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Biodegradable poly(asparagine) grafted with poly(caprolactone) and the effect of substitution on self-aggregation

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Abstract

Micelle-like aggregates formed with amphiphilic graft copolymers, poly(asparagine)-g-poly(caprolactone) (PAsn-g-PCL) were prepared through 'the precipitation and dialysis method' and their self-aggregation phenomena were investigated by light scattering, fluorescence probing, and TEM. The strong hydrophobic interaction of associated PCL grafts would facilitate the primary aggregate formation in a bimodal size distribution and significantly reduced CAC (critical aggregation concentration) with respect to DS (degree of substitution). Further, the size of aggregates increased as DS reduced apparently. The graft copolymer having a higher DS formed the more rigid or compact hydrophobic core in self-aggregates with polarization as well as smaller aggregates. When introduced in a basic condition at 37 °C, the effective diameter of aggregates increased and the scattering intensity reduced due to the degradation of PCL as time changed. When incubated in aqueous solution at 4 °C, the sample of DS 4.2 maintained without any size change for up to 20 days, while that of DS 1.2 increased up to the equilibrium diameter. But, the rapid growth of turbid state at DS 6.0 was observed within 2 weeks. After 25 days, the effective diameter of aggregates increased irrespective of DS, becoming a little turbid, and attributed to the swelling and degradation of hydrophobic domains with various morphogenic changes.

Keywords: Self-aggregate; Poly(asparagine); Poly(caprolactone); Degree of substitution; Degradation

1. Introduction

Micelle-like aggregates or nanoparticles formed with amphiphilic block or graft copolymers have recently been studied for a possible application of drug carriers [1–4]. Among them, interested are biocompatible block and graft copolymers including hydrophobically modified water-soluble polymers (HMP), which form self-assemblies, nano-sized micelle-like aggregates of various morphologies in an aqueous solution. The hydrophobic microdomains of self-agggregates could be formed through intra- and/or inter-molecular interactions in an aqueous solution and act as host systems for many hydrophobic molecules, while the hydrophilic corona or outer shell plays a role in avoiding the uptake by reticuloendothelial systems (RES). 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.

Poly(amino acid)s grafted with side chains would form water-soluble and biologically acceptable self-aggregates, and construct a hydrophobic core or a hydrophilic shell. Several block and graft copolymers containing a poly(amino acid) core-forming block have been employed as delivery vehicles for anti-cancer drugs [4–6]. In our previous works, nano-sized micelle-like aggregates formed poly(amino acid)s-based amphiphilic copolymers such as poly(aspartic acid)-g-alkyl [3] and poly(hydroxyethylaspartamide)-g-

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dehydroxycholic acid (PHEA-g-DHA) [8], and proteinoid-cholesterol [9] were studied on physiochemical properties and as carriers for hydrophobic drugs. Poly(amino acid)s' derivatives such as poly(aspartic acid) and poly(asparagine) are synthesized by acid-catalyzed thermal polycondensation of L-aspartic via poly(succinimide) (PSI) [8] and has fully biodegradable, water-soluble properties and toxicological suitability and have been proposed as a carrier in a synthesis of prodrug [12,13].

We have recently reported the synthesis of a new aminoacid-based copolymer of poly(asparagine) (PAsn) as a backbone and poly(caprolactone) (PCL), a semi-crystalline biodegradable polymer as grafted hydrophobic chains [9]. Also, the graft copolymers formed self-aggregates in aqueous solution were prepared through 'the precipitation and dialysis method'. In this paper, we investigated the self-aggregation of PAsn-g-PCL graft copolymer that has dependence on the degree of substitution (DS) of PCL groups. The structure of the self-aggregate and the hydrophobic effect of degradable PCL oligomers on the physicochemical properties are discussed.

2. Experiments

2.1. Materials

PSI, PAsn and PAsn grafted with PCL-diol (PAsn-g-PCL) were synthesized in our previous studies [9–11]. L-aspartic acid (Sigma), PCL-diol (mol. wt. 1250, Polyscience, Inc), phosphotungstic acid (TAAB), and sodium hydroxide (Junsei Chemical) were used as purchased. Fluorescent probe, pyrene (99%, optical grade) and diphenylhexatriene (DPH) were both purchased from Aldrich. *N*,*N*-dimethylformamide (DMF; Aldrich) and tetrahydrofuran (THF; Aldrich) were dried over molecular sieve 4 Å before use.

2.2. Sample preparation

The stable solution of self-aggregates of PAsn-g-PCL was prepared when water was added to the DMSO solution of PAsn-g-PCL [9]. First, the purified copolymer (10 mg) 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 = 8000–12000 g/mol).

2.3. Dynamic light scattering measurements

The sample solutions were filtered with 0.45 μm pore size membrane filters to remove dust prior to measurement. Light scattering measurement was performed for determining the size distribution with an apparatus from Brookhaven Instruments Inc. equipped with a diode laser of 523 nm. The scattering angle was fixed at 90° for dynamic light scattering (DLS), the effective diameter was obtained by the Stokes–Einstein relationship, and histograms were calculated with the NNLS routine. When the difference between the measured and calculated baselines was less than 0.2%, the correlation function was accepted.

2.4. Transmission electron microscopy

The morphology of self-aggregates was observed using a transmission electron microscopy (TEM) with the negative staining technique. A drop of self-aggregates solution (Conc.: 1.0 mg/ml, pH 6.8) containing 0.1% phosphotungstic acid (PTA) was placed on a copper grid coated with a carbon film. The grid was held horizontally for 30 s to allow aggregates to settle down and then vertically to allow excess fluid to drain. Observation was carried out at 80 kV with Philips CM200.

2.5. Fluorescence measurements

Critical aggregation concentration (CAC) was determined from steady-state fluorescence spectra using a Perkin–Elmer luminescence spectrometer with a bandwidth of 2.5 and 5.0 nm for excitation and emission. The excitation spectra were obtained using emission wavelength of 390 nm. The stock solution of pyrene (6.0×10^{-4} M) was prepared in acetone and dropped into the polymer solution. Then, the solution was sonicated with a bath-type sonifier (Branson) for 10 min, and induced equilibrium of pyrene and polymers. The mixture was incubated at room temperature for at least 24 h. The polarization of hydrophobic domain was determined with DPH as a fluorescent probe. The stock solution of DPH (2.0×10^{-3} M) was prepared in tetrahydrofuran (THF) and dropped into the polymer dispersion. The DPH concentration was adjusted to 2.0×10^{-6} M. Then, the polymer series with DPH was mixed by vortex and bubbled with nitrogen and incubated at 50 °C for at least 5 h. As the fluorescence quantum yield of DPH depends on the solvent polarity, DPH can be used as a sensitive probe to monitor the formation of hydrophobic domains. The fluorescence polarization of DPH was determined as the following equation.

$$\text{Fluorescence polarization} = \frac{(I_{VV} - I_{VH})}{(I_{VV} + I_{VH})}$$

where I_{VV} and I_{VH} are emission intensities when an emission polarizer is oriented parallel and perpendicular to an excitation polarizer, respectively. The emission intensities were recorded at 430 nm with excitation of 360 nm, varying the temperature of an aggregate solution from 25 to 60 °C.

2.6. Collapse behavior of self-aggregates

The collapse procedure of self-aggregates prepared by the simple hydrolysis of grafted PCL oligomers was monitored by a dynamic light scattering (DLS) and pH meter (Orion

310). All measurements were observed by in situ. The collapse of self-aggregates was performed at pH 11.7 when the solution concentration of NaOH was adjusted to 0.02 M, by adding 1.0 M NaOH solution to the sample solution (PBS: pH 7.4). The sample solution was incubated in a beaker at 37 °C and then picked out and measured after a fixed period of time.

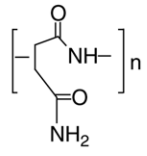
2.7. Other measurements

X-ray diffraction analysis was carried out using a Rigaku D/max-RB apparatus Cu Kα source (λ = 0.154 nm) with the powder samples. The DSC thermograms were investigated by using Thermal Analyzer 2000 (DuPont) at a heating rate of 10 °C/min under nitrogen.

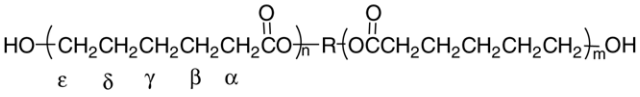
3. Results and discussion

3.1. Amphiphilic graft copolymers, PAsn-g-PCLs

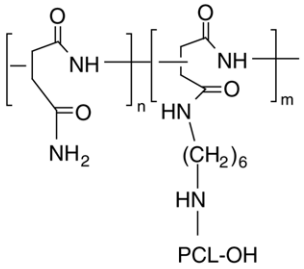
Amphiphilic graft copolymers composed of poly(asparagine) as the hydrophilic backbone and poly(caprolactone) as the hydrophobic segment were successfully synthesized [9]. For convenience, poly(asparagine) grafted with poly(caprolactone) groups, of which molecular structure can be seen in Fig. 1 was abbreviated to PAsn-g-PCLs according to the degree of substitution (DS) of PCL groups. The



Poly(asparagine)



Poly(caprolactone) -diol (mol.wt. 1250)



PAsn-g-PCL

Fig. 1. Molecular structures of poly(asparagine), poly(caprolactone)-diol, and poly(asparagine) grafted with poly(caprolactone) (PAsn-g-PCL).

Table 1
Characteristics of graft copolymers, PAsn-g-PCL series

Sample identification	PAsn-g-PCL1	PAsn-g-PCL2	PAsn-g-PCL3	PAsn-g-PCL4
Feed mole ratio[a]	97/3	94/6	91/9	88/12
DS[b] (%)	1.2	3.2	4.2	6.0
Weight fraction[c]	13.53	29.87	36.10	45.13
X_c[d]	0.50	3.67	6.14	10.83
T_m[e] (°C)	41.5	44.2	47.0	49.0

[a] Succinimide unit/PCL.
[b] DS determined by elemental analysis.
[c] Weight of PCL/weight of PAsn-g-PCL.
[d] Calculated with WXRD data.
[e] Measured by DSC at a heating rate of 10 °C/min.

DS is the mole percentage of the grafting unit with PCL per total succinimide unit. The DS in graft copolymers was calculated from the integral area of ^{13}C NMR peaks and the weight percentage of elemental analysis (EA). Table 1 showed the DS of PAsn-g-PCL series determined by EA. DS could be controlled by grafting of PCL onto the PAsn backbone. The crystallinity of the PAsn-g-PCL series became apparent with the increasing DS that the spatial confinement of the PCL graft facilitated the crystallization of PCL domains with neighboring PCL in the amorphous PAsn backbone. Also, the copolymers with DS (1.2, 3.2, 4.2, and 6.0%) were employed for the self-aggregation of PAsn grafted with PCL.

3.2. Self-aggregation

Micelle-like aggregates, PAsn-g-PCL aggregates were carefully prepared by 'the precipitation and dialysis method' in aqueous solutions. The size distribution of self-aggregates in the aqueous solution of PAsn-g-PCL series was bimodal when determined by DLS (Fig. 2(a)). The small ones are located near 20–30 nm, while the large ones vary between 100 and 200 nm. The population of small particles is dominant with small portions of large particle as shown Fig. 2(a). Also, the scattering intensities of large particles fluctuate significantly in all cases, while those of small ones remain unchanged. Practically, the intensity of large particles dramatically reduced as the incubation temperature of aggregates solution increased. The large particles may be secondary aggregates from primary small aggregates. It is assumed that the secondary large particles are formed by the intermolecular interaction of surface or bridging formation, which depends on the kinetic process of particle–particle interaction, while the primary small particles are formed by the intramolecular interaction between neighboring hydrophobic side chains in the same backbone [3]. Most theories of micelle formation are based on the equilibrium thermodynamic approach. However, if the T_g or T_m of insoluble groups in copolymer is higher than the process temperature, aggregates may be in a non-equilibrium state, dispersed in the frozen state of material [18]. In our system, the melting temperature (T_m) of PAsn-g-PCL series was detected around 35–45 °C due to the crystallinity of

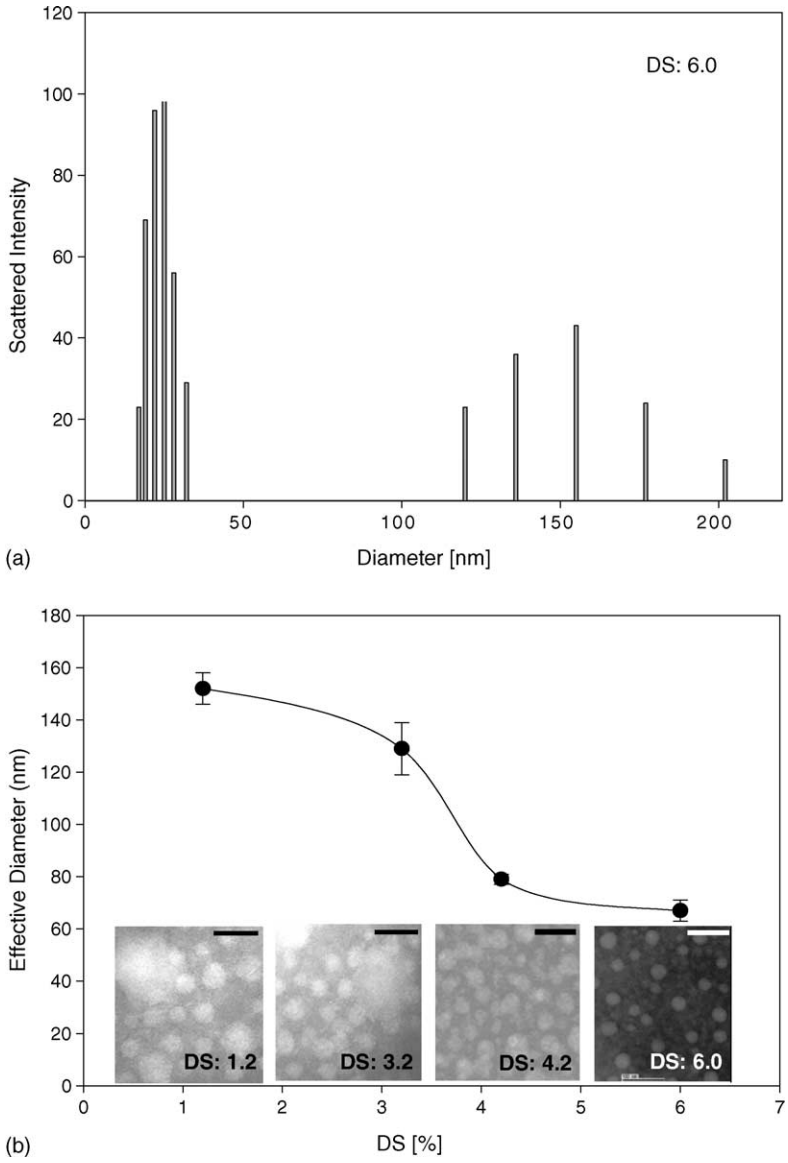


Fig. 2. (a) Particle size distribution (DS: 6.0%). (b) Effective diameters and TEM photographs as a function of DS (the bar represents 100 nm).

neighboring PCL in the PAsn backbone, as shown Table 1. Alternatively, the precipitation and dialysis of the polymer solution may force the copolymer to form self-aggregates by both intramolecular and intermolecular interactions, and to form simultaneously large secondary aggregates under the non-equilibrium thermodynamic condition.

Interestingly, though the size distribution was not monodisperse due to the presence of small primary and large secondary aggregates, the effective diameters were reduced as DS increased apparently because of the strong hydrophobic interaction of PCL (Fig. 2(b)). Such a reduction in size has been observed when alkyl or DHA were grafted. This indicates that the self-aggregation of the graft copolymer would be affected by the DS of PCL groups. It is believed that a higher DS would induce smaller aggregates and the lower aggregation number because of stronger hydrophobic interactions and higher packing density in self-aggregates. Fig. 2(b) shows the transmission electron micrograph of PAsn-g-PCL (Conc.: 0.1 wt.%) with negative staining. The picture clearly indicates a spherical shape of primary self-aggregates as light parts surrounded by the negative staining. Also, the population of primary particles is dominant in PAsp-g-PCL4 (DS: 6.0%). But, the evidence that supports the ‘secondary aggregate’ concept is the existing of large aggregates in TEM image of DS1.2 and 3.2.

The PCL core structure of PAsn-g-PCL was investigated with pyrene, whose vibrational structure of the fluorescence spectrum alters with the polarity of the environment [16]. Fig. 3(a) showed the intensity ratio (I_{336}/I_{333}) determined from fluorescence excitation spectra of pyrene in PAsn-g-PCL. At the low concentration, I_{336}/I_{333} was unchanged, indicating that polymer would not form aggregates in dilute

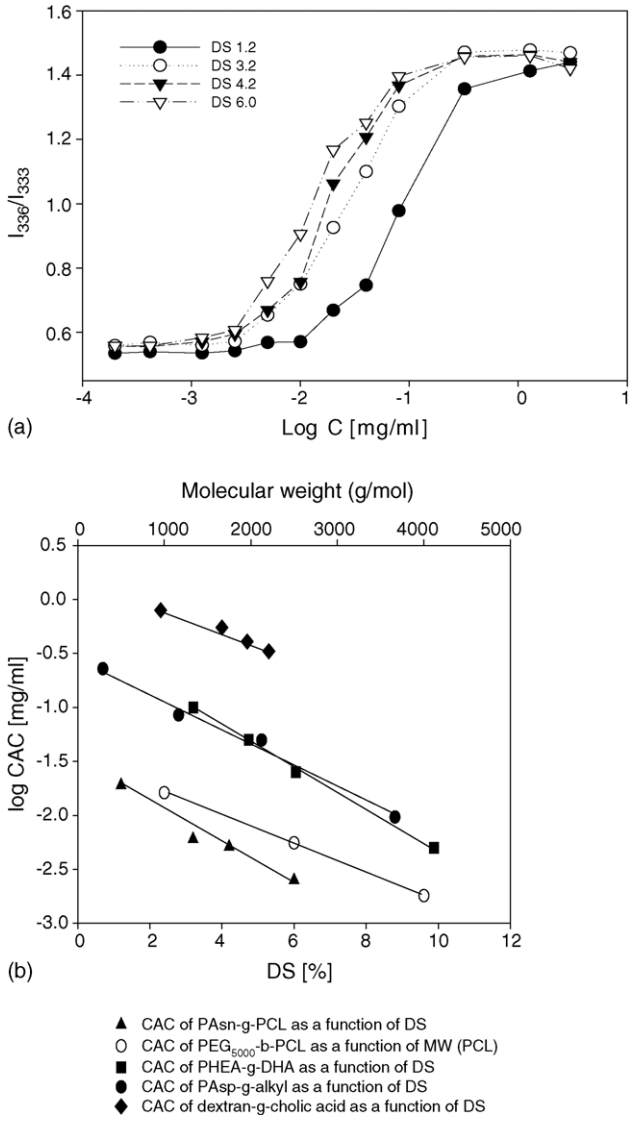


Fig. 3. (a) Variation of I_{336}/I_{333} with PAsn-g-PCL concentration in aqueous solution. (b) Critical aggregation concentration (CAC) of polyaminoacids grafted with hydrophobic moieties determined by emission fluorescence spectra of pyrene. The triangle represents this work.

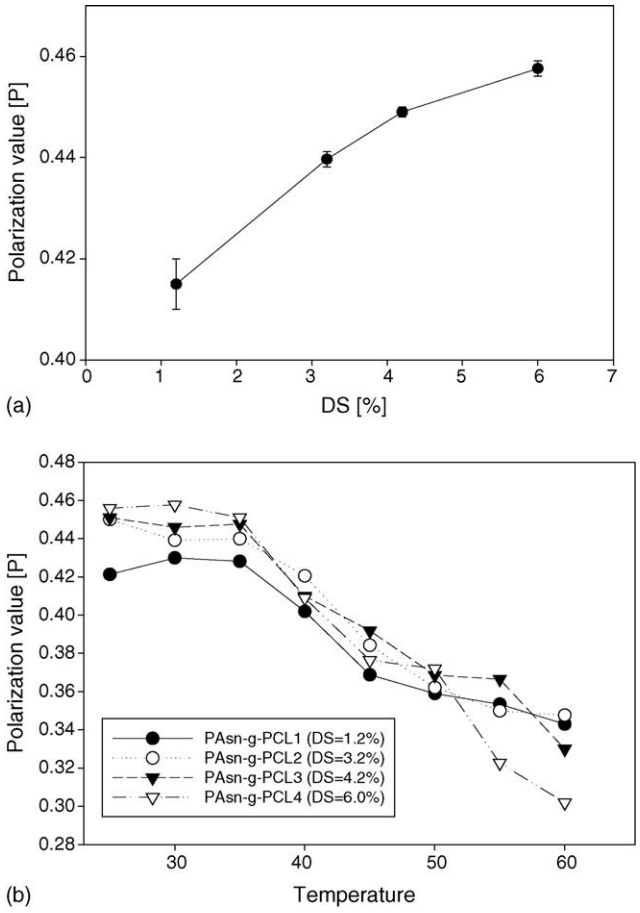


Fig. 4. (a) Polarization value of self-aggregates as a function of DS at 25 °C. (b) Polarization change of hydrophobic microdomains of PAsn-g-PCL series with varying temperature.

solution. As the concentration increased, the ratio rises in a sigmoidal shape. It is believed that the grafted PCL groups would start to form hydrophobic domains and then aggregates in the aqueous solution. The critical aggregation concentration (CAC) was determined as the intersections of extrapolating straight regression line segments between flat and rising region. Fig. 3(b) shows the CAC of PAsn-g-PCL series as well as other works [3,7,16,17]. The CAC of PAsn-g-PCL decreased logarithmically as the grafting density of PCL groups (DS) increased similar to the linear relationship between the CMC values and the hydrophobicity in surfactants. Since the PCL side chain forms a crystalline phase, it is expected that the graft copolymer would have a small CAC. In fact, the CACs of PAsn-g-PCL are as small as 1/10 of others such as PAsp-g-alkyl, PHEA-g-DHA, and dextran-g-cholicacid. But, it is noticeable that CACs of PAsn-g-PCL and PEG_{5000}-b-PCL have a similar value, even though the nature of hydrophilic parts and polymer type such as graft or block is not the same. In our systems, a lower CAC value may be originated from the nature of a crystalline PCL.

Fig. 4 showed the change of the fluorescence polarization of DPH partitioned into the hydrophobic domains of PAsn-g-PCL series. Fig. 4(a) and Table 2 showed that the fluorescence polarization of DPH exhibited slightly higher value as grafted PCL group (DS) increased at room temperature. Therefore, it is concluded that the graft copolymer having a higher DS could form a more rigid or compact hydrophobic core in self-aggregates as well as smaller aggregates as observed by DLS measurements. The almost constant polarization value was observed from 25 to 35 °C as shown Fig. 4(b). However the polarization value starts to decrease around 35 °C, suggesting that the hydrophobic core began to become loosely formed. This is a little lower than 45 °C, the melting temperature (Table 1) may be because the hydrophobic domain of aggregates in an aqueous solution is different from the crystalline state of PAsn-g-PCL in a bulk but has ordered

Table 2
The properties of self-aggregates in aqueous solutions as a function of DS

Sample identification	PAsn-g-PCL1	PAsn-g-PCL2	PAsn-g-PCL3	PAsn-g-PCL4
(1) Hydrodynamic diameter[a] (effective diameter)	45 ± 2 (152 ± 6)	36 ± 1 (129 ± 10)	30 ± 2 (79 ± 2)	24 ± 1 (67 ± 4)
(2) CAC[b] (mg/L)	19.0	6.0	5.1	2.5
(3) Polarization[c]	0.4213	0.4501	0.4510	0.4560

[a] Size of primary aggregates as a function of DS.
[b] Critical aggregation concentration measured at 25 °C.
[c] Polarization value measured at 25 °C.

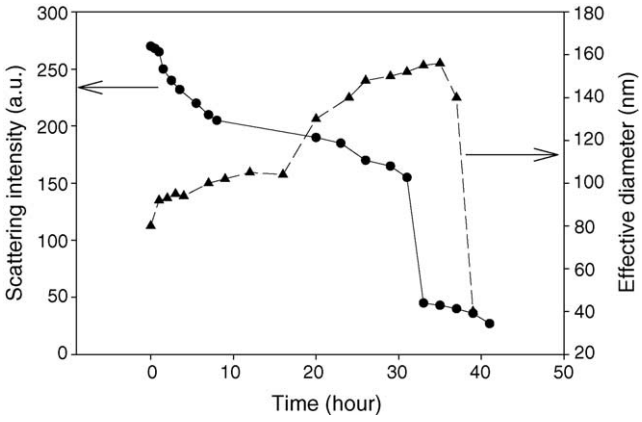


Fig. 5. Simple hydrolytic degradation behavior of PAsn-g-PCL in PBS solution: (0.02 M NaOH; temperature, 37 °C).

structures including an amorphous region. Fig. 4(b) showed that the polarization decreased gradually as the temperature increased due to the increase of mobility of hydrophobic domains in self-aggregates.

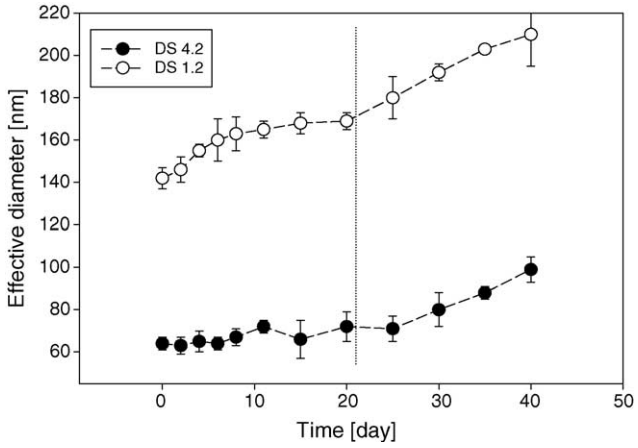


Fig. 7. Effective diameter change of self-aggregates with varying time in PBS solution. (Conc.: 1 mg/ml; temperature: 4 °C; DS: 1.2 and 4.2%).

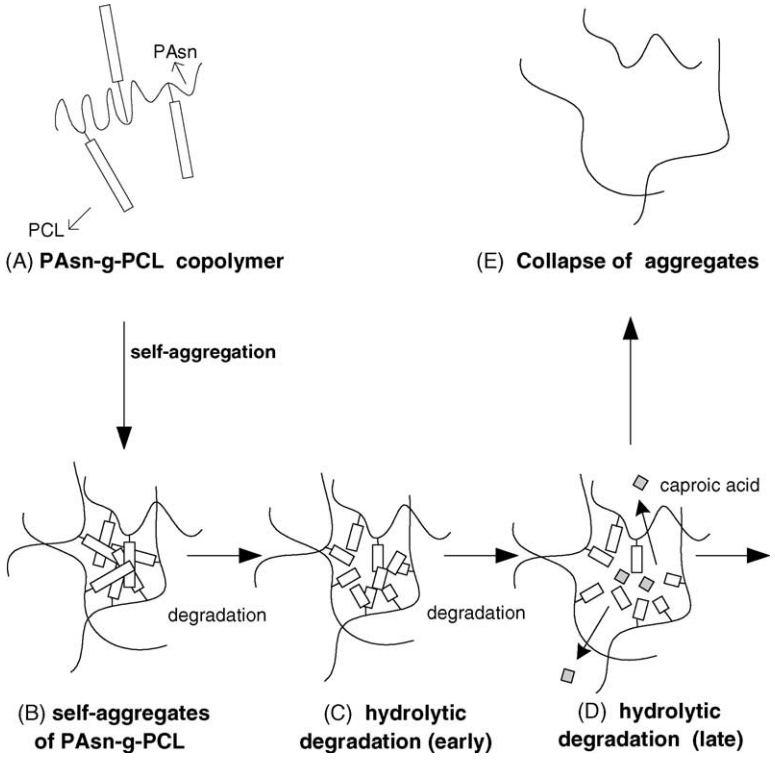


Fig. 6. A schematic representation of 'degradation behavior' of PAsn-g-PCL self-aggregates.

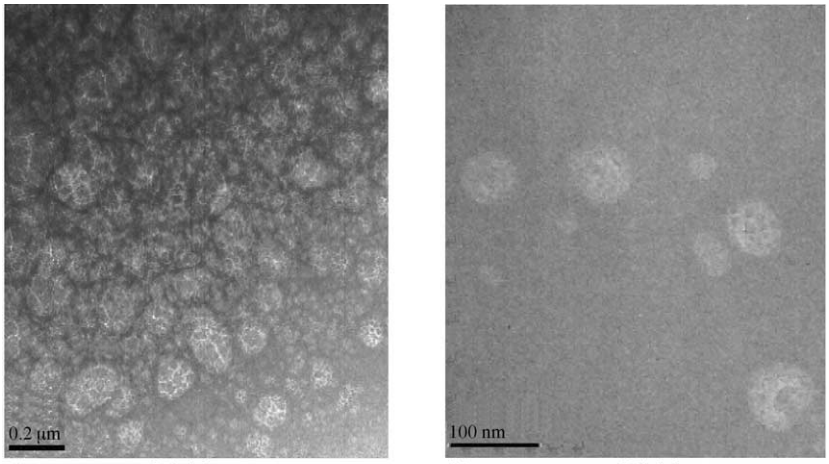


Fig. 8. TEM photographs of PAsn-graft-PCL self-aggregates ((a) DS 1.2%; (b) DS 4.2%) with swelling and degradation of PCL group after 2 months.

3.3. Collapse behavior of aggregates

PCL could be easily decomposed by the cleavage of ester bond linkage in an acidic or basic condition [14] but degraded slowly due to its hydrophobic properties of slow water penetration. Therefore, the collapse or degradation behavior of aggregates could be simulated with the hydrolytic degradation of the PCL groups in 0.02 M NaOH solution (PBS) at 37 °C. The kinetics and degradation in self-aggregates were observed with varying pH, scattering intensity and effective diameter. Fig. 5 showed the change of scattering intensity and effective diameter of aggregates (DS: 6.0%) with incubation time. In overall, the effective diameter increased and the scattering intensity reduced as time changed. It can be easily expected from the degradation of the PCL groups in a basic environment. Therefore, it is proposed that the collapse pattern of PAsn-g-PCL aggregates could be explained by the mechanism in Fig. 6. In a basic condition, aggregates begin loosely packed and then the size increased, because the molecular weight of PCL reduced. Also, the randomly degraded oligomer PCL may be placed not in water but in core due to the hydrophobicity. Nevertheless, oligomer PCL with the carboxylate end group [15] can facilitate the diffusion of water into the core in aggregates and then finally swell the hodrophobic core. Practically, the pH of solution reduced apparently from 11.7 to 11.3 and the reason can be easily deduced from the generation of caproic acid (CA). Also, since both the effective diameter and scattering intensity was dramatically reduced after 32 h, it is believed that there is the theoretical amount of the PCL contents required for aggregate formation.

3.4. Stability of aggregates

Fig. 7 showed the change of effective diameters of aggregates according to the incubation time in PBS solution at 4 °C. The sample of DS 4.2 maintained without any size change for up to 20 days, while that of DS 1.2 increased up to the equilibrium diameter. However, the rapid growth of turbid state at DS 6.0 was observed within 2 weeks (data not shown). The growth of turbid entities may be attributed to one of three different growth mechanisms: by swelling, or kinetic aggregation, or reformation with shape change of primary particles. Aggregates of DS 1.2 were loosely formed and reconstituted into a thermodynamically stable conformation as time changed. Especially, the samples of DS 4.2 and DS 1.2 started to become a little turbid and grow gradually in effective diameter after 25 days. Fig. 8 showed photographs of self-aggregates after 2 months observed by TEM. Comparing TEM images of Fig. 2, various morphogenic changes could be seen, as well as size changes. From the deformation images of aggregates, it is indicated that the increase of effective diameter may be attributed to the swelling and degradation of hydrophobic domains in aggregates or reconstitution with shape change.

4. Conclusion

Micelle-like aggregates formed with amphiphilic graft copolymers, PAsn-g-PCL were prepared through ‘the precipitation and dialysis method’. The strong hydrophobic interaction of PCL grafts would facilitate the primary aggregate formation in a bimodal size distribution and significantly reduce CAC with respect to DS. The graft copolymer having a higher DS formed more rigid or compact hydrophobic core in self-aggregates with polarization. The collapse procedure in aggregates was consistent with the tendency of the size versus. The aggregates increased in effective diameter with incubation time, and attributed to the swelling and degradation of hydrophobic domains with various morphogenic changes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.colsurfa.2005.05.019.

References

[1] M. Yokoyama, S. Fukkushima, R. Uehara, K. Okamoto, K. Kataoka, Y. Sakurai, T. Okano, J. Control. Release 50 (1998) 79.
[2] K. Kataoka, A. Harada, Y. Katasaki, Adv. Drug Delivery Rev. 47 (2001) 113.
[3] H.S. Kang, S.R. Yang, J.D. Kim, S.H. Han, I.S. Chang, Langmuir 17 (2001) 7501.
[4] H.S. Kang, J.D. Kim, S.H. Han, I.S. Chang, J. Control. Release 81 (2002) 135.
[5] G. Kwon, S. Suwa, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, J. Control. Release 29 (1994) 17.
[6] G. Kwon, M. Naito, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, J. Control. Release 48 (1997) 195.
[7] S.R. Yang, J.H. Jeong, K. Park, J.D. Kim, Colloid. Polym. Sci. 281 (2003) 852.
[8] S.K. Bae, J.D. Kim, J. Biomed. Mater. Res. A 64 (2003) 282.
[9] J.H. Jeong, H.S. Kang, S.R. Yang, J.D. Kim, Polymer 44 (2003) 583.
[10] M. Tomida, T. Nakato, M. Kuramochi, M. Shibata, S. Matsunami, T. Kakuchi, Polymer 38 (1997) 4733.
[11] G. Caldwell, N.W. Neuse, A.G. Perlwitz, J. Appl. Polym. Sci. 66 (1997) 911.
[12] C.J. Chang, G.J. Swift, J. Macromol. Sci. Pure A 36 (1999) 963.
[13] R. Chandra, R. Rustgi, Prog. Polym. Sci. 23 (1998) 1273.
[14] S. Gimenez, S. Ponsart, J. Coudane, M. Vert, J. Bioact. Compat. Pol. 16 (2001) 32.
[15] M. Wihelm, C.L. Zhao, Y. Wang, R. Xu, M.A. Winnik, J.L. Mura, G. Riess, M.D. Croucher, Macromolecules 24 (1991) 1033.
[16] K.K. Jette, D. Law, E.A. Schmitt, G.S. Kwon, Pharm. Res. 21 (2004) 1184.
[17] M. Nichifor, A. Lopes, A. Carpov, E. Melo, Macromolecules 32 (1999) 7078.
[18] J.A. Massey, K. Temple, L. Cao, Y. Rharbi, J. Raez, M.A. Winnik, I. Manners, J. Am. Chem. Soc. 122 (2000) 11577.