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Research Article

Preparation and Characterization of Self-Assembled Nanoparticles of Hyaluronic Acid-Deoxycholic Acid Conjugates

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Novel amphiphilic biopolymers were synthesized using hyaluronic acid (HA) as a hydrophilic segment and deoxycholic acid (DOCA) as a hydrophobic segment by a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide mediated coupling reaction. The structural characteristics of the HA-DOCA conjugates were investigated using ^{1}H NMR. Self-assembled nanoparticles were prepared based on HA-DOCA conjugates, and its characteristics were investigated using dynamic laser light scattering, transmission electron microscopy (TEM), and fluorescence spectroscopy. The mean diameter was about 293.5 nm with unimodal size distribution in distilled water. The TEM images revealed that the shape of HA-DOCA self-aggregates was spherical. The critical aggregation concentration (CAC) was in the range of 0.025–0.056 mg/mL. The partition equilibrium constant (K_v) of pyrene in self-aggregates solution was from 1.45×10^4 to 3.64×10^4. The aggregation number of DOCA groups per hydrophobic microdomain, estimated by the fluorescence quenching method using cetylpyridinium chloride, increased with increasing degree of substitution.

1. Introduction

Polymeric amphiphiles derived from hydrophobically modified soluble polymers have recently attracted much attention because of their potential application in drug delivery systems [1–3]. Such amphiphiles are able to spontaneously form micelles or nanoparticles via undergoing intra- and/or intermolecular association between hydrophobic moieties in aqueous environment. The hydrophobic parts form the core of the nanoparticles, which is a host system for various hydrophobic drugs, while the hydrophilic backbone forms corona or outer shells enwrapping the hydrophobic core. This shell prevents the inactivation of the encapsulated drug molecules by decreasing the contact with the inactivating species in the aqueous (blood) phase [4–6]. Furthermore, these polymeric nanoparticles exhibit unique characteristics, such as special rheological features, a rather narrow size distribution, considerable lower critical aggregation concentrations (CAC) than surfactant of low molecular weight, and thermodynamic stability [4, 7–9]. Recently, self-assembled nanoparticles based on natural polysaccharides have been of particular interest because of their good biocompatibility, biodegradability, reduced toxic side effects, and improved therapeutic effects [10–12].

Hyaluronic acid (HA) is a natural mucopolysaccharide that consists of alternating residues D-glucuronic acid and N-acetyl-D-glucosamine. HA plays a key role in the structure and organization of the extracellular matrix, transport of nutrients, a regulation of cell adhesion, morphogenesis, and modulation of inflammation [13–16]. The immunoneutrality and nontoxicity of HA make it as an attractive building block for new biocompatible and biodegradable polymers [17]. HA has been applied in drug delivery systems, tissue engineering, and viscous supplementation [18]. Its major advantages as a drug carrier consist of its ability to bind CD44, a specific membrane receptor frequently overexpressed on the tumor cell surface [19, 20]. That is, targeting of anticancer agents to tumor cells and tumor metastases can be accomplished by receptor-mediated uptake of complexes of these agents and HA. Nevertheless, the poor biomechanical properties of HA prevent generation of new biomaterials, a fact that has given rise to a variety of chemical modifications of HA for providing mechanically and chemically stable materials [21]. The resulting HA derivatives have

better physicochemical properties than the native polymer, but retaining the biocompatibility and biodegradability of native HA. Previous studies have been performed on several different HA derivatives, for drug delivery, such as films, hydrogels, bioconjugates, and microspheres [22–25]. But few studies about nano-scaled HA derivatives have been reported [26, 27]. Nanoparticles as drug carriers have been considered to provide opportunities for the site-specific delivery of drugs by the enhanced permeation and retention (EPR) effect, and they have the ability to dissolve hydrophobic agents and protect the bioactive drug from host [28–30].

Herein the hydrophobically modified HA nanoparticles with favorable physicochemical properties are promising as drug delivery system for the pharmaceutical applications. In this study, chemical conjugates of HA and DOCA were synthesized by covalent attachment of DOCA to HA through amide formation. Physicochemical characteristics of the amphiphilic HA-DOCA conjugates in aqueous phase were revealed by dynamic light scattering, transmission electron microscopy and fluorescence probe techniques. Deoxycholic acid is known to form micelles in water as a result of its amphiphilicity [31]. Thus, it is expected that HA, hydrophobically modified with deoxycholic acid, will induce self-assembled aggregates.

2. Experimental

2.1. Materials. Hyaluronic acid (HA) (average Mn = 16600 Da) was purchased from Freda Biochemical company (Shandong, China). Deoxycholic acids (DOCA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), N,N-dicyclohexyl carbodiimide (DCC), and cetylpyridinum chloride (CPC) were obtained from Sigma Chemical Co. (MO, USA). These reagents were all of analytical grade and used as received. Pyrene, as fluorescence probe was purchased from Sigma and purified by double recrystallization from absolute ethanol. Water was purified by distillation, deionization, and reverse osmosis (Milli-Q plus, Bedford, UK).

2.2. Synthesis of N-Deoxycholylethylenediamine (DOCA-NH_2). Firstly, the carboxylic group of deoxycholic acid was activated, as reported previously (11). In brief, DOCA (3.54 g) was mixed with DCC (2.40 g) and NHS (1.48 g) in 30 mL of THF. The feed mole ratio of DOCA, DCC and NHS were 1 : 1.2 : 1.2. The concentration of DCC and NHS was slightly higher than DOCA in order to activate DOCA completely. The mixture was allowed to react for 12 h at room temperature under a nitrogen atmosphere, and the precipitated dicyclohexylurea was removed by filtration. The filtered solution was poured into excessive hexane and the remaining NHS was dissolved in the hexane. The succinimido DOCA precipitate was filtered off and washed thoroughly with hexane, followed by vacuum-drying (DZF, Chemat, USA) at room temperature. The succinimido DOCA was stored at −20°C before use.

The N-deoxycholy-ethylenediamine (DOCA-NH_2) was synthesized by introducing ethylenediamine to the succinimido DOCA. The succinimido DOCA (5 g, 10 mmoL) was dissolved in DMF (20 mL) and the solution was slowly added dropwise into ethylenediamine solution (70 mL, 1 moL) in 250 mL beaker with separatory funnel (34731-00, Coleparmer, USA). The free mole ratio of succinimido DOCA and ethylenediamine was about 1 : 100. After reaction for 6 h, the mixture was precipitated in exceed distilled water. The filtered precipitation was carefully washed three times with distilled water and vacuum-drying at room temperature to obtain white powder DOCA-NH_2.

2.3. Preparation of HA-DOCA (HD) Conjugates. HA (0.1 g) was dissolved in formamide (5 mL) during gentle magnetic stirring, in a 20 mL beaker, at room temperature. Different amounts of EDC (the mole ratio to HA was 5 : 1, 10 : 1, 15 : 1, respectively) were thereafter added to the HA solution. Different amounts of DOCA-NH_2 (the mole ratio to HA was 10 : 1, 20 : 1, 40 : 1, respectively) were dissolved in DMF (5 mL) in a 20 mL beaker, by gentle heating (at 50°C) and added into the mixed solution of HA and EDC. The resulting solution was thereafter cooled at room temperature and then stirred under a nitrogen atmosphere for 24 h. The reaction mixture was extensively dialyzed against the excess amount of water/acetone (1v/3v-1v/1v) and distilled water for 3 days, followed by lyophilization.

Various HD conjugates were prepared by controlling the free mole ratios of DOCA-NH_2 to HA. The degree of substitution (DS), defined as the number of DOCA per one HA molecules, was determined by a titration method as previously reported (11). Briefly, HD conjugates (50 mg) were diffused in distilled water (20 mL) in a 50 mL beaker during magnetic stirring. After adding 0.05 mL of 0.1 N HCl, the solution was titrated with 0.01 N NaOH using microtitrator (PAX 100-3, Burkard, UK) while stirring and measuring the pH (Delta 320, Mettler Toledo, Switzerland).

The consumed amount of NaOH solution when the pH reached neutral value was recorded. By that, the DS of DOCA was calculated using the difference in the amounts of NaOH solution added to the solutions between standard HA solution and HD conjugate solutions.

2.4. Preparation of Self-Assembled Nanoparticles of HA-DOCA Conjugates. The HD conjugates were suspended in distilled water at 37°C for 24 h. The solution was then sonicated three times using a probe-type sonifier (Sonics Ultrasonic Processor, VC750) at 90 W for 2 min each, in which the pulse was turned off for 1 s with the interval of 5 s to prevent the increase in temperature. Solutions of different concentrations (0.0001–1 mg/mL) were obtained by diluting the stock solution with distilled water.

2.5. ^1H Nuclear Magnetic Resonance (NMR) Spectroscopy. The ^1H NMR spectra of the conjugates were obtained using a 500-MHz NMR (UNIYTINOVA-500 NMR, VARIAN) at 25°C. Samples of HA and HD conjugates were separately dissolved in solutions of D_2O or $CDCl_3$ (analytical reagent, Sigma), yielding a concentration of 10 mg/mL. The measurement conditions were as follows: a spectral window of 500 Hz, 32 k data points, a pulse angle of 30°, an acquisition

time of 2.03 s, and 32 scans with a delay of 1 s between scan [32].

2.6. Particle Size Distribution. To determine the average particle size and size distribution of self-aggregates, dynamic laser light scattering were performed using a helium ion laser system (Spectra Physics Laser Model 127-35). Three milliliters of self-aggregates suspension in distilled water (HD9: 1 mg/mL) was put into polystyrene latex cells and measured at a detector angle of 90°, a wavelength of 633 nm, and a temperature of 25°C.

2.7. Measurement of Fluorescence Spectroscopy. The fluorescence measurements were used to determine the critical aggregation concentration as previously described by Liu et al. [32]. Pyrene, used as a hydrophobic probe, was purified by repeated recrystallization from ethanol and vacuum-dried at 20°C. Purified pyrene was dissolved in pure ethanol (analytical reagent, Sigma) at the concentration of 0.04 mg/mL. About of 20 μL of the result in solution was added into a 20 mL test tube and the ethanol was evacuated under purging of nitrogen gas. Four milliliters of HD nanoparticles solution was subsequently added to the test tube, resulting in a final pyrene concentration of 1.0×10^{-6} M. The concentration of nanoparticles in the solution ranged from 1×10^{-4} mg/mL to 1 mg/mL. The mixture was incubated for 3 h in a water bath at 65°C and shaken in a SHA-B shaking water bath GuoHua company, Hebei, China overnight at 20°C. Pyrene emission spectra were obtained using a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu Co., Kyoto, Japan). For measurements of intensity ratios for the third and the first peaks (I_3/I_1) in the emission spectra for pyrene, the slit openings for excitation and emission were set at 15 and 1.5 nm, respectively. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 343 and 390 nm, respectively. The spectra were accumulated with an integration time of 3 s/nm.

The hydrophobicity of self-aggregates in this study was estimated by measuring the equilibrium constant (K_v), for partitioning of pyrene, between the water and nanoparticles phase as described by Wilhelm et al. [33],

$$\frac{F - F_{\min}}{F_{\max} - F} = \frac{K_v X_{\mathrm{DOCA}} (c - \mathrm{cac})}{1000 \rho_{\mathrm{DOCA}}}, \quad (1)$$

Where $F_{\max}$ and $F_{\min}$ are the intensity ratios (I_3/I_1) at high and low concentration ranges of self-aggregates in Figure 6, and F is the intensity ratios (I_3/I_1) at the intermediate HD nanoparticle concentration region. X_{DOCA} is the weight fraction of DOCA in the conjugates, c is the concentration of the HD nanoparticles, and ρ_{DOCA} is the density of the inner core of self-aggregates, assumed to be equal to the density of DOCA(1.31 g/mL) [34].

The aggregation number of an associating DOCA hydrophobic domain was estimated by using the steady-state fluorescence quenching technique. CPC was used as quencher dissolving in distilled water to different concentrations ($2.0 \times 10^{-7} - 8.0 \times 10^{-7}$ M). The CPC solution was added to the nanoparticle suspension with pyrene just before the measurement. Steady-state quenching data in a microheterogeneous system such as an aqueous nanoparticle suspension fit in the quenching kinetics in [35]

$$\ln\left(\frac{I_0}{I}\right) = \frac{[Q]}{[M]}, \quad (2)$$

Where I and I_0 are the fluorescence intensities, in the presence or absence of a quencher, respectively, $[Q]$ is the concentration of the quencher, and $[M]$ is the concentration of hydrophobic domains in a polymer self-aggregates. Thus, $[M]$ can be obtained from the slope of $\ln(I_0/I) = f([Q])$ and the aggregation number per single hydrophobic microdomain (N_{DOCA}) is given by

$$N_{\mathrm{DOCA}} = \frac{[\mathrm{DOCA}]}{[M]}. \quad (3)$$

2.8. Transmission Electron Microscopy (TEM). To measure the morphology and size distribution of the nanoparticles, swatches were prepared by dropping the sample solution (1 mg/mL) onto a Formvar-coated copper grid. The grid was held horizontally for 2 min to allow the molecular aggregates to deposit. The surface water was then removed by tapping with a filter paper (9 cm, Xinhua company, China), followed by air-drying. One drop of 2% uranyl acetate solution (analytical reagent, Sigma) was added to the grid to give a negative stain for nanoparticles. The grid was then allowed to stand for 3 min at room temperature before the excess staining solution was removed by draining as above. The dried grid containing the nanoparticles was visualized using a Philips EM 400 transmission electron microscope (Koninklijke Philips Electronics N.V, the Netherlands) at an acceleration voltage of 80 KV.

3. Results and Discussion

3.1. Synthesis and Characterization of HA-DOCA Conjugates. Firstly, we activated DOCA with DCC and NHS then introduced a primary amino group to DOCA using ethylenediamine. For the synthesis of HD conjugates, we chemically coupled DOCA-NH_2 to HA with EDC as a cross-linker. EDC is a so-called "zero-length" cross-linker, which gives an amide linkage without leaving a spacer molecule. By this coupling reaction, various HD conjugates were prepared by controlling the free mole ratios of DOCA-NH_2 to HA. The schemes for the introduction amine group to the carboxylic acid of DOCA and subsequent coupling reaction of HA and N-deoxycholylethylenediamine (DOCA-NH_2) are shown in Figure 1.

The presence of DOCA in HA was verified by the characteristic peaks of DOCA appearing in the ^{1}H NMR spectra. Figure 2 shows the ^{1}H NMR spectrum of HA and HD conjugates in different solvent systems including both D_2O and $CDCl_3$. The proton assignment of HA (Figure 2(a)) is as follows: δ2.0 =(3H, NHCO–CH3), δ3.3–3.9 = (1H, H-2, 3, 4, 5, and 6) [36]. The proton assignment of HD (Figure 2(b)) is as follows: δ0.67 = (3H, 18-CH3), δ0.85 = (3H, 19-CH3), δ0.99 = (3H, 21-CH3), δ1.06–2.32 = (25H, steroidal H), δ2.22 = (3H, –NH–CO–CH3) [37]. The presence of DOCA in HA was evaluated by the characteristic

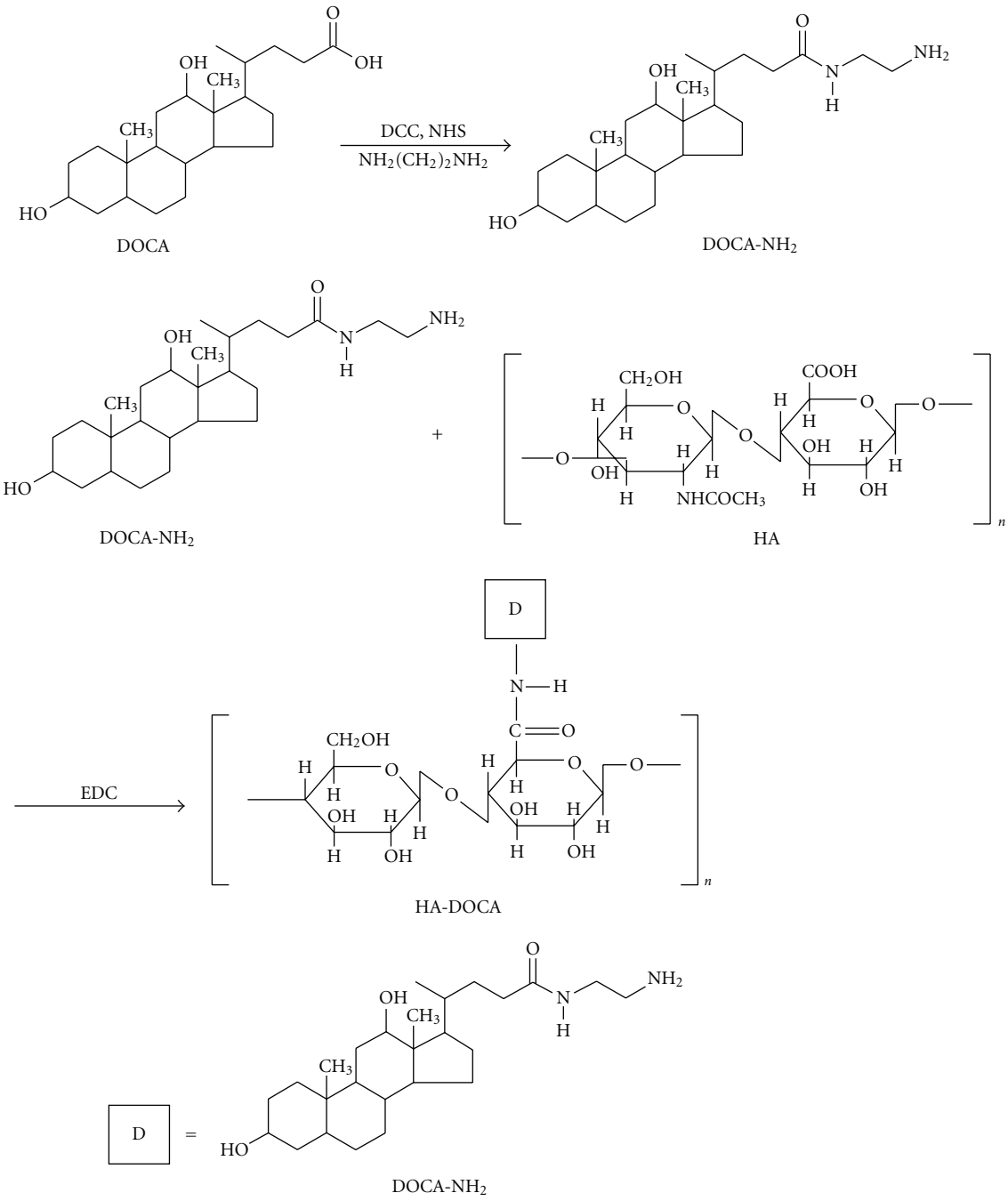

Figure 1: Synthesization scheme of HA-DOCA conjugates.

peaks of DOCA appearing at 0.85–2.3 ppm in the spectra (Figure 2(b)).

Various HD conjugates with different amounts of DOCA were prepared by changing the feed ratio of HA to DOCA. The results indicated that the degree of substitution (DS) of DOCA increases as the added feed ratio of DOCA and EDC increases. EDC could react with the carboxyl group of the HA to form an active ester intermediate which could chemically couple with DOCA-NH_2. The DS is in the range from 5.9 to 9.4 per one HA molecule in this experiment. The mean molecular weight and DS are summarized in Table 1.

3.2. Formation of Self-Aggregated Nanoparticles. The formation of self-assembled nanoparticles in an aqueous phase and the critical aggregation concentration (CAC) for HD self-aggregates were confirmed by the fluorescence technique with pyrene as a fluorescence probe. Pyrene was chosen as the fluorescence probe because its condensed aromatic structure is sensitive to polarity, and it produces distinctive excimer fluorescence under conditions of sufficiently high concentration and mobility [38]. When the self-aggregates are formed in an aqueous phase, pyrene molecules are preferentially located within or close to the hydrophobic

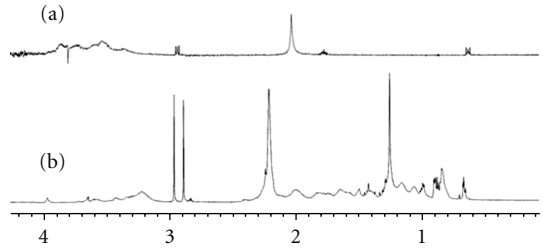

Figure 2: ^{1}H NMR spectra of HA in D_2O (a) and HD9 in $CDCl_3$ (b) at 25°C.

Table 1: General properties of hyaluronic acid-deoxycholic acid conjugates in distilled water.

Sample[a]	Feed ratio[b]	Mn[c]	DS	X[d](%)
HD6	1 : 10	18925	5.9	12.3
HD7	1 : 20	19594	7.6	15.3
HD9	1 : 40	20304	9.4	18.2

[a]HA-DOCA (HD) conjugates, where the number indicates the DS of DOCA. [b]Mole ratio of HA : DOCA-NH_2. [c]Number-average molecular weight, estimated from the titration results. [d]Weight fraction of DOCA in a HA molecule.

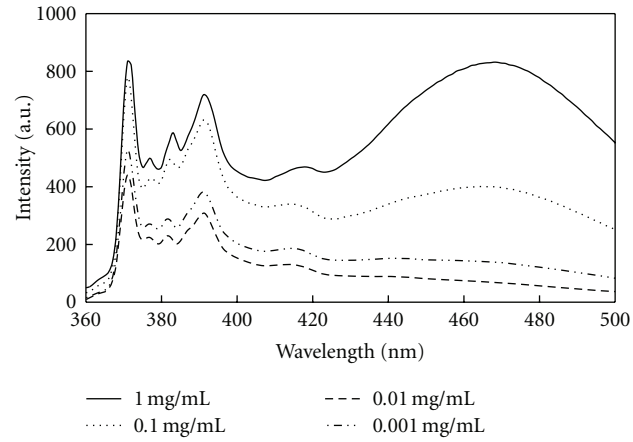

Figure 3: Fluorescence emission spectra of pyrene in the presence of HA-DOCA nanoparticles (HD9) at a fixed excitation wavelength of 343 nm. The concentrations of HA-DOCA conjugate were from 0.00001 to 1.0 mg/mL in distilled water with 1.0 μM of pyrene.

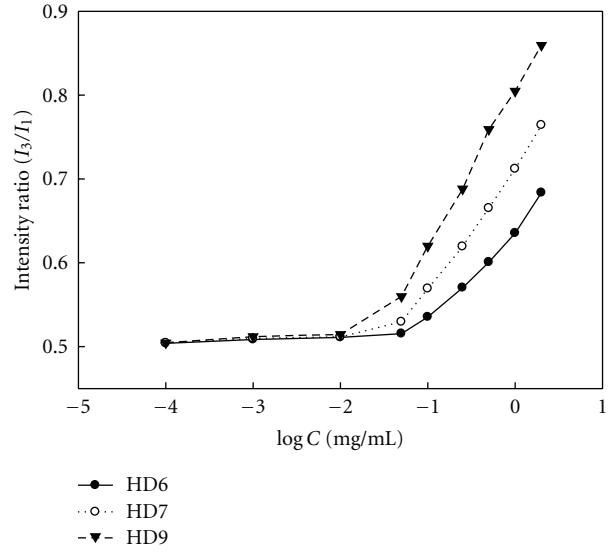

Figure 4: Intensity ratios (I_3/I_1) from pyrene emission spectra as a function of logarithm of HA-DOCA conjugate concentration in distilled water.

microdomain of the nanoparticles rather than to the aqueous phase. Consequently, there was a remarkable change in its fluorescence spectra, such as an increase in the quantum yield [38, 39]. Figure 3 shows the typical fluorescent emission spectra of pyrene in solutions of HD nanoparticles (sample HD9) with various concentrations. The fluorescent emission spectra of pyrene in solutions exhibit four predominant peaks, one for each concentration. The fluorescence intensities did remarkably increase with the increasing of self-aggregates concentrations especially for the first and third peaks suggesting that pyrene was shifted from polar water environment to less polar one because pyrene had a longer lifetime and a higher quantum yield in the nonpolar environment. The intensities of fluorescence increase substantially, suggesting the formation of nanoparticles and the incorporation of pyrene in the hydrophobic core of self-aggregates.

The partition of pyrene from aqueous to hydrophobic phase of nanoparticles causes the ratios of peak III (I_3) with peak I (I_1) to increase with the increasing of HD conjugates concentration especially above the CAC. Critical aggregation concentration (CAC), which is the threshold concentration of self-aggregate formation by intra- and/or intermolecular association, can be determined from the change of intensity ratio (I_3/I_1) of pyrene in the presence of polymeric amphiphiles. The intensity ratio of I_3/I_1 is sensitive to the polarity of the microenvironment where the pyrene exists: the larger the I_3/I_1 ratio, the less polar microenvironment of pyrene. Figure 4 exhibits the changes of the I_3/I_1 value as a function of logarithm of concentration of HD self-aggregates for samples of HD6, HD7 and HD9. At low concentrations, the I_3/I_1 value remains nearly unchanged meaning that there is a lack of hydrophobic nonpolar environment with no occurrence of assemblage. However, the intensity ratios begin to increase with further increase in concentration, indicating the occurrence of self-aggregation of HD resulting from intermolecular hydrophobic interactions between the DOCA groups. The CAC was taken as the intersection of a flat region in the low concentration extreme and sigmoid region in the crossover region. The CAC values determined for HD conjugates HD6, HD7 and HD9 are 0.056, 0.037 and 0.025 mg/mL, respectively, which agree well with the conjugate compositions as the DOCA group in conjugates is increased in the same order as listed in Table 2. The CAC values of deoxycholic acid modified HA are similar to that of the deoxycholic acid modified chitosans self-aggregates (CAC are in the range of 0.01–0.07 mg/mL) [5, 40], but much lower than the critical micelle concentration

Table 2: Microscopic characteristics of HA-DOCA self-aggregates determined by the fluorescence probe method.

sample	CAC[a] (mg/mL)	K_v[b] ($\times 10^4$)	N_{DOCA}[c]	N_{chain}[d]
HD6	0.056	1.45	17.6	2.98
HD7	0.037	2.83	27.8	3.66
HD9	0.025	3.64	43	4.57

[a]Critical aggregation concentration determined by pyrene emission spectra. [b]Binding equilibrium constant for pyrene in distilled water in the presence of HA-DOCA nanoparticles. [c]Aggregation number of DOCA moieties per one hydrophobic domain. [d]Number of HA-DOCA conjugate polymer chains per one hydrophobic domain.

(cmc) of low-molecular-weight surfactants (2.3 mg/mL for sodium dodecyl sulfate (SDS) in water and 1.0 mg/mL for deoxycholic acid in water) [41, 42]. The lower CAC values of the hydrophobic modified HA as compared with low-molecular-weight surfactants may be one of the important characteristics of polymeric amphiphiles, indicating the stability of HD self-aggregate in dilute condition.

3.3. Microscopic Structure of Self-Aggregates. Figure 4 shows that above the CAC, the intensity ratio increases due to the binding of pyrene is larger. This suggests that pyrene preferentially interacts with amphiphiles phase than to self aggregate and then distributes into the inner core of self-aggregates. The partition of pyrene between the aqueous and self-aggregates phase could reflect the hydrophobicity of the inner core of nanoparticles. The hydrophobicity is one important factor for its application as a drug carrier and it can be expressed as equilibrium constant K_v of pyrene, which is one of the critical parameters related to self-assembled nanoparticle stability. At the concentration range of HD conjugates from 0.0001 mg/mL to 1 mg/mL, the mean diameter and the size distribution factor of self-aggregates were not changed. Therefore, we assume a simple equilibrium, the equilibrium constant (K_v) for partitioning of pyrene between the water and micellar phases can be calculated according to the (1). Where the K_v values of pyrene can be determined using a plot of $(F - F_{min})/(F_{max} - F)$ versus the HD nanoparticle concentration as shown in Figure 5. Table 2 summarizes the K_v values for pyrene, and they are in the range of 1.45–3.64 $\times$ 10^4. As shown in Table 2, K_v values increase with increasing DS of DOCA in the conjugates, indicating the increase of hydrophobicity of inner core of the nanoparticles with the DS. On the basis of these K_v values, we determined that HD self-aggregates also formed less polar microdomains and that their hydrophobic cores were covered with hydrophilic HA shells. By comparing to the HD self-aggregates, other polymer amphiphiles based on poly (ethylene oxide) or poly (2-ethyl-2-oxazoline) as hydrophilic blocks have higher K_v values [33, 43]. Whereas the hydrophobicity of the interior core of HD self-aggregates is very similar to glycol chitosan nanoparticle bearing 5β-cholanic acid as a hydrophobic segment and heparin-DOCA nanoparticle, in addition it is higher than glycol chitosan-DOCA nanoparticle ($\sim 10^3$).

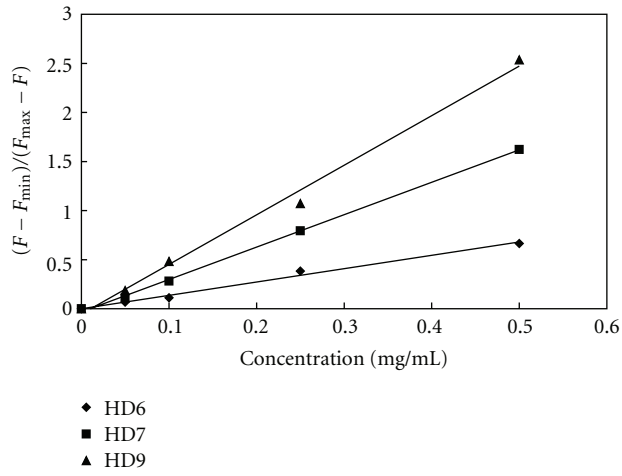

Figure 5: Plots of $(F - F_{min})/(F_{max} - F)$ versus concentration of HA-DOCA conjugate in distilled water.

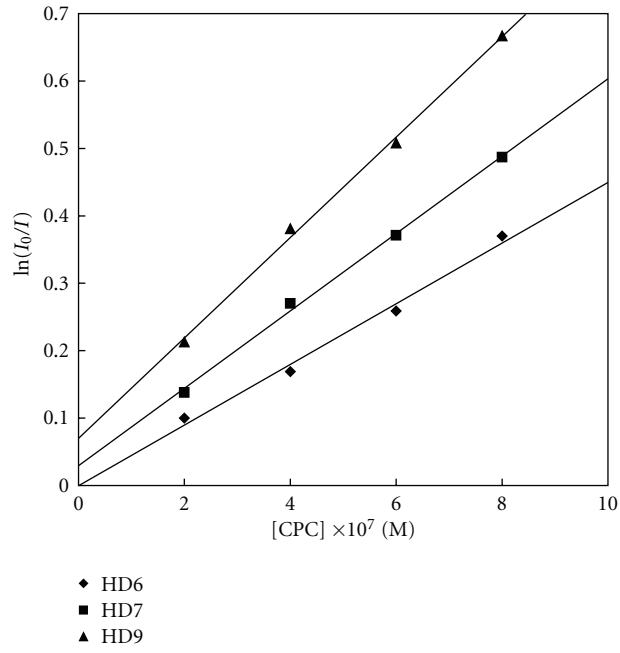

Figure 6: $\ln(I_0/I)$ of pyrene (1×10^{-6} M) fluorescence as a function of CPC concentration in the presence of HA-DOCA conjugates at the concentration of 0.5 mg/mL.

To investigate the associating number of hydrophobic moieties in one self-aggregate and to identify the microstructure of nanoparticles, a fluorescence quenching method was used which has been successfully applied to estimate the microstructure of polymeric amphiphiles [39, 44]. Figure 6 shows the intensity $[\ln(I_0/I)]$ of pyrene fluorescence as a function of CPC concentration in the presence of the HD conjugates at the concentration of 0.5 mg/mL in water. There is a good liner relationship with all the cases (correlation factor, $r = 0.9974$ (HD6), $r = 0.9974$ (HD7), $r = 0.9892$ (HD9)) and the date fit well to (2). Hence, the concentration of hydrophobic domains in self-aggregates could be obtained

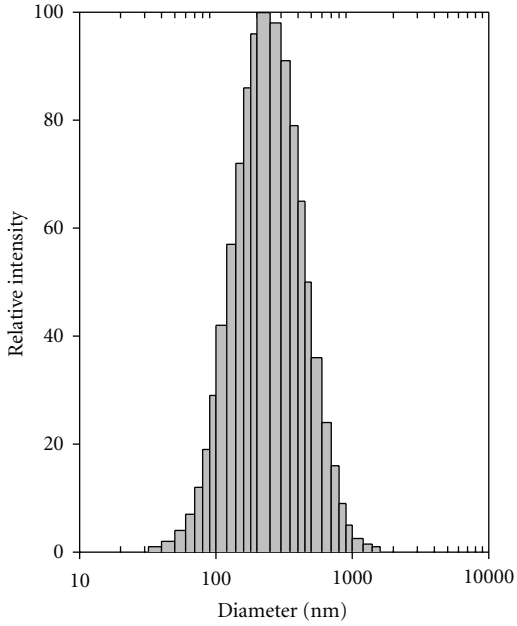

Figure 7: Size distribution of HA-DOCA nanoparticles (HD9, 1 mg/mL) prepared in distilled water.

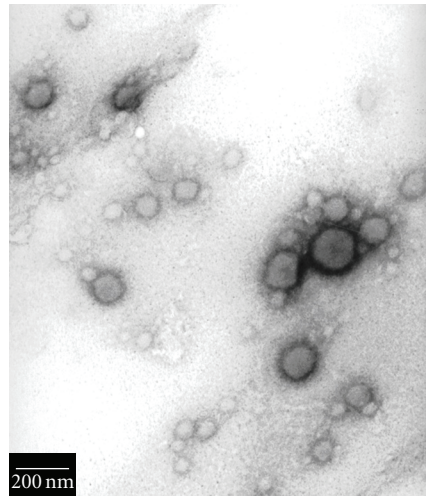

Figure 8: Transmission electron micrograph of self-aggregates based on HA-DOCA conjugate (HD9, 1 mg/mL).

from the slope of the straight line. The mean aggregation numbers of DOCA groups (N_{DOCA}) per single hydrophobic domain are calculated from (3) and the values are listed in Table 2. The aggregation number increase with increasing of the DS of DOCA which means that the conjugated DOCA groups may all interassociate to form hydrophobic microdomains. The N_{DOCA} values are much higher than the aggregation number of sodium deoxycholate in aqueous media (8 ± 2) [45]. The big aggregation number in self-aggregates is considered to result from the DOCA moieties didn't form dense hydrophobic interior core because of the limited mobility and steric hindrance of DOCA covalently attached to HA. The N_{chain} value is defined as the number of polymer chains per one hydrophobic domain. There are about 2.98–4.57 chains of HD conjugates forming one hydrophobic microdomain from the fluorescence quenching study. The N_{chain} values increased with the increase of DS of DOCA which means that compact hydrophobic domains may be formed with enhanced hydrophobicity. The increase of N_{DOCA} and N_{chain} values corresponds to the DS of DOCA which indicates the higher hydrophobicity resulting from the increasing DOCA moieties making the reinforced association between single polymer chains. From the microscopic characteristics of HD conjugate, it can be concluded that one HD nanoparticle could have multiple hydrophobic inter-cores. The results are very similar to the other hydrophobic modified polysaccharide biocopolymers with different fatty acids and bile acids [8, 9, 11, 44].

3.4. Characteristics of Self-Aggregated Nanoparticles. The self-aggregated nanoparticles of HD conjugates were produced by a simple sonication method in aqueous condition. Figure 7 shows the particle size and distribution of self-aggregates of HD conjugates in a distilled water solution determined by dynamic laser light scattering. A majority of nanoparticles size was in 100–600 nm with a mean hydrodynamic diameter of 293.5 nm as shown in Figure 7. The polydispersity factor of self-aggregates was 0.108 and it demonstrated a narrow particle size distribution. The mean diameter of each nanoparticle was reproducible with repeated experiments and it was not changed with respect to the sonication time that ranged from 3 to 20 min. The size of Nanoparticle was not significantly affected by changing concentration of HD conjugates. This implies that the interactions between self-aggregates are almost negligible.

TEM observation demonstrated the near spherical shape of self-aggregates with narrow size distribution, as shown in Figure 8. The mean diameter and size distribution of self-aggregates appeared to be a little different from the results obtained by dynamic light scattering measurement. For example, the size measured by the TEM methods was smaller than the laser light scattering method. This might be due to the different conditions for sample preparation. The TEM images depicted the size at the dried state of the sample, whereas the laser light scattering method involves the measurement of size in the hydrated state. In other words, the size determined by TEM is an actual diameter (dry state) of the nanoparticles, whereas the size measured by the dynamic laser scattering method is a hydrodynamic diameter (hydrated state) of the nanoparticles. Therefore, in the hydrated state, the nanoparticles will have a higher hydrodynamic volume due to solvent effect; hence, the size measured by the laser light scattering method was higher than the TEM method [32].

4. Conclusion

Novel polymeric amphiphiles, partially hydrophobic modified hyaluronic acid with deoxycholic acid as hydrophobic moieties in an aqueous solution, were studied. The diameters of self-aggregates were in the range of 100–600 nm with

a mean hydrodynamic diameter of 293.5 nm. The critical aggregation concentrations of HD conjugates depended on the DS of deoxycholic acid in a range of 0.025–0.056 mg/mL. The TEM images of self-aggregates showed a spherical shape. The K_v and N_{DOCA} values, confirmed by fluorescence probe studies, were substantially dependent on the DS of DOCA. Results demonstrate that a hydrophobic domain was composed of multiple HD polymer chains.

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