Water Soluble Poly(histamine acrylamide) with Superior Buffer Capacity Mediates Efficient and Nontoxic In Vitro Gene Transfection

SIBIN LUO,[1] RU CHENG,[1] FENGHUA MENG,[1] TAE GWAN PARK,[2] ZHIYUAN ZHONG[1]

[1]Biomedical Polymers Laboratory, Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China

[2]Department of Biological Sciences and Graduate School of Nanoscience and Technology, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea

Received 17 March 2011; accepted 11 May 2011
DOI: 10.1002/pola.24773
Published online 31 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Water-soluble cationic polymers, poly(histamine acrylamide)s (PHAs), with superior buffer capacity at the endosomal pH range were designed, prepared, and investigated for non-viral gene transfection. PHAs were obtained with molecular weights ranging from 9.2 to 28.7 kDa through controlled radical polymerization of histamine acrylamide (HA). Acid–base titration results displayed that all PHA polymers had a remarkably high buffer capacity of about 70% at pH 5.1–7.2. 12.7–28.7 kDa PHAs were able to effectively condense DNA into nano-sized (<220 nm) polyplexes with moderate positive surface charges (+13–+19 mV) at N/P ratios ≥10/1. CCK assays indicated that polyplexes of 12.7 and 17.5 kDa PHAs were non-toxic to COS-7 cells up to a tested N/P ratio of 20/1. Interestingly, the in vitro transfection using pCMV-Luc and pEGFP-C1 plasmid DNA as reporter genes showed that polyplexes of 12.7 kDa PHA formed at an N/P ratio of 20/1 mediated efficient transfection in COS-7 cells under 10% serum conditions, with transfection efficiencies comparable to that of 25 kDa polyethylenimine control. Their versatile design of structures, controlled synthesis, low cytotoxicity, and high transfection activity render PHA-based cationic polymers particularly interesting for the development of safe and efficient non-viral gene delivery systems. © 2011 Wiley Periodicals, Inc. J Polym Sci Part A: Polym Chem 49: 3366–3373, 2011

KEYWORDS: biological applications of polymers; gene delivery; imidazole; plasmid DNA; proton sponge effect; radical polymerization; water soluble polymers

INTRODUCTION The past decade has witnessed significant progress in the development of polymer-based gene delivery systems as they provide several advantages over their viral counterparts such as improved safety, low immune response, enabling repeated use, and ease of large-scale production.[1,2] Among many cationic polymers, polyethylenimine (PEI), poly (amido amine) (PAMAM) dendrimer, and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) are the most extensively studied for non-viral gene transfection.[3,4] Their superior transfection activity is most likely associated with their high endosomal pH buffer capacity which is hypothesized to facilitate the endosomal escape of DNA complexes via the "proton sponge effect."[5–7] In consistence with this, modification of high pKa cationic polymers including polylysine and chitosan with histidine or imidazole group that is known to possess a superior endosomal pH buffer capacity has been reported to bring about dramatic enhancement of transfection efficiency.[8–11] In the past years, several histidine-rich polymers and polypeptides have been developed for improved nucleic acid delivery.[12,13] For example, Kim et al. designed biodegradable poly(ethylene glycol)-*co*-poly(L-lysine)-*g*-histidine multiblock copolymers[14] and Asayama et al. prepared aminated or alkylated poly(1-vinylimidazole)[15,16] for non-viral transfection. Kichler et al. reported that histidine-rich amphipathic peptide promoted efficient delivery of DNA into different cell lines in which the transfection efficiency depended on the number and positioning of histidine residues in the peptide.[17] Yang et al. developed histidine-containing triblock-type oligopeptides for efficient DNA transfection.[18] It is interesting to note that histamine-based poly(β-amino ester) is among the most potent polymer carriers in the library of biodegradable poly(β-amino ester)s.[19,20] Engbersen et al. reported that histamine-based bioreducible poly(amido amine)s had a transfection activity comparable to 25 kDa PEI control.[21] It is equally important to note that besides good transfection activity, histidylated or imidazole-containing cationic carriers often exhibit low or no cytotoxicity,[12] which is likely due to their low cationic charge density.

Cationic vinyl polymers like PDMAEMA are a particularly versatile family of non-viral gene carriers.[22] For example, Hennink et al. reported that high molecular weight PDMAEMA (>300 kDa) mediates efficient gene transfection in various types of cells.[23,24] Unlike other cationic polymers

Correspondence to: Z. Zhong (E-mail: zyzhong@suda.edu.cn)

including PEI and PAMAM dendrimer, vinyl-type cationic copolymers can be conveniently prepared with controlled macromolecular structures, molecular weights and compositions by controlled radical polymerization techniques including reversible addition-fragmentation chain transfer (RAFT) polymerization. In the past years, different types of PDMAEMA-based polymers such as biodegradable PDMAEMA copolymers,[25–27] PHEMA-PDMAEMA-PEG-PDMAEMA-PHEMA pentablock copolymers,[28] copolymers of DMAEMA and *N*-vinyl-pyrrolidone (NVP),[29] copolymers of DMAEMA and aminoalkyl methacrylate,[30] and bioreducible PDMAEMA[31] have been synthesized and investigated for in vitro gene transfection. The unique control over synthesis renders cationic vinyl polymers remarkably versatile in vector design.

In this article, we report on a novel water-soluble cationic vinyl polymer, poly(histamine acrylamide) (PHA), with superior buffer capacity at the endosomal pH range for non-viral DNA transfection. The synthesis, DNA condensation, transfection activity, and cytotoxicity of PHA were investigated.

EXPERIMENTAL

Materials

25 kDa branched polyethylenimine (25 kDa PEI) and acryloyl chloride (96%) were purchased from Alfa Aesar and used as received. 2,2′-Azobisisobutyronitrile (AIBN, 98%, J&K) was recrystallized twice from methanol. Histamine dihydrochloride, dichloromethane, 2-mercaptoethanol, anhydrous methanol, HCl, NaOH, isopropanol, and anhydrous $MgSO_4$ were purchased from Sinopharm Chemical Reagent (Shanghai, China).

COS-7 cells were routinely maintained in a DMEM medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator with 5% CO_2. FBS (Fetal bovine serum) and DMEM (Dulbecco's Modified Eagle Medium) were obtained from Invitrogen (Carlsbad, CA) and penicillin/streptomycin solution was purchased from Sigma–Aldrich (St. Louis, MO). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). Luciferase assay kit was bought from Promega (Madison, WI).

Synthesis of Histamine Acrylamide Monomer

In a three-necked 100 mL flask equipped with a stirrer and two 25 mL dripping funnels, histamine dihydrochloride (2.01 g, 10.9 mmol) was dissolved in 10 mL of distilled water. The solution was cooled to 0−5 °C in an ice bath. A solution of acryloyl chloride (0.983 g, 10.9 mmol) in dichloromethane (10 mL) and an aqueous NaOH solution (3.27 M, 10 mL, 32.7 mmol) were simultaneously dropwise added under stirring over a period of 1 h. Then, the reaction mixture was allowed to be warmed to room temperature and stirred for additional 4 h. The aqueous phase was collected and freeze-dried. The residue was re-suspended in 50 mL of isopropanol and filtered. The filtrate was concentrated by rotary evaporation. The crude product was purified with column chromatography using isopropanol as an eluent. The collected fractions were dried over anhydrous $MgSO_4$ overnight. The evaporation of isopropanol yielded HA as a white solid.

Yield: 1.16 g (64.5%). FT-IR (cm^{-1}): 3234, 3135, 3022, 2930, 2898, 1656, 1577, 1453, 1360, 976. ^{1}H NMR (400 MHz, D_2O, δ): 2.80 (t, 2H, —CCH_2CH_2—), 3.51 (t, 2H, —CH_2CH_2NH—), 5.68−5.76 (d, 1H, vinyl methine proton), 6.08−6.27 (m, 2H, vinyl methylene protons), 6.91 (s, 1H, imidazole proton), 7.67 (s, 1H, imidazole proton). ^{13}C NMR (101 MHz, D_2O, δ): 28.8 (—CH=C(NH)*C*H_2CH_2—), 42.0 (—NH*C*H_2CH_2—), 119.8 (—NH*C*H=C(NH)CH$_2$—), 130.0 (CH$_2$=*C*H—), 132.9 (*C*H$_2$=CH—), 137.5 (—CH$_2$*C*(NH)=CH—), 138.6 (—NH—*C*H=N—CH$_2$—), 171.2 (—CH(*C*O)NH—). Anal. Calcd for $C_8H_{11}N_3O$: C 58.17, H 6.71, N 25.44; Found: C 57.50, H 6.61, N 24.91. TOF-MS (*m/z*): $[M + H]^+$ Calcd for $C_8H_{11}N_3O$, 165.09; Found, 165.09.

Synthesis of Poly(histamine acrylamide)

Poly(histamine acrylamide) (PHA) polymers were prepared by radical polymerization of HA in the presence of AIBN and 2-mercaptoethanol in methanol at 60 °C. In a typical example, under a nitrogen atmosphere, HA (0.201 g, 1.22 mmol), AIBN (2.00 mg, 12.2 μmol), 2-mercaptoethanol (0.523 mg, 6.69 μmol), and 3 mL methanol were added into a 10 mL Schlenk flask equipped with an magnetic stirrer. The flask after purging with nitrogen for 30 min was sealed and placed in an oil bath thermostated at 60 °C. After stirring for 48 h, 3 mL of aqueous HCl (0.5 M) was added. The polymer was purified through extensive dialysis against deionized water (MWCO 3.4 kDa). The polymer after lyophilization was collected in its HCl salt form as a white solid powder.

Yield: 76.4−95.6%. FT-IR (cm^{-1}): 3256, 3109, 3014, 2928, 2859, 1644, 1548, 1439, 1369. ^{1}H NMR (400 MHz, D_2O, δ): 1.52 (s, —CH_2CH(CO)—), 1.93 (s, —CH$_2$C*H*(CO)—), 2.74 (s, —CCH_2CH_2—), 3.29 (s, —CH$_2$CH_2NH—), 7.08 (s, imidazole proton), 8.35 (s, imidazole proton). ^{13}C NMR (101 MHz, D_2O, δ): 27.4 (—CH=C(NH)*C*H_2CH_2—), 37.7 (—*C*H$_2$CH(CO)—), 41.2 (—NH*C*H_2CH_2—), 45.3 (—CH$_2$*C*H(CO)—), 119.0 (—NH*C*H=C(NH)CH$_2$—), 134.5 (—CH$_2$*C*(NH)=CH—), 136.7 (—NH—*C*H=N—CH$_2$—), 179.1 (—CH(*C*O)NH—).

Polymer Characterization

^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) spectra were recorded on a Unity Inova 400 MHz nuclear magnetic resonance instrument using deuterated water (D_2O) as a solvent. Fourier transform infrared (FT-IR) spectra were recorded on NICOLET 6700 spectrophotometer (Thermo Scientific). The samples were prepared using KBr pellets. The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity (PDI) of PHA polymers were determined with a Waters 1515 gel permeation chromatography (GPC) instrument equipped with three ULTRAHYDROGEL columns (120, 250, and 1000 PKGD; M_w from 200 to 10^6 g/mol) following a INLINE precolumn and a differential refractive-index detector. The measurements were performed using 0.3 M NaAc aqueous solution (pH 4.4) as an eluent at a flow rate of 0.5 mL/min at 30 °C and a series of narrow poly(ethylene oxide)s as standard.

Buffer Capacity Measurements

The buffer capacity of PHA polymers was determined by acid–base titration assay over a pH range of 2.0–10.0, similar to previous reports.[30,32] Briefly, PHA polymer (0.1 mmol N) was dissolved in 5 mL of 150 mM NaCl aqueous solution to yield 20 mM nitrogen concentration. The pH of the polymer solution was first brought to 2.0, and then titrated with 0.1 M NaOH using a pH meter (DELTA 320). For comparison, 150 mM NaCl aqueous solution and 25 kDa PEI was also titrated in the same way. The buffer capacity defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.2 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 (0.1 M) required to bring the pH value of the polymer solution from 5.1 to 7.2, and N mol is the total moles of protonable amine groups (0.1 mmol).

Gel Retardation Assay

The DNA binding ability of PHA polymers were studied by agarose gel electrophoresis. The polymer/DNA complexes prepared at varying N/P ratios from 2/1 to 6/1 were electrophoresed through a 0.8% agarose gel with a current of 110 V for 40 min in TAE solution (40 mM Tris–HCl, 1 v/v% acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide. The final DNA concentration is 0.4 μg each well.

Preparation and Characterization of DNA Polyplexes

The polyplexes were prepared at varying N/P ratios ranging from 5/1 to 30/1. They were prepared by adding a HEPES buffer solution (20 mM, pH 7.0) of polymer (600 μL, various concentration) to a HEPES buffer solution (20 mM, pH 7.0) of plasmid DNA (150 μL, 37.5 μg/mL), followed by vortexing for 5 s and incubating at room temperature for 30 min. The size and surface charge of polyplexes were measured at 25 °C using a Zetasizer Nano ZS (Malvern Instruments) equipped with dynamic light scattering (DLS, 10 mW He-Ne laser, 633 nm wavelength) and a standard capillary cell, respectively. The measurements were performed in triplicate.

Transmission Electron Microscopy

The polyplex sizes were characterized using Tecnai G220 Transmission Electron Microscopy (TEM) operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of the polyplexes solution on the copper grid followed by staining with phosphotungstic acid.

In Vitro Transfection and Cell Viability Assays

Transfection experiments were performed in COS-7 cells using the pEGFP-C1 and pCMV-Luc plasmid DNA as reporter genes. Transfections were conducted using polyplexes formed at N/P ratios of 10/1, 15/1, and 20/1. The cells were plated in a 12-well plate (cell density: 1.5×10^5 cells/well) and maintained in DMEM medium 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 after rinsing with 1 mL of PBS were incubated for 4 h at 37 °C with 100 μL of polyplex dispersion (1.0 μg of plasmid DNA per well) and 400 μL of culture medium containing 10% serum, the polyplexes were removed, 1 mL of fresh culture medium was added, and the cells were cultured for additional 2 days.

The GFP expression level of cell lysates was analyzed with a spectrofluorophotometer (SLM-AMINCO 8100, SLM Instruments, Urbana, IL) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The Luciferase quantification was done using a commercial luciferase assay kit (Promega, Madison, WI) and a Lumat LB 9501 illuminometer (Berthold, Milbach, Germany). Luciferase transfection efficiency was expressed as relative light unit (RLU) per mg of protein. The polyplexes of 25 kDa PEI at its optimal N/P ratio of 10/1 were used as a reference. All the experiments were carried out in triplicate.

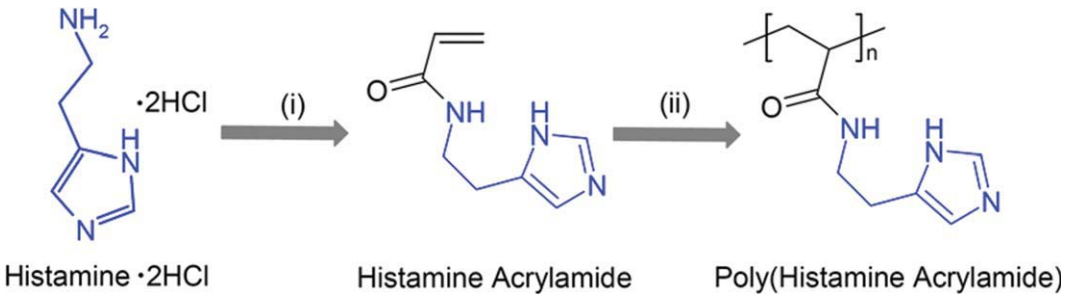
The cytotoxicity of polyplexes was evaluated with the same cell culture procedure as the transfection followed by CCK assay. The cells were incubated with CCK-8 reagent solution for 1.5 h before the measurement of absorption at 450 nm using a microplate reader (BIORAD, Model 550). The CCK value for the untreated cells (i.e., cells not exposed to transfection systems) was taken as 100% cell viability. Data are presented as average $\pm$ SD ($n = 5$).

RESULTS AND DISCUSSION

Synthesis and Characterization of PHA Polymers

The aim of this study was to develop novel water-soluble histamine-based cationic vinyl polymer, poly(histamine acrylamide) (PHA), for non-viral gene transfection. Unlike synthesis of polyhistidine, which requires protection and deprotection of imidazole amino groups,[33] PHA was synthesized in two straight steps, that is, (i) treatment of histamine with an equiv. molar amount of acryloyl chloride to yield histamine acrylamide (HA) and (ii) controlled radical polymerization of HA in methanol (Scheme 1). HA was obtained with 64.5% yield after purification with column chromatography. HA was readily dissolved in several highly polar solvents such as water, DMF, and different alcohols. ^{1}H NMR spectrum of HA in D_2O showed signals at δ 5.68−5.76, 6.08−6.27, and δ 2.80, 3.51, 6.91, 7.67, which were attributable to protons of acryloyl and histamine moieties, respectively [Fig. 1(A)]. The integral ratio between signals at δ 5.68−5.76 (assignable to vinyl methine proton) and 3.51 (assignable to methylene protons neighboring to the amino group of histamine moieties) was close to 1:2, supporting equivalent coupling of histamine and acryloyl chloride leaving imidazole amino groups intact. The structure of HA has been further confirmed by ^{13}C NMR [Fig. 1(B)], elemental analysis, and time-of-flight mass spectrometry (TOF-MS).

The radical polymerization of HA was performed in methanol at 60 °C for 2 days using AIBN as an initiator and 2-mercaptoethanol as a chain transfer agent. The polymer was purified by exhaustive dialysis against deionized water following acidification of the reaction mixture and isolated in its HCl salt form after lyophilization. The results of polymerization



SCHEME 1 Synthesis of histamine acrylamide monomer and poly(histamine acrylamide) polymer. Reaction conditions: (i) acryloyl chloride, H_2O/CH_2Cl_2, NaOH, r.t., 4 h; (ii) radical polymerization, 2-mercaptoethanol, AIBN, methanol, 60 °C, 48 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

are summarized in Table 1. The polymers were obtained with good yields (76.4−95.6%). ^{1}H NMR spectrum (in D_2O) showed clear signals at δ 2.74, 3.29, 7.08, and 8.35 assignable to the protons of histamine moieties and resonances at δ 1.52 and 1.93 due to the methylene and methine protons of the main chain, respectively [Fig. 2(A)]. The structure of PHA was corroborated by ^{13}C NMR [Fig. 2(B)]. FT-IR spectra showed broad signals at about 3200 cm^{-1} characteristic of N—H vibration and complete disappearance of absorption at 976 cm^{-1} due to vinyl bonds for PHA (Fig. 3). Gel permeation chromatography (GPC) measurements using 0.3 M NaAc aqueous solution (pH 4.4) as an eluent and a series of poly(-ethylene oxide)s as standards revealed that these PHAs had moderate polydispersities of 1.46–1.91 and importantly the number average molecular weights (M_n) of PHAs increased from 9.2 kDa to 28.7 kDa with increasing HA/2-mercaptoethanol molar ratios from 121/1 to 500/1 (Table 1), indicating that PHAs were synthesized in a rather well controlled manner.

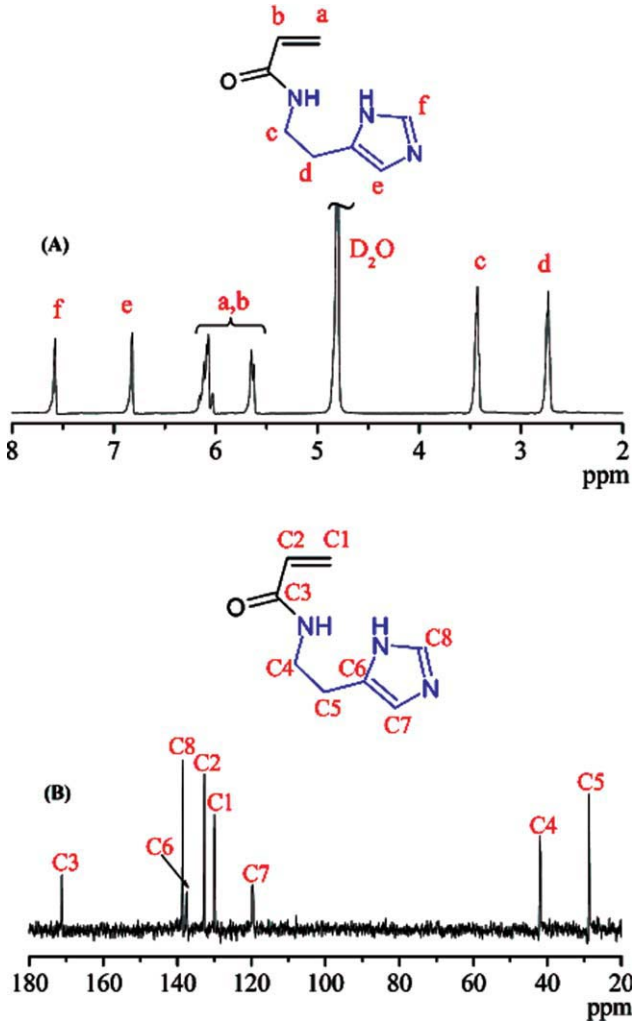


FIGURE 1 ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) spectra of HA monomer in D_2O. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We were interested in exploring water soluble histamine-based cationic polymers because of their high buffer capacity at the endosomal pH range. Here, the buffer capacity, defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.2, was determined from acid–base titration curves from pH 2.0 to pH 10. Interestingly, the results showed that all PHA polymers had remarkably high buffer capacities of about 70%, which were five-fold higher than 25 kDa branched PEI (14.2%; Fig. 4). This excellent buffer capacity of PHA might lead to effective disruption of endosomes via the "proton sponge effect," facilitating the

TABLE 1 Synthesis of Poly(histamine acrylamide)s by Radical Polymerization of Histamine Acrylamide[a]

Entry	Polymer	[HA]/[ME] mol/mol	GPC[b] ($\times10^{-3}$ g/mol) M_n	M_w	PDI	Yield (%)
1	9.2 kDa PHA	121/1	9.2	16.8	1.82	80.3
2	12.7 kDa PHA	182/1	12.7	18.6	1.46	95.6
3	17.5 kDa PHA	302/1	17.5	33.4	1.91	76.4
4	28.7 kDa PHA	500/1	28.7	49.4	1.72	85.0

[a] The radical polymerization of histamine acrylamide was carried out in the presence of 2-mercaptoethanol and AIBN in methanol at 60 °C for 48 h.
[b] Determined by GPC measurements (eluent: 0.3 M NaAc pH 4.4 aqueous solution, standards: polyethylene oxide, flow rate: 0.5 mL/min, 30 °C).

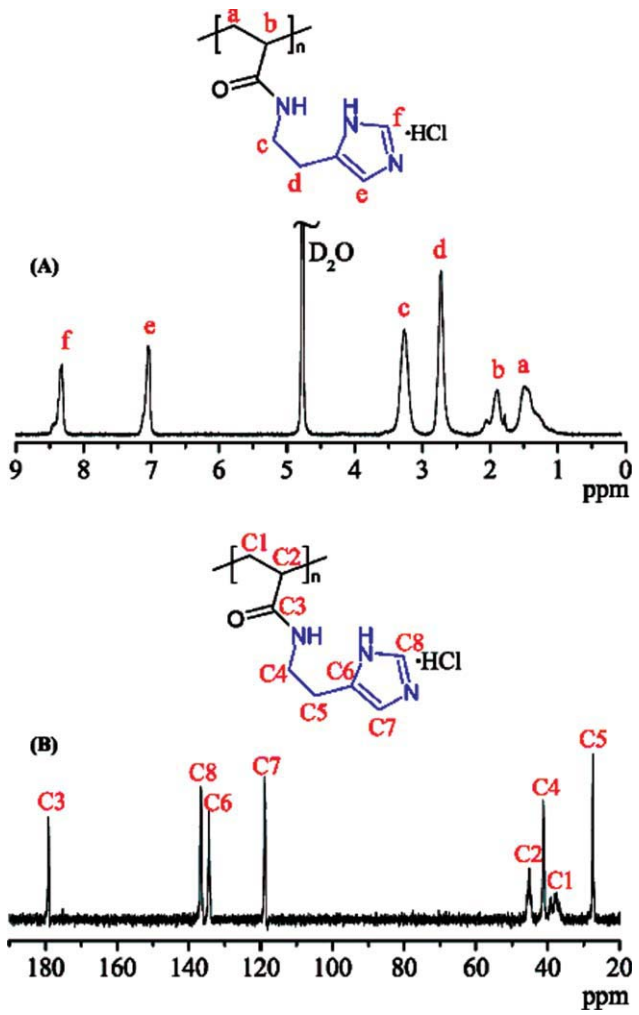


FIGURE 2 ^{1}H NMR (400 MHz) and ^{13}C NMR (101 MHz) spectra of PHA polymer in D_2O (Table 1, Entry 2). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

endosomal escape of DNA complexes.[6,34] In addition to "proton sponge effect," the strongly increased cationic charge of the polymer upon endosomal acidification could also contribute to the endosomal escape, due to increased disruptive interaction with the endosomal membrane.

Biophysical Characterization of PHA/DNA Polyplexes

The plasmid DNA condensation abilities of PHAs were studied using gel electrophoresis, dynamic light scattering (DLS), and zeta potential measurements. The plasmid DNA existed mainly in a supercoiled form. Interestingly, gel retardation assays showed that 9.2, 12.7, and 17.5 kDa PHAs were able to completely inhibit DNA migration at an N/P ratio of 5/1, and 28.7 kDa PHA effectively retarded DNA migration at an N/P ratio of 4/1 in 20 mM HEPES at pH 7.0 (Fig. 5). For DLS and zeta potential measurements, PHA polyplexes were prepared at varying N/P ratios from 5/1 to 30/1 in 20 mM HEPES at pH 7.0. Notably, the results showed that at N/P ratios $\leq$15/1, the sizes of PHA polyplexes decreased with increasing molecular weights from 9.2 to 17.5 kDa, whereas

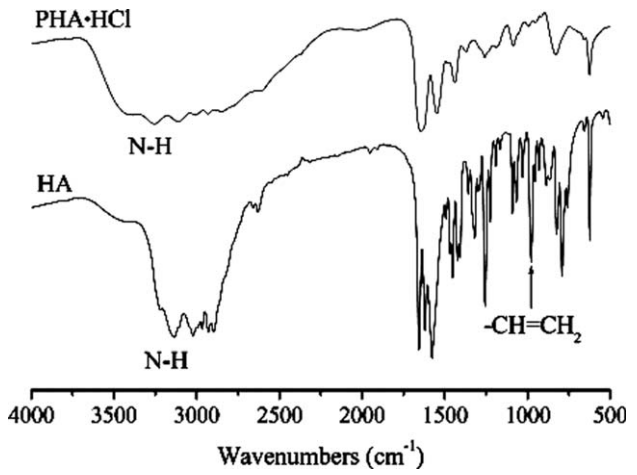


FIGURE 3 FT-IR spectra of HA monomer and PHA polymer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

17.5 and 28.7 kDa PHAs condensed DNA into comparable particle sizes at the same N/P ratios [Fig. 6(A)]. For all PHA polymers, the polyplex sizes also decreased with increasing N/P ratios, in which 12.7, 17.5, and 28.7 kDa PHAs were able to condense DNA into nano-sized particles (<220 nm) at an N/P ratio of 10/1. For example, polyplexes of 12.7 and 17.5 kDa PHAs formed at N/P ratios of 10/1, 15/1 and 20/1 had particle sizes of about 214, 143, 123 nm and 112, 98, 88 nm, respectively [Fig. 6(A)]. TEM image of 12.7 kDa PHA/DNA polyplexes formed at an N/P ratio of 20/1 showed an average diameter of ca. 100 nm (Fig. 7), which was in good

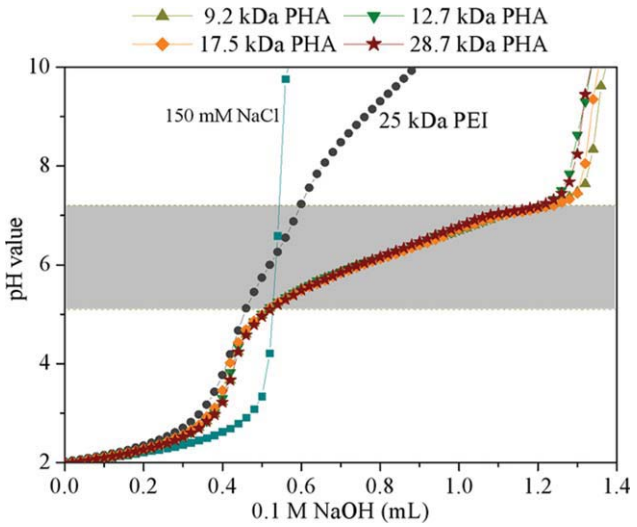


FIGURE 4 Acid–base titration curves of PHA polymers in 150 mM NaCl aqueous (from pH 2 to pH 10 with 0.1 M NaOH). For comparison, the titration curves of 150 mM NaCl aqueous solution and 25 kDa PEI are also presented. The buffer capacities were 70.5%, 69.4%, 71.2%, 69.3%, and 14.2% for 9.2 kDa PHA, 12.7 kDa PHA, 17.5 kDa PHA, 28.7 kDa PHA, and 25 kDa PEI, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

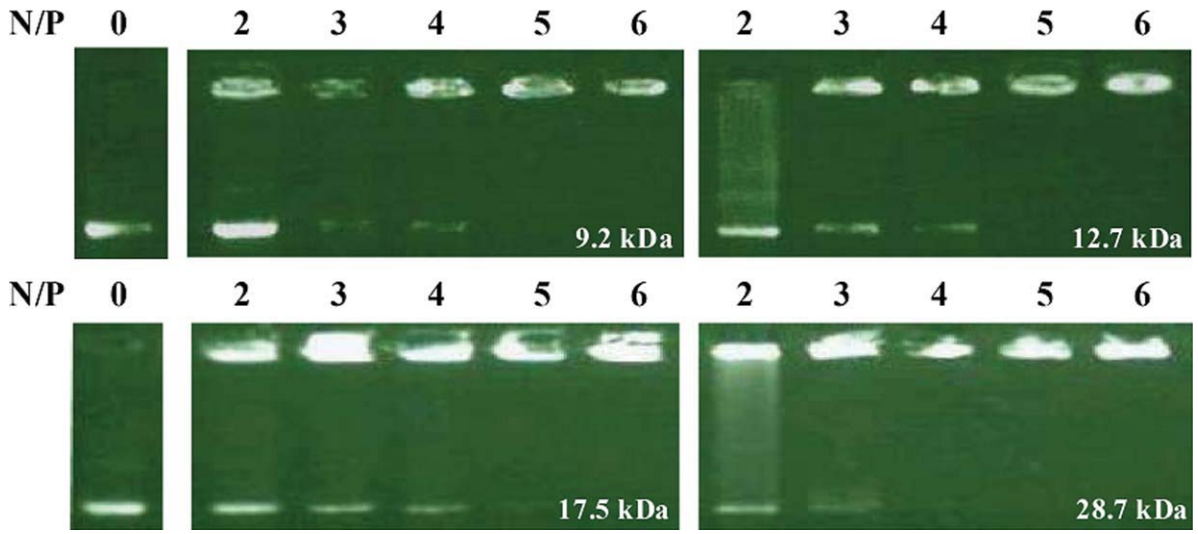


FIGURE 5 Agarose gel electrophoresis of PHA/DNA complexes formed at N/P ratios varying from 2/1 to 6/1 (20 mM HEPES, pH 7.0).

agreement with that determined by DLS. Zeta potential analyses revealed that all nano-sized (<220 nm) polyplexes had moderate positive surface charges (+13–+19 mV), in which both PHA molecular weights and N/P ratios appeared to have not much influence on polyplex surface charges [Fig. 6(B)].

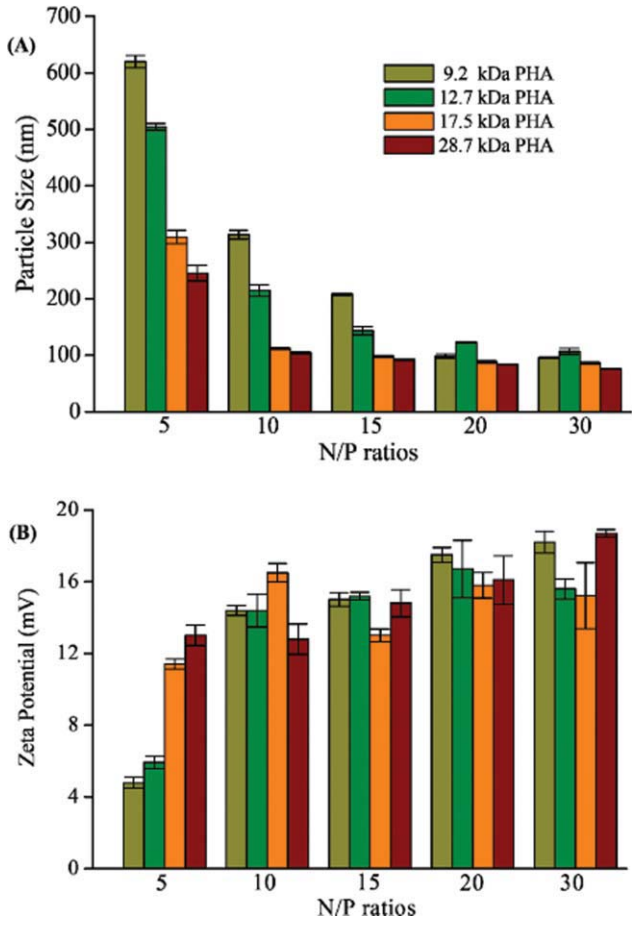


FIGURE 6 The average particle sizes (A) and zeta potentials (B) of PHA polyplexes prepared at N/P ratios from 5/1 to 30/1 (20 mM HEPES, pH 7.0). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In Vitro Cytotoxicity and Transfection

On the basis of the above biophysical characterization results, 12.7 and 17.5 kDa PHAs, which formed nano-sized polyplexes at and above an N/P ratio of 10/1, were selected for the DNA transfection studies. We first evaluated the cytotoxicity of PHA polyplexes using CCK assays in COS-7 cells in 10% serum conditions. Interestingly, the results indicated that polyplexes of both 12.7 and 17.5 kDa PHAs prepared at N/P ratios varying from 10/1, 15/1 to 20/1 were practically non-toxic to COS-7 cells (ca., 100% cell viabilities; Fig. 8). It has been often observed that histidylated or imidazole-containing gene carriers have low cytotoxicities.[12] Engbersen

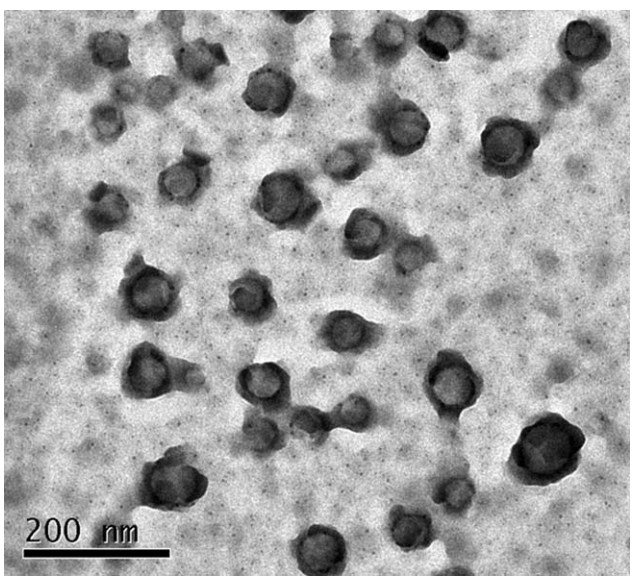


FIGURE 7 TEM image of 12.7 kDa PHA/DNA complexes formed at an N/P ratio of 20/1.

et al. reported that histamine-based poly(amido amine)s were basically non-toxic to cells.[21] The low cytotoxicity of histidylated or imidazole-containing gene carriers is most likely due to a minimal charge density of imidazole amino group. The detailed toxicity investigation on different polycations by Kissel and coworkers revealed that cationic charge density is one of the key parameters for cell damage.[35]

The in vitro transfection activity of PHA polyplexes was studied in COS-7 cells using two reporter genes, that is, pEGFP-C1 and pCMV-Luc plasmid DNA, under the identical conditions as for CCK assays. The transfection results using pCMV-Luc plasmid DNA showed that the transfection activity of 12.7 kDa PHA polyplexes increased with increasing N/P ratios from 10/1 to 20/1, with transfection efficiency at its optimal formulation (i.e., N/P 20/1) reached a level comparable to that of 25 kDa PEI [Fig. 9(A)]. In addition, polyplexes of 17.5 kDa PHA displayed similar transfection efficiency to those of 12.7 kDa PHA, indicating that PHA molecular weight does not significantly influence its transfection activity. The transfection studies using pEGFP-C1 plasmid DNA showed that 12.7 kDa PHA polyplexes formed at N/P ratios of 15/1 and 20/1 had transfection efficiencies slightly higher than 25 kDa PEI [Fig. 9(B)]. It is remarkable that this low molecular weight PHA while non-toxic mediates efficient in vitro gene transfection. For many cationic polymer-based gene carriers, satisfactory transfection performance is observed only for high molecular weight polymers, for example, PDMAEMA was reported with a molecular weight of over 300 kDa,[24] branched PEI with a molecular weight of 25 kDa or higher,[36] and PAMAM dendrimer with six generation.[4] These high molecular weight polycations, nevertheless, showed also high cytotoxicity. The superior transfection activity of low molecular weight PHA is likely associated with its good DNA condensation ability to form

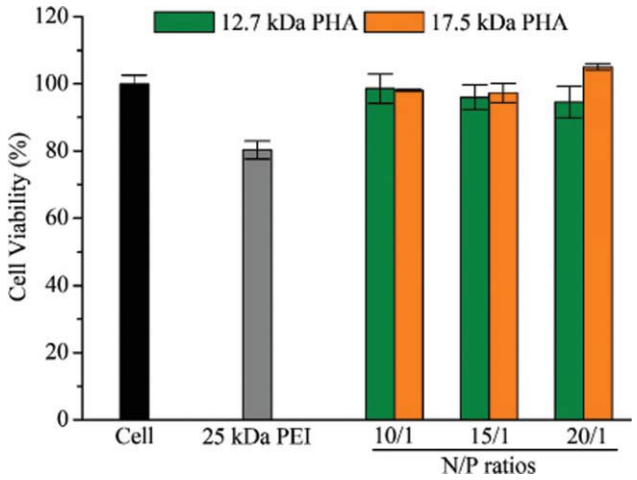


FIGURE 8 Cytotoxicity of polyplexes based on 12.7 kDa and 17.5 kDa PHAs formed at N/P ratios ranging from 10/1 to 20/1 in COS-7 cells. 25 kDa PEI/DNA complexes prepared at an N/P ratio of 10/1 was used as a control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

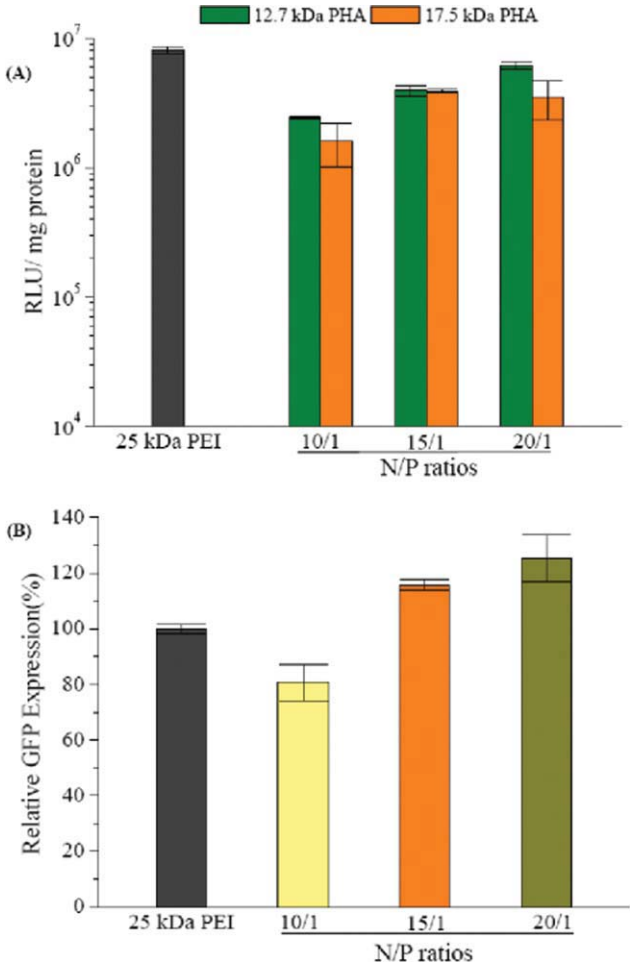


FIGURE 9 Transfection efficiencies of PHA polyplexes in COS-7 cells at different N/P ratios of 10/1, 15/1, and 20/1 in 10% serum conditions. 25 kDa PEI at an N/P ratio of 10/1 was used as a control. (A) 12.7 kDa and 17.5 kDa PHAs, pCMV-Luc plasmid DNA; (B) 12.7 kDa PHA, pEGFP-C1 plasmid DNA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

nano-sized polyplexes and exceptional buffer capacity. In addition, PHA like other vinyl polymers (e.g., PDMAEMA) presents a significant advantage over PEI and PAMAM dendrimers with its facile synthesis through controlled radical (co)polymerization. Their versatile and controlled synthesis, low cytotoxicity, and high transfection activity render PHA-based cationic polymers particularly interesting for the development of safe and efficient non-viral gene delivery systems.

CONCLUSIONS

We have demonstrated that novel water-soluble cationic poly (histamine acrylamide) (PHA) polymers with superior buffer capacity at endosomal pH range mediate efficient while nontoxic in vitro gene transfection. These histamine-based vinyl polymers can be readily obtained with controlled molecular weights through radical polymerization of

histamine acrylamide (HA). It is remarkable that low molecular weight PHA (e.g., 12.7 kDa PHA) presents a transfection activity comparable to 25 kDa PEI control. The versatile and controlled synthesis, low cytotoxicity, and high transfection activity render PHA-based cationic polymers particularly interesting for the development of safe and efficient non-viral gene transfer agents.

This work was supported by National Natural Science Foundation of China (NSFC 20874070, 50803043, 50703028, 50973078, and 20974073), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (08KJB150016), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, Program of Innovative Research Team of Soochow University, and the A3 (China–Japan–Korea) Program on Gene Delivery from the National Research Foundation of Korea (NRF), Republic of Korea.

REFERENCES AND NOTES

1 Mintzer, M. A.; Simanek, E. E. Chem Rev 2009, 109, 259–302.

2 Mastrobattista, E.; van der Aa, M.; Hennink, W. E.; Crommelin, D. J. A. Nat Rev Drug Discov 2006, 5, 115–121.

3 Park, T. G.; Jeong, J. H.; Kim, S. W. Adv Drug Deliv Rev 2006, 58, 467–486.

4 Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. Nat Rev Drug Discov 2005, 4, 581–593.

5 Boussif, O.; Lezoualch, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. Proc Natl Acad Sci USA 1995, 92, 7297–7301.

6 Cho, Y. W.; Kim, J. D.; Park, K. J Pharm Pharmacol 2003, 55, 721–734.

7 Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. J. Gene Med 2005, 7, 657–663.

8 Benns, J. M.; Choi, J. S.; Mahato, R. I.; Park, J. S.; Kim, S. W. Bioconjug Chem 2000, 11, 637–645.

9 Roufai, M. B.; Midoux, P. Bioconjug Chem 2001, 12, 92–99.

10 Chang, K. L.; Higuchi, Y.; Kawakami, S.; Yamashita, F.; Hashida, M. Bioconjug Chem 2010, 21, 1087–1095.

11 Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R. Proc Natl Acad Sci USA 2001, 98, 1200–1205.

12 Midoux, P.; Pichon, C.; Yaouanc, J. J.; Jaffres, P. A. Br J Pharm 2009, 157, 166–178.

13 Pichon, C.; Goncalves, C.; Midoux, P. Adv Drug Deliv Rev 2001, 53, 75–94.

14 Bikram, M.; Ahn, C. H.; Chae, S. Y.; Lee, M. Y.; Yockman, J. W.; Kim, S. W. Macromolecules 2004, 37, 1903–1916.

15 Asayama, S.; Sekine, T.; Kawakami, H.; Nagaoka, S. Bioconjug Chem 2007, 18, 1662–1667.

16 Asayama, S.; Hakamatani, T.; Kawakami, H. Bioconjug Chem 2010, 21, 646–652.

17 Kichler, A.; Leborgne, C.; Marz, J.; Danos, O.; Bechinger, B. Proc Natl Acad Sci USA 2003, 100, 1564–1568.

18 Seow, W. Y.; Yang, Y. Y. Adv Mater 2009, 21, 86–90.

19 Akinc, A.; Lynn, D. M.; Anderson, D. G.; Langer, R. J Am Chem Soc 2003, 125, 5316–5323.

20 Greenland, J. R.; Liu, H. N.; Berry, D.; Anderson, D. G.; Kim, W. K.; Irvine, D. J.; Langer, R.; Letvin, N. L. Mol Ther 2005, 12, 164–170.

21 Lin, C.; Zhong, Z. Y.; Lok, M. C.; Jiang, X. L.; Hennink, W. E.; Feijen, J.; Engbersen, J. F. J. Bioconjug Chem 2007, 18, 138–145.

22 Dubruel, P.; Schacht, E. Macromol Biosci 2006, 6, 789–810.

23 Verbaan, F. J.; Klouwenberg, P. K.; van Steenis, J. H.; Snel, C. J.; Boerman, O.; Hennink, W. E.; Storm, G. Int J Pharm 2005, 304, 185–192.

24 van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Crommelin, D. J. A.; Hennink, W. E. J Control Release 1998, 53, 145–153.

25 Jiang, X.; Lok, M. C.; Hennink, W. E. Bioconjug Chem 2007, 18, 2077–2084.

26 Zhu, C. H.; Jung, S.; Luo, S. B.; Meng, F. H.; Zhu, X. L.; Park, T. G.; Zhong, Z. Y. Biomaterials 2010, 31, 2408–2416.

27 Funhoff, A. M.; van Nostrum, C. F.; Janssen, A.; Fens, M.; Crommelin, D. J. A.; Hennink, W. E. Pharm Res 2004, 21, 170–176.

28 Xu, F. J.; Li, H. Z.; Li, J.; Zhang, Z. X.; Kang, E. T.; Neoh, K. G. Biomaterials 2008, 29, 3023–3033.

29 van de Wetering, P.; Schuurmans-Nieuwenbroek, N. M. E.; van Steenbergen, M. J.; Crommelin, D. J. A.; Hennink, W. E. J Control Release 2000, 64, 193–203.

30 Zhu, C. H.; Jung, S.; Si, G. Y.; Cheng, R.; Meng, F. H.; Zhu, X. L.; Park, T. G.; Zhong, Z. Y. J Polym Sci Part A: Polym Chem 2010, 48, 2869–2877.

31 You, Y. Z.; Manickam, D. S.; Zhou, Q. H.; Oupicky, D. J Control Release 2007, 122, 217–225.

32 Wang, Y.; Zheng, M.; Meng, F.; Zhang, J.; Peng, R.; Zhong, Z. Biomacromolecules 2011, 12, 1032–1040.

33 Lee, E. S.; Na, K.; Bae, Y. H. J Control Release 2003, 91, 103–113.

34 Park, I. K.; Singha, K.; Arote, R. B.; Choi, Y. J.; Kim, W. J.; Cho, C. S. Macromol Rapid Commun 2010, 31, 1122–1133.

35 Fischer, D.; Li, Y. X.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. Biomaterials 2003, 24, 1121–1131.

36 Lungwitz, U.; Breunig, M.; Blunk, T.; Gopferich, A. Eur J Pharm Biopharm 2005, 60, 247–266.