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Endolytic, pH-Responsive HPMA-*b*-(L-Glu) Copolymers Synthesized via Sequential Aqueous RAFT and Ring-Opening Polymerizations†

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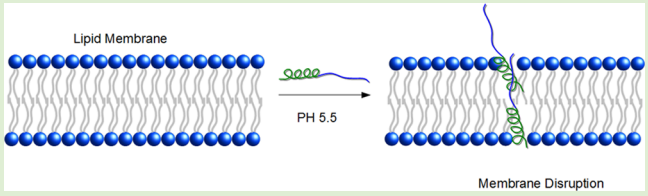
Supporting Information

ABSTRACT: A facile synthetic pathway for preparing block copolymers with pH-responsive L-glutamic acid segments for membrane disruption is reported. Aqueous reversible addition−fragmentation chain transfer (*a*RAFT) polymerization was first used to prepare biocompatible, nonimmunogenic poly[*N*-(2-hydroxypropyl)methacrylamide]. This macro chain transfer agent (CTA) was then converted into a macroinitiator via simultaneous aminolysis and thiol−ene Michael addition using the primary amine substituted *N*-(3-aminopropyl)methacrylamide. This macroinitiator was subsequently utilized in the ring-opening polymerization of the *N*-carboxyanhydride monomer of γ-benzyl-L-glutamate. After deprotection, the pH-dependent coil-to-helix transformations of the resulting HPMA-*b*-(L-Glu) copolymers were monitored via circular dichroism spectroscopy. HPMA segments confer water solubility and biocompatibility while the L-glutamic acid repeats provide reversible coil-to-helix transitions at endosomal pH values (∼5−6). The endolytic properties of these novel [HPMA-*b*-(L-Glu)] copolymers and their potential as modular components in drug carrier constructs was demonstrated utilizing red blood cell hemolysis and fluorescein release from POPC vesicles.



INTRODUCTION

Remarkable progress has been made over the past decade in the design of polymeric vehicles in order to achieve cellular targeting,[1] increased blood circulation,[2−4] drug/gene protection,[5−7] reduced cytotoxicity,[8−11] and so on.[12] Despite these advances, a number of challenges remain for efficacious gene/drug delivery.[13,14] An ideal carrier should provide protection and solubility during circulation as well as a mechanism for targeting and entry into specified cells. Once delivered, the drug/gene/carrier complex must overcome other critical barriers including trafficking to the lysosome, where the cargo can be degraded, or transport outside the cell into the extracellular milieu. A promising approach for an ideal polymeric drug vehicle is inclusion of a modular segment promoting disruption of the endosomal membrane at an appropriate time, allowing drug/gene release into the cytoplasm.

Some polymeric carriers rely on an osmotic swelling mechanism ("proton sponge effect") to escape the endosome. Alternatively, poly(amido amines), prepared by Duncan[15−17] and Wagner,[18] and poly(aspartamides), prepared by Kataoka and co-workers,[19−21] exploit the enhanced buffering capacity of pendant and backbone amines to facilitate endosomal swelling and rupture. While these systems demonstrate improved efficacy both in vitro and in vivo, the toxicity of amines is still a concern as is the necessity of a "charge-shielding" block such as PEG or HPMA. To alleviate unwanted electrostatic effects and undesirable toxicity, Convertine et al.[22−24] prepared endosomolytic block copolymers containing *N*,*N*-(2-dimethylaminoethyl)methacrylate (DMAEMA), propacrylic acid, and butyl methacrylate (BMA). DMAEMA served to bind siRNA, while propacrylic acid masked the hydrophobic BMA. At endosomal pH, propacrylic acid segments are protonated, thus increasing the hydrophobicity of the block copolymer and destabilizing the endosomal membrane.

Naturally occurring peptides and proteins provide several pathways for endosomal disruption via hydrophobic alignment between the α-helix and bilayer surface, usually resulting in electrostatic interactions of the membrane that promote permeability.[25] Such pH-responsive coil-to-helix transitions are attractive features of these biopolymers that offer opportunities for synthetic mimicry. Peptides based on melittin,[26] a component of bee venoms, as well as the lytic amino-terminus of the influenza virus HA-2[27] have been conjugated to polymeric vehicles increasing pDNA and siRNA efficacy both in vitro and in vivo. To realize a nature-inspired mimic, *N*-carboxyanhydride (NCA) polymerization[28−30] has been used to prepare synthetic peptides that undergo stimuli-responsive conformational changes into α-helices, β-sheets, and other ordered structures.[31] These conformational changes are facile and reversible, and they have been utilized for triggered drug release in vitro.[32]

In recent years our research has centered on the development of a modular drug/siRNA delivery platform, capitalizing

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on the attributes afforded by aqueous RAFT polymerization[33] for synthesis of biologically relevant systems.[34,35] For example, homo- and block copolymers of (3-guanidinopropyl)-methacrylamide (GPMA) were shown to serve efficiently as cell-penetrating mimics of natural peptides.[36] Folate targeting groups for receptor-mediated endocytosis have also been attached to both interpolyelectrolyte siRNA complexes[37] and amphiphilic diblock copolymers with disulfide-bound, pendant siRNA.[38] While siRNA uptake and trafficking to the endosome occurred with both of these charge-neutral delivery vectors, only the former showed significant gene knockdown. We attribute negligible gene suppression to inability of the latter construct as designed to escape the endosome. Herein, we report the controlled synthesis, characterization, and endolytic activity of a series of HPMA-*b*-(L-Glu) copolymers specifically designed to elicit membrane disruption. To our knowledge, this facile synthetic approach using sequential RAFT polymerization and aminolysis to produce a telechelic, amine-functional macroinitiator followed by NCA polymerization and hydrolysis has not been previously reported. The pH-dependent coil-to-helix transitions of the L-glutamic acid (L-Glu) block were followed by circular dichroism. Membrane disruption was demonstrated by red blood cell hemolysis and fluorescein release from POPC vesicles.

EXPERIMENTAL SECTION

Materials. All reagents were purchased from Sigma and used without further purification unless otherwise noted. *N*-(3-aminopropyl)methacrylamide (APMA) was purchased from Polysciences, while 4,4′-azobiscyanovaleric acid (V-501; Wako) was recrystallized twice from methanol. 4-Cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CEP)[24] and *N*-(2-hydroxypropyl)methacrylamide (HPMA)[39] were prepared as previously reported. For reactions requiring nitrogen, ultrahigh purity nitrogen (purity ≥99.998%) was used. Spectra/Por regenerated cellulose dialysis membranes (Spectrum Laboratories) with a molecular weight cutoff of 12−14 kDa were used for dialysis.

Synthesis of γ-Benzyl-L-glutamate-NCA. γ-Benzyl-L-glutamate (10 g, 42.1 mmol) was added to a flame-dried reaction flask equipped with a stir bar and placed onto a Schlenk line; the flask was evacuated under reduced pressure followed by the introduction of nitrogen. Approximately 100 mL of THF was added to yield a final concentration of ∼0.1 g/mL, and the slurry was stirred under nitrogen for 20 min at 75 °C. Triphosgene (6 g, 20.2 mmol) dissolved in 10 mL of THF was added to the mixture under a nitrogen atmosphere, and a drying tube was attached to the reaction flask. The reaction was allowed to proceed for ∼1 h, as THF slowly evaporated to give a final volume of ∼20−30 mL. After cooling, the mixture was precipitated into hexanes. The recovered precipitate was then redissolved in THF; 2−3 g of decolorizing charcoal was added and the mixture was allowed to stir overnight to remove residual hydrochloric acid. The mixture was then passed through a Celite column to remove charcoal, reprecipitated into hexanes, and cooled overnight. The resulting white powder was collected and dried under vacuum overnight to produce γ-benzyl-L-glutamate-NCA, with standard yields between 75 and 85%: ^{1}H NMR (DMSO-d_6) δ 9.15−9.05 (ring, COOH-N**H**, s), 7.40−7.25 (Ar, s), 5.15−5.05 (ring, COOH-C**H**-CH$_2$, t), 2.60−2.40 (COOH-C**H**$_2$, d), 2.2−1.80 (COOH-CH$_2$-C**H**$_2$, m).

MacroCTA of Poly(HPMA). The macro chain transfer agent (CTA) was prepared employing V-501 as the primary radical source and CEP as the chain transfer agent at 70 °C. To a 50 mL round-bottomed flask HPMA (2.86 g, 0.02 mol) dissolved in acetate buffer (pH 5.2, 0.27 M acetic acid, and 0.73 M sodium acetate) was added and diluted to a final volume of 20 mL ($[M]_o$ = 1 M). The round-bottomed flask was septum-sealed and purged with nitrogen for 1 h prior to polymerization. The macroCTA was reacted at a $[M]_o/[CTA]$ ratio = 400/1, while the [CTA]/[I] ratio was kept at 5/1, and the polymerization was allowed to proceed for 3.5 h before being quenched with liquid nitrogen followed by exposure to air. The macroCTA was isolated by dialysis (pH 3−4) at 4 °C and recovered by lyophilization yielding 1.6 g (93%).

Poly(HPMA) End-Capping with APMA. The macroCTA was converted into a macroinitiator via simultaneous aminolysis and thiol−ene Michael addition with APMA. The reaction is as follows. poly(HPMA) (305 mg, 9.5 μmol) was combined with APMA (170 mg, 0.95 mmol) in a septum sealed scintillation vial equipped with a stir bar, then dissolved in 5 mL of DI H_2O, and the pH elevated to 10 with 0.1 M NaOH. The reaction temperature was maintained at 70 °C for 48 h. The macroinitiator was isolated by dialysis in DI H_2O for 48 h and recovered by lyophilization, yielding 300 mg (98%).

Poly(HPMA) Chain Extension with γ-Benzyl-L-glutamate-NCA. Poly(HPMA)-NH_2 was used to initiate ring-opening polymerization (ROP) of the γ-benzyl-L-glutamate-NCA, thereby yielding poly(HPMA-*b*-(benzyl-L-glutamate)) (poly(HPMA-*b*-BLGA)) block copolymers. A typical reaction is as follows. In a 25 mL, round-bottomed flask, poly($HPMA_{220}$-NH_2) (50 mg, 1.56 μmol) macroinitiator was dissolved in 5 mL of dry DMF. γ-Benzyl-L-glutamate-NCA (10.3 mg, 38.9 μmol) was also dissolved in 5 mL of dry DMF and was immediately added via a glass, gas-tight syringe to the macroinitiator solution. Reactions were carried out for 5 days at 0 °C under a nitrogen atmosphere.[40] The polymer was precipitated into ether, redissolved in chloroform, and reprecipitated into ether to eliminate unreacted NCA. Benzyl protecting groups were removed by hydrolysis in a 50:50 mixture of trifluoroacetic acid (TFA) and hydrobromic acid (HBr)[40] at room temperature, followed by dialysis and lyophilizaton to yield pH-responsive poly[HPMA-*b*-(L-Glu)] copolymers. Recovered yields were ∼95%.

Copolymer Characterization. All polymers were characterized by aqueous size exclusion chromatography (ASEC) using an eluent of 20 wt % acetonitrile/0.05 M Na_2SO_4 (aq) at a flow rate of 0.300 mL/min at 25 °C, TOSOH Bioscience, LLC TSKgel columns (4 and 6 μm), a Polymer Laboratories LC1200 UV/vis detector, a Wyatt Optilab DSP interferometric refractometer (λ = 690 nm), and a Wyatt DAWN-DSP multiangle laser light scattering (MALLS) detector (λ = 633 nm). Absolute molecular weights and polydispersities were calculated using the Wyatt Astra (version 4) software. ASEC chromatograms obtained for poly($HPMA_{220}$) (before and after end-group conjugation) and poly[$HPMA_{220}$-*b*-(L-Glu)] block copolymers are shown in Figures 1 and 3. The d*n*/d*c* measurements for poly(HPMA) and poly[HPMA-*b*-(L-Glu)] were performed with a Wyatt Optilab DSP interferometric refractometer (λ = 690 nm) at 35 °C and determined using Wyatt DNDC (version 5.90.03). Conversions for the macroCTA and the chain extension with γ-benzyl-L-glutamate-NCA were determined by

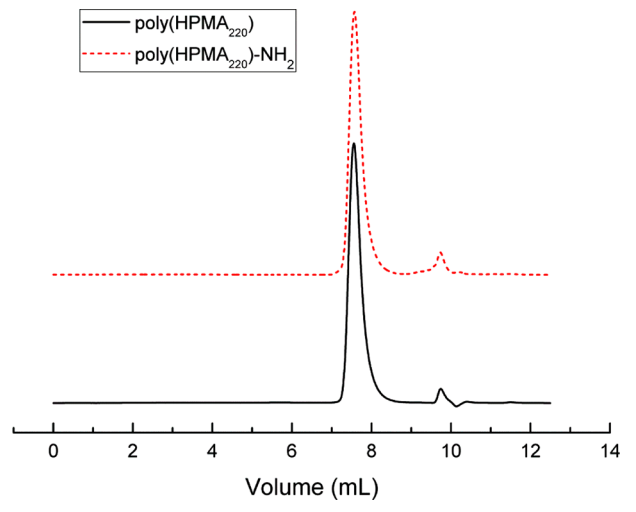
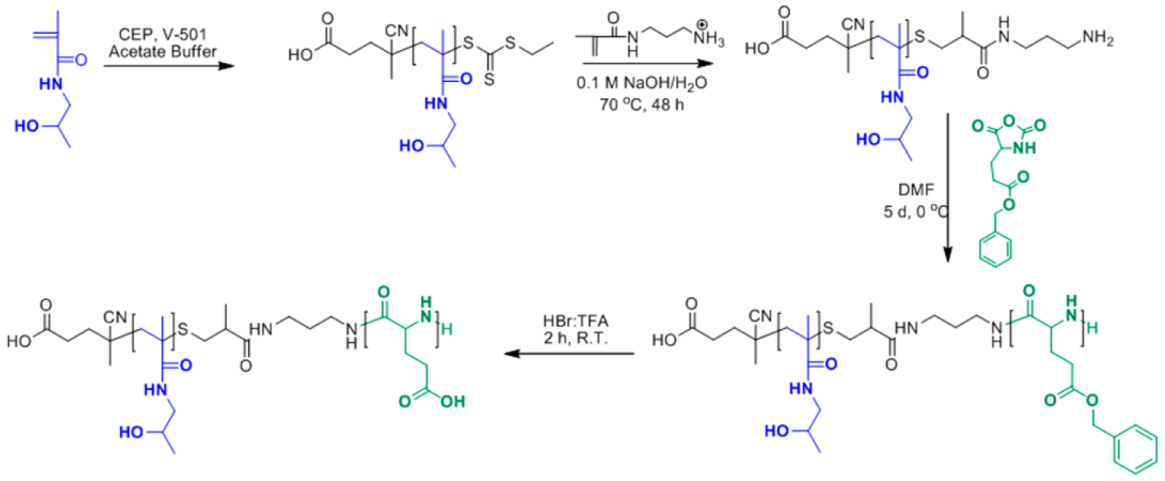


Figure 1. ASEC-MALLS of poly($HPMA_{220}$) (black) and poly-($HPMA_{220}$)-NH_2 (red). Poly($HPMA_{220}$)-NH_2 is offset for clarity.

Scheme 1. Synthetic Pathway for the Preparation of HPMA-*b*-(L-Glu) Copolymers



comparing the area of the monomeric UV signal detected at 274 nm at t_0 to the area at t_x using a Polymer Laboratories LC1200 UV/vis detector.

Copolymer compositions were determined with a Varian Mercury^{PLUS} 300 MHz spectrometer in deuterated DMSO supplemented with 15 wt % TFA, and spectra were recorded with a delay time of 2 s. ^{1}H NMR was used to determine the copolymer composition of poly(HPMA-*b*-BLGA) copolymers by integration of the relative intensities of the methyne-proton resonances of HPMA at 3.75 ppm and the aromatic-proton resonances of BLGA at 7.2 ppm.

Circular Dichroism of poly[HPMA-*b*-(L-Glu)]s. The ellipticity of the synthesized copolymers was determined utilizing a Jasco J-815 circular dichroism spectropolarimeter. Samples were dissolved in DI H_2O, and the pH was adjusted with 0.1 M HCl. Final sample concentrations ranged from 0.3−0.5 mg/mL, and solutions were allowed to equilibrate for 1 day prior to measurement. The spectra were obtained with a scan rate of 10 nm/min, a 0.5 nm bandwidth, and a time constant of 2 s. The signal-to-noise for all spectra was increased by averaging three scans. The formation of α-helices was determined by monitoring the presence of the characteristic double minima at 220 and 208 nm.[41]

Fluorescein-POPC (fPOPC) Liposome Preparation and Dye Release Studies. The preparation of dye-loaded POPC liposomes followed a standard literature procedure.[42] In a 25 mL round-bottomed flask, 5 mg of POPC was dissolved in 10 mL of chloroform. Then the chloroform was removed by rotary evaporation, and subsequently, the flask was placed under a high vac for 8 h. The resulting film was hydrated with PBS at the appropriate pH containing fluorescein (40 mM). The film was subjected to five freeze−pump−thaw cycles, and subsequently extruded (20 passes) through two stacked 200 nm pore PC membranes at 40 °C. Finally, free fluorescein was removed via a Sephadex-25 column eluted with PBS (20 mM Pi, 150 mM NaCl) at the appropriate pH. The resulting fPOPC liposomes possessed hydrodynamic radii of 90 nm with PDIs < 0.2 (data not shown). Block copolymers (200 μg/mL) were incubated with fPOPC at the appropriate pH for 1 h at room temperature. The mixture was then vortexed, and the fluorescence intensities measured using a PTI spectrofluorometer. Measurements were acquired with a 460 nm excitation (isosbestic point) and 520 nm emission. The slit widths were adjusted for each pH and ranged from 0.5 to 0.3 mm (pHs 4.0−7.4). All fluorescein dye release experiments were performed in triplicate with Triton-X100 (0.1 wt %) utilized as the positive control, and the percent release was determined using eq 1, in which $F(T)$ is the fluorescence observed when incubated with Triton-X100, F(P) is the fluorescence observed when incubated with HPMA-*b*-(L-Glu) copolymers, and $F(C)$ is the fluorescence observed with nothing added to the prepared POPC lipids.

$$\%\text{release} = \left[\frac{F(T) - F(P)}{F(T) - F(C)}\right] \times 100 \quad (1)$$

Red Blood Cell Hemolysis Assay. Bovine blood was drawn into vacutainers containing EDTA. The blood was centrifuged, plasma decanted, and washed with 150 mM NaCl (three times). Finally, the red blood cells (RBC) were resuspended in PBS (10 mM Pi, 150 mM NaCl) at either pH 7.4 or pH 5.5 to mimic physiological and endosomal pHs, respectively. Varying concentrations (10−400 μg/mL) of HPMA-*b*-(L-Glu) copolymers (100 μL) were incubated with 100 μL of RBC for 1 h at 37 °C. After incubation, the solution was centrifuged, and the supernatant was monitored at 541 nm for the presence of hemoglobin. All hemolysis experiments were performed in triplicate. Triton-X100 (0.1 wt %) was utilized as the positive control, and the percent release was determined using eq 2, in which $A(T)$ is the absorbance observed when the liposomes are incubated with Triton-X100, A(P) is the absorbance observed when incubated with HPMA-*b*-(L-Glu) copolymers, and $A(C)$ is the absorbance observed with nothing added to the red blood cells.

$$\%\text{release} = \left[\frac{A(T) - A(P)}{A(T) - A(C)}\right] \times 100 \quad (2)$$

RESULTS AND DISCUSSION

Synthesis of HPMA Homopolymer and Chain-End Functionalization. An HPMA homopolymer of DP 220 was prepared via aqueous RAFT polymerization (Scheme 1) in order to yield a water-soluble, biocompatible segment with telechelic[43,44] functionality appropriate for further modification to a macroinitiator for block copolymerization. The polymerization was carried out in acetate buffer (pH 5.2) at 70 °C using 4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CEP) and 4,4′-azobis(4-cyanopentanoic acid) (V-501) as the CTA and initiator, respectively. The resulting HPMA macro-CTA was end-capped with a primary amine containing monomer *N*-(3-aminopropyl)methacrylamide (APMA) via simultaneous aminolysis and thiol−ene Michael addition. To ensure quantitative thiol−ene coupling, the pH was elevated (>10) to deprotonate the amine, and the reaction was allowed to proceed at elevated temperature (70 °C) for an extended period of time (48 h.) End-capping efficiency was determined via a ninhydrin assay[45] (see Supporting Information), and primary amine incorporation exceeded 96%. Aqueous size exclusion chromatography (ASEC) was used to determine

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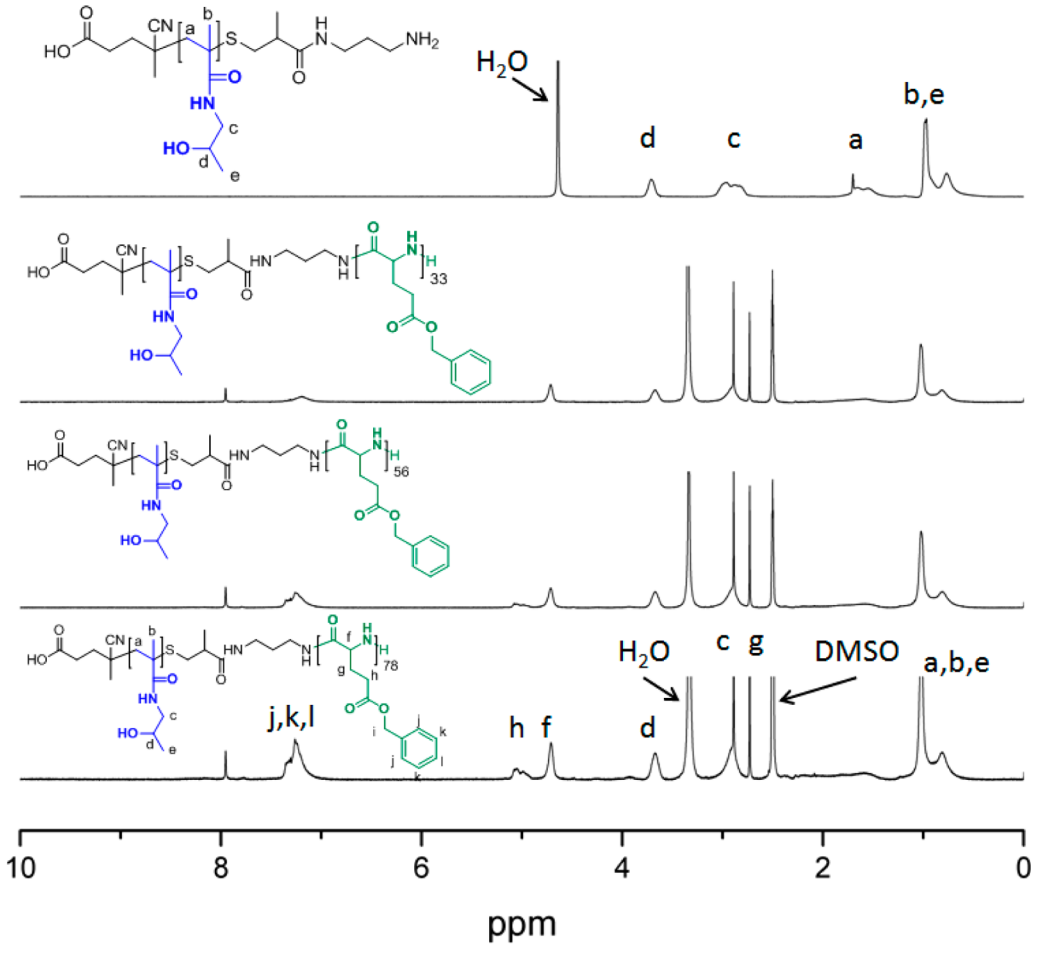

Figure 2. ^{1}H NMR of poly(HPMA$_{220}$)-NH$_2$ and poly(HPMA$_{220}$-*b*-BLGA). The poly(HPMA$_{220}$)-NH$_2$ spectrum was recorded in D$_2$O, while those of poly(HPMA$_{220}$-BLGA)s were recorded in DMSO-d_6 supplemented with TFA (15 wt %).

Table 1. Molecular Weight (M_n, M_w), Polydispersity (PDI), Composition, Conversion, and d*n*/d*c* Values for HPMA-*b*-(L-Glu) Copolymers

sample	$M_{n,Th}$[a] (kDa)	$M_{n,exp}$[b] (kDa)	M_w[b] (kDa)	PDI[b]	composition[c]	% conv.[d]	d*n*/d*c*[e]
poly(HPMA$_{220}$)-CTA	30.6	32.1	34.5	1.08	100:0	53	0.170
poly[HPMA$_{220}$-*b*-(L-Glu$_{33}$)]	34.8	37.8	44.6	1.18	87:13	96	0.145
poly[HPMA$_{220}$-*b*-(L-Glu$_{56}$)]	39.1	42.1	50.5	1.20	79:21	98	0.145
poly[HPMA$_{220}$-*b*-(L-Glu$_{78}$)]	42.8	45.6	53.4	1.17	75:25	94	0.144

[a]Theoretical M_n, ($M_{n,Th}$), calculated from conversion (ρ) using $M_{n,Th} = ([M]_o/[CTA]) \times M_{w,monomer} \times \rho + M_{w,CTA}$. [b]As determined by aqueous SEC-MALLS. [c]As determined by ^{1}H NMR. [d]Conversions were determined by comparison of the UV signal at 274 nm of the monomer at t_0 to that at t_x. [e]Determined by Wyatt Optilab DSP interferometric refractometer (λ = 690 nm).

PDIs, molecular weights, and macro disulfide coupling; the ASEC chromatograms (Figure 1) are shown prior to and after chain-end functionalization with APMA, indicating narrow distributions (PDIs < 1.1).

Synthesis of (HPMA-*b*-Glu) Block Copolymers. Utilizing poly(HPMA$_{220}$)-NH$_2$ as a macroinitiator, a series of chain extensions was accomplished via ring-opening polymerization of *N*-carboxyanhydride, γ-benzyl-L-glutamate-NCA, in DMF. By altering the [M]/[I] ratio, where [M] = γ-benzyl-L-glutamate-NCA (benzyl-protected glutamic acid) and [I] = P(HPMA)-NH$_2$, a range of block lengths was targeted. In order to prevent anticipated side reactions,[28,46−49] the polymerizations were conducted at 0 °C under an atmosphere of nitrogen. Copolymer compositions for the block copolymers were determined using ^{1}H NMR by comparing the relative intensities of the aromatic-protons resonances of benzyl L-glutamate units at 7.2 ppm to the methyne-proton resonances of the HPMA repeats at 3.75 ppm. Spectra of the poly(HPMA$_{220}$)-NH$_2$ macroinitiator and three copolymers with benzyl-L-glutamate blocks of DP 33, 58, and 78 are shown in Figure 2. Deprotection of benzyl-L-glutamate units was accomplished under acidic conditions at room temperature using a 50:50 mixture of TFA and HBr (see Supporting Information).[40,50] Copolymer molecular weights, PDIs, compositions, and d*n*/d*c* values are presented in Table 1. Size exclusion chromatograms (Figure 3) indicate successful chain extension with shifts to lower elution volume as the polymerization progressed. The copolymer molecular weights determined directly by ASEC-MALLS correlate well with those calculated from NMR compositional data; PDI values are

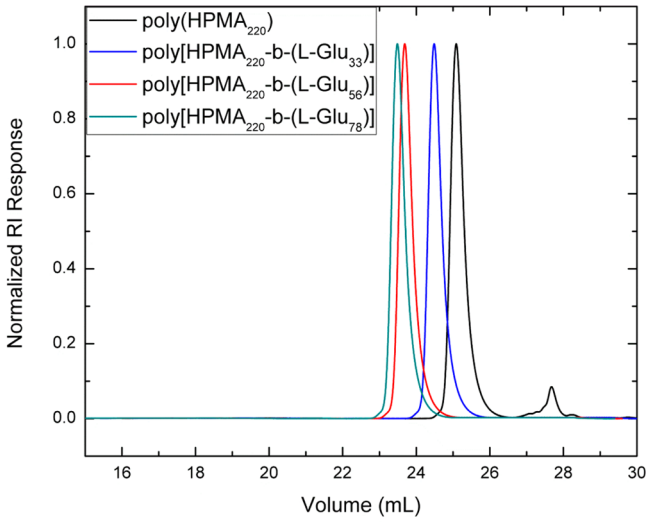

Figure 3. ASEC-MALLS of poly(HPMA$_{220}$) (black), poly[HPMA$_{220}$-*b*-(L-Glu$_{78}$)] (cyan), poly[HPMA$_{220}$-*b*-(L-Glu$_{56}$)] (blue), and poly-[HPMA$_{220}$-*b*-(L-Glu$_{33}$)] (red).

narrow, ranging from 1.08−1.20. The deviations in experimental and theoretical M_n values are relatively small and may be attributed to incomplete conversion of the HPMA macroCTA to the amine-terminated macroinitiator upon addition of APMA.

***α*-Helix Formation.** Circular dichroism[41] is an exceptionally valuable technique for ascertaining structural information of proteins,[51] nucleic acids,[52] and chiral self-assemblies.[53] By measuring the differences between left-handed and right-handed absorbances of chiral or asymmetric species, insight into the secondary structure can be gained. The utility of the HPMA-*b*-(L-Glu) copolymers, according to our synthetic design, lies in the pH-responsive L-glutamic acid block, which upon protonation is expected to self-assemble into an *α*-helix. Ultimately, these *α*-helices should mimic those discussed in the Introduction and disrupt the integrity of lipid membranes. Figure 4 illustrates the pH-dependence of the coil-to-helix transition for each block copolymer as well as for poly-(HPMA$_{220}$). Not surprisingly, poly(HPMA$_{220}$) alone shows no evidence of *α*-helix structure. However, as the pH is reduced, the CD spectra indicate pronounced development of *α*-helices for both poly[HPMA$_{220}$-*b*-(L-Glu$_{56}$)] (Figure 4C) and poly-[HPMA$_{220}$-*b*-(L-Glu$_{78}$)] (Figure 4D); however, poly[HPMA$_{220}$-*b*-(L-Glu$_{33}$)] with the lowest glutamic acid block length (Figure 4B) exhibits a spectrum lacking discernible evidence of helix formation. As the pH is further lowered, the characteristic signal of the copolymers with longer helical L-Glu blocks becomes even more pronounced; eventually, the signal is lost at

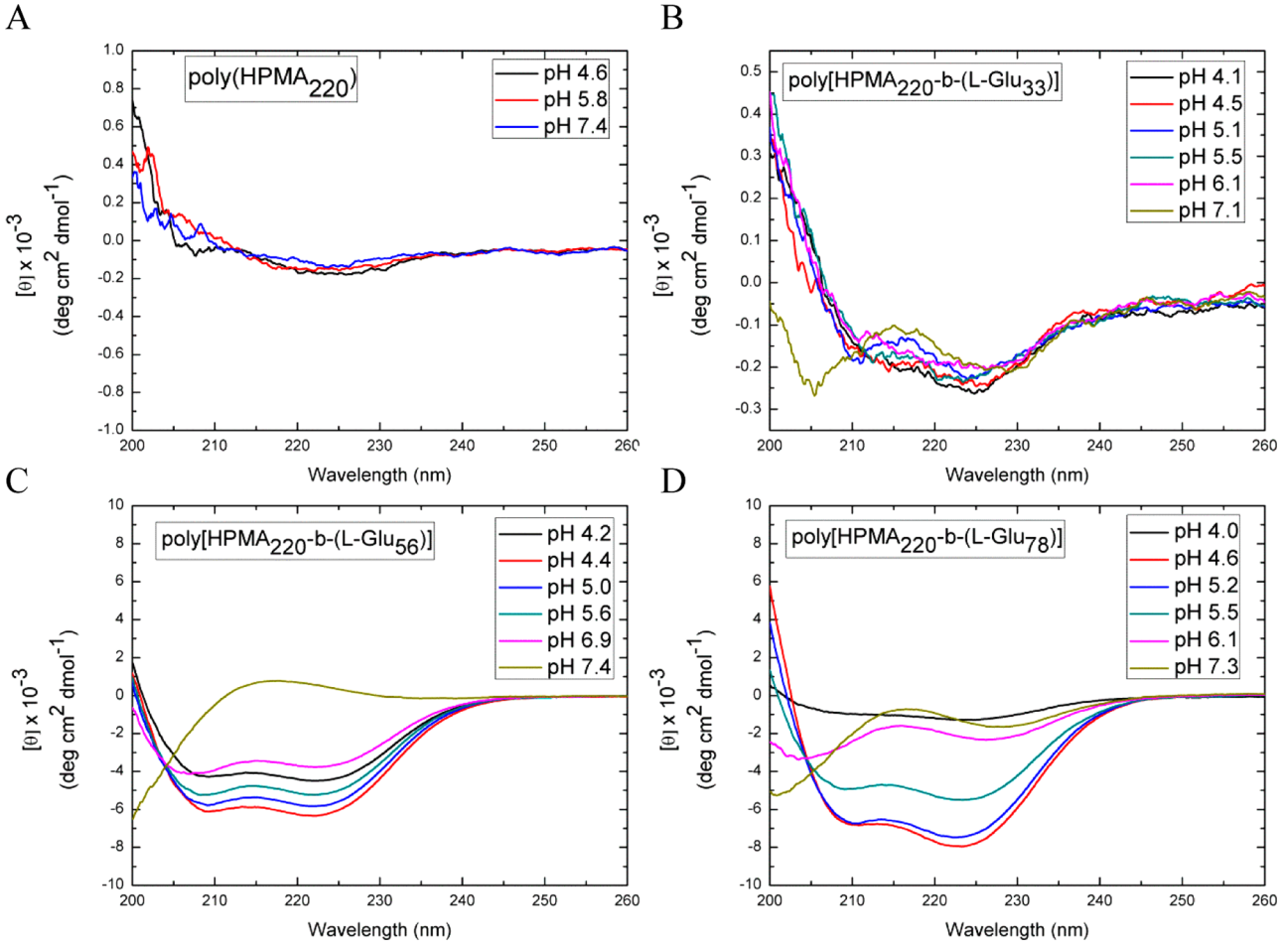

Figure 4. Mean residue ellipticity as a function of pH for (A) poly(HPMA$_{220}$), (B) poly[HPMA$_{220}$-*b*-(L-Glu$_{33}$)], (C) poly[HPMA$_{220}$-*b*-(L-Glu$_{56}$)], and (D) poly[HPMA$_{220}$-*b*-(L-Glu$_{78}$)].

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pH values below 4.5 due to precipitation and onset of flocculation.

Fluorescein Release from POPC Lipid Membranes and Red Blood Cell Hemolysis. Leakage of fluorescent dyes from artificially prepared liposomes is a commonly used assay to elucidate membrane−particle interactions, and it is widely employed in the study of antimicrobial peptides/polymers.[54−58] This technique is well suited for studying membrane disruption at endosomal conditions. By varying the pH, the endolytic activity with respect to α-helical content was investigated using fluorescein loaded 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (POPC) lipids (fPOPC). HPMA-*b*-(L-Glu) (200 μg/mL) copolymers were incubated with fPOPC liposomes for 1 h prior to measurement. Figure 5 shows the extent of fluorescein

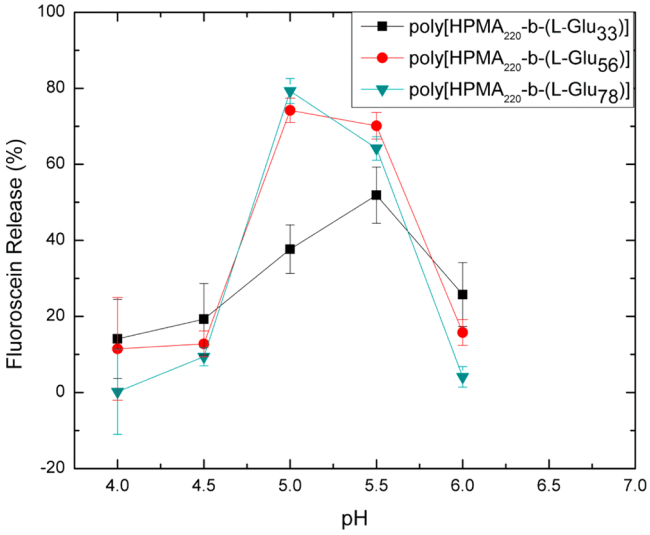

Figure 5. Fluorescein release from POPC liposomes as a function of pH. fPOPC liposomes were incubated 1 h with poly[HPMA$_{220}$-*b*-(L-Glu$_{33}$)] (black), poly[HPMA$_{220}$-*b*-(L-Glu$_{56}$)] (red), and poly[HPMA$_{220}$-*b*-(L-Glu$_{78}$)] (cyan). Triton-X100 was utilized as the positive control. Error bars represent the standard deviation.

release from liposomes as a function of pH for each HPMA-*b*-(L-Glu) copolymer as well as for the HPMA macroinitiator normalized to L-Glu content. No fluorescein release is observed until pH values are progressively lowered below 6 (see Supporting Information). For the block copolymers with L-Glu DP values of 78 and 56, maxima representing 80 and 70% release, respectively, occur at pH 5.0; Only ~30% release was observed for the copolymer with DP 33. These values are consistent with CD data. As the pH drops, L-glutamate units are converted to α-helix forming L-glutamic acid, and the extent of fPOPC membrane leakage is related to helical block content.

The endolytic characteristics of these stimuli-responsive copolymers were also investigated utilizing a red blood cell hemolysis assay at pH 5.5 (see Supporting Information for pH 7.4). Figure 6 shows % hemolysis as a function of block copolymer concentration, normalized for L-Glu units. As expected, poly(HPMA$_{220}$) shows no hemolytic activity, while poly[HPMA$_{220}$-*b*-(L-Glu$_{56}$)] and poly[HPMA$_{220}$-*b*-(L-Glu$_{78}$)] show notable concentration-dependent release profiles, reaching values of nearly 90% after 1 h of incubation. On the other hand, poly[HPMA$_{220}$-*b*-(L-Glu$_{33}$)], which has a much shorter block length, displays substantially lower hemolysis across the concentration range. These results seem to be in agreement

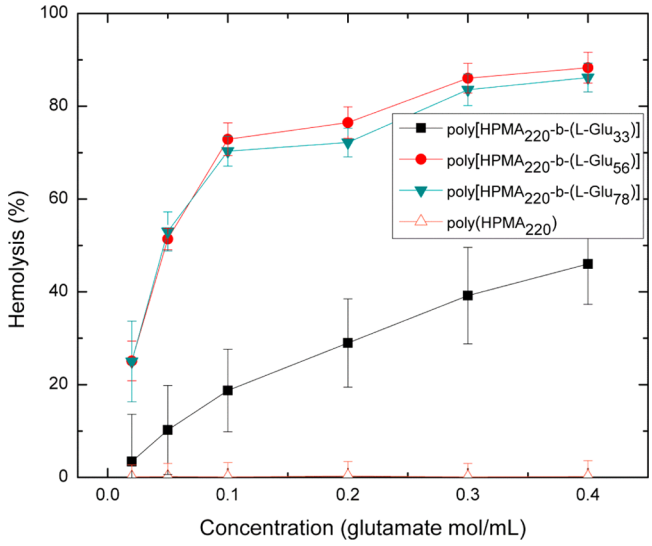

Figure 6. Percent hemolysis as a function of copolymer concentration at pH 5.5. Triton-X100 was utilized as the positive control, and the error bars represent the standard deviation.

with previous studies, indicating a helical block length dependence on membrane destabilization.[25]

CONCLUSIONS

HPMA-*b*-(L-Glu) copolymers with controlled structures, narrow PDIs, and target molecular weights were prepared by sequential aqueous RAFT and ring-opening (NCA) polymerizations followed by postreaction hydrolysis. The block copolymers with tailored L-glutamic acid sequences allow formation of membrane-disruptive helical segments at biorelevant pH values. Red blood cell hemolysis and fPOPC release studies were performed, and at moderate concentrations and sufficient block lengths (α-helical content), pH-dependent hemoglobin, and fluorescein release occurred. It is anticipated that the facile synthetic approach reported here will allow further development of modular drug/gene carriers for the efficient endosomal release of anticancer drugs. While we have shown membrane disruption with these novel HPMA-*b*-(L-Glu) copolymers, future studies will be necessary to evaluate efficiency and mechanistic pathways of drug/gene delivery in vitro.

ASSOCIATED CONTENT

Supporting Information

The determination of APMA functionalization, fluorescein release at pH 7.4, and hemolysis release at 7.4, and the ^{1}H NMR for the deprotection of poly(HPMA-*b*-BGLA) is included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

†This is paper number 154 in a series entitled "Water-Soluble Polymers."

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