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ORIGINAL CONTRIBUTION

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Self-aggregates of hydrophobically modified poly(2-hydroxyethyl aspartamide) in aqueous solution

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Abstract Dehydrocholic acid (DHA) grafted poly(2-hydroxyethyl aspartamide) (PHEA)s were successfully synthesized and their self-aggregates in aqueous solution were characterized by fluorescence spectra and light scattering. PHEA was obtained by a simple reaction of ethanolamine with synthesized poly(succinimide) (PSI), and then PHEA-g-DHA was synthesized through an ester linkage between DHA and PHEA. The degree of substitution (DS) of DHA groups, defined by grafted mole%, was determined from both ^{1}H-NMR and elemental analysis. The grafting reaction of DHA was retarded up to almost 10 mole% feed ratio of DHA/PHEA, but increased linearly above the threshold ratio. Nano-size self-aggregates in aqueous solution were examined with four DSs less than 10. As DS increased, the critical aggregation concentrations (CAC) of polymers were continuously reduced and the size of primary aggregates reduced to as small as 40 nm in diameter. When stored, the sonicated aggregates of high DS were destabilized, apparently forming large aggregates with small curvatures. The formation of irreversible interfused secondary structures would be induced by the curvature change or aggregation of primary particles. A simple calculation indicates that a small change of separation between grafted DHA groups may induce the large curvature shift, in fact, sphere-to-planar surface transition.

Keywords Hydrophobically modified water-soluble polymer · Self-aggregate · Poly(2-hydroxyethyl aspartamide) · Dehydrocholic acid · Degree of substitution

Introduction

Hydrophobically modified water-soluble polymers and their self-aggregates in aqueous solution have been studied for a possible application as drug carriers [1, 2]. The hydrophobic microdomains of self-agggregates can be formed through intra- and/or intermolecular interactions in aqueous solution and act as host systems for many hydrophobic molecules. Among the amphiphilic polymers, polysaccharides with steroids have been paid some interest, such as pullulan with cholesterol [3, 4], chitosan with deoxycholic acid [5, 6], and dextran with cholic or deoxycholic acid [7].

Poly(amino acid)s grafted with side chains would form water-soluble and biologically acceptable self-aggregates, and construct a hydrophobic core [8] or a hydrophilic shell [9], depending on the hydrophilicity of side chain. In particular, poly(aspartic acid) grafted with hydrophobic side chains forms polymer micelles with a negatively charged surface [9, 10, 11]. Poly(2-hydrox-

ethyl aspartamide) (PHEA), obtained by a simple reaction of ethanolamine with poly(succinimide) [12, 13], has favorable toxicological properties (i.e., lack of toxicity, antigenicity, and immunogenicity) and has been proposed as a plasma expander in the biomedical field and as a carrier in a synthesis of prodrug. However, few reports have appeared on their self-assembled systems for the application to delivery carriers.

In this paper, we report on PHEA modified with dehydrocholic acid (DHA) and its aggregate formation. Dehydrocholic acid forms a hydrophobic core that exhibits a better biocompatibility in biological applications and interacts favorably with proteins, enzymes, or lipids [14]. The formation, structure, and stability of self-aggregates of PHEA-g-DHA will be investigated by varying the degrees of substitution (DS) of DHA moieties.

Experimental

Materials

L-Aspartic acid (Sigma), mesitylene (Aldrich), sulfolane (Aldirich), phosphoric acid (85%, Junsei) and 2-aminoethanol (Sigma) were used as received. *N,N*-dimethylformamide (Merk) was dried over molecular sieve 4A before use. Dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) were purchased from Aldirich. Dehydrocholic acid (Sigma) was used without further purification. Fluorescent probe, pyrene (99%, optical grade), was purchased from Aldrich and used as received.

Synthesis of PHEA derivatives

The molecular structures and all synthesis routes are shown in Fig. 1. Poly(succinimide) (PSI) was synthesized by a thermal condensation of aspartic acid with phosphoric acid as previously

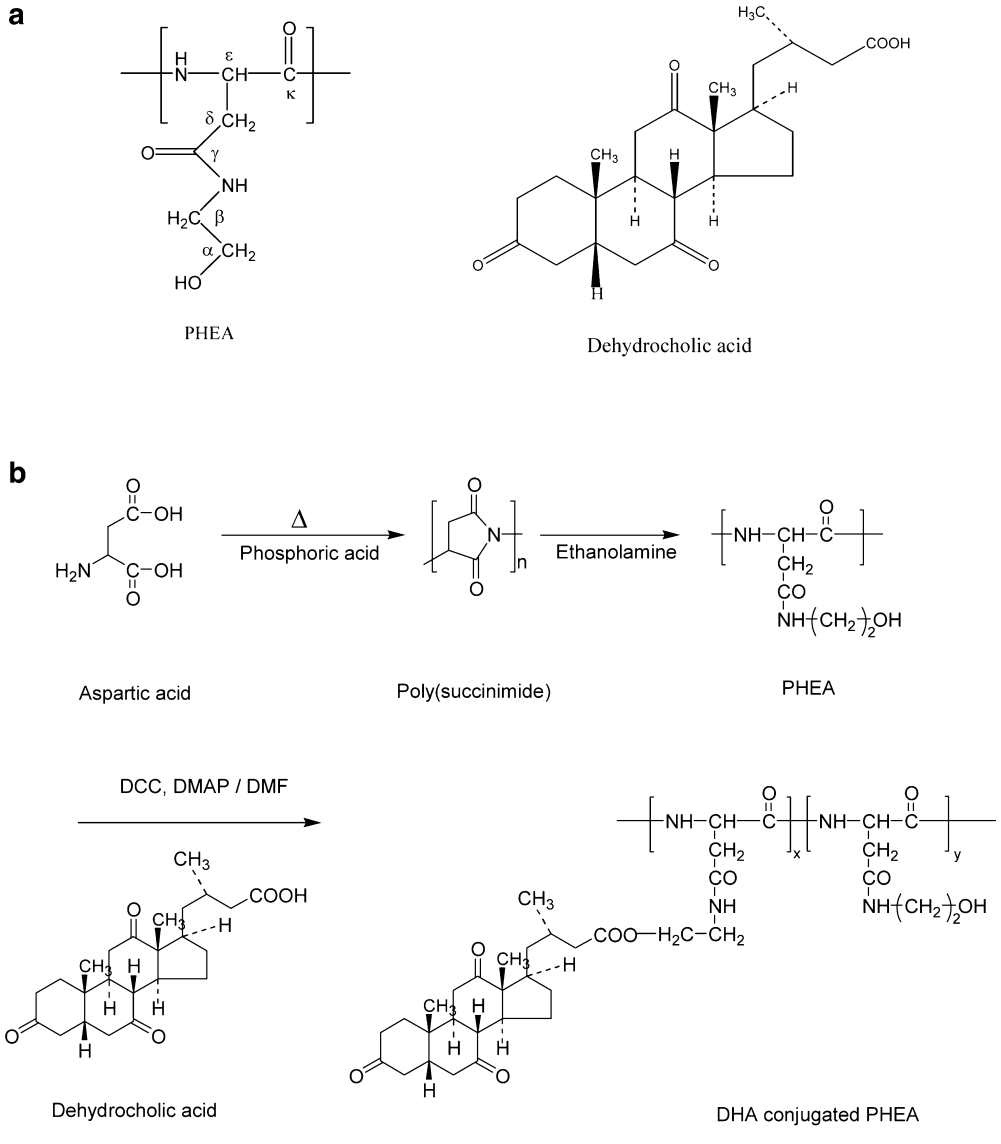

Fig. 1a–b Molecular structure and synthetic scheme. **a** Molecular structure of poly(2-hydroxyethyl aspartamide) and dehydrocholic acid. **b** Synthesis of PHEA and PHEA-g-DHA

reported [9, 10, 11] and its ring was opened with 2-aminoethanol to give PHEA. Purified PSI (10 g, 103 mmol equiv. as succinimide units) was dissolved in 80 mL of DMF and then 15 mL of 2-aminoethanol was added dropwise (1.2 equivalents of succinimide unit). The reaction temperature was kept at 30 °C for 6 h. The reaction mixture was precipitated in 1:1 ethanol/ethyl ether (5 equivalent volume of DMF), followed by washing with methanol and dried in vacuo. The structure of PHEA is illustrated in Fig. 1a and confirmed by NMR analysis. ^{1}H-NMR(D_2O): $\delta = 3.70(\alpha, 2H)$; $3.40(\beta, 2H)$; $2.86(\delta, 2H)$; $4.80(\epsilon, 1H$, overlapped with solvent peak) ^{13}C-NMR(D_2O): $\delta = 60.5(\alpha)$, $42.4(\beta)$, $37.5(\delta)$, $51.2(\epsilon)$, $172.5(\gamma,\kappa)$. The molecular weight of PHEA was determined by GPC analysis, eluted by a mixture of 0.1 M $NaNO_3$/tris buffer solution of pH 8 at a flow rate of 0.3 mL/min, with Ultrahydrogel Linear and Ultrahydrogel 120(Waters) column in series. The number average molecular weight (Mn) was 23 800, the weight average molecular weight (Mw) was 33 500 and the poly dispersity (PD) was 1.41. The yields of product were 80–90%.

DHA was coupled to PHEA by a DCC-mediated reaction through the formation of ester linkages. DCC, DMAP, and synthesized PHEA (0.80 g, 5 mmol equiv. as monomer units) were added to the solutions of different amounts of DHA (0.5–1.5 mmol) in 20 mL of dried DMF under stirring at 30 °C. The reaction mixtures were stirred under N_2 atmosphere for 2 days. Insoluble products were filtered out and the clear reaction mixtures were poured into 200 mL ethyl ether. The precipitated polymers were separated by filtration and extensively washed with acetone to remove ungrafted DHA, then dried in vacuo. The DS of DHA was determined by both ^{1}H-NMR and elemental analysis.

Preparation of PHEA-g-DHA self-aggregates

Purified PHEA-g-DHA was suspended in deionized water under gentle shaking, followed by sonication for 10 min with a bath-type sonifier (Sonics & Materials) at room temperature and gave an optically clear solution. The clear solution of self-aggregates was filtered through a 0.45 μm filter (Whatman) to remove dust. The solutions of different concentrations were obtained by diluting the 1 wt% stock solution with deionized water.

Measurements

The dynamic light scattering (DLS) measurement was performed with an apparatus from Brookhaven Instruments equipped with a diode laser(523 nm) to obtain the size distribution. The scattering angle was fixed at 90° and the particle size and size distribution were calculated using NNLS (non-negative least squares) algorithms. When the difference between the measured and calculated baselines was less than 0.2%, the correlation function was accepted. The turbidity of aqueous solutions of PHEA-g-DHA was measured at 500 nm using an UV/VIS spectrometer.

Steady-state fluorescence spectra were measured using a Perkin-Elmer luminescence spectrometer. Excitation and emission slit widths were maintained at 2.5 and 5.0 nm, respectively. A stock solution of pyrene (6.0×10^{-4} M) was prepared in acetone and dropped into the polymer dispersion until the pyrene concentration in the final solution was 6×10^{-7} M. Then, the mixture was sonicated for 1 min using a probe-type sonifier and incubated at room temperature for at least 24 h. Critical aggregation concentration (CAC) was determined from the excitation spectra obtained with the emission monochromator set at 390 nm. The intensities of peaks at 333 nm and 336 nm were monitored to deduce the formation of hydrophobic aggregates. The polarity of aggregate cores was also obtained from the emission spectra with an excitation monochromator set at 335 nm, and the first-to-third vibrational band ratios were measured from the spectra.

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

Results and discussions

Synthesis

An amphiphilic graft copolymer, composed of PHEA as a hydrophilic backbone and DHA as a hydrophobic segment, was successfully synthesized performing the esterification of PHEA with DHA in the presence of DCC as a coupling reagent and DMAP as a catalyst. As shown in Fig. 2a, the ^{1}H-NMR spectra of PHEA-g-DHA show the additional sharp peaks at 1.30, 0.98, and 0.73 ppm due to the characteristic methyl protons of DHA. There is a new peak at 3.98 ppm, which originates from the methylene proton at -CH_2OH of reacted PHEA with DHA. This peak, representing the occurrence of esterification, can be used to verify the grafting of DHA and to determine the degree of substitution (DS) of DHA groups. As shown in Fig. 2b, the height of new peaks and the three characteristic methyl proton peaks of DHA increase with increasing the ratio of DHA/PHEA in the reaction mixtures. Table 1 shows the results of elemental analysis of products and DS determined from both ^{1}H NMR and elemental analysis. As shown in Fig. 3, DS increases with the feed ratio of DHA to PHEA. The reaction efficacy (ratio of mole DHA bound to mole DHA in reaction mixture) was about 0.5 at higher than 7% of the feed mole ratio of DHA/PHEA. The retardation of grafting reaction at low concentrations was also observed, as reported in the grafting of alkyl groups [9, 10]. In general, however, the esterification reaction can be affected by reaction conditions such as solvent, temperature, and coupling reagent [15].

The solutions of PHEA grafted with DHA less than 9.88 mol% were optically clear at polymer concentrations less than 10 mg/mL as shown in Fig. 4, while the solutions of PHEA grafted with DHA more than 12 mol% were observed to be strongly turbid. It seems that the highly grafted PHEA derivatives form very large aggregates in aqueous solution, apparently due to the high density of DHA groups with a large exclusive volume. It is believed that PHEA-g-DHA copolymers with DS more than 12 mol% could not form the layer curvature enough to stabilize self-aggregates.

Formation of self-aggregates

PHEA-g-DHA forms self-aggregates in aqueous solution, as do the other hydrophobically modified

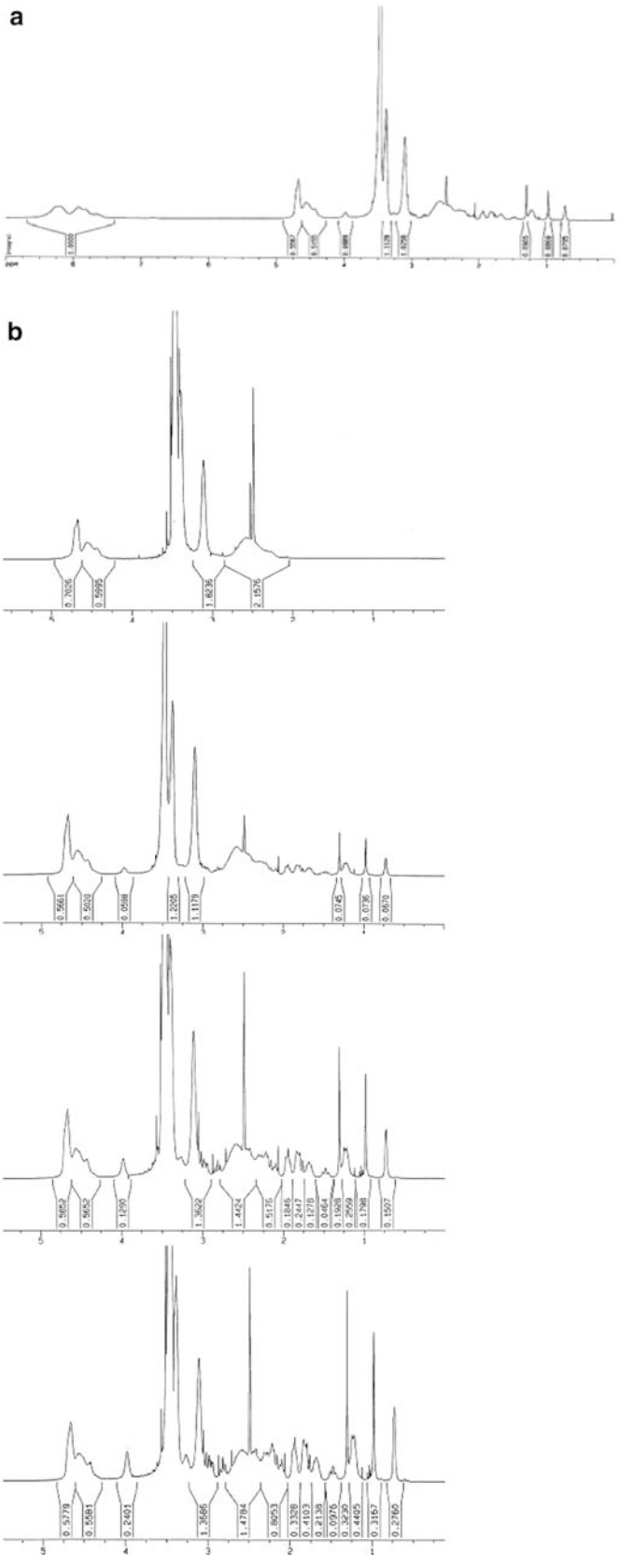

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Fig. 2 a ^{1}H-NMR spectra of PHEA-g-DHA (DMSO-*d6*). **b** ^{1}H-NMR series of PHEA-g-DHA. Feed ratio DHA/PHEA 0%,15%, 20%, and 30%

water-soluble polymers, due to intra- and/or intermolecular hydrophobic interactions of conjugated hydrophobes [16 ,17, 18, 19, 20]. The aggregates build hydrophobic domains in aqueous solution, which can solubilize small hydrophobic molecules.

Pyrene is a pericondensed polyaromatic substance whose vibrational structure of the fluorescence spectrum alters with the polarity of the environment [17, 18]. Fig. 5 shows the change of total fluorescence intensity of pyrene and the shift of the (0,0) band at 333 nm to 336 nm. Figure 6a shows the change of intensity ratio (I_{336}/I_{333}) of pyrene in the presence of PHEA-g-DHAs, indicating that DHA groups abruptly start to form self-aggregates. The critical aggregation concentrations (CAC) of polymers, which are the intersections of extrapolating straight regression line segments, decrease with DS, as if the hydrophobic nature of surfactants reduces the critical micellization concentration (CMC).

Figure 6b shows the CACs of PHEA-g-DHA series as well as other works [7, 10]. The linear relationship between DS and log CAC was confirmed as described elsewhere [11]. The hydrophobic DHA was expected to have lower CAC than other bile acids like cholic acid and deoxycholic acid. In the dextran backbone, deoxycholic acid has lower CAC than cholic acid [15]. In fact, DHA is significantly hydrophobic enough to reduce the CAC to 0.1 mg/mL at DS = 3.21. Further, the reduction of CAC significantly depends on the nature of backbone chain, i.e., chitosan modified with deoxycholic acid [5] has lower CAC than dextran [15]. It is noticeable that two peptide linkages of the non-ionic PHEA and ionic poly(aspartic acid) have a similar range, even if the side chain is not the same.

Figure 7 shows the intensity ratio of the third to the first vibronic peaks (I_3/I_1) of pyrene to the net concentrations of DHA in aqueous solutions. If DHA is free from the main chain or independent of DS, the intensity ratio should be a single curve as appeared in low DS. The increased DS reduces the main chain segment length per DHA (in fact, the hydrophilic unit) and results in the large intensity ratio (I_3/I_1) of emission spectra [17, 18, 19, 20]. This means that PHEA-g-DHA of high DS forms a hydrophobic domain at at lower concentration of polymer than does that of low DS. In the low range of DS (i.e., 3.21, 4.74), the neighboring DHAs are free from the chain stiffness, but aligned only by the molecular interaction between the DHA groups. As DS increases (i.e., 6.05, 9.88), the size of interlinked backbone units per DHA decreases by 100/DS and the segments become hydrophobic. The hydrophobic chains are enforced to pack near the backbone chain forming a diffuse DHA

Table 1 Degree of substitution(DS) of DHA groups in PHEA-g-DHA by elemental analysis

Feed mole%	12	15	18	20	30
N (weight %)	15.74	15.13	14.55	13.76	12.13
C (weight %)	45.66	46.27	46.48	49.37	51.61
H (weight %)	7.0	7.0	6.99	7.17	7.35
DS (mole %)	3.21	4.74	6.05	9.88	16.36
DS (weight %)	7.78	11.24	14.08	21.82	33.24
Sample identification	DS 3.21	DS 4.74	DS 6.05	DS 9.88	DS 16.36

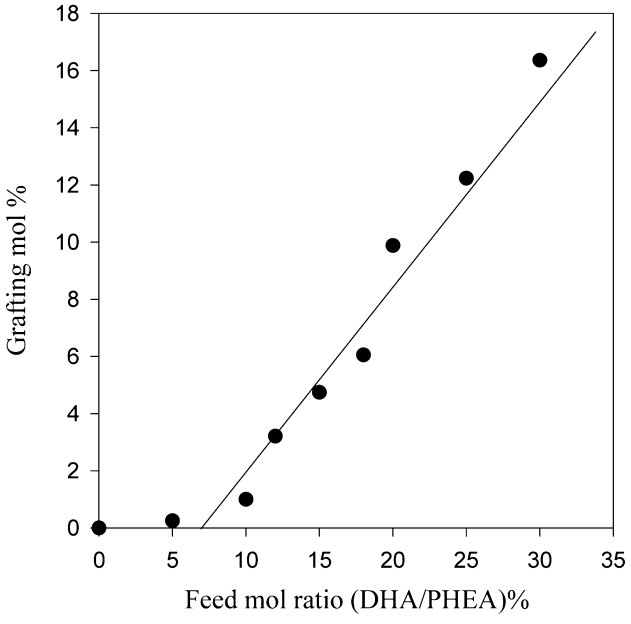

Fig. 3 The degree of substitution (DS) with the feed mole ratio of DHA/PHEA in the reaction medium. All reactions were carried out at 30 °C for 2 days

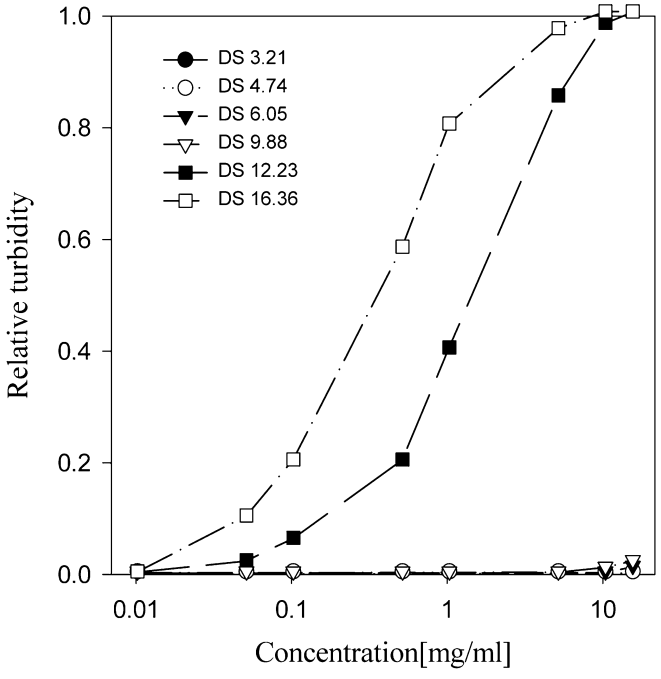

Fig. 4 Relative turbidities of PHEA-g-DHA solutions with varying polymer concentrations by a UV/VIS spectrometry

layer in hydrophobic domains. Therefore, the deviation of ratio curves at high DS from those of low DHA will give information on chain stiffness of backbone and unit size. Further, the ratios I_3/I_1 below CAC are close to 0.63, which is characteristic for pyrene in water, while the large entrapment of pyrene at high concentrations may be attributed to the hydrophobic regions rearranged by chain stiffness and packing density of side chains.

Size and structure

Figure 8 shows the scattering intensities of PHEA and PHEA-g-DHA solutions with varying concentrations and DSs. The intensities of scattered light of PHEA-g-DHA solutions increase with polymer concentrations, while those of PHEA solutions show no change at all concentrations. However, such an intensity of scattered light may be coupled with several factors such as the number of aggregates, and their size and shape [21].

The PHEA solutions and the PHEA-g-DHA solutions below CAC do not form self-aggregates of the range of $\lambda/20$ in aqueous solutions. Therefore, the solutions may have a critical DS or smooth transition of solutions toward highly scattering entities as DS increases. The grafting of DHA significantly reduces the rotational and translational degree of freedom, and generates attraction forces between neighboring DHA groups. At low DS, the driving force to aggregation will be attributed to only the attraction, which assembles loosely packed aggregates, and the grafted DHA acts as if it is a free surfactant of DHA-segment blob [22, 23]. These aggregates may have similar refractive indices at internal and external environments or, if present, the difference would be marginally small. The loose aggregates may become dense as DS increases. At high DS, the grafted DHA was not free, but bounded to the main

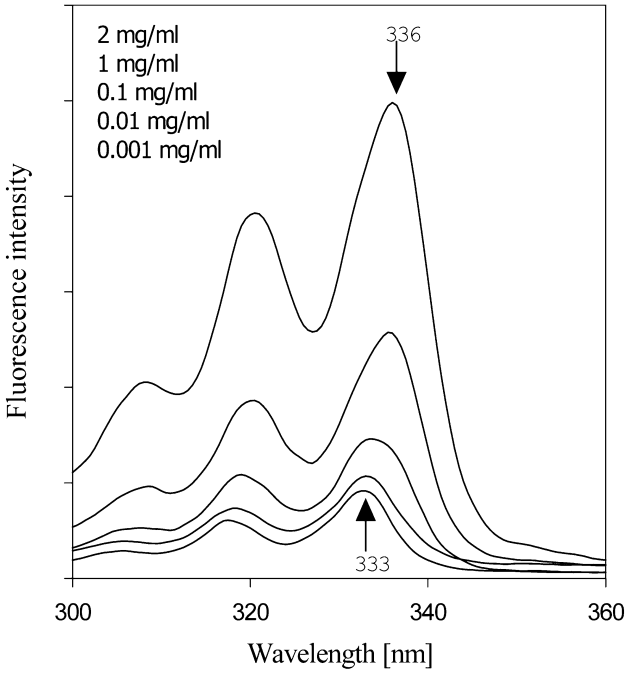

Fig. 5 Fluorescence excitation spectra of DS 6.05 with varying polymer concentrations

chain with a small blob. The DHA will be tightly packed and immobilized as if it is a solid phase. Therefore, the scattering intensities at low DS are attributed to the increase of packing density, while those at higher DS are attributed to the increase in the number of densely packed aggregates before the curvature changes.

Figure 9a shows the size distribution of self-aggregates in aqueous solutions of PHEA-g-DHA determined by dynamic light scattering of 523 nm wavelength (λ). The aggregates of PHEA-g-DHA were significantly polydispersed in size, coexisting with the primary and secondary particles. Figure 10 showed the clear existence of primary aggregates with a size of less than 50 nm at DS 4.74. It is interesting that the radius of gyration R_G of a random coil peptide with an equivalent length was about 30–50 nm [24]. However, it is not clear whether the primary particles are mainly formed by the hydrophobic interaction between DHA molecules or by the peptide stiffness.

It is also observed that the large particles exist in the form of secondary aggregates of smaller ones. The scattering intensities of large particles fluctuate significantly in all cases, while those of small ones remain unchanged. Therefore, it is assumed that the secondary large particles are formed by the interparticle interaction of surface or bridging formation, which depends on the kinetic process of particle–particle interaction. However, the formation of secondary aggregates may have more complicated processes such as the interaction between primary particles, the sharing of DHA in a single chain with different primary particles, or kinetically-frozen states. If the Tg of insoluble blocks is higher than the process temperature, aggregates may be in a non-equilibrium state, dispersed in the frozen state of material [25, 26]. Therefore, it is noticed that primary aggregates would be self-aggregated by both intramolecular and intermolecular interactions of grafted DHA, while the large secondary aggregates are formed by interparticle interactions or bridging of small primary self-aggregates.

The population of primary particles is dominant as shown in Fig. 9a and the TEM photograph (Fig. 10). Figure 9b shows the hydrodynamic diameters (2R) of primary aggregates of PHEA-g-DHA. The small primary aggregates apparently exhibit a minimum behavior in DS 9.88. The size reduction may be attributed to the large curvature K created by both the chain stiffness and strong hydrophobic interactions of neighboring DHA groups. Assume that the curvature $K(=2/R)$ of primary aggregates can be calculated by a geometric relation of $2(1\text{-}D_1/D_2)/L$, where D_1 and D_2 are the molecular diameters of inner hydrophobic and outer hydrophilic layers and L is the thickness of layer (or length of side chain). If D_2 is equal to or approaches D_1, then K converges to zero, forming a planar surface and destabilizing the polymer micelle-like aggregates. Here, the diameter of hydrophobic layer D_1 depends on the side group dimension and interaction energy, while the diameter of hydrophilic group, D_2, is a function of DS and chain flexibility.

Let the side groups be uniformly distributed and their interaction energy constant. Then, the molecular alignment and curvature of polymer aggregates could be evaluated with respect to DS, by taking the lattice parameters similar to the orthorhombic crystal structure of cholic acid (a ,b, c = 16.447, 8.394, 16.993) [27] and the bond length of fully extended trans peptide bond by n×3.8 Å [24]. Therefore, it is recognized that at least four units of backbone are required for this distance. In fact, the highest DS of tightly packed side chain can be estimated by DS (= 100/4 = 25) and, therefore, the fully extended distance of four amide linkages is 15.2 Å. In solutions, the distance between neighboring DHAs can be extended by the inclusion of solvent; for example, when ethanol is included, the b can be extended from 8.4 Å to 11.8 Å [27]. Then, the diameter of aggregates will be 18.6 nm. But if D_1 were extended from 11.8 Å to 14.0 Å (for example), the diameter would become 40 nm, equivalent to the diameter of primary aggregates.

At DS = 10, the extended distance of the amino acid chain between graft points is 38 Å and b may be greater than 11.8 Å. The appearance of turbidity indicates that the curvature approaches zero near DS = 10. Since the DHA separation D_1 is not much different from 11.8 Å, the length of hydrophilic group (D_2) is at least about 4 backbone units distance (15.2 Å). Therefore, it is estimated that four units distance of backbone and distance

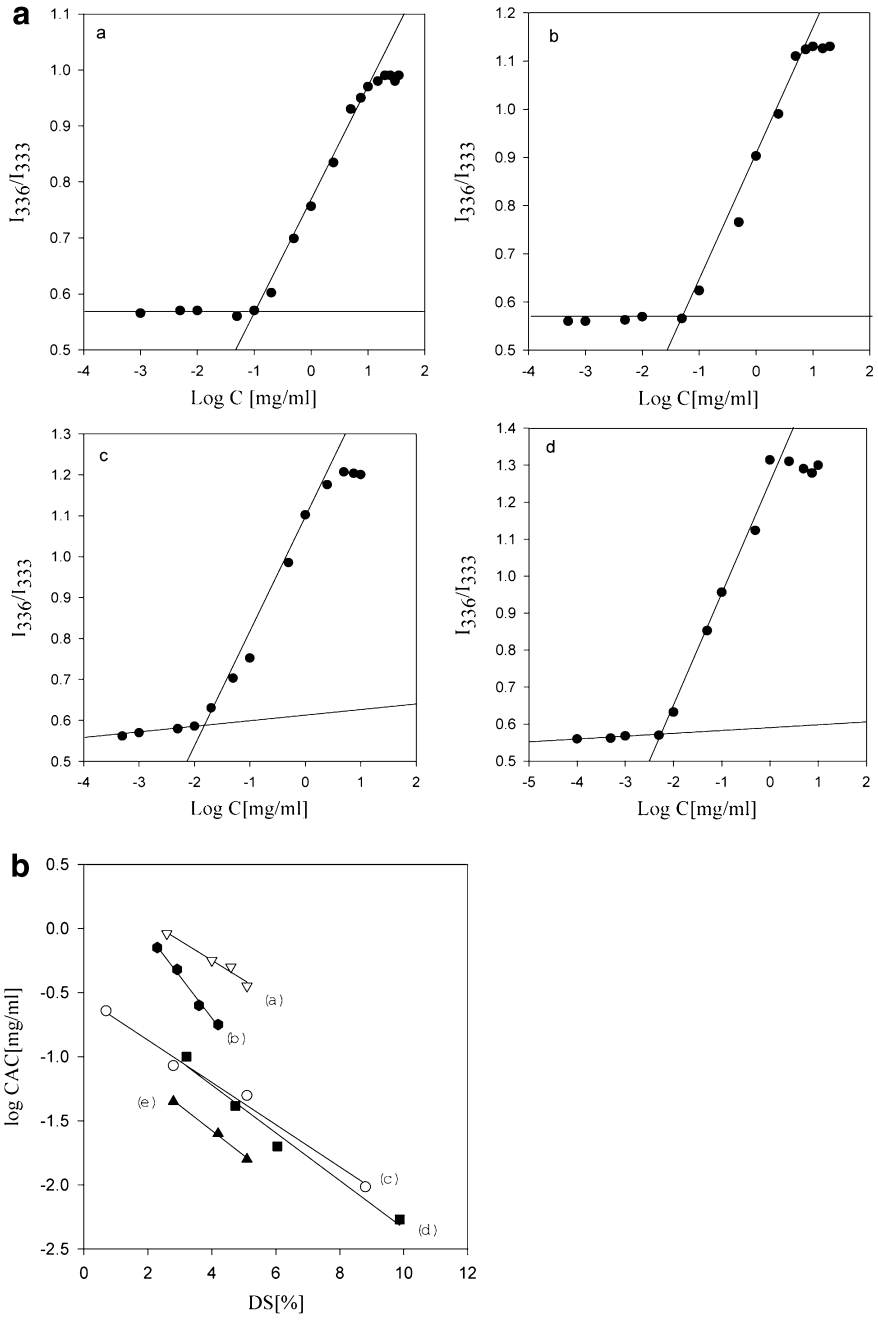

Fig. 6a–b Critical aggregation concentration (CAC) of PHEA-g-DHA. **a** I_{336}/I_{333} ratio of PHEA-g-DHA with varying concentrations (*a* DS 3.21, *b* DS 4.74, *c* DS 6.05, and *d* DS 9.88). **b** CAC of PHEA-g-DHA as a function of DS: *a* dextran modified with cholic acid [7], *b* dextran modified with deoxycholic acid [7], *c* PASP modified with alkyl chain [9], *d* present work, *e* chitosan modified with deoxycholic acid [5]

between DHAs form a parallelism of curvature, but that 6 units form a flexible looping. For DS < 10, excess units over four units may form a flexible loop, balance the curvature, and then decrease particle size. For DS > 10, excess units will be below 6 units, and become rigid and unstable. The rigidity of chain and excluded volume of side chain limit the direct interaction between side chains and form the curvature $K \rightarrow 0$ and, therefore, apparently form large aggregates. On the other hand, when DS is close to zero, the backbone units form a flexible loop, while the b parameter or distance between side groups remains unchanged, forming a flower-shape aggregate.

Stability of particles

Figure 11 shows the change of effective diameters of self-aggregates with time. The samples of DS 3.21 and DS 4.74 were maintained without any size change for up to 2 months while those of DS 6.05 and DS 9.88 increased to the equilibrated size. Especially, the sample of DS

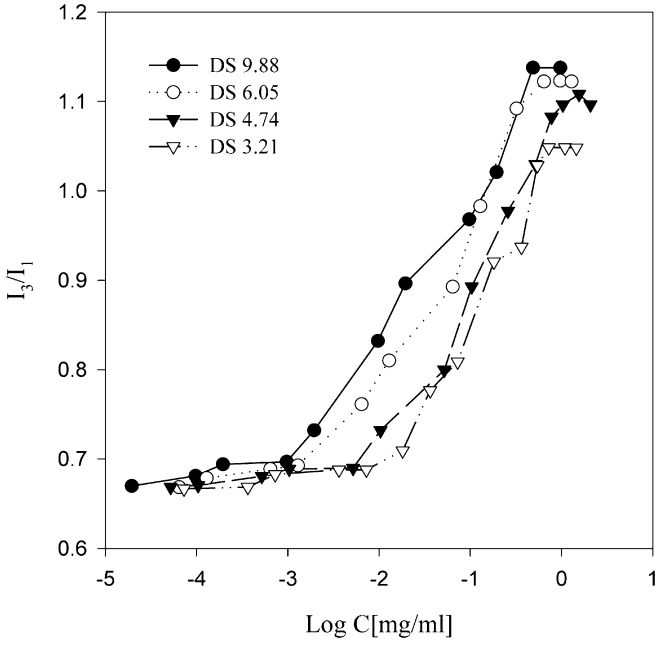

Fig. 7 Fluorescence emission I_3/I_1 ratio of PHEA-g-DHA series to the net concentrations of DHA

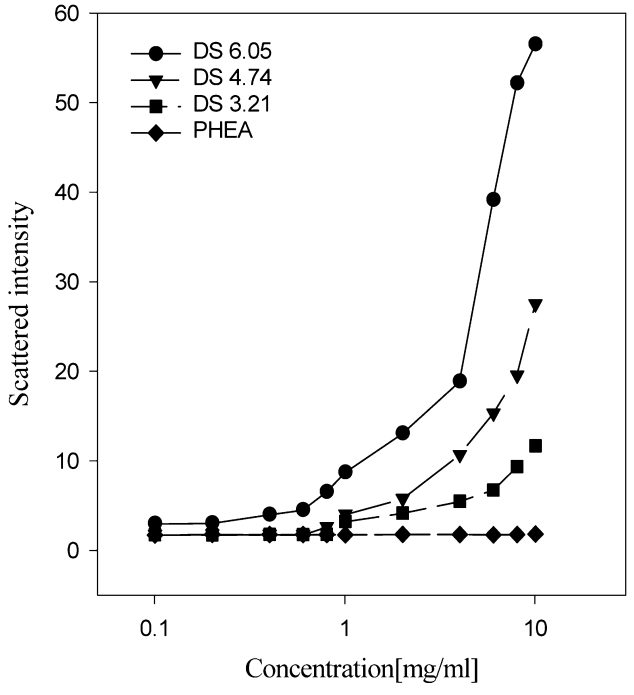

Fig. 8 Light scattering behavior of PHEA-g-DHA as a function of concentration and DS

9.88 showed a fast increase in size and became a little turbid within one week. Since all PHEA particles have hydroxyl groups, the surface properties are not much different when the primary particles are aggregated.

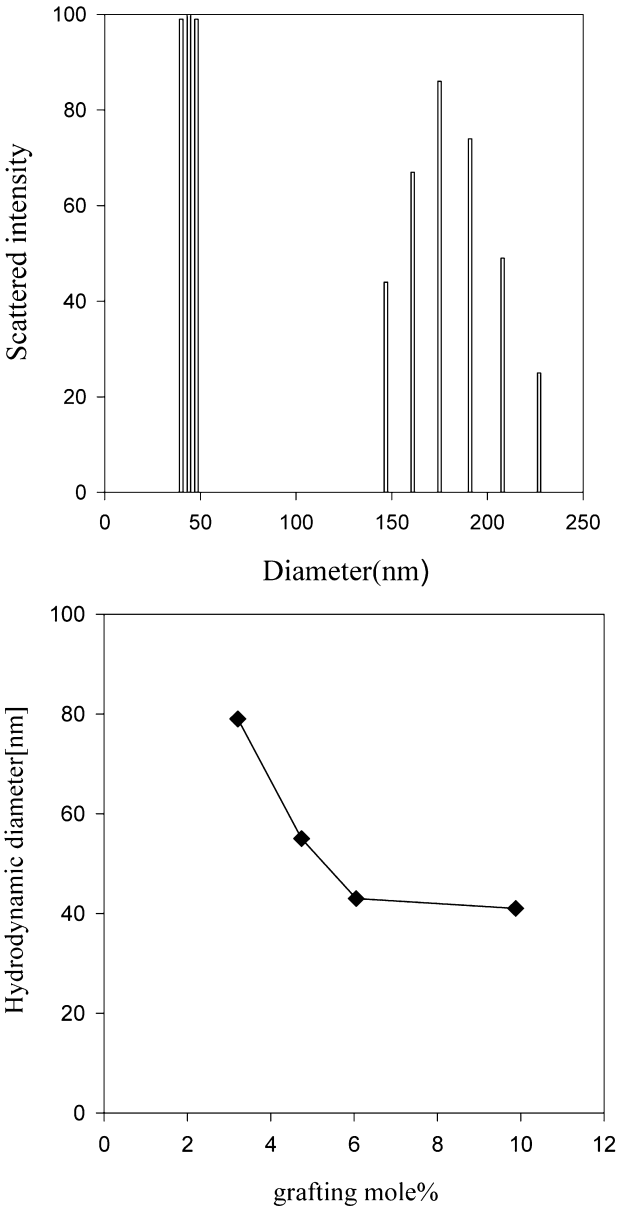

Fig. 9a–b Particle size distribution. **a** Bimodal size distribution of DS 6.05 (C = 1 mg/mL). **b** Size of primary aggregates as a function of DS

However, the rapid growth of turbid state at DS 9.88 was observed in sonicated solutions. 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. The swelling would be induced by the molecular interaction leading to the reduction of curvature K or sphere-to-planar surface transition. The kinetic aggregation forms the large particles by the inter-particle attraction of primary particles, while the shape change may be the result of coupling of two phenomena. At present, how-

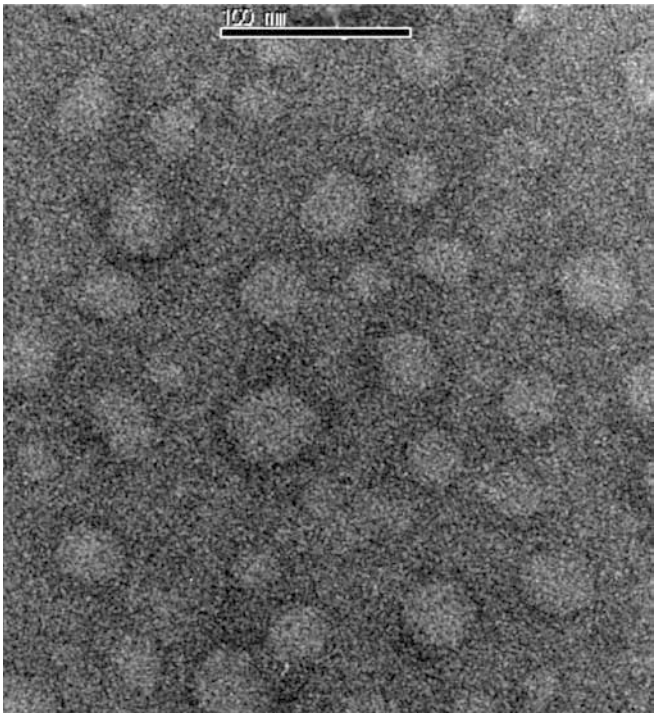

Fig. 10 TEM photograph of PHEA-g-DHA self-aggregate (DS 4.74)

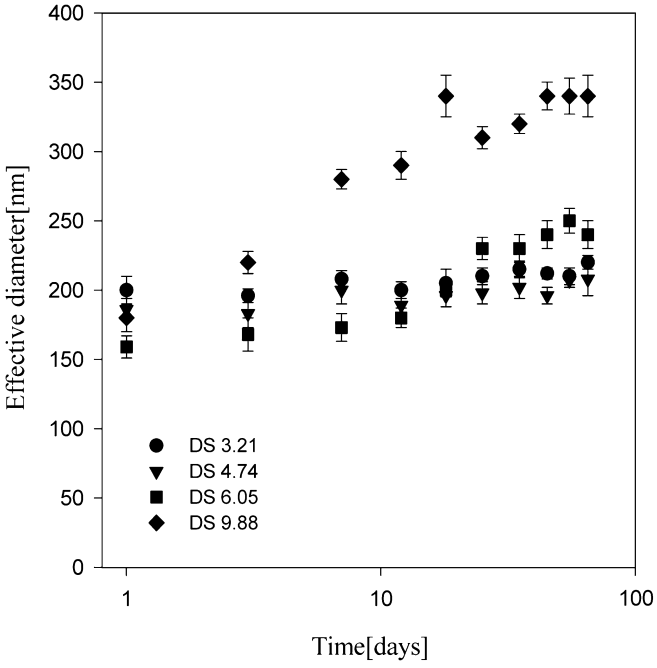

Fig. 11 Effective diameter change of self-aggregates of PHEA-g-DHA with time

ever, it is not possible to distinguish the mechanisms of growth corresponding to each DS.

Sonication may enhance the particle–particle dispersion in solution and also affects the aggregation to a tightly packed layer. If the tightly packed layer were swelled with solvent, it could increase the distance D_1 and result in the curvature reduction. If it swells one or two angstrom in D_1, the curvature approaches to zero. The slow increase of size at low DS may be the result of swelling. If the intrinsic interaction energy between side-chains overcomes the stress from the hydrophilic group, the primary particles become stable. In this case, the formation of secondary particles from primary particles is simply a kinetic phenomena and can be redispersed upon a mild mechanical agitation at medium DS. At high DS, however, the inclusion of solvent between side chains may change the curvature of primary particles close to $K = 0$, transferring to a new planar surface, and the aggregated secondary particles may grow intermixed as if it is bicontinuous. If the bending stress of the stiff backbone and the repulsive excluded volume of chain are too large to maintain their curvature, the aggregates are forced to reform their new size distribution after sonication. Therefore, it is believed that the slight modification of highly packed DHA groups induced the mechanical stress and reformed secondary aggregates into a new type.

Conclusion

Dehydrocholic acid (DHA) grafted poly(2-hydroxyethyl aspartamide) (PHEA) was successfully synthesized through ester linkage and these amphiphilic polymers formed self-aggregates in aqueous solution in the range of DS less than 10mole%. The critical aggregation concentrations of self-aggregates(CAC) were determined by fluorescence spectroscopy and decreased with DS. The size distribution of self-aggregates was bimodal, and the primary particles were about 40–80 nm, decreased with DS. After sonication and storage, the size distribution of self-aggregates changed with time. At low DS, the size may increase by swelling, and at medium DS, the solution remains in the most stable state, while at higher DS, the solution turns turbid. Such a rapid transition can be attributed to the formation of irreversible interfused secondary structure by both the curvature change and aggregation of primary particles. Therefore, the interaction of neighboring DHA groups stabilizes the primary self-aggregates at low DS, while at high DS, the stiffness of the PHEA backbone destabilizes the primary aggregates to form irreversible interfused large aggregates.

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