Cationic Methacrylate Copolymers Containing Primary and Tertiary Amino Side Groups: Controlled Synthesis via RAFT Polymerization, DNA Condensation, and *In Vitro* Gene Transfection

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ABSTRACT: A versatile family of cationic methacrylate copolymers containing varying amounts of primary and tertiary amino side groups were synthesized and investigated for *in vitro* gene transfection. Two different types of methacrylate copolymers, poly(2-(dimethylamino)ethyl methacrylate)/aminoethyl methacrylate [P(DMAEMA/AEMA)] and poly(2-(dimethylamino)ethyl methacrylate)/aminohexyl methacrylate [P(DMAEMA/AHMA)], were obtained by reversible addition-fragmentation chain transfer (RAFT) copolymerization of dimethylaminoethyl methacrylate (DMAEMA) with *N*-(*tert*-butoxycarbonyl)aminoethyl methacrylate (Boc-AEMA) or *N*-(*tert*-butoxycarbonyl)aminohexyl methacrylate (Boc-AHMA) followed by acid deprotection. Gel permeation chromatography (GPC) measurements revealed that Boc-protected methacrylate copolymers had M_n in the range of 16.1–23.0 kDa and low polydispersities of 1.12–1.26. The copolymer compositions were well controlled by monomer feed ratios. Dynamic light scattering and agarose gel electrophoresis mea-

surements demonstrated that these PDMAEMA copolymers had better DNA condensation than PDMAEMA homopolymer. The polyplexes of these copolymers revealed low cytotoxicity at an *N/P* ratio of 3/1. The *in vitro* transfection in COS-7 cells in serum free medium demonstrated significantly enhanced (up to 24-fold) transfection efficiencies of PDMAEMA copolymer polyplexes as compared with PDMAEMA control. In the presence of 10% serum, P(DMAEMA/AEMA) and P(DMAEMA/AHMA) displayed a high transfection activity comparable with or better than 25 kDa PEI. These results suggest that cationic methacrylate copolymers are highly promising for development of safe and efficient nonviral gene transfer agents. © 2010 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 48: 2869–2877, 2010

KEYWORDS: biological applications of polymers; copolymers; DNA polyplexes; gene delivery; living radical polymerization; nanoparticles; PDMAEMA; RAFT polymerization

INTRODUCTION In the past decade, polymer-based gene delivery systems have attracted a tremendous amount of attention for gene transfer due to many advantages they offer over their viral counterparts including improved safety, low immune responses, enabling repeated uses, and ease of production.[1–5] However, polymeric vectors are associated with several extracellular and intracellular barriers such as inadequate protection of DNA from enzymatic degradation, poor cellular uptake, insufficient endosomal escape, and/or inefficient intracellular release of DNA, which result in usually low to moderate transfection efficiencies.[6,7] Unlike poly(L-lysine) (PLL) and chitosan, which contain only primary amines, polyethylenimine (PEI) and poly(amido amine) (PAMAM) dendrimer (two best polymeric transfection agents) contain multivalent amine groups with distinct pK_a values.[8–10] The amine groups with a high pK_a (e.g., primary

amine) render effective complexation and protection of DNA, whereas amine groups with a low pK_a (e.g., secondary or tertiary amine) are hypothesized to facilitate endosomal escape through proton sponge mechanism.[11–13] Langer and Cho reported that transfection activity of PLL and chitosan can be largely augmented by introduction of imidazole functions ($pK_a \sim 6.0$).[14,15] It has been shown by different groups on various types of cationic polymers that combination of assorted amine groups with a wide buffer range gives rise to best transfection activity.[16–22] The endosomal escape of polyplexes is, however, not always enhanced by polymers buffering at low pH.[23]

It is interesting to note that high molecular weight ($M_w > 300$ kDa) poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) which contains solely tertiary amines mediates efficient transfection in various types of cells.[24–26] PDMAEMA is shown to
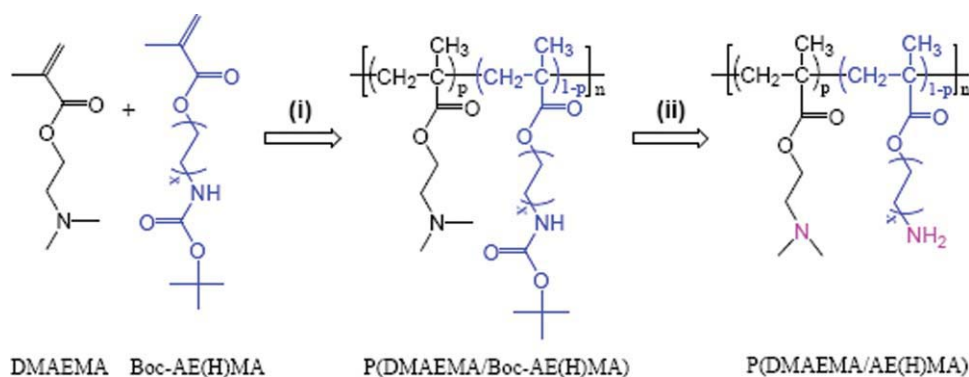
SCHEME 1 Synthetic pathway to cationic methacrylate copolymers containing primary and tertiary amino side groups ($x = 1$: AEMA; $x = 3$: AHMA). Conditions: (i) RAFT polymerization, dioxane, 70 °C, 40 h; (ii) de-protection, r.t., TFA, HCl. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

display high buffer capacity at endosomal pH.[27] However, PDMAEMA is not readily biodegradable, which may render toxicity problems when high molecular weight polymer is used. In the past years, bioreducible PDMAEMA polymers,[28,29] biodegradable PDMAEMA copolymers,[30–33] PHEMA-PDMAEMA-PEG-PDMAEMA-PHEMA pentablock copolymers,[34] and copolymers of DMAEMA and N-vinyl-pyrrolidone (NVP)[35] have been investigated for DNA condensation and/or in vitro transfection. Unlike other cationic polymers including PEI and PAMAM dendrimer, PDMAEMA copolymers can be conveniently prepared with controlled macromolecular structures and compositions by living radical polymerizations including reversible addition-fragmentation chain transfer (RAFT) polymerization.[36,37]

In this article, we report a versatile family of DMAEMA-based copolymers that contain varying amounts of primary and tertiary amino side groups for enhanced gene transfection (Scheme 1). These copolymers were designed with relatively low molecular weights ($M_n < 25$ kDa) and DMAEMA as the major component, thereby retaining a good buffer capacity at endosomal pH. The incorporation of primary amino functions was intended to increase their charge density at physiological pH, which on one hand may improve DNA condensation ability and on the other hand may enhance water solubility and colloidal stability of DNA polyplexes. In this study, two series of well-defined PDMAEMA copolymers, based on aminoethyl methacrylate (AEMA) or aminohexyl methacrylate (AHMA), were prepared by controlled radical polymerization approach. The influences of copolymer structure and composition on DNA condensation, transfection activity and cytotoxicity were investigated.

EXPERIMENTAL

Materials

2-(N,N-dimethylamino)ethyl methacrylate (DMAEMA, 97%, Alfa Aesar) was purified by passing through a basic alumina column before use. 4-Cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to a reported procedure for 4-(4-cyanopentanoic acid) dithiobenzoate.[38] Azobisisobutyronitrile (AIBN, 99%, J & K Chemical) was recrystallized twice from hexane and methanol, respectively. Dichloromethane (DCM) was dried by refluxing over CaH$_2$ and distilled before use. 2-Aminoethanol (Sinopharm Chemical Reagent Co. Ltd.), 6-amino-1-hexanol (97%, Aldrich),

di-tert-butyl dicarbonate (99%, J & K Chemical), triethylamine (TEA, 99%, Alfa Aesar), methacryloyl chloride (Wuxi Chemical), and trifluoroacetic acid (TFA, 99%, Aldrich) were used as received.

Synthesis of N-(tert-butoxycarbonyl)aminoethyl methacrylate (Boc-AEMA)

Under a nitrogen atmosphere and vigorously stirring, to a solution of 2-aminoethanol (8.6 g, 143.1 mmol) in 100 mL of anhydrous DCM at 0 °C was added dropwise a solution of di-tert-butyl dicarbonate (15.3 g, 70.1 mmol) in 70 mL of DCM. The reaction mixture was warmed to r.t. and reacted for additional 20 h. The reaction mixture was filtered, the filtrate was concentrated, and the residue was dissolved in 100 mL saturated NaCl solution. The resultant aqueous solution was acidified to pH 4–5 and extracted with ethyl acetate (3 × 40 mL). The collected organic fractions were dried over Na$_2$SO$_4$ overnight and ethyl acetate was evaporated to yield N-(tert-butoxycarbonyl)-2-aminoethanol as a colorless oil. Yield: 71.8%.

^{1}H NMR (300 MHz, CDCl$_3$): δ 1.44 (s, 9H, —C(CH$_3$)$_3$), 2.47 (s, 1H, —OH), 3.28 (m, 2H, —CONHCH$_2$—), 3.70 (m, 2H, —CH$_2$OH), 4.96 (br.s, 1H, —NHBoc).

To a solution of N-(tert-butoxycarbonyl)-2-aminoethanol (8.1 g, 50.3 mmol) and TEA (15 mL, 103.8 mmol) in 75 mL of anhydrous DCM at 0 °C was added dropwise a solution of methacryloyl chloride (9.8 mL, 100.9 mmol) in 25 mL of DCM. The reaction mixture was warmed to r.t. and reacted for additional 24 h. After removing white precipitate, the filtrate was washed with water (3 × 100 mL) and dried overnight over Na$_2$SO$_4$. The resultant solution was concentrated and purified through a silica gel column with petroleum ether/ethyl acetate (7/1, v/v) to yield Boc-AEMA as a white solid. Yield: 62.9%.

^{1}H NMR (300 MHz, CDCl$_3$): δ 1.44 (s, 9H, —C(CH$_3$)$_3$), 1.95 (s, 3H, —CH$_3$), 3.44 (m, 2H, —CH$_2$NHBoc), 4.20 (t, 2H, —COOCH$_2$—), 4.77 (br.s, 1H, —NHBoc), 5.59/6.13 (s, 2H, CH$_2$=CH).

Synthesis of N-(tert-Butoxycarbonyl)aminohexyl methacrylate (Boc-AHMA)

Under a nitrogen atmosphere and vigorously stirring, to a solution of 6-amino-1-hexanol (3.3 g, 28.4 mmol) in 30 mL of anhydrous DCM at 0 °C was added dropwise a solution of di-tert-butyl dicarbonate (7.1 g, 32.4 mmol) in 20 mL of

DCM. The reaction mixture was warmed to r.t. and reacted for additional 20 h. The reaction mixture was then diluted with 60 mL diethyl ether and successively washed with sodium phosphates buffer (3 × 30 mL, pH 5.4), saturated sodium dicarbonate solution (1 × 50 mL), and saturated NaCl solution (1 × 50 mL). The organic phase was dried overnight over Na$_2$SO$_4$ and concentrated by rotary evaporation to yield N-(tert-butoxycarbonyl)-6-amino-1-hexanol as a white solid. Yield: 71.5%.

^{1}H NMR (300 MHz, CDCl$_3$): δ 1.43 (s, 9H, —C(CH$_3$)$_3$), 1.75 (s, 1H, —OH), 1.56 (m, 2H, CH$_2$CH$_2$OH), 1.47 (m, 2H, —CH$_2$CH$_2$ NHBoc), 1.35 (m, 4H, —NHCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$ OH), 3.63 (t, 2H, HOCH$_2$—), 3.10 (m, 2H, —CH$_2$NHBoc), 4.54 (br, 1H, —NHBoc).

To a solution of N-(tert-butoxycarbonyl)-6-amino-1-hexanol (4.3 g, 19.6 mmol) and TEA (6 mL, 41.5 mmol) in 50 mL of anhydrous DCM at 0 °C was added dropwise a solution of methacryloyl chloride (3.8 mL, 39.1 mmol) in 10 mL of DCM. The reaction mixture was warmed to r.t. and reacted for additional 24 h. After removing white precipitate, the filtrate was washed with water (3 × 100 mL) and dried overnight over Na$_2$SO$_4$. The resultant solution was concentrated and purified through a silica gel column with petroleum ether/ethyl acetate (5/1, v/v) to yield Boc-AHMA as a pale yellow oil. Yield: 56.9%.

^{1}H NMR (300 MHz, CDCl$_3$): δ 1.44 (s, 9H, —C(CH$_3$)$_3$), 1.67 (m, 2H, CH$_2$CH$_2$OCO—), 1.50 (m, 2H, —CH$_2$CH$_2$NHBoc), 1.37 (m, 4H, —NHCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$OCO—), 1.94 (s, 3H, —CH$_3$), 4.13 (t, 2H, —CH$_2$OCO—), 3.10 (m, 2H, —CH$_2$NHBoc), 4.52 (br., 1H, —NHBoc), 5.55/6.09 (s, 2H, CH$_2$=CH).

Synthesis of DMAEMA-Based Copolymers
RAFT Polymerization
The copolymerization of DMAEMA with Boc-AEMA or Boc-AHMA was carried out using CPADN as a RAFT agent and AIBN as a radical source. In a typical example, under a nitrogen atmosphere, DMAEMA (0.92 g, 5.83 mmol), Boc-AEMA (0.33 g, 1.46 mmol), CPADN (12.0 mg, 0.0365 mmol), AIBN (0.6 mg, 0.0036 mmol), and 2.5 mL dioxane were charged into a Schlenk flask. The polymerization was performed at 70 °C for 40 h. The resulting copolymer was isolated by precipitation in cold hexane, filtration and drying in vacuo. Yield: 72–84%.

^{1}H NMR (300 MHz, CDCl$_3$) of P(DMAEMA/Boc-AEMA): δ 4.06, 3.36, 1.81–1.95, 1.45, 1.04/0.88 (poly(Boc-AEMA)); 4.06, 2.58, 2.28, 1.81–1.95, 1.04/0.88 (PDMAEMA). ^{1}H NMR (300 MHz, CDCl$_3$) of P(DMAEMA/Boc-AHMA): δ 3.91, 3.09, 1.35–1.62, 1.45, 1.04/0.88 (poly(Boc-AHMA)); 4.07, 2.58, 2.29, 1.80–1.93, 1.04/0.88 (PDMAEMA).

Acid Deprotection
Under vigorously stirring, to a solution of 0.9 g of P(DMAEMA/Boc-AEMA) or P(DMAEMA/Boc-AHMA) copolymer in 4 mL of DCM was added dropwise 4 mL of TFA. After 2 h reaction at room temperature, 2 mL of concentrated HCl (12 M) was added and stirred for 30 min. The resulting co-

polymer was isolated by precipitation in acetone, filtration, and drying in vacuo. Yield: 75–90%.

^{1}H NMR (300MHz, D$_2$O) for P(DMAEMA/AEMA): δ 4.36, 3.35, 2.02, 1.11/0.94 (PAEMA); 4.36, 3.55, 2.96, 2.02, 1.11/0.94 (PDMAEMA). ^{1}H NMR (300MHz, D$_2$O) for P(DMAEMA/AHMA): δ 3.98, 2.99, 1.99, 1.67, 1.41, 1.11/0.94 (PAHMA); 4.36, 3.56, 2.99, 1.99, 1.11/0.94 (PDMAEMA).

Polymer Characterization
^{1}H NMR spectra of copolymers were recorded on an INOVA 300 MHz nuclear magnetic resonance instrument using CDCl$_3$ or D$_2$O as a solvent. The chemical shifts were calibrated against solvent signal of CDCl$_3$ or D$_2$O. The molecular weight and polydispersity (PDI) of copolymers were determined with a Waters 1515 gel permeation chromatography (GPC) instrument equipped with 500, 10^3 and 10^4 Å columns (MZ-Gel SD plus) and a differential refractive index detector (RI2414). The measurements were performed using DMF containing 0.05 M LiBr as eluent at a flow rate of 0.8 mL/min at 30 °C and a series of polystyrene as standards.

Buffer Capacity Measurements
The buffer capacity of PDMAEMA copolymers was determined by acid–base titration.[39] Briefly, polymer was dissolved in 150 mM NaCl aqueous solution to yield 20 mM nitrogen concentration. The pH of the solution was firstly brought to 2.0, and then titrated with 0.1 M NaOH. The buffer capacity defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.4 was calculated from equation:

$$\text{Buffer capacity} (\%) = 100(\Delta V_{\text{NaOH}} \times 0.1\text{M})/\text{N mol}.$$

wherein ΔV_{NaOH} is the volume of NaOH solution required to bring the pH value of the copolymer solution from 5.1 to 7.4, and N mol is the total moles of protonable amine groups (0.1 mmol).

Preparation and Characterization of DNA Polyplexes
The polyplexes were prepared by adding a HEPES buffer solution (400 μL, 20 mM, pH 7.4) of polymer with the desired concentration to a HEPES buffer solution (400 μL, 20 mM, pH 7.4) of plasmid DNA (20 μg/mL), which resulted in polyplexes with N/P ratios ranging from 3/1 to 9/1. The dispersions were vortexed for 5 s and incubated at r.t. for 15 min before ξ-potential and size measurements were carried out. The ξ-potential and hydrodynamic diameters of polyplexes prepared at an N/P ratio of 3/1 were determined at 25 °C using a Zetasizer Nano ZS Instruments (Malvern) equipped with a standard capillary electrophoresis cell and dynamic light scattering (DLS, 10 mW He-Ne laser, 633 nm wavelength), respectively. The measurements were performed in triplicate.

Gel Retardation Assay
The DNA binding ability of PDMAEMA copolymers was studied by agarose gel electrophoresis. The polymer/DNA complexes prepared at varying N/P ratios from 0.75/1 to 3/1 were electrophoresed through a 1% agarose gel containing

TABLE 1 Synthesis of P(DMAEMA/Boc-AEMA) and P(DMAEMA/Boc-AHMA) Copolymers[a]

Entry	Polymer	f_{DMAEMA}[b] (%)	Yield (%)	F_{DMAEMA}[c] (%)	GPC[d] M_n (kDa)	PDI
1	P(DMAEMA/Boc-AEMA) 91/9	90	84.2	91	16.1	1.19
2	P(DMAEMA/Boc-AEMA) 79/21	80	81.1	79	18.2	1.16
3	P(DMAEMA/Boc-AEMA) 70/30	70	71.9	70	21.8	1.12
4	P(DMAEMA/Boc-AHMA) 91/9	90	81.7	91	16.1	1.20
5	P(DMAEMA/Boc-AHMA) 79/21	80	75.3	79	16.7	1.26
6	P(DMAEMA/Boc-AHMA) 72/28	70	72.7	72	23.0	1.21
7	PDMAEMA	100	92.6	100	13.4	1.26

[a] RAFT polymerization conditions: [monomer]$_{total}$/[CPADN]/[AIBN] = 200/1/0.1, dioxane, 70 °C, 40 h.
[b] Molar fraction of DMAEMA in the feed.
[c] Molar fraction of DMAEMA units in the resulting copolymers determined by ^{1}H NMR.
[d] Determined by GPC measurements (eluent: DMF containing 0.05 M LiBr, standards: polystyrene, flow rate: 0.8 mL/min, 30 °C).

ethidium bromide at 100 V in TAE solution (40 mM Tris-HCl, 1 v/v % acetic acid, and 1 mM EDTA).

In Vitro Transfection and Cell Viability Assays

Transfection experiments were performed in COS-7 cells using the plasmid pCMV-Luc as a reporter gene. Transfections were conducted using polyplexes formed at N/P ratios of 3/1, 6/1, and 9/1. The cells were plated in a 12-well plate (cell density 1.5×10^5 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2 until 70% confluency. In a standard transfection experiment, the cells were rinsed with PBS and incubated with 100 μL of polyplex dispersion (i.e., 1 μg of plasmid DNA per well) and 400 μL of culture medium with or without 10% serum for 4 h at 37 °C. Next, the polyplexes were removed, 1 mL of fresh culture medium was added, and the cells were cultured for 43 h. Luciferase quantification was done using a commercial luciferase assay kit (Promega, Madison, WI) and a Lumat LB 9501 illuminometer (Berthold, Milbach, Germany). Transfection efficiency was expressed as relative light unit (RLU) per mg of protein. A 25 kDa bPEI/DNA formulation prepared at an optimal N/P ratio of 10/1 was used as a reference.

The cytotoxicity of polyplexes was evaluated with the same cell culture procedure as the transfection with 10% serum, followed by CCK assay. The cells were incubated with CCK-8 reagent solution for 3.5 h before measurement of absorption at 450 nm using a microplate reader (BIORAD, Model 550). The CCK value for the untreated cells (i.e., cells not exposed to transfection systems) was taken as 100% cell viability.

RESULTS AND DISCUSSION

#### Synthesis of DMAEMA-Based Copolymers

Cationic methacrylate copolymers containing varying amounts of primary and tertiary amino side groups were synthesized by RAFT copolymerization of DMAEMA with Boc-AEMA or Boc-AHMA followed by acid deprotection (Scheme 1). RAFT polymerization is a versatile yet controlled free radical polymerization technique that has been exploited for synthesis of well-defined functional polymers and copolymers.[40,41] Boc-AEMA and Boc-AHMA monomers were readily obtained from ethanolamine and hexanolamine through selective protection of amino group with Boc and subsequent methacrylation with methacryloyl chloride. The copolymerization was performed in dioxane at 70 °C for 2 days using CPADN. CPADN is a versatile RAFT agent, through which we have obtained degradable PDMAEMA-PCL-PDMAEMA triblock copolymers.[31] The total monomer-to-CPADN mole feed ratio was set at 200/1 and mole fraction of DMAEMA in the feed was varied from 70%, 80% to 90%.

The copolymerization results are summarized in Table 1. ^{1}H NMR spectrum of P(DMAEMA/Boc-AEMA) showed clearly resonances characteristic of DMAEMA and Boc-AEMA units [Fig. 1(A)]. The copolymer compositions could be calculated by comparing the intensities of signals at δ 2.56 and 3.38, which were assignable to methylene protons neighboring to the amino group of PDMEMA and methylene protons next to the amide group of P(Boc-AEMA), respectively. In a similar way, compositions of P(DMAEMA/Boc-AHMA) copolymers could also be determined [Fig. 1(B)]. Interestingly, our results pointed to excellent control over copolymer compositions (Table 1). The copolymer compositions had little change with increasing conversions, indicating similar reactivities of DMAEMA with Boc-AEMA or Boc-AHMA. Furthermore, GPC curves showed that all copolymers had a unimodal distribution with M_n in the range of 16.1–23.0 kDa and low polydispersities of 1.12–1.26 (Table 1). These copolymers were subsequently treated with CF_3COOH/CH_2Cl_2 (1:1 v/v), to yield a freely water soluble product. Notably, ^{1}H NMR spectra (Fig. 2) showed complete disappearance of signal at δ 1.44 that is attributable to the methyl protons of Boc group, indicating quantitative deprotection. The mole fraction of PDMAEMA in the resulting copolymers, however, was not changed upon acid deprotection (Fig. 2), supporting successful preparation of P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers. For comparison, we have also obtained PDMAEMA homopolymer (M_n = 13.4, PDI = 1.26) using the same procedure (Table 1, entry 7).

Buffer Capacity of Methacrylate Copolymers

It has been reported that the high transfection activity of PEI is intimately related to its buffer capacity at endosomal pH,
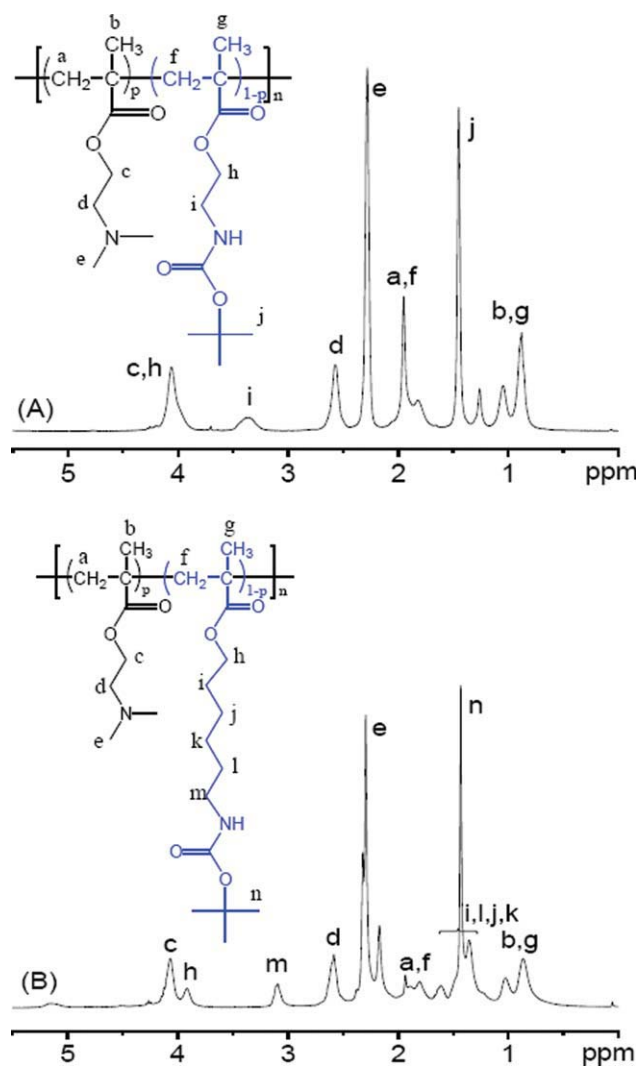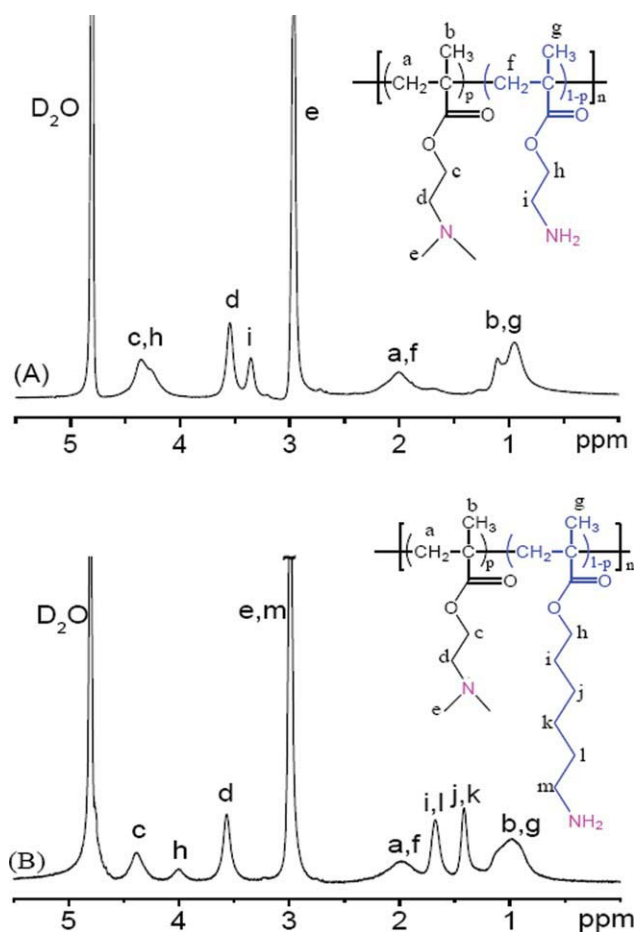
FIGURE 1 ¹H NMR spectra (300MHz, CDCl₃) of P(DMAEMA/Boc-AEMA) 70/30 copolymer (A) and P(DMAEMA/Boc-AHMA) 72/28 copolymer (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

which facilitates endosomal escape of DNA polyplexes by "proton sponge effect."[11–13] The buffer capacity of these methacrylate copolymers, defined as the percentage of amino functions becoming protonated in the pH range from pH 7.4 to 5.1, was determined by acid-base titration. The results showed that the buffer capacity of methacrylate copolymers in general decreased with increasing content of AEMA or AHMA (Fig. 3). This is in line with our expectation since AEMA and AHMA units present only primary amino groups that usually have a high pK_a. It should be noted, nevertheless, that the buffer capacity of these methacrylate copolymers remains rather high, ranging from 26.6% to 53.1%, as DMAEMA units are still the major component (70–91 mol %).
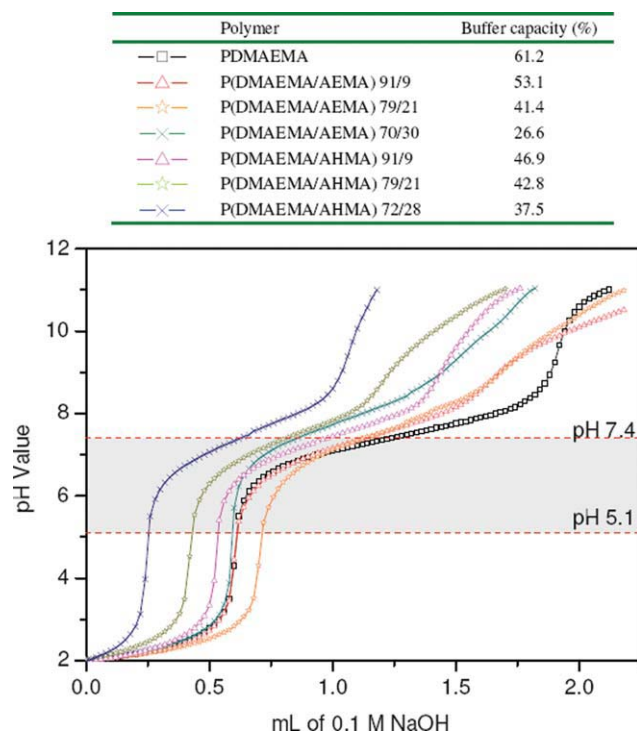
Biophysical Characterization of DNA Polyplexes

To investigate influences of AEMA or AHMA units on DNA complexation behaviors, polyplexes of P(DMAEMA/AEMA)

and P(DMAEMA/AHMA) copolymers were prepared at an N/P ratio of 3/1 in HEPES buffer (20 mM, pH 7.4). DLS measurements showed that all copolymers could effectively condense plasmid DNA to give small sized polyplexes with mean diameters of 60–110 nm for P(DMAEMA/AEMA) and 60–75 nm for P(DMAEMA/AHMA) [Fig. 4(A)]. The sizes of P(DMAEMA/AEMA) polyplexes decreased from 110 nm to 60 nm with increasing contents of AEMA from 9 to 30%, which is in accordance with our hypothesis that incorporation of primary amino groups will result in enhanced DNA condensation. Notably, P(DMAEMA/AHMA) copolymers with only 9% AHMA were shown to condense DNA into ∼60 nm particles. It has been reported that cationic polymers with certain hydrophobicity often lead to small-sized DNA particles.[42] In comparison, polyplexes of PDMAMEA homopolymer at an N/P ratio of 3/1 had somewhat larger size of ∼120 nm. ζ-potential measurements displayed that polyplexes of P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers had positive surface charges of +15 mV and +26 mV, respectively, wherein content of AEMA or AHMA did not seem to play a significant role in ζ-potentials of polyplexes [Fig. 4(B)]. Agarose gel electrophoresis

FIGURE 2 ¹H NMR spectra (300MHz, D₂O) of P(DMAEMA/AEMA) 70/30 copolymer (A) and P(DMAEMA/AHMA) 72/28 copolymer (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

| Polymer	Buffer capacity (%)
—□— PDMAEMA	61.2
—△— P(DMAEMA/AEMA) 91/9	53.1
—☆— P(DMAEMA/AEMA) 79/21	41.4
—×— P(DMAEMA/AEMA) 70/30	26.6
—△— P(DMAEMA/AHMA) 91/9	46.9
—☆— P(DMAEMA/AHMA) 79/21	42.8
—×— P(DMAEMA/AHMA) 72/28	37.5

FIGURE 3 Acid–base titration curves for PDMAEMA copolymers (at a concentration of 20 mM nitrogen) titrated by 0.1 M NaOH.

showed that P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers were capable of condensing DNA at and above an N/P ratio of 1.5/1 or 0.75/1 depending on contents of AEMA and AHMA, whereas for PDMAEMA complete DNA retardation was observed only at a higher N/P ratio of 3/1 (Fig. 5). These results fully confirmed that incorporation of primary amines into PDMAEMA is an effective approach to enhance its DNA complexation capacity.

In Vitro Cytotoxicity and Transfection

The cytotoxicity of polyplexes based on P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers was evaluated by CCK assay using COS-7 cells at different N/P ratios ranging from 3/1 to 9/1. Interestingly, P(DMAEMA/AEMA) copolymers displayed in general comparable or improved cell viability as compared with PDMAEMA under otherwise the same conditions, in which P(DMAEMA/AEMA) copolymer with 9% AEMA revealed minimal cytotoxicity up to an N/P ratio of 9/1 (Fig. 6). P(DMAEMA/AHMA) copolymers showed practically similar level of cytotoxicity with respect to PDMAEMA at N/P ratios of 3/1 and 6/1, and the copolymer composition appeared to have little influence on toxicity. It should be noted that polyplexes of all copolymers revealed a low cytotoxicity at an N/P ratio of 3/1, in which approximately 80% or above cell viability was observed (Fig. 6).

The *in vitro* transfection activity of polyplexes based on PDMAEMA copolymers in COS-7 cells was studied using the plasmid pCMV-Luc as a reporter gene. Remarkably, polyplexes of all copolymers demonstrated much better
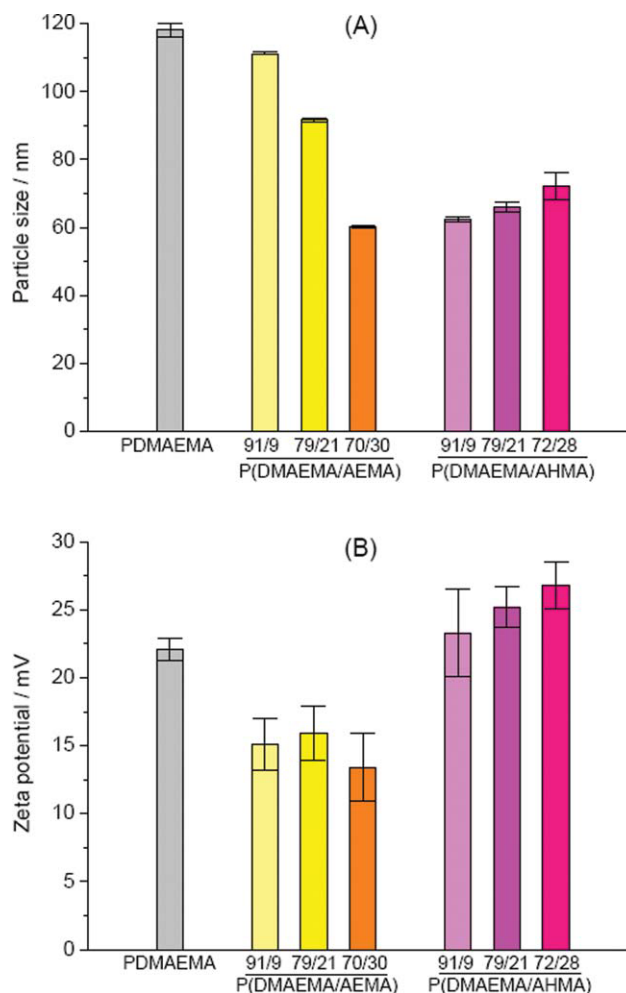
FIGURE 4 Average particle size (A) and zeta potential (B) of DNA polyplexes based on P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers at an N/P ratio of 3/1.
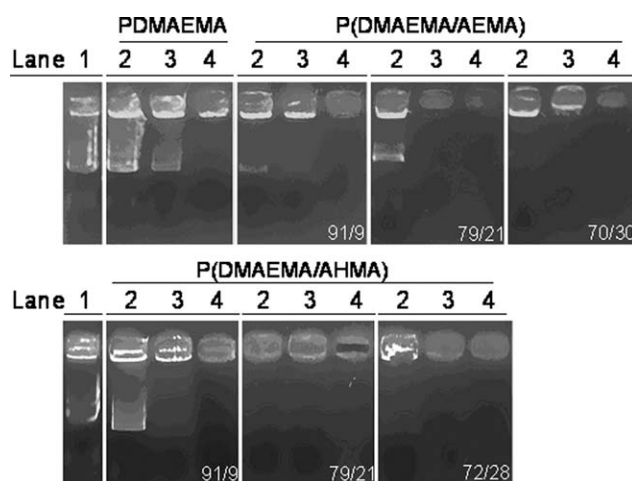

FIGURE 5 Agarose gel electrophoresis of polymer/DNA complexes based on P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers at varying N/P ratios. Lane 1 is free DNA; Lanes 2–4 correspond to N/P ratios of 0.75, 1.5, and 3, respectively.
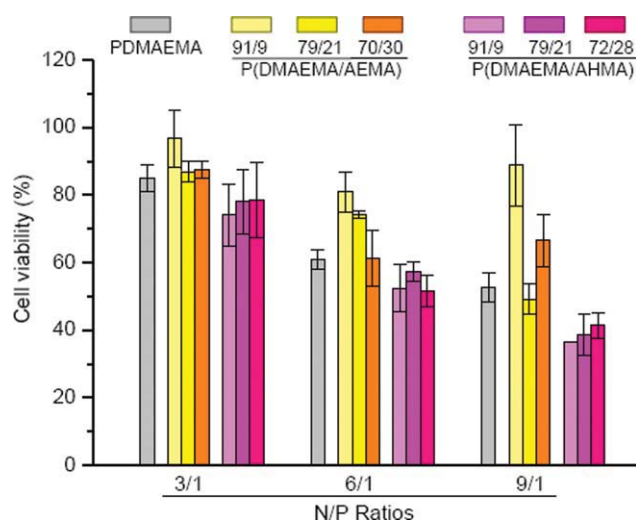
FIGURE 6 Cell viabilities of DNA polyplexes based on P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers in COS-7 cells at varying N/P ratios from 3/1 to 9/1 in 10% serum condition. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
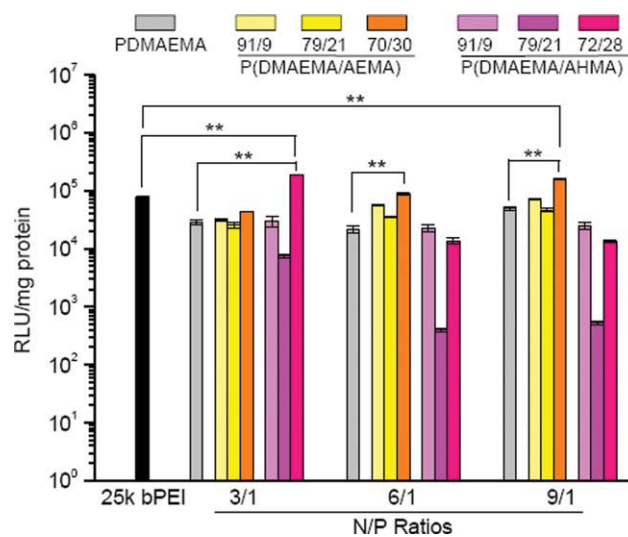
transfection efficiencies than those of PDMAEMA control at the same N/P ratios in serum free medium (Fig. 7). The transfection activity seemed to follow the order P(DMAEMA/AHMA) > P(DMAEMA/AEMA) > PDMAMEA, in which polyplexes of P(DMAEMA/AHMA) displayed up to 24-fold higher transfection efficiency as compared with those of PDMAEMA. The elevated transfection of P(DMAEMA/AHMA) polyplexes is most likely due to their small particle sizes, high surface charges, and enhanced association with cellular membranes
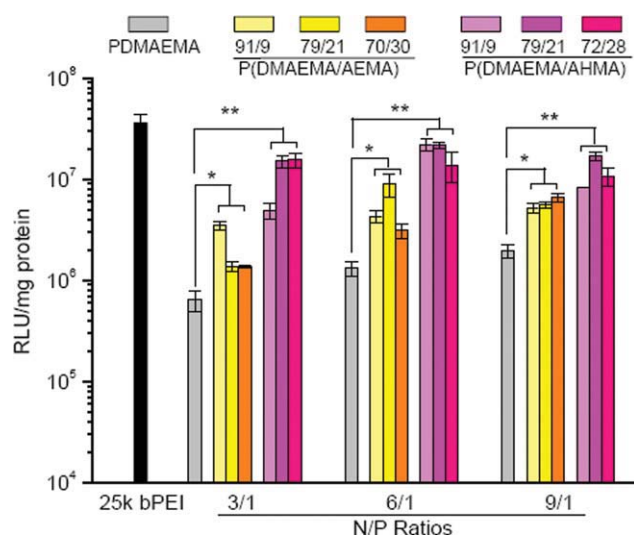
via the hydrophobic interactions. DLS measurements showed that polyplexes of P(DMAEMA/AHMA) 91/9 and P(DMAEMA/AEMA) 91/9 formed at an N/P ratio of 3/1 following 4 h incubation with transfection medium had average diameters of 280 nm and over 400 nm, respectively. Kim, Langer, and Feijen reported separately that linear and branched poly(β-amino ester)s composed of relatively hydrophobic units exhibit best transfection activity.[20,42,43] It should be noted that P(DMAEMA/AHMA) has achieved high transfection at a low N/P ratio of 3/1. In comparison, high molecular weight PDMAEMA ($M_w > 300$ kDa) and 25 kDa PEI afforded optimal transfections at an N/P ratio of 6/1 and 10/1, respectively.[44]

Interestingly, when transfection was carried out in the presence of 10% serum, polyplexes of P(DMAEMA/AEMA) 91/9 formed at N/P ratios of 6/1 and 9/1, which had minimal cytotoxicity (Fig. 6), showed transfection activity comparable with 25 kDa PEI control. Moreover, two copolymers, P(DMAEMA/AHMA) 72/28 and P(DMAEMA/AEMA) 70/30, mediated significantly more efficient transfection at an N/P ratio of 3/1 and 9/1, respectively, than 25 kDa PEI (Fig. 8). Ulbrich and Seymour have reported previously that copolymer of DMAEMA and AEMA has a higher transfection activity than PDMAEMA, likely due to their combination of primary and tertiary amines.[45] The polymers were obtained by conventional free radical polymerization and had high molecular weights and broad molecular weight distributions. The aim of this study was to develop structurally well-defined low molecular weight cationic DMAEMA copolymers for enhanced transfection. It is remarkable that low molecular weight DMAEMA copolymers prepared by controlled RAFT polymerization have transfection activities higher than or comparable to 25 kDa PEI, which is

FIGURE 7 Transfection efficiencies of DNA polyplexes based on P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers in COS-7 cells at N/P ratios of 3/1, 6/1, and 9/1 in serum-free condition. 25k bPEI at an N/P ratio of 10/1 was used as a control. (Student's t-test, *$p < 0.05$, **$p < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 8 Transfection efficiencies of DNA polyplexes based on P(DMAEMA/AEMA) and P(DMAEMA/AHMA) copolymers in COS-7 cells at N/P ratios of 3/1, 6/1, and 9/1 in 10% serum condition. 25k bPEI at an N/P ratio of 10/1 was used as a control. (Student's t-test, *$p < 0.05$, **$p < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

known as one of the best nonviral gene transfer agents. These DMAEMA copolymers may be developed for safe and highly efficient gene transfection.

CONCLUSIONS

We have demonstrated that PDMAEMA copolymers containing varying amounts of primary amino side groups are a versatile family of cationic polymers that mediate significantly enhanced gene transfection as compared with PDMAEMA homopolymer under otherwise the same conditions. These methacrylate copolymers have several unique features: (i) they can be readily prepared with controlled compositions and narrow molecular weight distributions using RAFT polymerization method followed by acid deprotection; (ii) they have improved water solubility and can effectively condense DNA into small sized particles due to presence of primary amine groups; and (iii) they have relatively low molecular weights (<25 kDa) and afford optimal transfection efficiencies at a low N/P ratio of 3/1. We are convinced that cationic methacrylate copolymers with versatile design of structures and controlled synthesis have a great potential in development of safe and efficient non-viral gene transfer agents.

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