Self-Aggregation Behavior of Amphiphilic Polyaspartamide Derivatives Containing Cholesterol Moieties

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ABSTRACT: Biodegradable amphiphilic copolymers were successfully synthesized by the conjugation of various densities of hydrophobic biocompatible cholesterol (Chol) moieties onto poly(2-hydroxyethyl aspartamide) and poly(*N*-isopropylaminoethyl-*co*-2-hydroxyethyl aspartamide). These were obtained from polysuccinimde, the thermal polycondensation product of L-aspartic acid, via a ring-opening reaction with multifunctional pendant groups, including ethanolamine and *N*-isopropylethylenediamine (NIPEDA). Copolymers containing 5–30 mol % Chol showed self-aggregation behavior in aqueous solution, as evidenced by the dynamic light scattering measurement of their particle size distribution. The average particle size of these copolymers increased linearly with increasing Chol content. Moreover, the presence of secondary amine groups in the poly(2-hydroxyethyl aspartamide)–NIPEDA system made the conjugation more efficient; however, these also seemed to accelerate the degradation of the copolymers in an aqueous medium. The degradation behavior and pH dependence of the particle size of these copolymers in aqueous solution were also examined. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 1685–1693, 2011

Key words: biodegradable; graft copolymers; micelles; self-assembly; solution properties

INTRODUCTION

Polymeric micelles built from amphiphilic copolymers can form nanoscale self-assemblies with a hydrophobic core surrounded by a hydrophilic corona because of the intramolecular and/or intermolecular interactions of hydrophobic segments in aqueous media.[1] These materials have recently attracted much attention in the development of drug-delivery systems (DDSs) because of their nanoscale size, ability to solubilize hydrophobic drugs in large amounts, and ability to achieve site-specific delivery. Many investigations have reported that these self-assemblies, used as DDSs, can reduce unwanted toxic side effects, prolong circulation time, reduce uptake by the reticuloendothelial system, and enhance the therapeutic index of drugs.[2–5]

For DDS applications, the hydrophobic domain has a dual role: first, hydrophobicity forces the polymer molecules to assemble in an aqueous environment, and second, the hydrophobic nature can be used to entrap hydrophobic drugs; the outer hydrophilic shell prevents direct contact between the solvent and the insoluble core. The thermodynamic and kinetic stabilities of such micelles can be controlled by the mass ratio of hydrophobic and hydrophilic segments. More importantly, many stimuli-responsive groups have been introduced into polymer structures to establish smart micellar drugs carriers that show temperature, pH, electric, and enzyme sensitivities.[6–9]

Polymeric amphiphiles can be synthesized by several methods, including the copolymerization of a water-soluble monomer with a hydrophobic comonomer[10] or the attachment of hydrophobic moieties such as long alkyl chains or bulky cholesterol (Chol) derivatives to a water-soluble polymer backbone.[11,12] Among hydrophobically modified water-soluble polymers, poly(amino acid) and its derivatives have received some interest because they are degradable and pH-responsive, can be further modified, and have a high biocompatibility.[13]

Poly(aspartic acid) (PAsp) is one typical nontoxic, nonantigenic, and biodegradable poly(amino acid). It is expected to be an attractive candidate for conventional nonbiodegradable polymers in biomedical fields. The pharmaceutical importance of PAsp for the delivery of poorly soluble drugs has been reported in the literature.[14,15] PAsp modified with a

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hydrophobic moiety is a promising delivery system for poorly soluble drugs, as it entraps drugs into hydrophobic domains of aggregates; therefore, the analysis of the aggregation behavior by association of the hydrophobic part can provide useful information about the structure and physicochemical properties of aggregates for the development of delivery devices. Poly(2-hydroxyethyl aspartamide) (PHEA) is another important and well-known polymer, which can be derived by the coupling of polysuccinimide (PSI) with ethanolamine (EA). PHEA has been proposed as a potential plasma extender and carrier for macromolecular prodrugs because of its unique properties, including its biodegradability, water solubility, multipoint drug attachment, nontoxicity, nonantigenicity, and biocompatibility. Several groups have reported studies on hydrophobically modified PAsp and its derivatives. For example, Kang et al.[15] reported the self-assembly behavior of PAsp grafted with a long alkyl chain, whereas Yang et al.[16] studied PHEA modified with dehydrocholic acid and its aggregate formation.

We examined the synthesis of amphiphilic graft copolymers with poly(2-hydroxyethyl aspartamide) and cholesterol side chains (PHEA–Chol or PHEAC) in this study. Additionally, an *N*-isopropylethylenediamine (NIPEDA) group was introduced into the PHEA-Chol system because its hydrophilic nature helped reduce the overall hydrophobicity of the PHEA–Chol system. Moreover, the secondary amino groups were expected to show pH-dependent properties within this system. Both copolymer systems showed self-aggregation behavior in aqueous solution, and their physicochemical and degradable properties were also investigated.

EXPERIMENTAL

Materials and measurements

L-Aspartic acid (>98%), *o*-phosphoric acid (>98%), *N*,*N*′-dimethylformamide (DMF; anhydrous, 99.8%), chloroform (>99.8%), EA (>99%), NIPEDA (>98%), cholesterol chloroformate (CC; >97%), and triethylamine (TEA; >99.5%) were all purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification.

The ^{1}H-NMR spectra were recorded on a Bruker AMX-500 spectrometer (Karlsruhe, Germany) with deuterium oxide (D_2O) and hexadeuterated dimethyl sulfoxide (DMSO-d_6) as the solvents.

The particle size and change in the copolymer solution (1 wt %) were determined with an ELS-Z2 (Otsuka Electronics, Osaka, 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 was then filtered with a 0.45-μm pore-sized syringe filter disc to remove the oversized material after the polymer was totally dissolved.

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

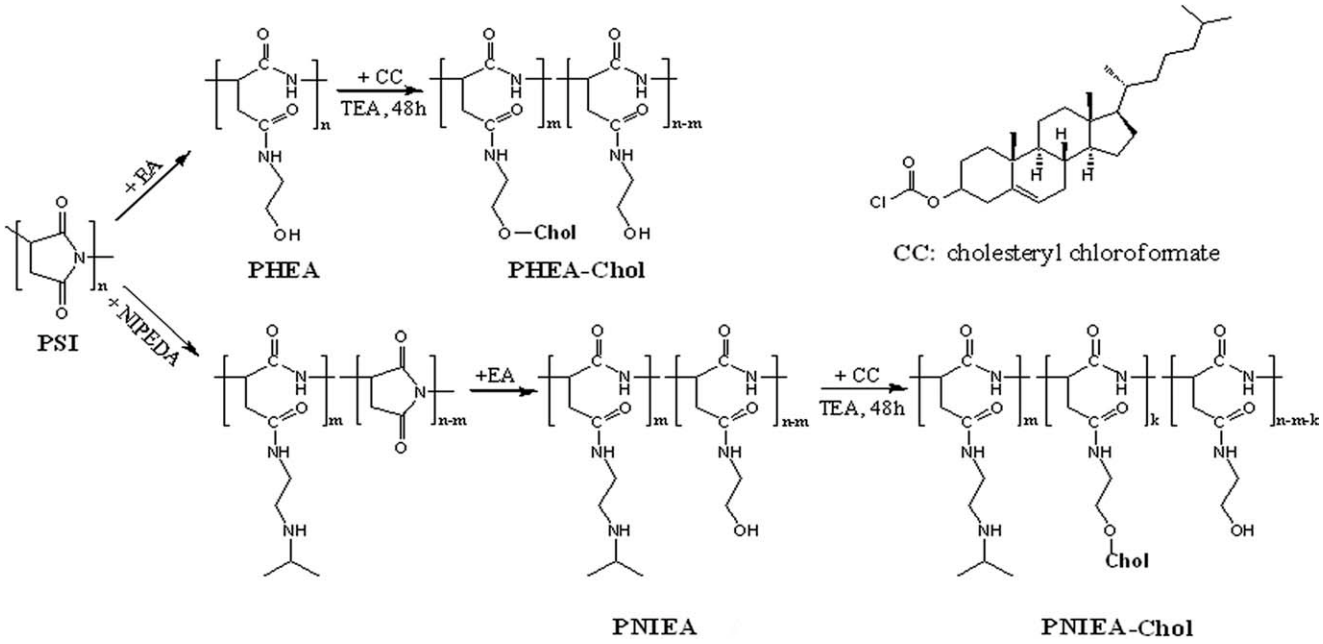
The critical micelle concentration (cmc) was determined with a fluorescence spectrometer (Aminco Bowoman Series 2; Aminco Bowoman, Urbana, IL) with pyrene as the hydrophobic fluorescence probe. The excitation spectra of pyrene with a slit width of 2.5 nm were recorded from 300 to 360 nm with an emission wavelength of 390 nm at 25°C. A stock solution of pyrene in tetrahydrofuran was poured into a phosphate buffered saline (PBS) solution containing different amounts of the polymer, where 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.

Preparation of PSI

L-Aspartic acid (20 g) and *o*-phosphoric acid (20 g) were blended and mixed at a low temperature. The mixture was put into a flask, which was immersed in an oil bath with a heat controller and connected to a rotary evaporator rotating at a speed of about 90–100 rpm. We carried out the reaction by increasing the temperature from 25 to 200°C and slowly decreasing the pressure to full vacuum. About 5 h after the maximum temperature was reached, the reaction was completed. DMF was added to dissolve the collected brown polymer (PSI). The solution was precipitated with 1.5 L of methanol and washed with a large amount of deionized water until it was neutral (pH 6–7, as measured by a pH meter). The solid residue was dried at 60°C for 2 days *in vacuo.* The prepared PSI had a reduced viscosity of 0.45 dL/g in DMF. The weight-average molecular weight of PSI was measured to be 172,000 g/mol by gel permeation chromatography with polystyrene standards and DMF as the eluent.

Synthesis of PHEA and PHEA–Chol

PSI (1 g) was dissolved in DMF in a three-necked flask, and an excess amount of EA was added dropwise (1.3 equiv of succinimide units). The reaction was kept at 30°C under continuous stirring for 12 h. The reaction mixture was precipitated in 1:1 ethanol/ethyl ether; this was followed by washing with



Scheme 1 Synthesis of PHEA–Chol and PNIEA–Chol.

copious amounts of methanol to obtain the final product, which was dried *in vacuo* for 2 days.

PHEA and different amounts of CC were dissolved in DMF and chloroform, respectively. Before mixing, TEA was added as a catalyst. The reaction was carried out under stirring at room temperature for 48 h. The mixture was precipitated and washed with copious amounts of ethyl ether. Finally, the PHEA–Chol product was dried *in vacuo* for 2 days.

The structure of PHEA and PHEA–Chol is illustrated in Scheme 1 and was confirmed by ^{1}H-NMR analysis.

Synthesis of PNIEA and PNIEA–Chol

PSI (0.5 g) was dissolved in DMF before it was added to a three-necked flask, which was placed in a water bath at 40°C. Different amounts of NIPEDA were added to the solution, which was subjected to continuous stirring for 24 h. Then, an excess amount of EA was added dropwise, and the solution was stirred for another 24 h. The product (PNIEA) was precipitated in 1:1 ethanol/ethyl ether, washed with copious amounts of methanol, and then dried *in vacuo* for 2 days.

PNIEA and different amounts of CC were dissolved in DMF and chloroform, respectively. Before they were mixed together, TEA was added as a catalyst. The reaction was carried out under stirring at room temperature for 48 h. The mixture was precipitated and washed with a large quantity of ethyl ether. Finally, the PNIEA–Chol product was dried *in vacuo* for 2 days.

The structures of PNIEA and PNIEA–Chol are illustrated in Scheme 1 and were confirmed by ^{1}H-NMR analysis.

RESULTS AND DISCUSSION

Preparation and characterization of polyaspartamides with grafted Chol moieties

Novel amphiphilic polyaspartamide derivatives with alcohol and *N*-isopropylamine pendant groups containing Chol moieties (PHEA–Chol and PNIEA–Chol) were successfully synthesized, and their structures and characteristics were confirmed by ^{1}H-NMR spectroscopy. As shown in Figure 1, the C and D peaks were characterized as the two methylene groups of the EA pendant group in PHEA. In Figure 2, peaks C, D, E, and F were assigned to the NIPEDA pendant group, and peaks G and H were related to the two methylene protons of EA. As shown in both figures, the methyl protons of Chol appeared around 0.60–0.68 ppm, that is, peak i, which was chosen for the integral calculation of the Chol content in the copolymer. (Chol had a lot of protons that overlapped each other. Only one methyl group occurred at 0.6–0.68 ppm and was observed separately from other the protons of Chol and the polymer backbone. Therefore, we chose this as the reference peak for calculating the Chol content.)

Tables I and II show the details of the chemical composition of PHEA, PNIEA, and their hydrophobically modified products, PHEAC and PNIEA–Chol (PNIEAC). All of the chemical contents were analyzed from ^{1}H-NMR spectra. As shown in Table II, the feed and real Chol contents were calculated on the basis of the number of —OH units in each copolymer. The number of cholesterol molecules per copolymer chain (CPC) was calculated on the basis of number of repeat polyaspartamide (PolyAspAm)

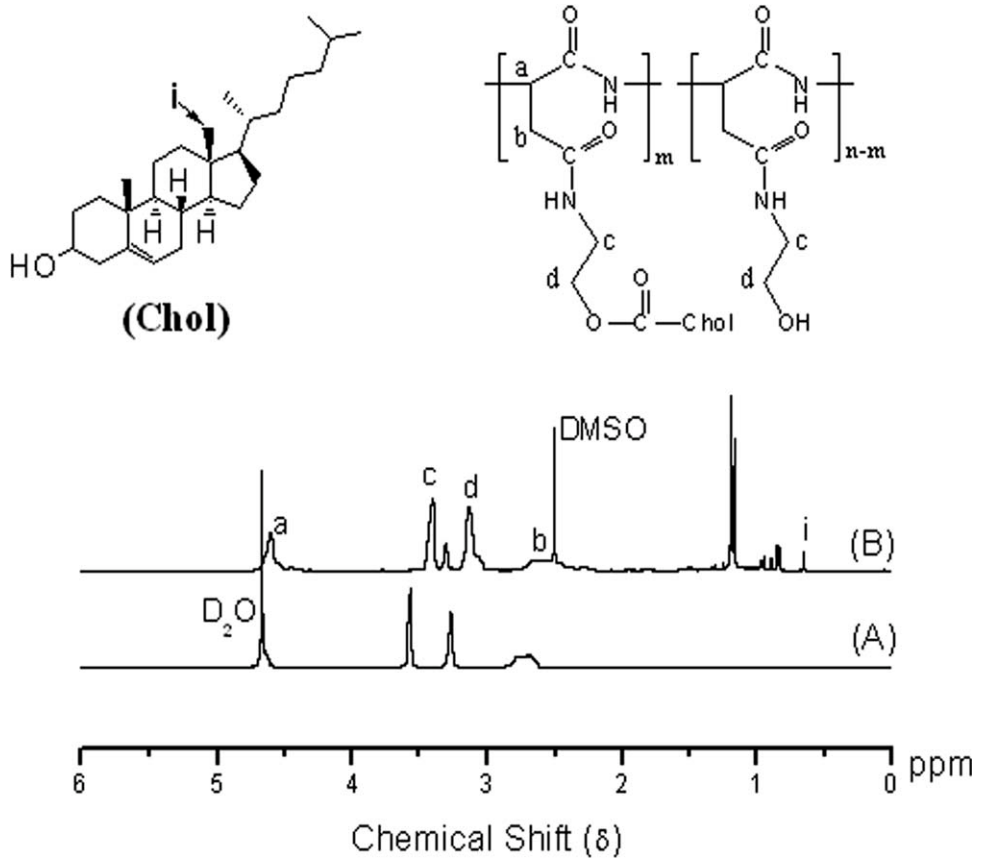


Figure 1 ^{1}H-NMR spectra of (A) PHEA and (B) PHEA–Chol.

units. The conjugation reaction gave a low yield due to (1) a difference in the hydrophilic and hydrophobic properties between the copolymer chain and the Chol pendant and (2) the high reactivity of the chloroformate group with moisture, even in the DMF solvent.[17] In the case of the PNIEA–Chol system with NIPEDA groups, the efficiency of the reaction increased, probably because of the catalytic effect of neighboring secondary amine groups.

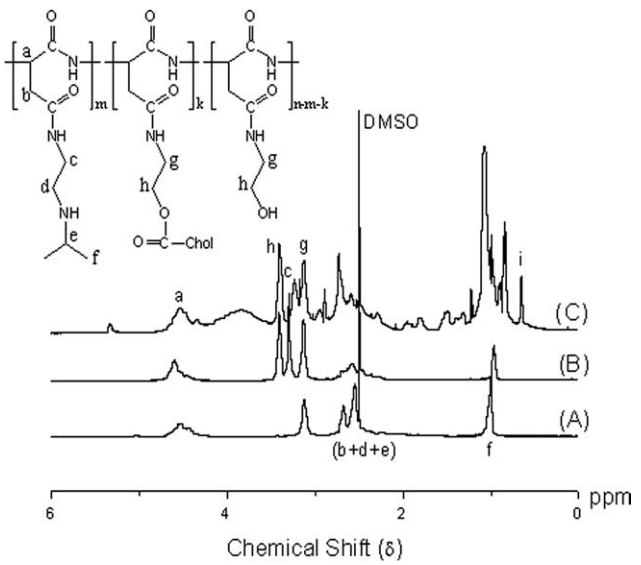


Figure 2 ^{1}H-NMR spectra of (A) PolyAspAm(NIPEDA), (B) PolyAspAm(NIPEDA/EA), and (C) PolyAspAm (NIPEDA/EA)–Chol.

Micelle formation of the polyaspartamide derivatives with grafted Chol moieties

The goal of our synthesis was to produce amphiphilic polymers containing Chol that could show micelle formation in aqueous solutions for applications as drug carriers for drugs with poor water solubility. Figures 3 and 4 show the ^{1}H-NMR spectra of PHEA–Chol and PNIEA–Chol in two different solvents, that is, D_2O and DMSO-d_6. In DMSO-d_6, the intensity of Chol protons was better than it was in the D_2O solvent. This is because, in D_2O, the copolymer formed a micelle structure with the hydrophobic core and an

TABLE I
Chemical Compositions of PHEA and PNIEA as Confirmed from ^{1}H-NMR Spectra

	EA content	NIPEDA content
PHEA	100	—
PNI30EA	70	30
PNI50EA	50	50

TABLE II
Chemical Compositions of the PHEAC and PNIEAC Samples

	Feed Chol content[a]	Real Chol content[a]	CPC	Average particle size (nm)
PHEAC10	10	1.6	20	74
PHEAC15	15	2.5	31	82
PHEAC20	20	3.9	48	125
PNI30EAC10	10	4.8	42	—
PNI30EAC15	15	6	52	186
PNI30EAC20	20	8	70	224
PNI30EAC30	30	10	87	842
PNI50EAC10	10	3.5	22	—
PNI50EAC15	15	5	31	29
PNI50EAC20	20	14	87	43
PNI50EAC30	30	16	99	118

[a] Values based on the number of —OH units in each copolymer.

outer, hydrophilic corona; this prevented some signals coming from Chol molecules. Thus, the Chol peak intensities were weaker than they appeared in the organic solvent (DMSO-d_6), in which the micelle forms did not occur.

Also, Figures 5 and 6 show the particle size distributions of PHEA and PNIEA grafted with Chol, which were analyzed by dynamic light scattering (DLS). Increasing the Chol content led to an increase in the hydrophobic core diameter; therefore, the micelle size increased when the copolymer was grafted with more Chol. With the PHEA–Chol series, the average particle size increased from 74 nm at 10% Chol to 125 nm at 20% Chol in water and showed a slight increase when the polymer was dissolved in PBS solution. Although the PNIEA–Chol series showed no micelle formation at a Chol content below 10%, above this value, the micelle size increased as the Chol content increased from 15 to 30%. On the other hand, an increase in the NIPEDA content in the copolymers from 30 to 50% resulted

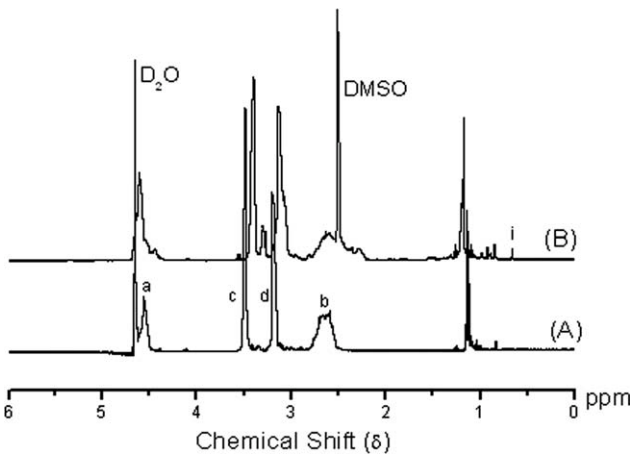


Figure 3 ^{1}H-NMR spectra of PHEA–Chol in different solvents: (A) D_2O and (B) DMSO-d_6.

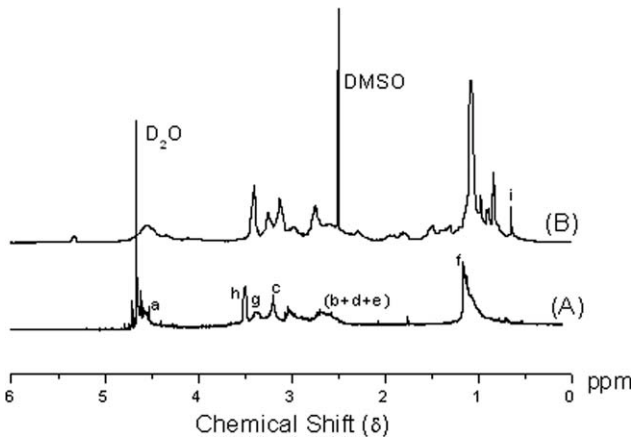


Figure 4 ^{1}H-NMR spectra of PolyAspAm(NIPEDA/EA)–Chol in different solvents: (A) D_2O and (B) DMSO-d_6.

in a decrease in the average particle size of the PNIEA–Chol system because of the enhanced hydrophilicity of the system due to the NIPEDA pendants. The PNIEA–Chol copolymer, however, was found to be unstable in aqueous systems, and these properties are discussed in more detail later.

A typical FE-SEM morphology of PHEA–Chol is shown in Figure 7 and confirms the micelle formation of the copolymer in aqueous solution. We observed that spherical particles were formed, and the size was a little smaller than that detected by DLS. This is because DLS measured the size in the hydrated state wherein the particles were surrounded by a water-swollen hydrophilic outer shell, whereas FE-SEM depicted the size of the solid sample in the dried state.

Figure 8 shows the typical cmc of the PHEA and PNIEA series grafted with Chol. The cmc is defined

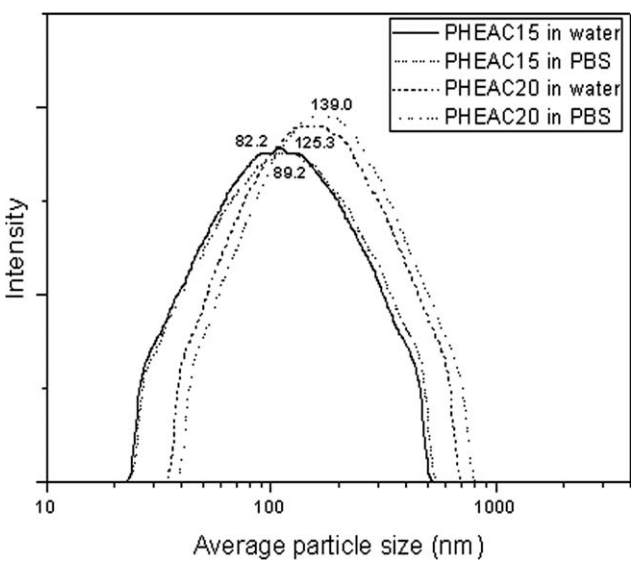


Figure 5 Particle size distribution of different contents of PHEAC in water and in PBS solution.

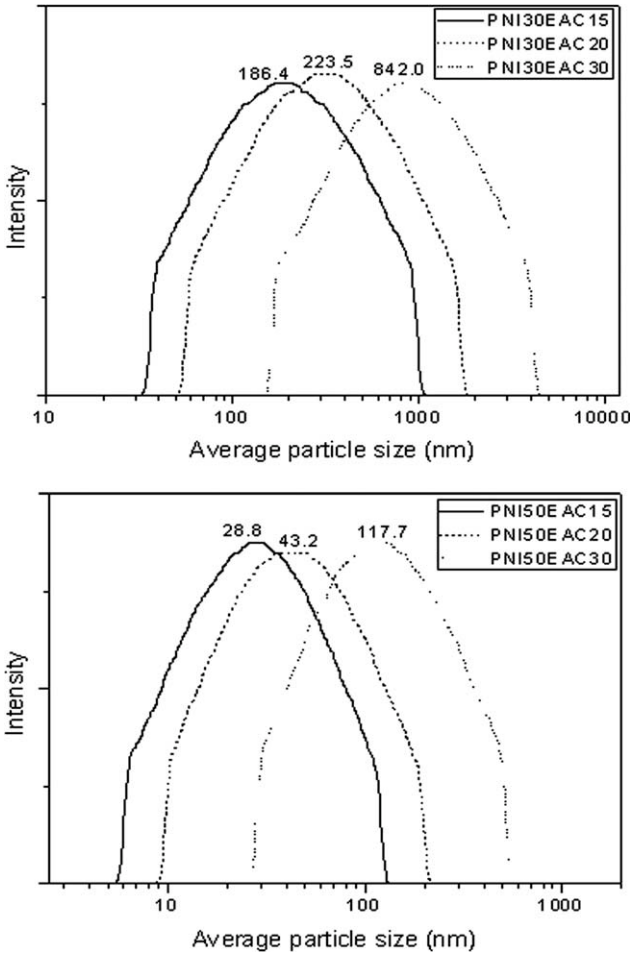


Figure 6 Particle size distribution of different contents of PNIEAC in water.

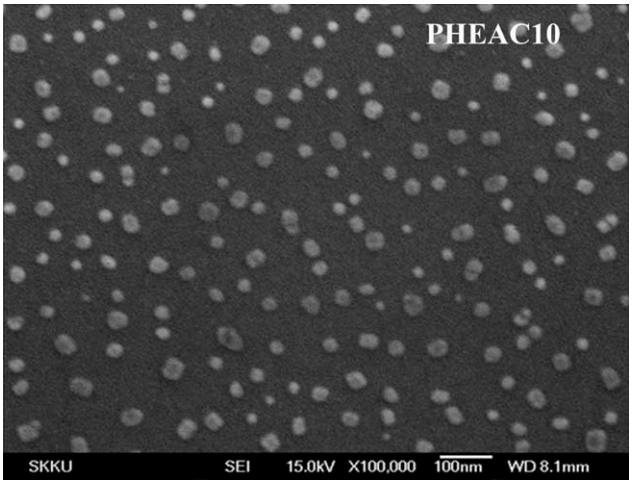


Figure 7 FE-SEM image of PHEA–Chol.

as the concentration of surfactants above which micelles are spontaneously formed. With the introduction of surfactants (or any surface active materials) into the system, they will initially partition to the interface; this reduces the system free energy by (1) lowering the energy of the interface and (2) removing the hydrophobic parts of the surfactant from contact with water. Subsequently, when the surface coverage by the surfactants increases and the surface free energy (surface tension) has decreased, the surfactants start aggregating into micelles; this, again, decreases the system free energy by decreasing the contact area of hydrophobic parts of the surfactant with water. In this study, micelle formation was monitored with pyrene as a hydrophobic probe. The cmc value of both the PHEAC and PNIEAC series was about 0.01 mg/mL, which was a reasonably low concentration; this suggested that micelles could form and remained in an extremely dilute solution.

With the presence of hydrophobic Chol moieties grafted onto the hydrophilic polymer chain, it was

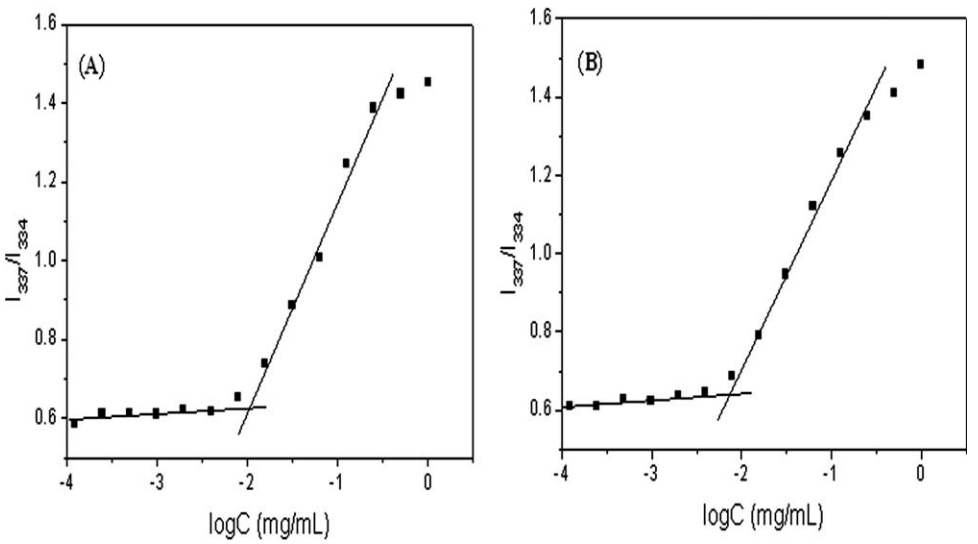


Figure 8 cmc of (A) PHEAC15 and (B) PNI30EAC15. C is the polymer concentration and I_{337}/I_{334} is intensity ratio from pyrene excitation spectra.

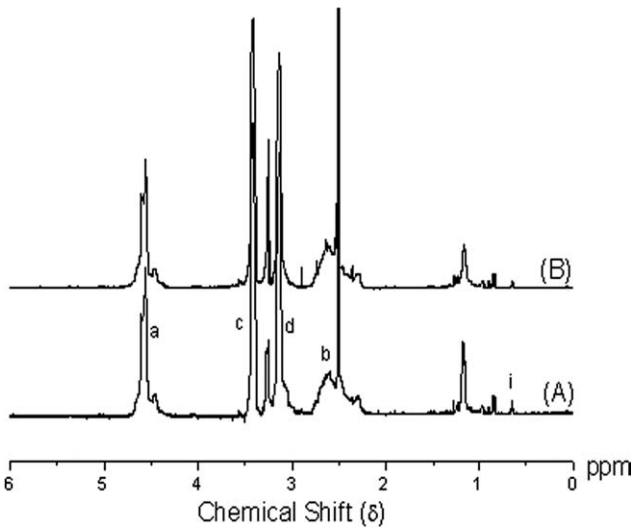


Figure 9 ^{1}H-NMR spectra of PHEAC: (A) as prepared and (B) after 2 weeks.

necessary for the two systems to undergo micelle formation to reduce interfacial energy. Thus, Chol was prevented from coming into contact with water by the polymer chains, which acted as an outer hydrophilic shell and allowed Chol to join the hydrophobic core. Therefore, a higher content of Chol in the copolymer led to micelle formation at lower concentrations and resulted in a lower cmc value.

Stability of the particles in aqueous solution

The particles of the PHEA–Chol series in water were relatively stable with a small change in the average particle size up to several weeks. After about 2 weeks, some flocks in the solution were observable by the naked eye. When they were filtered and reanalyzed, we found that very little change in size was detected by DLS (data not shown), whereas the content of Chol was slightly decreased, as evidenced by ^{1}H-NMR (Fig. 9) analysis.

Compared to the relatively stable PHEA–Chol system, the average particle size decreased quite rapidly in the PNIEA–Chol series. As we discussed previously, the presence of the *N*-isopropylamine group in PNIEA caused an increase in Chol conjugation, but on the other hand, it seemed to accelerate the hydrolysis process in aqueous media; this led to a reduction in the particle size over a short time. Changes in the particle size distribution of the PNIEA–Chol series as function of time are shown in Figure 10. The particle size of this system decreased to several 10s of nanometers over a few days, and finally, the particles could not be detected by DLS because of their low intensity. Additionally, the decreasing Chol content was confirmed by ^{1}H-NMR,

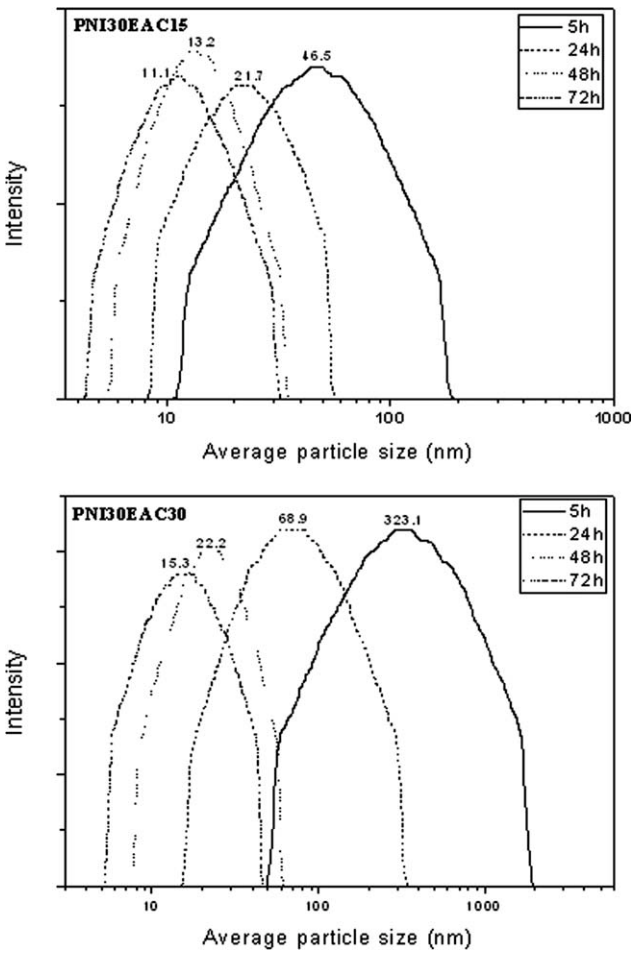


Figure 10 Change in the particle size distribution of PNI30EAC as a function of time.

and these results are shown in Figure 11. The faster degradation phenomenon in the PNIEA–Chol system may have been due to the presence of neighboring

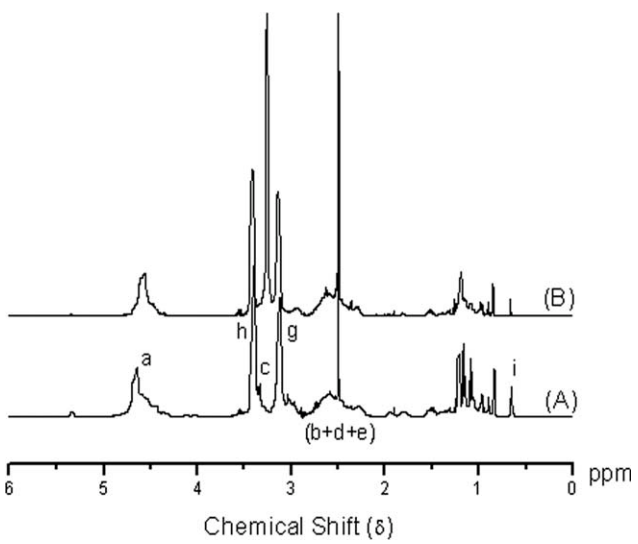
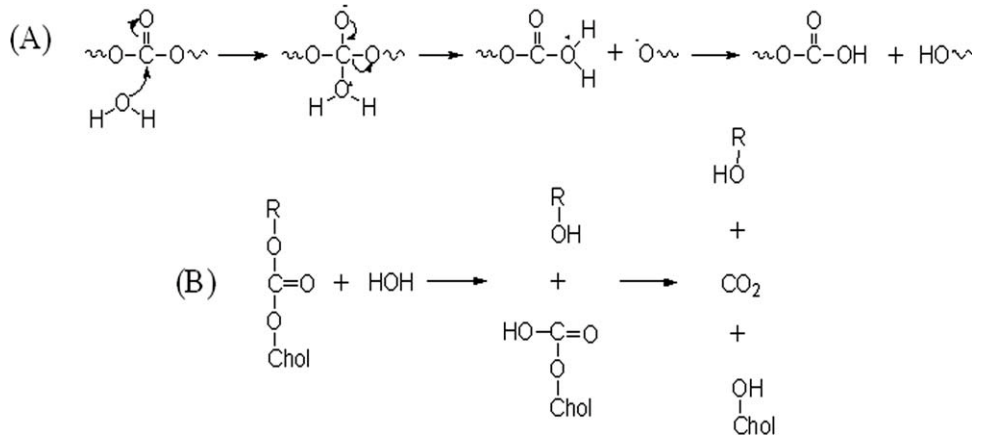


Figure 11 ^{1}H-NMR spectra of PNIEAC: (A) as prepared and (B) after 2 weeks.



Scheme 2 Mechanism of the degradation of polyaspartamide derivatives grafted with Chol moieties in aqueous solution.

secondary amine groups on the polymer, which could catalyze the hydrolytic cleavage of the carbonate linkage between the polymer and cholesteryl moiety.

The change in the average particle size, the appearance of isolated particles, and the reduced Chol content in the dissolved polymer could be

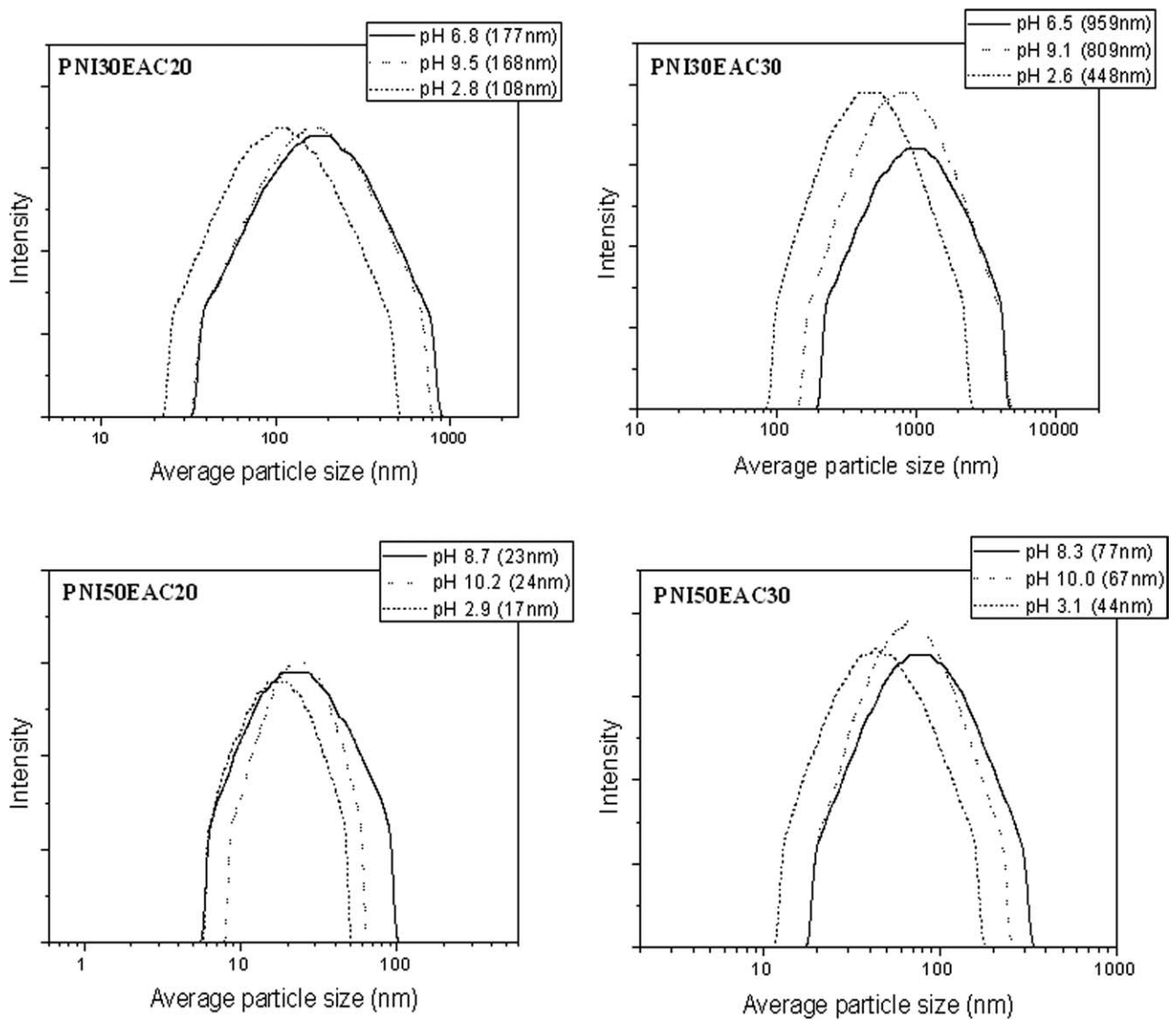


Figure 12 Particle size distribution of (A) PNI30EAC15 and (B) PNI50EAC20 at various pH values.

explained by the degradation of the polymer accompanying the detachment of the Chol moiety from the polymer backbone via the hydrolysis of carbonate linkages. This hydrolytic degradation reaction is represented in Scheme 2. The hydrolysis of the carbonate group initially gave two products containing —OH and a chemically unstable —OCOOH end group, which further decomposed to provide a stable —OH group by releasing CO_2. By this process, the original PHEA (or PNIEA) and water-insoluble Chol molecule must finally have been formed.

pH-dependent particle size distribution of the PNIEA–Chol series

In addition to the hydrophilic property of PNIEA, which increased the solubility of the PNIEA–Chol system in water, the presence of amine groups in this system resulted in a pH-dependant particle size distribution, as shown in Figure 12. All the copolymers were totally dissolved in water (1 wt % concentration), and the initial pH values were recorded; the micelle size was measured by DLS. In the next step, NaOH or HCl was added to the solution to make a more alkaline or acidic environment, and the pH values and micelle size were recorded again. As shown in the figure, the particle size in alkaline media was equivalent or a little smaller than that of the as-prepared solution. The protonation of the amine group at lower pH made the copolymer system more hydrophilic or less hydrophobic and resulted in an increased copolymer solubility or decreased average particle size of the micelles through molecular reorganization. Here, the change in the size of particles as a function of pH was more pronounced in the PNI50EAC series than in the PNI30EAC series.

CONCLUSIONS

A series of amphiphilic polyaspartamide derivatives (PHEA and PNIEA) grafted with Chol were successfully synthesized, and their self-aggregation behavior to form nanosized particles in an aqueous solution was investigated. The cmc values were in the range of 0.01 mg/mL, and the average particle size of these copolymers increased linearly with increasing Chol content. The presence of an *N*-isopropylamine moiety in the copolymer led to a higher conjugation degree of Chol moieties onto the polymer chain by the potential catalytic effect of neighboring secondary amino groups. The PNIEA–Chol copolymer system showed pH-dependency in its particle size distributions, with smaller sizes in an acidic solution than in an alkaline solution. The stability in aqueous solutions was also investigated and showed that the PNIEA–Chol system had a relatively faster degradation compared to the PHEA–Chol system. These novel amphiphilic copolymers have the potential to be used in various biomedical applications, including novel DDSs.

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