloxycarbonyl ornithine) (PEI-P(ZOrn)$_n$) is as follows: To a solution of ZOrn-NCA (1.00 g, 3.40 mmol) in 30 mL of dry DMF was added a proportional PEI (600 Da) as an initiator under argon. The reaction solution was stirred for 72 h at 30 °C, and then precipitated by addition of excess diethyl ether. The precipitate was collected, dissolved in DMF, and re-precipitated by adding diethyl ether. ^1H NMR (300 MHz, $CDCl_3$, δ): 7.30 (br s, $C_6H_5CH_2$), 4.99 (s, $C_6H_5CH_2$), 3.95 (br s, COCHNH), 3.11 (br s, $CH_2CH_2CH_2NHCOO$), 2.55–2.39 (m, NCH_2CH_2N), 1.94–1.28 (m, $CHCH_2CH_2CH_2NH$).

PEI-polyornithines (PEI-P(Orn)$_n$) were synthesized by removing the benzyloxycarbonyl (Z) groups on PEI-P(ZOrn)$_n$. PEI-P(ZOrn)$_n$ polymers were treated with 4 equiv of HBr (33% in HOAc) respect to Z groups in CF_3COOH at 0 °C for 1.5 h. The product was precipitated with excess diethyl ether and dried in vacuo. ^1H NMR (300 MHz, D_2O, δ): 4.24 (br s, COCHNH), 3.75–3.11 (m, NCH_2CH_2NHCO), 2.91 (br s, $CH_2CH_2CH_2NH_2$), 2.65 (m, NCH_2CH_2N), 1.66 (br s, $CH_2CH_2CH_2NH_2$).

2.3. Guanidinium-modified PEI-poly(ornithine) (G-PEI-P(Orn)$_n$)

PEI-P(Orn)$_{71}$ (100 mg) was dissolved in 6 mL of ammonia solution in a 15 mL centrifuge tube. To this tube a known amount of *O*-methylisourea hemisulfate was added to the PEI-P(Orn)$_{71}$ solution. The solution was stirred at 65 °C for 1.5 h. Then the mixture was transferred to an MWCO 3500 Da dialysis bag and dialyzed against deionized water for 2 days. The product was obtained by lyophilization to give a dry solid mass. ^1H NMR (300 MHz, D_2O, δ): 4.25 (br s, COCHNH), 3.75–3.11 (m, NCH_2CH_2NHCO), 2.96 (br s, $CH_2CH_2CH_2NH$), 2.65 (m, NCH_2CH_2N), 1.74–1.59 (m, $CHCH_2CH_2CH_2NH$). ^{13}C NMR (75 MHz, D_2O, δ): 173.8 (COCHNH), 156.8 ($NHC(NH)NH_2$), 53.4 (COCHNH), 40.6 ($CHCH_2CH_2CH_2NH$), 28.3 ($CHCH_2CH_2CH_2NH$), 24.5 ($CHCH_2CH_2CH_2NH$).

2.4. Cytotoxicity assay

The cytotoxicity was evaluated on the basis of an MTT assay on 293T cells. 293T cells were seeded in 96-well plates at an initial density of 5000 cells per well in 100 μL of DMEM complete medium. The cells were allowed to grow for 24 h. The PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_n$ solutions were added to the media. Each dosage was replicated in 4 wells. Treated cells were incubated at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 for 24 h. MTT reagent (20 μL in PBS, 5 mg mL^{-1}) was added to each well, and the cells were incubated for 4 h at 37 °C. The liquid in each well was removed and 150 μL of DMSO was added to each well to dissolve the crystals. The absorbance at 570 nm in each well was recorded using a spectrophotometer Multiskan Go (Thermo Scientific). Cell viability was calculated according to the following equation: Cell viability (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$, where OD_{sample} is the absorbance of the solution of the cells cultured with the polymer; OD_{blank} is the absorbance of the medium; $OD_{control}$ is the absorbance of the solution of the cells cultured with the medium only.

MTT assay on L929 cells was carried out by the similar way, except RPMI 1640 medium was used instead of DMEM medium.

2.5. Agarose gel retardation assay

Designed amounts of PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_n$ aqueous solutions were added slowly to 25 μL of pGL3 solutions (40 μg mL^{-1} in 40 mM Tris–HCl buffer solution), and then the polyplexes were diluted to a total volume of 1 mL with 150 mM NaCl and vortexed for 15 s. The mixture was incubated at room temperature for 30 min for the polyplex formation. The polyplexes at various polymer/DNA (w/w) ratios mixed with 1 μL of 6 × loading buffer were loaded on 0.7% (w/v) agarose gel containing GelRed and electrophoresed with Tris-acetate (TAE) running buffer at 80 V for 80 min. DNA was visualized with a UV lamp using a Vilber Lourmat imaging system (France).

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