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Synthesis and characterization of poly(L-glutamic acid)-*block*-poly-(L-phenylalanine)

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ABSTRACT

Poly(γ-benzyl L-glutamate)-*block*-poly(L-phenylalanine) was prepared via the ring opening polymerization of γ-benzyl L-glutamate *N*-carboxyanhydride and L-phenylalanine *N*-carboxyanhydride using *n*-butylamine·HCl as an initiator for the living polymerization. Polymerization was confirmed by ^{1}H-nuclear magnetic resonance spectroscopy and matrix assisted laser desorption ionization time of flight mass spectroscopy. After deprotection, the vesicular nanostructure of poly(L-glutamic acid)-*block*-poly (L-phenylalanine) particles was examined by transmission electron microscopy and dynamic light scattering. The pH-dependent properties of the nanoparticles were evaluated by means of ζ-potential and transmittance measurements. The results showed that the block copolypeptide could be prepared using simple techniques. Moreover, the easily prepared PGA–PPA block copolypeptide showed pH-dependent properties due to changes in the PGA ionization state as a function of pH; this characteristic could potentially be exploited for drug delivery applications.

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1. Introduction

Polypeptides with well-defined nano-architectures have attracted considerable attention as tools for biomedical applications such as drug delivery systems and tissue engineering [1–7]. Amphiphilic polypeptides containing both hydrophilic and hydrophobic polypeptide segments can form a variety of structures, including micelles, fibers, and vesicles, via intermolecular or intramolecular hydrogen bonding, hydrophobic interactions and π–π stacking [8–15].

Holowka et al. [14] reported the composition-dependent architecture of poly(L-lysine)-*b*-poly(L-leucine). Specifically, they showed that this polypeptide forms sheets, fibers, vesicles or micelles depending on the hydrophilic–hydrophobic balance within the molecule. The α-helical structure of poly(L-leucine) and its intermolecular hydrogen bonding drive spontaneous self-assembly. The synthesis and properties of poly(L-lysine)-*b*-poly (L-phenylalanine) were reported by Sun et al. [15]. Hydrophobic interactions and hydrogen bonding of poly(L-phenylalanine) within poly(L-lysine)-*b*-poly(L-phenylalanine) were reported to induce the formation of large vesicles regardless of the block lengths.

The aims of this study was to synthesize poly(L-glutamic acid)-*b*-poly(L-phenylalanine) (PGA–PPA) from *N*-carboxyanhydride (NCA) monomers and to form pH-dependent PGA–PPA particles. Polypeptide synthesis from NCA is the most common method [16,17], but there are some limitations when synthesizing block copolypeptides. In particular, the polymerization of γ-benzyl L-glutamate NCA (BLG-NCA), when initiated by a primary amine, yields poly (γ-benzyl L-glutamate) (PBLG), a precursor polypeptide of PGA. However, PBLG with an amine end group undergoes intramolecular amination between the N-terminal position and benzyl ester group to form pyroglutamate [11,16–18], which makes further polymerization impossible. And the self-assemble property of block copolypeptide is a quite unique phenomenon even though there are some reported studies [11,19]. To solve the problem mentioned above polymerization of BLG-NCA was initiated by *n*-butylamine·HCl and further block copolymerization was carried out using L-phenylalanine NCA (Phe-NCA) in the study.

This study provided a simple synthetic method to fabricate PGA based block copolypeptide without using inconvenient techniques.

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In addition, our study about pH-dependent destabilization of PGA–PPA vesicles would be helpful to find applications as drug delivery systems that need pH-dependent release profile (Scheme 1).

2. Experimental section

2.1. Materials

γ-glutamic acid benzyl ester, L-phenylalanine, solvents and other reagents were purchased from Sigma–Aldrich, Korea. Anhydrous *N*,*N*-dimethylformamide (DMF, 99.9%) was used without further purification. Triphosgene was obtained from TCI Chemicals. BLG-NCA and Phe-NCA were synthesized according to the method reported by Daly et al. [20]. *n*-Butylamine·HCl was prepared by treating *n*-butylamine with 0.1 M HCl in diethyl ether followed by drying in a vacuum oven at room temperature for 1 day.

2.2. Synthesis

Before polymerization, BLG-NCA was reacted with an excess of the initiator, *n*-butylamine or *n*-butylamine·HCl, to determine if the HCl end-capped primary amine effectively prevented the side reactions observed when *n*-butylamine was used as the initiator. In detail, BLG-NCA (0.02 g) and 2 molar equivalents of either *n*-butylamine or *n*-butylamine·HCl were dissolved in DMF-d_7 and reacted for 1 day at room temperature. The resulting solutions were analyzed without purification by ^{1}H NMR.

PGA–PPA was polymerized as follows. First, BLG-NCA (5 g) and 10 mol% *n*-butylamine·HCl of monomer were transferred to a Schlenk tube and dissolved in anhydrous DMF (10 mL). After reaction for 1 day at room temperature, the product was precipitated in excess diethyl ether and filtered off. The acquired white powder was washed 2–3 times with diethyl ether and dried in a vacuum oven for 1 day. The prepared PBLG (5 g) and 5 molar

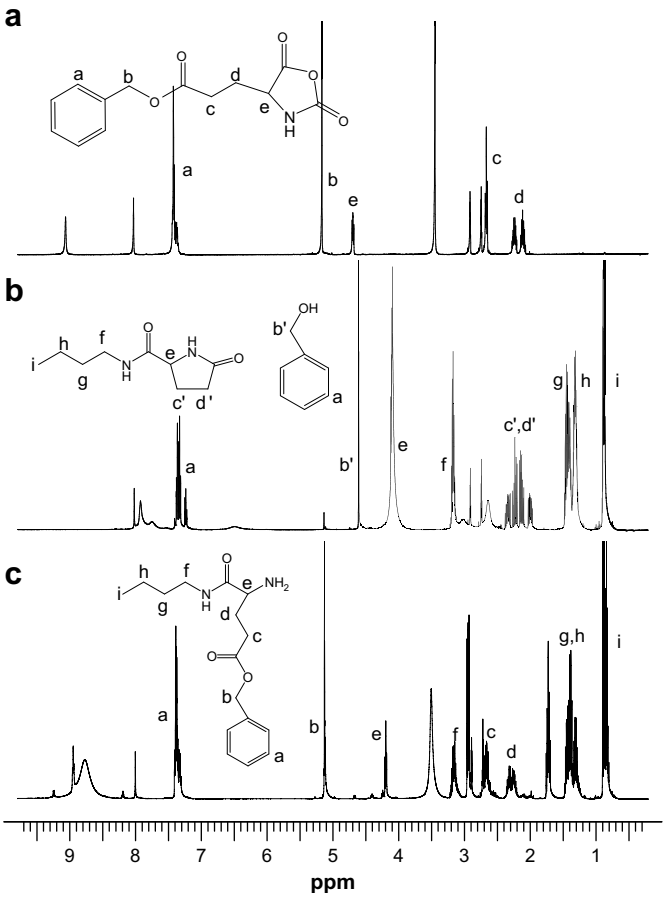
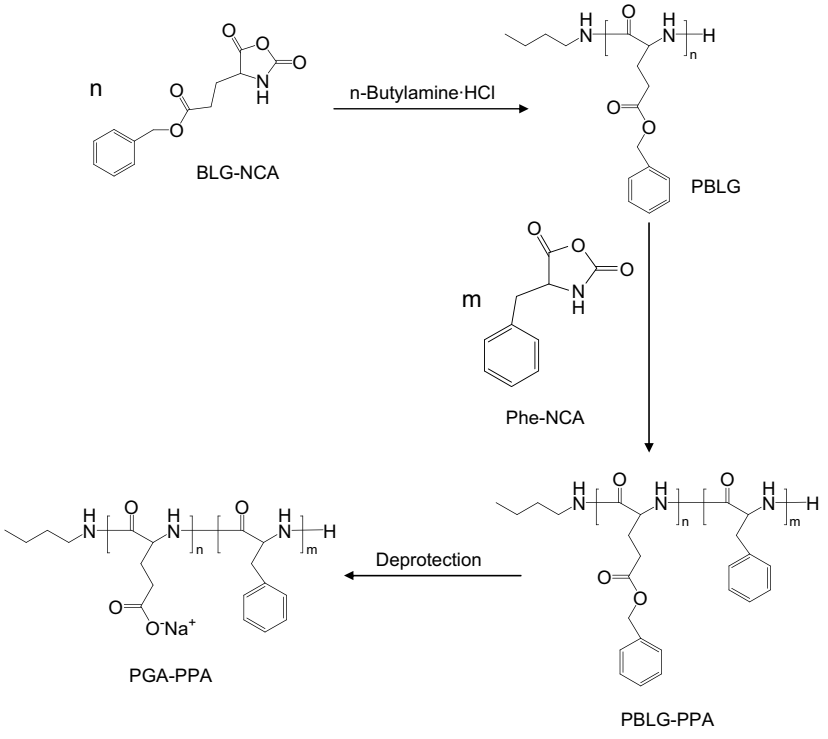


Fig. 1. ^{1}H NMR spectra and proposed structures of (a) BLG-NCA, (b) reactant with *n*-butylamine and (c) reactant with *n*-butylamine·HCl.



Scheme 1. Synthesis of PGA–PPA block copolypeptide.

equivalents of Phe-NCA were dissolved in anhydrous DMF and reacted at room temperature for 1 day. The reaction mixture was then precipitated in diethyl ether and PBLG–PPA was obtained by filtration. To synthesize PGA–PPA, a protection group, the benzyl ester of PBLG, was removed with a 9:9:2 trifluoroacetic acid/methanesulfonic acid/anisole (v/v/v) mixture at room temperature and precipitated in diethyl ether. After filtering and drying, the filtrate was dispersed in 1 M NaOH aqueous solution and dialyzed through a dialysis membrane bag (Spectra/Por MWCO 3500) in deionized water for 1 day. After 2 days of freeze drying, PGA–PPA was collected.

2.3. Peptide analysis

The synthesized polypeptides were characterized by ^{1}H NMR spectroscopy (500 MHz JNM-LA FT-NMR) with TFA-*d* and TFA-*d* with 0.1% methanesulfonic acid as the solvents for PBLG and PGA–PPA, respectively. MALDI-TOF MS (Voyager-DE™ STR Bio-spectrometer) spectra were collected using 2,5-dihydroxybenzoic acid as the matrix and $CHCl_3$ as the solvent.

2.4. Particle analysis

PGA–PPA particles were prepared by dissolving the PGA–PPA to deionized water at a concentration of 1 mg/mL. The nanostructure was analyzed using a transmission electron microscope (Philips CM 200) operated at an acceleration voltage of 80 kV, after negatively staining the solution with 2% (w/v) uranyl acetate. The critical aggregate concentration (CAC) was determined using a fluorescence spectrometer (Aminco Bowoman Series 2; Aminco Bowoman, Urbana, IL) with pyrene as the hydrophobic dye [21]. The pH dependencies of the properties of the PGA–PPA particles were examined by analyzing each particle solution at different pH values using UV/VIS spectroscopy at 400 nm (S4100, Scinco, Korea), ζ-potential measurements (ELS-Z2, Otsuka Electronics, Japan). Dynamic light scattering (DLS) data were collected at 90° with a Malvern PCS100 spectrogoniometer and Brookhaven BI-9000AT digital autocorrelator equipped with a He–Ne laser (633 nm). The CONTIN algorithms were used in the Laplace inversion of the autocorrelation function to obtain the size distribution of the particles. The mean diameter was evaluated using the Stokes–Einstein equation.

3. Results and discussion

3.1. Evidence of living polymerization

Prior to polymerization, BLG-NCA was reacted with 2 molar equivalents of the initiator, *n*-butylamine or *n*-butylamine·HCl, to determine if the HCl end-capped primary amine effectively prevents side reactions. As shown in Fig. 1(a) and (b), when BLG-NCA was reacted with *n*-butylamine, the $-CH_2-$ proton signal of benzyl ester at 5.17 ppm (b) was shifted to a peak at 4.59 ppm (b′) assigned to the $-CH_2$ protons of benzyl alcohol, indicating that nucleophilic substitution had occurred. The product was pyroglutamate, which is produced by amination.

Fig. 1(c) shows the ^{1}H NMR spectrum of the product of a reaction of BLG-NCA with 2 molar equivalents of *n*-butylamine·HCl. Unlike the reaction with *n*-butylamine, there was no shift

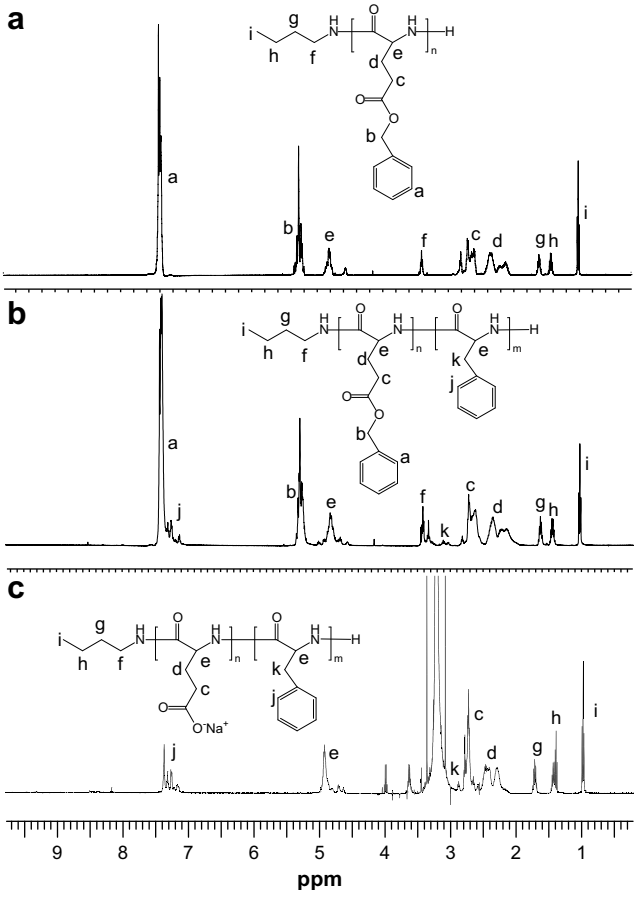


Fig. 2. ^{1}H NMR spectra of (a) PBLG, (b) PBLG–PPA and (c) PGA–PPA.

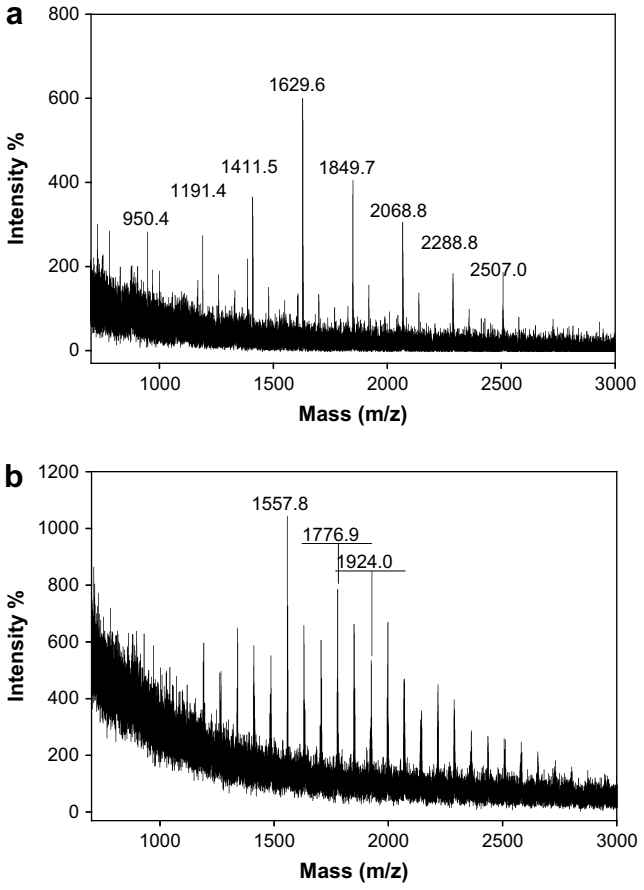


Fig. 3. MALDI-TOF MS spectra of (a) PBLG and (b) PBLG–PPA.

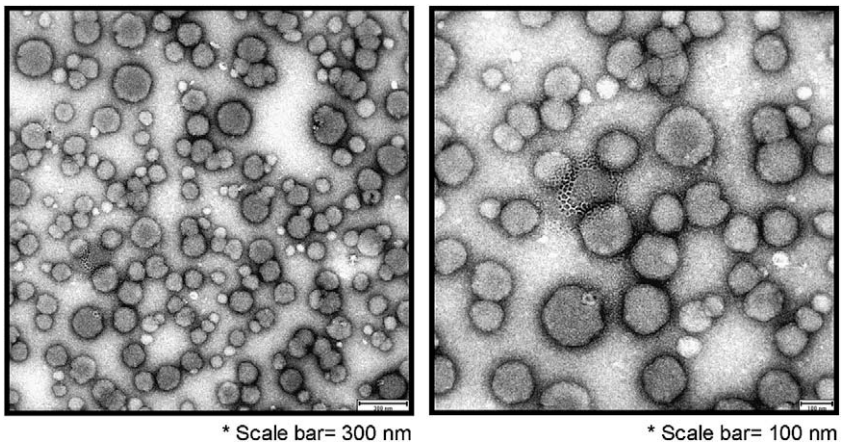


Fig. 4. TEM image of PGA–PPA particles.

of the $-CH_2-$ (b, 5.17 ppm) proton signal of the benzyl ester, demonstrating that nucleophilic substitution had not occurred. In addition, the reaction caused a shift in the $-CH_2-$ proton signal of the *n*-butylamine·HCl initiator from 2.96 ppm to 3.19 ppm (f). Analysis of the reactant was quite difficult because the ^{1}H NMR spectrum was measured using a mixture of the reactant and unreacted initiator. However, the integration ratio confirmed a precise 1:1 molar ratio between the integration values of the reacted initiator, $-CH_2-$ (f, 3.19 ppm), and the reacted monomer, $-CH_2-$ (b, 5.14 ppm). This confirmed that the HCl-capped primary amine effectively hindered the side reactions, including the formation of pyroglutamate. The present findings thus confirm that *n*-butylamine·HCl can be used to initiate BLG-NCA polymerization.

3.2. ^{1}H NMR of polypeptides

The PBLG obtained by the polymerization of BLG-NCA using *n*-butylamine·HCl as an initiator was characterized by ^{1}H NMR using TFA-*d* as the solvent. The molecular weight was calculated from the integrated value of the ^{1}H NMR data. The $-CH_2-$ proton signal (5.17 ppm) of benzyl ester and $-CH_3$ proton signal (0.90 ppm) of *n*-butylamine were used for PBLG. Theoretically, PBLG with 10 repeat units should have been synthesized, but the calculated number of repeat units was 7. This can be explained by the reaction rate being slow due to an equilibrium between *n*-butylamine and *n*-butylamine·HCl when the NCA monomer is initiated by a HCl end-capped primary amine [18].

If the end group of PBLG is a primary amine, it must react with the other added monomer. To evaluate this possibility, PBLG initiated by *n*-butylamine·HCl was reacted with Phe-NCA, with a target repeat unit number of 5. The characteristic peaks for PPA appeared after adding Phe-NCA to PBLG. The formation of PBLG–PPA was confirmed from the ^{1}H NMR spectrum (Fig. 2), which showed peaks corresponding to the $-CH_2-$ protons of PBLG (b, 5.14 ppm) and PPA (k, 3.05 ppm). However, calculating the molecular weight was difficult due to interference between the peaks and the insolubility of the PPA block, even in TFA [22]. To confirm the length of the PPA portion, the ^{1}H NMR spectra of PGA–PPA were analyzed using TFA-*d* containing 5 vol% methanesulfonic acid. From the phenyl proton signal of PPA, $-CH_2$ proton signal of PGA and $-CH_3$ proton signal of *n*-butylamine, the final structure was confirmed to have 7 units of glutamic acid and 2 units of phenylalanine.

3.3. MALDI-TOF MS of polypeptides

The MALDI-TOF MS spectrum of PBLG (Fig. 3a) showed a major peak at 1629.6 Da, which is consistent with the calculated mass of 1607.8 for the 7-mer. In the spectrum, the difference between each pair of adjacent peaks was approximately 219 Da which corresponds to glutamic acid. In reality, polymerized PBLG was not a perfect homopeptide which has a little broad distribution because it was polymerized from a NCA monomer. Indeed, PBLG was a mixture containing 4–10-mers.

The MALDI-TOF spectrum of PBLG–PPA (Fig. 3b) showed complicated peaks. Consideration of the spectrum indicates that

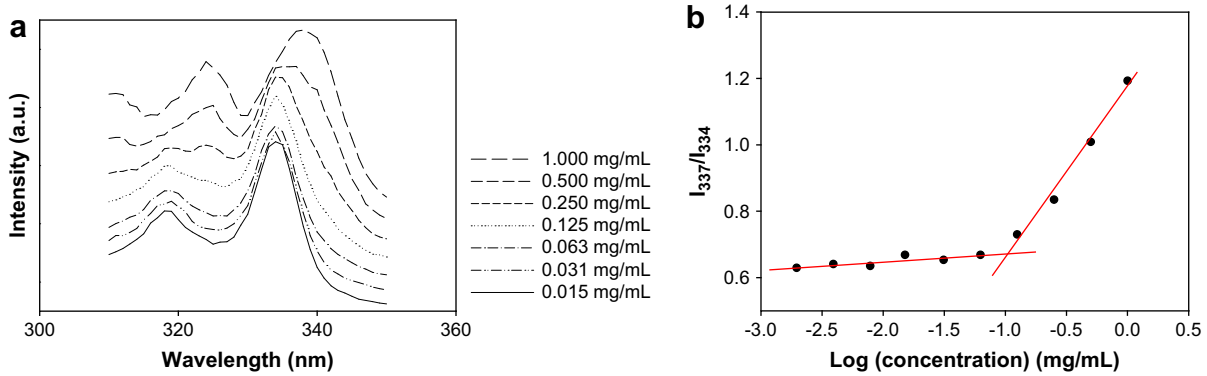


Fig. 5. (a) Excitation spectra of PGA–PPA solution in the presence of pyrene as a function of concentration and (b) plots of the intensity ratio I_{337}/I_{334} vs. log (concentration).

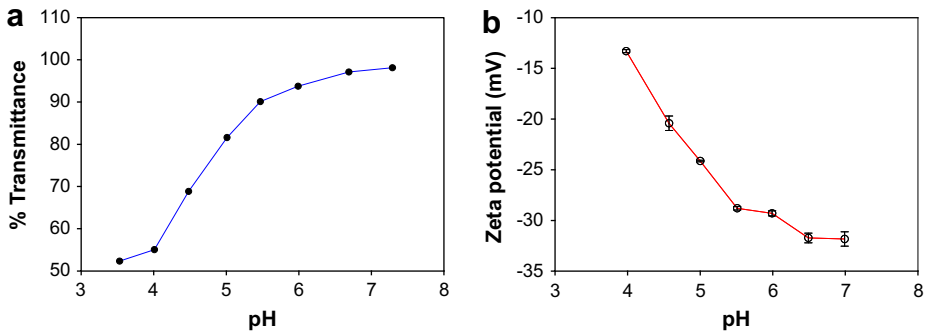


Fig. 6. (a) Transmittance and (b) ζ-potential of a PGA–PPA solution as a function of pH.

1–3-mer PPA chains might be attached to PBLG. The 1777.0 Da peak for PBLG–PPA is close to the 1629.6 Da result for 7-mer PBLG plus a 1-mer PPA, which has a calculated mass of 1776.8. Therefore, the peak at 1777.0 Da for PBLG–PPA is assigned to a copolypeptide consisting of a PBLG 7-mer and PPA 1-mer. Similarly, the peak at 1924.0 Da for PBLG–PPA is assigned to a copolypeptide comprised of a PBLG 7-mer and PPA 2-mer. Using the same method, the hypothesized PPA unit was below 3-mer. These results are similar to the ^{1}H NMR results, suggesting 2-mer on average.

3.4. Particle analysis

The morphology of the PGA–PPA particles was analyzed by TEM. As shown in Fig. 4, the rim of each particle appears dark with a thin line around the sphere. Therefore, the particles are considered to be vesicles. Although the PPA block is shorter than the PGA block, it is believed that a π–π interaction, including a stacking interaction between the aromatic moieties of peptides, favors the formation of a vesicular structure, as found in Phe-Phe dipeptide [8,9]. The CAC of the vesicle confirmed using fluorescence techniques [21] was found to be 0.11 mg/mL (see intersection of the two lines of best fit in Fig. 5(b)).

The pH dependencies of the turbidity and surface charge of the particles were determined by UV–vis spectroscopy and ζ-potential measurements, respectively. As shown in Fig. 6(a), the particle solution was transparent at pH >6.0, indicating that the nanosized particles are stable in this pH range. However, the solution became translucent at pH <6.0. As the pH was decreased to 5.5, aggregated particles could be observed with the naked eye. The ζ-potential results gave some indication as to why the particle stability changes according to pH. As shown in Fig. 6(b), the PGA–PPA particles have a negative surface charge, which means that PGA is fully ionized above pH 6.0. However, after PGA is deionized with decreasing pH, interparticle hydrogen bonding increases, resulting in agglomeration.

Evidence of pH-dependent agglomeration was also observed by DLS (Fig. 7). Above pH 6.0, the particle size (hydrodynamic diameter) was about 150 nm uniformly. However, the particle size increased when the pH was decreased to below 6.0. Between pH 5.0 and 6.0, PGA was not fully deionized; hence, not all the particles had aggregated and their average size was about 300 nm with a little agglomerates. Below pH 5.0, however, PGA was in a fully deionized state, resulting in significant agglomeration. Overall, these particles exhibited pH-dependent properties that

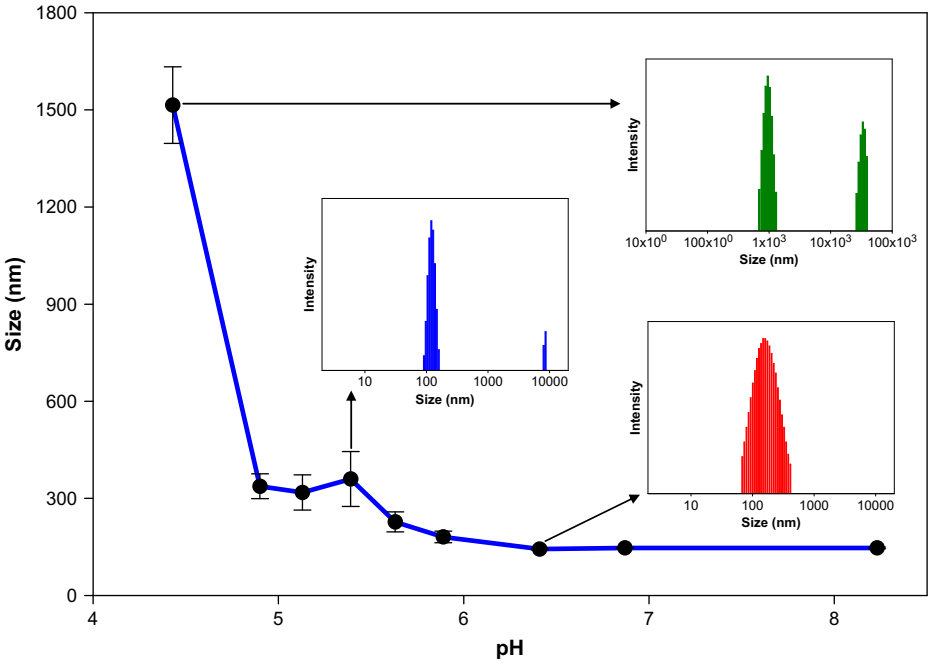


Fig. 7. Change in the particle size of a PGA–PPA solution as a function of pH. The small box indicates the size distribution at an arbitrary pH.

can be potentially applicable to pH-dependent drug delivery systems.

4. Conclusions

The living polymerization of an NCA monomer was examined and it is found that use of an HCl end-capped primary amine as the initiator can effectively prevent side reactions, including the pyroglutamate formation happening when *n*-butylamine is used as the initiator. Using *n*-butylamine·HCl as an initiator, PGA–PPA block copolypeptide could be prepared conveniently without special techniques such as the use of a transition metal complex, hazard catalyst, high vacuum apparatus and inconvenient purification method. The present results thus establish that the block copolypeptide can be prepared using a simple synthetic method without using inconvenient techniques. This new method can be also applied to prepare various composition of the block copolypeptide from the ring opening polymerization of NCA-monomers. Moreover, the easily prepared PGA–PPA block copolypeptide forming vesicular structure showed pH-dependent properties due to changes in the PGA ionization state as a function of pH; this characteristic could potentially be exploited in endosomal or lysosomal delivery systems [23–25].

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