Carbohydrate Polymers 74 (2008) 220–227

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Preparation and characterization of self-assembled nanoparticles of the novel carboxymethyl pachyman-deoxycholic acid conjugates

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ARTICLE INFO

Article history:
Received 12 November 2007
Received in revised form 19 February 2008
Accepted 20 February 2008
Available online 6 March 2008

Keywords:
Carboxymethyl pachyman
Deoxycholic acid
Self-aggregates

ABSTRACT

Various novel carboxymethyl pachyman-deoxycholic acid conjugates (CMPD) were synthesized using carboxymethylated pachyman (CMP) as a hydrophilic segment and Deoxycholic Acid (DOCA) as a hydrophobic segment. The degree of DOCA substitution (DS) in CMPD conjugates, which was determined by elemental analysis, can reach to 30.0, 49.2, or 54.9 DOCA groups per hundred sugar residues of CMP. Structural characteristics of these CMPD conjugates were investigated by using ^{1}H NMR, dynamic light scattering, zeta potential, transmission electron microscopy (TEM) and fluorescence spectroscopy. The CMPD conjugates provided apparently smaller monodispersed self-aggregates in water, with mean diameters decreasing with increasing of DOCA DS in the range of 98–158 nm. Zeta potentials of the CMPD self-aggregated nanoparticles indicated that the nanoparticles were covered with negatively charged CMP shells. TEM images demonstrated that the nanoparticles were spherical in shape. The critical aggregation concentration (cac) of the CMPD nanoparticles (1.55×10^{-2}–5.89×10^{-3} mg/mL) was comparatively low which implies that the CMPD self-assembled nanoparticles form at low concentration in aqueous solution.

1. Introduction

Recently, various polymeric amphiphiles have received increasing attention because of their special physicochemical and morphological characteristics in water (Gaucher et al., 2005; Kataoka, Harada, & Nagasaki, 2001; Nishikawa, Akiyoshi, & Sunamoto, 1996). These polymeric amphiphiles consisting of hydrophilic and hydrophobic segments can form micelles or nanoparticles via non-covalent association arising from intra and/or intermolecular interactions among hydrophobic segments in aqueous medium. Hydrophobic cores form inside hydrophilic outer shells in the micelles or nanoparticles. Thus, the inner core can serve as a microcontainer for various substances such as hydrophobic anticancer drugs (Kim et al., 2006; Park et al., 2006; Sant, Smith, & Leroux, 2005). Many polymeric amphiphiles have been designed and synthesized, and their physicochemical characteristics have been extensively reported (Benita & Levy, 1993; Jones & Leroux, 1999; Kang & Leroux, 2004; Qiu & Bae, 2007; Torchilin, 2001). Of all the amphiphilic polymers, natural polysaccharides which possess properties such as non-toxicity and biodegradability have been meticulously researched in recent years. Modified with hydrophobic moieties, such as long alkyl chains and cholesterol groups, polysaccharides, such as chitosan, dextran, pullulan and curdlan can form nanodisperse micelles in aqueous media (Akiyoshi, Deguchi, Tajima, Nishikawa, & Sunamoto, 1997; Akiyoshi et al., 1998; Na, Park, Kim, & Bae, 2000; Rodrigues et al., 2003; Sunil, Nadagouda, & Tejraj, 2004). A great deal of effort has been expended to investigate and characterize the properties of polysaccharide conjugates, especially to develop their promising potential for biotechnology and medicine applications.

Pachyman, a fungus polysaccharide, is a naturally occurring linear polysaccharide composed of 1,3-β-linked D-glucose units and is produced by a sclerotium of *Poria cocos* (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; Wang, Zhang, Li, Hou, & Zeng, 2004), one of the most important herbs in China and many other Asian countries (Fig. 1). *Poria cocos* is not only a common health-promoting food in the Chinese diet, but also one of the widely used ingredients in the prescription of traditional medicines. Pachyman, the main component of *Poria cocos*, is well known for its diuretic (Narui, Takahashi, Kobayashi, & Shibata, 1980), mitogenic, complement activating (Yamada et al., 1992), anti-inflammatory (Schinella, Tournier, Prieto, Mordujovich de Buschiazzo, & Rios, 2002) and immunoactive properties (Wang, Wen, & Hu, 1995). Meanwhile, many have investigated the mechanism of anti-tumor activity exhibited by the polysaccharide extracted from *Poria cocos*, which indicates that the polysaccharides may enhance the host defense mechanism through activation of immune system (Lee & Jeon, 2003; Yu &

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doi:10.1016/j.carbpol.2008.02.014

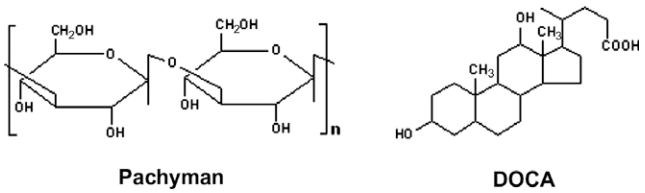

Fig. 1. Chemical structures of a repeat unit of pachyman and DOCA.

Tseng, 1996). Due to its unique properties, pachyman shows great promise in the food, medical and pharmaceutical fields (Xiao et al., 2007).

Various pachyman derivatives have been investigated, such as carboxymethylated, sulfated, hydroxyethylated, hydroxylpropylated and methylated derivatives (Wang & Zhang, 2006; Wang, Zhang, & Ruan, 2004; Wang et al., 2004; Xiao et al., 2007). Among these, the carboxymethylated derivative of pachyman (CMP) has been widely investigated due to its good water solubility and anti-tumor activity (Hamuro, Yamashita, Ohsaka, Maeda, & Chihara, 1971; Wang et al., 2004). The anti-tumor activities of CMP has been attributed to stimulation of a cell mediated immune response like other 1,3-β-linked D-glucan derivatives but not a direct cytotoxic action (Maeda & Chihara, 1973). Due to its anti-tumor activity, CMP like other fungal polysaccharides (Na et al., 2000) may act as a new delivery carrier for anti-tumor drugs.

In this study, we designed a novel self-assembled CMPD conjugate by introducing DOCA moieties (Fig. 1) into the CMP molecule. By varying the ratio of raw materials, A series of CMPD conjugates with different DS values were prepared. The physicochemical properties of these CMPD aggregates were characterized by using ^{1}H NMR, dynamic light scattering, zeta potential, transmission electron microscopy (TEM) and fluorescence spectroscopy. In addition, the relationship between the conjugate DS value and the nanoparticles size, and the factors affecting the size and the cac of self-aggregate were also investigated.

2. Experimental methods

2.1. Materials

Pachyman, the average molecular weight of 2.2×10^5, extracted with 0.5 M NaOH aqueous solution from the sclerotium of *Poria cocos* was used (Wang et al., 2004). DOCA was purchased from Sigma Co. (St. Loius, MO, USA). 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide (EDC) was obtained from Pukang Chemical Co. (Zhejiang, China). *N*-hydroxyl succinimide (NHS) and pyrene were purchased from Aldrich Co. (Milwaukee WI). All other chemical reagents were analytical grade and obtained from commercial sources.

2.2. Carboxymethylation of pachyman (CMP)

Pachyman was carboxymethylated according to the method previously reported (Wang et al., 2004). Briefly, a suspension of 1.2 g pachyman in a mixture of 20 mL 20% NaOH and 50 mL isopropanol was stirred at an ice bath for 3 h. Then, a mixture of 10.5 g chloroacetic acid, 20 mL 20% NaOH and 50 mL isopropanol was slowly added with stirring. The reaction was continued at room temperature for 3 h and then at 50 °C for 3 h. After the solution was cooled to room temperature, 0.5 M HCl was added to adjust pH to 7.0 and then dialyzed at 4 °C by a regenerated cellulose tube (Mw cut-off 8000) against tap water for 7 days and then distilled water for 4 days. The resulting solution was concentrated by rotary evaporator at reduced pressure below 40 °C. Finally, the carboxymethylated pachyman was lyophilized by using a lyophilizer to give the product. CMP was identified by using Fourier transform infrared spectrophotometer (FT-IR, Spectrum One, Perkin-Elmer, USA), and ^{1}H NMR and ^{13}C NMR (D_2O) (Bruker DPX spectrometer, 400 MHz). The degree of substitution (DS) of the CMP sample was 0.9 U of carboxymethyl group per glucose determined by a method as previously described (Eyler, Klug, & Diephuis, 1947).

2.3. Synthesis of N-deoxycholylethylenediamine (DOCA-NH_2)

DOCA (3.0 g, 7.6 mmol) was mixed with DCC (2.0 g, 9.9 mmol) and NHS (1.1 g, 9.9 mmol) in 30 mL of THF. The mixture was reacted for 12 h at room temperature under nitrogen atmosphere, and then the precipitated dicyclohexylurea was removed by filtration. The filtrate was precipitated in *n*-hexane. The succinimido DOCA precipitate was filtered off and washed thoroughly with *n*-hexane, followed by vacuum-drying at room temperature. *N*-deoxycholyl-ethylenediamine (DOCA-NH_2) was synthesized by introducing ethylenediamine to the succinimido DOCA. The succinimido DOCA (3 g, 6 mmol) was dissolved in DMF (10 mL), and then the solution was added dropwise into ethylenediamine (40 mL, 0.6 mol) solution. After 6 h at room temperature, the reaction mixture was precipitated in distilled water. The white powder DOCA-NH_2 was obtained after washing three times with distilled water and drying in a vacuum.

2.4. Preparation and characterization of CMP-DOCA-NH_2 (CMPD) conjugates

DOCA-NH_2 was coupled to CMP by an EDC/NHS-mediated coupling reaction. The ratios of reactants varied as shown in Table 1 to obtain CMPD30, CMPD49 and CMPD55 of increasing hydrophobicity.

CMP (0.1 g) was dissolved in formamide (5 mL) by gently heating. Different amounts of EDC and NHS were mixed with CMP solution at room temperature, followed by the addition of different amounts of DOCA-NH_2 dissolved in DMF (5 mL). The resulting solution was stirred at room temperature under a nitrogen atmosphere for 24 h. After the reaction mixture was precipitated in excessive acetone, the precipitate was carefully washed with acetone and THF respectively to remove excessive DOCA-NH_2, followed with drying under vacuum. The dried CMPD conjugates were dissolved in water, dialyzed against distilled water and lyophilized then produced the white powder.

The chemical structure of CMPD conjugate was confirmed by using FT-IR spectrophotometer (KBr pellets) and ^{1}H NMR, which was operated in D_2O/CD_3OD (1.5/1, v/v). The degree of substitution (DS), defined as the number of deoxycholic acid groups per 100 glucose units of CMPD sample, was determined by elemental analysis.

2.5. Preparation of self-assembled nanoparticles of CMPD conjugates

Each CMPD conjugate was suspended for 2 min in distilled water under gently shaking. Then the CMPD solution was soni-

Table 1
Synthesis of CMPD conjugates with different DS of the DOCA moiety

Samples[a]	Feed mole ratio[b]	Elemental analysis (%)			DS[c]
		C	H	N	
CMPD30	1:0.5:0.5:0.5	43.24	7.10	2.39	30.0
CMPD49	1:0.8:0.8:0.8	46.13	7.44	3.06	49.2
CMPD55	1:1:1:1	48.08	7.67	3.28	54.9

[a] CMP bearing DOCA, in which the number indicates the DS of DOCA.
[b] Mole ratio of CMP:EDC:NHS:DOCA-NH_2.
[c] Degree of substitution of DOCA moiety.

cated three times using a probe-type Sonifier (Automatic Ultrasonic Processor, UH-500A, China) at 70 W for 1 min each, in which the pulse was turned off for 2 s with the interval of 5 s to stop the increase in temperature. The dispersion of self-aggregates was passed through a membrane filter (pore size: 0.45 μm, Millipore) and stored at room temperature.

2.6. Self-aggregation behavior of CMPD

The self-aggregate property of CMPD conjugates and their critical aggregation concentration (cac) were estimated by the probe fluorescence technique (Wilhelm et al., 1991) in which pyrene was used as a hydrophobic probe. The pyrene solution (3.0×10^{-2} M in THF), which had been stored at 4 °C prior to use, was added to the distilled water to get a pyrene concentration 12×10^{-7} M, and THF was removed by using a rotary evaporator at 30 °C for 2 h. This solution was mixed with the CMPD solution to obtain polymer concentration that ranging from 1.0×10^{-4} to 1.0 mg/mL, resulting in a pyrene concentration at 6.0×10^{-7} M. The excitation spectra of pyrene was recorded in the range of 300–360 nm using a Fluorescence spectrophotometer (LS-55, Perkin-Elmer, USA) at the emission wavelength (λ_{em}) of 390 nm and an integration time at 1.0 s/nm. The slit openings for excitation and emission were set at 3.0 and 2.5 nm, respectively. For the measurement of the emission intensity of pyrene, the excitation wavelength (λ_{ex}) was 336 nm.

2.7. Size distribution and zeta potential measurement

The mean diameter and zeta potential of the CMPD self-aggregated nanoparticles in water were measured by using a Zetasizer Nano ZS (Malvern Instruments). The concentration of the CMPD conjugates was constantly at 1.0 mg/mL.

2.8. Transmission electron microscopy (TEM)

TEM was performed by using a transmission electron microscope (JEM-100CX II, Jeol Company, Japan), operating at an acceleration voltage of 100 kV. In order to measure the morphology and the size distribution of nanoparticles, a drop of sample solution (1 mg/mL) was placed onto a 300 mesh copper grid coated with carbon. After deposition for 2 min, the grid was tapped with filter paper to remove surface water, followed with air-drying. The nanoparticles, deposited on the grid, were then negatively stained by 2 wt% uranyl acetate solution.

3. Results and discussion

3.1. Synthesis and characterization of CMPD

As pachyman is insoluble in water, the carboxymethylated derivative of pachyman was fabricated first, for it is well known that the carboxymethyl substitution is considered as a good way to improve the solubility of many polysaccharides (Jin, Zhang, Yin, & Nishinari, 2006; Zhang, Cheung, Zhang, Chiu, & Ooi, 2004). DOCA-NH_2, another material as a hydrophobic segment in CMPD conjugates, was produced by using ethylenediamine and DOCA. Then the product was covalently attached to CMP in the presence of "zero length" crosslinker of EDC/NHS. Detailed schemes for the preparation of CMPD conjugate are shown in Fig. 2. By changing the feed ratio of DOCA-NH_2 to CMP, various CMPD conjugates with different DS values were prepared.

Fig. 3 shows the IR spectra of pachyman, CMP and CMPD. Compared with the IR spectrum of native pachyman (Fig. 3a), the absorption band at 1601 cm^{-1} in CMP spectrum (Fig. 3b) was the carboxylic characteristic peak due to the asymmetrical COO^- stretching vibration, which indicates that the pachyman was substituted by the carboxymethyl groups. What is more, the absorption band at 1650 cm^{-1} in CMPD conjugate spectrum (Fig. 3c) increased more evidently, which indicates that the CMPD conjugate was produced by the new amide linkages between CMP and DOCA-NH_2. Through the ^1H NMR spectra, complete structural resolution of both the CMP (Fig. 4a) and the CMPD conjugate (Fig. 4b) could be observed in D_2O and D_2O/CD_3OD (1.5/1, v/v), respectively. The characteristic peaks of DOCA appearing at 0.65–2.5 ppm in Fig. 4b could prove the presence of DOCA in CMPD conjugate.

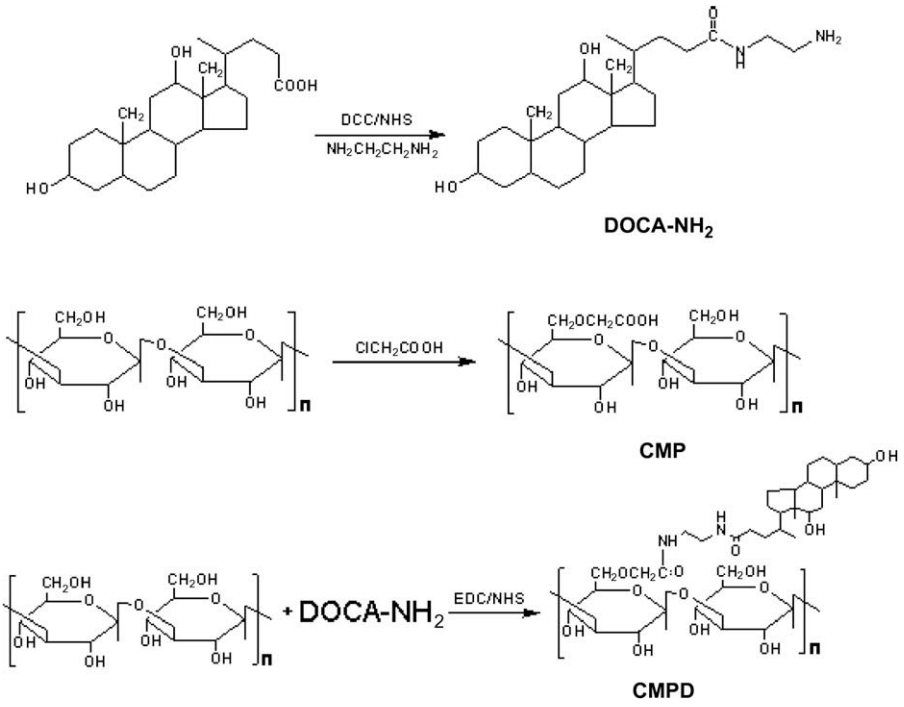

Fig. 2. Synthesis schemes of CMP and CMPD.

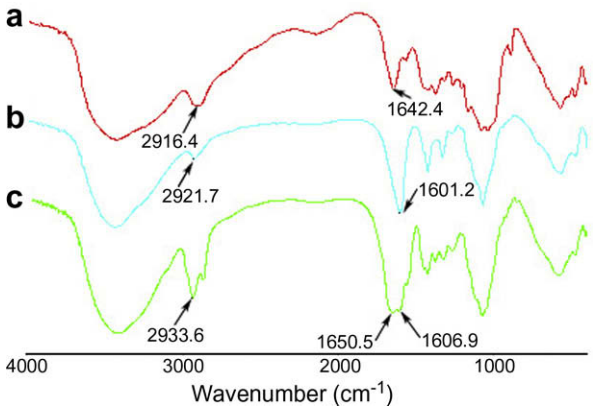

Fig. 3. IR spectra of (a) pachyman, (b) CMP, (c) CMPD.

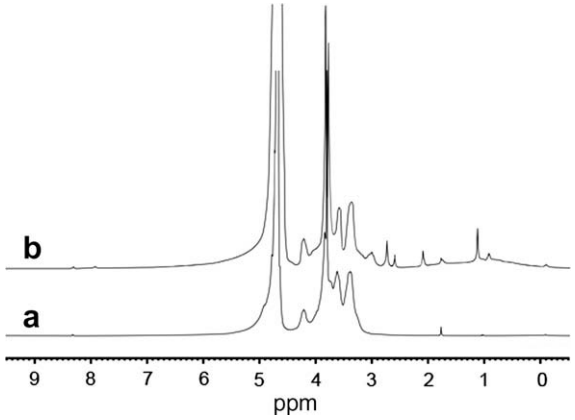

Fig. 4. ^{1}H NMR spectra of (a) CMP and (b) CMPD.

As listed in Table 1, various CMPD conjugates with different amounts of DOCA were prepared by changing the feed ratio of DOCA to CMP. As determined by elemental analysis, the DS of CMPD conjugate, which is defined as the number of deoxycholic acid groups per 100 glucose units of CMP, can reach to 55. In our work, the EDC-mediated coupling reaction took place in a homogeneous solution phase made up of formamide and DMF. As this mixed solvent is suitable for the solution of both CMP and DOCA-NH_2, the DS value obtained in this condition was not only notably higher than that of many other DOCA modified amphiphilic polymers (Lee, Jo, Kwon, Kim, & Jeong, 1998a; Park et al., 2004), but also was apparently higher than that of many cholesterol modified or 5-cholanic acid modified polymeric amphiphiles (Kwon, Park, Chung, Kwon, & Jeong, 2003; Lee et al., 1998a).

In previous reports, considering the different solubility between the hydrophilic polymer segment and the hydrophobic DOCA segment, people always try this coupling reaction in a mixed solvent, such as H_2O/THF (Wang, Liu, Jiang, & Zhang, 2007) or H_2O/CH_3OH (Kwon et al., 2003) systems, for there is no shared common solvent for both polymer and DOCA-NH_2. In our study, in order to synthesize the CMPD conjugates, we tried the reaction in H_2O/THF mixed solvent first, because DOCA-NH_2 is insoluble in distilled water, but well soluble in THF for its hydrophobicity. Several repeated experiments indicated that the DS value is not only determined by the ratio of the native materials of CMP and DOCA-NH_2, but also significantly depending on the solvent system (composition of H_2O/THF mixture). In order to obtain higher DS value, larger amounts of DOCA-NH_2 are always required for the reaction. Nevertheless, due to the poor solubility of hydrophobic segment in H_2O, many DOCA-NH_2 will be precipitated from the solvent system. On the other hand, if we increase the THF ratio in the mixed solvent to improve the solubility of DOCA-NH_2, CMP will be precipitated. So it is very difficult to dissolve both the CMP and DOCA-NH_2 thoroughly by controlling the ratio of H_2O to THF. Hence the DS value of the CMPD conjugates is always limited in this solvent system. And the similar result was also obtained in the H_2O/CH_3OH system. However, in our following experiments, this problem was successfully resolved by using the formamide and DMF solvent system instead.

When changing the feed amount of the DOCA-NH_2 and crosslinker, the CMPD conjugates with various DS values can be easily prepared. CMPD conjugates with DS value 30, 49, 55 were obtained simply by increasing the amount of DOCA-NH_2 and crosslinker continuously. However, in the following experiment, we found that even if the double molar amount of DOCA-NH_2 to the CMP was added to the reaction, and the reaction was prolonged to 48 h, the maximum DS value we obtained was only 59. It is just slightly higher than 55, which was obtained by adding equivalent molar amount of DOCA-NH_2 to CMP, and only 24 h for the reaction. Such a little increase in DS value indicates that when the DS reaches to a certain value, it becomes difficult to enhance the DS value markedly, even by adding more reactive reagents or prolonging the reaction time. Some probable reasons for this phenomenon may be: 1st, the DS value limitation of carboxymethyl groups in CMP; 2nd, the steric hindrance caused by many substituted DOCA-NH_2 molecules in CMPD conjugate.

3.2. Self-aggregation of CMPD

The self-aggregated nanoparticles of CMPD conjugates were produced by a simple sonication method in aqueous condition. The size and size distribution of the CMPD self-assembled nanoparticles with different DS values in the aqueous medium were measured by DLS. It is well known that the size of polymeric micelles is one of the major factors affecting the application and property of polymeric micelles (Davis, 1997). Aliabadi and Lavasanifar (2006) suggested that polymeric micelles with the size range in 20–100 nm showed great promise in solubilization and controlling delivery for hydrophobic drugs. Greish, Fang, Inutsuka, Nagamitsu, and Maeda (2003) also reported that polymeric micelles in a size range <200 nm can reduce non-selective reticuloendothelial system (RES) scavenge and show enhanced permeability and retention effect (EPR effect) at solid tumor sites for passive targeting. For the CMPD conjugates, as shown in Table 2, the mean size of the nanoparticles is in the range of 98–158 nm, depending on the DS value. Accompanying with the DS value increasing, the size of self-assembled nanoparticles decrease, which indicates that more compact hydrophobic cores have formed. In several repeated experiments, the mean diameter of each conjugate was reproducible and it didn't change during the sonication time from 1st to 30th minute.

It's worthy to know that the mean diameter of the CMPD micelles was apparently smaller than many other self-assembled amphiphilic polysaccharide polymers, such as deoxycholic acid-

Table 2
Characterization of CMPD self-aggregated nanoparticles

Sample	cac[a] (mg/mL)	d[b] (nm)	μ_2/I^2	ζ[c] (mV)
CMPD30	1.55×10^{-2}	158 ± 11.5	0.096	−20.1
CMPD49	8.31×10^{-3}	111 ± 4.7	0.053	−19.4
CMPD55	5.89×10^{-3}	98 ± 1.0	0.079	−19.0

[a] Critical aggregation concentration determined from I_{337}/I_{334} data.
[b] Mean diameter in water measured by dynamic light scattering.
[c] The potential of the CMPD nanoparticles in distilled water at 1 mg/mL.

modified chitosan (159–180 nm), 5α-cholanic acid-modified glycol chitosan (211 nm) (Kwon et al., 2003; Lee, Jo, Kwon, Kim, & Jeong, 1998b). Due to the higher substitution of deoxycholic group in the CMPD molecule, the aggregate tends to form much more compact inner core within the nanoparticles, thus resulting in much smaller size.

Meanwhile, by varying the concentrations from 0.5 to 2.0 mg/mL, the size of the CMPD nanoparticles can not be significantly affected, which suggests that the interparticle interactions between self-aggregates is nearly negligible. The polydispersity factors (μ_2/Γ^{-2}) of self-aggregates, estimated by the cumulant method, were fairly low (0.053–0.096), implying highly monodispersed nanoparticles (Harada & Kataoka, 1995). Fig. 5 shows the size distribution of the self-assembled nanoparticals of CMPD55 conjugates. Interestingly, a notably size distribution difference exists between the CMPD product obtained by lyophilization and the sample produced by dialysis without lyophilization process. For example, the CMPD 49 reaction solution after dialysis was divided into two parts. The size of the first part and its diluted ones detected directly are only about 50 nm, in contrast, the size of the other part determined after the process of lyophilization and resolved to the same concentration as the first half part, can reach to 100 nm. Such a notable change arising from the difference of the post-treatment method was also detected in other samples, which suggests that the freeze-drying process may lead to the change of intramolecular structure of CMPD conjugates. However, the exact reason for this phenomenon is now still in need of our further research.

The ξ potential of the self-aggregates in distilled water is negative, nearly −20 mV, which indicates that negatively charged CMP covers the self-aggregates, though the charge is quite weak. With the DOCA DS value increasing, the ξ potential decrease (Table 2). This change can give another evidence that high amounts of COO^- group in CMP were substituted by DOCA, hence result in the reduce of the negative charge on the shells. Nevertheless, the negatively charged CMPD shells still retain the electrostatical stability effect, which could keep the nanoparticles in a long-term stability in the aqueous phase. This could be confirmed by the phenomenon that the size distribution of sample solution in size measurement did not notably change after keeping the sample solution for several weeks (data is not given).

The TEM result is shown in Fig. 6, which indicates that CMPD conjugates formed nanoparticles and the shape of these nanoparticles are spherical. However, their diameter appeared a little different from the results determined by dynamic laser light scattering measurement. This may be ascribed to the different state of the aggregates. Just as pointed out in other researches (Jiang, Quan, Liao, & Wang, 2006; Liu, Desai, Chen, & Park, 2005; Wang, Liu, Weng, & Zhang, 2007; Wang et al., 2007), the size determined by TEM is the actual diameter (dry state) of the nanoparticles, whereas the size measured by laser light scattering method is the hydrodynamic diameter (hydrated state). Therefore, because of the solvent effect of the samples in the hydrated state, the size measured by laser light scattering method is larger than the size observed by TEM method.

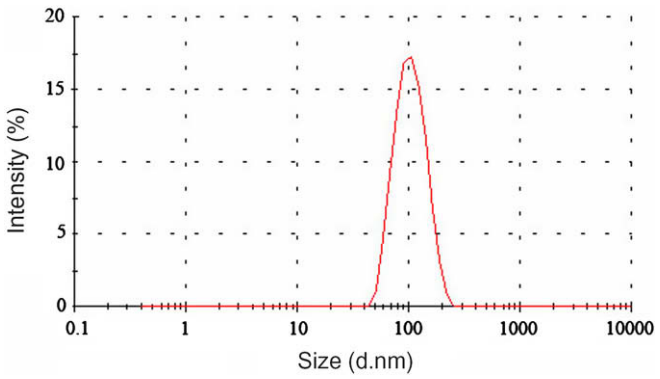

Fig. 5. Size distribution of CMPD55.

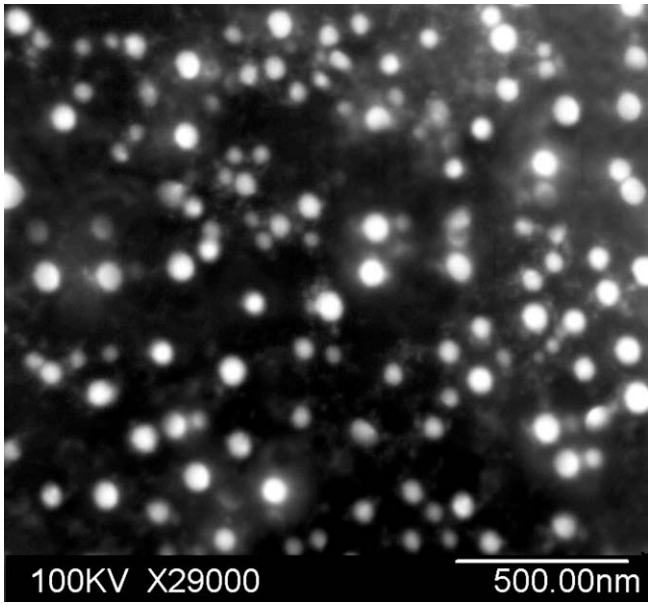

Fig. 6. TEM images of CMPD55.

3.3. Fluorescence probe studies

The fluorescence probe technique is used to study the self-aggregation behavior of CMPD on a molecular level, where pyrene is chosen as a fluorescence probe. Due to its poor solubility and self-quenching, pyrene shows only small fluorescence intensity in polar environment. But when the hydrophobic micro-domains form, it strongly emits radiation because the pyrene molecules prefer to being close to those hydrophobic micro-domains. Therefore, pyrene is often used as a fluorescence probe to monitor the self-aggregation behavior of surfactants or polymers (Ananthapadmanabhan, Goddard, Turro, & Kuo, 1985; Glushko, Thaler, & Karp, 1981).

Fig. 7a shows the fluorescence excitation spectra of pyrene incorporated into various concentrations of CMPD55 self-assembled nanoparticles water solution after sonication. No significant change in the total fluorescence intensity was detected at low concentration range of those CMPD conjugates. With the concentration increasing, however, the fluorescence intensity increased markedly, reflecting the partitioning of pyrene into the hydrophobic microdomains of self-aggregates. Also, the partitioning of pyrene brought in the shift of the main peak, (0, 0) band, from 334 to 337 nm.

Fig. 7b shows the intensity ratio (I_{337}/I_{334}) of the pyrene excitation spectra versus the logarithm of the concentration of CMPD55. Critical aggregation concentration (cac), the threshold concentration of self-aggregate formation by intra- or intermolecular association, was determined from the crossover point at the low concentration ranges. The cac value of the CMPD conjugates in the range of (1.55×10^{-2}–5.89×10^{-3} mg/mL) (Table 2) are lower than that of low molecular weight surfactants (e.g., 1.0 mg/mL for deoxycholic acid and 2.3 mg/mL for sodium dodecyl sulfate (Nagasaki et al., 1998) in water), and even much lower than that of other polymeric amphiphiles (Kim, Lee, Kwon, Chung, & Jeong, 2002; Kim et al., 2000; Kratohvil, Hsu, & Kwok, 1986; Kwon et al., 1993; Lee et al., 1998b), including the deoxycholic acid-modified (4.47×10^{-2}–1.32×10^{-2} mg/mL) (Lee et al., 1998a, 1998b) and

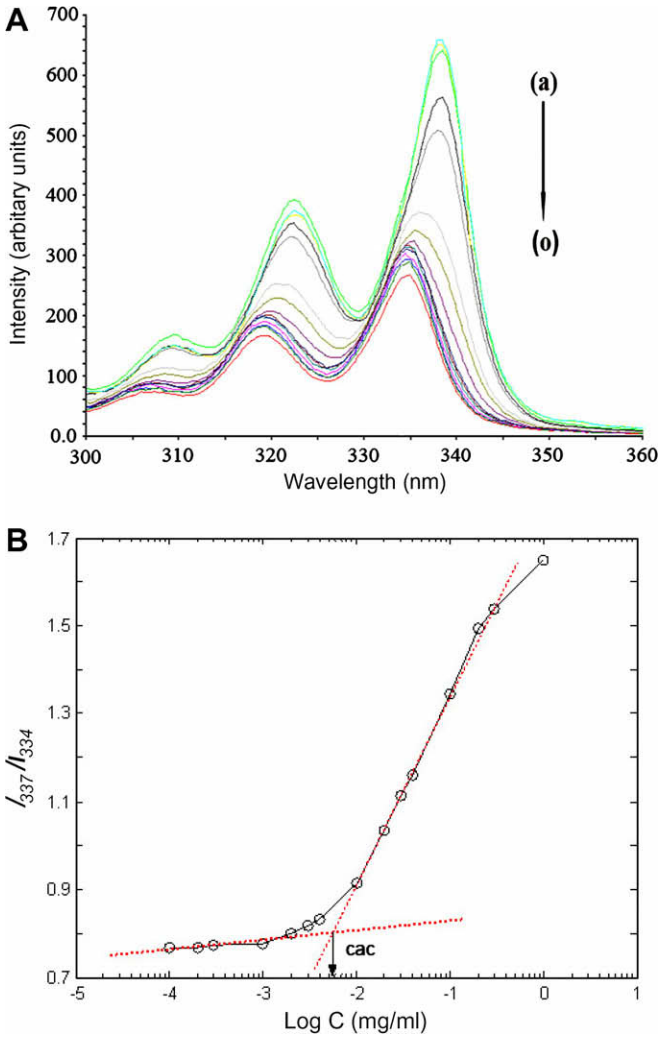

Fig. 7. (A) Excitation spectra of pyrene (6.0×10^{-7} mol/L) in CMPD55 solution with concentrations (mg/mL) of (a) 1.0, (b) 0.3, (c) 0.2, (d) 0.1, (e) 0.04, (f) 0.03, (g) 0.02, (h) 0.01, (i) 0.004, (j) 0.003, (k) 0.002, (l) 0.001, (m) 0.0003, (n) 0.0002 and (o) 0.0001 in distilled water. (B) Change of intensity ratio (I_{337}/I_{334}) from excitation spectra of pyrene (6.0×10^{-7} mol/L) with various concentrations of CMPD55.

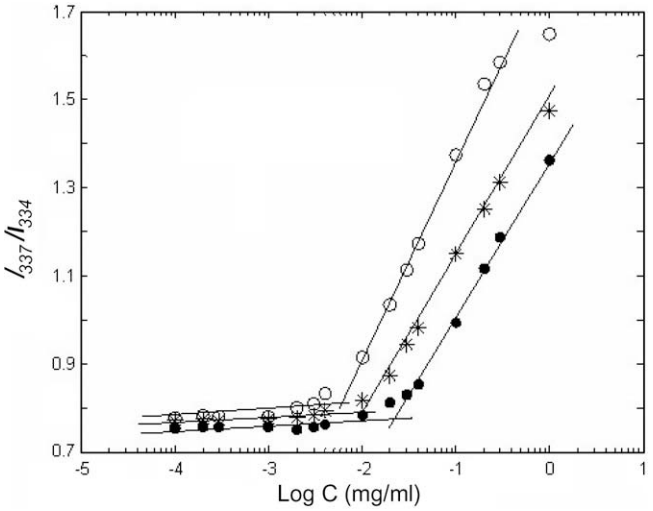

Fig. 8. Intensity ratios (I_{337}/I_{334}) from pyrene excitation spectra as a function of CMPD conjugate concentration in distilled water; CMPD30 (●), CMPD49 (*), and CMPD55 (○).

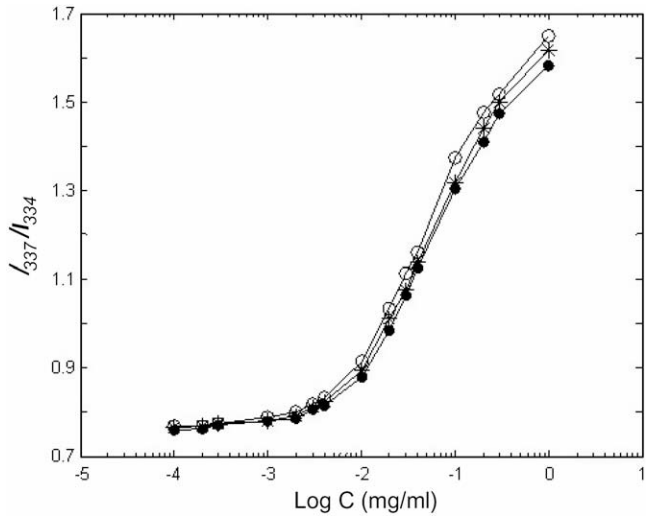

Fig. 9. Intensity ratios (I_{337}/I_{334}) from pyrene excitation spectra as a function of CMPD conjugate concentration in distilled water; CMPD56 (●), CMPD58 (*), CMPD59 (○).

cholesterol-modified aggregates (1.16×10^{-2} mg/mL) (Wang et al., 2007), which suggests that these CMPD self-aggregates are easy to form in a low concentration by highly substitution of DOCA in the CMPD molecules. Compared with cac value of low-molecular-weight surfactants and many other self-aggregates, the lower cac value of the modified pachyman may be one of the main characteristics for polymeric amphiphiles; i.e., a small amount of the pachyman derivatives can form self-aggregates and maintain the stability in dilute condition.

The cac value of DS 30, 49, and 55 CMPD conjugates are 1.55×10^{-2}, 8.31×10^{-3} and 5.89×10^{-3} mg/mL, respectively. It is easy to find that the cac value of the CMPD conjugates decreases with the hydrophobic DOCA content increasing (Fig. 8). By introducing large amount of deoxycholic acid moieties into the polymer, the cac value of the CMPD conjugates can be further reduced for the reason that the hydrophobicity was enhanced. Na et al. (2000) also reported that the cac of self-assembled nanoparticles can be controlled by the amount of DS. Fig. 9 shows the intensity ratios (I_{337}/I_{334}) from pyrene excitation spectra of other three CMPD conjugates with higher DS values. Their cac values are 5.62×10^{-3}, 5.37×10^{-3} and 3.89×10^{-3} mg/mL, respectively, which is another example indicating that when the DS value slightly increase, the cac will decrease.

4. Conclusions

CMPD conjugates based on a novel polymeric amphiphile by grafting hydrophobic DOCA molecules to the pachyman derivatives with high DS values have been prepared and they can form nanoparticles via self-aggregation in water. These nanoparticles show notable difference to many other DOCA modified polymers in much smaller size and cac value which are the mainly desirable characters for the self-aggregate amphiphiles. Therefore, this novel kinds of CMPD conjugates presents considerable potential functions in pharmaceutical and biomedical development. Meanwhile, this study also provides a new thought to utilize natural polysaccharide of pachyman and the further investigations for the CMPD conjugates as an anti-cancer drug carrier are active in progress.

Acknowledgments

This work was financially supported by the Chen Guang Foundation of Scientific and Technologic Council of Wuhan (Grant No. 20055003059-21), and Hubei Province Gongguan Foundation of Science and Technology (Grant No. 2006AA301B22).

References

Akiyoshi, K., Deguchi, S., Tajima, H., Nishikawa, T., & Sunamoto, J. (1997). Microscopic structure and thermoresponsiveness of a hydrogel nanoparticle by self-assembly of a hydrophobized polysaccharide. *Macromolecules, 30*, 857–861.

Akiyoshi, K., Kobayashi, S., Shichibe, S., Mix, D., Baudys, M., Kim, S. W., et al. (1998). Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: Complexation and stabilization of insulin. *Journal of Controlled Release, 54*, 313–320.

Aliabadi, H. M., & Lavasanifar, A. (2006). Polymeric micelles for drug delivery. *Expert Opinion on Drug Delivery, 3*(1), 139–162.

Ananthapadmanabhan, K. P., Goddard, E. D., Turro, N. J., & Kuo, P. L. (1985). Fluorescence probes for critical micelle concentration. *Langmuir, 1*, 352–355.

Benita, S., & Levy, M. Y. (1993). Submicron emulsions as colloidal drug carriers for intravenous administration: Comprehensive physicochemical characterization. *Journal of Pharmaceutical Sciences, 82*(11), 1069–1079.

Chihara, G., Hamuro, J., Maeda, Y., Arai, Y., & Fukuoka, F. (1970). Antitumour polysaccharide derived chemically from natural glucan (pachyman). *Nature, 225*, 943–944.

Davis, S. S. (1997). Biomedical applications of nanotechnology – Implications for drug targeting and gene therapy. *Trends in Biotechnology, 15*, 217–224.

Eyler, R. W., Klug, E. D., & Diephuis, F. (1947). Determination of degree of substitution of sodium carboxymethylcellulose. *Analytical chemistry, 19*(1), 24–27.

Gaucher, G., Dufresne, M.-H., Sant, V. P., Kang, N., Maysinger, D., & Leroux, J. C. (2005). Block copolymer micelles: Preparation, characterization and application in drug delivery. *Journal of Controlled Release, 109*, 169–188.

Glushko, V., Thaler, M. S., & Karp, C. D. (1981). Pyrene fluorescence fine structure as a polarity probe of hydrophobic regions: Behavior in model solvents. *Archives of Biochemstry Biophysics, 210*, 33–42.

Greish, K., Fang, J., Inutsuka, T., Nagamitsu, A., & Maeda, H. (2003). Macromolecular therapeutics: Advantages and prospects with special emphasis on solid tumour targeting. *Clinical Pharmacokinetics, 42*(13), 1089–1105.

Hamuro, J., Yamashita, Y., Ohsaka, Y., Maeda, Y. Y., & Chihara, G. (1971). Carboxymethylpachymaran, a new water soluble polysaccharide with marked antitumour activity. *Nature, 233*, 486–487.

Harada, A., & Kataoka, K. (1995). Formation of polyion complex micelles in an aqueous milieu from a pair of oppositely-charged block polymers with poly(ethylene glycol) segments. *Macromolecules, 28*, 5294–5299.

Jiang, G. B., Quan, D. P., Liao, K. R., & Wang, H. H. (2006). Preparation of polymeric micelles based on chitosan bearing a small amount of highly hydrophobic groups. *Carbohydrate Polymers, 66*, 514–520.

Jin, Y., Zhang, H., Yin, Y., & Nishinari, K. (2006). Comparison of curdlan and its carboxymethylated derivative by means of Rheology, DSC, and AFM. *Carbohydrate Research, 341*(1), 90–99.

Jones, M. C., & Leroux, J. C. (1999). Polymeric micelles – A new generation of colloidal drug carriers. *Journal of Pharmaceutics and Biopharmaceutics, 48*, 101–111.

Kang, N., & Leroux, J. C. (2004). Triblock and star-block copolymers of *N*-(2-hydroxypropyl)methacrylamide or *N*-vinyl-2-pyrrolidone and D,L-lactide: Synthesis and self-assembling properties in water. *Polymer, 45*(26), 8967–8980.

Kataoka, K., Harada, A., & Nagasaki, Y. (2001). Block copolymer micelles for drug delivery: Design, characterization and biological significance. *Advanced Drug Delivery Reviews, 47*, 113–131.

Kim, C., Lee, S. C., Kang, S. W., Kwon, I. C., Kim, Y.-H., & Jeong, S. Y. (2000). Synthesis and the micellar characteristics of poly(ethylene oxide)-deoxycholic acid conjugates. *Langmuir, 16*(11), 4792–4797.

Kim, C., Lee, S. C., Kwon, I. C., Chung, H., & Jeong, S. Y. (2002). Complexation of poly(2-ethyl-2-oxazoline)-block-poly (ε-caprolactone) micelles with multifunctional carboxylic acids. *Macromolecules, 35*(1), 193–200.

Kim, J. H., Kim, Y. S., Kim, S., Park, J. H., Kim, K., Choi, K., et al. (2006). Hydrophobically modified glycol chitosan nanoparticles as carriers for paclitaxel. *Journal of Controlled Release, 111*, 228–234.

Kratohvil, J. P., Hsu, W. P., & Kwok, D. I. (1986). How large are the micelles of di-α-hydroxy bile salts at the critical micellization concentrations in aqueous electrolyte solutions? Results for sodium taurodeoxycholate and sodium deoxycholate. *Langmuir, 2*, 256–258.

Kwon, G., Naito, M., Yokoyama, M., Okano, T., Sakurai, Y., & Kataoka, K. (1993). Micelles based on AB block copolymers of poly(ethylene oxide) and poly(beta-benzyl L-aspartate). *Langmuir, 9*(4), 945–949.

Kwon, S., Park, J. H., Chung, H., Kwon, I. C., & Jeong, S. Y. (2003). Physicochemical characteristics of self-assembled nanoparticles based on glycol chitosan bearing 5α-cholanic acid. *Langmuir, 19*, 10188–10193.

Lee, K. Y., & Jeon, Y. J. (2003). Polysaccharide isolated from *Poria cocos* sclerotium induces NF-kappa B/Rel activation and iNOS expression in murine macrophages. *International Immunopharmacology, 3*(10), 1353–1362.

Lee, K. Y., Jo, W. H., Kwon, I. C., Kim, Y.-H., & Jeong, S. Y. (1998a). Structural determination and interior polarity of self-aggregates prepared from deoxycholic acid-modified chitosan in water. *Macromolecules, 31*(2), 378–383.

Lee, K. Y., Jo, W. H., Kwon, I. C., Kim, Y.-H., & Jeong, S. Y. (1998b). Physicochemical characteristics of self-aggregates of hydrophobically modified chitosans. *Langmuir, 14*(9), 2329–2332.

Liu, C. G., Desai, K. G. H., Chen, X. G., & Park, H. J. (2005). Linolenic acid-modified chitosan for formation of self-assembled nanoparticles. *Journal Agriculture Food Chemical, 53*, 437–441.

Maeda, Y. Y., & Chihara, G. (1973). The effects of neonatal thymectomy on the antitumour activity of lentinan, carboxymethylpachymaran and zymosan, and their effects on various immune responses. *International Journal of Cancer, 11*, 153–161.

Na, K., Park, K. H., Kim, S. W., & Bae, Y. H. (2000). Self-assembled hydrogel nanoparticles from curdlan derivatives: Characterization, anti-cancer drug release and interaction with a hepatoma cell line ($HepG_2$). *Journal of Controlled Release, 69*, 225–236.

Nagasaki, Y., Okada, T., Scholz, C., Iijima, M., Kato, M., & Kataoka, K. (1998). The reactive polymeric micelle based on an aldehyde-ended poly(ethylene glycol)/poly(lactide) block copolymer. *Macromolecules, 31*, 1473–1479.

Narui, T., Takahashi, K., Kobayashi, M., & Shibata, S. (1980). A polysaccharide produced by laboratory cultivation of *Poria cocos* wolf. *Carbohydrate Research, 87*, 161–163.

Nishikawa, T., Akiyoshi, K., & Sunamoto, J. (1996). Macromolecular complexation between bovine serum albumin and the self-assembled hydrogel nanoparticle of hydrophobized polysaccharides. *Journal of the American Chemical Society, 118*, 6110–6115.

Park, J. S., Han, T. H., Lee, K. Y., Han, S. S., Hwang, J. J., Moon, D. H., et al. (2006). *N*-acetyl histidine-conjugated glycol chitosan self-assembled nanoparticles for intracytoplasmic delivery of drugs: Endocytosis, exocytosis and drug release. *Journal of Controlled Release, 115*, 37–45.

Park, K., Kim, K., Kwon, I. C., Kim, S. K., Lee, S., Lee, D. Y., et al. (2004). Preparation and characterization of self-assembled nanoparticles of heparin-deoxycholic acid conjugates. *Langmuir, 20*, 11726–11731.

Qiu, L. Y., & Bae, Y. H. (2007). Self-assembled polyethylenimine-graft-poly (ε-caprolactone) micelles as potential dual carriers of genes and anticancer drugs. *Biomaterials, 28*, 4132–4142.

Rodrigues, J. S., Santos-Magalhães, N. S., Coelho, L. C. B. B., Couvreur, P., Ponchel, G., & Gref, R. (2003). Novel core(polyester)-shell(polysaccharide) nanoparticles: Protein loading and surface modification with lectins. *Journal of Controlled Release, 92*, 103–112.

Sant, V. P., Smith, D., & Leroux, J. C. (2005). Enhancement of oral bioavailability of poorly water-soluble drugs by poly(ethylene glycol)-block-poly(alkyl acrylate-co-methacrylic acid) self-assemblies. *Journal of Controlled Release, 104*(2), 289–300.

Schinella, G. R., Tournier, H. A., Prieto, J. M., Mordujovich de Buschiazzo, P., & Rios, J. L. (2002). Antioxidant activity of anti-inflammatory plant extracts. *Life Sciences, 70*(9), 1023–1033.

Sunil, A. A., Nadagouda, N. M., & Tejraj, M. A. (2004). Recent advances on chitosan-based micro- and nanoparticles in drug delivery. *Journal of Controlled Release, 100*, 5–28.

Torchilin, V. P. (2001). Structure and design of polymeric surfactant based drug delivery systems. *Journal of Controlled Release, 73*(2), 137–172.

Wang, S. X., Wen, Y. Y., & Hu, C. X. (1995). Immunoactivities of the polysaccharides from Morus-alba, Chlamydomonas-mexicana and Poria-cocos. *Phytotherapy Research, 9*(6), 448–451.

Wang, Y. F., & Zhang, L. N. (2006). Chain conformation of carboxymethylated derivatives of (1–3)-β-D-glucan from *Poria cocos* sclerotium. *Carbohydrate Polymers, 65*, 504–509.

Wang, Y. F., Zhang, L. N., Li, Y. Q., Hou, X. H., & Zeng, F. B. (2004). Correlation of structure to antitumor activities of five derivatives of a β-glucan from *Poria cocos* sclerotium. *Carbohydrate Research, 339*, 2567–2574.

Wang, Y. F., Zhang, L. N., & Ruan, D. (2004). Preparation and structure of five derivatives of β-(1–3)-D-glucan isolated from *Poria cocos* sclerotium. *Chinese Journal of Polymer Science, 22*, 137–145.

Wang, Y. F., Zhang, M., Ruan, D., Shashkov, A. S., Kilcoyne, M., Savage, A. V., et al. (2004). Chemical components and molecular mass of six polysaccharides isolated from the sclerotium of *Poria cocos*. *Carbohydrate Research, 339*, 327–334.

Wang, Y. S., Liu, L. R., Jiang, Q., & Zhang, Q. Q. (2007). Self-aggregated nanoparticles of cholesterol-modified chitosan conjugate as a novel carrier of epirubicin. *European Polymer Journal, 43*, 43–51.

Wang, Y. S., Liu, L. R., Weng, J., & Zhang, Q. Q. (2007). Preparation and characterization of self-aggregated nanoparticles of cholesterol-modified *O*-carboxymethyl chitosan conjugates. *Carbohydrate Polymers, 69*, 597–606.

Wilhelm, M., Zhao, C., Wang, Y., Xu, R., Winnik, M. A., Mura, J., et al. (1991). Poly(styrene–ethylene oxide) block copolymer micelle formation in water: A fluorescence probe study. *Macromolecules, 24*, 1033–1044.

Xiao, Y. L., Liang, S. C., Qiu, G. F., Wu, J. Y., Zhang, J. B., & Hu, X. M. (2007). Preparation, characterization and tableting properties of two new pachyman-

based pharmaceutical aids: I. disintegrants in dispersible tablets. *Polymers for Advanced Technologies, 18*, 268–274.

Yamada, H., Kiyohara, H., Takemoto, N., Zhao, J. F., Kawamura, H., Komatsu, Y., et al. (1992). Studies on immunologically active substances from kampo medicine, juzen-taiho-to. 3. mitogenic and complement activating activities of the herbal components of juzen-taiho-to. *Planta Medica, 58*(2), 166–170.

Yu, S. J., & Tseng, J. M. (1996). Fu-Ling, a Chinese herbal drug, modulates cytokine secretion by human peripheral blood monocytes. *International Journal of Immunopharmacology, 18*(1), 37–44.

Zhang, M., Cheung, P. C. K., Zhang, L., Chiu, C., & Ooi, V. E. C. (2004). Carboxymethylated β-glucans from mushroom sclerotium of Pleurotus tuber-regium as novel water-soluble anti-tumor agent. *Carbohydrate Polymers, 57*(3), 319–325.