Effects of Branching Strategy on the Gene Transfection of Highly Branched Poly(β-amino ester)s

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Abstract Highly branched poly(β-amino ester)s (HPAEs) have emerged as one type of the most viable non-viral gene delivery vectors, both in vitro and in vivo. However, the effects of different branching strategies on the gene transfection performance have not yet been explored. Here, using triacrylate (B3) and diamine (B4) as the branching monomers, a series of HPAEs were synthesized via the “A2 + B3 + C2” and “A2 + B4 + C2” strategies, respectively. Results show that the branching strategy plays a pivotal role in dictating the physiological properties of the HPAE/DNA polyplexes and thus leads to obviously different cell viability and transfection efficiency. Comparatively, HPAEs synthesized via the “A2 + B3 + C2” branching strategy are more favorable for DNA transfection than that synthesized via the “A2 + B4 + C2” strategy. This study may provide new insights into the development of HPAEs based non-viral DNA delivery system.

Keywords Gene therapy; Non-viral gene vector; Highly branched polymer; Branching strategy

INTRODUCTION

Gene therapy, the process that specific genetic materials are transported into the target cells to restore the production of functional proteins or suppress the expression of undesired proteins, has become one of the most promising treatments for various inherited or acquired diseases. Naked DNA and RNA can be degraded by widely existing nuclease enzymes. Therefore, to improve the gene therapy efficacy, DNA and RNA are usually condensed or encapsulated by gene delivery vectors to form nano-sized polyplexes, which not only protects them from enzymatic degradation, but also facilitates cellular uptake of the formed polyplexes.

In general, gene delivery vectors can be categorized to viral and non-viral vectors. Viral vectors such as adeno-associated virus (AAV) usually manifest a high gene transfection capability. However, limitations associated with potential safety concerns, difficulties in large scale preparation, and high production cost may compromise their broad application in clinic. Comparatively, non-viral gene vectors have a higher safety profile, can be synthesized in large scales at a cost-effective manner, and thus have attracted much attention over the past decade. Of the numerous non-viral vectors, cationic polymers are particularly promising for translation from bench to bedside due to their high polymer structure flexibility and functionality tailorbility. Poly(ethylene imine) (PEI), poly(aminodiamine) (PAMAM), poly(dimethylaminoethyl acrylate) (PDMAEMA), poly(lysine) (PLL), etc. have been intensively developed and optimized as effective non-viral gene delivery vectors, in terms of high safety and efficient transfection. For example, by introducing a dipicolylamine-based disulfide-containing zinc(II) coordinative module into low molecular weight PEI, the gene transfection efficiency in primary and stem cells was improved by up to 2 orders-of-magnitude. Similarly, after fluorination, PAMAM dendrimers showed superior gene transfection efficiency and biocompatibility in comparison with Lipofectamine 2000 and SuperFect.

Among the diverse cationic polymers, linear poly(β-amino ester)s (LPAEs), first developed by Langer and colleague in 2000, are one type of the most efficient non-viral gene delivery vectors. LPAEs are usually synthesized via the con-
jugate addition of amines to diacylates under mild reaction conditions. The primary amines in the terminus and tertiary amines on the backbone can impart LPAEs positive charges and strong proton buffering capacity to facilitate DNA condensation and polyplex escape from endosomes through the "proton sponge effect." Importantly, the multiple ester groups on the backbone are biodegradable by hydrolysis under physiological environment, which can not only promote DNA release from the polyplexes, but also reduce the accumulative cytotoxicity after transfection. Utilizing high-throughput technology, more than 2350 LPAEs with different chemical compositions and terminal groups were synthesized and screened for gene transfection by Anderson et al. The top-performing LPAEs synthesized from 5-amino-1-pentanol, 1,4-butanediol diacrylate and end-capped with diacrylamides can even rival virus in transfection capability. Mechanistic studies revealed that the terminal groups play a critical role in dictating the overall gene transfection performance of LPAEs.

Despite the great progress, however, for LPAEs, the linear structure with only two terminal groups may limit further improvement of their gene transfection performance. In contrast, branched polymers with a three-dimensional (3D) structure and multiple terminal groups may be of additional advantages for gene delivery. Nevertheless, the synthesis of branched polymers has been a long-standing challenge due to the intrinsic gelation proneness during polymerization. For the synthesis of branched poly(β-amino ester), several strategies were developed by Park and Liu et al. However, these strategies either necessitate a catalyst and high pressure or are heavily dependent on the utilization of AA’A+ type special amines. In order to develop a generalizable and controllable strategy for highly branched PAEs (HPAEs) synthesis, in 2015, our group proposed the "A2 + B3 + C2" type Michael addition strategy using triacrylate as the branching monomer: primary amine (A2 type monomer), triacrylate (B3 type monomer), and diacrylate (C2 type monomer) were copolymerized in a one-pot manner, which not only keeps the viable monomer combination derived from LPAEs synthesis, but also gives rise to a branched structure. Using the "A2 + B3 + C2" strategy, 22 HPAEs with different monomer combinations and branching degrees were synthesized without gelation. In vitro gene transfection study shows that in comparison with the corresponding LPAEs, HPAEs showed up to 8521-fold higher gene transfection efficiency over 12 cell types, especially at low polymer/DNA weight (W/W) ratios. In vivo gene transfection using recessive dystrophic epidermolysis bullosa (RDEB) knockout and graft mouse models demonstrates that the optimized HPAEs can effectively deliver collagen VII (C7) encoding COL7A1 gene to restore C7 expression in the base membrane zone over 10 weeks without inducing any obvious inflammation. Mechanistic studies further revealed that branching imparts HPAEs a stronger DNA binding affinity and higher proton buffering capacity, which leads to the formation of more compact polyplexes with higher cellular uptake efficiency and endosomal escape ability. Using an iterative optimization strategy, we further showed that HPAEs are capable of delivering nerve growth factor (NGF) encoding DNA to promote neurite outgrowth, highlighting their potential application in the treatment of neural degenerative diseases, such as Parkinson’s disease. HPAEs have demonstrated their application in gene transfection and branching has proven to be one of the most critical parameters dictating the transfection efficiency. Up to now, all the HPAEs have been synthesized via the "A2 + B3 + C2" strategy by using triacrylate as the branching monomer. The effects of different branching strategies and branching monomers have never been explored. Herein, using triacrylate and diacrylate as the branching monomer respectively, we report the synthesis of HPAEs via the "A2 + B3 + C2" and "A2 + B4 + C2" Michael addition strategies, and compare their performance in gene transfection. It has been shown that the different strategies substantially affect the DNA condensation, polyplex size, zeta potential, cellular uptake, and eventually the gene transfection efficiency and safety profile of HPAEs. These results would provide new inspirations for the development of HPAEs based non-viral gene delivery vectors.

**EXPERIMENTAL**

**Materials**

For HPAEs synthesis and characterization, 4-amino-1-butanol, 1,4-butanediol diacrylate, trimethylolpropane triacrylate, 1,3-diaminopropane, 1-(3-aminopropyl)-4-methylpiperazine, dimethylsulfoxide (DMSO), dimethylformamide (DMF), lithium bromide (LiBr), tetrahydrofuran (THF), diethyl ether, deuterated chloroform (CDCl3), and sodium acetate buffer (pH = 5.2, 3.0 mol/L) were purchased from TCI, Sigma-Aldrich, and VWR. Polyelectrolytes such as PEI, Mw = 25 kDa were purchased from Qiagen and Sigma-Aldrich. Green fluorescence protein plasmid (GFP) and human β-galactosidase (β-gal) were purchased from Invitrogen. For cell culture and gene transfection, cell culture media, trypsin-EDTA, 4',6-diamidino-2-phenylindole (DAPI), paraformaldehyde, Triton X-100, fetal bovine serum (FBS), phosphate buffered saline (PBS), and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich and Life Technologies. Commercial gene transfection reagents SuperFect and branched polyethylene imine (PEI, Mw = 25 kDa) were purchased from Qiagen and Sigma-Aldrich. Green fluorescence protein-plasmid (GFP-DNA) and cell secreted Gaussia Princeps luciferase plasmid (Gluc DNA) were obtained from New England Biolabs UK, and its expansion, isolation and purification were performed using the Giga-Prep (Qiagen) kits as per protocols. BioLux Gaussia Luciferase Assay Kit purchased from New England Biolabs and Alamarblue purchased from Invitrogen were used as per standard protocols.

**HPAE Synthesis and Characterization**

Two HPAEs with a relatively low molecular weight and a relatively high molecular weight (named as HPAE-1 and HPAE-2, respectively) were synthesized via the "A2 + B3 + C2" Michael addition strategy using trimethylolpropane triacrylate as the branching monomer. Briefly, 4-amino-1-butanol, 1,4-butanediol diacrylate, and trimethylolpropane triacrylate were synthesized without gelation.
dissolved in DMSO at an overall monomer concentration of 500 mg/mL, and the mixture was then bubbled using argon for 10 min followed by reacting at 90 °C under magnetic stirring. Molecular weight of the polymers was monitored with gel permeation chromatography (GPC). When the weight-average molecular weight ($M_w$) was approaching to target values, the reactions were stopped by cooling down to room temperature and diluting with THF. Next, 1-(3-aminopropyl)-4-methylpiperazine was added to end-cap the polymers at room temperature for 24 h. For purification, the polymers were precipitated with excess diethyl ether three times, and then dried in vacuum oven for 24 h. Similarly, two HPAEs with a relatively low molecular weight and a relatively high molecular weight (named as HPAE-3 and HPAE-4, respectively) were also synthesized via the "A2 + B4 + C2" strategy by using 1,3-diaminopropane as the branching monomer.

**GPC Measurements**
The $M_w$ number-average molecular weight ($M_n$), polydispersity index (PDI), and Mark-Houwink plot of HPAEs were determined with a PL-GPC 50 Integrated GPC system equipped with a refractive index detector (RI), a viscometer detector (VS DP), and a dual angle light scattering detector (LS 15° and 90°). To monitor the molecular weight of polymers in the polymerization process, a volume of 50 μL of the reaction mixture was taken and diluted with 1 mL of DMF, and then filtered through a 0.22 μm filter. Measurements were carried out using DMF (plus 0.1% LiBr) as the eluent solution at a flow rate of 1 mL/min at 50 °C. To measure the molecular weight and Mark-Houwink plot of the final products, HPAEs were dissolved in DMF to 1 mg/mL and then filtered through a 0.22 μm filter. GPC measurements were carried out as above.

**NMR Measurements**
Chemical composition and purity of HPAEs were verified by nuclear magnetic resonance ($^{1}H$-NMR and $^{13}C$-NMR). HPAEs were dissolved in CDCl$_3$ and NMR measurements were conducted on a Varian Spectrometer (400 MHz). The chemical shifts were reported in parts per million (ppm) relative to the response of the solvent.

**Gel Electrophoresis**
Aagarose gel electrophoresis was used to determine the DNA condensation ability of HPAEs. Polymers were first dissolved in DMSO to form stock solution (100 mg/mL). According to the $W/W$ ratios, 1 μg of DNA solution and the required amount of HPAE stock solution were diluted to 20 μL with sodium acetate buffering (pH = 5.2, 0.025 mol/L), respectively. Afterwards, the DNA solution was added into the HPAE solution and vortexed for 15 s. The mixture was then kept undisturbed for 10–15 min for polyplex formation. Later on, 20 μL of the polyplex solution was loaded into the agarose gel (1%) containing SYBR Safe DNA Stain. Electrophoresis was conducted at 100 mV for 60 min.

**Polyplex Size and Zeta Potential Measurements**
2 μg of DNA was used for each sample preparation. Polyplexes were prepared as mentioned above. After diluting with PBS to 1.5 mL, the polyplex solution was transferred to the corresponding cuvettes. Size and zeta potential measurements were carried out with a Malvern Instruments Zetasizer (Nano-2590) equipment with a scattering angle of 90°. All experiments were repeated at least four times.

**Cell Culture**
Human cervical cancer cells (HeLa) purchased from ATCC were cultured in Dulbecco’s modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin (P/S) and 10% FBS. Human adipose derived mesenchymal stem cells (hADSC) purchased from Invitrogen were cultured in MesenPRO RS medium containing basal medium, growth supplements, and 1% P/S. RDEBK cells kindly provided by Dr. Fernando Larcher (CIEMAT, Spain) were cultured in keratinocyte cell basal medium (KBM-Gold) containing the supplements and 1% P/S. All the cells were cultured in a humid incubator under standard cell culture conditions.

**Polyplex Cellular Uptake**
DNA was labelled with Cy3 fluorescence dye. Cellular uptake of polyplexes was investigated by visualizing the polyplex distribution inside cells with a fluorescent microscope. HeLa cells were seeded in 96-well plate at a density of 3000 cells/well and incubated overnight to attach. Polyplexes were prepared as above and diluted with DMEM without FBS. The cell culture medium in the wells was removed and polyplex solution containing 0.1 μg of labelled DNA was added gently. The cells were incubated for another 4 h before washing with HBSS three times to remove the untaken polyplexes. And then, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with DAPI before visualizing and imaging with an Olympus IX81 fluorescence microscope.

**Gene Transfection Studies**
HeLa, hADSC, and RDEBK cells were seeded in 96-well plates at a density of 1.0 × 10$^4$ cells/well and cultured until 70–80% confluence. Polyplexes were prepared as mentioned above and 0.5 μg of GFP DNA or Gluc DNA was used for each sample preparation. The polyplex solution was then diluted with FBS containing cell culture media to 100 μL. After removal of the supernatant in the cell culture plate, the polyplex solution was added, and the cells were cultured for another 48 h. To quantify the Gluc expression efficiency, 20 μL of the cell supernatant was taken and mixed with 50 μL of Gluc assay working solution (prepared according to manufacturer’s protocol). The relative light unit (RLU) of the fluorescence of the mixture was determined with a SpectraMax M3 Plate Reader; the excitation and emission were 485 and 525 nm, respectively. To visualize GFP expression, cells were washed with HBSS and then observed using an IX81 fluorescence microscope.

**Alamarblue Assay**
The metabolic activity of cells after transfection was measured with Alamarblue assay. Briefly, medium in the cell culture plate was removed and cells were washed with HBSS. And then, 100 μL of 10% Alamarblue solution (diluted with HBSS) was added and the cells were incubated for another 2 h. After that, the Alamarblue solution was transferred to a fresh flat-bottomed 96-well plate. The fluorescence intensity was measured with an emission at 590 nm. Cells without any treatment were used as controls and plotted as 100% viable.
Statistical Analysis
All transfection data were analyzed using Graphpad Prism version 8 (GraphPad Software). A one-way ANOVA was performed, followed by Dunnett method as the post hoc test. P values < 0.05 were considered to be statistically significant. All transfection experiments were performed in quadruplicate unless otherwise stated, with error bars indicating ± SD.

RESULTS AND DISCUSSION
Synthesis of HPAEs via Different Branching Strategies
In previous studies, we have demonstrated that the introduction of B3 type monomer trimethylolpropane triacrylate to poly(β-amino ester)s (PAEs) can significantly enhance the gene transfection efficiency. In comparison with triacylate, there are four reaction sites in primary diamines, which would be more favorable for the formation of branched structure. Therefore, 1,3-diaminopropane was selected as a presentative B4 type branching monomer for the synthesis of HPAEs via the "A2 + B4 + C2" strategy and compare with those synthesized via the well-established "A2 + B3 + C2" strategy in gene transfection (Scheme 1). Given that gene transfection efficiency and safety of cationic polymers are usually related to molecular weight, HPAE-1 (HPAE-3) with a relatively low molecular weight and HPAE-2 (HPAE-4) with a relatively high molecular weight were synthesized, respectively. The monomer feed ratios for the synthesis of the four HPAEs are outlined in Tables S1–S3 (in the electronic supplementary information, ESI). GPC was used to monitor the increase of molecular weight in the polymerization process. It was found that no matter trimethylolpropane triacrylate or 1,3-diaminopropane was used as the branching monomer, no gelation was observed. When the $M_w$ of the reaction mixtures was approaching to 10 kDa or 25 kDa (Fig. S1 in ESI), the reaction was stopped and then excess 1-(3-aminopropyl)-4-methylpiperazine was added to end-cap the vinyl terminated base polymers. After purification, HPAE-1, HPAE-2, HPAE-3, and HPAE-4 had $M_w$ of 14.7, 28.6, 10.5, and 55.0 kDa, respectively.

Scheme 1 Illustration of the synthesis of HPAEs via the "A2 + B3 + C2" and "A2 + B4 + C2" branching strategies, respectively.

Fig. 1 (a) GPC traces and (b) Mark-Houwink plots of HPAEs after end-capping; HAPEs can condense DNA to formulate nano-sized polyplexes.

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Mark-Houwink plot measurements further showed that the HPAEs had an alpha (α) value of 0.28, 0.28, 0.18, and 0.19, respectively, all of which were below 0.5 indicating their highly branched structures. Comparatively, the α values of HPAE-3 (0.18) and HPAE-4 (0.19) were much lower than those of the HPAE-1 (0.28) and HPAE-2 (0.28) (Fig. 1b), demonstrating the more branched structures of HPAEs derived from the utilization of 1,3-diaminopropane as the branching monomer. Chemical structures and compositions of all the HPAEs were further validated with NMR (Fig. S2 in ESI). There are multiple tertiary amines on the backbone of HPAEs. Under acidic conditions such as in sodium acetate buffer (pH = 5.2), they were able to protonate and thus endowed HPAEs with DNA condensation ability through the electrostatic interaction, which was confirmed with gel electrophoresis. In general, HPAE-1 and HPAE-2 did not exhibit obvious difference from HPAE-3 and HPAE-4 in DNA condensation. As shown in Fig. 2(a), over the tested HPAE/DNA W/W ratios ranging from 0.5/1 to 8/1, all the HPAEs could condense DNA efficiently without any DNA band shift along the agarose gel. In most of the cases, DNA was condensed by HPAEs tightly and the SYBR Safe DNA Gel Stain was not able to access the condensed DNA. Especially, even at the lowest W/W ratio of 0.5/1, all the HPAEs were still able to condense DNA completely. The effective DNA condensation by HPAEs led to the formulation of nano-sized polyplexes, physiological properties of which (size and zeta potential) were measured with dynamic light scattering (DLS) (Figs. 2b and 2c). In PBS, at the W/W ratios of 30/1 and 60/1, the HPAE-3/DNA polyplexes manifested a slightly bigger size than the HPAE-1/DNA counterparts. However, at the W/W ratio of 90/1, a slightly smaller size of HPAE-3/DNA polyplexes was observed. In contrast, at all the three tested W/W ratios, the HPAE-4/DNA polyplexes exhibited much smaller sizes than the HPAE-2/DNA counterparts. Particularly, at the W/W ratio of 90/1, size of the HPAE-4/DNA polyplexes was only around 200 nm, less than half of that of the HPAE-2/DNA counterparts (around 550 nm). With regards to zeta potential, all the polyplexes exhibited a positive surface charge ranging from +1 mV to +11 mV, which is in agreement with the gel electrophoresis results, further confirming the complete condensation of DNA by HPAEs. At the W/W ratios of 30/1 and 60/1, HPAE-3/DNA and HPAE-4/DNA polyplexes had more positive zeta potentials than the corresponding HPAE-1/DNA and HPAE-2/DNA counterparts did. However, at the W/W ratio of 90/1, despite that both of the polyplexes had the highest zeta potential, a reverse trend was observed: HPAE-3/DNA and HPAE-4/DNA polyplexes showed slightly lower zeta potentials than the HPAE-1/DNA and HPAE-2/DNA counterparts. The obvious differences in size and zeta potential may lead the polyplexes to exhibit different cellular uptake behaviors.
Cellular Uptake of HPAE/DNA Polyplexes

Cellular uptake of polyplexes is the first barrier of gene transfection and would directly affect the following intracellular fates of the polyplexes, and therefore is critical for efficient gene transfection.\[42\] Cellular uptake of the four different HPAE/DNA polyplexes was further investigated using a fluorescence microscope. To visualize polyplex distribution inside cells, DNA was labelled with the red fluorescence dye Cy3. HeLa cells were incubated with the Cy3 labelled polyplexes at the W/V ratio of 60/1 for 4 h in the absence of serum. After washing, fixation and permeabilization with HBSS, paraformamide, Triton X-100 in sequence, the cell nucleuses were stained with the blue fluorescence dye DAPI. Fluorescence images of HeLa cells after incubation with the different HPAE/DNA polyplexes are shown in Fig. 3. In general, all the polyplexes exhibited a high cellular uptake efficiency. This is well in agreement with the small size and positive zeta potential of polyplexes, as determined by the DLS (Fig. 2). Comparatively, HPAE-3/DNA and HPAE-4/DNA polyplexes exhibited a relatively high cellular uptake efficiency, as indicated by the many more red polyplexes inside the cells. In addition, the HPAE-1/DNA and HPAE-2/DNA polyplexes were more aggregated compared with the HPAE-3/DNA and HPAE-4/DNA counterparts, which is possibly due to the slightly lower zeta potentials (Fig. 2c). Further analyzing polyplexes distribution inside the cells showed that HPAE-1/DNA and HPAE-2/DNA polyplexes were mainly aggregated around the nucleus, as indicated by the white arrows. Similar results were also observed in previous studies in which HPAEs were also synthesized via the “A2 + B3 + C2” strategy using trimethylolpropane triacrylate as the branching monomer.\[28,33\] In contrast, HPAE-3/DNA and HPAE-4/DNA polyplexes distributed more evenly inside the cells. The sharp contrast indicates that HPAE-1/DNA and HPAE-2/DNA polyplexes may have potential application in nucleus targeting delivery of genes and/or drugs. Considering that the main difference between the HPAEs lies with the utilization of different branching monomers (trimethylolpropane triacrylate and 1,3-diaminopropane), these results highlight the significance of branching strategy in determining polyplex cellular uptake efficiency and nucleus targeting ability.

![Fig. 3](https://doi.org/10.1007/s10118-020-2393-y)
In vitro Gene Transfection Efficiency and Safety of HPAE/DNA Polyplexes

To test the gene transfection capability and applicability of the different HPAE/DNA polyplexes, a cancer cell line HeLa, a diseased skin keratinocyte RDEBK, and human adipose derived mesenchymal stem cells hADSC were used. The reporter gene GFP DNA was first used to qualitatively evaluate the gene transfection potency of HPAE/DNA polyplexes. SuperFect and PEI have similar dendritic structures to HPAE, so they were used as the positive controls. As shown in Fig. 4, in HeLa cells, at the W/W ratio of 60/1, HPAE-1 mediated much higher GFP expression than HPAE-3, while HPAE-2 and HPAE-4 showed similar gene transfection efficiency. These results indicate that HPAEs synthesized via the “A2 + B3 + C2” branching strategy were more favorable for HeLa transfection. In RDEBK, a similar trend in gene transfection capability was observed: HPAE-1 and HPAE-3 showed similar level of GFP expression, while HPAE-2 mediated much higher gene transfection efficiency, as evidenced by the many more GFP positive cells observed. hADSC are notorious for gene transfection using non-viral gene vectors. Indeed, all the HPAEs showed much lower GFP expression in hADSC in comparison with the HeLa and RDEBK. However, HPAE-1 and HPAE-2 were still more potent than HPAE-3 and HPAE-4. To further confirm the gene transfection capability of the different HPAEs, Gluc DNA was used and the Gluc activity of cells after transfection was quantified with Gluc assay at three different W/W ratios (30/1, 60/1, and 90/1); commercial gene transfection reagents SuperFect and branched PEI (M_w = 25 kDa) were used as the positive controls. As shown in Fig. 5(a), in HeLa cells, both HPAE-1/DNA and HPAE-2/DNA polyplexes mediated high gene transfection efficiency without inducing obvious cytotoxicity, as validated by the Alamarblue assay. Comparatively, cells treated with HPAE-3/DNA polyplexes at the W/W ratio of 60/1 showed the highest Gluc activity. In constrast, although HPAE-3/DNA and HPAE-4/DNA polyplexes had a similar transfection capability to that of the HPAE-2/DNA counterparts, only 20%–30% cell viability was preserved at the W/W ratios of 60/1 and 90/1. Similarly, in RDEBK (Fig. 5b), HPAE-1/DNA and HPAE-2/DNA mediated higher Gluc activity while maintaining > 80% cell viability, especially at the W/W ratios of 60/1 and 90/1. Interestingly, in hADSC (Fig. 5c), HPAE-4/DNA polyplexes showed the most robust gene transfection ability at the W/W ratios of 60/1 and 90/1. Particularly, at the W/W ratio of 60/1, HPAE-4/DNA polyplexes mediated high gene transfection efficiency while preserving over 60% hADSC viability, highlighting its potential application in stem cell gene transfection. Taking the GFP expression and Gluc activity of cells after transfection together, it can be concluded that HPAEs synthesized via the “A2 + B3 + C2” branching strategy were more favorable for gene transfection than the ones prepared from the “A2 + B4 + C2” strategy, especially given the obviously different safety profiles of the polymers. Despite that the physiological properties of polyplexes derived from the polymers synthesized via the “A2 + B4 + C2” strategy were more favorable for cellular uptake, the overall gene transfection efficiency was lower. We speculate this is possibly derived from the different hydrophobicity of the polymers: in comparison with the B4 monomer (diamine), the B3 monomer (triacrylate) is more hydrophobic, and therefore, the relatively high hydrophobicity of the polymers synthesized from the “A2 + B3 + C2” strategy would be more favorable for gene transfection, as reported previously. However, in some cases
(such as in hADSC), HPAEs synthesized via the “A2 + B4 + C2” branching strategy also showed good balance between gene transfection efficiency and cytotoxicity.

**CONCLUSIONS**

In this study, using triacrylate and diamine as the branching monomers, HPAEs were synthesized via the “A2 + B3 + C2” and “A2 + B4 + C2” branching strategies, respectively, and compared in their gene transfection performance. HPAEs synthesized from the both strategies could effectively condense DNA to formulate polyplexes. However, obviously different physiological properties, such as size and zeta potential, were observed. Cellular uptake study further

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revealed that the “A2 + B3 + C2” branching strategy would impart the HPAEs a stronger nucleus targeting ability than the “A2 + B4 + C2” strategy. In three different cell types, HPAEs synthesized via the “A2 + B3 + C2” strategy mediated high gene transfection efficiency while inducing much lower cytotoxicity. Our study demonstrates that “A2 + B3 + C2” strategy may be more favorable for enhancing the gene transfection of PAE based gene delivery vectors.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at http://dx.doi.org/10.1007/s10118-020-2393-y.

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REFERENCES

1. Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. Design and development of polymers for gene delivery. Nat. Rev. Drug Discov. 2005, 4, 581–593.
2. Nguyen, J.; Szoka, F. C. Nucleic acid delivery: the missing pieces of the puzzle. Acc. Chem. Res. 2012, 45, 1153–1162.
3. Behr, J. P. Synthetic gene-transfer vectors. Acc. Chem. Res. 1993, 26, 274–278.
4. Srivinas, R.; Samanta, S.; Chaudhuri, A. Cationic amphiphiles: promising carriers of genetic materials in gene therapy. Chem. Soc. Rev. 2009, 38, 3326–3338.
5. Mintzer, M. A.; Simanek, E. E. Nonviral vectors for gene delivery. Chem. Rev. 2009, 109, 259–302.
6. Guo, X.; Huang, L. Recent advances in non-viral vectors for gene delivery. Acc. Chem. Res. 2013, 45, 971–979.
7. Boussif, O.; Lezouac'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethyleneimine. Proc. Natl. Acad. Sci. 1995, 92, 7297–7301.
8. Liu, H.; Wang, H.; Yang, W.; Cheng, Y. Disulfide cross-linked low generation dendrimers with high gene transfection efficacy, low cytotoxicity, and low cost. J. Am. Chem. Soc. 2012, 134, 17680–17687.
9. Zhao, T.; Zhang, H.; Newland, B.; Aied, A.; Zhou, D.; Wang, W. Significance of branching for transfection: synthesis of highly branched degradable functional poly(dimethylaminoethyl methacrylate) by vinyl oligomer combination. Angew. Chem. Int. Ed. 2014, 53, 6095–6100.
10. Zhou, D.; Li, C.; Hu, Y.; Zhou, H.; Chen, J.; Zhang, Z.; Guo, T. The effects of a multifunctional oligomer and its incorporation strategies on the gene delivery efficiency of poly(L-lysine). Chem. Commun. 2012, 48, 4594.
11. Li, C.; Guo, T.; Zhou, D.; Hu, Y.; Zhou, H.; Wang, S.; Chen, J.; Zhang, Z. A novel glutathione modified chitosan conjugate for efficient gene delivery. J. Control Release 2011, 154, 177–188.
12. Liu, S.; Zhou, D.; Yang, J.; Zhou, H.; Chen, J.; Guo, T. Bioreducible zinc(II)-coordinated polyethyleneimine with low molecular weight for robust gene delivery of primary and stem cells. J. Am. Chem. Soc. 2017, 139, 5102–5109.
13. Wang, M.; Liu, H.; Li, L.; Cheng, Y. A fluorinated dendrimer achieves excellent gene transfection efficacy at extremely low nitrogen to phosphorus ratios. Nat. Commun. 2014, 5, 3053.
14. Lynn, D. M.; Langer, R. Degradable poly(β-aminoster): synthesis, characterization, and self-assembly with plasmid DNA. J. Am. Chem. Soc. 2000, 122, 10761–10768.
15. Green, J. J.; Langer, R.; Anderson, D. G. Yields insight into nonviral gene delivery. Acc. Chem. Res. 2008, 41, 749–759.
16. Akinc, A.; Lynn, D. M.; Anderson, D. G.; Langer, R. Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery. J. Am. Chem. Soc. 2003, 125, 5316–5323.
17. Ettoukhly, A. A.; Siegwart, D. J.; Abali, C. A.; Rajan, J. S.; Langer, R.; Anderson, D. G. Effect of molecular weight of amine end-modified poly(β-aminoster) on gene delivery efficiency and toxicity. Biomaterials 2012, 33, 3594–3603.
18. Anderson, D. G.; Lynn, D. M.; Langer, R. Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery. Angew. Chem. Int. Ed. 2003, 42, 3153–3158.
19. Green, J. J.; Zugates, G. T.; Tedford, N. C.; Huang, Y. H.; Griffith, L. G.; Lauffenburger, D. A.; Sawicki, J. A.; Langer, R.; Anderson, D. G. Combinatorial modification of degradable polymers enables transfection of human cells comparable to adenovirus. Adv. Mater. 2007, 19, 2836–2842.
20. Lee, C. C.; MacKay, J. A.; Fréchet, J. M. J.; Szoka, F. C. Designing dendrimers for biological applications. Nat. Biotechnol. 2005, 23, 1517–1526.
21. Voit, B. I.; Lederer, A. Hyperbranched and highly branched polymer architectures—synthetic strategies and major characterization aspects. Chem. Rev. 2009, 109, 5924–5973.
22. Lim, Y. B.; Kim, S. M.; Lee, Y.; Lee, W. K.; Yang, T. G.; Lee, M. J.; Suh, H.; Park, J. C. Cationic hyperbranched poly(aminoster): a novel class of DNA condensing molecule with cationic surface, biodegradable three-dimensional structure, and tertiary amine groups in the interior. J. Am. Chem. Soc. 2001, 123, 2460–2461.
23. Wu, D.; Liu, Y.; Chen, L.; He, C.; Chung, T. S.; Goh, S. H. 2A+ βBB approach to hyperbranched poly(aminoster). Macromolecules 2005, 38, 5519–5525.
24. Liu, Y.; Wu, D.; Ma, Y.; Tang, G.; Wang, S.; He, C.; Chung, T.; Goh, S. Novel poly(aminoster)s obtained from Michael addition polymerizations of trifunctional amine monomers with diacylates: safe and efficient DNA carriers. Chem. Commun. 2003, 20, 2630–2631.
25. Wu, D.; Liu, Y.; Jiang, X.; He, C.; Goh, S. H.; Leong, K. W. Hyperbranched poly(aminoster)s with different terminal amine groups for DNA delivery. Biomacromolecules 2006, 7, 1879–1883.
26. Huang, J. Y.; Gao, Y.; Cutlar, L.; O’Keeffe-Ahern, J.; Zhao, T.; Lin, F.; Zhou, D.; Mcmahon, S.; Greiser, U.; Wang, W.; Wang W. Tailoring highly branched poly(β-aminoster)s: a synthetic platform for epidermal gene therapy. Chem. Commun. 2015, 51, 8473–8476.
27. Zhou, D.; Cutlar, L.; Gao, Y.; Wang, W.; O’Keeffe-Ahern, J.; McMahan, S.; Duarte, B.; Larcher, F.; Rodriguez, J. B.; Greiser U.; Wang, W. Highly branched poly(β-aminoster): synthesis and application in gene delivery. Biomacromolecules 2015, 16, 2609–2617.
28. Zhou, D.; Cutlar, L.; Gao, Y.; Wang, W.; O’Keeffe-Ahern, J.; McMahan, S.; Duarte, B.; Larcher, F.; Rodriguez, J. B.; Greiser U.; Wang, W. The transition from linear to highly branched poly(β-aminoster)s: branching matters for gene delivery. Sci. Adv. 2016, 2, e1600102.
29. Zhou, D.; Gao, Y.; Aied, A.; Cutlar, L.; Igoucheva, O.; Newland, B.; Alexeev, V.; Greiser, U.; Utito, J.; Wang, W. Highly branched poly(β-aminoster)s for skin gene therapy. J. Control Release
Cutlar, L.; Zhou, D.; Duarte, B.; Greiser, U.; Larcher, F.; Wang, W. A non-viral gene therapy for treatment of recessive dystrophic epidermolysis bullosa. *Exp. Dermatol.* **2016**, *25*, 818−820.

Zeng, M.; Zhou, D.; Alshehri, F.; Lara-Sáez, I.; Lyu, Y.; Creagh-Flynn, J.; Xu, Q.; Zhang, J.; Wang W. Efficient and robust highly branched poly(β-amino ester)/minicircle COL7A1 polymeric nanoparticles for gene delivery to recessive dystrophic epidermolysis bullosa keratinocytes. *ACS Appl. Mater. Interfaces* **2019**, *11*, 30661−30672.

Zhou, D.; Gao, Y.; Ahern, J. O.; Xu, Q.; Huang, X.; Greiser, U.; Wang, W. Highly branched poly(β-amino ester) for non-viral gene delivery: high transfection efficiency and low toxicity achieved by increasing molecular weight. *Biomacromolecules* **2016**, *17*, 3640−3647.

Liu, S.; Gao, Y.; Zhou, D.; Greiser, U.; Guo, T.; Wang, W. Biodegradable highly branched poly(β-amino ester)s for targeted cancer cell gene transfection. *ACS Biomater. Sci. Eng.* **2017**, *3*, 1283−1286.

Sperling, L. H. *Introduction to physical polymer science*, 4th ed.; John Wiley & Sons, 2005.

Anderson, D. G.; Peng, W.; Akinc, A.; Hossain, N.; Kohn, A.; Padera, R.; Langer, R.; Sawicki, J. A. A polymer library approach to suicide gene therapy for cancer. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 16028−16033.

Newland, B.; Zheng, Y.; Jin, Y.; Abu-Rub, M.; Cao, H.; Wang, W.; Pandit, A. Single cyclized molecule versus single branched molecule: a simple and efficient 3D ‘knot’ polymer structure for nonviral gene delivery. *J. Am. Chem. Soc.* **2012**, *134*, 4782−4789.

Xiang, S.; Tong, H.; Shi, Q.; Fernandes, J. C.; Jin, T.; Dai, K.; Zhang, X. Uptake mechanisms of non-viral gene delivery. *J. Control. Release* **2012**, *158*, 371−378.

Liu, Z.; Zhang, Z.; Zhou, C.; Jiao, Y. Hydrophobic modifications of cationic polymers for gene delivery. *Prog. Polym. Sci.* **2010**, *35*, 1144−1162.

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