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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.

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. ^{1}H 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. ^{1}H 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. ^{1}H 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. ^{1}H 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).

2.6. Particle size and zeta potential measurements

The particle size and zeta potential were measured with a Nano-ZSZEN3600 (Malvern) zetasizer. The polyplexes at various polymer/DNA (w/w) ratios were prepared by adding appropriate volume of polymer to 1.0 μg of pGL3 DNA solution. The polyplexes were incubated at room temperature for 30 min. Then the polyplexes were diluted with pure water, DMEM without containing FBS, or DMEM containing 10% FBS to 1 mL for size measurements and zeta potential measurements.

2.7. In vitro transfection efficiency

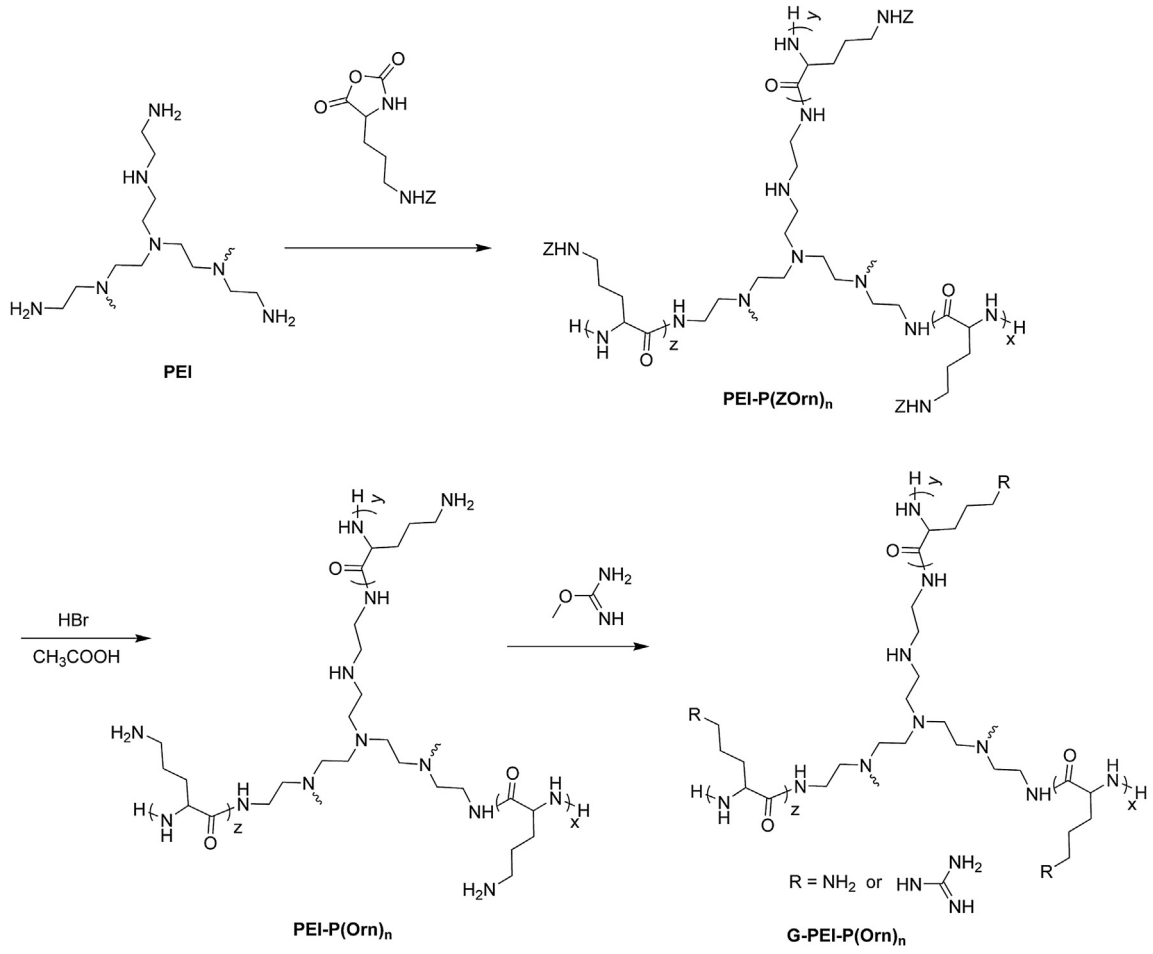
The in vitro transfection efficiency of the PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_n$ was evaluated in 293T cells, using pGL3 plasmid. Cells were seeded at a density of 70,000 cells/well into 24-well plates. They were incubated at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 for 24 h. The medium in each well was replaced with 1 mL of DMEM without containing FBS or 1 mL of DMEM containing 10% FBS. The polyplexes containing 1.0 μg of pGL3 at various polymer/DNA (w/w) ratios were added to each well and incubated with cells for 4 h at 37 °C. Then the medium was replaced with fresh DMEM complete medium and the cells were incubated for another 48 h. After incubation, cells were lysed with 200 μL of cell lysis buffer. The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega, USA). The relative light units (RLU) were normalized by the total protein concentration of the cell extracts, and the total protein was measured with a BCA protein assay kit (Pierce, USA). Luciferase activity was expressed as RLU/mg protein.

3. Results and discussion

3.1. Preparation and characterization of star polyornithines PEI-P(Orn)$_n$

The strategy for the preparation of star polyornithines PEI-P(Orn)$_n$ is shown in Scheme 1. First, PEI-P(ZOrn)$_n$ was obtained by grafting polyornithine arms on the branched PEI core through the ROP of ZOrn-NCA initiated by the amino groups of PEI. Then, the benzyloxycarbonyl protective groups were removed by acidolysis with a solution of HBr in trifluoroacetic acid.

Molecular weights of PEI-P(ZOrn)$_n$ were measured by gel permeation chromatography coupled with multi-angle laser light scattering (GPC-MALLS) in DMF. The n values in PEI-P(ZOrn)$_n$ star polymers were calculated according to their number average molecular weights (M_n), *i.e.*, PEI-P(ZOrn)$_{29}$, PEI-P(ZOrn)$_{42}$, and PEI-P(ZOrn)$_{71}$ have an M_n of 7900, 11,000, and 18,200, respectively. As shown by the data in Table 1, control of molecular weights was achieved through the use of specific feed ratios of the NCA monomer to PEI initiator. The deprotection of PEI-P(ZOrn)$_n$ was confirmed by ^{1}H NMR. The strong benzyl signal at 7.2 ppm disappeared, and the signal at 3.95 ppm of the polyornithine chain shifts to 4.24 ppm after deprotection because of the leaving of the benzoxycarbonyl group from the amino group.



Scheme 1. Schematic representation of the preparation of PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_n$.

Table 1
Synthesis and molecular weights of PEI-P(ZOrn)$_n$.

PEI-P(ZOrn)$_n$	Feed			Molecular weights		
	PEI (g)	ZOrn-NCA (g)	NCA/PEI (molar ratio)	M_n[a] (10^3)	M_w[a] (10^3)	M_w/M_n
PEI-P(ZOrn)$_{29}$	0.1280	1.9615	31.5	7.9	8.6	1.09
PEI-P(ZOrn)$_{42}$	0.0788	2.0123	52.5	11.0	12.7	1.15
PEI-P(ZOrn)$_{71}$	0.0430	1.5368	73.4	18.2	20.2	1.11

[a] Molecular weights were determined by GPC-MALLS in DMF.

3.2. Guanidination of PEI-poly(ornithine)

Star polymer PEI-P(Orn)$_{71}$, which demonstrated the highest transfection efficiency among the PEI-P(Orn)$_n$ series, was guanidinated with *O*-methylisourea hemisulfate to give the guanidinium-modified G-PEI-P(Orn)$_{71}$. The content of guanidine groups was determined by elemental analysis, on the basis that the guanidinated polymers have higher N/C ratios than the originals. The amino groups of ornithine were transformed to guanidino groups at two different percentages (34% and 60%), and the resulted guanidinium-modified polymers are represented as 34% G-PEI-P(Orn)$_{71}$ and 60% G-PEI-P(Orn)$_{71}$, respectively. These polymers were used to investigate the possible effect of amino/guanidino ratio on DNA condensation, cytotoxicity and transfection efficiency.

3.3. Cytotoxicity of PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_n$

MTT assay was carried out to evaluate the cytotoxicity of the PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_{71}$ polymers in 293T cells. The results are shown in Fig. 1A. The IC_{50} values of PEI-P(Orn)$_{29}$, PEI-P(Orn)$_{42}$, and PEI-P(Orn)$_{71}$ were 110, 45, and 17 μg mL^{-1}, respectively, indicating that the cytotoxicity increases with the increase of molecular weight. Under the same conditions, the IC_{50} value of 25 kDa PEI was detected to be 7 μg mL^{-1}. Therefore, the cytotoxicity of PEI-P(Orn)$_n$ was only slightly lower than that of PEI with a similar molecular weight. Noticeably, the cytotoxicity of PEI-P(Orn)$_n$ could be remarkably lowered by guinidination of the amino groups. The IC_{50} of 34% G-PEI-P(Orn)$_{71}$, and 60% G-PEI-P(Orn)$_{71}$ was elevated to 26 μg mL^{-1} and 51 μg mL^{-1}, respectively, from 17 μg mL^{-1} of PEI-P(Orn)$_{71}$.

Cytotoxicity of the polymers in L929 mouse fibroblasts was also evaluated by MTT assay. The results are shown in Fig. 1B. The cytotoxicity of the PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_{71}$ polymers in L929 cells was obviously higher than that in 293T cells. Similar trend was observed in L929 cells that the cytotoxicity increases with the increase of molecular weight and the cytotoxicity could be lowered by guinidination.

The cytotoxicity of cationic polymers was probably caused by polymer aggregation on cell surfaces impairing the important membrane function. The contents of amino groups increased with the increase of molecular weight, resulting to increased positive charges. The polymers with higher molecular weights more seriously impaired cell membranes that are negatively charged. Guanidination of amino groups results in delocalization of the positive charge which leads to reduction in toxicity [45]. Further, guanidino groups form bidentate hydrogen bonding with the constituents present in cell membrane rather than electrostatic interactions alone which could cause cell damage [46–47]. After amino groups in PEI-P(Orn)$_{71}$ were converted to guanidino groups, the electrostatic force was weakened due to partial role of hydrogen bonding of guanidine in binding cell membrane. It is deemed that the depressed electrostatic interaction is beneficial to the improvement of biocompatibility of vector.

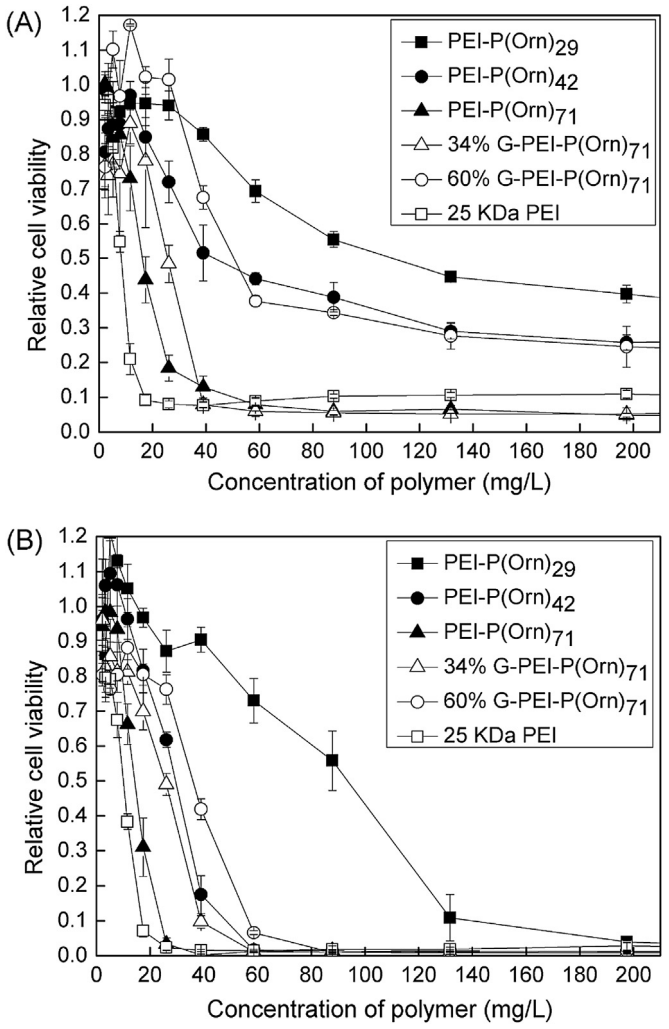


Fig. 1. Relative cell viability of 293T cells (A) and L929 cells (B) at 24 h after the addition of the polymers.

3.4. Characterization of PEI-P(Orn)$_n$/pDNA and G-PEI-P(Orn)$_{71}$/pDNA polyplexes

The polyplexes of PEI-P(Orn)$_n$ and G-PEI-P(Orn)$_{71}$ with plasmid pGL3 were prepared by mixing their aqueous solutions at chosen polymer/DNA weight ratios (w/w). The PEI-P(Orn)$_n$/pGL3 and G-PEI-P(Orn)$_{71}$/pGL3 polyplexes were characterized by agarose gel retardation, particle size, and zeta potential.

The ability of PEI-P(Orn)$_n$ series to interact with DNA was characterized by agarose gel retardation. As shown in Fig. 2, the concentration of the polymer to retard DNA reduced with the molecular weight increasing. The required polymer/DNA weight ratios to retard DNA were 0.3, 0.2 and 0.15 for PEI-P(Orn)$_{29}$, PEI-P(Orn)$_{42}$, and PEI-P(Orn)$_{71}$, respectively. The star polymer could bind DNA at a very low polymer/DNA ratio in contrast to the linear PLL ($M_n = 4$ kDa) that needs a high weight ratio of 1 [48]. 25 kDa PEI as a very efficient polymer could completely retard DNA at weight ratio of 0.39, or equivalently at N/P ratio of 3 [49]. This suggests that the star PEI-P(Orn)$_n$ polymers bind the plasmid DNA as efficiently as PEI of similar molecular weights.

Guanidinated G-PEI-P(Orn)$_{71}$ polymers exhibit lower DNA-binding strength relative to their non-guanidinated counterparts. The required weight ratios for PEI-P(Orn)$_{71}$, 34% G-PEI-P(Orn)$_{71}$, and 60% G-PEI-P(Orn)$_{71}$ to completely retard the DNA were 0.15,

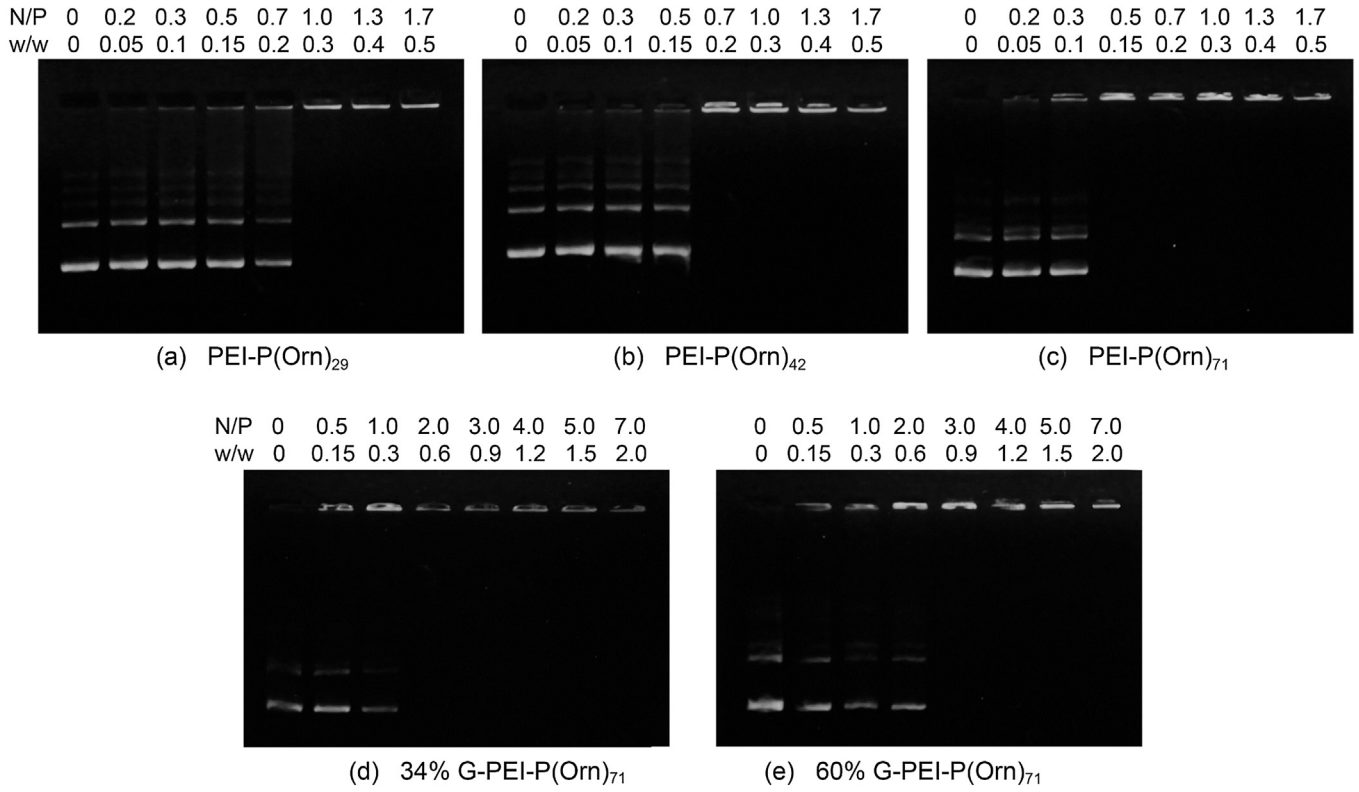


Fig. 2. Agarose gel electrophoresis assay of the polyplexes of pGL3 with (a) PEI-P(Orn)$_{29}$, (b) PEI-P(Orn)$_{42}$, (c) PEI-P(Orn)$_{71}$, (d) 34% G-PEI-P(Orn)$_{71}$, and (e) 60% G-PEI-P(Orn)$_{71}$.

0.3, and 0.6, respectively. Guanidination results in delocalization of the polymer charge which leads to weaker interaction between polymer and DNA [45]. As reported previously, guanidine has an attenuated interaction with DNA than amine does [47,50].

The particle sizes of the polyplexes in pure water were determined by DLS and shown in Fig. 3. The particle size decreased with the increase of the polymer/DNA ratio and was from 400 to 160 nm in the polyplexes formed by PEI-P(Orn)$_n$ and DNA. G-PEI-P(Orn)$_{71}$ formed polyplexes with a size in the range of 150–300 nm.

Surface charge of DNA polyplexes is another important parameter affecting the efficiency of cellular uptake. Zeta potentials of the polyplexes in various media were shown in Fig. 4. For PEI-P(Orn)$_n$ series and G-PEI-P(Orn)$_{71}$ in pure water (Fig. 4A), the polyplexes were positively charged and the zeta potentials of increased with the polymer/DNA ratio increase. At polymer/DNA weight ratio of 20, zeta potentials trended to reach a plateau of 34 mV. In DMEM medium (Fig. 4B), the zeta potentials were much lower than those in pure water, probably because the surface charge was partially neutralized by the amino acids and other electrolytes in the medium. In the 10% FBS-containing DMEM medium, zeta potential was found to become negative. This is possibly due to negatively charged serum proteins in FBS that adsorb to the positively charged polyplexes and made them a net negative surface charge.

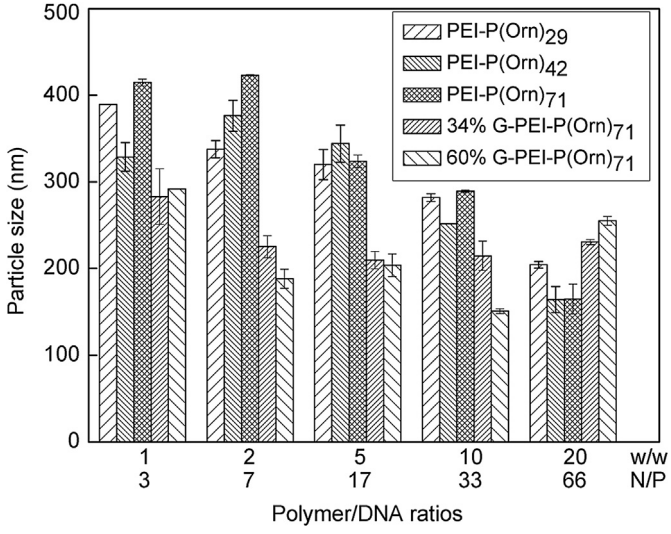


Fig. 3. Particle size of the polymer/pGL3 polyplexes in water measured by DLS.

The effect of media type on particle size and stability over time was shown in Fig. 5. The results demonstrated that PEI-P(Orn)$_{71}$/DNA and 60% G-PEI-P(Orn)$_{71}$/DNA particles in DMEM without containing FBS were significantly larger than those in DMEM containing 10% FBS. It was also demonstrated that in DMEM without containing FBS, these particles were unstable and particle sizes increased over time. In contrast, particles were stable over time in DMEM containing 10% FBS serum, presumably, from negative electrostatic repulsions. The sizes of 60% G-PEI-P(Orn)$_{71}$/DNA particles were significantly smaller than the sizes of PEI-P(Orn)$_{71}$/DNA particles in both DMEM without containing FBS and DMEM containing 10% FBS serum. The size of the gene delivery complex is known to dramatically affect transfection efficacy. Small particles that are stable in serum are typically preferred in gene transfection.

3.5. *In vitro transfection efficiency*

The gene delivery efficiency of PEI-P(Orn)$_n$ series and G-PEI-P(Orn)$_{71}$ was evaluated by luciferase expression of pGL3 plasmid DNA in 293T cells. The amount of pGL3 plasmid DNA was 1.0 μg in vitro transfection assay. Polyplexes were prepared at polymer/DNA weight ratios ranging from 1 to 20. Branched 25 kDa PEI at its

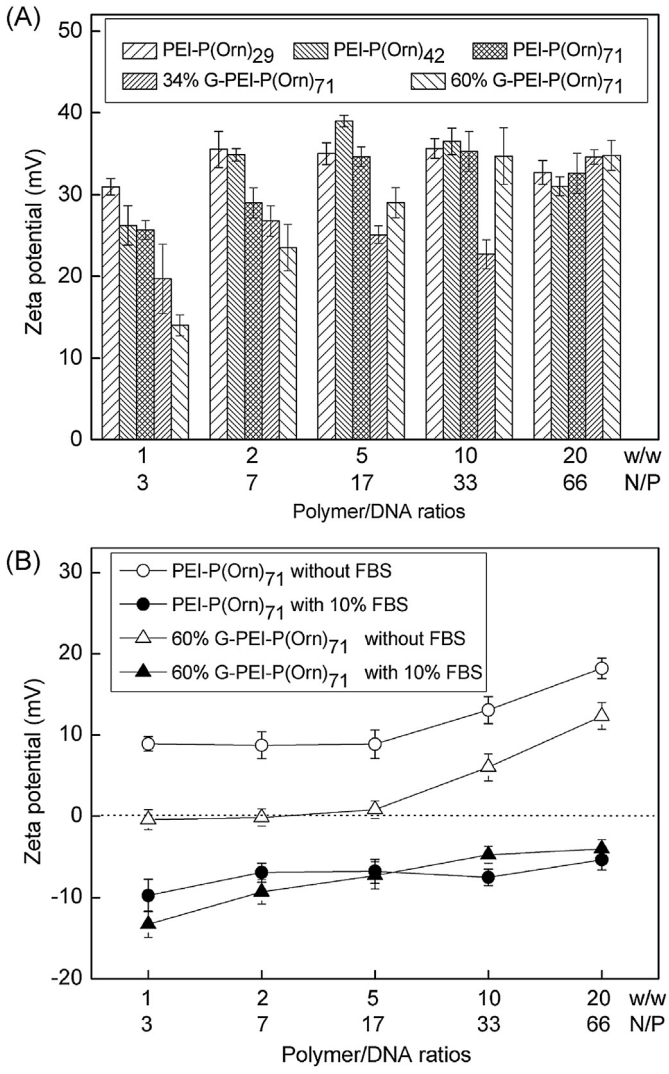


Fig. 4. Surface charge of polymer/pGL3 polyplexes at various weight ratios with 1 μg of pGL3 in pure water (A) and in DMEM medium with or without FBS (B).

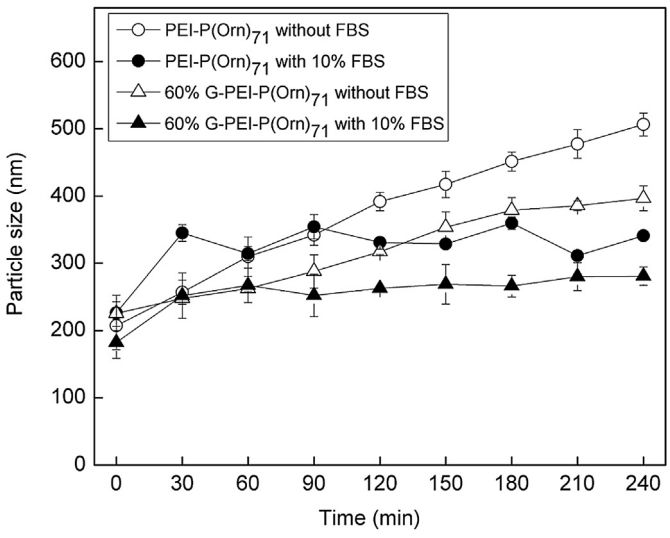


Fig. 5. Polymer/pGL3 polyplexes (20 w/w) particle size and stability in DMEM with or without containing FBS.

optimal N/P ratio of 10 (equivalently at weight ratio of 1.3) was used for comparison.

Fig. 6 shows the luciferase expression of 293T cells in the pGL3 transfection mediated by PEI-P(Orn)$_n$ series and G-PEI-P(Orn)$_{71}$ in the serum-free medium. The PEI-P(Orn)$_{71}$/pGL3 polyplexes at w/w polymer/DNA weight ratio of 2 exhibited the highest efficiency, while PEI-P(Orn)$_{29}$/pGL3 and PEI-P(Orn)$_{42}$/pGL3 reached their maxima at weight ratios of 5 and 2, respectively. At its optimal ratio of 2, PEI-P(Orn)$_{71}$ achieved nearly the same luciferase expression of PEI 25 kDa. The polyplexes prepared at higher ratios led to reduced efficiency, possibly ascribed to the increased cytotoxicity and tight interaction with DNA as revealed by gel agarose gel electrophoresis that the polymer retarded DNA at very low ratios. 34% G-PEI-P(Orn)$_{71}$ and 60% G-PEI-P(Orn)$_{71}$ reached their highest efficiency at ratio of 2. In contrast to the other polymers, 60% G-PEI-P(Orn)$_{71}$ did not significantly reduce efficiency at polymer/DNA weight ratios higher than the optimal value of 2. The *P*-values calculated by *T*-test by comparing the data of 60% G-PEI-P(Orn)$_{71}$ with PEI-P(Orn)$_{29}$, PEI-P(Orn)$_{42}$, PEI-P(Orn)$_{71}$, and 34% G-PEI-P(Orn)$_{71}$ are 0.011, 0.012, 0.015, and 0.052, respectively. The statistical calculation reveals that the efficiency changing trend of 60% G-PEI-P(Orn)$_{71}$ along with polymer/DNA ratio changes is significantly different from that of the non-guanidinated polymers. These results are consistent with the tendency of cytotoxicity difference of the polymers, suggesting that cytotoxicity may be an important cause for the efficiency decrease at higher ratios.

It is important to investigate the influence of serum on the transfection efficiency since therapeutic gene transfection is in vivo. Fig. 7 shows luciferase expression of the pGL3-transfected 293T cells mediated by PEI-P(Orn)$_n$ series and G-PEI-P(Orn)$_{71}$ in the medium containing 10% serum. Compared to the results in serum-free medium, the maximum transfection efficiency achieved by PEI-P(Orn)$_n$ series as well as 25 kDa PEI was reduced by approximate 10 times. In the case of G-PEI-P(Orn)$_{71}$, though the efficiency was also significantly reduced at lower polymer/DNA ratios, it was retained or even enhanced at higher polymer/DNA ratios of 10 and 20.

These multi-arm star polymers have a flexible structure in some extent similar to the degraded polyamidoamine dendrimers. At neutral pH, electrostatic repulsion between protonated primary amines at branch chains cause the star polymer an extended conformation, and when the amino groups are charge neutralized

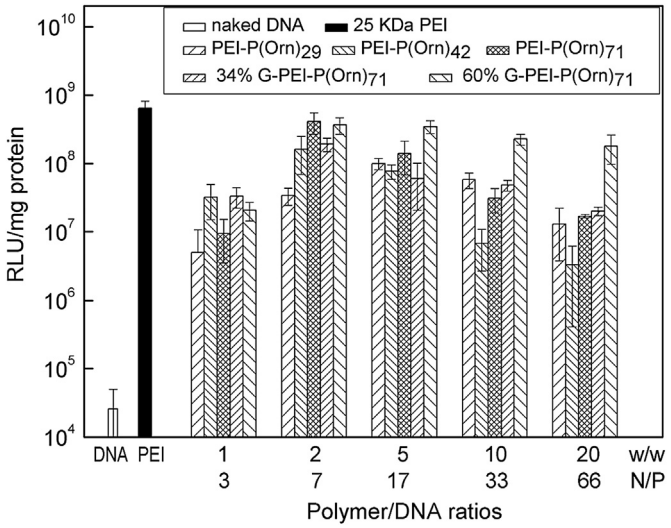


Fig. 6. In vitro transfection efficiency of the polyplexes of pGL3 with the polymers in 293T cells in serum-free medium. The transfection efficiency of 25 kDa PEI was obtained at an optimal weight ratio of 1.3.

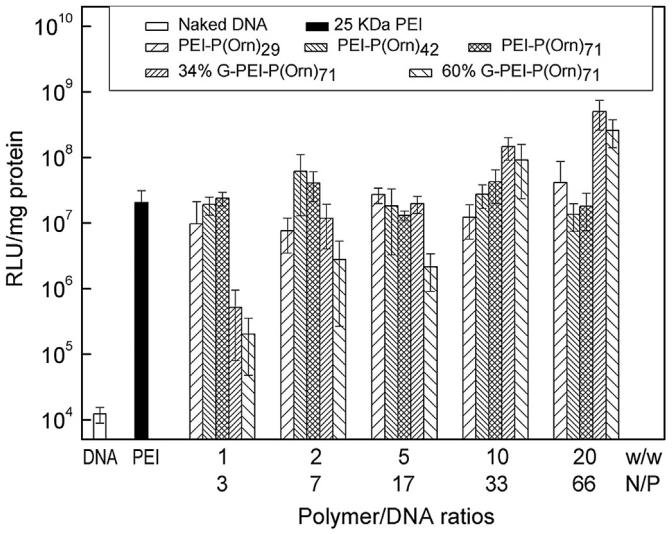


Fig. 7. In vitro transfection efficiency of the polyplexes of pGL3 with the polymers in 293T cells in 10% serum-containing medium. The transfection efficiency of 25 kDa PEI was obtained at an optimal weight ratio of 1.3.

by DNA, the conformation turned to a compact form. When taken up in endosome, as charge on the polymer increased because of the lower pH, less polymer is required to maintain charge neutralization of the DNA. The excess polymer swells due to the electrostatic repulsion that causes endosome rupture and release the DNA. Guanidination of the side amino group of ornithine forms arginine which is responsible for the biological activity of cell penetrating peptides (CPPs) [51–52]. Highly basic guanidino groups can undergo protonation at physiological pH, generating a delocalized positive charge density for interaction with negatively charged biomolecules including phospholipids in the lipid bilayers, which play an important role in facilitating cellular uptake and protein transduction [53–54]. The enhancement of transfection efficiency in medium containing 10% serum might be related to the cell function raised by the addition of serum and appropriate ratio of amino to guanidino groups.

4. Conclusions

In closing, multi-arm star polymers PEI-P(Orn)$_n$ with various molecular weights were synthesized by polymerization of ornithine NCA with PEI as an initiator. Then, guanidino groups were conjugated to PEI-P(Orn)$_{71}$ resulting the guanidinated star polymers G-PEI-P(Orn)$_{71}$. For PEI-P(Orn)$_n$ series, the ability of retarding DNA in gel electrophoresis increased with the increase of molecular weight. Compared to PEI-P(Orn)$_n$, G-PEI-P(Orn)$_{71}$ had remarkably lower cytotoxicity owing to the guanidination of amino groups. PEI-P(Orn)$_n$ mediated the transfect of pGL3 in 293T cells efficiently at low polymer/DNA ratios in serum-free medium. Noticeably, G-PEI-P(Orn)$_{71}$ mediated the gene transfection about 10 times more efficiently than 25 kDa PEI at a polymer/DNA (w/w) ratio of 20 in the medium containing 10% serum. The high efficiency and improved serum-compatibility of the guanidinium-modified star polyornithines make them promising for gene delivery.

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