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PAPER

The synthetic tuning of clickable pH responsive cationic polypeptides and block copolypeptides†

Amanda C. Engler,[a] Daniel K. Bonner,[ab] Hilda G. Buss,[a] Eva Y. Cheung[a] and Paula T. Hammond*[a]

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A series of pH responsive synthetic polypeptides has been developed based on an *N*-carboxyanhydride ring opening polymerization combined with a facile and versatile click chemistry. Poly(γ-propargyl L-glutamate) (PPLG) homopolymers and poly(ethylene glycol-*b*-γ-propargyl L-glutamate) (PEG-*b*-PPLG) block copolymers were substituted with various amine moieties that range in pK_a and hydrophobicity, providing the basis for a library of new synthetic structures that can be tuned for specific interactions and responsive behaviors. These amine-functionalized polypeptides have the ability to change solubility, or reversibly self-assemble into micelles with changes in the degree of ionization; they also adopt an α-helical structure at biologically relevant pHs. Here we characterize the pH responsive behavior of the new polypeptides and the hydrolysis of the ester containing amine side chains. We examine the reversible micellization with block copolymers of the polypeptides and nucleic acid encapsulation that demonstrate the potential use of these materials for systemic drug and gene delivery.

Introduction

Synthetic polypeptides have received attention because of their unique structural properties and biocompatibility.[1–4] Like their naturally occurring analogs, these molecules have a poly(amino acid) backbone and possess the ability to fold into stable secondary structures. Helical structures, in particular, allow for proteins to optimally display surface moieties that dictate cell signaling and molecular docking.[5] This property gives synthetic polypeptides an advantage over most traditional polymers that can only adopt a random coil structure. A considerable amount of research has been performed on synthetic polypeptides to better understand the complex features of proteins and to gain insight into their secondary structures.[6–10] Synthetic polypeptides can be synthesized on a large scale by the ring opening polymerization (ROP) of *N*-carboxyanhydrides (NCA) formed from naturally occurring amino acids. These simple homopolypeptides are able to arrange into or change their secondary structure based on solution conditions.[7–10] Although these macromolecules' secondary structure can be controlled to some extent, we are limited by the given side chain, which dictates polymer function, structure, and responsive behavior to temperature or pH among many other properties.

Recently, we reported a new approach to the manipulation of synthetic polypeptide composition and function through the introduction of a new NCA polymer, poly(γ-propargyl L-glutamate) (PPLG),[11] which contains a pendant alkyne group that can be reacted with an azide by the alkyne–azide cycloaddition click reaction.[11,12] This synthetic strategy allows for the convenient and efficient functionalization of a polypeptide without the need for protection and deprotection steps. To demonstrate the efficiency of polymer modification, we used a model PPLG-*g*-PEG system, for which we were able to attain a "grafting onto" efficiency of over 96%.[11] Since our initial report, other research groups have extended this platform methodology of combining NCA polymerization and click chemistry side chain modification. Chen *et al.* used PPLG to click on several different azide functionalized monosaccharides to form glycopolypeptides.[13] Tang and Zhang reported the synthesis of poly(γ-azidopropyl-L-glutamate), which was functionalized with alkyne containing mannose moieties *via* the alkyne–azide cycloaddition click reaction.[14] Sun and Schlaad developed thiol–ene clickable polypeptides, where they synthesized poly(D,L-allylglycine) and clicked on thiol functionalized sugars.[15] Huang *et al.* synthesized poly(D,L-propargylglycine) and clicked on azide containing protected galactose, using alkyne–azide cycloaddition click chemistry.[16]

By employing our PPLG platform for the click chemistry of amino-functional groups, we have developed several new pH responsive macromolecules. A unique aspect of these new amine-functionalized polypeptides is the ability to buffer and in some cases, undergo a solubility phase transition with degree of ionization, while adopting an α-helical structure over biologically relevant pHs. These polymers include both poly(γ-propargyl L-glutamate)

[a]*Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA, USA. E-mail: hammond@mit.edu; Fax: +1 617-258-8991; Tel: +1 617-253-4562*
[b]*Division of Health Science and Technology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA, USA*

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(PPLG) based homopolymers and poly(ethylene glycol-*b*-γ-propargyl L-glutamate) (PEG-*b*-PPLG) block copolymers substituted with various amine moieties that range in pK_a and hydrophobicity, providing the basis for a library of new synthetic structures that can be tuned for specific interactions and responsive behaviors.

The new PPLG based cationic polypeptides have the potential to be used for many different applications. Polypeptides have been investigated as smart molecules in lipid membranes,[17–19] liquid crystals used in optical storage and display devices,[4] vehicles for drug and gene delivery,[3,20–29] anti-fouling coatings,[30] components for tissue engineering and biosensors, and synthetic mimics of naturally occurring molecules.[1,3,31–34] We have characterized the pH responsive behavior of the new polypeptides, the pH-dependent hydrolysis rate of the ester containing amine side chains, and have performed preliminary experiments that demonstrate the potential use of these new materials for systemic drug and gene delivery. More specifically, for pH responsive drug delivery, one could design a micellar system that forms stable micelle drug carriers in the blood stream and normal tissue at extracellular conditions (pH 7.00–7.45)[35] but destabilizes in the endosome (early endosome pH 5.5–6.3 and late endosome pH < 5.5)[36] or in hypoxic regions of tumors (pH approaching 6.0),[35] to release the drug. To achieve this behavior, a pH responsive polypeptide is needed that is fully soluble at endosomal or tumor pH and insoluble at extracellular pH. We have determined the solubility behavior of the amine functionalized PPLG and the self-assembly behavior of the amine functionalized PEG-*b*-PPLG as a function of pH. For gene delivery, it is critical that the polymers complex with siRNA or DNA to form protective polymer–gene complexes (polyplexes); these polyplexes must escape the endosomal compartments into which they are initially trafficked upon internalization.[37,38] One such mode of endosomal escape is through the so-called "proton sponge effect", in which the basic polymer buffers the endosome during acidification, leading to osmotic swelling and rupture.[37,39] The buffering capacity of the new polypeptides has been explored using titrations to determine the pH range at which these polymers buffer. In addition, siRNA complexation studies have been performed to determine if these polymers complex with siRNA to form polyplexes.

A unique aspect of these new polypeptides is that there is an ester linkage between the amine and the polymer backbone. These ester side chains can be hydrolyzed, leaving behind a carboxylic acid moiety, thus creating a charge shifting polymer (shifting with hydrolysis from positive to negative net charge). We have examined the rate of hydrolysis of the ester side chain at various pH conditions and the role of the shift in overall polymer charge plays on disrupting the secondary structure. This hydrolysis and overall shift in charge from positive to negative could play a role in improving the safety and biodegradability of these substituted poly(γ-glutamic acid) based polymers,[40–42] and may also aid in the delivery and subsequent unpackaging and release of nucleic acid based cargos that are delivered using these systems.

Experimental

Materials

L-(+)-Glutamic acid, 99% minimum, was purchased from EMD Chemicals. 3-Dimethylaminopropylchloride hydrochloride, 99%, was purchased from Arcos Organics. Sunbright® amine terminated poly(ethylene glycol) was purchased from NOF Corporation. siRNA was purchased from Dharmacon RNAi Technologies and QuantiT Ribogreen RNA Reagent was purchased from Invitrogen. All other chemicals were purchased from Sigma Aldrich. All materials were used as received.

Methods

^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker 400 MHz FT-NMR spectrophotometer. Gel permeation chromatography measurements were carried out using a Waters Breeze 1525 HPLC system equipped with two Polypore columns operated at 75 °C, a series 2414 refractive index detector, a series 1525 binary HPLC pump, and a 717 plus autosampler. Waters' Breeze Chromatography Software Version 3.30 was used for data collection as well as data processing. DMF with 0.01 M LiBr was the eluent for analysis, and samples were dissolved at 4–6 mg mL^{-1} in DMF. The average molecular weight of the sample was calibrated against narrow molecular weight poly(methyl methacrylate) standards.

Acid–base titrations were performed on all amine functionalized PPLG. 3 mL of 10 mM amine in 125 mM NaCl was adjusted to a pH of 3 using 1 M HCl. The solution was titrated with 10 μL aliquots of 0.1 M NaOH, measuring the pH with each addition. For polymers where precipitation was observed, UV/Vis measurements were obtained at 600 nm to monitor the solution turbidity. UV/Visible measurements were carried out on an Agilent Technologies G3172A spectrometer.

Critical micelle concentration measurements of the diblock polymers in aqueous solutions at different pH values were performed by fluorescence spectroscopy using a pyrene probe. Fluorescence peak intensity emission ratios (373 nm/384 nm) were plotted against the logarithm of polymer concentrations to determine CMC as the onset of micellization.[43] Fluorescence spectroscopy was carried out on a Horiba FluoroLog®-3 spectrofluorometer at 25 °C. Stock solutions of pyrene at 5×10^{-7} M in water or pH buffer were prepared. Polymer samples were dissolved in the stock pyrene solution and diluted to specific concentrations.

Tapping-mode atomic force microscopy (AFM) measurements were conducted in air with a Dimension 3100 system (Digital Instruments, Santa Barbara, CA) operated under ambient conditions. The samples were prepared for AFM analysis by spin coating a silicon wafer with a polymer solution at a concentration of 1 mg mL^{-1} in Milli-Q water with the pH adjusted using 0.1 M NaOH.

Circular dichroism (CD) spectroscopy of polymer solutions was carried out using an Aviv model 202 CD spectrometer. Measurements were performed at 25 ± 0.1 °C, sampling ever nm with a 3–5 s average time over the range of 195–260 nm (bandwidth = 1.0 nm). Measurements were taken using a cell with a 1 mm path length. Samples were prepared at a concentration of 0.5–1 mg mL^{-1} in either buffer solutions or Milli-Q water with pH adjusted using 0.1 M NaOH and 0.1 M HCl solutions.

Ester hydrolysis samples were prepared by dissolving polymer in a stock solution at 10 mg mL^{-1} for homopolymer and 20 mg mL^{-1} for diblock copolymer. The stock solutions were then diluted with pH buffer to a concentration of 0.5 mg mL^{-1} for homopolymer and 1 mg mL^{-1} for diblock copolymer. At various

time points, samples were analyzed by CD. Samples were also freeze dried, reconcentrated in D_2O to 2.5 mg mL^{-1} for homopolymers and 3.75 mg mL^{-1} for diblock copolymers, acidified with trifluoroacetic acid to stop hydrolysis, and analyzed by ^{1}H-NMR.

Ribogreen assays (QuantiT Ribogreen RNA Quantification Reagent, Invitrogen) were performed to determine the complexation efficiency of the polymers with siRNA. Ribogreen is a cyanine dye that is nearly non-fluorescent when unbound to RNA, but exhibits a >1000 fold enhancement in fluorescence when bound to RNA. When siRNA is complexed (*e.g.* by a polymer), it is unavailable to bind to Ribogreen and thus the fluorescence signal decreases relative to uncomplexed siRNA.[44] 25 μL of siRNA at 0.006 mg mL^{-1} was aliquoted into wells of a 96 well plate and the appropriate amount of polymer was added to attain the desired polymer : siRNA ratio (*N*/*P*) in a total volume of 50 μL. After allowing 10 minutes for complexation, 20 μL of the complex solution was added to a black, flat-bottomed, polypropylene 96-well plate containing 100 μL of Ribogreen (diluted 1 : 200 in water per manufacturer instructions). The fluorescence of each well was measured on a Perkin Elmer Plate 1420 Multilabel plate reader and the fraction of uncomplexed siRNA was determined by comparing the fluorescence of the polymer complexes with the fluorescence of a free siRNA control. For the heparin destabilization titrations, heparin (167 IU mg^{-1}) was dissolved in a stock solution at 0.5 IU mL^{-1} and added to polyplex/ribogreen solutions.

Synthesis of γ-propargyl L-glutamate hydrochloride. L-Glutamic acid (15 g, 102 mmol) was suspended in propargyl alcohol (550 mL) under argon. Chlorotrimethylsilane (28.5 mL, 224 mmol) was added dropwise to the suspension over 1 hour. The resulting solution was stirred at room temperature for two days until there was no undissolved L-glutamic acid. The reaction solution was precipitated into diethyl ether giving a white solid. The crude product was removed by filtration, dissolved in boiling isopropanol, and precipitated into diethyl ether. The product was filtered, washed with diethyl ether, and dried under vacuum to yield 19.13 g (84.5%). ^{1}H-NMR (400 MHz, D_2O) δ = 2.20 (m, 2H, CH_2), 2.63 (dt, 2H, CH–CO), 2.86 (t, 1H, C≡CH), 4.05 (t, 1H, CH), 4.69 (d, 2H, CH_2CO).

Synthesis of *N*-carboxyanhydride of γ-propargyl L-glutamate (PLG–NCA). γ-Propargyl L-glutamate hydrochloride (6 g, 27 mmol) was suspended in dry ethyl acetate (190 mL). The solution was heated to reflux and triphosgene (2.67 g, 9 mmol) was added. The reaction solution was refluxed for 6 hours under nitrogen. The reaction solution was cooled to room temperature and any unreacted γ-propargyl L-glutamate hydrochloride was removed by filtration. The reaction solution was then cooled to 5 °C and washed with 190 mL of water, 190 mL of saturated sodium bicarbonate, and 190 mL of brine all at 5 °C. The solution was then dried with magnesium sulfate, filtered, and concentrated down to viscous oil (4.53 g, 79.2% yield). ^{1}H-NMR (400 MHz, $CDCl_3$) δ = 2.20 (dm, 2H, CH_2), 2.49 (t, 1H, C≡CH), 2.58 (t, 2H, CH–CO), 4.39 (t, 1H, CH), 4.68 (d, 2H, CH_2CO), 6.5 (s, 1H, NH).

Synthesis of poly(γ-propargyl L-glutamate) initiated by heptylamine. A typical procedure for the polymerization is as follows. To a flame dried Schlenk flask, heptylamine (14.5 μL, 0.0980 mmol) and DMF (8 mL) were combined under Ar. In a separate vial, PLG–NCA (1.552, 7.35 mmol) was dissolved in dimethylformamide (DMF) (8 mL) and added to the reaction flask. The reaction mixture was stirred for three days at room temperature. The polymer was precipitated into diethyl ether and removed by centrifugation (0.823 g, 67.0% recovered, by ^{1}H-NMR n = 75, by DMF GPC M_w = 14 100, PDI = 1.09). ^{1}H-NMR (400 MHz, [D_6] DMF) δ = 2.28 (br m, 2H, CH_2), 2.55 (br m, 2H, CH–CO), 3.38 (br m, 1H, C≡CH), 4.09 (br m, 1H, CH), 4.76 (br m, 2H, CH_2CO), 8.5 (br m, 1H, NH). COSY ^{1}H-NMR shown in the ESI†.

Synthesis of poly(ethylene glycol)-*b*-poly(γ-propargyl L-glutamate). A typical procedure for the polymerization is as follows. A round bottom flask was rinsed with acetone and oven dried. In a glove box, PEG–NH_2 (0.900 g, 0.180 mmol) was dissolved in DMF (9 mL) in a round bottom flask. PLG–NCA (0.950 g, 4.50 mmol) was dissolved in dry DMF (9 mL) added to the reaction flask. The reaction mixture was stirred for three days at room temperature. The reaction solution was rotovaped and dried under high vacuum to remove the DMF. To remove any residual PLG–NCA and DMF, the polymer was redissolved in dichloromethane precipitated into diethyl ether and removed by centrifugation (1.45 g, 87.9% recovered, by ^{1}H-NMR n = 23, by GPC PDI = 1.09). ^{1}H-NMR (400 MHz, [D_6] DMF) δ = 2.28 (dm, 2H, CH_2 PPLG), 2.55 (dm, 2H, CH–CO PPLG), 3.38 (m, 1H, C≡CH PPLG), 3.59 (s, 4H, CH_2CH_2 PEG), 4.09 (m, 1H, CH PPLG), 4.76 (m, 2H, CH_2CO PPLG), 8.5 (m, 1H, NH PPLG).

Synthesis of 2-bromo-*N*-methylethanamine hydrobromide. 2-Bromo-*N*-methylethanamine hydrobromide was synthesized following the protocol presented by Schutte *et al.*[45] Briefly, in a round bottom flask, 48% w/w HBr (30 mL) was cooled in an ice bath to 4 °C and 2-(methylamino)ethanol (10 mL, 125 mmol) was added dropwise. H_2O and HBr were distilled off and the crude product solution was cooled to 60 °C. The solution was slowly added to a solution of cold acetone, where it precipitated out to form a white solid. The precipitant was removed, washed with cold acetone, and dried under high vacuum (16.46 g, 60.4% yield). ^{1}H-NMR δ (400 MHz, D_2O) 3.69 (t, 2H, $BrCH_2$), 3.50 (t, 2H, CH_2N), 2.75 (s, 3H, CH_3).

General synthesis of amino azides. *Organic azides can be EXPLOSIVE! A guide to safe handling and storage of organic azides can be found in "Click Chemistry: Diverse chemical function from a few good reactions" by Kolb et al.*[12] The shorthand notation for each side group used in this article is provided in parentheses after the specific chemical name. Amino azides were synthesized using the protocol presented by Carboni *et al.*[46] A representative example, 3-dimethylamino-1-propylchloride hydrochloride (10 g, 63 mmol) and sodium azide (8.22 g, 126 mmol) were dissolved in water (1 mL $mmol^{-1}$) and heated at 75 °C for 15 h. The reaction mixture was cooled in an ice bath and NaOH (4 g) was added. The solution phase separated and the organic phase was removed. The aqueous phase was extracted with diethyl ether twice. The organic layers were combined, dried with $MgSO_4$, and concentrated down to an oil (6.60 g, 80.8%

yield). 3-Azido-*N*,*N*-dimethylpropan-1-amine (dimethylpropanamine) ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.30 (t, 2H, N_3CH_2), 2.30 (t, 2H, CH_2N), 2.17 (s, 6H, $N(CH_3)_2$), 1.71 (m, 2H, $N_3CH_2CH_2$). 2-Azidoethanamine (primary amine) ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.32 (t, 2H, N_3CH_2), 2.83 (t, 2H, CH_2NH_2), 1.45 (s, 2H, NH_2). 2-Azido-*N*-methylethanamine (secondary amine) ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.45 (t, 2H, N_3CH_2), 2.72 (t, 2H, CH_2NH), 2.39 (s, 3H, CH_3), 1.28 (s, 1H, NH). 2-Azido-*N*,*N*-dimethylethanamine (dimethylethanamine) ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.32 (t, 2H, N_3CH_2), 2.47 (t, 2H, CH_2N), 2.24 (s, 6H, $N(CH_3)_2$). 2-Azido-*N*,*N*-diethylethanamine (diethylamine) ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.25 (t, 2H, N_3CH_2), 2.62 (t, 2H, CH_2CH_2N), 2.52 (q, 2.54, $N(CH_2CH_3)_2$), 1.00 (s, 6H, $(CH_2CH_3)_2$). *N*-(2-Azidoethyl)-*N*-isopropylpropan-2-amine (diisopropylamine) ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 3.01 (t, 2H, N_3CH_2), 2.98 (m, 2H, $N(CH(CH_3)_2)_2$), 2.62 (t, 2.54, CH_2N), 0.99 (d, 12H, $(CH_2(CH_3)_2)_2$).

General synthesis of substituted PPLG. A typical procedure started with a feed ratio of alkyne/azide/CuBr/PMDETA equal to 1/1.2/0.1/0.1. The PPLG (0.0750 g, 0.45 mmol alkyne repeat units), amino azide (0.069 g, 0.54 mmol 3-azido-*N*,*N*-dimethylpropan-1-amine), and PMDETA (9.4 μL, 0.045 mmol) were all dissolved in DMF (3 mL). The solution was degassed by bubbling argon through the solution for 20 minutes. CuBr catalyst (0.0064 g, 0.045 mmol) was added, and the reaction solution was stirred at room temperature, under argon. Once the reaction was complete, the reaction solution was purified by dialysis against water acidified by HCl (pH < 4) for 2–3 days, followed by dialysis with Milli-Q water to remove acid before freeze drying. The final polymer was a white solid. For ^{1}H-NMR δ (400 MHz, D_2O) see the ESI†.

Results and discussion

Polymer synthesis

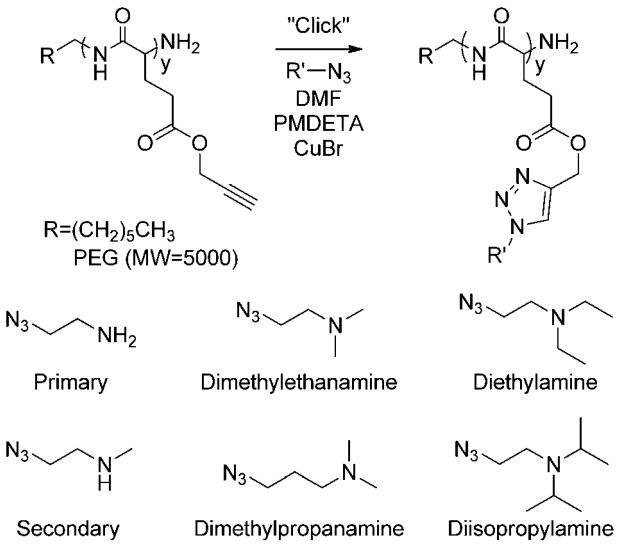
The PPLG polymers were prepared as previously described.[11] Briefly, γ-propargyl L-glutamate was reacted with triphosgene to form the NCA. PPLG and PEG-*b*-PPLG were prepared by ring opening polymerization in dimethylformamide (DMF) at room temperature by initiation with heptylamine and PEG–NH_2 (M_w = 5000), respectively. Table 1 summarizes the stoichiometric feed ratio of each polymerization, the degree of polymerization characterized by ^{1}H-NMR, and the molecular weight and molecular weight distribution characterized by gel permeation chromatography (GPC) with DMF with 0.01 M LiBr as the carrier solvent. The narrow polydispersities (1.09–1.25) and reaction feed ratio compared to the degree of polymerization by ^{1}H-NMR indicate that the polymerization is well controlled. Furthermore, this polymerization route allows for high molecular weight polymers with a degree of polymerization as high as 140. As indicated by Poché *et al.*, a high degree of polymerization can be obtained if the NCA monomer purity is high; the washing strategy employed in this NCA monomer preparation does significantly improve the monomer purity by removing residual HCl.[47]

Six different amine moieties ranging in pK_a (1°, 2°, and 3° amines) and hydrophobicity (dimethylethanamine, dimethylpropanamine, diethylamine, and diisopropylamine) were attached to four different molecular weight PPLG backbones and a PEG-*b*-PPLG diblock copolymer through the copper-mediated 1,3 cycloaddition between the alkynes on the PPLG backbone and the azide bearing amine groups, shown in Scheme 1. The labels under each side group are used to refer to each polymer bearing that side group. The specific chemical name for each side group can be found in the Experimental section. The PPLG was coupled with azido amines using CuBr/PMDETA as a catalyst in DMF with a molar ratio of alkyne/azide/CuBr/PMDETA equal to 1/1.2/0.1/0.1. After the reaction was complete, the polymer was purified by dialysis against water acidified with HCl (pH ≤ 4) to remove any unreacted amino azides and the copper catalyst.

Table 1 Summary of polymerization feed NCA-monomer/initiator, degree of polymerization by ^{1}H-NMR, molecular weight and polydispersity determined by DMF GPC with PMMA standards

Polymer	Feed ratio	DP by NMR	DMF GPC with PMMA standards		
			M_n	M_w	PDI
PPLG	25	30	6100	7600	1.25
PPLG	50	56	12 700	14 100	1.11
PPLG	75	75	17 900	19 400	1.09
PPLG	150	140	42 900	50 000	1.17
PEG–NH_2	—	—	10 000	11 500	1.14
PEG-*b*-PPLG	25	23	14 600	15 800	1.08

The polymer structures were confirmed using ^{1}H-NMR. Representative ^{1}H-NMR spectra of the diethylamine and diisopropylamine substituted PPLG compared to the ^{1}H-NMR spectra of PPLG are shown in Fig. 1. For all amine groups, the coupling efficiency was near quantitative, as indicated by the disappearance of the PPLG alkyne peak (a, 3.4 ppm) and ester peak (b, 4.7 ppm) and the appearance of a new ester peak (k, 5.2 ppm) and the triazole ring peak (m, 8.15 ppm). Furthermore, the peak integration for all samples tested was as expected for near quantitative substitution without hydrolysis of the ester group on



Scheme 1 Functionalization of PPLG by the alkyne–azide cycloaddition click reaction and the pH responsive side groups.

the polymer side chains. Representative ^{1}H-NMR for the remaining amine functionalized PPLG and PEG-*b*-PPLG can be found in the ESI†.

Investigation of polymer buffering and solubility

To investigate pH responsiveness and the buffering behavior of these polypeptide systems, titrations were performed on all polymers. Polymers were dissolved in 125 mM NaCl at 10 mM polypeptide–amine (molarity based on repeat unit), titrated with increasing pH to a pH of 10–10.5 using 0.1 M NaOH, and subsequently titrated with decreasing pH using 0.1 M HCl. After titrations were complete, representative samples were freeze dried, dissolved in D_2O, and analyzed using ^{1}H-NMR. From the ^{1}H-NMR, the spectra were nearly identical to those obtained before titrations, indicating that hydrolysis did not occur during the 2–3 hour titration process (see ESI†). Representative titrations with increasing pH are shown in Fig. 2, where Fig. 2A consists of the dimethylethanamine polymers at varying degrees of polymerization, and Fig. 2B consists of titrations of each polymer side functional group with a degree of polymerization of NCA backbone of 75 (titrations of additional polymers can be found in the ESI†). All polymers appear to have strong buffering capacity in the pH range of 5–7.35, which scales with the pH range of typical extracellular tissue to late endosomal pH.[35,36] The diisopropylamine polypeptide exhibits the sharpest buffering transition at pH 5.25; the diethylamine polymer also buffers in this range, but with a broader transition that has a midpoint at the slightly higher pH of approximately 6.5. The primary and secondary amine functional polymers interestingly exhibit similar broad buffering behavior beginning at pH 5.5 with a midpoint at 7.25. One would typically expect buffering at higher pH for primary and secondary amines (pK_a approximately 9–11),[48,49] although some buffering is observed in these polymers from pH 8 to 10. Polyelectrolytes typically exhibit broad buffering behavior and shifted pK_a values due to segmental charge repulsion. For the dimethyl substituted amines, the dimethylethanamine exhibits buffering behavior starting at the same pH as the primary and secondary amines with a midpoint falling between 6.5 and 7.0. These values are consistent with the series of polymers with ethylene linker groups to the tetrazole ring; whereas, the dimethylpropanamine polymer exhibits buffering at higher pHs. The additional carbon between the amine group and the triazole ring results in a higher pK_a for the dimethylpropanamine. This shift in pK_a could be the result of the amine group being further removed from the electron withdrawing triazole ring or from the decreased crowding experienced by the amine group. All of the polymers exhibit a small amount of buffering at the start of the titration curve, at pH 3–4;

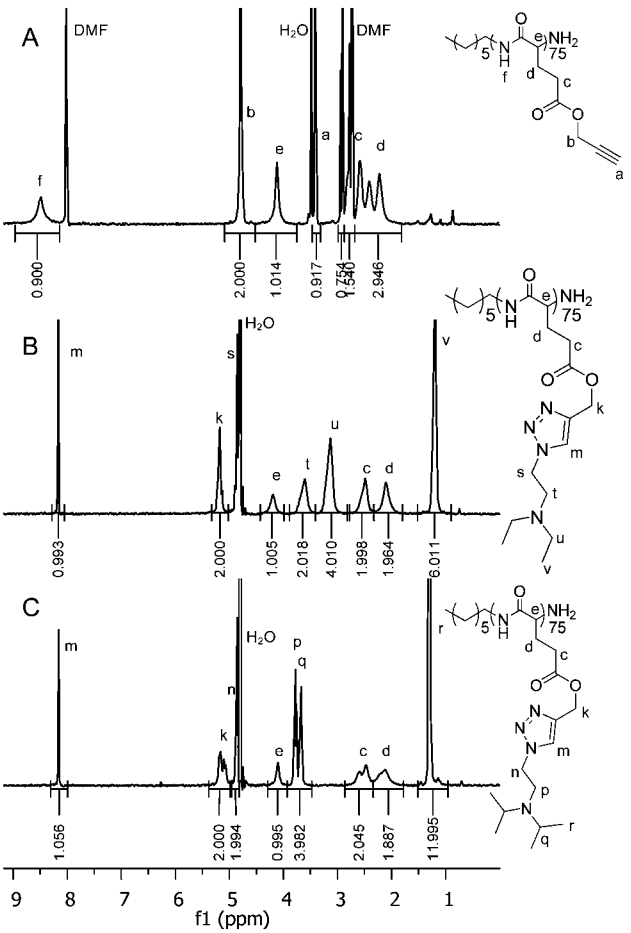


Fig. 1 (A) PPLG in d_7 DMF, (B) PPLG functionalized with diethylamine in D_2O, and (C) ^{1}H-NMR of PPLG functionalized with diethylamine in D_2O. The PPLG backbone has a degree of polymerization of 75.

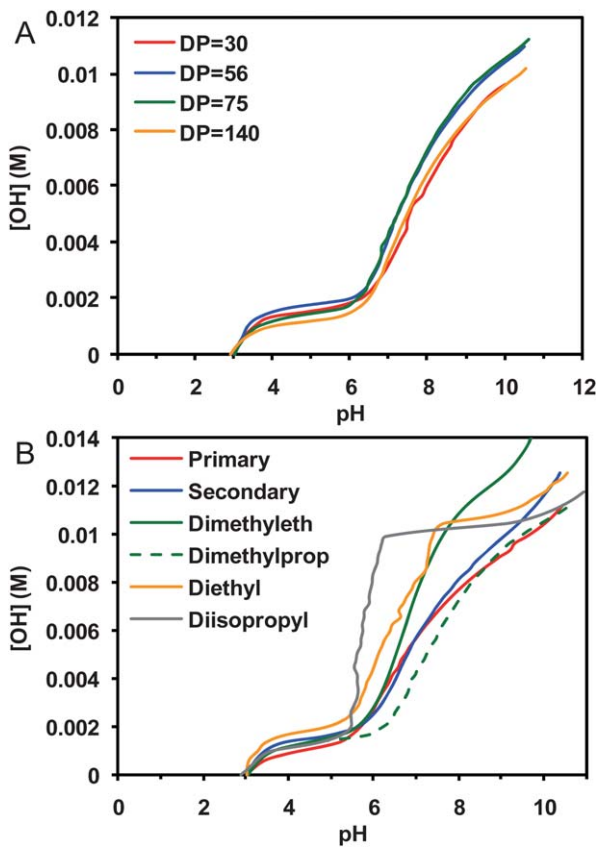


Fig. 2 Titrations of polymers at a concentration of 10 mM using 0.1 M sodium hydroxide. (A) PPLG polymers functionalized with dimethylethanamine with varying degrees of polymerization, and (B) PPLG polymers with a degree of polymerization of 75.

the buffering in this region could be a result of the triazole ring generated during the click reaction; triazoles exhibit pK_a's of less than 3.0.[48,50] The polymer buffering appears to have little dependence on polymer molecular weight, as indicated in Fig. 2A.

The primary, secondary, and dimethyl polypeptides remain water soluble over the entire pH range; however, as the cationic diethylamine and diisopropylamine functionalized polypeptides are titrated from acidic to basic conditions, the amines become deprotonated. The resulting uncharged polypeptide is no longer soluble in water, leading to precipitation of the polypeptide from aqueous solution. For the diethylamine and diisopropylamine functionalized PPLG, polymer precipitation was observed at various pH values depending on polymer molecular weight. To determine the pH where precipitation occurs, turbidity measurements were performed on the diethylamine and diisopropylamine functionalized PPLGs by monitoring polymer solution transmission at 550 nm, shown in Fig. 3. When the polymers begin to precipitate out of solution, there is a sharp drop in transmission. For the diethylamine functionalized PPLG (Fig. 3, solid lines), precipitation occurred between 6.80 and 7.45 depending on the degree of polymerization and for the diisopropylamine functionalized PPLG (Fig. 3, dashed lines), precipitation occurred between 5.23 and 5.59. These values are consistent with the titration data shown in Fig. 2 and in the ESI†. In general we see the anticipated trend that increased molecular weight leads to precipitation at lower pH values and higher degrees of ionization of the polymer functional group. It is notable that the diethylamine series is more sensitive to molecular weight than the diisopropylamine series, which seems to approach a limiting minimum pH value for precipitation. This result may be due to the greater hydrophobicity of the diisopropylamine group as opposed to the diethylamine, which would lead to a lower degree of solubility of the amine side chain and a decreased dependence on molecular weight.

The pH transition observed for both the diisopropylamine and diethylamine functionalized polymers can be utilized for the design of a pH responsive drug carrier in which the responsive PPLG block would be the interior, pH responsive block of a micellar system. To determine if the precipitation pH could be tuned, a 50 : 50 mixture of diethylamine and diisopropylamine side groups was attached to PPLG (DP = 140) to generate a random copolymer. As shown in Fig. 3 (dotted-gray line), the copolymer precipitation pH falls between the precipitation pH values observed for the diethylamine and diisopropylamine substituted PPLG (DP = 140), indicating that the pH responsiveness of the amine substitute PPLG block can be fine tuned by changing the ratio of side groups. One could also envision using this strategy to incorporate side groups that will improve the loading of a specific drug or increase polymer–gene complexation efficiency. The buffering and the precipitation behavior was found to be fully reversible, as indicated by reverse titrations that were performed on all polymers (reverse titrations can be found in the ESI†). For the completely water soluble primary, secondary, and dimethyl polymers, the reverse titration curve has the same shape as the original titration with no signs of hysteresis. For tertiary amine polymers that precipitated out of solution, hysteresis was often observed for the larger degrees of polymerization, such that the pH value for which the polymers re-dissolved was often lower than the value observed for precipitation. For the shortest degree of polymerization (DP = 30), the polymers returned to solution at nearly the same pH as when the precipitation was initially observed (Fig. 4).

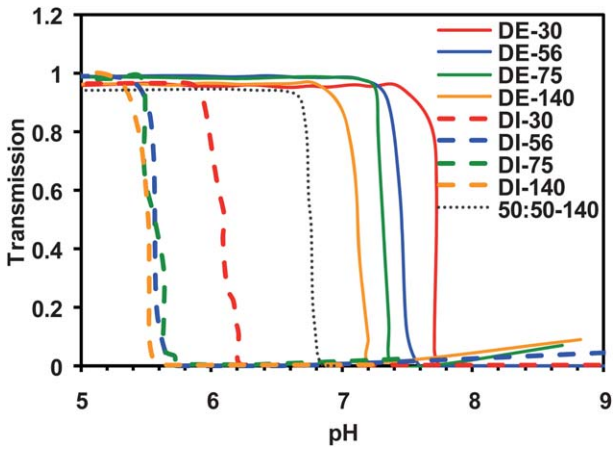


Fig. 3 Transmission as a function of pH for all diethylamine and diisopropylamine functionalized polymers. Diethylamine is abbreviated DE and diisopropylamine is abbreviated DI.

Functionalized PEG-*b*-PPLG self-assembly

The self-assembly of PEG-*b*-PPLG functionalized with diethylamine and diisopropylamine was studied as a function of pH. The critical micelle concentration (CMC) was determined for PEG-*b*-PPLG in water and amine functionalized PEG-*b*-PPLG in buffer solutions at pH 9 and pH 5.5. The CMC was determined by fluorometry using a pyrene probe. A representative example of the diisopropylamine functionalized PEG-*b*-PPLG is shown in Fig. 5 (information for diethylamine substituted PEG-*b*-PPLG can be found in the ESI†). As shown in Fig. 5A, there is a clear break in the emission ratio indicating a CMC for the amine functionalized PEG-*b*-PPLG in pH 9 buffer. In pH 5.5 buffer, no break in emission ratio was observed for the functionalized polymer, indicating that these macromolecules do not self-assemble at all at this acidic pH, but remain completely

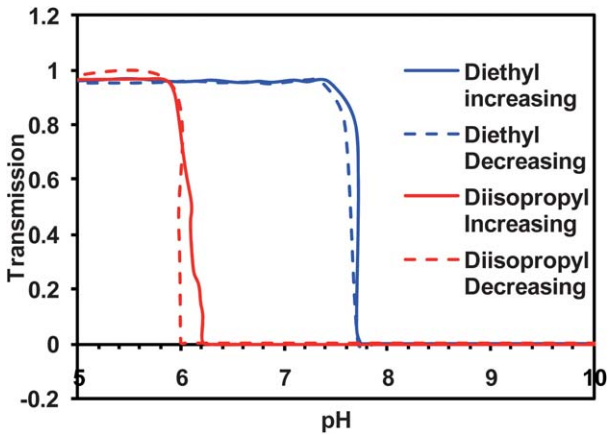


Fig. 4 Transmission as a function of increasing and decreasing pH for diethylamine and diisopropylamine with DP = 30.

soluble in water. The observed CMC values for all diblock polymers tested (Table 2) are of the same order of magnitude of PEG-*b*-PBLA[21] and are several orders of magnitude lower than Pluronic micelle CMC values.[51] To further verify that the self-assembled structures were micelles, AFM was performed on diethylamine and diisopropylamine substituted PEG-*b*-PPLG cast from a water solution adjusted with 0.1 M NaOH to pH ≈ 9. Spherical micelles were observed for the amine substituted PEG-*b*-PPLG; Fig. 5B shows an AFM image of the diisopropylamine functionalized diblock copolymer. The micelles are thus able to form at moderate to high pH, but become completely destabilized at low pH, making them of interest for drug release in which a pH triggered rapid disassembly of drug carrier can be designed to take place within acidic compartments to release a drug.

Secondary structure

Circular dichroism (CD) was used to probe the secondary structure of the various polymers as a function of pH. Polymer dissolved at 1 mg mL^{-1} was brought down to a pH of 3, titrated to a pH higher than 10, and then immediately titrated back to a pH of 3. A sample CD titration of a secondary amine polypeptide with DP = 75 is shown in Fig. 6. When initially brought down to a pH of 3, the sample adopts a mixture of α-helix and random coil conformations, as indicated by the minimum at 222 nm, which is characteristic of an α-helix and the second, more negative minimum at 204 nm, which is indicative of a combination of α-helix and random coil. As the sample pH is increased, the sample adopts an all α-helical structure at high pH values (pH > 6.36), as indicated by the minima at 208 nm and 222 nm.[52] When the pH is decreased stepwise back down to acidic pH, this α-helical structure transitions back to a mixture of α-helix and random coil. The α-helix to random coil transition correlates well with the pK_a observed in the polymer titrations. In summary, the α-helix structure appears to correlate with the uncharged polymer backbone; as the backbone becomes charged, the helical structure becomes reversibly disrupted and exhibits some random coil structure.

Table 2 CMC values for PEG-*b*-PPLG in water and amine functionalized PEG-*b*-PPLG in pH 5 and pH 9 buffer

	Solvent	CMC/mg mL^{-1}	CMC/M
PEG-*b*-PPLG	MQ water	3.75×10^{-4}	3.74×10^{-8}
Diethylamine	pH 9 buffer	1.11×10^{-2}	7.49×10^{-7}
	pH 5.5 buffer	—	—
Diisopropylamine	pH 9 buffer	1.05×10^{-3}	7.38×10^{-8}
	pH 5.5 buffer	—	—

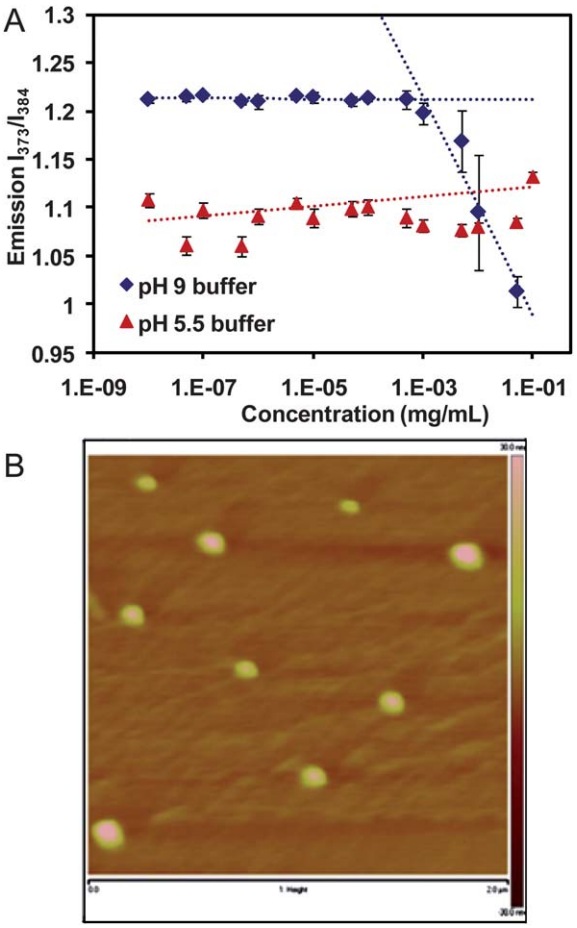


Fig. 5 (A) CMC determination by fluorometry using a pyrene probe for the diisopropylamine substituted PEG-*b*-PPLG in pH 5.5 and 9 buffer and (B) AFM images of diisopropylamine substituted PEG-*b*-PPLG at pH 8.88. The AFM images are 2 by 2 μm with a height range from −30 μm to 30 μm.

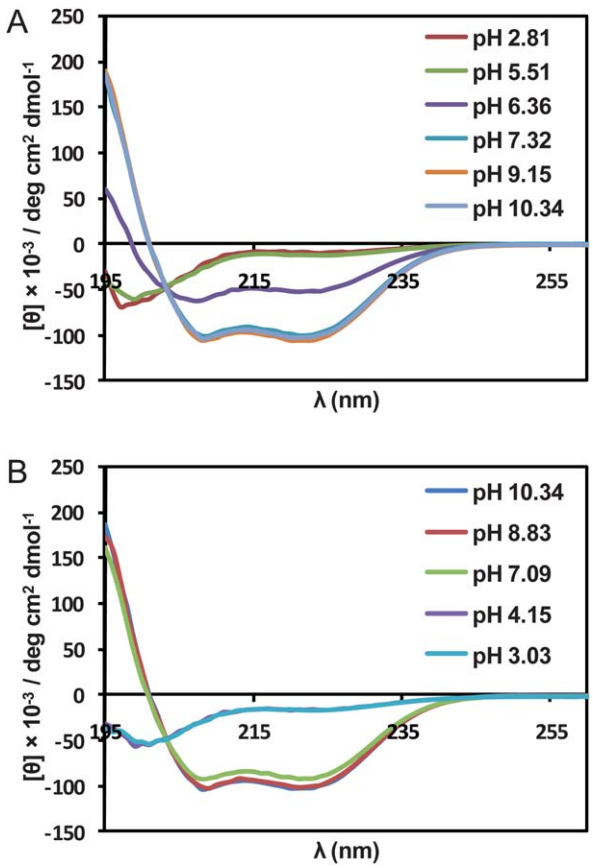


Fig. 6 (A) Increasing pH CD titrations and (B) decreasing pH CD titrations for secondary amine, DP = 75.

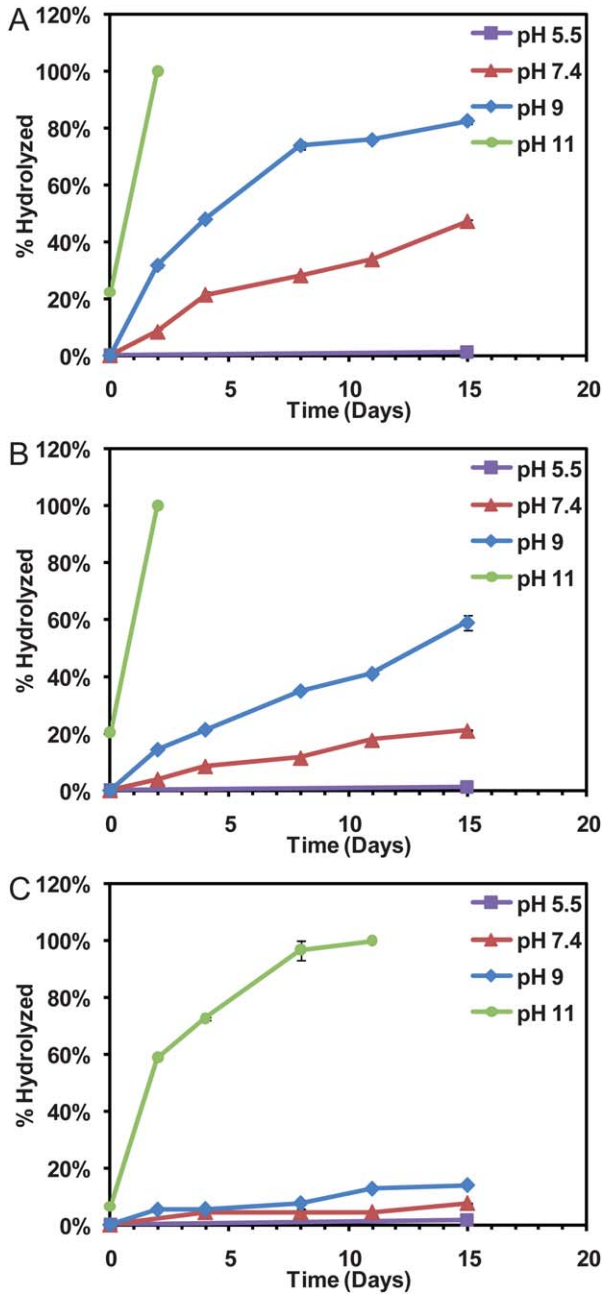


Fig. 7 (A) Percentage of ester side chains hydrolyzed as a function of time for PPLG (DP = 75) functionalized with secondary amine, (B) PEG-*b*-PPLG functionalized with diethylamine, and (C) PEG-*b*-PPLG functionalized with diisopropylamine.

Impact of pH on side chain hydrolysis

The functional groups introduced along the PPLG backbone are esters that can undergo hydrolysis under basic conditions, yielding the loss of the amino side group and the introduction of the carboxylate anion, thus introducing negative charge to the polyelectrolyte backbone. Slow or moderate changes in the polypeptide backbone may be of interest for drug delivery, gene delivery, tissue engineering, and coating applications.[30,42,53,54] Specifically, for systemic use, positively charged polymers such as poly(L-lysine) and poly(ethylene imine) often exhibit significant cytotoxicity.[41] The introduction of a mechanism that eliminates the multivalent positive charge and transforms the polymer into the benign and naturally occurring negatively charged poly-(γ-glutamic acid), which enhances the long-term biocompatibility of these polymers.[40,41]

To determine the side chain ester hydrolysis rate and the change in polymer secondary structure, ^{1}H-NMR and CD measurements were taken at various time points and pH conditions. Polymer samples (PPLG DP = 75 with secondary amine and PEG-*b*-PPLG with diethylamine and diisopropylamine) were dissolved in various pH buffers to a concentration of 0.5–1 mg mL^{-1} and left to hydrolyze at room temperature. From ^{1}H-NMR, the amount of ester hydrolyzed was determined by comparing the peak integration of the triazole peak from the ester side chain (8.15 ppm) to the integration of a new triazole peak from the alcohol side chain byproduct (8.07 ppm). A sample ^{1}H-NMR for PEG-*b*-PPLG functionalized with diethylamine hydrolyzed at pH 9 is shown in the ESI†. When the polyamide backbone, which maintains an α-helical structure when at equilibrium at all pH conditions investigated (pH 7.4, 9, and 11), undergoes hydrolysis, a glutamic acid residue is generated. Poly(γ-glutamic acid), like poly(L-lysine), maintains an α-helix in the uncharged state, and is a random coil in the charged state;[55] thus as hydrolysis occurs at more basic conditions we observe the loss of the α-helical polymer structure. Circular dichroism at 222 nm was observed to determine the change in secondary structure as a function of time. At 222 nm, a shift from a strong negative value towards a small positive value is indicative of a secondary structure shift, in this case, a shift from an α-helix to a random coil (representative full spectra and plots for PPLG functionalized with secondary amine at pH 7.4 and pH 9 can be found in the ESI†).

The results of the ester hydrolysis study are shown in Fig. 7 and 8. Representative ester hydrolysis plots for PPLG (DP = 75) functionalized with secondary amine and PEG-*b*-PPLG functionalized with diethyl and diisopropylamine are shown in Fig. 7A–C, respectively. In Fig. 8, the CD value observed at 222 nm is plotted at various pH values as a function of time for PPLG (DP = 75) functionalized with secondary amine. For all polymers, the rate of ester hydrolysis was highest at pH 11 and was increasingly slower as the pH was decreased. For example, in

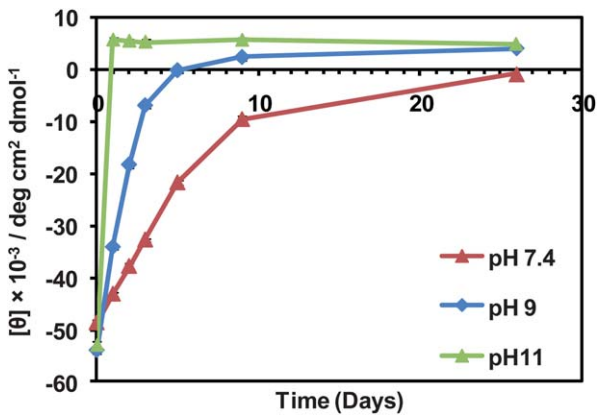


Fig. 8 CD value observed at 222 nm at various pH values as a function of time for PPLG (DP = 75) functionalized with secondary amine.

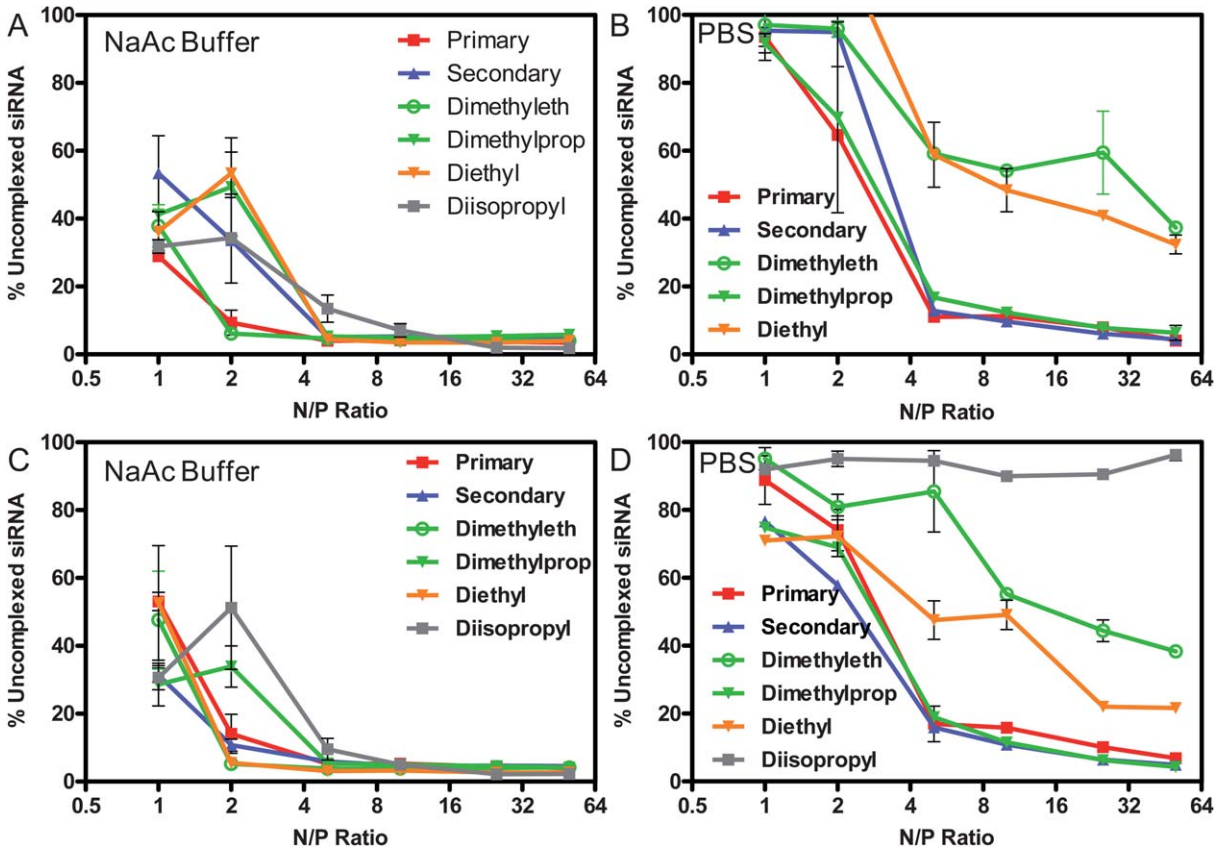


Fig. 9 Percentage of uncomplexed siRNA as a function of siRNA : polymer (*N*/*P*) ratio for each amine substituted PPLG for degree of polymerization 140 (A and B) and 75 (C and D). Polyplexes were formed in either sodium acetate buffer (A and C) or PBS (B and D). The DP140 diisopropylamine sample was insoluble in PBS.

all cases complete hydrolysis was observed at pH 11 (at 2 days for the secondary amine and diethylamine and 11 days for the diisopropylamine), but at pH 5.5 after 15 days, all samples were less than 2% hydrolyzed. When comparing the ester side chain hydrolysis between polymers, the rate of hydrolysis at pH 7.4, 9, and 11 was fastest for PPLG functionalized with secondary amine and slowest for PEG-*b*-PPLG functionalized with diisopropylamine. For the diblock polymers, the polypeptide is encapsulated as the inner core of a micelle, and is partially protected from hydrolysis, thus greatly slowing the rate of hydrolysis.

When looking at the secondary structure of PPLG (DP = 75) functionalized with secondary amine (Fig. 8), the polymer adopts a random coil after 1 day (24 hours) in pH 11 buffer solution, at pH 9, the polymer gradually adopts a random coil over several days, and at pH 7.4 the polymer primarily maintains an α-helical structure for multiple days. When compared to the ^{1}H-NMR data, at pH 11, the ester side chains have completely hydrolyzed in two days, leaving poly(γ-glutamic acid) which is in a random coil conformation. For pH 9, at day 4, the polymer is 50% hydrolyzed, and the polymer structure is nearly all random coil. This observation indicates that not all the ester side chains need to be hydrolyzed for the α-helix to be disrupted. Similar CD trends were observed for all PPLG (DP = 75) and PEG-*b*-PPLG polymers tested and can be found in the ESI†. In summary, we can control the rate of ester degradation and the rate of α-helix disruption by changing the side chain functionality.

siRNA complexation studies

Studies have been performed to determine if the amine functionalized homopolymers complex siRNA into protective polyplexes. Polymers were mixed with siRNA at various PPLG polymer to siRNA charge ratios (*N*/*P*) ranging from 1 : 1 to 50 : 1 in either sodium acetate buffer (pH 5.5) or PBS (pH 7.4). Ribogreen was used to determine the complexation efficiency of each polymer at the various ratios, shown in Fig. 9. As shown in Fig. 9A and C, all amine functionalized PPLG homopolymers prevent dye access to more than 90% of siRNA at charge ratios above 4 : 1 in sodium acetate. Additionally PPLGs with primary amine substituents are able to completely complex siRNA at a charge ratio that is two-fold lower, indicating the strength of primary amines for complexation. At the higher pH of PBS (7.4), fewer amines are charged, particularly in the case of the dimethylethanamine, diethylamine, and diisopropylamine substituents, leading to looser complexes and greater dye access. This manifests itself both at low polymer : siRNA ratios for all of the polymers, and most notably for the dimethylethanamine, diethylamine, and diisopropylamine PPLGs (see Fig. 9B and D). While these tertiary amine substituents may be useful for stimulating endosomal escape, the copolymers with primary and tertiary amines are more likely to exhibit properties that enable full encapsulation of siRNA and buffering effects *in vivo*.

Polyplexes can be disrupted by the addition of a competing polyanion, such as heparin. In Fig. 10A, PPLGs (DP = 140) with

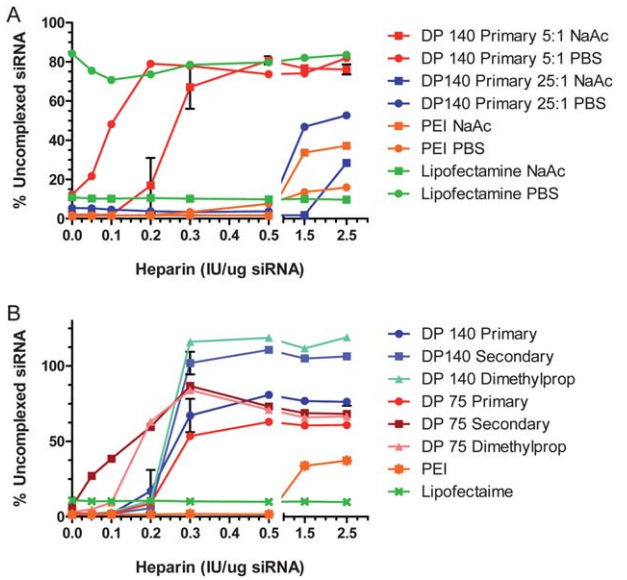


Fig. 10 Percentage of uncomplexed siRNA as a function of added heparin for various complexation conditions. (A) Complexes were formed in pH 5.5 sodium acetate buffer (squares) or PBS (circles) at two different polymer : siRNA ratios (*N*/*P*). (B) PPLGs with primary (circle), secondary (square), or dimethylpropanamine (triangle) substitutions were complexed in sodium acetate buffer prior to dissociation with heparin.

primary amine substituents were complexed at low (5 : 1) and high (25 : 1) polymer : siRNA ratios (*N*/*P*) in either sodium acetate or PBS, along with PEI and Lipofectamine 2000 as controls. As anticipated, relatively low levels of heparin were required to dissociate PPLG complexes formed at 5 : 1 as compared with those complexes formed at the 25 : 1 *N*/*P* ratio. PPLG complexes formed in PBS were more easily disrupted than those formed at low pH, most likely because those formed at low pH contained more highly charged amines, and were thus more tightly complexed. Fig. 10B demonstrates this concept with different amine substituents. In the DP75 polymers (red), the tertiary amine in the dimethylpropanamine group forms a looser polyplex and is disrupted more readily than the secondary and primary amines. However, for DP140, the dimethylpropanamine polyplexes begin to dissociate with the same amount of added heparin as the primary and secondary polyplexes, indicating that molecular weight is also a factor in polyplex stability. In summary, the siRNA complexation behavior of these systems is tunable, and can be altered through the introduction of different buffering amine functionalities, molecular weight and pH conditions of complexation. Further studies of these systems as siRNA and gene delivery vehicles are ongoing.

Conclusion

We have developed a new library of pH responsive polypeptides based on the combination of NCA polymerization and alkyne–azide cycloaddition click chemistry. PPLG homopolymers and PEG-*b*-PPLG block copolymers were substituted with various amine moieties that range in pK_a and hydrophobicity, that can be tuned for specific interactions and responsive behaviors. We have demonstrated that these new amine-functionalized polypeptides change solubility, or self-assemble into micelles for the case of diblock polymers, with degree of ionization and adopt an α-helical structure at biologically relevant pHs. The impact of side chain hydrolysis was also explored to determine the hydrolysis rate as a function of pH and the impact of hydrolysis on polymer side chain conformation. These properties are of interest for a number of applications, here we have performed preliminary experiments that demonstrate that these polymers are strong candidates for drug and gene delivery.

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