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Polymer micelle-like aggregates of novel amphiphilic biodegradable poly(asparagine) grafted with poly(caprolactone)

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Abstract

Novel amphiphilic graft copolymers composed of poly(asparagine) (PAsn) as the hydrophilic backbone and poly(caprolactone) (PCL) as the hydrophobic segment were successfully synthesized by grafting PCL–HMDs to poly(succinimde). After tosylating PCL-diol with *p*-toluenesulfonylchloride (TsCl), tosylated poly(caprolactone) (PCL–OTs) was then reacted with hexamethylenediamine (HMD). The reaction of the amine terminated PCL with poly(succinimide) (PSI) and the following aminolysis resulted in poly(aspargine)-*graft*-poly(caprolactone) (PAsn-*g*-PCL). The degree of substitution (DS) and grafting reaction was confirmed by ^{13}C NMR, FT-IR spectroscopy and elemental analysis. X-ray diffraction and DSC thermogram showed that the crystalline domain originated from PCL became apparent with an increase of DS. The amphiphilic comb-type graft polymer formed self-aggregates in aqueous solution when precipitated and dialysed against distilled water. Strong hydrophobic interaction of associated PCL grafts facilitated primary aggregate formation with DS, significantly reducing critical aggregation concentration and secondary aggregates also appeared in DLS measurements. Self-aggregates showed a bimodal size distribution originated from the self-aggregation and kinetically controlled particle aggregation, although the smaller primary aggregates was predominant. Spherical and dispersed aggregates of about 20 nm in diameter were observed by a transmission electron microscope.

Keywords: Poly(caprolactone); Poly(asparagine); Self-aggregates

1. Introduction

Micelle-like aggregates formed with amphiphilic copolymers have been recently receiving much attention as carriers for hydrophobic drugs [1–4]. The amphiphilic block or graft copolymers have been found to form self-assemblies, nano-sized micelle-like aggregates of various morphologies in aqueous solution. Their hydrophobic parts form the core of a micelle as an incorporation site of lipophilic drugs, while the hydrophilic corona or outer shell plays a role in avoiding the uptake by reticuloendothelial systems. Further, these nano-sized aggregates have the advantages of having a fairy narrow size distribution, a low critical aggregation concentration (CAC), a slow rate of dissociation, and a high drug-loading capacity in biotechnological and pharmaceutical applications.

Several block and graft copolymers have been employed, such as poly(lactic acid), poly(caprolactone), poly(styrene), benzyl esters of poly(aspartic acid) and poly(glutamic acid) as hydrophobic segments [5–9], and as poly(ethylene glycol), poly(acrylic acid), and poly(*N*-isopropylacrylamide) as hydrophilic segments [5,6,10]. In our previous works, poly(aspartic acid)-based amphiphilic copolymers and their self-aggregates such as of poly(aspartic acid)-*g*-alkyl [2,11,12] and poly(hydroxyethylaspartamide)-*g*-dehydroxycholic acid (PHEA-*g*-DHA) [13], and proteinoid-cholesterol [14] were synthesized by acid-catalyzed thermal polycondensation of L-aspartic acid via poly(succinimide) (PSI), which produced a regular poly(aspartic acid) backbone of high molecular weight and complete biodegradability [15–17]. Poly(aspartic acid) has fully biodegradable, water-soluble properties and toxicological suitability such as lack of toxicity, antigenicity, and immunogenicity [18].

In this paper, we report the synthesis of a new

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amphiphilic graft copolymer of poly(asparagine) (PAsn) as a backbone and poly(caprolactone) (PCL), a semi-crystalline biodegradable polymer [19–23] as grafted hydrophobic chains. This PAsn has an amide linkage, which is fully biodegradable, water soluble polymer and can be used as drug carriers, while few studies have been reported on poly(asparagine) among these poly(amino acid)s. Its characteristics as a graft copolymer were determined by various analytical techniques including gel permeation chromatography, elemental analysis (EA), differential scanning calorimeter and X-ray diffractometry. Also, the effect of the degree of substitution (DS) of PCL on the self-association properties of PAsn-*g*-PCL in aqueous solution will be discussed. The stable self-aggregates were prepared by a precipitation and dialysis method and were observed with DLS, fluorescence and transmission electron microscope (TEM).

2. Experiment

2.1. Materials

L-aspartic acid (Sigma), PCL-diol (mol. wt. 1250, Polyscience, Inc), *p*-toluene-sulfonylchloride (TsCl; Aldrich), hexamethylene diamine (HMD; Sigma), triethylamine (Et_3N; Sigma), trimethylamine hydrochloride ($Me_3N{\cdot}HCl$; Aldrich), ammonium hydroxide (Aldrich), and ethanolamine (Sigma) were used as purchased. Mesitylene (Aldrich), phosphoric acid (85%, Junsei), sulfolane (Aldrich), tetrahydrofuran (THF; Aldrich) and dimethyl sulfoxide (DMSO; Junsei) were used without further purification. *N*,*N*-Dimethylformamide (DMF; Aldrich) was dried over Molecular Sieve 4 Å before use. The dimethyl sulfoxide-d_6 (DMSO-d_6) used for NMR experiments was received from Aldrich.

2.2. Synthesis of precursors; PSI and modified PCL-diol

All synthesis routs are shown in Fig. 1. A suspension of L-aspartic acid (25 g, 0.188 mol) and an acid catalyst (phosphoric acid: 9.4 mmol) in the solvent (100 g: mesitylene/sulfolane ratio of 7/3) was refluxed at 180 °C under N_2 atmosphere. Water generated in the reaction mixture was continuously removed using a Dean-stark trap with a reflux condenser. After 10 h, the reaction mixture was precipitated in excess methanol and then washed with water until it was neutral. The precipitate, PSI was washed with methanol and dried at 80 °C in vacuo. The structure of PSI ($M_w = 51,520$) was confirmed by ^1H NMR analysis. The peaks at 2.7, 3.2 and 5.3 ppm were assigned to the methylene and methine protons of the succinimide unit, respectively.

Most of alcohols are smoothly tosylated through the *p*-toluenesulfonylation (tosylation) process at 0–5 °C within 1 h [24,25] while poly(caprolactone) was tosylated at 18–20 °C for 1 day. A slightly excess amount of TsCl was reacted with PCL-diol to obtain monotosylated PCL. PCL-diol was tosylated using a mixture of TsCl/Et_3N/catalytic $Me_3N{\cdot}HCl$ as the reagent. TsCl (12 mmol) in THF (20 ml) was dropwisely added to a stirred solution of PCL-diol (12 mmol), Et_3N (60 mmol) and $Me_3N{\cdot}HCl$ (1.2 mmol) in THF (20 ml) at 18–20 °C, and the mixture was stirred for 1 day. The reaction process was monitored by TLC (ethyl acetate/hexane = 1:3 by volume). Insoluble products were filtered out and the clear reaction mixture was poured into a bath of excess ethyl ether at 0 °C and then the precipitated product, poly(caprolactone)–toluenesulfonylchloride (PCL–OTs) was dried in *vacuo*. Subsequently, HMD (0.25 g) was dissolved in DMF (10 ml), and synthesized PCL–OTs (2.0 g) was dissolved in DMF (20 ml). The PCL–OTs solution was slowly dropped into the HMD solution [26]. The mixture was stirred for 6 h at 18–20 °C, and precipitates, poly(caprolactone)–hexamethylenediamine (PCL–HMD) washed with ethyl ether, and dried in vacuo.

2.3. Synthesis of PAsn grafted with PCL

Synthesized PCL–HMD (0.6 g) in 15 ml DMF was added to the solution of PSI (0.43 g) in DMF (10 ml) [15] under stirring at 60 °C. The reaction mixture was stirred at 60 °C for 2 days and slowly cooled to room temperature. After insoluble products were filtered out, the solution was added dropwise to a 5.07N NH_4OH solution (0.84 ml) to aminolyze the residual succinimide unit of PSI. After stirring for 5 h at room temperature, the reaction mixture was precipitated in excess ethyl ether. The precipitate, poly(asparagine)-*g*-poly(caprolactone) (PAsn-*g*-PCL) was filtered and then dried in vacuo.

2.4. Preparation of self-aggregates

The stable solution of self-aggregates, PAsn-*g*-PCL (10 mg) was prepared when water was added to the DMSO solution of PAsn-*g*-PCL. First, the copolymer was dissolved in a common solvent (DMSO, 1.0 ml) for both segments and then water (10 ml), which is a precipitant for PCL segments but a good solvent for PAsn segments, was added to induce the aggregation of graft copolymers. The mixed solution of graft copolymer was stirred and then dialyzed for 2 days against distilled water using a dialysis membrane (MWCO = 8000–12,000 g/mol).

2.5. Measurement methods

^1H NMR and ^{13}C NMR spectra were measured on a Bruker AMX500 spectrometer (Germany). Samples were dissolved in DMSO-d_6 or $CDCl_3$ for NMR analysis. *Infrared spectra* were measured on a Shimadzu FTIR 8000 Series spectrophotometer. The molecular weight of PSI was determined by a *gel permeation chromatography* (GPC) equipped with Ultrahydrogel Linear and Ultrahydrogel 120

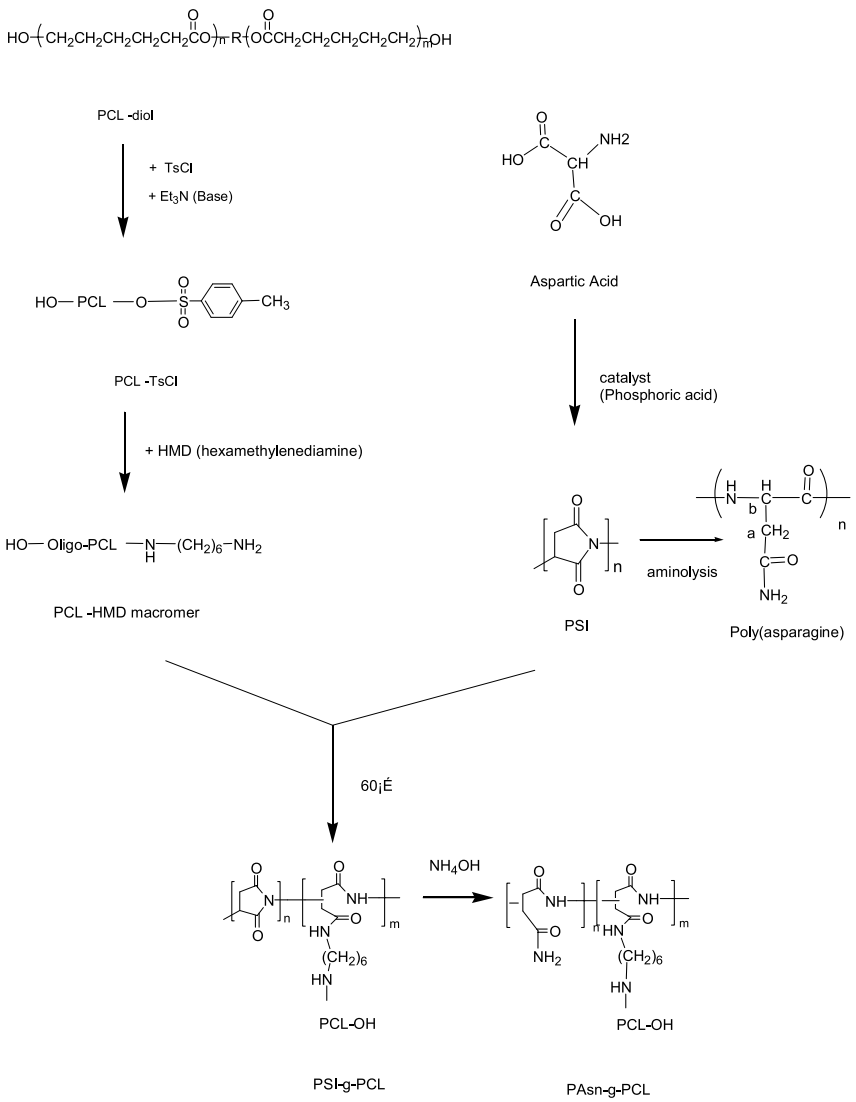

Fig. 1. The overall synthetic scheme of PAsn-*g*-PCL from L-aspartic acid and PCL-diol.

columns in series. The eluent was DMF containing 20 mmol/l of LiBr and the flow rate was 1.0 ml/min. The GPC was calibrated using standard samples of polystyrene (P-82, Shodex). *X-ray diffraction analysis* was carried out using a Rigaku D/max-RB apparatus Cu K_α source ($\lambda = 0.154$ nm) with the powder samples. The thermal properties were investigated by using *differential scanning calorimetry* (DSC) with Thermal Analyzer 2000 (DuPont) at a heating rate of 10 °C/min under nitrogen. The size distribution was measured by a *dynamic light scattering* (DLS, Brookhaven Instruments Inc.) equipped with a He–Ne laser at a scattering angle of 90° (by the Stokes–Einstein relationship). The particle size and size distribution were calculated using NNLS (non-negative least squares) algorithms. For measuring CAC, *steady-state fluorescence spectra* were measured using a Perkin–Elmer luminescence spectrometer with a bandwidth of 2.5 nm for excitation and emission [4]. The morphology of the self-aggregates was observed using a *transmission electron microscopy* (TEM). A drop of self-aggregates solution containing 0.1% phosphotungstic acid (PTA, negative staining) was placed on a copper grid coated with carbon film. The grid was held horizontally for 30 s to allow the aggregates to settle and then vertically to allow excess fluid to drain. Observation was carried out at 80 kV with Philips CM200.

3. Results and discussion

3.1. Synthesis and characterization

Synthesis of PAsn-g-PCL. The overall synthetic scheme is shown in Fig. 1. A graft copolymer, PAsn-*g*-PCL, was synthesized in three steps. After synthesizing precursors of PCL–OTs, PCL–HMD, and PSI, the PCL was grafted in a form of PSI-*g*-PCL, and then aminolyzed the ungrafted sites to PAsn-*g*-PCL. In the first step, the tosylated PCL was synthesized with PCL-diol and TsCl. The reaction conditions

and results of tosylation are summarized in Table 1. All trials were conducted at the fixed PCL-diol/TsCl molar ratio (1:1). DMF as solvent (trials 5–8) retarded the tosylating reaction. As shown Table 1, trial 4 resulted in the optimum reaction condition of tosylation using the TsCl/Et_3N/catalytic Me_3N·HCl. As the tosylating reaction (in trial 4) proceeds, the products were monitored by TLC. The R_f value on silica (ethyl acetate/hexane = 1:3 by volume) was 0.67 in the initial stage of the reaction for unreacted TsCl, but 0.05 in the final stage for PCL–OTs. TLC monitoring offers that the tosylation of PCL-diol was completed within 24 h. It is confirmed that only one TsCl was attached to one end of PCL-diol by NMR analysis. In the second step, PCL–HMD was easily prepared with HMD and PCL–OTs. During the final step, it was possible to graft the PCL–HMD with PSI. Thus, PCL–HMD had a relatively higher efficiency of graft reaction in PAsn-*g*-PCL synthesis than the direct grafting of PCL-diol did. Fig. 2 shows that the grafting efficiencies of PCL–HMD were doubled of PCL-diol at a fixed feed mole ratio.

Characterization of PAsn-g-PCL. The FT-IR spectrum of PCL-diol showed a strong hydroxyl peak around 3500 cm^{-1} and a very sharp peak at 1700 cm^{-1} for the ester groups of the PCL repeat unit (Fig. 3(a)). When PCL-diol reacted with TsCl, the hydroxyl peak around 3500 cm^{-1} decreased in PCL–OTs and at the same time, two sharp new peaks appeared at 1600 cm^{-1} for the backbone vibration of the benzene ring and at 694 cm^{-1} for the bending vibration of the benzene ring (Ts groups of PCL–HMD). In the FT-IR spectrum of PCL–HMD, the benzene ring peak disappeared. At the same time, a new broad band for the amide ($-NH_2$ in primary amides) groups appeared around 3300 cm^{-1}. These results indicate that the PCL hydroxyl end groups are changed to amide groups. As shown in Fig. 3(b), the FT-IR spectrum of PAsn-*g*-PCL shows a peak at around 1700 cm^{-1} for the ester groups of the PCL repeat unit, as well as the carbonyl peak ($-CONH_2$ of PAsn) at around 1650 cm^{-1}, while the peak for the hydroxyl groups of PCL at 3500 cm^{-1} overlapped with the

Table 1
Tosylation of PCL-diol with *p*-toluenesulfonylchloride

$$HO-PCL-OH \xrightarrow[24\ h]{room\ temp.} HO-PCL-OTs$$

Trial	PCL-diol (equiv)	TsCl	Et_3N	Me_3N·HCl	Solvent	Yield[a] (%)
1	1.0	1.0	1.0		THF	40
2	1.0	1.0	1.0	0.1	THF	51
3	1.0	1.0	5.0		THF	72
4	1.0	1.0	5.0	0.1	THF	85
5	1.0	1.0	1.0		DMF	18
6	1.0	1.0	1.0	0.1	DMF	37
7	1.0	1.0	5.0		DMF	46
8	1.0	1.0	5.0	0.1	DMF	53

All reaction were carried out at 18 °C for 24 h.
[a] Tosylate yield was obtained by ^1H NMR peak assignments.

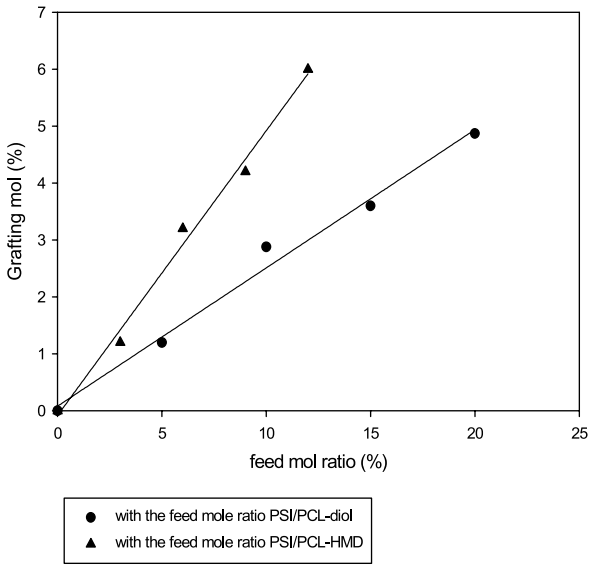

Fig. 2. DS with the feed mol ratio PCL-diol/PSI and PCL–HMD/PSI. All reactions were carried out at 60 °C for 2 days.

peak of the PAsn amide groups. Fig. 4(a) shows a typical ^{13}C NMR spectrum of PAsn and PCL-diol in DMSO-d_6. PSI was easily aminolyzed in ammonium hydroxide for conversion into PAsn. At ^{13}C NMR spectrum of PAsn, the

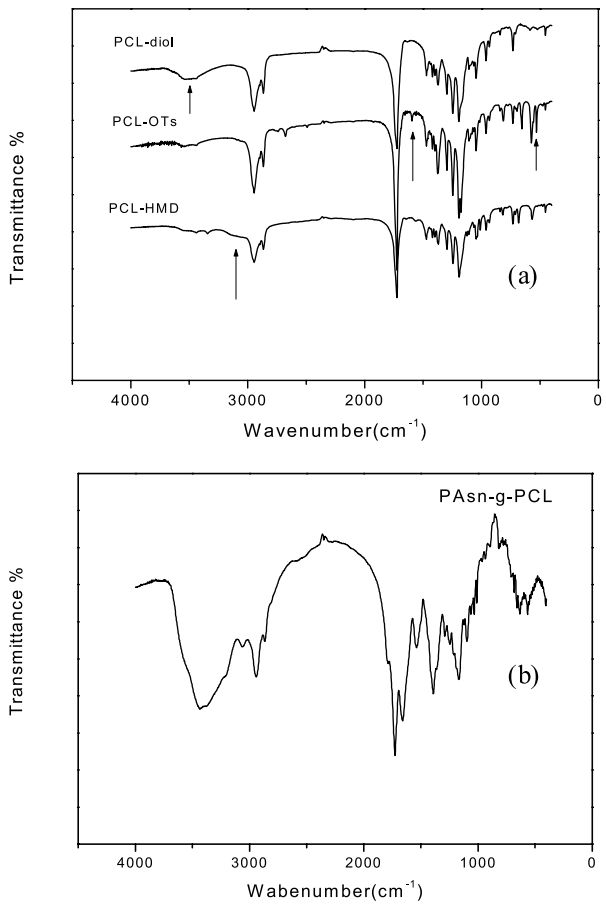

Fig. 3. The FTIR spectrum of PCL, PCL–OTs, PCL–HMD and PAsn-*g*-PCL.

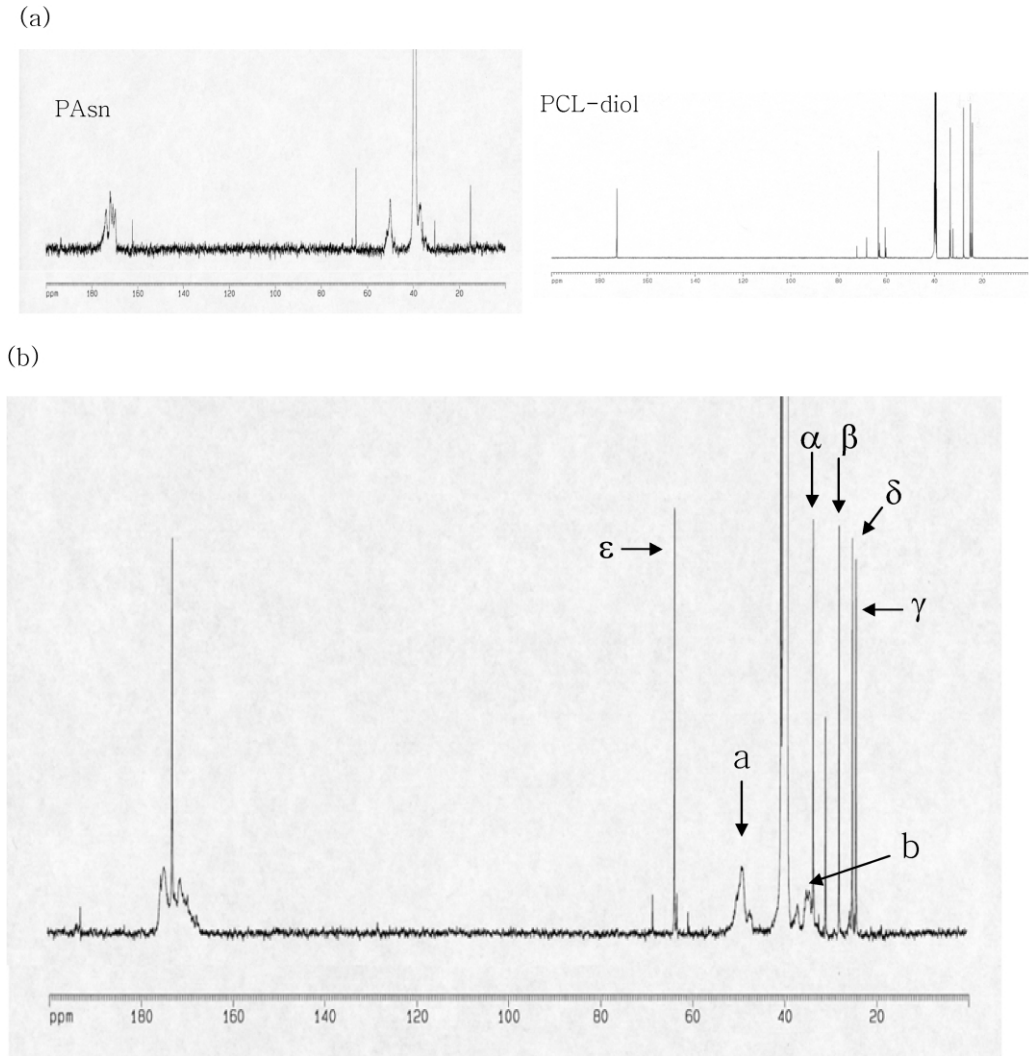

Fig. 4. ^{13}C NMR spectra of (a) PAsn and PCL-diol (b) ^{13}C NMR spectra series of PAsn-*g*-PCL (DS: 1.2%).

methylene and methine carbons of the succinimide unit disappeared and the chemical shifts at 37 and 50 ppm were assigned to the methylene and methine carbons of the asparagine unit, respectively. The carbonyl peaks are observed at 170–173 ppm. The ^{13}C NMR spectrum of PAsn-*g*-PCL was shown in Fig. 4(b) in comparison with that of PAsn and PCL-diol (Fig. 4(a)). Peaks at 172.8, 33.29, 24.82, 23.97, 27.74 and 63.41 ppm were assigned to methylene carbon in PCL-diol unit, respectively, while peaks at 30.79, 35.80 and 170–173 ppm (marked with symbols) were assigned to methylene, methine and carbonyl carbone in the PAsn unit, respectively.

3.2. Microstructures and properties

The molecular structure of PAsn-*g*-PCL, i.e. DS, was determined with the peak intensity in ^{13}C NMR spectra and the weight percentage of the element in EA. DS can be controlled by grafting of PCL–HMD onto the PAsn backbone. DS is the mole percent of the grafting unit with PCL per total succincimide unit. The DS in the graft copolymers was calculated from the integral signal area of ^{13}C NMR peaks and the weight percentage of EA. Table 2 shows DS (1.2, 3.2, 4.2 and 6.0%) of the PAsn-*g*-PCL series determined by only EA.

The relationship between crystallinity and composition was studied by X-ray diffraction and DSC measurements. Fig. 5 shows the X-ray diffraction patterns of PAsn-*g*-PCL with a typical X-ray diffraction pattern of PCL-diol and PAsn. The orthorhombic crystalline structure of PCL-diol is involved in the diffraction pattern [27,28]. The *d*-spacings of PCL-diol are 0.4179 (nm) (110 plane) and 0.3777 (nm) (200 plane). The WAXD pattern of PCL-diol has major peaks at $2\theta = 21.24$, 21.85 and 23.53°, but that of PAsn has not. As shown in Fig. 5(b) which depicts the PAsn-*g*-PCL WAXD data, the peaks at $2\theta = 21.24$ and 23.53° increased as PCL contents increased because the crystalline domain of PCL increased. The crystalline domain fraction, X_c(%) of copolymers calculated from X-ray data is shown in Table 3. From the WAXD data, it is proven that the

Table 2
EA data (%)

Sample identification	PAsn-*g*-PCL1	PAsn-*g*-PCL2	PAsn-*g*-PCL3	PAsn-*g*-PCL4
N (wt%)	16.7738	14.8604	14.3993	11.9978
C (wt%)	44.7394	47.9117	49.1552	50.8204
H (wt%)	6.3835	6.6793	6.8276	6.4318
DS[a] (mol%)	1.2	3.2	4.2	6.0

[a] Weight ratio of carbon (measurement) $= \frac{\text{carbon\_weight}}{\text{total\_weight}}$(theoretical).

Table 3
DS of PCL in PAsn-*g*-PCL

	Feed mole ratio[a]	DS[b]	Weight fraction[c]	X_c (%)[d]
PAsn	100/0	–	–	–
Pasn-*g*-PCL1	97/3	1.2	13.53	0.50
PAsn-*g*-PCL2	94/6	3.2	29.87	3.67
PAsn-*g*-PCL3	91/9	4.2	36.10	6.14
PAsn-*g*-PCL4	88/12	6.0	45.13	10.83

[a] Succinimide unit/PCL–HMD.
[b] DS determined by EA.
[c] Weight of PCL/(weight of PCL + weight of PAsn).
[d] X_c (%) calculated with WXRD data.

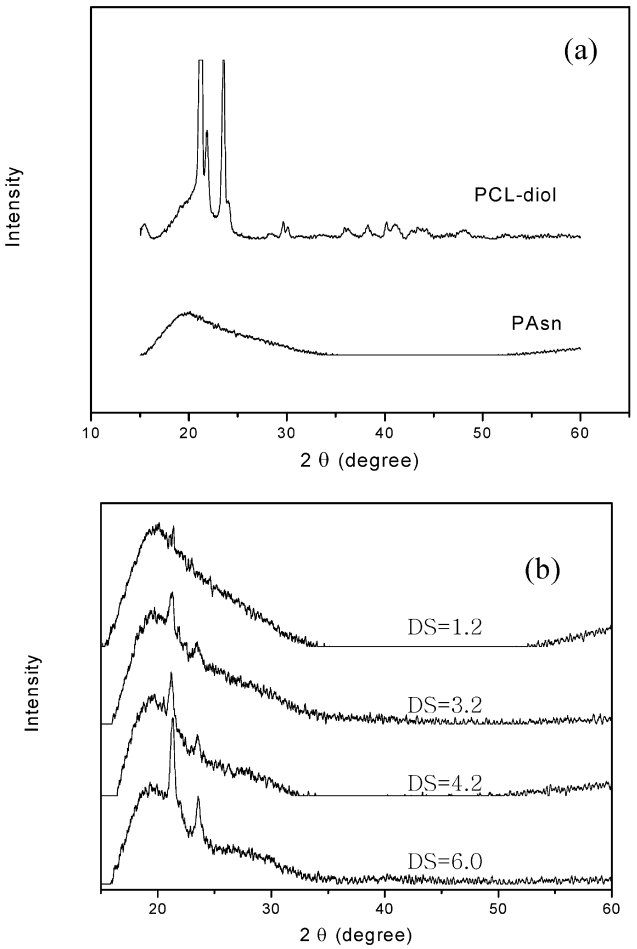

Fig. 5. X-ray diffraction patterns of (a) PCL-diol and PAsn (b) PAsn-*g*-PCL.

PAsn-*g*-PCL graft copolymer contained two kinds of structure, the crystalline structure of PCL and the amorphous structure of PAsn.

The thermal properties of PAsn-*g*-PCL were investigated with DSC, since PAsn has only a T_g in low temperature due to its amorphous structure. The DSC thermogram of PAsn-*g*-PCL was scanned from room temperature to 100 °C as shown in Fig. 6(b). Fig. 6(b) shows a melting temperature (T_m) detected around 35–45 °C that corresponds to the melting temperature of the PCL crystalline phase. Poly(caprolactone) (PCL) is a synthetic semi-crystalline polymer having a melting point (T_m) of ~60 °C and a glass transition temperature (T_g) of −60 °C [19]. Fig. 6 shows that T_m and ΔH_m apparently increases as the DS increased. Therefore, it is consistent with WXRD data that the crystallinity of the PAsn-*g*-PCL

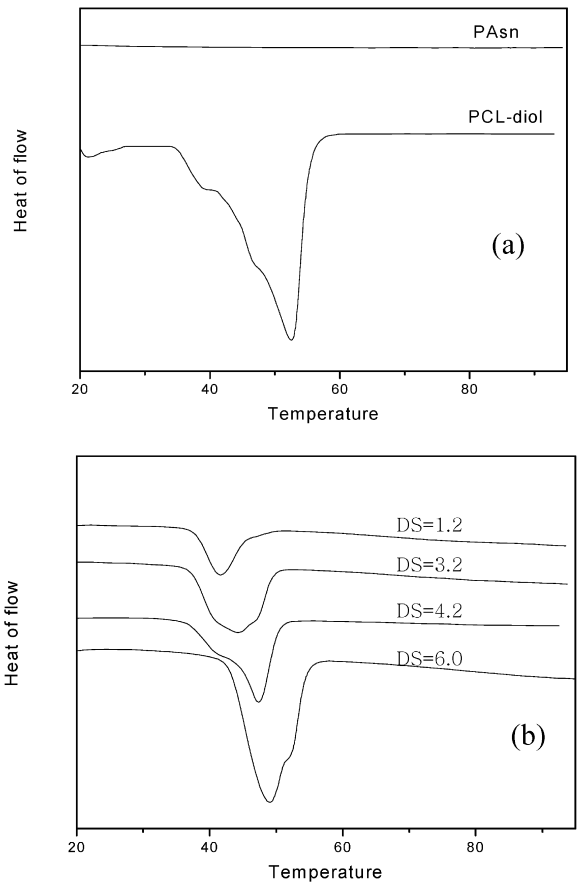

Fig. 6. DSC thermograms of (a) PAsn, PCL-diol and (b) PAsn-*g*-PCL.

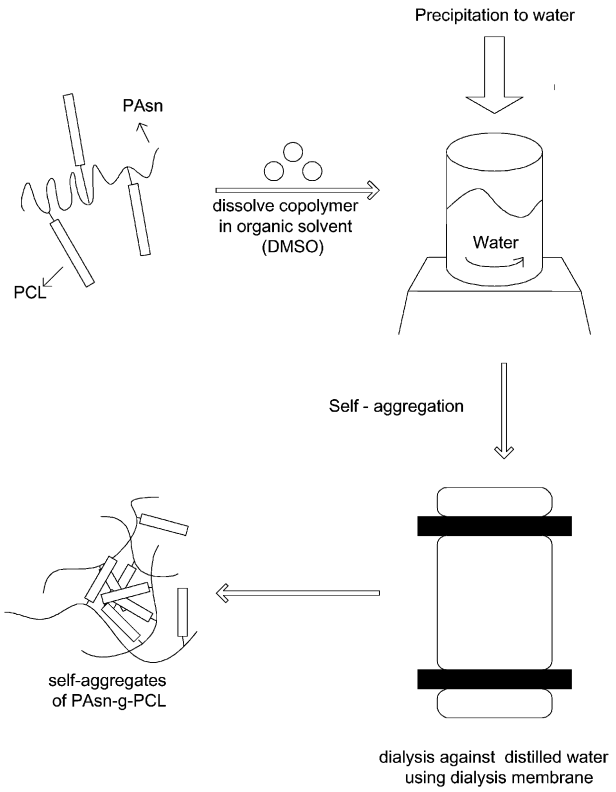

Fig. 7. A schematic of 'the precipitation and dialysis method' of aggregates preparation.

series became apparent with the increasing DS. Furthermore, it is apparent that the spatial confinement of the PCL graft facilitated the crystallization of PCL domains with neighboring PCL in the PAsn backbone.

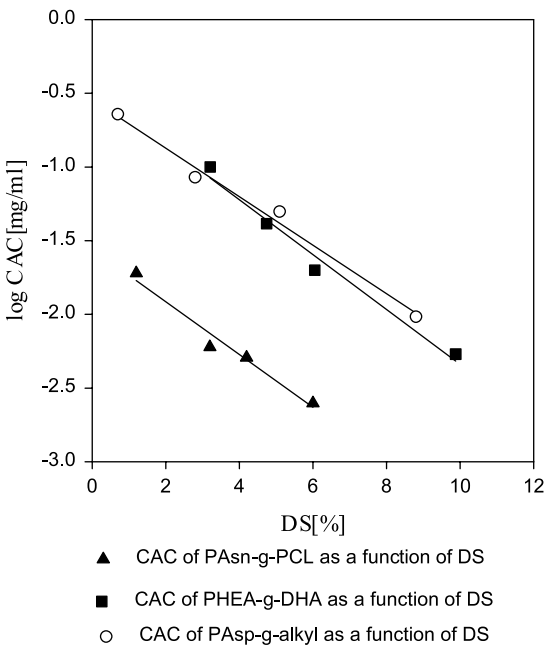

Fig. 8. CAC of polyaminoacids grafted with hydrophobic moieties determined by emission fluorescence spectra of pyrene [4,13] The triangle represents this work.

3.3. Self-aggregates

The amphiphilic graft copolymer forms self-aggregates in aqueous solution. PAsn-*g*-PCL aggregates were carefully prepared by 'the precipitation and dialysis method' (Fig. 7). The CACs, determined by an emission fluorescence spectroscopy of pyrene [4,13] were 19, 6, 5.1, and 2.5 mg/l for DS, 1.2, 3.2, 4.2 and 6.0, respectively. The CAC decrease as the grafting density of PCL groups increases, similar to how the increasing hydrophobicity of low molecular weight surfactants reduces the CMC value. Fig. 8 shows the CAC of three polyaminoacid-graft copolymers of PAsp-*g*-alkyl [4], PHEA-*g*-DHA [13] and PAsn-*g*-PCL. In fact, the CACs of PAsn-*g*-PCL are as small as 1/10 of the others. Since the PCL side chain forms a crystalline phase, it is expected that the graft copolymer may have a small CAC. On the other hand, the backbone linkage of the polymer originated with the same polysuccinimide condensed by aspartic acid. Though direct comparison of these polyaminoacids is not possible, the ring opening with different functional groups will produce different interactions with water; The acid and hydroxyl group in the side chain of aminoacids, for example, is more hydrophilic than the amine group is.

The DLS measurements showed a bimodal size distribution as shown in Fig. 9(a). The small one is located near 20–30 nm, while the large one varies between 100 and 200 nm. The large particles may be secondary aggregates

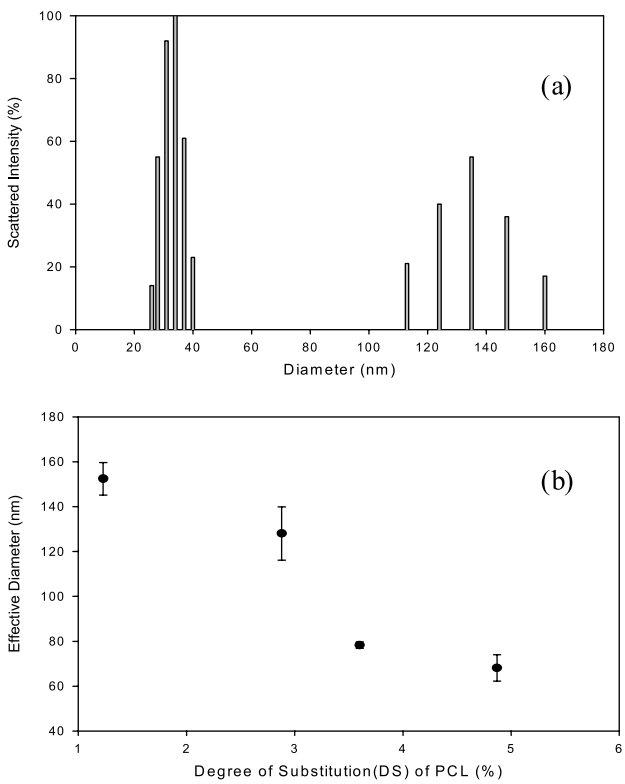

Fig. 9. Particle size distribution. (a) Bimodal size distribution of PAsn-*g*-PCL. (b) Effective diameter as a function of DS.

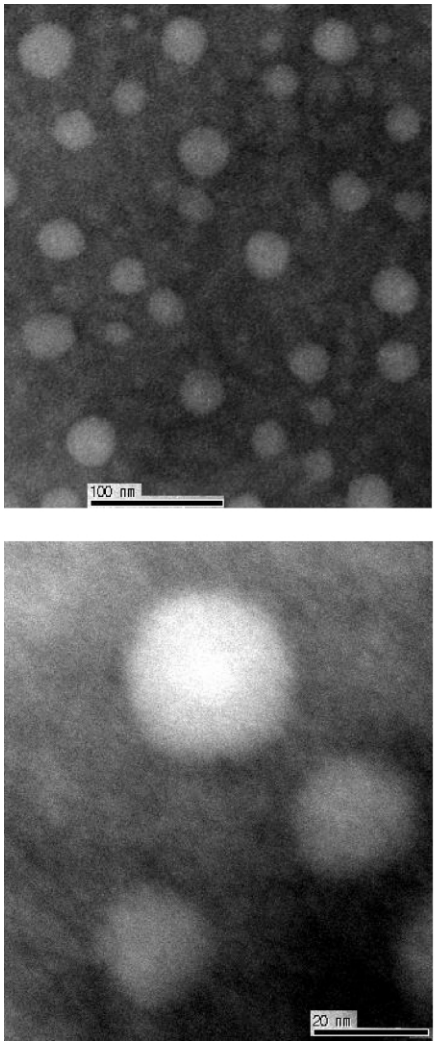

Fig. 10. TEM photographs of PAsn-*g*-PCL4 self-aggregates (DS = 6.0%, 0.1% w/w) with negative staining (PTA 0.1%) (a) magnified by 230K (b) magnified by 1000K.

from primary small aggregates. Alternatively, the precipitation and dialysis of the polymer solution may force the aggregate to form under the non-equilibrium condition because of the long chain of hydrophobic PCL. Interestingly, Fig. 9(b) showed that the effective diameters were reduced as DS increased apparently because of the strong hydrophobic interaction of PCL. Such a reduction in size has been observed when alkyl or DHA were grafted [4, 11–13]. This indicates that the self-association of the graft copolymer is affected by the DS of PCL groups; It is believed that the graft copolymer having a higher DS, forms smaller aggregates, due to stronger hydrophobic interactions of grafted PCL groups with increases in the packing density of PCL groups in self-aggregates.

Fig. 10 shows the transmission electron micrograph of the PAsn-*g*-PCL 4 (DS = 6.0%, 0.1% w/w) with negative staining (PTA 0.1%). The picture clearly indicates a spherical shape of self-aggregates as light parts surrounded by the negative staining.

4. Conclusion

Novel amphiphilic biodegradable graft copolymer, PAsn-*g*-PCL was synthesized with a relatively high yield through modified PCL-diol via controlled grafting of PCL–HMD to PAsn backbone. The grafting copolymers with the DS of PCL exhibited two domains of crystalline and amorphous phase by WAXD and DSC measurements. The crystalline domain of PAsn-*g*-PCL graft copolymer increased with DS. The graft copolymers formed self-aggregates in aqueous solution through the precipitation and dialysis method. Strong hydrophobic interaction of associated PCL grafts facilitated primary aggregate formation with DS, significantly reducing CAC; Secondary aggregates also appeared in DLS measurements. The spherical shape of self-aggregates was confirmed with TEM by the negative staining.

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