Micellar Solubilization and In Vitro Release of Silymarin in the self-Aggregates of an Amphiphilic Derivative of Chitosan

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Summary: The amphiphilic derivatives of chitosan, (2-hydroxyl-3-butoxyl)-propylcarboxymethyl-chitosan (HBP-CMCHS), can form micelles with the inner core of hydrophobic segments and the outer shell of hydrophilic segments. The typical poor water-soluble drug silymarin was solubilized in the HBP-CMCHS micellar by physical entrapped method. Results showed that the solubilizing capacity was enhanced by increasing the concentration of HBP-CMCHS with the same dosage of silymarin. Silymarin-loaded micellar system of HBP-CMCHS was characterized by TEM and DLS. TEM photograph revealed that the micelles were spherical and silymarin was solubilized in the cores of the spherical polymeric micelles. DLS showed that after solubilization the size of the micelles became bigger. *In vitro* tests showed that silymarin was slowly released from micellar solution and the release lasted up to 40 h by means of the dialysis method.

Keywords: chitosan; micelles; self-aggregate; silymarin; solubilization

Introduction

In recent decades, significant efforts have been devoted to develop novel polymeric carriers that can form compact micellar structure in an aqueous milieu, which can be used as peptide delivery system, gene delivery system, or drug delivery system [1,2]. Amphiphilic polymers can self-aggregate to form micelles with the inner core of hydrophobic segments and the outer shell of hydrophilic segments. [3,4] The inner core is created by association of the hydrophobic portions due to their cohesive hydrophobic interactions with each other in aqueous media, while the outer hydrophilic portions surround the inner hydrophobic core as a hydrated shell. The hydrophobic inner core is solubilized by the hydrophilic shell, which prevents the inactivation of core-encapsulated molecules by decreasing the contact with the inactivating species in the aqueous phase. [4–6]

Amphiphilic polymeric micelles have attracted much attention in drug delivery because of their ability to solubilize hydrophobic molecules, small particle size, good thermodynamic solution stability, and extended release of various drugs, which was loaded into the hydrophobic core of polymeric micelles by chemical conjugation or physical entrapment utilizing various interactions such as hydrophobic interactions, ionic interactions and hydrogen bonding. [6–8] The hydrophobic core serves as a reservoir from which drug is released slowly over prolonged periods of time. [8,9] Being in a micellar form, the drug is well protected from possible inactivation under the effect of biological surroundings, it does not provoke undesirable side effects, and its bioavailability is usually increased. [10]

In the biomedical materials field, polymeric micelles must possess several specific properties to be of use, including biocompatibility, biodegradability, target specificity, and stability in the body. [1,11] Therefore

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in recent years, self-assemblies based on naturally occurring polymers have been of particular interest. [1,2,11] Chitosan, the *N*-deacetylated derivative of chitin, has attracted significant interest in the broad range of scientific areas, including biomedical, agricultural, and environmental fields. [1,2,12] Recently, much attention has been paid to chitosan as a drug or gene carrier because of its biocompatibility and biodegradability. [1,12–14] However, the extended applications of chitosan are frequently limited because it is insoluble in biological solution (pH 7.4), which has stimulated studies to prepare water-soluble chitosan. Many kinds of soluble chitosan derivatives were prepared [1,13], and some of them can form aggregates in solution, especially for the amphiphilic derivatives. [13–16] These micelles or nanoparticles can be used as peptide delivery system, gene delivery system, or drug delivery system. [12–14]

In our previous report, we synthesized a series of amphiphilic derivatives of carboxymethylchitosan (CMCHS) prepared by reaction of CMCHS with alkyl glycidol ether. [15,16] And their application properties such as surface activity, foaming property, emulsifying ability, and moisture retention have been studied. The aim of this work was to evaluate one of the hydrophobic modified derivative, (2-hydroxyl-3-butoxyl) propyl- carboxymethylchitosan (HBP-CMCHS), as potential drug carrier using Silymarin as an example. Silymarin is one of the effective drugs against a wide range of liver diseases, but its clinical applications have been hindered by its low solubility. [17] In this study, Silymarin-loaded HBP-CMCHS micelles were prepared by physical entrapped method, and investigated by TEM photograph and DLS measurement. *In vitro* release profiles were also tested by means of the dialysis method.

Materials and Methods

Materials

Chitosan was provided by the Jinan Haidebei Biochemical Co. Ltd., China, with deacetylation degrees of 91% and viscosity average molecular weight of 175000 D. Silymarin was obtained from Hangzhou zhongxiang Pharmaceutical Co. Ltd., China. Butyl glycidol ether (BGE) was supplied from Shanghai Resin. Co., China. Pyrene was purchased from Sigma Company (>99%). All commercially available solvents and reagents were used without further purification.

Synthesis of HBP-CMCHS

HBP-CMCHS synthesis method was followed as reported before. [15] Briefly, As the first step, 5 g Chitosan was added in the mixture of 80 ml 2-propanol and 22 g of aqueous NaOH (45% w/w) solution while stirring, then added dropwise 14.5 g monochloroacetic acid desloved in 20 mL 2-propanol solution, kept 50 °C for 4 h while stirring. The product was filtered and washed with 80% ethanol and then 2-propanol twice and filtered to get carboxymethylchitosan (CMCHS). CMCHS was dispersed in 2-propanol, and 10 times of BGE (mol ratio to CHS anhydroglucose unit) was added dropwise in the dispersion, kept 55 °C for 18 hours while stirring. The products were filtered and washed with acetone, and then dried in a vacuum at 50 °C. For water-soluble products, the precipitates were redissolved in deionized water, dialyzed against deionized water and freeze-dried.

Elemental analysis of C, H and N were performed using a PE-240C analyzer (P.E. Company, USA). The degree of deacetylation of CHS (x), carboxymethyl substitution degree of CMCHS (y) and BGE substitution degree of HBP-CMCHS (z) were calculated according to the C/N ratio. The substitution degrees of carboxymethylation and BGE in this paper were estimated to be 0.8 and 0.24 according to the following formula:

$$\text{CHS}: \text{C}/\text{N} = 12 \times [6 + 2 \times (1\text{-x})] / 14$$

$$\text{CMCHS}: \text{C}/\text{N} = 12 \times [6 + 2 \times (1\text{-x}) + 2\,\text{y}] / 14$$

$$\text{HBP-CMCHS}: \text{C}/\text{N} = C/N = 12 \times [6 + 2(1 - x) + 2y + 7z] / 14$$

Preparation of Silymarin-Loaded Micelles

HBP-CMCHS was dissolved in phosphate buffer saline (PBS, pH = 6.8) with stirring for 30 min, then ethanol solution including definite amount of silymarin was added dropwise in the solution. In order to form micelles, the solution was sonicated with ultrasonication (KQ-100D, 40 kHz, 100 W, Qunshan Ultrasonic Co. Ltd., China) for 30 min. To remove free silymarin and ethanol, the solution was dialyzed against PBS (pH = 6.8) overnight and the micellar solution was centrifuged at 3000 rpm for 15 min to remove insoluble silymarin. The supernatant was filtered with membrane filter (pore size: 0.8 μm), and silymarin-loaded HBP-CMCHS micellar solution was obtained. A series of micellar solutions were obtained by varying the mixing ratio of HBP-CMCHS and silymarin. Free micelles were prepared by adding ethanol without silymarin, using the above method.

Measurement of Fluorescence Spectroscopy

Critical micelle concentration (cmc) of HBP-CMCHS was determined by using pyrene as a hydrophobic probe with fluorescence spectroscopy (RF-540 PC, Japan). [18] Briefly, a known amount of pyrene in methanol was added to each of a series of 10 mL tubes and the methanol was evaporated, then 10 mL of various concentrations of HBP-CMCHS solutions were added to each tube (the final concentration of pyrene was 6×10^{-7} mol L^{-1}), sonicated for 30 min at room temperature and kept for at least 2 h. The solutions were filtered with a 0.8 μm pore-sized filtration membrane. Fluorescence emission spectrum was measured at excitation wavelength of 335 nm, emission wavelength was 350 to 550 nm for excitation spectra. The hydrophobic index, I_1/I_3, was calculated as the ratio of the intensities at the first (374 nm) and the third (385 nm) vibrational peaks of monomeric pyrene in the pyrene emission spectra.

Solubilization Amount of Silymarin in Micelles

The amount of silymarin in micelle was determined by spectrophotometry. Silymarin solution with different concentration in 50% (v/v) ethanol was prepared with definite amount of silymarin dissolved in ethanol at first, followed by diluted with same volume of PBS (pH = 6.8) solution. The absorbance of silymarin solutions was analyzed at the maximum absorption wavelength of 287 nm by UV spectrophotometry (UV-260, Shimadzu, Japan) with the 50% (v/v) ethanol/PBS solution as reference. Calibration curves were obtained for the analysis of the concentration of Silymarin.

The resulting silymarin-loaded micelles solution was taken and diluted with ethanol and PBS buffer to 50% (v/v) ethanol solution. The UV absorbance was measured at 287 nm, and the corresponding concentration of silymarin was counted according to the standard curve.

Micelle Size and Size Distribution

The average size and the size distribution of the polymeric micelles were estimated by dynamic light scattering (DLS) by Zetasizer 3000HS instrument (Malvern Instruments, UK) equipped with temperature-controlled cell holder [6]. The intensity of scattered light was detected at room temperature. All measurements were made after the supernatant solution was filtered with a PTFE filter having an average pore size of 0.8 μm.

Transmission Electron Microscopy (TEM) Observation

The morphology of polymeric micelles was observed by Transmission electron microscopy (TEM) with JEM-100 CX II (Jeol Ltd., Japan) at 75 kV. A drop of the sample solution was placed onto a mesh copper grid coated with carbon. About 1 min after deposition, the grid was tapped with a filter paper to remove surface water and negatively stained using a 2% phosphotungstic acid solution. After 1 min, excess fluid was removed, the surface air-dried for 5 min and the grid loaded in the transmission electron microscope. [5]

In vitro Drug Release Studies

The silymarin-loaded polymer micelle was introduced into a dialysis membrane bag

(MWCO= 8000-10,000), and the whole bag was placed in the solution of PBS (pH 6.8) and continuously stirred with magnetic stirrer at 200 rpm to ensure uniform distribution. The temperature of the entire diffusion cell assembly was maintained at $37 \pm 0.5\,^{\circ}C$, using a recirculating water jacket [10]. Samples was taken for UV analysis from the media outside the dialysis bag at predetermined time intervals, and the whole medium was replaced with the same volume of fresh PBS. The absorbance of the sample was measured by spectrophotometry and the amount of silymarin was obtained through the standard curve.

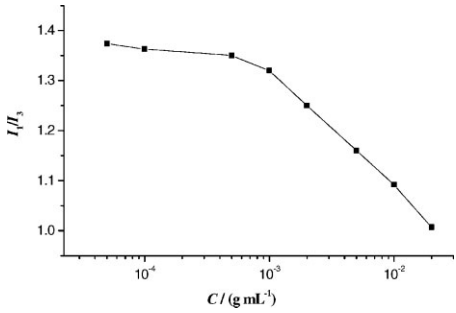

Figure 1.
I_1/I_3 value of HBP-CMCHS as a function of HBP-CMCHS concentration.

Results and Discussion

As an amphiphilic polymer, HBP-CMCHS can form micelle in PBS buffer solution. At very low concentration, the polymers only exist as single chains, while as the concentration increases to a critical value called the critical micelle concentration (cmc), polymer chains start to associate to form micelles in such a way that the hydrophobic chain associate with each other to form inner core, while the outer hydrophilic carboxymethylchitosan portions surround the inner hydrophobic core as a hydrated shell.

The self-assembled micelle formation was determined by fluorescence probe techniques, with the intensity ratio of I_1/I_3 from the emission spectra of pyrene to monitor the polarity of the surrounding microenvironment. [18] Figure 1 shows the I_1/I_3 value of pyrene in solution as a function of HBP-CMCHS concentration. When the concentration of HBP-CMCHS is above $0.5\,mg\,mL^{-1}$, the I_1/I_3 value gradually decreases, indicating that the microenvironment around pyrene is getting more hydrophobic. It can be interpreted as the formation of micelle with the hydrophobic aggregate core getting more compact.

Solubilization of Silymarin in HBP-CMCHS Micelles

In order to determine the amount of silymarin in micelles, standard curve of silymarin was made under the maximum absorption wavelength of 287 nm in advance. In the 50% (v/v) ethanol/PBS buffer solution, the standard equation was $A = -0.03872 + 0.03384C$, $C = 2 \sim 22\,\mu g\,mL^{-1}$ and $R = 0.99946$. The resulting silymarin-loaded micelles solution was taken out and diluted to 50% ethanol (v/v) ethanol/PBS buffer solution. The UV absorbance was also measured at 287 nm, and the corresponding concentration of silymarin was counted according to the standard curve of silymarin.

Silymarin loading capacity of HBP-CMCHS micelle was evaluated by varying the initial concentration of HBP-CMCHS and added amount of silymarin, and the corresponding particle size was determined by DLS method. Results were summarized in Table 1.

Silymarin is a hydrophobic drug with aqueous solubility of $0.04\,mg\,mL^{-1}$ at 25°C. As shown in the table, the solubilization capacity of silymarin dramatically increases with increasing the concentration of HBP-CMCHS or the adding amount of silymarin. As the concentration of HBP-CMCHS was $10\,mg\,mL^{-1}$, the solubilized silymarin concentration can increase more than 13 times of that dissolved in water.

Morphology of the Micelles

The appearance of blank and silymarin loaded polymeric micelles were observed by TEM photograph. Figure 2.a showed that the $2\,mg\,mL^{-1}$ blank micelle before

Table 1.
Particle Size and Silymarin loading Capacity of HBP-CMCHS Micelles.

Initial Concentration (mg mL^{-1}) HBP-CMCHS / Silymarin	Particle size (nm)	Silymarin Concentration (mg.mL^{-1})
0 / 1		0.040
2 / 0	300	——
2 / 1	310	0.27
2 / 2	319	0.30
5 / 0	332	——
5 / 1	354	0.35
5 / 2	643	0.44
10 / 0	462	——
10 / 1	502	0.42
10 / 2	753	0.53

solubilization were as approximately spherical shapes with hydrophobic cores (white core without stained by phosphotungstic acid) and hydrophilic shell (black shell being stained). Figure 2.b showed that the cores became black as a result of the solubilization of silymarin, indicating that the drug was solubilized in the inner core of HBP-CMCHS micelle. When the concentration of HBP-CMCHS increased to 10 mg mL^{-1}, the separated micelles can associate with each other with the interaction of the hydrophilic shell to form aggregate micelles, resulting in the size of the micelle increase. Solubilization of silymarin in the inner core makes the micelles aggregate and become bigger.

The Diameter Distribution of the Polymeric Micelles

The diameter and distribution of the polymeric micelles were measured by Zetasizer 3000HS instrument. Figure 3 showed the particle size distributions of HBP-CMCHS based on DSL intensity. The mean particle size of blank HBP-CMCHS micelles was about 460 nm as the concentration of HBP-CMCHS is 10 mg mL^{-1}. Solubilization of silymarin into micelles made the particle much bigger, and the particle size distribution get much dispersive. At the same concentration of HBP-

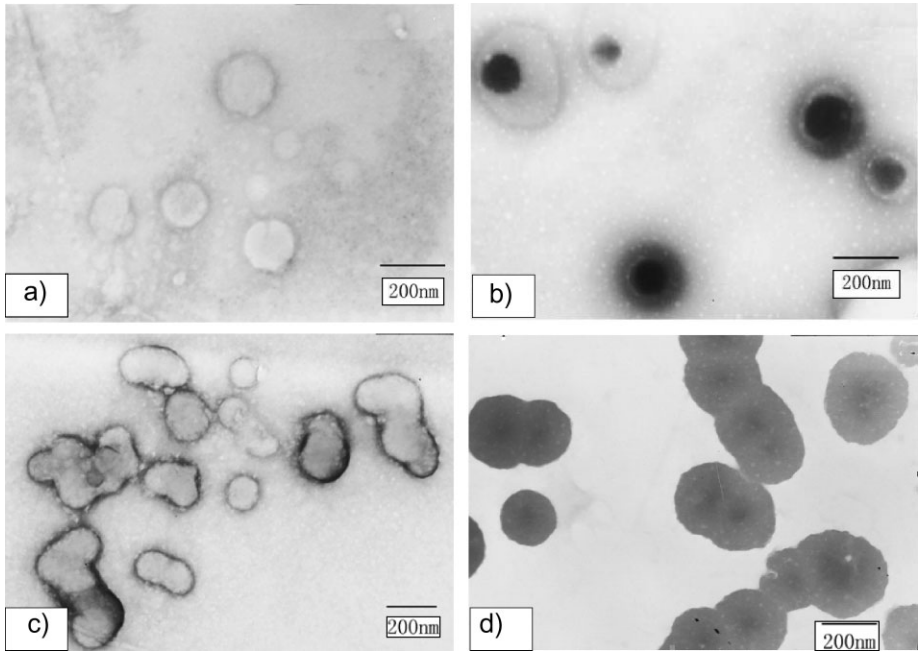

Figure 2.
Morphology of blank and silymarin-loaded micelle observed by Transmission electron micrograph (a) 2 mg mL^{-1} blank micelle; (b) 2 mg mL^{-1} silymarin-loaded micelle (2/2); (c) 10 mg mL^{-1} blank micelle; (d) 10 mg mL^{-1} silymarin-loaded micelle (10/2).

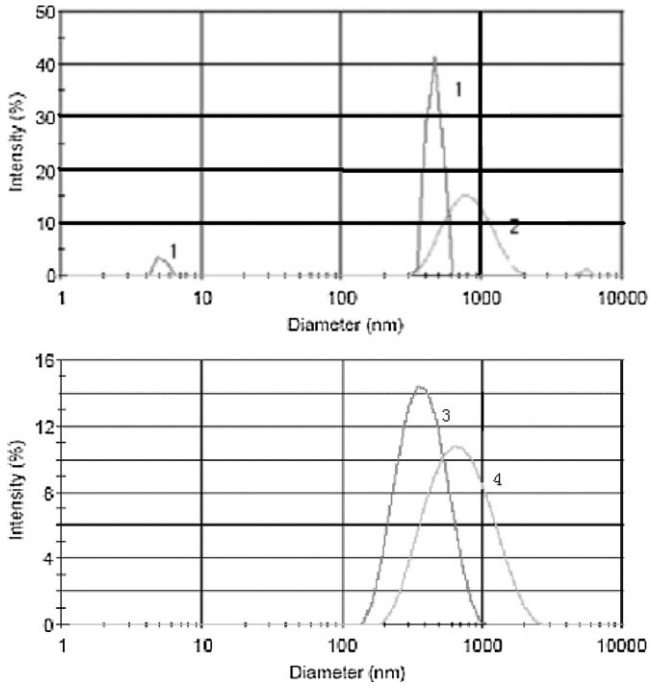

Figure 3.
Particle size distribution based intensity of blank and silymarin-loaded HBP-CMCHS micelles with different initial concentration. (1) HBP-CMCHS / silymarin (10/0); (2) HBP-CMCHS / silymarin (10/2); (3) HBP-CMCHS / silymarin (5/1); (4) HBP-CMCHS / silymarin (5/2).

CMCHS, the more silymarin solubilized, the bigger size of the particle.

The micelles size measured by dynamic light scattering was bigger than those visualized by TEM, due to the different measurement mechanism. But it was consistent that after solubilization the micelles became bigger than blank micelles.

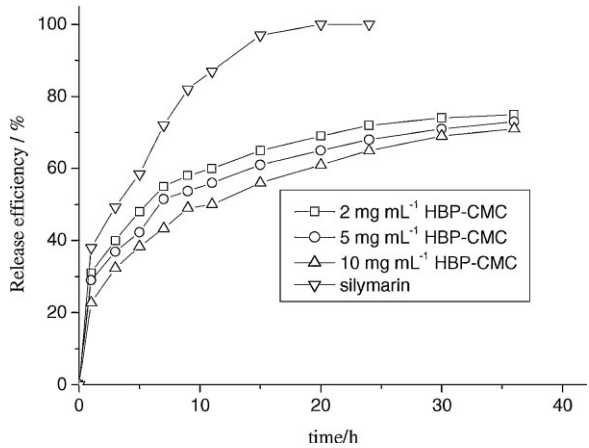

Figure 4.
In vitro cumulative release profiles of silymarin at 310 K in only PBS (pH 6.8) solution and different concentration of HBP-CMCHS micelles.

Drug Release

Drug release from polymeric micelles is a rather complicated process and can be affected by many factors, including polymer degradation, molecular weight, binding affinity between the polymer and the drug. [7] Generally, micelle-incorporated drugs are slowly released from an intact micelle. [8,9] However, drug molecules located within or adjacent to the corona can be quickly released, and thus be responsible for the ''fast release'' component of the net release curve. The phase state of the drug can also be important for its association with a micelle; if the drug is not dissolved in the core, but exists as a separate phase inside the core, this property can hinder drug release from the micelle. [9]

Figure 4 shows *in vitro* cumulative release profiles of silymarin from HBP-CMCHS micelles prepared by using the initial drug concentration 2 mg mL^{-1}. As shown in Figure 4, silymarin release from the micelles was slow and showed sustained release characteristics over 40 h relative to the free silymarinin PBS solution. 60% percent of silymarin was released within 20 h at 37 °C, afterwards, the release rate was slow down and it was released about 70% after 40 h. Drug-loading studies revealed that slow release of hydrophobic drugs from HBP-CMCHS-based polymeric micelles could allow accumulation of polymeric micelles at targeted sites with minimal drug loss and localized drug release. [8]

Conclusion

The novel chitosan derivative HBP-CMCHS can form polymeric micellar system which has a comparative solubilization capacity for the typical poor water-soluble drug silymarin. The micelles were spherical and silymarin was solubilized in the cores of the spherical polymeric micelles, resulting in the size of the micelles became bigger. *In vitro* tests showed that silymarin was slowly released from micellar solution and the release lasted up to 40 h. Taking the advantage of the high solubilization capacity and slowly released profile, the micellar system of the modified chitosan seems a promising nanocarrier for some of the insoluble drugs.

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