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Amphiphilic cholesteryl grafted sodium alginate derivative: Synthesis and self-assembly in aqueous solution

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Abstract

A novel water soluble amphiphilic sodium alginate derivative grafted with 3 cholesteryl groups per 100 hexuronic acid residues (CSAD-3) has been first synthesized with *N*,*N*′-dicyclohexylcarbodiimide as a coupling agent and 4-(*N*,*N*′-dimethylamino)pyridine as a catalyst at room temperature. The result of fluorescence analysis shows that CSAD-3 forms self-aggregates in 0.15 mol/L aqueous NaCl solution and its critical aggregation concentration (CAC) is 0.33 g/L. The value of CAC decreases with increase of the concentration of aqueous NaCl solution. The CSAD-3 self-aggregates are able to encapsulate hydrophobic compounds like pyrene. The hydrodynamic diameter of CSAD-3 self-aggregates in aqueous NaCl solution is 136 nm determined by dynamic light scattering measurement. The oblate particles with the size about 100–200 nm are observed in the negatively dyed TEM image of CSAD-3 self-aggregates.

Keywords: Sodium alginate; Cholesterol; Amphiphilic; Self-assembly; Critical aggregation concentration

1. Introduction

Alginate is a linear anionic polysaccharide consisted of two kinds of hexuronic acid residues including 1,4-β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, which are arranged in repeating GG (MM) blocks or alternating MG blocks (Scheme 1) (Kang, Jeon, Lee, & Yang, 2002). As a natural biopolymer, alginate has been found increasing biotechnological and biomedical applications in view of its several advantages, such as high biocompatibility, biodegradability, non-toxicity, non-immunogenicity, chelating ability, and the possibility of chemical modification. In particular, the sodium alginate hydrogels crosslinked by calcium ions are widely used for the encapsulation of cells, proteins, oligonucleotides or DNA (Ferreiro, Tillman, Hardee, & Bodmeier, 2002; Gombotz & Wee, 1998; Quong, Neufeld, Skjak-Braek, & Poncelet, 1998; Simpson et al., 2006;Vandenberg, Drolet, Scott, & de la Noue, 2001), and also as the scaffolds for cartilage tissue engineering (Iwasaki et al., 2004; Wang et al., 2003). However, the crosslinking structures are easy destroyed in biological buffers containing chelators of calcium ions or monovalent electrolytes. As a result, the hydrogels will lose most of their initial mechanical and swelling properties within a few hours (Rastello De Boisseson et al., 2004).

Recently, Dellacherie (Leonard, Rastello de Boisseon, Hubert, & Dellacherie, 2004; Leonard, Rastello de Boisseon, Hubert, Dalencon, & Dellacherie, 2004) reported that the microparticles prepared by amphiphilic derivatives of sodium alginate bearing with long alkyl chains, could stably exist in aqueous NaCl solution and even could encapsulate proteins for several days. In addition, the hydrophobically modified alginate formed strong hydrogels in aqueous solution used as protein carriers with specific controlled release properties. The research of Hall (Broderick et al., 2006) showed that calcium ion crosslinked hydrogels of amphiphilic alginate derivative linked with butyl chains was capable of encapsulating both

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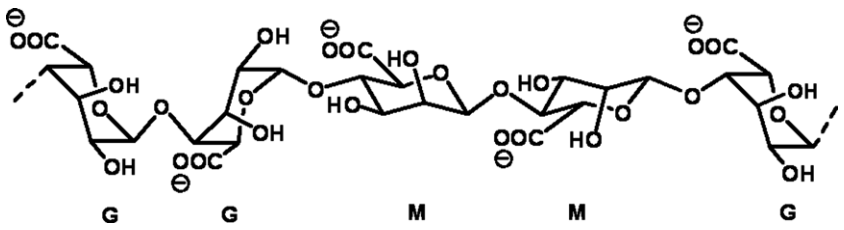


Scheme 1. Chemical structure of alginate.

hydrophobic and hydrophilic molecules, and retained the gelling and non-toxic properties of native alginate. These works suggest that the preparation of amphiphilic alginate derivatives may open up the prospects for new areas of research in biotechnology and pharmacology.

We considered cholesteryl as the hydrophobic segments rather than alkyl chains, because cholesteryl possesses much better bio-compatibility and potential interaction with cholesteryl receptors on cell surface, and stronger ability to drive self-assembly of cholsteryl containing polymers (Liu, Pramoda, Yang, Chow, & He, 2004). Akiyoshi and Sunamoto (Akiyoshi, Deguchi, Moriguchi, Yamaguchi, & Sunamoto, 1993; Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1997; Kuroda, Fujimoto, Sunamoto, & Akiyoshi, 2002) reported that pullulan, a polysaccharide, modified with rigid and bulky hydrophobic cholesteryl groups (CHP) showed a stronger tendency for self-association than did the usual long alkyl chains. CHP formed stable nanoparticles with diameters of 20–30 nm in water via intermolecular self-association of cholesteryl groups even in the lower content less than 5 cholesteryl per 100 glucose residues. Their studies further motivated us to investigate a cholesteryl modified amphiphilic sodium alginate derivative as a new kind of biomaterial.

In this work, the cholesteryl grafted amphiphilic sodium alginate derivative (CSAD) is synthesized at room temperature by the aid of *N*,*N*′-dicyclohexylcarbodiimide (DCC), which is commonly used as a coupling agent for synthesis of polypeptides or proteins. The self-assembly of CSAD in aqueous NaCl solution is characterized with pyrene fluorescence, dynamic light scattering and TEM. To the best of our knowledge, it is the first time to report such an amphiphilic sodium alginate derivative.

2. Experimental

2.1. Materials

Sodium alginate, purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai), was purified twice by dissolving in distilled water, filtered, precipitated with ethanol and dried in a vacuum at 40 °C. Its weight average molecular weight and number average molecular weight are 1.2×10^5 and 7.9×10^4 g/mol, respectively, and its polydispersity of 1.5 determined by gel permeation chromatography (GPC). The contents of mannuronic acid and guluronic acid residues are 65% and 35%, respectively, as determined by circular dichroism analysis (Morris, Rees, & Thom, 1980). Formamide (FA) and dimethyl formamide (DMF) were dried with the molecular sieve and vacuum distilled just before use. Water-free *p*-toluenesulfonic acid (*p*TSA) was prepared by drying under vacuum at 120 °C for 30 min and then was stored in dried DMF. Cholesterol and DCC were bought from Acros, and pyrene from Fluka company. Pyrene was purified by twice re-crystallization in ethanol and dried under vacuum. Other reagents were used without further purification.

2.2. Synthesis of the cholesteryl grafted sodium alginate derivative (CSAD)

Water-free *p*TSA (0.324 g, 1.70 mmol) was added to the mixture of sodium alginate (1.00 g, 5.05 mmol of hexuronic acid residues) and 38 ml of FA/DMF (10/9, v/v) solvents. The sodium alginate was partially protonated by stirring at 50–60 °C for 30 min into a suspension (Srokova, Tomanova, Ebringerova, Malovikova, & Heinze, 2004; Vogt, Klemm, & Heinze, 1996). The reaction between the carboxylic acid (–COOH) groups of protonated sodium alginate and the hydroxyl of cholesterol (it has only 1 –OH) was carried out at room temperature for 24 h, after adding of DCC (0.400 g, 1.94 mmol), 4-(*N*,*N*′-dimethylamino)pyridine (DMAP, 0.475 g, 3.89 mmol) and a solution of chloroform (2 ml) containing cholesterol (0.66 g, 1.70 mmol). The product was purified by adding 200 ml of ethanol, stirring at 40–50 °C and centrifugation, and then the precipitate was dried in vacuum at 40 °C for 24 h. The solid product was dissolved in the distilled water and neutralized by adding 1.5% Na_2CO_3 solution. The solution was dialyzed against the distilled water for 3 days and lyophilized to get the pure CSAD product.

2.3. Preparation of self-aggregates in aqueous NaCl solution

The optically clear solutions of CSAD and the parent sodium alginate were prepared with the concentration of 1.1 and 4.0 g/L in 0.15 mol/L aqueous NaCl solution, respectively. All sample solutions were stored for more than 24 h for self-assembly.

2.4. FTIR spectroscopy

FTIR measurement was carried out on a FT-IR Analyzer (Nicolet/Nexus 670, USA) at a 4 cm^{-1} resolution using

KBr pellets. FTIR (KBr, cm^{-1}) of CSAD product: 3266 (ν_{-OH}), 2920 (ν_{-CH}), 1733 ($\nu_{-C=O}$ of –COOR), 1599 [$\nu_{as(-C=O)}$ of –COONa], 1411 [$\nu_{s(-C=O)}$ of –COONa], 1093 and 1033 (ν_{C-O-C}), 877 (δ_{-CH} on pyrano-ring), 808 (characteristic of mannuronate acid residues) (Mackie, 1971; Wu, 1994; Zhang, 1999).

2.5. ^1H NMR spectroscopy

^1H NMR spectra were obtained on a Nuclear Magnetic Resonance Spectrometer (Mercury-Plus 300, Varian, USA) at 75 °C using 5 mm NMR tube. The samples were dissolved in D_2O (99.9%, Cambridge Isotope Laboratories, Inc.) to a concentration approximately 10 mg/ml. The HDO signal was eliminated by using the standard Varian Presat sequence. DMF was used as the internal standard with $\delta_H = 2.97$ ppm.

2.6. Fluorescence measurement

Fluorescence measurement was carried out on a Spectrofluorophotometer (RF-5301PC, Japan) using pyrene as a fluorescence probe. The excitation wavelength was chosen as 330 nm and the slit width was 10 nm. The fluorescence emission spectra were recorded in the range of 350–500 nm with the slit width of 3 nm. A pyrene stocking solution of different NaCl content was prepared by adding NaCl to a saturated aqueous pyrene solution. Samples were dissolved in the stocking pyrene/NaCl solution, and then diluted to the desired concentrations (Tian, Yam, Zhou, Tat, & Uhrich, 2004). All sample solutions were stored for more than 48 h to ensure pyrene was completely entrapped into the hydrophobic microdomains.

2.7. Dynamic light scattering (DLS) measurement

The average hydrodynamic diameter (D_h) distribution of the CSAD self-aggregates in 0.15 mol/L aqueous NaCl solution was detected by DLS using Dynamic/Static Laser Scattering System (Brooken Haven BI-200SM, USA). The DLS experiment was carried out with a wavelength of 532.0 nm and an angle of detection of 90° at 25 °C.

2.8. Particle size analysis

The particle size distribution of the self-aggregates of the parent sodium alginate in 0.15 mol/L aqueous NaCl solution was analyzed using a particle size analyzer (MasterSizer 2000, Marven, Britain).

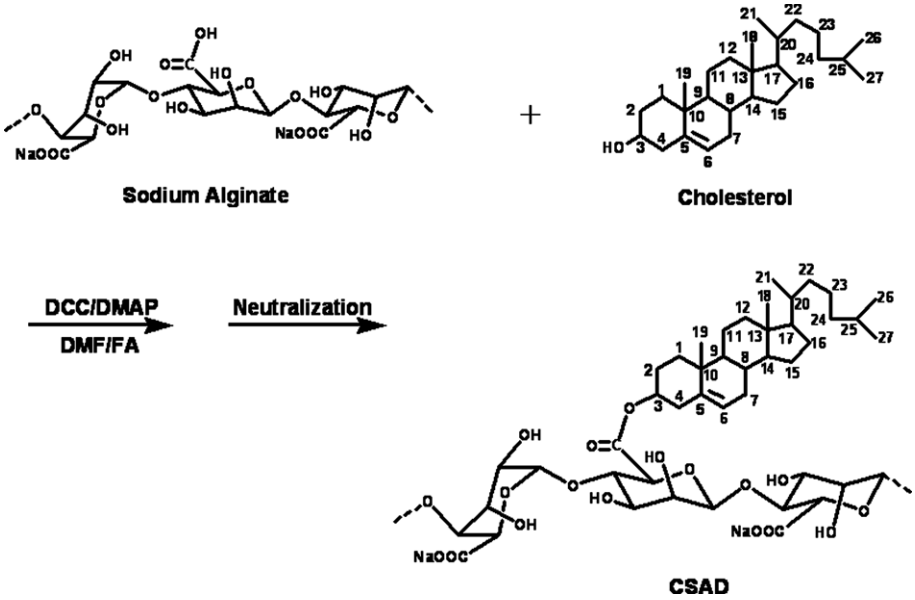
2.9. Transmission electron microscopy (TEM)

The solution of the CSAD self-aggregates was dropped on a 200 mesh copper grid deposited by carbon, dried in the air, and then negative dyed in a phosphotungstic acid solution (about 2 wt%) for 5–6 min. TEM observation was carried out on a transmission electron microscope (JEOL JEM-2010HR, JEOL Ltd., Japan).

3. Results and discussion

3.1. Synthesis and structural characterization of CSAD

Approximate, 1/3 of sodium carboxylate groups (–COONa) of alginate were protonated into –COOH groups by water-free *p*TSA. In order to avoid the hydrolysis of the glycosidic bonds, a mixture dipolar-aprotic solvent containing dried FA and DMF was used as reaction



Scheme 2. The main process of synthesis of CSAD.

medium. The resulting –COOH groups were esterified with –OH groups on cholesterol by aid of DCC as a coupling agent at room temperature (Scheme 2). DMAP was used to remove water produced form esterification reaction. Hot ethanol was added to purify the product of sodium alginate derivative from the un-reacted cholesterol monomer, which is soluble in ethanol. The remaining –COOH from the product were converted to –COONa by neutralization with dilute Na_2CO_3 solution and finally CSAD product was obtained.

The FTIR spectrum of CSAD is shown in curve (a) of Fig. 1. The absorption at 1733 cm^{-1} indicates the presence of ester bonds. However, such vibration was not seen in the FTIR spectrum of the referenced sodium alginate (curve b), which was treated according to the same procedure of synthesis of CSAD in absence of cholesterol. It suggests that the esterification between sodium alginate and cholesterol predominates, while the intermolecular ester-cross-linking of alginate chains is minimal, even though DCC and DMAP are good reagents for coupling of –COOH and –OH groups.

The ^1H NMR spectrum of CSAD is illustrated in Fig. 2A. In contrast with the spectrum of the before mentioned referenced sodium alginate (Fig. 2B): (1) several broad peaks from 1.0 to 2.5 ppm can be attributed to the protons of the cholesteryl graft, except of H3 and H6 (Abraham, Fisher, & Loftus, 1999); (2) the signals from the chemical shifts of 3.5–6.0 ppm are assigned to the methine protons of hexuronic acid residues of alginate main chains (Zhang et al., 2004) and the H3 and H6 of the cholesteryl moiety (Abraham et al., 1999).

The degree of substitution (DS) of CSAD is defined as the average numbers of cholesteryl groups ester-linked to hexuronic acid residues ($N_{chol}/N_{G(M)}$). For determination of DS, we make the assumption that the integrations of H3 and H6 on cholesteryl grafts are negligible.

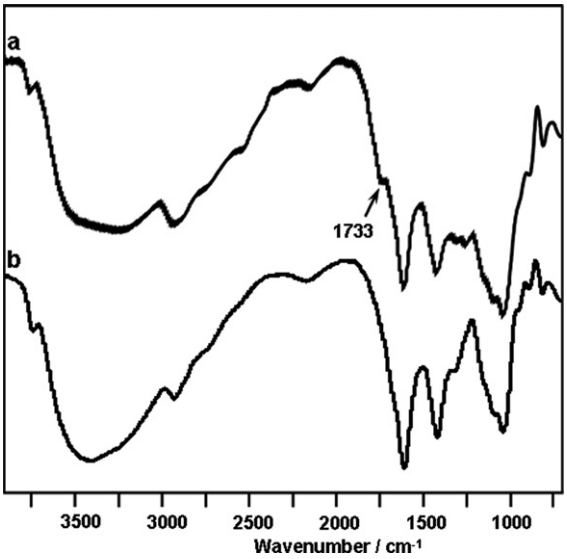


Fig. 1. FTIR spectra of (a) CSAD and (b) the reference sodium alginate.

Consequently, according to the relations of $N_{chol} = N_{chol\text{-}H}/43$ and $N_{G(M)} = N_{G(M)\text{-}H}/5$ evaluated from the chemical structure of CSAD in Scheme 2, DS is finally described as Eq. (1).

$$DS = (5I_{chol\text{-}H})/[43I_{G(M)\text{-}H}] \quad (1)$$

where, $N_{chol\text{-}H}$ and $I_{chol\text{-}H}$ are the number and integration of the protons on cholesteryl grafts except H3 and H6, while $N_{G(M)}$ and $I_{G(M)\text{-}H}$ corresponding to the number and integration of the protons on alginate main chains.

In this work, the DS value of CSAD is 0.03, indicating that, on the average, there are 3 cholesteryl groups ester-linked to 100 hexuronic acid residues. Thus synthesized CSAD is named CSAD-3 in the following.

In summary, a cholesteryl grafted sodium alginate derivative has been synthesized. Cholesteryl can be grafted to the partial protonated sodium alginate chains undergoing the esterification at room temperature for one day. This process is more simple and moderate than that of CHP, in which cholesteryl was linked to the pullulan chains through the bridge bonds of diisocyanate groups by two synthesis steps and reacted at 80 °C for more than two days (Akiyoshi et al., 1993).

3.2. Self-assembly of CSAD-3 and the parent sodium alginate in aqueous solution

Pyrene is one of the effective probes to detect the formation of hydrophobic microdomains by intra- or intermolecular associations of amphiphilic copolymers. The intensity of the first band at 372 nm (I_1) increases with increase of the polarity of solvent, while the intensity of the third band at 383 nm (I_3) is stronger in the non-polar solvent but stable in various conditions. Accordingly, the ratio value of I_1/I_3 demonstrates the micro-environmental polarity surrounding pyrene molecules (Nouvel et al., 2004). I_1/I_3 ratio decreases after the formation of hydrophobic microdomains, where pyrene is included. The corresponding onset concentration of copolymer is called a critical aggregation concentration (CAC). The CAC value is a principal thermodynamic parameter for polymer aggregation and is usually used to evaluate the thermodynamic stability of the aggregates in aqueous solutions (Kataoka, Harada, & Nagasaki, 2001; Tian et al., 2004).

In the pyrene fluorescence spectra of CSAD-3 in 0.15 mol/L aqueous NaCl solution (Fig. 3A), I_1 deceases with increase of the CSAD-3 concentration but I_3 is almost constant. It is obvious that I_1/I_3 ratios of CSAD-3 decrease from 1.8 to about 1.1 after a plateau at low concentrations (curve (b) in Fig. 3B), illustrating that the micro-environmental polarity around pyrene changes from high polarity medium to hydrophobic microdomains of the aggregates. The value of CAC was evaluated from the inflection point of the curve (b) as 0.33 g/L. On the other hand, the I_1/I_3 ratios of the parent sodium alginate also exhibit decrease with increase of concentration [curve (a) in Fig. 3B], implying that pyrene is included in hydrophobic microdomains

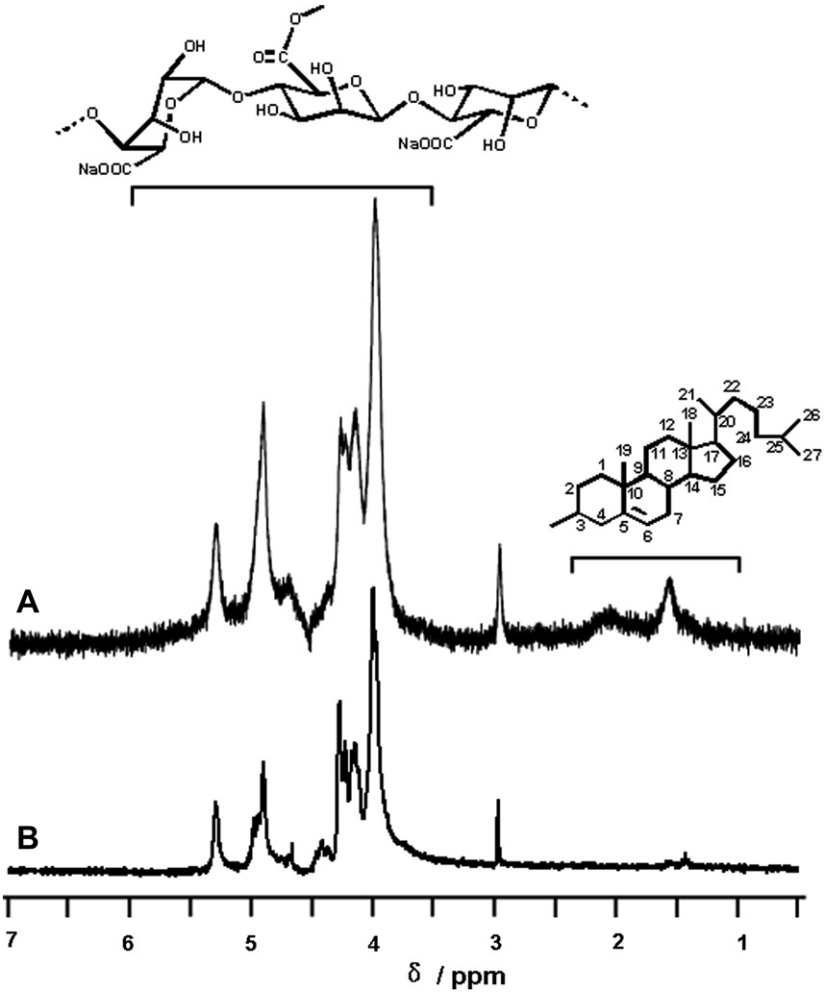


Fig. 2. ^{1}H NMR spectra of (A) CSAD and (B) the reference sodium alginate in D_2O solvent.

of the sodium alginate skeleton. It demonstrates that the parent sodium alginate also forms aggregates in aqueous NaCl solution. However, its CAC value of 3.2 g/L is much higher than that of CSAD-3 in the same concentration of aqueous NaCl solution. These results are consistent with the fact that the hydrophobic alkyl groups modified sodium alginate has a lower CAC value than its parent sodium alginate (Pelletier, Hubert, Lapicque, Payan, & Dellacherie, 2000).

The lower CAC value of CSAD-3 illustrates the effectiveness of hydrophobic cholesteryl grafts for stabilizing aggregates. There are several intra- and intermolecular interactions controlling the CSAD-3 self-aggregates in aqueous NaCl solution, including the hydrophobic interaction between cholesteryl grafts, the hydrogen bonding among hydrophilic sodium alginate backbones, the hydrogen bonding of hydrophilic sodium alginate backbones and water molecules, and the electrostatic repulsive interaction between anionic $-COO^{-}$. The driving force for self-assembly is the hydrophobic interaction between cholesteryl grafts (Liu et al., 2004; Yusa, Kamachi, & Morishima, 1998). Two kinds of hydrogen bonds also tend to stabilize the aggregates (Liu et al., 2004). However, the electrostatic repulsive interaction between anionic $-COO^{-}$ keeps the alginate main chains separated and leads to disassembly. In contrast, the aggregates of the parent sodium alginate lack of the important driving force for self-assembly, which is the hydrophobic interaction between cholesteryl grafts. Therefore, the CSAD-3 aggregates are more stable than those of the parent sodium alginate.

On the other hand, the CAC value of CSAD-3 decreases to 0.22 g/L in 1.5 mol/L aqueous NaCl solution [curve (c) in Fig. 3B], demonstrating the effectiveness of the ionic strength of aqueous NaCl solution for stabilizing aggregates, too. It is probably because that the electrostatic repulsive interactions between anionic $-COO^{-}$ of the CSAD-3 self-aggregates are reduced in higher concentrations of aqueous NaCl solution, corresponding to an increase of hydrophobic interaction between cholesteryl grafts.

3.3. Size and morphology of self-aggregates

The hydrophobic interaction between cholesteryl groups of the CSAD-3 self-aggregates can be further elucidated by comparison of the size of two kinds of aggregates. The D_h

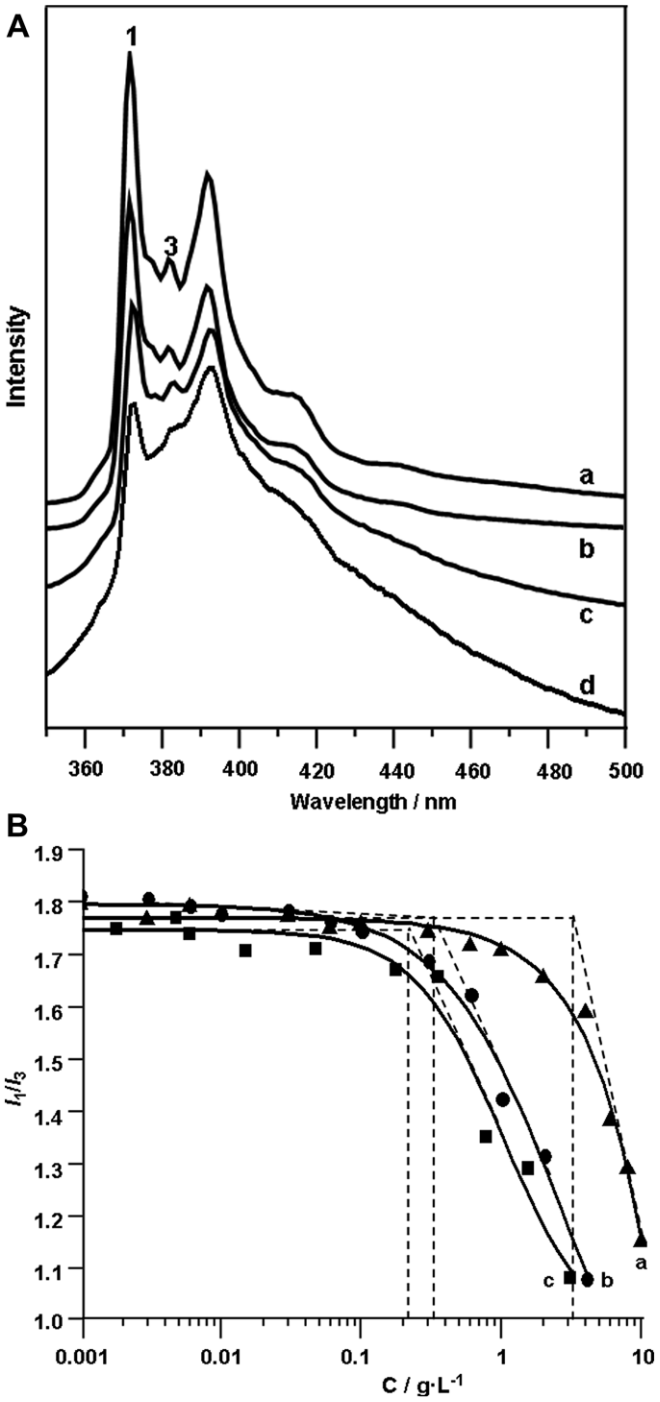


Fig. 3. (A) pyrene fluorescence spectra of CSAD-3 in 0.15 mol/L aqueous NaCl solution (1: the first band at 372 nm, and 3: the third band at 383 nm): (a) 0.001 g/L, (b) 0.01 g/L, (c) 2 g/L and (d) 4 g/L; (B) pyrene fluorescence intensity ratios (I_1/I_3) as a function of the concentration of: (a) the parent sodium alginate and (b) CSAD-3 in 0.15 mol/L aqueous NaCl solution, (c) CSAD-3 in 1.5 mol/L aqueous NaCl solution.

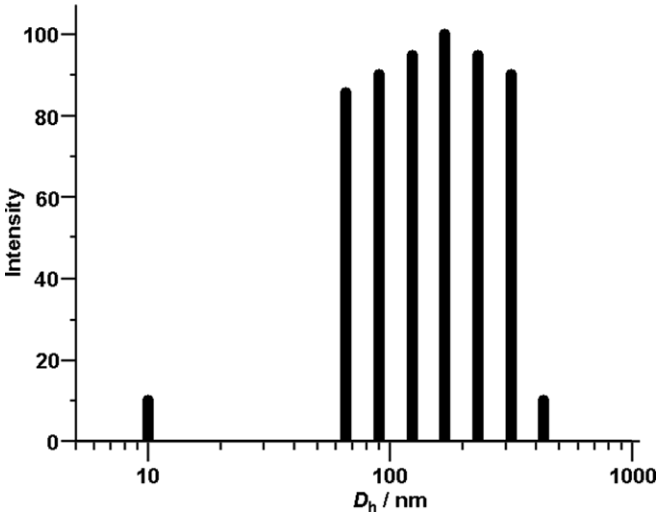


Fig. 4. Hydrodynamic diameters (D_h) distribution of the CSAD-3 self-aggregates in 0.15 mol/L aqueous NaCl solution.

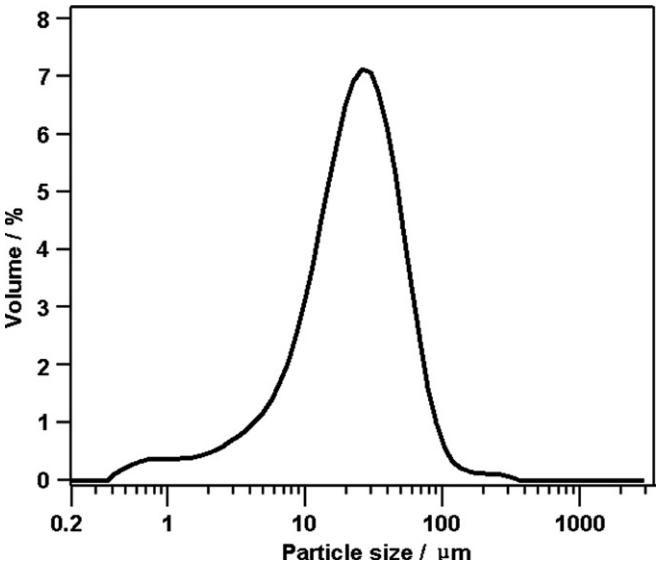


Fig. 5. The particle size distribution of self-aggregates of the parent sodium alginate in 0.15 mol/L aqueous NaCl solution.

value of the CSAD-3 aggregates is 136 nm from DLS measurement (Fig. 4). In contrast, the mean particle diameter of the aggregates of sodium alginate is much lager as 31 μm (Fig. 5), determined by laser diffraction particle size analysis. It is of prime interesting to emphasize here that the size of the aggregate of CSAD-3 is much smaller than that of the parent sodium alginate. The result gives an evidence of the presence of the intra- and intermolecular hydrophobic interaction between cholesteryl grafts, which causes the CSAD-3 chains to self-associate into compact aggregates. The TEM image of CSAD-3 self-aggregates exhibits some oblate particles with the size about 100–200 nm in Fig. 6, which matches with the result of DLS.

In summary, CSAD-3 forms more stable and compact nano-scale self-aggregates in aqueous NaCl solution than the parent sodium alginate, and the stability of self-aggregates results from the intra- and intermolecular hydrophobic interaction between cholesteryl grafts. CSAD is therefore potentially promising as a new kind of biomaterial. The nano-aggregates of CSAD could be used for

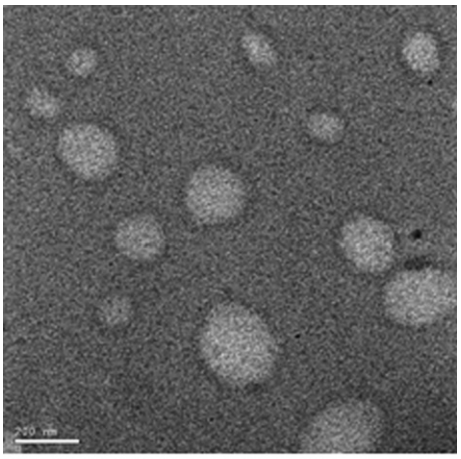
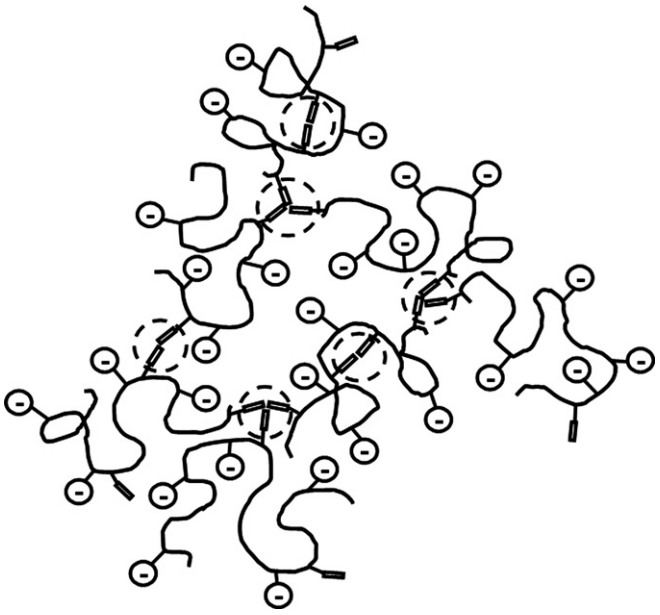


Fig. 6. TEM image of the CSAD-3 self-aggregates.

controlled release of drugs and growth factors in pharmacology and tissue engineering applications. In this work, the CSAD self-aggregates were able to encapsulate the hydrophobic compound pyrene. Further studies to examine the ability of self-aggregates to encapsulate and release hydrophobic drugs, hydrophilic peptides and proteins are under investigation in our laboratory.

A speculated model for the hydrophobic interaction between cholesteryl groups of CSAD-3 aggregates by self-assembly in aqueous NaCl solution is illustrated in Scheme 3. The cholesteryl grafts associate through the intra- and intermolecular hydrophobic interactions to form some hydrophobic microdomains, which are surrounded by the loose anionic alginate chains.



Scheme 3. A speculated model for the hydrophobic interaction between cholesteryl grafts of the CSAD-3 self-aggregates.

4. Conclusion

A novel water soluble amphiphilic cholesteryl grafted sodium alginate has been synthesized at room temperature, with DCC as a coupling agent and DMAP as a catalyst. It self-assembles into the more stable and compact nano-aggregates through the intra- and intermolecular hydrophobic interactions between cholesteryl grafts in aqueous NaCl solution, compared with the parent sodium alginate. The CSAD-3 self-aggregates are able to encapsulate the hydrophobic compound pyrene.

Acknowledgements

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