

Note

Protection of Photoactivity of Photosensitizers by Amphiphilic Polysaccharide Micelles^{*}

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Abstract Photosensitizer (photosan)-encapsulated micelles were prepared by self-assembly of Photosan with two amphiphilic polysaccharide derivatives, including the cholesteryl conjugated sodium alginate derivative (CSAD) and the deoxycholic acid group conjugated chitosan derivative (DA-Chit). The results of UV-Vis and fluorescence spectroscopy indicated that the hydrogen bonding strength dominated the micelle formation. Methyl viologen was used as a model quencher of photosan. Using the steady-state fluorescence technology, the quenching constants were determined to be 2.05, 1.88 and 0.30 L/mmol for the free photosan, the photosan-CSAD micelle and the photosan-DA-Chit micelle, respectively. This suggested that photosan was protected from quenching of methyl viologen by the polysaccharide micelles. In addition, the protection effect of the photosan-DA-Chit micelle was significantly stronger than that of the photosan-CSAD micelle. The photosan-DA-Chit micelle is thus anticipated for protection of photoactivity of photosan during the blood circulation process *in vivo*.

Keywords: Chitosan; Sodium alginate; Photosensitizer; Photoactivity; Quenching.

INTRODUCTION

Polysaccharides are an important class of natural biomacromolecules with good biocompatibility, biodegradability and nontoxicity^[1]. In recent years, amphiphilic polysaccharide derivatives have received increasing attention in the drug delivery system^[2]. They tend to form micelles by self-assembly, which consist of hydrophobic multicores and a hydrophilic shell, allowing for the achievement of low Gibb's energy in water^[2–5]. The micelles may encapsulate water-insoluble and water-soluble drugs, and the drugs are thus delivered efficiently to living tissues and then are released sustainedly^[2, 6].

Photodynamic therapy (PDT) has recently received much attention as a safe, minimally invasive and tissue selective treatment of cancer and other diseases^[7]. During the PDT process, a photosensitizer, as a photoactive porphyrin-based molecule, is intravenously administered into the patient. Following light activation at the targeted tissues, reactive oxygen species, including singlet oxygen (¹O₂), superoxide (O₂^{•−}) and peroxide anions (O₂^{2−}), are generated to oxidize subcellular organelles and other biomolecules, leading to light-induced cell death^[8].

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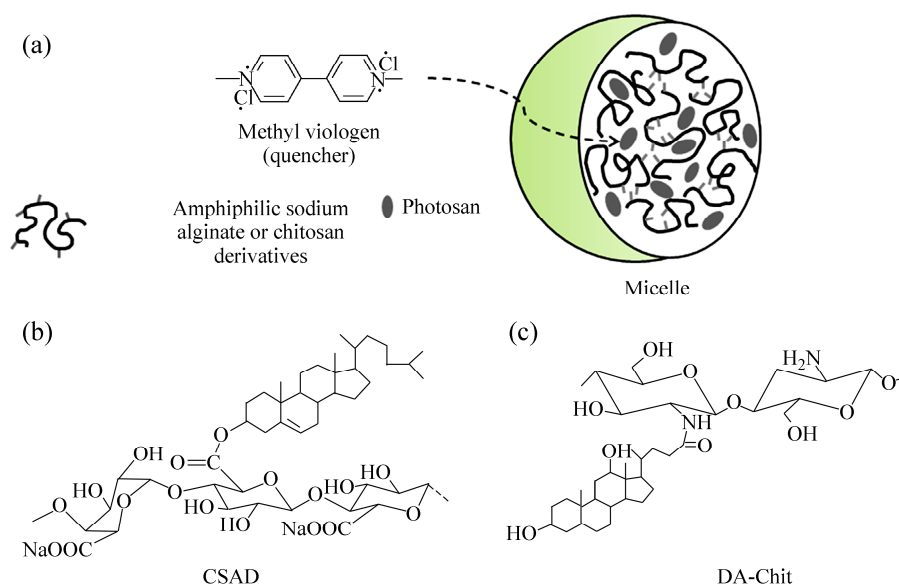
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At present, the main focus of this research field is to prepare photosensitizer-encapsulated micelles for increasing water-solubility, reducing aggregation, and improving targeting property of photosensitizers^[7, 9–11]. On the other hand, the photoactivity of photosensitizers may decrease *via* quenching induced by compounds from food and drugs (*e.g.* vitamin K₃) during the blood circulation process *in vivo*^[12–14]. However, this has not been paid more attention, which is also an essential problem in clinical applications. It is thus important to protect photoactivity of photosensitizers against quenchers.

Photosan (also called photofrin) is a mixture of oligomers formed by ether ester linkages of up to 8 porphyrin units containing sodium carboxylate groups. It has been approved as a water-soluble photosensitizer in clinical applications^[7, 15]. In this work, the protection of photoactivity of photosan by two amphiphilic polysaccharide micelles with different electro-negativities was investigated using fluorescence spectroscopy. Methyl viologen was used as a model quencher of photosan (Scheme 1). The micelles were prepared by self-assembly of photosan with two amphiphilic polysaccharide derivatives, including the cholesteryl conjugated sodium alginate derivative (CSAD) and the deoxycholic acid group conjugated chitosan derivative (DA-Chit) (Scheme 1).



Scheme 1 (a) A speculated model for the quenching effect of methyl viologen on photosan encapsulated in amphiphilic polysaccharide micelles; (b) and (c) Chemical structures of the CSAD and DA-Chit derivatives

EXPERIMENTAL

Materials

Chitosan with a weight average molecular weight of 4.5×10^5 g/mol and the deacetylation degree of 90.0% was bought from Shanghai Bo'ao Biological Technology Co., Ltd (Shanghai, China). Sodium alginate, purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), was purified twice by dissolving in the distilled water, filtrating, precipitating with ethanol and drying in a vacuum at 40 °C. Its weight average molecular weight and number average molecular weight are 1.2×10^5 g/mol and 7.9×10^4 g/mol, respectively, and its polydispersity of 1.5 was determined by gel permeation chromatography. Cholesterol, *N,N'*-dicyclohexylcarbodiimide (DCC), 4-(*N,N'*-dimethylamino)pyridine (DMAP) and methyl viologen were purchased from Acros Organics (Janssens Pharmaceuticaaan, Belgium). *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) was purchased from Shanghai Medpep Co., Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO) was acquired from Aladdin Reagent Company (Shanghai, China), and was dried by

soaking in molecular sieves and calcium hydride for a week before use. Photosan (Porfimer sodium), a freeze-dried injectable powder, was purchased from SeeLab Inc. (Wesselburen, Germany).

Synthesis of the Amphiphilic CSAD Derivative

Sodium alginate was protonated in an aqueous HCl solution (2 mol/L). The resultant alginate acid precipitate was purified by washing with distilled water and lyophilized. The CSAD derivative was then synthesized based on our previous work^[16]. Briefly, alginic acid (1.0 g, 5.68 mmol of uronic acid unit) was dissolved in 35 mL of water-free DMSO at room temperature overnight. And then the solutions, including cholesterol (0.40 g, 1.03 mmol) in 2 mL chloroform and DCC (0.24 g, 1.16 mmol) and DMAP (0.14 g, 1.13 mmol) in 15 mL of DMSO, were added. The reaction was allowed to proceed at room temperature for 24 h, followed by purification with ethanol. The resultant product was dissolved in distilled water and neutralized by adding NaHCO₃ solution (4%, *W/V*). The solution was dialyzed against the distilled water for 3 days and lyophilized to get the CSAD derivative. FTIR: 3266 cm⁻¹ (vibration of —OH), 2920 cm⁻¹ (vibration of —CH<, —CH₂—), 1733 cm⁻¹ (vibration of —C=O of carboxylic ester group, —COOR), 1600 and 1414 cm⁻¹ (asymmetric and symmetric stretching vibrations of —C=O of sodium carboxylate group, —COONa), 1093 and 1033 cm⁻¹ (vibration of —C—O—C— of glucosidic bond); ¹H-NMR (D₂O): δ = 5.40 (glucose unit, H1), δ = 3.5–4.5 (glucose unit, H2–H6), δ = 1.0–2.5 (protons of cholesteryl group). DS = 0.03 (DS: the degree of substituted residues per sugar unit, determined by the integration of protons of substituted residues and polysaccharides).

Synthesis of the Amphiphilic DA-Chit Derivative

The DA-Chit derivative was synthesized following the method used in our previous work^[17]. In brief, chitosan (1.0 g, 6.21 mmol of glucosamine unit) was dissolved in a 40 mL of 1% aqueous acetic acid solution and diluted with 46 mL of ethanol. Deoxycholic acid (0.85 g, 2.17 mmol) and EDC (0.62 g, 3.26 mmol) were dissolved in 8 mL and 6 mL ethanol, respectively. After the solutions of deoxycholic acid and EDC were added to the chitosan solution in the order list under stirring, the reaction was allowed to proceed at room temperature for 24 h. The reaction mixture then was neutralized by the dropwise addition of ethanol/ammonia solution (7/3, *V/V*) and precipitated with 300 mL of ethanol, followed by centrifugation (3500 r/min, 10 min). The resultant precipitate was dissolved in distilled water, dialyzed against the distilled water. FTIR: 3266 cm⁻¹ (vibration of —OH, —NH₂), 2927 and 2872 cm⁻¹ (vibration of —CH<, —CH₂—), 1660 cm⁻¹ (secondary amide I band: stretching vibration of —C=O), 1566 cm⁻¹ (secondary amide II band: deformation vibration of —NH< and stretching vibration of —C—NH—), 1093 and 1033 cm⁻¹ (vibration of —C—O—C— of glucosidic bond); ¹H-NMR (D₂O): δ = 2.8–6.0 (proton of chitosan), δ = 0.6–2.5 (protons of deoxycholic acid group). DS = 0.03.

Preparation of Amphiphilic Polysaccharide-encapsulated Photosan Micelles

For the preparation of the DA-Chit-encapsulated photosan (photosan-DA-Chit) micelle, the DA-Chit derivative (30.0 mg) was dissolved in 15 mL of PBS (pH 6.2) with stirring at room temperature overnight, prior to the addition of 15 mL of PBS (pH 6.2) with photosan (150 μ g/mL). The resulting mixture was stirred at room temperature in the dark flask for 24 h, followed by a period of dialysis in PBS (pH 6.2) in darkness for 1 day to get ride of the un-encapsulated photosan. In the same way, the CSAD-encapsulated photosan (photosan-CSAD) micelle was prepared. A working curve linking the photosan concentration in PBS (pH 6.2) and a wavelength of 362 nm was used to evaluate the amount of photosan in the solution outside of dialysis bag ($R^2 = 0.998$), which was analyzed on a UV-Vis spectrophotometry (UV-3150, Shimadzu, Japan). The amount of photosan encapsulated in the micelle was calculated from the reduction in the photosan concentration. The encapsulating capacity of photosan in the micelle was then calculated using Eq. (1).

$$\text{Encapsulating capacity (\%)} = [(A-B)/C] \times 100 \quad (1)$$

where A is the total weight of photosan used, B is the weight of un-encapsulated photosan, and C is the weight of CSAD or DA-Chit. Consequently, the encapsulating capacities of the photosan-CSAD and photosan-DA-Chit micelles were determined to be 2.5% and 3.8%, respectively. The UV-Vis spectra of photosan before and after being encapsulated in the micelles were obtained in PBS (pH 6.2) with photosan concentration of 2 μ g/mL.

Steady-state Fluorescence Measurements

Steady-state fluorescence measurements were carried out on a Combined Fluorescence Lifetime and Steady State Spectrometer (FLSP920, Edinburgh Instruments Ltd., Edinburgh, UK). The fluorescence emission spectra were obtained with the maximum excitation wavelength at 395 nm, the scanning wavelength range of 550–750 nm, and exciting and emission slits of 4 nm and 5 nm, respectively.

The protection effect of photoactivity of photosan by amphiphilic polysaccharide micelles was evaluated using the steady-state fluorescence-quenching technique^[18]. Methyl viologen was used as a fluorescence quencher to quench photosan fluorescence. A methyl viologen stock solution (2 mmol/L) was prepared in PBS (pH 6.2). After the methyl viologen stock solutions with different volumes were added to the 10.0 mL vials, the free photosan solutions were added until the final concentration of photosan was 5 µg/mL. In the same way, the mixture solutions of methyl viologen and the amphiphilic polysaccharide-encapsulated photosan micelles were prepared. All mixture solutions were stirred, kept aside in darkness for 2 h, and then analyzed by fluorescence spectroscopy. The quenching constant (k_{sv}) was then calculated by Eq. (2) according to the Stern-Volmer theory^[18].

$$F_0/F = 1 + k_{sv} [Q] \quad (2)$$

where F_0 and F are the fluorescence emission intensities in the absence and presence of a quencher, $[Q]$ is the molar concentration of the quencher.

RESULTS AND DISCUSSION

Formation Mechanism of Amphiphilic Polysaccharide-encapsulated Photosan Micelles

In the UV-Vis spectrum of the free photosan (Fig. 1A), the absorption peaks at 364 nm and in the range of 450–650 nm are assigned to the characteristic absorption peaks of porphyrin rings including the Soret band and the Q-bands^[7]. Interestingly, it was found that the Soret band red shifted to 371 and 403 nm (Fig. 1), after photosan was encapsulated in the CSAD and DA-Chit micelles, respectively. Figure 1(B) shows the fluorescence emission spectra of the free photosan and the photosan encapsulated in micelles. The fluorescence emission spectra of the photosan-CSAD micelle and the free photosan were similar as shown in Fig. 1(B). However, the

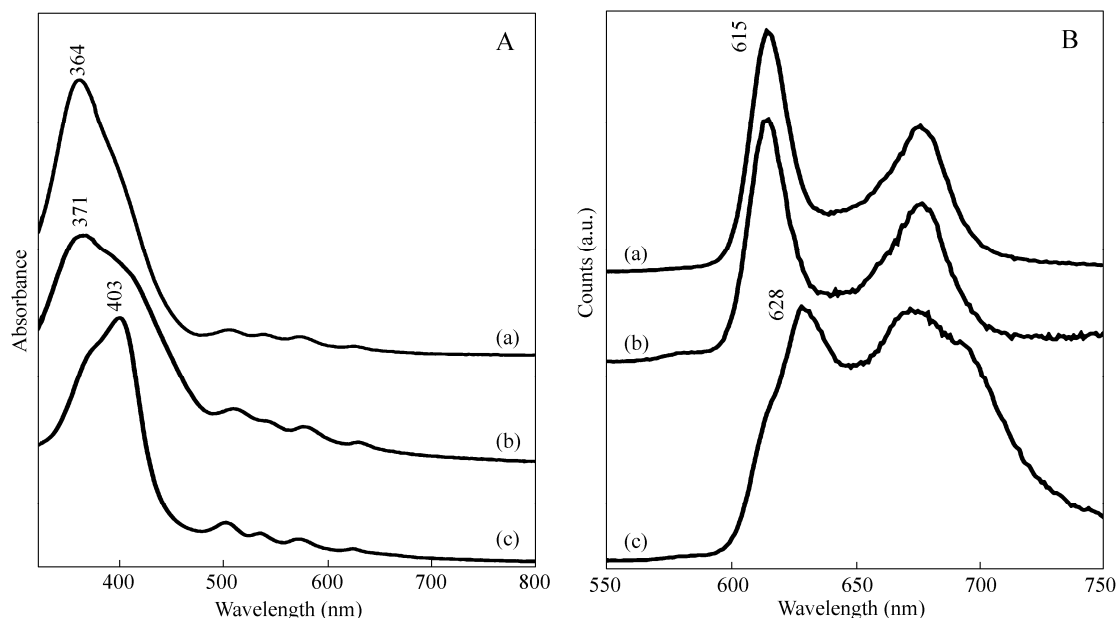


Fig. 1 (A) UV-Vis absorption spectra and (B) fluorescence emission spectra of (a) the free photosan, (b) the photosan-CSAD micelle, (c) the photosan-DA-Chit micelle in PBS (pH 6.2) (with photosan concentration of 2 µg/mL for all samples)

maximum emission wavelength of the photosan-DA-Chit micelle red shifted from 615 nm to 628 nm (Fig. 1B), compared with that of the free photosan. These results suggest that the different polysaccharide micelle circumstances obviously affect the spectroscopic property of photosan.

With the aid of the literature^[19–21], we assumed that the red shift of absorption and fluorescence spectra of photosan occurred as a consequence of hydrogen bond formation between the polysaccharide derivatives and photosan in the micelles. In addition, the electrostatic attraction interaction between the DA-Chit derivative and photosan is advantageous to enhancement of such hydrogen bonding strength, while the case of the CSAD derivative and photosan is opposite. As a result, the absorption and fluorescence spectra of the photosan-DA-Chit micelle shift to the longer wavelength. Therefore, the results of UV-Vis and fluorescence spectroscopy indicated that the hydrogen bonding strength dominated the micelle formation.

Protection of Photoactivity of Photosan by Amphiphilic Polysaccharide Micelles

The interaction of photosan with methyl viologen was thus evaluated to investigate the protection effect of photosan by the micelles using the steady-state fluorescence technology. It was found that the fluorescence intensity of photosan decreased with increasing the concentration of methyl viologen as shown in Figs. 2(a)–2(c), suggesting that the photosan fluorescence was quenched by methyl viologen. The fluorescence intensity of peak at 615 nm was recorded for both free photosan and the photosan-CSAD micelle, and that at 628 nm for the photosan-DA-Chit micelle.

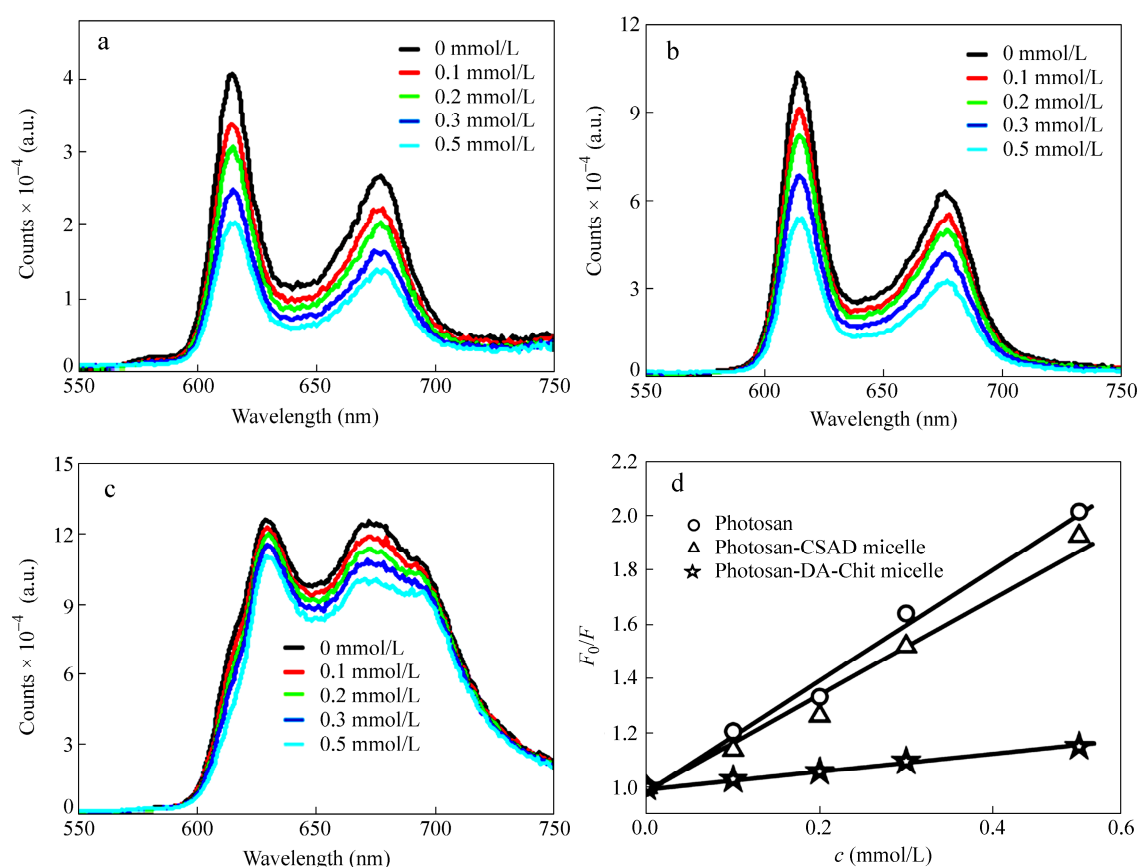


Fig. 2 Fluorescence emission spectra of photosan quenched by methyl viologen with different concentrations: (a) the free photosan, (b) the photosan-CSAD micelle and (c) the photosan-DA-Chit micelle; (d) Variation of $\ln(F_0/F)$ with the concentration of methyl viologen (with the final photosan concentration of 5 $\mu\text{g/mL}$ for all samples, $R^2 = 0.99$)

As shown in Fig. 2(d), the k_{sv} values were determined to be 2.05, 1.88 and 0.30 L/mmol for the free photosan, the photosan-CSAD micelle and the photosan-DA-Chit micelle according to Eq. (2). This suggested that photosan was protected from quenching of methyl viologen by the polysaccharide micelles, and the protection effect of the DA-Chit micelle was significantly stronger than that of the CSAD micelle. The stronger shielding of methyl viologen could come from the stronger hydrogen bonding strength between the DA-Chit derivative and photosan. As a result, photosan was more tightly restricted in the photosan-DA-Chit micelle, which prevented methyl viologen from penetrating.

CONCLUSIONS

The photosan-DA-Chit and photosan-CSAD micelles were prepared for the protection of photoactivity of photosan. The results of UV-Vis and fluorescence spectroscopy indicated that the hydrogen bonding strength dominated the micelle formation. Photosan was protected from quenching of methyl viologen by the polysaccharide micelles, and the protection effect of the photosan-DA-Chit micelle was significantly stronger than that of the photosan-CSAD micelle. The photosan-DA-Chit micelle is thus anticipated for protection of photoactivity of photosan during the blood circulation process *in vivo*.

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