Articles

Synthesis and Characterization of Novel Amino Acid-conjugated Poly(aspartic acid) Derivatives

Seung Il Kim, Seok Kee Min,† and Ji-Heung Kim*

*Department of Chemical Engineering, Sungkyunkwan University, Suwon, Gyonggi 440-746, Korea. *E-mail: kimjh@skku.edu*
†CHARMZONE CO. LTD, Taejang-dong, Wonju, Gangwon 220-120, Korea
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Novel poly(aspartic acid) derivatives conjugated with L-lysine moieties and their amphiphilic analogs were synthesized and characterized. The chemical structures of these polymers were confirmed using FT-IR and ^{1}H-NMR spectroscopy. The physicochemical properties of amphiphilic copolymers were characterized using an electrophonetic light scattering spectrophotometer (ELS) and transmission electron microscopy (TEM). These results indicated a stable nanoparticle formation within aqueous media. These polymers have potential applications in the pharmaceutical and cosmetic fields as delivery vehicles for bioactive molecules.

Key Words : Nanoparticle, Poly(aspartic acid), Amphiphilic copolymer, L-Lysine

Introduction

The importance of polymeric materials incorporating biodegradability and biocompatibility is increasingly been recognized for various biomedical applications. Thus, the macromolecular design and synthesis of these polymers has been extensively studied in recent years. Among these polymers, poly(amino acid), which has a protein-like amide linkage, is known to be biodegradable and has been used for medical, cosmetic, and other industrial materials.[1] Poly-(aspartic acid), PASP, is a promising water-soluble and biodegradable polyamide that can be obtained from the hydrolysis of polysuccinimide (PSI),[2,3] the thermal poly-condensation product of L-aspartic acid.[4-6]

Recent studies of amphiphilic poly(aspartic acid) derivatives have been reported by several research groups.[7-11] Amphiphilic graft copolymers have attracted considerable interest because of their various industrial applications and relatively easy preparation methods compared to block copolymers. Due to their amphiphilic characteristics, block and graft copolymers containing both hydrophobic and hydrophilic components can be used to stabilize dispersions, emulsions, and polymer blends. They can also be used for surface modification, drug delivery carriers, nano-reactors, etc.[12,13]

Amino acids are used in various fields including nutrition, pharmaceuticals, cosmetics, agrochemicals, among others. L-lysine is one of the most important amino acids, and has been known to have various biological functions including collagen cell growth and antiviral activity.[14] Poly(L-lysine) (PLL), on the other hand, has been widely used as a gene delivery carrier due to its strong ability to protect DNA from nuclease attack.[15]

The covalent immobilization of bioactive compounds onto functionalized polymer surfaces has seen rapid growth in the past decade within the biomedical, textile, microelectronic, bioprocessing, and food packaging industries. Bioactive compounds can be natural or synthetic, and are defined as compounds which catalyze or elicit a specific response within a given biological system.[16] While pharmaceutical applications may require only minute amounts of bioactive compound to be effective, there is often a desire to maximize the amount of bioactive compound per unit area. This can be accomplished by grafting a polyfunctional agent onto the surface, thus increasing the number of available reactive functional groups per unit surface area. Carbodiimides are most commonly used as coupling reagents to obtain an amide linkage between a carboxylate and an amine or a phosphor-amidate linkage between a phosphate and an amine.

Novel biodegradable graft copolymers based on poly-(aspartic acid) that contained pendent L-lysine moiety were synthesized in this work. L-lysine components conjugated to the polymer backbone can provide a specific biological function *in vivo* and a unique pH-sensitive property.[17,18] The physicochemical properties of the amphiphilic copolymers in aqueous media were characterized by electrophonetic light scattering spectrophotometer (ELS) and transmission electron microscopy (TEM).

Experimental

Materials. L-Aspartic acid (98+%), *o*-phosphoric acid (98%), L-lysine (97+%), 1-hexadecylamine (98%, HDA), *N,N*-dimethylformamide (99.8% anhydrous, DMF), dimethyl sulfoxide (99.9+% ACS reagent, DMSO), *N,N'*-dicyclo-hexyl carbodiimide (99%, DCC), and buffer pH solution were purchased from Aldrich Chemical Co. All other chemicals purchased were of high quality and used without

further purification. Dialysis membrane (Spectra/pore4 with MWCO 3500, 12000-14000) was used to eliminate any unreacted monomers and solvent.

Measurements. ^{1}H-NMR spectra were recorded on a Varian Unity Inova 500NB using D_2O or DMSO-d_6 as the solvent. The FT-IR spectra were obtained on a Bruker Tensor27. The particle size distribution in aqueous solutions (0.1 wt %) with various pHs was determined using ELS-Z2 (ELS-8000, Otsuka Electronics, Japan) with a laser light wavelength of 638 nm and a scattering angle of 165°. The polymer product was dispersed by magnetic stirring in aqueous solution and then filtered using a 0.45 μm syringe filter disc to remove oversized material. Aqueous electrophoresis measurements were also carried out using ELS-Z2 (ELS-8000, Otsuka Electronics, Japan). The pH of the solution was adjusted by addition of either dilute HCl or NaOH solution. The morphology of the PASP-HDA-Lys nanoparticle was measured using a transmission electron microscope (TEM, Phillips CM200), operated at an acceleration voltage of 80 kV, after negatively staining the nanoparticle with 2% (wt./vol.) uranyl acetate.

Synthesis of Poly(aspartic acid) (PASP). L-Aspartic acid and 98% *o*-phosphoric acid (50:50 wt. ratio) were put into a blender and mixed at low temperature. The mixture was then placed in a round bottom flask and stirred under reduced pressure at 200 °C for 5 h. The reaction mixture was cooled, and DMF was added to dissolve the brown viscous melt completely. The resulting solution was precipitated into an excess MeOH and the precipitate was washed several times with distilled water. The product was finally dried at 80 °C in vacuum for three days to obtain PSI in white powder form.[19]

PSI was dispersed in distilled water and then sodium hydroxide solution (equivalent to succinimide residue) was dropped into PSI dispersion while maintaining the solution pH below 10.8 in an ice bath. After the mixture was stirred overnight, hydrochloric acid (equivalent to succinimide residue) was dropped into the solution until the pH of solution stabilized at pH 4.0. The resulting solution was filtered, dialyzed (using membrane MWCO 12,000-14,000), and finally freeze-dried to obtain white solid polymer.

^{1}H-NMR (500 MHz, D_2O): δ 2.5-3.1 (s, 2H, CH-*CH*$_2$-CO-NH), 4.56-4.7 (s, 1H, NH-*CH*-CO-CH_2).

Synthesis of L-Lysine-conjugated Poly(aspartic acid), (PASP-Lys). Poly(aspartic acid) and DCC (1.5 equivalent of PASP residue) were dissolved in DMF/water mixed-solvent in a three-neck flask. The solution was stirred for 3 h in a water bath at 60 °C, and then a molar equivalent of L-lysine was added. After reacting for 24 h, the solution was precipitated in 8-fold acetone and then centrifuged. The powdery product was dissolved in distilled water and dialyzed using membrane (MWCO 12,000-14,000) to remove low molecular weight impurities. Finally, the dialysis product was freeze-dried (Yield 84%).

^{1}H-NMR (500 MHz, D_2O): δ 2.4-2.9 (br, 2H, CH-*CH*$_2$-CO-NH), 4.35-4.6 (br, 1H, NH-*CH*-CO- CH_2), 2.9-3.0 (t, 2H, NH- *CH*$_2$- CH_2- CH_2- CH_2-CH), 1.7-1.9 (m, 2H, NH-CH_2- *CH*$_2$- CH_2- CH_2-CH), 1.3-1.5 (m, 2H, NH- CH_2- CH_2- *CH*$_2$- CH_2- CH_2-CH), 1.55-1.7 (m, 2H, NH- CH_2- CH_2- CH_2- *CH*$_2$-CH), 3.6-3.7 (t, 1H, NH- CH_2- CH_2- CH_2-CH_2-*CH*-COOH).

Synthesis of Hexadecylamine-graft PASP, (PASP-HDA). PASP-HDA was prepared from PSI-HDA by hydrolysis, and the PSI-HDA was obtained *via* aminolysis reaction of PSI with hexadecylamine according to the procedure described below; PSI and hexadecylamine were dissolved in DMF (30 mL) in a three-neck flask. The solution was stirred at 70 °C for 7 h. After reaction, the solution was precipitated into 8-fold methanol and the precipitate was washed with fresh methanol several times. The product was filtered and then dried at 25 °C in vacuum. The hexadecylamine-graft PSI (PSI-HDA) prepared above was dispersed into 20 mL of water. 0.1 N sodium hydroxide solution was slowly dropped into the PSI-HDA dispersion, thus keeping the solution pH below 10.8 at room temperature. After the mixture was stirred overnight, 0.1 N HCl was dropped into the solution until the pH of solution remained at 4.0 in an ice bath. After that, the solution was precipitated in 8-fold acetone and then centrifuged. The powdery product was dissolved in distilled water and purified by dialysis using membrane (MWCO 12,000-14,000). Finally, the dialyzed product was freeze-dried.

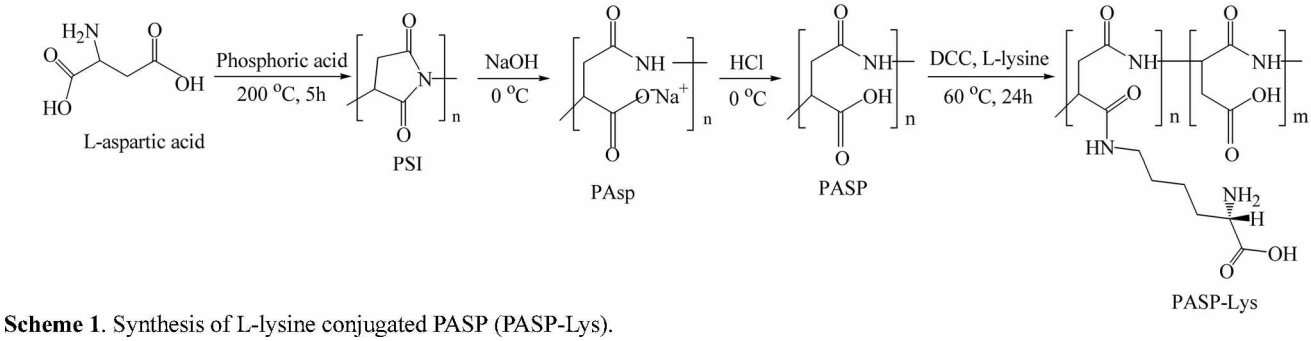
^{1}H-NMR (500 MHz, DMSO-d_6): δ 2.8-3.1 (d, 2H, CH-*CH*$_2$-CO-NH), 4.32-4.7 (s, 1H, NH-*CH*-CO-CH_2), 1.3-1.5 (d 2H, NH-*CH*$_2$-$(CH_2)_{14}$-CH_3), 1.1-1.3 (s, 2H, NH-CH_2-(*CH*$_2$)$_{14}$-CH_3), 0.75-0.98 (s, 3H, NH-CH_2-$(CH_2)_{14}$-*CH*$_3$).

Synthesis of L-Lysine-conjugated PASP-HDA (PASP-HDA-Lys). PASP-HDA and DCC (1.5 equivalent of PASP residue) were dissolved in DMSO/water mixed-solvent in a three-neck flask. The solution was stirred for 3 h in a water bath at 40 °C, and then L-lysine (1.0 equivalent of PASP residue) was added. After reaction, 100 mL distilled water was added, stirred for 10min, and then the mixture was filtered. The filtrate was dialyzed using membrane (MWCO 3,500) to remove unreacted monomers and residual solvent. Finally, the dialysis product was filtered and freeze-dried to obtain white solid polymer.

^{1}H-NMR (500 MHz, D_2O): δ 2.5-2.8 (br, 2H, CH-*CH*$_2$-CO-NH), 4.32-4.7 (br, 1H, NH-*CH*-CO-CH_2), 2.9-3.0 (t, 2H, NH-*CH*$_2$-CH_2-CH_2-CH_2-CH), 1.7-1.9 (m, 2H, NH-CH_2-*CH*$_2$-CH_2-CH_2-CH), 1.3-1.5 (m, 2H, NH-CH_2-CH_2-*CH*$_2$-CH_2-CH), 1.55-1.7 (q, 2H, NH-CH_2-CH_2-CH_2-*CH*$_2$-CH), 3.6-3.7 (t, 1H, NH-CH_2-CH_2-CH_2-CH_2-*CH*-COOH), 2.5-2.8 (2H, NH-*CH*$_2$-$(CH_2)_{14}$-CH_3), 1.1-1.3 (m, 2H, NH-CH_2-(*CH*$_2$)$_{14}$-CH_3), 0.75-0.98 (t, 3H, NH-CH_2-$(CH_2)_{14}$-*CH*$_3$).

Results and Discussion

Synthesis and Characterization of L-Lysine-conjugated Poly(aspartic acid), (PASP-Lys). PSI, polysuccinimide, was prepared using the procedure previously detailed in the experimental section of this paper. The resulting PSI powder was dispersed into pure water, titrated with a molar equivalent of NaOH, and then acidified by a molar equivalent of



Scheme 1. Synthesis of L-lysine conjugated PASP (PASP-Lys).

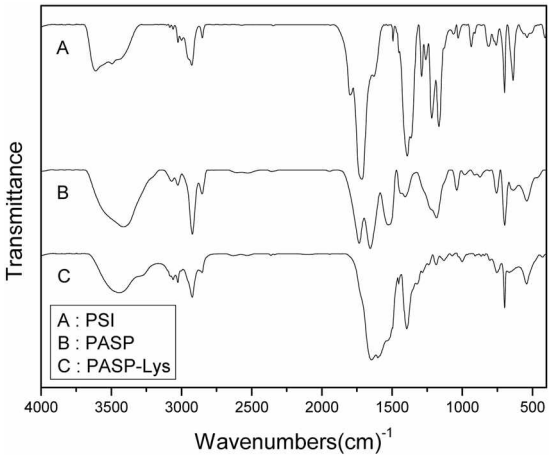


Figure 1. FT-IR spectra of PSI (A), PASP (B), and PASP-Lys (C).

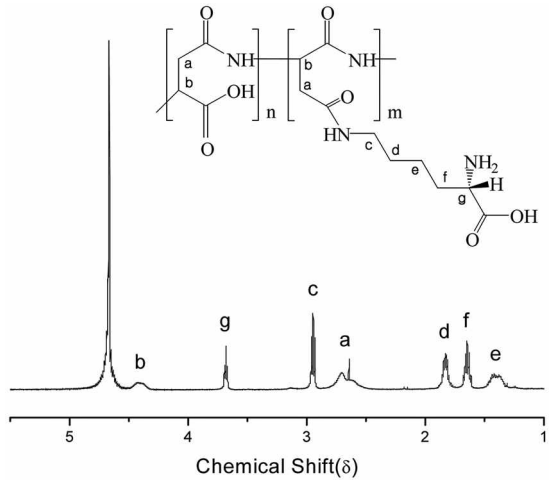
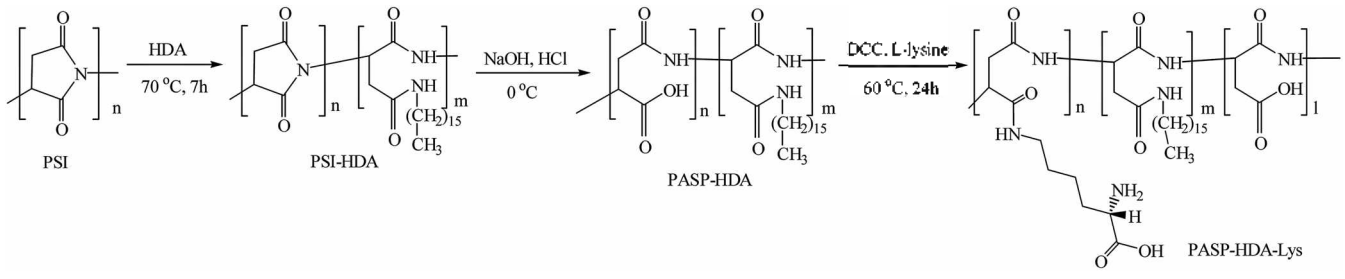


Figure 2. ^{1}H-NMR spectrum of PASP-Lys.

HCl to obtain poly(aspartic acid). L-lysine was conjugated to poly(aspartic acid) using DCC as the coupling reagent (Scheme 1). The FT-IR spectra of (A) PSI, (B) PASP, and (C) PASP-Lys are shown in Figure 1. Spectrum (A) shows characteristic absorption bands of the imide ring at 1727 cm^{-1} and 1393 cm^{-1}. Spectrum (B) shows the characteristic absorption peak of the amide at 1610 cm^{-1} and 1530 cm^{-1}, and a broad absorption band around 1240 cm^{-1} corresponding to the carboxylic acid pendent groups. In the spectrum of (C), a change in the absorption bands at 1500-1700 cm^{-1} region was observed by the introduction of an amino acid moiety to the pendants, and the broad absorption band around 1240 cm^{-1} on the spectrum (B) was found to be almost disappeared. The structure of PASP-Lys was also confirmed by ^{1}H-NMR measurement as shown in Figure 2. The proton peaks of **c**, **d**, **e**, **f**, and **g** were assigned to each methylene and methine protons of lysine, respectively. The DS (degree of substitution) of lysine was calculated by comparing the peak intensity of the methine proton of the polyaspartamide backbone at **b** with that of the lysine moiety at **g**. The DS of lysine moiety per aspartic acid unit was approximately 82%.

Synthesis and Characterization of L-Lysine-conjugated PASP-HDA (PASP-HDA-Lys). PASP-HDA-Lys, an amphiphilic poly(aspartic acid) derivative, was prepared from the reaction of PASP-HDA with L-lysine (see Scheme 2). The precursor polymer, PASP-HDA, was prepared from the aminolysis reaction of PSI with HDA and the following hydrolysis reaction. From the ^{1}H NMR analysis, the HDA content was determined to be 23% of repeating units. The carboxylic group of PASP-HDA was then coupled with the primary amine of L-lysine using DCC, a condensing reagent, to obtain PASP-HDA-Lys.[20,21] Figure 3 shows the FT-IR



Scheme 2. Synthesis of L-lysine conjugated PASP-HDA (PASP-HDA-Lys).

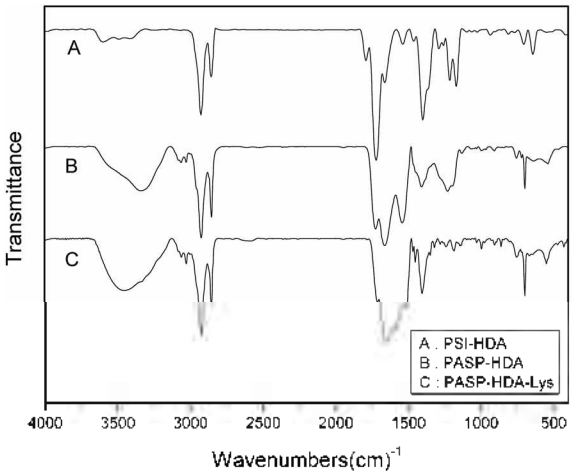


Figure 3. FT-IR spectra of PSI-HDA (A), PASP-HDA (B), and PASP-HDA-Lys (C).

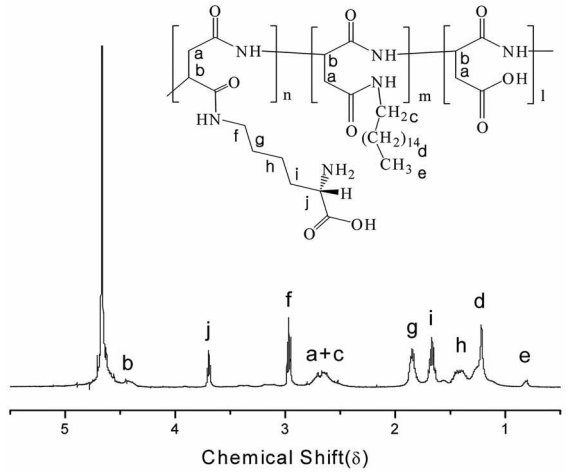


Figure 4. ^{1}H-NMR spectrum of PASP-HDA-Lys.

spectra of (A) PSI-HDA, (B) PASP-HDA, and (C) PASP-HDA-Lys, respectively. Spectrum (A) showed characteristic absorption bands of imide ring at 1727 cm^{-1} and 1393 cm^{-1}, as well as the absorption peak of the alkyl group at 2950 cm^{-1}. Spectrum (B) of PASP-HDA showed the characteristic absorption bands of amide group at 1610 cm^{-1} and 1530 cm^{-1}. Spectrum (C) showed multiple absorption bands of amide and carboxylate groups at 1500-1700 cm^{-1}, with a strong and broad absorption bands of hydroxyl and amine groups at 3200-3600 cm^{-1}. The structure of PASP-HDA-Lys was also confirmed by ^{1}H-NMR analysis as the spectrum is shown in Figure 4. The proton peaks of **f**, **g**, **h**, **i**, and **j** were assigned to the methylene and methine protons of L-lysine moiety, and proton peaks of **c**, **d**, and **e** were assigned to the

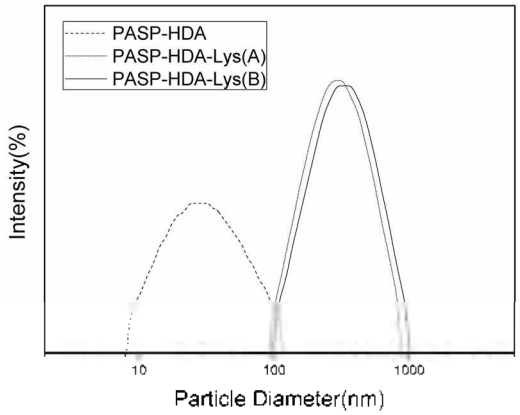


Figure 5. Particle size distributions of PASP-HDA, PASP-HDA-Lys(A), and PASP-HDA-Lys(B) in PBS 7.4.

methylene and methyl protons of the HDA pendant, respectively. The DS of lysine was calculated by comparing the integral area of the methyl proton (**e**) of HDA with the methine proton (**j**) of lysine. Both the FT-IR and ^{1}H-NMR analyses indicated that the PASP-HDA-Lys copolymer was successfully prepared.

Physicochemical Properties of PASP-HDA-Lys in Aqueous Media. The composition and physicochemical properties of several amphiphilic PASP derivatives are summarized in Table 1. The particle size distribution of amphiphilic PASP-HDA and PASP-HDA-Lys (A, B) was measured by DLS, as shown in Figure 5. The average diameter of PASP-HDA was about 28 nm. After L-lysine conjugation, the particle size of PASP-HDA-Lys increased to an average size of around 200 nm. The average diameters of nano-

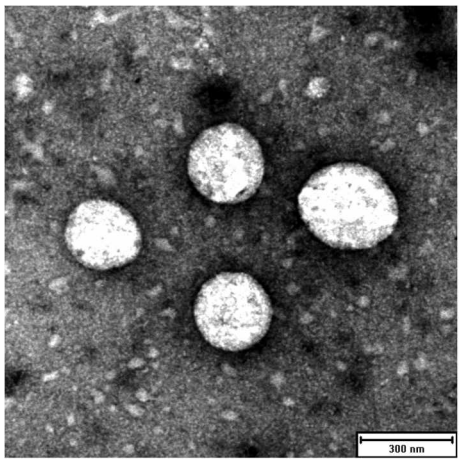


Figure 6. Transmission electron microscopy (TEM) image of PASP-HDA-Lys(B) particle formed in distilled water at 25 °C, 0.1 wt%.

Table 1. Conditions and Results of Polymerization

Sample	Solvent	Temp. (°C)	Degree of substitution (HDA)	Degree of substitution (lysine)	Average Diameter (nm)	Yield (%)
PASP-HDA	Water	25	23%	0%	28	81
PASP-HDA-Lys(A)	DMSO/water (2/1)	40	23%	16%	229	72
PASP-HDA-Lys(B)	DMSO/water (2/1)	40	23%	35%	193	76

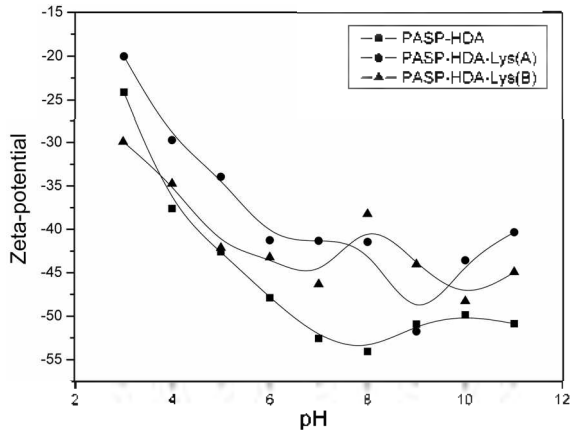


Figure 7. Zeta potentials of PASP-HDA, PASP-HDA-Lys(A), and PASP-HDA-Lys(B) aggregates at various pH in distilled water.

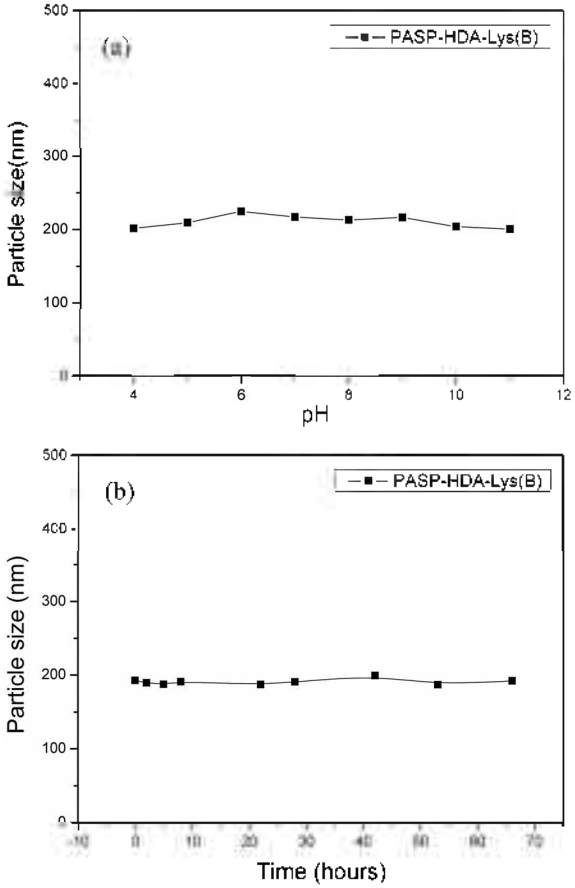


Figure 8. The stability of nanoparticles of PASP-HDA-Lys(B) in distilled water at various pH (a) and in PBS 7.4 at 25 °C as a function of time (b).

particles were 230 nm and 193 nm for the PASP-HDA-Lys (A) and (B), respectively. With L-lysine conjugation, the hydrophilic and hydrophobic balance should be changed to allow molecular reorganization of polymers, resulting in a significant increase of the particle size. Figure 6 shows a typical TEM image of a PASP-HDA-Lys (B) particle formed in distilled water (0.1 wt% concentration) at 25 °C. Spherical particles with a diameter of approximately 250 nm were clearly observed. The zeta potential measurement for each dilute solution (0.1 wt% concentration) of PASP-HDA, PASP-HDA-Lys (A), and PASP-HDA-Lys (B) was carried out while evaluating the particle charge distribution at different pHs (Figure 7). The potentials of these polymer solutions were measured to be negative with increasing negativity with an increase of pH, implying greater ionization of carboxylic pendent groups with increasing pHs. The same decreasing potential was observed for L-lysine-conjugated derivatives, even though the potential values were somewhat less negative. Interestingly, some protrusion in zeta potential curves was observed near pH 8-9 (pKa of $-NH_2$ of L-lysine is 8.95), which was likely to be caused by the presence of zwitterionic amino acid moieties on the surface of the polymeric particles and their minute buffering effect.

Figure 8a shows the average particle size as a function of the pH of the medium. At a region of pH 11-4, only a marginal change in particle size was observed, indicating that the particles remain relatively stable with the sizes of 220-180 nm. When the pH of solution dropped below pH 4, however, polymer precipitation has occurred. This may be due to protonation of carboxylic groups, resulting in the insolubilization of polymers in the aqueous medium. Figure 8b shows particle size as a function of storage time, demonstrating that the nanoparticles remain stable over a long period of time. These observations indicate that satisfactory colloidal stability in aqueous solutions can be achieved *via* steric and physicochemical stabilization of a given amphiphilic copolymer system. Studies on the biocompatibility and the *in vitro* cell activity of these L-lysine conjugated polymers are currently under investigation.

Conclusions

Novel amphiphilic biodegradable graft copolymers based on poly(aspartic acid) with pendent L-lysine moieties were synthesized, and their chemical structures were confirmed by FT-IR and ^{1}H-NMR spectroscopy. L-lysine-conjugated PASP-HDA possessed a nanoparticle form in aqueous media and remained stable over a long period of time at different pHs, as evidenced by ELS and TEM measurements. These amino acid-conjugated biocompatible polymers have potential applications in pharmaceutical and cosmetic fields.

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