Hyperbranched Poly(amino ester)s with Different Terminal Amine Groups for DNA Delivery

Decheng Wu,[†,‡] Ye Liu,[*,†] Xuan Jiang,[§] Chaobin He,[†] Suat Hong Goh,[‡] and Kam W. Leong[§,#]

Institute of Materials Research and Engineering, 3 Research Link, Singapore 117602, Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Division of Johns Hopkins in Singapore, Singapore 138669, Singapore, and Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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Hyperbranched poly(amino ester)s containing tertiary amines in the core and primary, secondary, and tertiary amines in the periphery, respectively, were evaluated for DNA delivery in vitro. The same core structure facilitated the investigation on the effects of the terminal amine type on the properties of hyperbranched poly(amino ester)s for DNA delivery. The hydrolysis of the poly(amino ester)s was monitored using ^{1}H NMR. The results reflected that the terminal amine type had negligible effects on the hydrolysis rate but was much slower than that of linear poly(amino ester)s, probably due to the compact hyperbranched spatial structure preventing the accessibility of water. In comparison with PEI 25 K, the hyperbranched poly(amino ester)s showed much lower cytotoxicity in Cos7, HEK293, and HepG2 cells. Gel electrophoresis indicated that poly(amino ester)s could condense DNA efficiently, and the zeta potentials and sizes of the complexes formed with different weight ratios of hyperbranched poly(amino ester)s and DNA were measured. Remarkably, all the hyperbranched poly(amino ester)s showed DNA transfection efficiency comparable to PEI 25 K in Cos7, HEK293, and HepG2 cells regardless of the terminal amine type. Therefore, the terminal amine type had insignificant effects on the hydrolysis rate, cytotoxicity, DNA condensation capability, and in vitro DNA transfection efficiency of the hyperbranched poly(amino ester)s.

Introduction

It still is a formidable challenge but vital to the success of gene therapy to prepare safe and efficient gene vectors.[1] Viral vectors and nonviral vectors have been explored for gene therapy; however, nonviral vectors have recently attracted more and more interest because of the increasing concerns of the potential immunogenic problems related to viral vectors.[1−4] Various polymers have been explored for preparation of nonviral vectors; however, biodegradable polymers are preferred for long-term biocompatibility and safety.[3−15] Among the polymers exploited, the poly(amino ester) is one category of the most promising biodegradable polymers for the preparation of safe and efficient nonviral gene vectors.[12−15] The screening of thousands types of linear poly(amino ester)s containing tertiary amines in the backbones identified several promising candidates with good in vitro or in vivo DNA transfection efficiency with low cytotoxicity.[12] Some of the hyperbranched poly(amino ester)s with tertiary amines in the core and primary amines in the periphery prepared via the AB_m approach also showed good DNA delivery properties.[13]

We have set up an approach to linear poly(amino ester)s containing secondary and tertiary amines in the backbones for DNA delivery based on the Michael addition polymerization of trifunctional amines and diacrylates.[14] On the basis of a clear understanding of the mechanisms of the Michael addition polymerization of trifunctional amines,[16] we have designed and synthesized two types of hyperbranched poly(amino ester)s: one type has amine constitutions similar to polyethylenimine (PEI) showing a DNA transfection efficiency close to that of PEI,[15] and the other type contains tertiary amines in the core with the terminal group easily tunable to different types of amines and widely adjustable chemistry.[17] Here the degradability, cytotoxicity, and in vitro DNA transfection efficiency of the second type of hyperbranched poly(amino ester)s with different terminal amine groups are reported, and the effects of the terminal amine type are investigated.

Experimental Procedures

Materials. 1,4-Butanediol diacrylate (BDA, 90%), 1-(2-aminoethyl)-piperazine (AEPZ, 99%), *N*-methyl piperazine (MPZ, 99%), benzyl 1-piperazine carboxylate (ZPZ, 98%), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and DMSO (99.9%) were purchased from Aldrich. Acetone was obtained from Tedia, and 37.5% hydrochloric acid was received from J. T. Baker. BDA was purified by vacuum distillation (90 °C/50 Pa) in the presence of a little amount of hydroquinone. After being amplified in *Escherichia coli* and purified according to the supplier's protocol (Qiagen, Hilden, Germany), plasmid DNA (pCMV-Luc) (Elim Biopharmaceuticals) was resuspended in TE buffer and kept in aliquots at a concentration of 1 mg/mL for use. DMSO was dried using molecular sieves, and other reagents were used as received.

Synthesis of Protonated Poly(amino ester)s. The neutral hyperbranched poly(amino ester)s with different terminal amines (i.e., poly-(BDA2-AEPZ1)-AEPZ (absolute M_n: 69 780; PDI: 3.90), poly(BDA2-AEPZ1)-PZ (absolute M_n: 60 430; PDI: 3.80), poly(BDA2-AEPZ1)-MPZ (absolute M_n: 62 520; PDI: 3.70) as described in Scheme 1)

* To whom correspondence should be addressed. E-mail: ye-liu@imre.a-star.edu.sg.

† Institute of Materials Research and Engineering.

‡ National University of Singapore.

§ Division of Johns Hopkins in Singapore.

Department of Biomedical Engineering, Johns Hopkins University School of Medicine.

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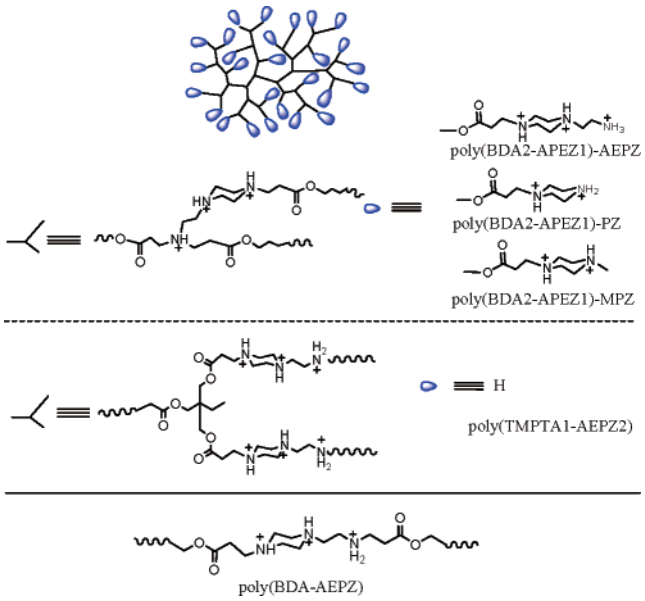
Scheme 1. Structure of Linear and Hyperbranched Poly(amino ester)s Obtained via Michael Addition Polymerization of Trifunctional Amines with Diacrylates and Triacrylates



were synthesized as previously reported.[17a] Briefly, the vinyl terminated hyperbranched poly(amino ester)s were obtained by the Michael addition polymerization of AEPZ with a double molar BDA in DMSO at 70 °C under an argon stream. Then, the terminal group was tuned to the primary, secondary, or tertiary amine, respectively.

Protonated poly(amino ester)s were obtained through hydrochlorination of the neutral hyperbranched poly(amino ester)s. In a typical process, 0.5 g of a pure poly(amino ester) was dissolved in 10 mL of acetone, and the solution was added dropwise into the mixture of 100 mL of acetone and 5 mL of 37.5% HCl. The white precipitate was collected and dried under vacuum at 65 °C for 7 days.

Cytotoxicity Assay of Protonated Poly(amino ester)s. Cos7, HEK293, and HepG2 cells were cultured in DMEM supplemented with 10% FBS at 37 °C, 10% CO_2, and 95% relative humidity. For the cell viability assay, polymer solutions were prepared in serum supplemented tissue culture medium. pH and osmolarity of the preparations were routinely measured and adjusted to pH 7.4 and 280−320 mosm/kg. The cells (10 000 cells/well) were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany). After overnight incubation, the culture medium was replaced with 100 μL serial dilutions of the polymers, and the cells were incubated for another 12 h. Then medium with polymer extraction was aspirated and replaced by 100 μL of DMEM without serum to minimize the change of aggregate formation between the charged sites of proteins and polymer before adding MTT assays. 20 μL of sterile filtered MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) stock solution in phosphate buffered saline (PBS) was added to each well. After 4 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μL/well DMSO (BDH laboratory Supplies) and measured spectrophotometrically in an ELISA reader (Model 550, Bio-Rad) at a wavelength of 570 nm. The spectrophotometer was calibrated to zero absorbance using culture medium without cells. The relative cell growth (%) related to control cells containing cell culture medium without polymer was calculated by $[A]_{test}/[A]_{control} \times 100\%$. All the tests were performed in quadruplicate.

Formation and Analysis of DNA/Poly(amino ester) Complexes. Plasmid DNA (pCMV-Luc) was diluted to a chosen concentration (usually 0.5−1.0 μg/μL) in 5% glucose under vortexing. Various amounts of 0.1−1 μg/μL solution of protonated poly(amino ester)s in 5% glucose were added slowly to the DNA solutions. The amount of poly(amino ester) added was calculated based on a chosen weight ratio of polymer to DNA. After the solution was incubated at ambient temperature for 30 min with gentle vortexing, the polymer/DNA complexes formed were mixed with a loading buffer and loaded onto a 1% agarose gel containing ethidium bromide. Gel electrophoresis was run at room temperature in HEPES buffer (20 mM, pH 7.2) at 80 V for 60 min. DNA bands were visualized using an UV (254 nm) illuminator. The particle sizes and zeta potentials of the complexes of DNA/polymer (DNA: 20 μg/mL; protonated polymer: 10−1200 μg/mL in HEPES buffer (20 mM, pH 7.2)) were measured at 25 °C using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY, 15 mW laser, incident beam = 676 nm).

DNA Delivery In Vitro. The in vitro transfection efficiency of the protonated poly(amino ester) was evaluated in Cos7, HEK293, and HepG2 cells. Cells were seeded for 24 h prior to transfection into 24-well plates (Becton-Dickinson, Lincoln Park, NJ) at a density of 5×10^4 per well with 0.5 mL of complete medium. At the time of transfection, 100 μL of poly(amino ester)/DNA complexes with various weight ratios at a DNA dose of 2 μg/well was added dropwise into each well and incubated with the cells for 4 h at 37 °C. Meanwhile, PEI 25 K was tested with a well-recognized optimum N/P ratio of 10:1 for a comparison. The medium was replaced with 0.5 mL of fresh complete medium, and cells were further incubated for 48 h. After the medium was drawn out and the well was washed with 0.3 mL of 1 × PBS, cells were permeabilized with 200 μL of 1 × cell lysis buffer (Promega Co., Madison, WI). After being frozen at −78 °C for 20 min followed by returning to room temperature for two cycles, the complexes were collected. After centrifugation (15 000/5 min at 4 °C), the samples were ready for testing. The luciferase activity in cell extracts was measured using a luciferase assay Kit (Promega Co., Madison, WI) on a single-well luminometer (Berthold Lumat LB 9507) for 10 s. The light units (LU) were normalized against protein concentration in the cell extracts, which were measured using a protein assay kit (Bio-Rad Labs, Hercules, CA). All the transfection tests were performed in quadruplicate.

Results and Discussion

Hyperbranched Poly(amino ester)s Applied. In our works, hyperbranched poly(amino ester)s containing tertiary amines in the core and different types of amines in the periphery were prepared via the $2A_2 + BB'B''$approach (i.e., the Michael addition polymerization of trifunctional amines with a double molar diacrylate followed by tuning of the terminal vinyl group).[17] The chemistry of the hyperbranched poly(amino ester)s could be adjusted easily through adopting different diacrylate and trifunctional amines; therefore, a potential library of hyperbranched poly(amino ester)s can be produced. Here, the hyperbranched poly(amino ester)s were produced from 1,4-butanediol diacrylate (BDA) and 1-(2-aminoethyl) piperazine (AEPZ). After the terminal groups were tuned to primary, secondary, and tertiary amines as reported,[17a] three kinds of water-soluble protonated hyperbranched poly(amino ester)s (i.e., poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ as described in Scheme 1) were prepared. The same core of the three hyperbranched poly(amino ester)s facilitates the evaluation of the effects of the terminal amine type on the DNA delivery property, which has been carried out rarely.

Degradability and Cytotoxicity. The protonated poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ are degradable in aqueous solution through hydrolysis of the ester groups. The hydrolysis processes of these polymers were monitored using ^{1}H NMR as reported.[14,15,17b] After hydrolysis of the ester group, the peaks attributed to the protons attached to the α and β carbons in the 1, 4-butanediol butane shifted from ca. 4.15 and 1.70 ppm to ca. 3.50 and 1.55 ppm, respectively. Therefore, the degree of hydrolysis could

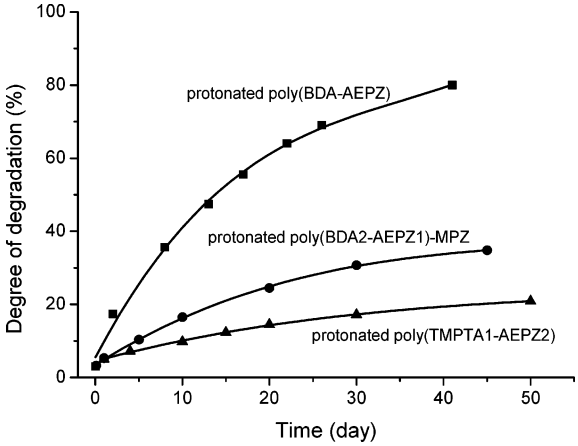


Figure 1. Comparison of the hydrolysis profiles of protonated hyperbranched poly(BDA2-AEPZ1)-MPZ, hyperbranched poly(TMPTA1-AEPZ2), and linear poly(BDA-AEPZ) in aqueous solutions.

be monitored by the change in the ratio of the integral intensities of the three peaks, $I_{4.15}/(I_{1.70} + I_{1.55})$. The results showed that the three poly(amino ester)s have almost the same hydrolysis profile. Figure 1 shows a typical hydrolysis profile, that of poly(BDA2-AEPZ1)-MPZ. Therefore, the terminal group exposes a negligible effect on the hydrolysis profiles, and the internal spatial structure determines the hydrolysis rate. In addition, Figure 1 indicates that only 35% of the ester groups in hyperbranched poly(BDA2-AEPZ1)-MPZ was destroyed after 45 days; however, around 80% of the ester groups in linear poly(BDA-AEPZ) were broken after 40 days.[14] This further verified the hydrolysis retardation function of the compact spatial core of the hyperbranched poly(amino ester)s as observed in our previous work.[15,17b] This phenomenon should be caused by the lesser water accessibility of the ester group in the hyperbranched structure. Furthermore, the hydrolysis profile of these three kinds of protonated poly(amino ester)s was compared with another type of protonated hyperbranched poly(amino ester), poly(TMPTA1-AEPZ2) (TMPTA: trimethylol-propane triacrylate).[15] As shown in Figure 1 as well, the hydrolysis of poly(BDA2-AEPZ1)-MPZ was apparently faster than that of poly(TMPTA1-AEPZ2). This should be caused by the more compact spatial structure adjacent to the ester group in poly(TMPTA1-AEPZ2) than in the poly(BDA2-AEPZ1) series (comparing their structures described in Scheme 1), assuming that the two types of hyperbranched poly(amino ester)s have a similar degree of branching (DB).

Cytotoxicity of the protonated poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ was evaluated in HEK293, Cos7, and HepG2 cells using the standard MTT/thiazolyl blue dye reduction method, and the results are presented in Figure 2. As compared to PEI ($M_n = 25$ K), these protonated poly(amino ester)s are much less cytotoxic. For example, as reflected in Figure 2A, the IC_{50} of PEI was lower than 30 μg/mL in HEK293 cells; however, the cell viability remained above 60% when the concentrations of protonated hyperbranched poly(amino ester)s were up to 250 μg/mL. The cytotoxicity of cationic polymers was probably caused by polymer aggregation on cell surfaces impairing the important membrane functions; also, polymers may interfere with critical intracellular processes of cells, especially as primary amine was reported to disrupt PKC function through disturbing protein kinase activity.[18,19] Nevertheless, Figure 2A−C reflects that no significant difference was observed in the cytotoxicity profile of the poly(amino ester)s with different terminal amine groups even for poly(BDA2-AEPZ1)-AEPZ containing abundant terminal primary amines. Hence, the terminal group posed insignificant effects on the cell viability, and the low cytotoxicity of the poly(amino ester)s should result from the degradability similar to other types of poly(amino ester)s.

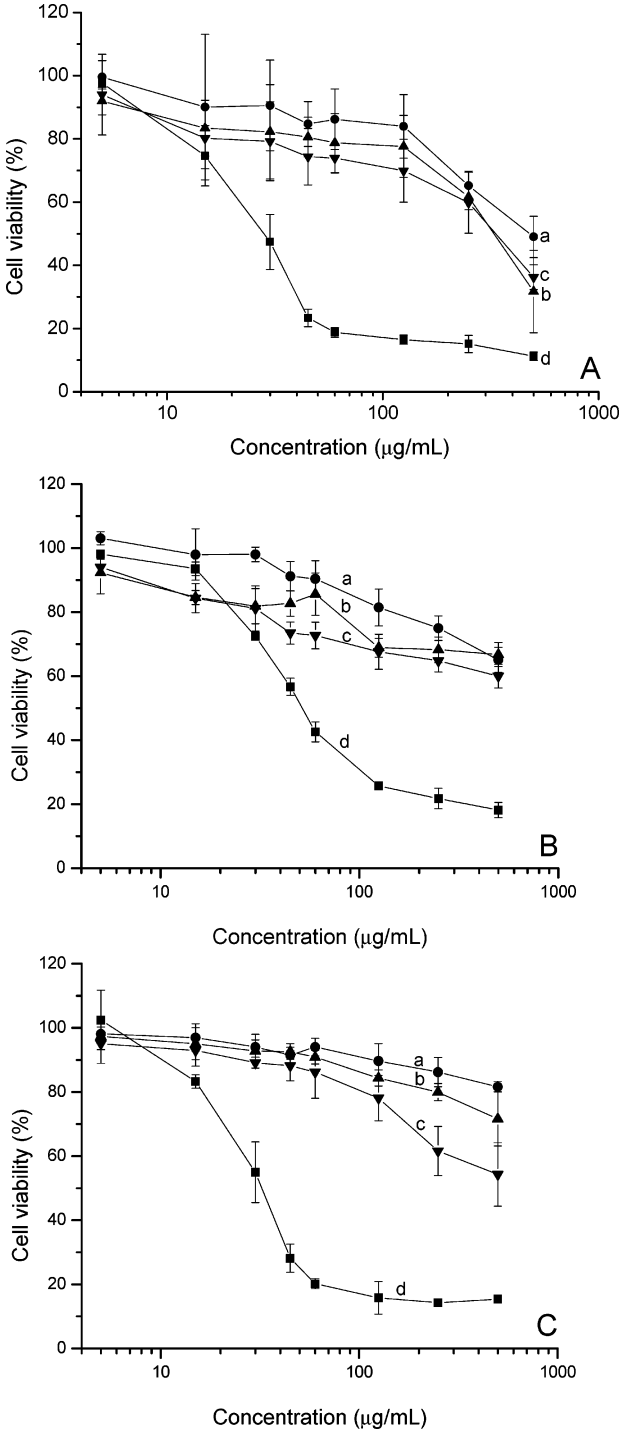


Figure 2. Comparison of the cytotoxicity of (a) protonated poly(BDA2-AEPZ1)-MPZ; (b) protonated poly(BDA2-AEPZ1)-AEPZ; (c) protonated poly(BDA2-AEPZ1)-PZ; and (d) PEI ($M_n = 25$ K) in (A) HEK293; (B) Cos7; and (C) HepG2 cells. Values were presented as mean ± standard deviation ($n = 4$).

DNA Condensation Capability and Transfection Efficiency In Vitro. All the protonated hyperbranched poly(amino ester)s could condense DNA efficiently. As shown in Figure 3, the gel eletrophoresis results reflected that the migration of DNA was retarded completely when the weight ratios (polymer/DNA) were higher than 1, 2, and 2 for poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ, re-

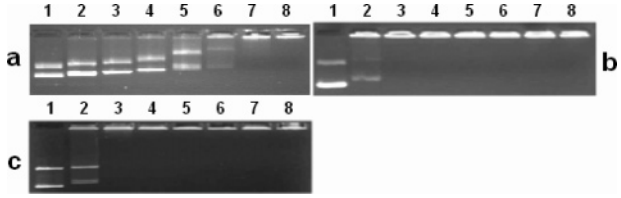


Figure 3. Agarose gel electrophoresis retardation of DNA by protonated poly(amino ester)s. Lane numbers correspond to different DNA/polymer weight ratios. (a) DNA/poly(BDA2-AEPZ1)-AEPZ: (1) 1:0 (DNA only), (2) 1:0.1, (3) 1:0.2, (4) 1:0.4, (5) 1:0.6, (6) 1:0.8, (7) 1:1.0, and (8) 1:1.2; (b) DNA/poly(BDA2-AEPZ1)-MPZ; and (c) DNA/poly(BDA2-AEPZ1)-PZ: (1) 1:0 (DNA only), (2) 1:1, (3) 1:2, (4) 1:3, (5) 1:4, (6) 1:6, (7) 1:8, and (8) 1:10.

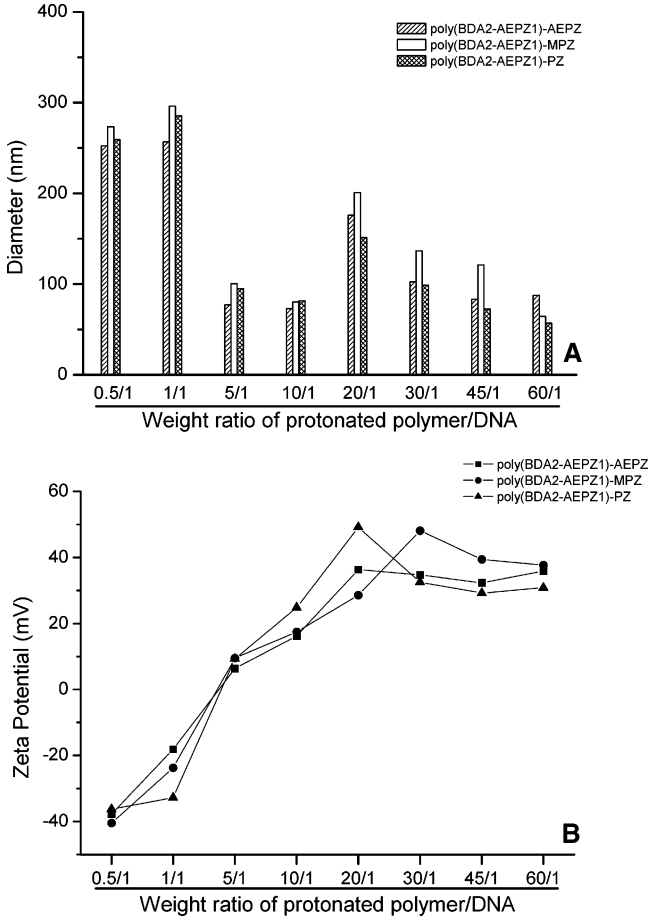


Figure 4. (A) Average effective diameters and (B) average zeta potentials of the complexes formed from DNA and protonated poly(amino ester)s with different weight ratios.

spectively. Poly(BDA2-AEPZ1)-AEPZ with primary terminal amines showed the strongest DNA condensation capacity, which should be due to the smaller steric hindrance of primary amine facilitating the ionic interaction with DNA.

The particle sizes and zeta potentials of these protonated polymer/DNA complexes were measured. As shown in Figure 4A, when the weight ratio of polymer to DNA is more than 5, the sizes of complexes of DNA and all the poly(amino ester)s were below 200 nm. The formation of small particles should be attributable to the positive surface charges as reflected in Figure 4B preventing aggregation of the complexes. When the weight ratio of polymer/DNA was 1:1 or 0.5:1, the complexes had a negatively or less positively charged surface, which might result in the formation of larger complexes due to aggregation.

The transfection efficiency of the DNA complexes from the protonated hyperbranched poly(amino ester)s was evaluated in

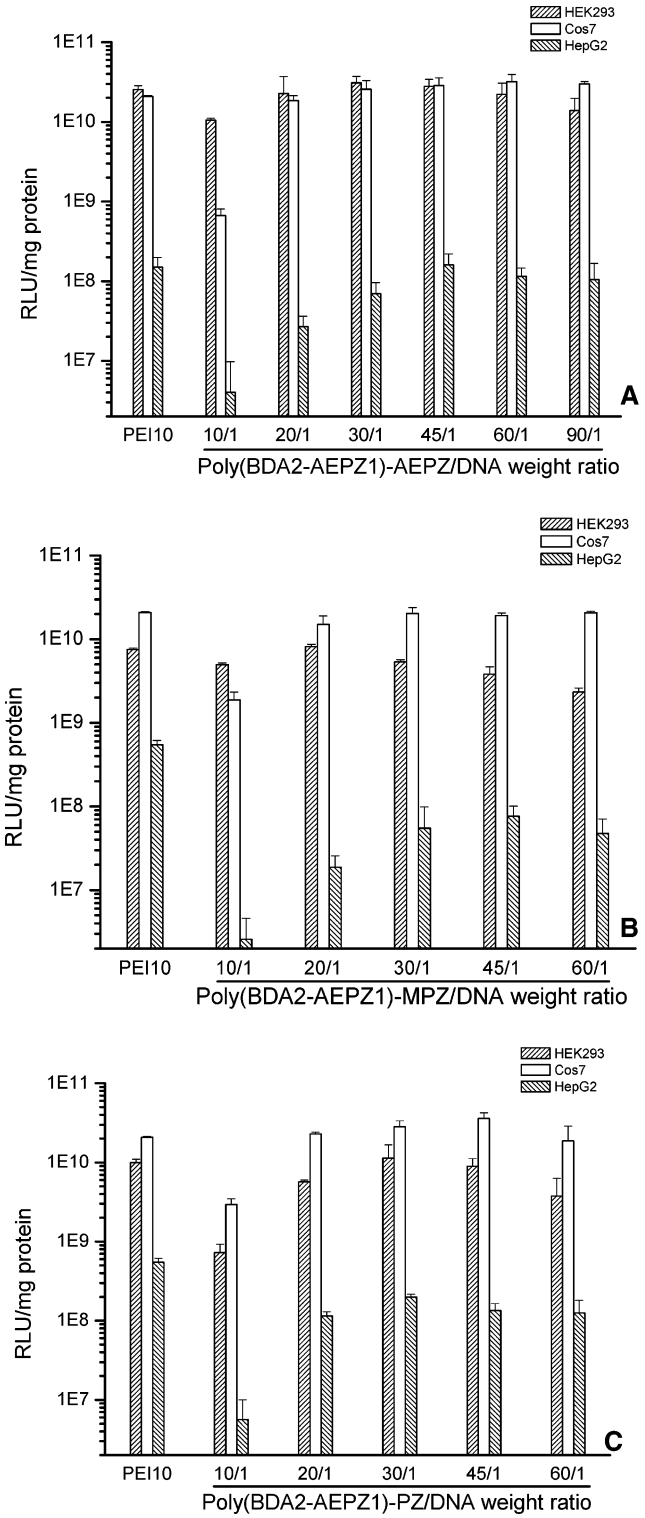


Figure 5. Transfection efficiencies of the complexes of DNA (pCMV-Luc) and (A) protonated poly(BDA2-AEPZ1)-AEPZ; (B) protonated poly(BDA2-AEPZ1)-MPZ; and (C) protonated poly(BDA2-AEPZ1)-PZ in HEK293, Cos7, and HepG2 cells in comparison with those of PEI ($M_n = 25$ K). The transfection efficiencies of PEI were obtained under an optimal N/P ratio of 10:1. Values were presented as mean ± standard deviation ($n = 4$).

HEK293, Cos7, and HepG2 cells. The results were summarized in Figure 5. All these hyperbranched poly(amino ester)s, poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ, showed the highest transfection efficiencies when the weight ratios of polymer to DNA ranged from 30:1 to 60:1. Remarkably, the transfection efficiencies obtained were

comparable to those of the control experiments employing PEI (M_n = 25 K) obtained under optimal conditions in HEK293 and Cos7 cells. In HepG2 cells, the protonated poly(BDA2-AEPZ1)-AEPZ still showed a transfection efficiency similar to that of PEI, and the values obtained for poly(BDA2-AEPZ1)-PZ and poly(BDA2-AEPZ1)-MPZ were ca. 30 and 15% of those for PEI, respectively. All transfection efficiencies were obtained in the absence of chloroquine, a commonly used weak base to enhance in vitro transfection through facilitating the release of DNA vectors from endosomes. The good transfection efficiency of all the hyperbanched poly(amino ester)s with different terminal groups should be attributed to the proton sponge effect formed by the tertiary amines in the core and the amines in the periphery. The proton sponge effect facilitated the escape of the complexes from endosomes.[6a,6b,20] The biodegradability of these poly(amino ester)s may also favor the release of DNA after the complexes entered cells, which also enhances transfection efficiency. Moreover, the results also indicated that the type of the terminal amine had insignificant effect on the transfection efficiency.

Conclusion

The protonated hyperbranched poly(amino ester)s (i.e., poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ) were degradable and showed good DNA condensation capacity and low cytotoxicity. The hydrolysis of hyperbranched poly(amino ester)s, poly(BDA2-AEPZ1)-AEPZ, poly(BDA2-AEPZ1)-MPZ, and poly(BDA2-AEPZ1)-PZ, was slower than that of linear poly(amino ester), which might be ascribed to the compact hyperbranched spatial structures protecting the ester groups from hydrolysis. The positively charged complexes with a size below 200 nm could be obtained when the weight ratio of the polymer to DNA was higher than 5 and the complexes showed a DNA transfection efficiency comparable to those of PEI 25 K. All these properties make these hyperbranched poly(amino ester)s promising for safe and efficient gene delivery for gene therapy. Also, it was clearly indicated that the terminal amine type had less effects on the hydrolysis, cytotoxicity, DNA condensation capability, and in vitro DNA transfection efficiency of these hyperbranched poly(amino ester)s.

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