Synthesis and Characterization of Novel Thermo- and pH-Responsive Copolymers Based on Amphiphilic Polyaspartamides

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ABSTRACT: Novel amphiphilic, thermo- and pH-responsive polyaspartamides showing both double-responsive (pH and temperature) behavior and a sol-gel transition were prepared and characterized. The polyaspartamide derivatives were synthesized by the successive aminolysis reactions of polysuccinimide using both hydrophobic *N*-alkylamine (laurylamine, octylamine, hexylamine) and hydrophilic *N*-isopropylethylenediamine. The composition of each component was analyzed by ^{1}H NMR. At the intermediate composition range, the system showed a lower critical solution temperature behavior in water. The transition temperature (pH dependent) could be modulated by changing the alkyl chain length and graft composition. The temperature dependence of the nanoparticle size distribution of the polyaspartamide derivatives was also examined. The critical micelle concentration of the copolymers in a phosphate-buffered saline (pH 7.4) solution ranged from 6 to 20 μg/L. In addition, physical gelation, i.e., a sol-gel transition, was observed in a concentrated solution. These novel double-responsive and injectable hydrogels have potential biomedical applications such as drug delivery systems and tissue engineering. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 998–1004, 2009

Key words: thermo- and pH-responsive polymer; polyaspartamides; amphiphilic copolymer; LCST; sol-gel transition

INTRODUCTION

There has been increasing interest in stimuli-responsive systems over the last few decades.[1] In addition, the promising potential of stimuli-responsive polymers and hydrogels has attracted considerable attention in the field of novel drug delivery systems, cell encapsulation, and tissue engineering.[1–6] Among them, polymer systems that undergo a phase transition in response to environmental stimuli such as temperature and pH have been widely investigated for gene or tumor targeting delivery, separation, and other medical devices.[5,7–15] Poly(*N*-isopropyl acrylamide) (PNIPAAm) is a typical thermosensitive polymer that undergoes a rapid and reversible hydration–dehydration change through the lower critical solution temperature (LCST). PNIPAAm, NIPAAm-containing copolymers, and hydrogels have been widely used in controlled drug delivery systems and various bio-related applications.[16–20]

Recently, injectable polymeric hydrogels have become quite attractive in biomedical applications such as novel drug delivery and tissue engineering.[6] The polymers are loaded with bioactive molecules or cells in an aqueous solution and are transformed into physically crosslinked hydrogels *in situ* as a result of external stimuli such as temperature, pH, and light.[10,11] Among them, thermoresponsive hydrogels that can undergo rapid transformation from a liquid form to a gel state at body temperature without any additives have been studied extensively. However, most reported injectable hydrogels are nonbiodegradable, which may limit their use in the biomedical field.[6]

Polypeptides and their related synthetic poly (amino acid)s have become important because of their desirable properties such as biocompatibility and biodegradability, which are useful for various bio-related industries. Poly(aspartic acid) (PASP) is a water-soluble and biodegradable polyamide, which can be produced from the hydrolysis of polysuccinimide (PSI), which is the polycondensate of the L-aspartic acid monomer. Poly(*N*-2-hydroxyethyl-DL-aspartamide) (PHEA) is another derivative polymer that is obtained by coupling PSI with ethanolamine, and has been proposed as a potential plasma extender and a material for drug delivery as macromolecular prodrugs, polymeric micelles, and nanoparticles. The attachment and chemical modification of pendent groups either via an aminolysis reaction to PSI

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or by a secondary reaction through the hydroxyl or carboxylic groups of PASP and PHEA can provide a variety of biodegradable functional polymers with specific properties. Extensive studies on this class of material were performed by Giammona et al. and other groups.[21–30] In addition, Kobayashi and Watanabe et al. recently reported some thermosensitive systems based on polyaspartamide derivatives.[23–26] However, aforementioned groups did not report any pH-responsive property. In our previous study, novel biodegradable thermoresponsive polyaspartamides with hydrophobic dodecylamine and hydrophilic *N*-isopropylethylamine (NIPEDA) were synthesized and characterized.[30]

As part of an ongoing study aimed at identifying materials showing both double-responsive behavior and a sol-gel transition in an aqueous solution, this study prepared a series of amphiphilic polyaspartamide derivatives by a successive aminolysis reaction of PSI using both a hydrophobic alkyl group (HA, OA, LA) and a hydrophilic *N*-isopropyl group.

MATERIALS AND METHODS

Materials

L-Aspartic acid (98+%), *o*-phosphoric acid (98%), *N*-isopropylethylenediamine (NIPEDA, 98%), dodecylamine (laurylamine, LA, 98%), octylamine (OA, 99%), hexylamine (HA, 99%), *N,N*-dimethylformamide (DMF, anhydrous 99.8%), phosphate-buffered saline (PBS, pH 7.4) were purchased from the Aldrich Chemical (Korea) and were used as received. Diethylether (99%) and buffer solutions (pH 5–10) were obtained from DaeJung Chemical (Korea). All the other chemicals were of high quality and were used without purification.

Synthesis of amphiphilic polyAspAm(alkyl/NIPEDA) derivatives

PSI was prepared and purified according to a previously reported procedure.[30,31] A typical procedure used to prepare the amphiphilic polyaspartamide copolymers is as follows: 0.5 g of PSI was dissolved in 10 mL DMF in a three-neck round flask equipped with a nitrogen inlet and outlet. Forty-five mol % (based on succinimide unit) of alkylamine (LA, OA, HA) was then added dropwise at 0°C. The reaction flask was placed in a 70°C water bath and stirred for 6 h. Fifty-five mol % (excess) of NIPEDA was slowly added to the solution and stirred at 30°C for 24 h. The final solution was then precipitated into 300 mL of cold ethyl ether. The filtered precipitate of the PolyAspAm(alkyl/NIPEDA) was then dried at 25°C in a vacuum (yield: 74–85%). ^{1}H NMR of (500 MHz, DMSO): δ 2.5–3 (m, 5H, CH—CH_2—CO—NH—CH_2—CH_2—NH—CH—$(CH_3)_2$), 4.5–4.7 (m, 1H, NH—CH—CO—CH_2), 3.2–3.4 (br, 2H, NH—CH_2—CH_2—NH—CH—$(CH_3)_2$), 0.92–1.12 (br, 6H, NH—CH_2—CH_2—NH—CH—$(CH_3)_2$), 2.95–3.08 (br, 2H, NH—CH_2—CH_2—$(CH_2)_m$—CH_3), 1.32–1.47 (br, 2H, NH—CH_2—CH_2—$(CH_2)_m$—CH_3), 1.11–1.27 (br, mH, NH—CH_2—CH_2—$(CH_2)_m$—CH_3), 0.8–0.76 (br, 3H, NH—CH_2—CH_2—$(CH_2)_m$—CH_3) (see Fig. 1).

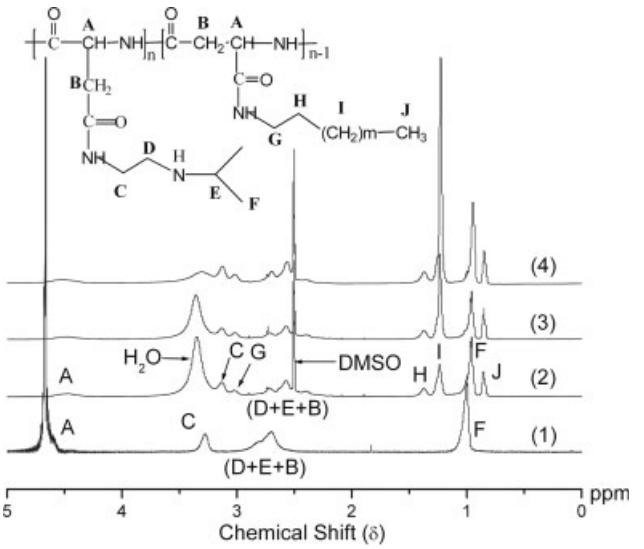


Figure 1 ^{1}H NMR spectra of PolyAspAm(NIPEDA) (1), PolyAspAm(HA/NIPEDA) (2), PolyAspAm(OA/NIPEDA) (3), and PolyAspAm(LA/NIPEDA) (4).

Measurements

^{1}H NMR and FTIR spectroscopy

The ^{1}H NMR spectra were recorded on a Unity Inova-500 (Varian, Palo Alto, CA) spectrometer using D_2O and DMSO-d_6 as the solvent. The FTIR spectra were obtained on a Perkin–Elmer FTIR spectrometer (model SPECTRUM 2000; Perkin–Elmer, Norwalk, CT).

LCST measurements

The LCSTs of the polymer in a buffer solution (pH 6–10) or distilled water were measured using a UV–visible spectrophotometer (Libra S22; Biochrom, Cambridge, UK) equipped with a cell holder and a temperature controller that can heat a sample at a rate of 1°C/min. The change in transmittance as a function of temperature was observed from a visible source at 500 nm using a 1 wt % polymer concentration. The LCST in this study was defined as the temperature of 90% light transmittance.

Acid–base titration

The pK_a value of the polymer was measured using the titration method. A 1 wt % solution of the

polymer was titrated to pH 10.5 with 1*N* NaOH and then back-titrated with 0.1*N* HCl in a given volume increment (100 μL).

Determination of particle size and the size distribution

The particle size and the changes in the PolyAspAm(LA/NIPEDA) copolymer solutions (1 wt %) with temperature were determined using a ELS-Z2 (Otsuka Electronics, Japan) with a laser light wavelength of 638 nm and a scattering angle of 165°. The polymer product was dispersed in aqueous solutions by magnetic stirring and then filtered using a 0.45-μm pore-sized syringe filter disc to remove the oversized material. The temperature was increased in 1°C increments at temperatures ranging from 15 to 35°C, and the reading was taken after standing for 1 min at each temperature.

Field emission scanning electron microscopy

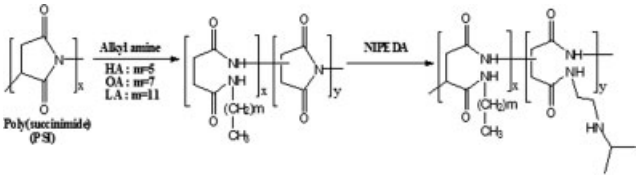
The nanoparticle (or micelle) morphology was observed by field emission scanning electron microscopy (FE-SEM, JSM6700F; JEOL, Japan). The sample was prepared by placing a droplet of the copolymer solution onto a glass slide. The sample was then dried overnight and coated with Pt using a plasma sputtering method (Ion sputter coater HC-21, JEOL-108auto).

Fluorescence spectroscopy

The critical micelle concentration (CMC) was determined using a fluorescence spectrometer (Aminco Bowoman Series 2; Aminco Bowoman, Urbana, IL) with pyrene as the hydrophobic fluorescence probe. The excitation spectra of pyrene with a slit width of 2.5 nm were recorded from 300 to 360 nm using an emission wavelength of 390 nm at 25°C. A stock solution of pyrene in THF was poured into a PBS solution containing different amounts of the polymer. The final pyrene concentration was 1×10^{-6} *M*. The solvent was removed by rotary evaporation at 50°C for 6 h. The concentrations of the polymer solution ranged from 1×10^{-6} to 1 mg/mL.

Sol-gel transitions

The sol-gel transition of the PolyAspAm(LA/NIPEDA) copolymer in PBS (pH 7.4) was determined using the vial inverting method. A PolyAspAm(LA/NIPEDA) copolymer solution (0.3 g) with a predetermined concentration was prepared in a 20-mL vial at 10°C. The vial containing the copolymer solution was immersed in a circulation water bath that had been thermostated at 10°C. The temperature was increased in 1°C increments and the vial was thermally equilibrated for 5 min after each increase. A gel state was considered when there was no flow within 10 s after inverting the vial.



Scheme 1 Synthesis of the amphiphilic polyaspartamide derivatives.

RESULTS AND DISCUSSION

Synthesis and characterization of amphiphilic polyaspartamide copolymers

The synthesis of PSI, the precursor polymer, has been well described in the literature. Novel amphiphilic polyaspartamide derivatives with a *N*-isopropylamine pendant were prepared from PSI using a successive nucleophilic ring opening reaction of both hydrophobic alkylamine (LA, OA, and HA) and hydrophilic NIPEDA (Scheme 1).

The reaction was carried out in anhydrous DMF, in which the succinimide group of PSI was reacted quantitatively to form the *N*-substituted aspartamide unit. The composition of the prepared copolymer was analyzed by ^{1}H NMR spectroscopy. Figure 1 shows the ^{1}H NMR spectra of the PolyAspAm(NIPEDA) (1), PolyAspAm(HA/NIPEDA) (2), PolyAspAm(OA/NIPEDA) (3), and PolyAspAm(LA/NIPEDA) (4) copolymer, respectively. As shown in Figure 1, the proton peaks C and F were assigned to the NIPEDA pendants, and the G, H, I, and J peaks were assigned to the methylene and terminal methyl protons of the alkyl amine pendant. The composition of each group in the polyaspartamide copolymer was determined from the integration ratio between peaks F and J.

Figure 2 shows the FTIR spectra of PSI (A), PolyAspAm(HA/NIPEDA) (B), PolyAspAm(OA/NIPEDA) (C), and the PolyAspAm(LA/NIPEDA) copolymer (D). All three polyaspartamide derivatives (B, C, and D) show characteristic strong bands at 1649 cm^{-1} (amide I), 1545 cm^{-1} (amide II), and 3305 cm^{-1} (—NH—), which correspond to the aspartamide backbone structure. The alkylene absorption band corresponding to the CH_2 stretch at 2950 cm^{-1} band increased gradually with increasing content of the alkylene moiety. FTIR and ^{1}H NMR analyses confirmed the successful preparation of the polyaspartamide derivatives from the aminolysis reaction of PSI.

LCST behavior of aqueous solutions

At the intermediate composition ranges of the PolyAspAm(alkyl/NIPEDA) copolymers, the polymer

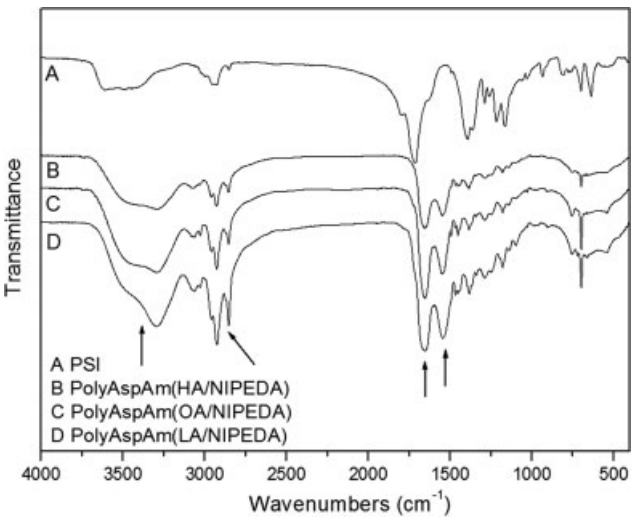


Figure 2 FTIR spectra of PSI (A), PolyAspAm(HA/NIPEDA) (B), PolyAspAm(OA/NIPEDA) (C), and PolyAspAm(LA/NIPEDA) (D).

solution exhibited thermally responsive phase separation. The phase transition temperatures were determined from the temperature dependence of light transmittance measured from a 1 wt % aqueous solution at 500 nm using UV spectroscopy. The temperature at 90% light transmittance of the polymer solution was defined as the LCST. Figure 3 shows the LCST of the three different series of polyaspartamides as a function of the alkyl content. The transition temperature in this copolymer system could be modulated by changing the alkyl chain length and graft composition. The copolymers exhibited a relatively sharp LCST phase transition in the temperature range of 20–60°C. Moreover, the LCSTs changed rather sensitively with changes in the alkyl content. This LCST behavior in these thermoresponsive polymers was attributed to a balance between hydrophilic and hydrophobic interactions and the resulting hydrogen-bonding interactions between water molecules and the polymer chain. The transition temperature decreased linearly with increasing content and chain length of the hydrophobic alkyl part.

From the acid–base titration of PolyAspAm(LA/NIPEDA), the aqueous polymer solution was found to exhibit a rather weak buffering region at pH 7.2–8.4. The pK_a of PolyAspAm(LA/NIPEDA) was $\sim$ 7.8 according to the mid-point of the pH buffering region in the titration curve.

The pH dependence of the phase transition was investigated using PolyAspAm(LA/NIPEDA) sample with 41 mol % LA (Fig. 4). At pH 10, the LCST was observed at $\sim$ 25°C. The LCST shifted toward higher temperature with decreasing pH, e.g., 36°C, 54°C, and 74°C at pH 9, 8, and 7, respectively. On the other hand, no LCST behavior was observed from the polymer solution at pH 6 up to 90°C. At pH $<$ 6, protonation of the secondary *N*-isopropyl amine groups will lead to electrostatic repulsion causing the polymer to be more soluble in water. In addition, the polymer–water interactions increase with increasing ionization.[14,31–33] At higher pH of solution (pH $>$ pK_a), the hydrophobic interaction between polymers increases with increasing temperature, and phase separation occurs at a certain temperature. As a result, it can be deduced that the hydrophobicity increases with increasing pH. The particle size distribution and the temperature-responsive behavior of the PolyAspAm(LA/NIPEDA) were analyzed by dynamic light scattering. Figure 5 shows the particle size distribution at different temperatures (the aqueous solution exhibited pH of $\sim$ 10). As described earlier, this specific copolymer

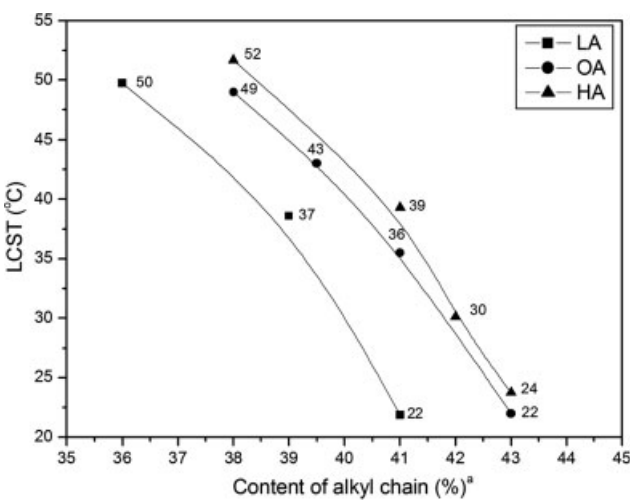


Figure 3 LCSTs of 1 wt % PolyAspAm(alkyl/NIPEDA) solutions with different alkyl contents.

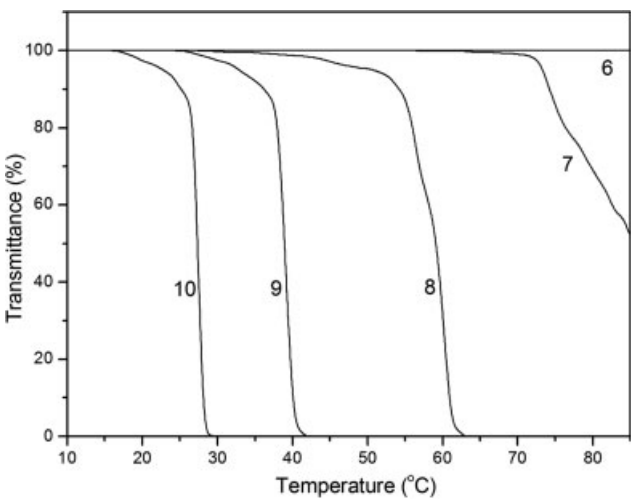


Figure 4 Effect of pH on the phase transmittance behavior of PolyAspAm(LA/NIPEDA) (wavelength: 500 nm; 1 wt % solution; heating rate: 1°C/min; LA content: 41 mol %).

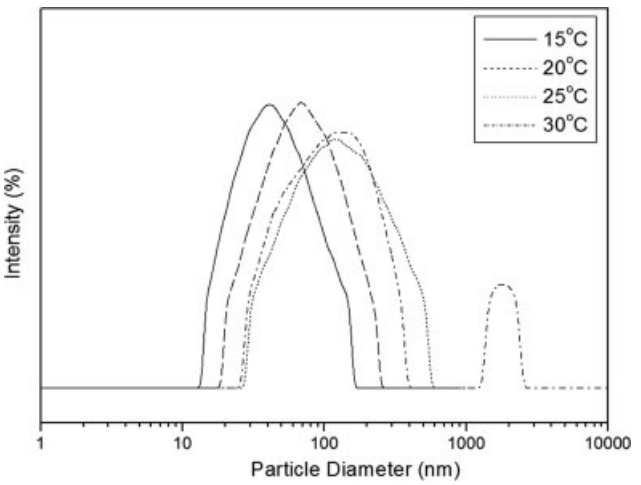


Figure 5 Temperature dependence of the micelle size distribution of PolyAspAm(LA/NIPEDA) in water (LA content: 41 mol %).

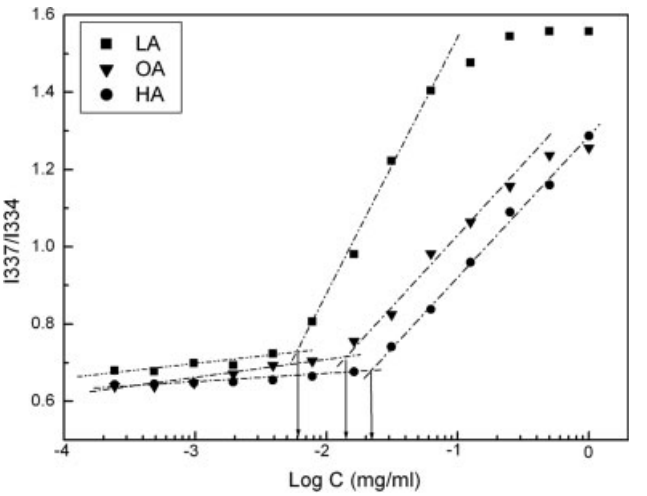


Figure 7 Plot of intensity ratios I_{337}/I_{334} from the excitation spectra versus log C of PolyAspAm(alkyl/NIPEDA) in PBS (pH 7.4) (alkyl content: 41 mol %).

exhibited a LCST at $\sim$ 25°C. At 15°C, which is below the phase transition, a narrow particle size distribution with an average diameter of $\sim$ 30 nm was observed. With increasing temperature to 20 and 25°C, the distribution shifted to a larger size with a slight increase in the size distribution. At 30°C, which is above the observed LCST, large particles (>1 mm) were detected along with main size of 100–200 nm, indicating partial precipitation through the aggregation of small-sized nanoparticles.

CMC and nanoparticle formation in aqueous solution

To further examine the formation of nanosized particles (or micelles), the CMC of the aqueous copolymer solution was determined using fluorescence spectroscopy. Below the CMC, the pyrene was dissolved in PBS (pH 7.4), which is a medium of high polarity. When micelles are formed, the pyrene molecule partitions preferentially into the hydrophobic domain in the micellar core, and it experiences a nonpolar environment. Figure 6 shows the fluorescence excitation spectra of the pyrene at various polymer concentrations. A red shift of pyrene in the excitation spectrum was observed with increasing copolymer concentration, which was attributed to the preferential partition of pyrene into the hydrophobic alkyl-group segments. Figure 7 shows intensity ratio of I_{337}/I_{334} as a function of the polymer concentration. The ratio increased slightly with increasing concentration at the lower concentration range, but increased rapidly above a certain concentration. The CMC value was determined from the intersection of two tangents to the parts of the curve corresponding to the left and right sides of the inflection point. The CMC values of the sample LA, OA, and HA were determined to be 0.006, 0.014, and 0.021 mg/L, respectively, with a decreasing tendency with increasing chain length of the hydrophobic alkyl group.

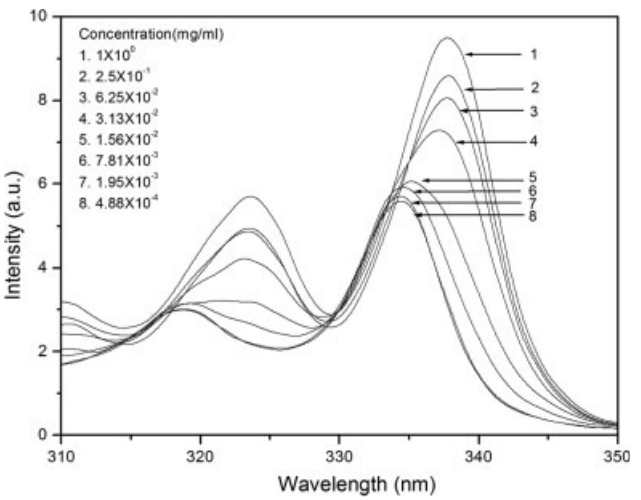


Figure 6 Excitation spectra of pyrene (6.0×10^{-6} M) in PBS (pH 7.4) in the presence of PolyAspAm(LA/NIPEDA) (LA content: 41 mol %).

Monodispersed spherical particles (or micelles) with 15–20 nm diameter were clearly observed in the FE-SEM image from the same PolyAspAm(LA/NIPEDA) prepared in distilled water (shown in Fig. 8).

Sol-gel transition in concentrated solutions

In addition, physical gelation, i.e., the sol-gel transition, was observed in the concentrated solutions of this copolymer system, which was determined using the vial inverting method. The effect of the polymer concentration on the temperature range of the sol-

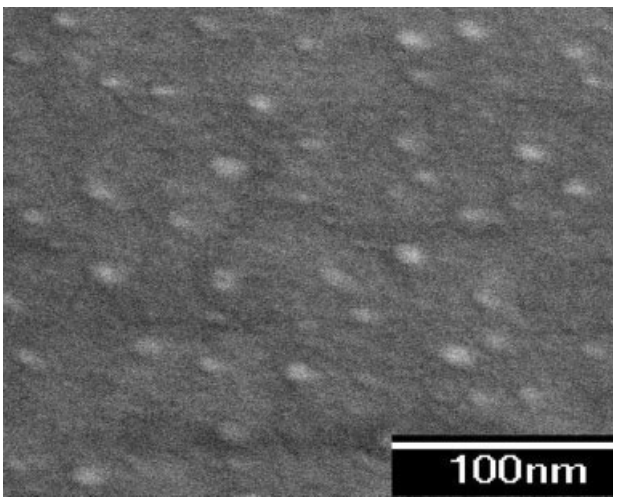


Figure 8 FE-SEM image of the amphiphilic PolyAspAm(LA/NIPEDA) nanoparticles formed in distilled water at room temperature (LA content: 41 mol %).

gel-sol phase transition of amphiphilic PolyAspAm(LA/NIPEDA) was examined, and the results are shown in Figure 9. When the temperature was increased, the clear polymer solution turned into nonflowing gel state. No sol-gel transition was observed below 10 wt %. At the higher polymer concentrations, the gel phase was found in the wider temperature range. On the other hand, only a small dependence of the sol-gel transition temperature on the polymer concentration was observed. In particular, the sol-gel phase transition occurs near body temperature, suggesting the potential of this amphiphilic copolymer system as an injectable gel for biomedical applications.

Scheme 2 shows a schematic diagram of the self-assembly and stimuli-responsive changes in the amphiphilic polyaspartamide copolymers in an aqueous solution. The mechanism of the sol-gel-sol

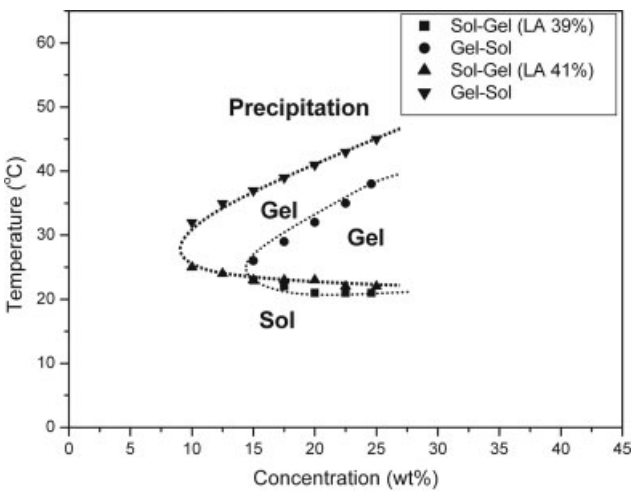
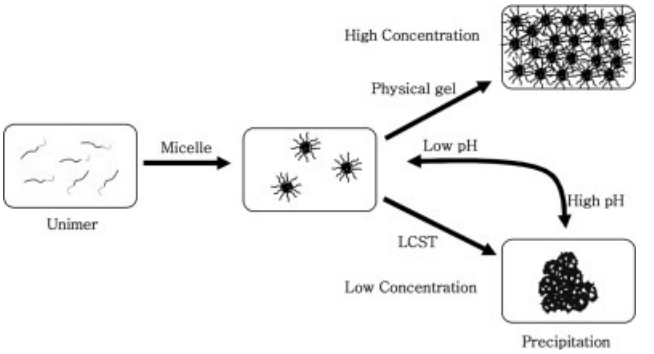


Figure 9 Sol-gel phase diagrams of amphiphilic PolyAspAm(LA/NIPEDA) solutions at various concentrations.



Scheme 2 Schematic diagram of the thermo- and pH-responsive PolyAspAm(alkyl/NIPEDA) in an aqueous solution system.

transition and phase separation of an aqueous solution is believed to be micellar expansion accompanied by an increase in the aggregation number driven by hydrophobic forces.[6] In addition, the pH-dependent phase transition was attributed to the ionization equilibrium of the basic isopropyl amine groups by varying the pH of the medium.

CONCLUSIONS

As part of an ongoing study aimed at identifying materials with both a double-responsive behavior and a sol-gel transition in aqueous solution, novel amphiphilic biodegradable copolymers based on polyaspartamide derivatives were prepared by a successive aminolysis reaction of PSI using both hydrophobic and hydrophilic groups. The LCSTs were controlled by changing the alkyl chain length, graft composition, and pH. In addition, the sol-gel transition was observed from the concentrated polymer solutions (10–25 wt) near body temperature. These novel stimuli-responsive polymers and injectable hydrogels have potential biomedical applications including targeted drug delivery and tissue engineering.

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