

Preparation and Characterization of Camptothecin Grafted Chitosan Oligosaccharide Nanomicelles

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ABSTRACT

Water soluble chitosan oligosaccharide (CHO) was hydrophobically modified with camptothecin (CPT) in different molar ratios of CPT, and characterized by FT-IR and ¹H-NMR spectroscopy. Nanomicelles prepared by these copolymers were analyzed for size, zeta, and length of time they remain stable. The critical micelle concentration (CMC) and dissociation of micelles at physiological pH and acidic pH were also investigated. FT-IR and ¹H-NMR spectroscopy approved chemical modification of CHO. Fluorometry study showed that the CMC of grafted copolymer decreased from 27.2×10 to 6.1×10 g/L with an increasing feeding ratio from 2.5 to 7.5. Particle size of CPT-CHO-2.5 and CPT-CHO-7.5 was 539 ± 152.15 and 197.8±66 nm, respectively. The dissociation study showed that the micelles are stable, and the disassembly was not observed in water at 37°C neither at pH of 7.4 nor at pH of 5.5. These phenomena make CPT-CHO micelles as stable polymeric micelles for drug delivery.

Introduction

Recently, polymeric micelles have attracted increased interest because of their potential utilization as a drug carrier system. Poorly-water-soluble hydrophobic drugs can be placed in the hydrophobic core of the micelles, and the hydrophilic surface interacts with aqueous phase molecules to improve the solubility of the drug. Moreover, the polymeric micelles show a desirable stability in aqueous medium regardless of the amount of hydrophobic drug content incorporated into the inner core of the micelles. Furthermore, polymeric micelles in a size range below 200 nm reduces the non-selective reticuloendothelial system's (RES) ability to scavenge, in addition it shows an enhanced permeability and retention (EPR) effect [1].

In the novel approach, polymeric micelles have attracted considerable attention to the potential advantages of hydrophobic drugs (as a core of micelle) that are covalently linked to a polymer. The conjugation of hydrophobic drugs with water-soluble polymers improves their aqueous solubility and decreases their renal clearance. Some studies have shown that covalently linked hydrophobic drugs not only

form the hydrophobic core of micelle but also function in the targeted cell. It has been also reported that using an anticancer drug as hydrophobic part of the micelle can allow the co-deliver of two or even more anticancer drugs to cancer cells [2].

20(S)-camptothecin (CPT) is a quinoline alkaloid which shows extreme cytotoxicity properties [3]. The topoisomerase I (TOPO I) enzyme is identified as the cellular target of camptothecin and its analogues [4]. The complex formed between CPT, TOPO I, and DNA, prevents DNA re-ligation which results in DNA damage and cellular death. Hence, unlike many other anti-cancer drugs, which prevent tumor cell proliferation by binding the DNA, CPT destroys the DNA strand during replication, and shows a significant anti-tumor activity in a wide spectrum of malignancies [3, 5]. There are two forms of camptothecin formed in aqueous medium (lactone and carboxylate). The lactone form of CPT shows more anti-cancer activity (almost 10 fold greater) than the carboxylate form. Moreover, there is equilibrium between them in aqueous medium (Fig. 1) [6].

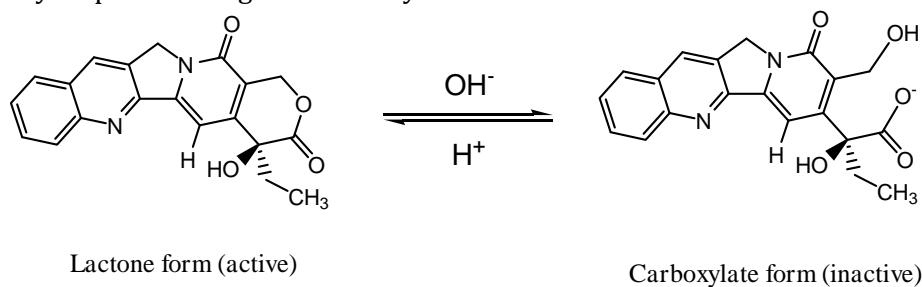


Fig.1. Chemical structure of camptothecin (CPT); two forms depending on the pH value, namely an active lactone form below pH5 and an inactive carboxylate form at basic pHs.

A number of poor characteristics of this highly potent anti-cancer drug have limited its clinical applications, such as: high aqueous insolubility, low stability of the lactone form at physiological pH, and severe systemic toxicities, which include myelo-suppression, vomiting, diarrhea, and hemorrhagic cystitis [7-9].

In past decades, researchers have synthesized water soluble compounds of camptothecin to improve its pharmaceutical profile, such as: irinotecan, topotecan, and 9-amino camptothecin

[10]. These analogues were widely tested in the early 1990's [11]. Despite improving their water-solubility, there remains significant disadvantages in these analogues profiles including; instability of the lactone form, short half-life in blood circulation, and a number of unresolved toxic effects.

An alternative approach is to conjugate CPT to polymers, in order to enhance water solubility and to maintain CPT in a closed lactone form in the

plasma compartment, by covalently linking the 20(S)-hydroxyl group to the polymer [12-14].

A wide range of materials, such as; natural or synthetic polymers, lipids, surfactants and dendrimers, have been employed as drug carriers or conjugates [15-18]. Among these, polysaccharides have received increasing attention because of their outstanding physical and biological properties [19].

Chitosan, a linear amino polysaccharide, composed of randomly distributed (1→4) linked D-glucosamine and N-acetyl-D-glucosamine units, is obtained through the deacetylation of chitin, a wide spread natural polysaccharide that is found in the exoskeleton of crustaceans, such as crabs and shrimps [20]. This cationic polysaccharide has drawn increasing attention within pharmaceutical and biomedical applications, owing to its abundant availability, unique mucoadhesivity, inherent pharmacological properties, and other beneficial biological properties, such as; biocompatibility, biodegradability, non-toxicity, and low-immunogenicity [20-22]. The physicochemical and biological properties of chitosan are greatly influenced by its molecular weight and degree of deacetylation. Unlike chitosan, its hydrolyzed products and chitosan oligosaccharides (CHO) are readily soluble in water due to their shorter chain lengths and free amino groups in D-glucosamine units [23]. The low viscosity and greater solubility of CHO at neutral pH have attracted the interest of many researchers who have utilized chitosan in its oligosaccharide form. Recent advances have provided insights into the health benefits of CHO, including lowering blood cholesterol [24], and enhancing antitumor properties [25].

However, chitosan oligosaccharide (CHO) cannot form micelles by itself. Therefore in this study, we induced self-assembly character by attaching camptothecin as a hydrophobic element to the backbone of CHO, and CPT-CHO micelles were synthesized and characterized.

Materials and Methods

Materials

Camptothecin (CPT), succinic anhydride (SUCC), dimethyl amino pyridine (DMAP), diisopropylcar-

bodiimide (DIPC), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were supplied by Sigma-Aldrich, USA. Cellulose membrane filter of 0.45 µm pore size was supplied by Orange, France. The chitosan oligosaccharide (90% deacetylated; verified by ¹HNM0052, Mw = 8.6 kDa; verified by CTO-20 AC-Shimadzu high performance liquid chromatography using refractive index detector and Ultrahydrogel 250 column, and PEG as standard) was supplied by Yuhuan Marine Biochemistry Co., Ltd., Zhejiang, China. Dialysis bag (MW cut-off 3,500 Da, cellu.sep®, USA), Dichloromethane (DCM) and other solvents were supplied from Merck, Germany.

Methods

Synthesis of camptothecin-20-Succinic acid (CPT-SUCC)

CPT-SUCC was prepared as reported by Greenwald et al. [26], and its structure was confirmed by FT-IR and ¹H NMR (fig. 2). Firstly, Succinic anhydride (25.83 mg, 0.2583 mmol) was dissolved in 50 ml anhydrous DCM at 0 °C, then DIPC (39.996 µl, 0.2583 mmol), DMAP (21.04 mg, 0.1722mmol) and CPT (30mg, 0.0861mmol) added to the solution. After two hours solution was smoothly warmed to the room temperature. Reaction continued for 18 h under constant stirring and nitrogen atmosphere condition. After precipitating of the product with 200 ml cold diethyl ether, the precipitant was dissolved in DCM and washed with 50 ml 1.0 N HCL three times. DCM fraction was dried by vacuum evaporation to achieve CPT-SUCC.

Synthesis of CPT-20-SUCC-Chitosan oligosaccharide (CPT-20-SUCC-CHO)

The triple conjugate (CPT-20-SUCC-CHO) was synthesized by conjugating the carboxylic acid group of CPT-20-SUCC to the amine group of CHO using molar ratios of 2.5% and 7.5% of CPT-20-SUCC to CHO in the presence of EDC/NHS (fig. 3). CPT-20-SUCC (5.38 mg, 0.0125mmol, 2.5%), (16.13 mg, 0.0375 mmol, 7.5%), was dissolved in

50 ml anhydrous DCM, EDC (7.16 mg, 0.0375 mmol, 2.5%), (21.4875 mg, 0.1125 mmol, 7.5%), and NHS (4.315 mg, 0.0375 mmol, 2.5%), (12.947 mg, 0.1125 mmol, 7.5%), added to the solution, under constant stirring and nitrogen atmosphere allowed to the reaction continued for 6 hours, in order to activate carboxyl group of CPT-20-SUCC-COOH. After that, poured chitosan (81 mg, 0.5 mmol) into the solution and let the reaction

continue for 72 h. To remove impurities and unreacted CPT-20-SUCC, the reaction suspension was washed by 20 ml DCM/MeOH (4:1) for three times and supernatant was discarded by centrifugation (8500 rpm, 10 min). In order to remove urea by-product of EDC, the precipitant was dialyzed in deionized water (0°C) for 3 h, precipitated polymer dried by vacuum evaporation [27].

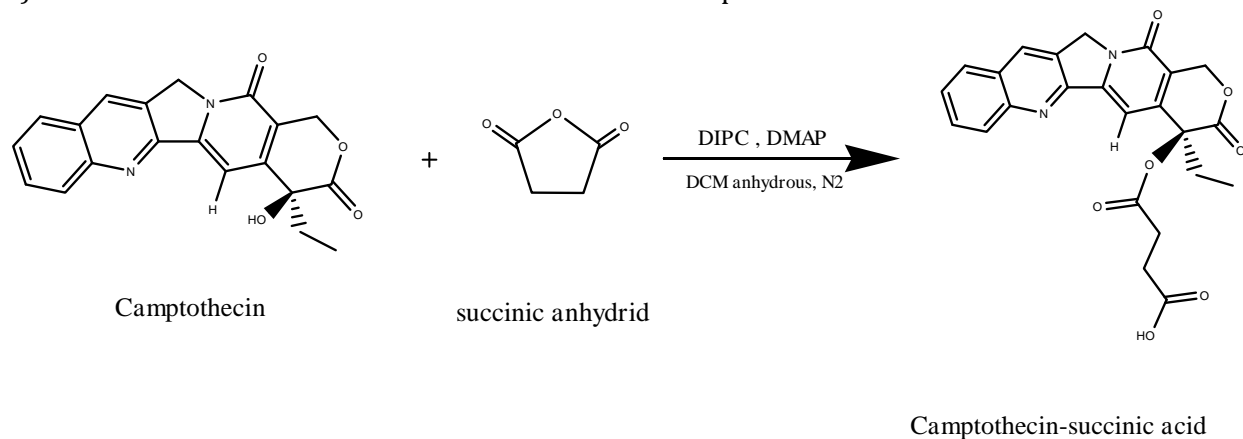


Fig. 2. Schematic diagram of Camptothecin-Succinic acid synthesis.

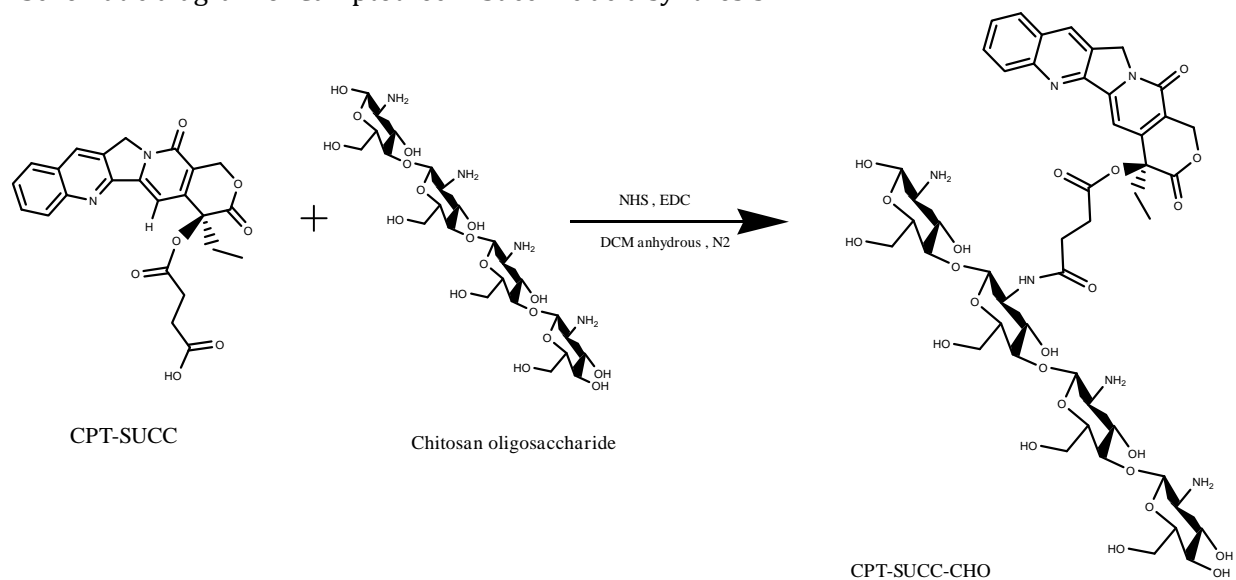


Fig. 3. Schematic diagram of Camptothecin-Succinic acid-Chitosan oligosaccharide synthesis.

FT-IR and ^1H NMR analysis

FT-IR spectra were recorded on Fourier-transform infrared spectrometer (IR prestige 21, Shimatzu, Japan) using KBr discs.

A ^1H NMR analysis was performed using Bruker Biospin (AC-400, Germany). 5 mg/mL of CPT-CHO dissolved in D_2O , and CPT dissolved in deuterated DMSO for the NMR spectrometry.

Particle size

The mean particle size of the bare-micelles and CPT-loaded polymeric micelles was determined in triplicate at 25 °C by using the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The micelles samples were diluted with distilled water [28].

Critical micelle concentration (CMC) determination

The CMC of CPT-20-SUCC-CHO 2.5% and 7.5% was determined using a spectrofluorometer with Nile Red as fluorescence probe. 1×10^{-5} M of Nile Red was prepared in methanol and a series of small vials were each added 100 μ L of the Nile Red solution. After the MeOH was evaporated, 4 mL of the CPT-20-SUCC-CHO 2.5% and 7.5% water solution with concentrations ranging from 1.22×10^{-4} mg/mL to 0.125 mg/mL was added to each vial. The vials were sonicated at room temperature for 1 h, and 30 min the solution fluorescence emission at the wavelength of 550 nm (excitation wavelength: 490 nm) of each vial was measured. The CMC was determined by the intersection of the two linear portions of the curve of the fluorescence intensity vs. the logarithmic concentration of the surfactant.

Dissociation of micelle

To study disassembly of micelle at different pHs, release of camptothecin as a function of dissociation was evaluated. For achieve this purpose, a calibration curve was drawn and release was studied as mentioned in following subsections.

Calibration curve

The fluorescent intensity of the camptothecin (excitation: 349 nm, emission: 428 nm) was measured using a fluorescent spectrophotometer (LS 55 230 V, spectrofluorophotometer, PerkinElmer, UK) to construct a standard calibration curve (Fig.6).

Releasing profile

Release of CPT from drug-linker-polymer triple conjugate micelles measured using a dialysis membrane with 3500 Da cut-off pore size, at phosphate buffer pH: 7.4 and acetate buffer pH: 5.5. Each type of measurement repeated three times. 50 ml of phosphate buffer used as medium at 37 °C under constant stirring, and 1 ml of CPT-polymer micelles placed in dialysis bag then put in the medium. All of the terms were prepared exactly same as pH: 7.4 for release in acetate buffer medium (pH: 5.5). Samples of the solution were analyzed by spectrofluorophotometer [29].

Results and Discussion

Characterization of CPT-SUCC

FT-IR analysis indicated successful synthesis of CPT-SUCC. The peak at 1157 cm^{-1} , was attributed to the C-O stretch bond of CPT which disappeared, and the intensity of the peak was attributed to the C=O stretch of CPT, carboxylic acid of SUCC and 20-OH ester at 1732 cm^{-1} which increased (Fig 4b). The intensity of the C-H stretch also increased which was related to introducing CH₂ to CPT by SUCC. ¹H-NMR spectra of CPT-SUCC showed characteristic peaks of CPT at (8.708,a), (8.176,d), (7.9,e), (7.727,b), (7.365,c), (6.79,j), (5.442,g), (5.305,f), (3.396,OH), (1.882, h), (1.133, k & l), and (0.893, i).

The peak at 6.561 ppm, related to 20-OH of CPT, almost disappeared and a new peak at 2.42 ppm was attributed to 20-OH ester which appeared, and this indicated the coupling of SUCC to CPT (Fig. 5a).

Characterization of CPT-SUCC-CHO

In the FT-IR spectra of CPT-CHO, characteristic peaks of CPT-SUCC ester was observed at 1739.79 cm^{-1} and the intensity of the amide peak (1627 cm^{-1}) increased significantly while a decrease was observed in the amine peak of CHO at 1523 cm^{-1} (Fig 4d).

The ¹H-NMR spectra of CPT-CHO showed characteristic peaks of CHO at (1.918, m), (0.936, b), (2.950, d), (3.5 to 4.0, e, f, g, h, i, j, k) and (4.442, l), while the peak at (7.912, a), and (6.773, c) ppm was attributed to the quinoline of CPT (Fig 5, b).

The degree of substitution of the drug on the polymer was calculated by $^1\text{H-NMR}$ as 1.9%.

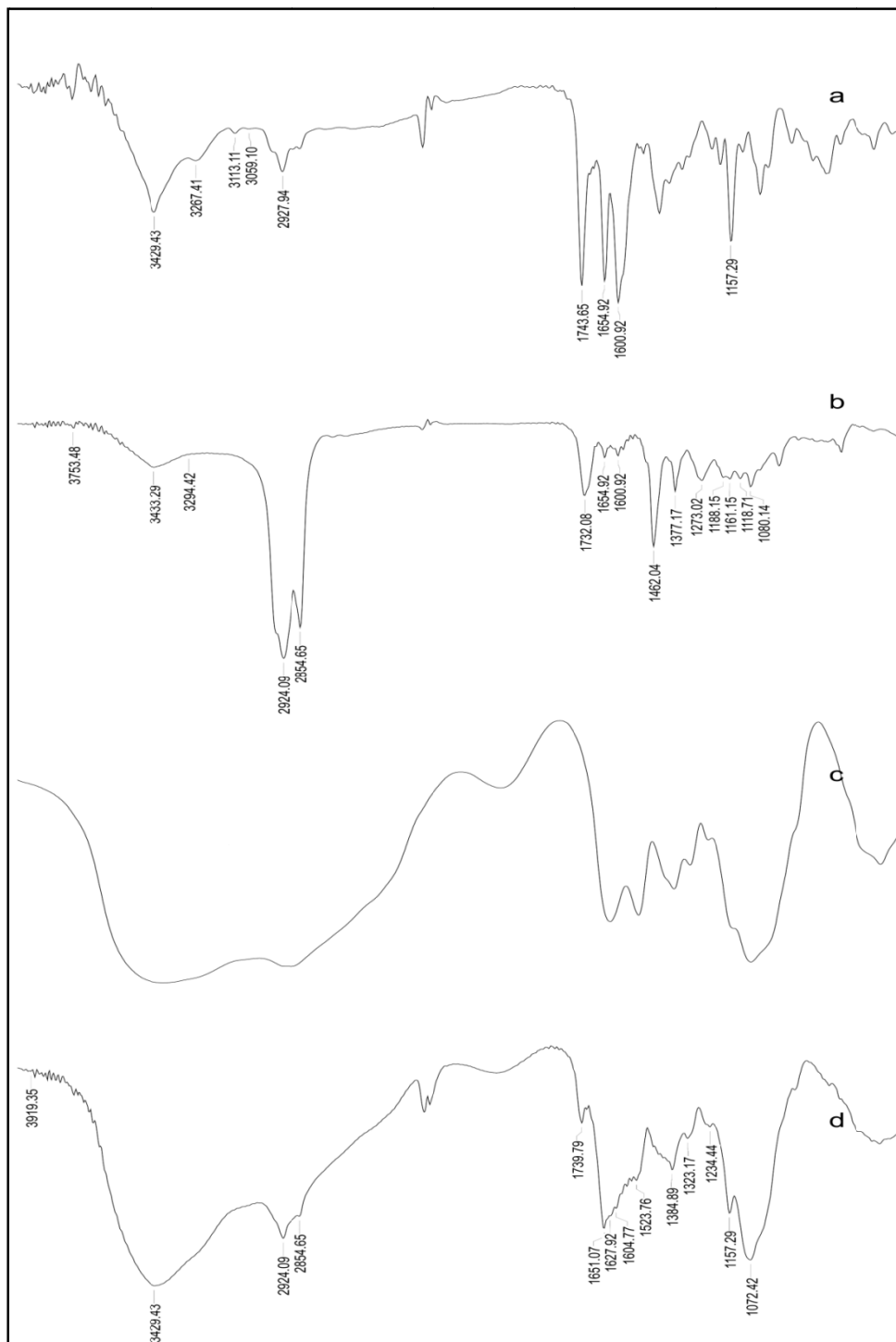


Fig. 4. The FT-IR spectrum of CPT (a), CPT-SUCC (b), CHO (c) and CPT-SUCC-CHO (d)

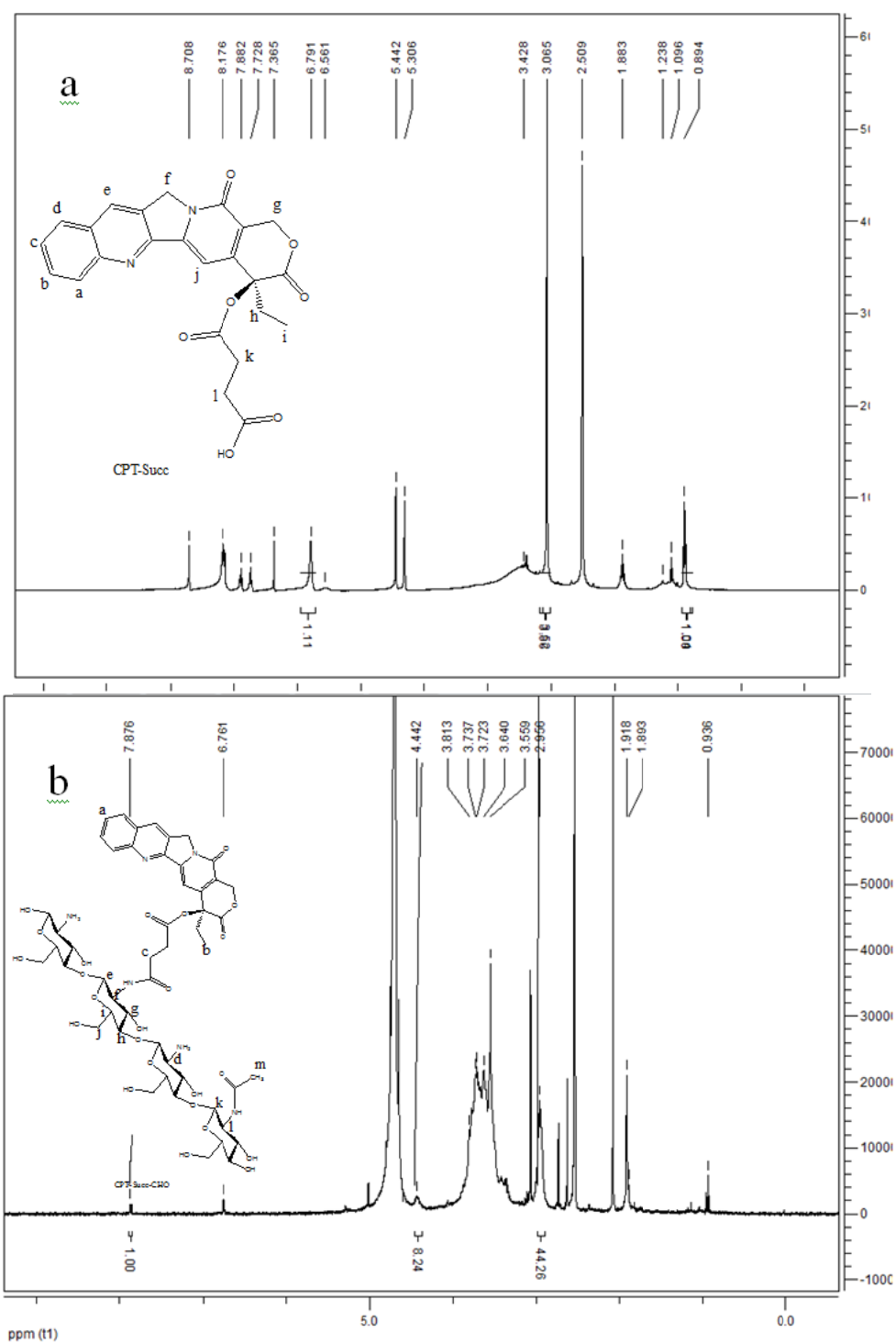


Fig. 5. the ¹H NMR spectrum of CPT-SUCC (a) and CPT-SUCC-CHO (b)

Size Determination

Particle sizes of CPT-CHO-2.5 and CPT-CHO-7.5 were 539 ± 152.15 and 197.8 ± 66 nm, respectively. These results showed that increasing the ratio of the grafting hydrophobic part of the grafted copolymer reduced the size of the micelles, which is attributed to enhanced hydrophobic interaction. In fact, with this increase in polymer hydrophobicity, the micelles formed a more tightly packed core. Our results are in agreement with results from Fattahi et al [2].

Critical micelle concentration (CMC)

In order to determine the critical micelle concentration (CMC) of the two ratios of grafted copolymer micelles (2.5%, 7.5%), fluorescence measurements were carried out using Nile red as a fluorescent probe. Nile red was used because its fluorescence spectrum is sensitive to the polarity of the environment, and in addition, its emission has no overlap with the excitation and emission spectra of camptothecin, while the spectrum of another well-known fluorescence probe, pyrene, has an overlap with the camptothecin spectrum. With an increase in grafted polymer concentrations, the total fluorescent intensity increased. The intensity of the Nile red emission spectra was used to determine the CMC of grafted copolymers in water. A plot of the intensity of the Nile red emission spectra against the logarithm of the polymer concentration is shown in Fig. 6. The CMC value can be determined at the polymer

concentration onset of the Nile red emission increase. The CMC value of the two ratios of grafted copolymer 2.5% and 7.5% were 27.2×10^{-3} g/L, and 6.1×10^{-3} g/L, respectively. This result indicates that a higher degree of substitution results in a lower CMC. The CMC of CPT-CHO micelles was in the range of the CMC of polymeric micelles which is typically in the order of 10^{-6} to 10^{-7} M, and three orders of magnitude higher than a low molecular weight surfactant [30].

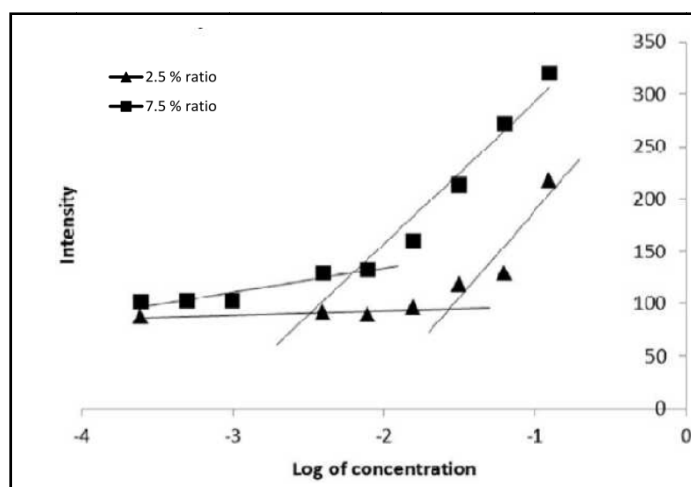


Fig. 6. Intensity of Nile red emission peaks at 520 nm versus log of concentration.

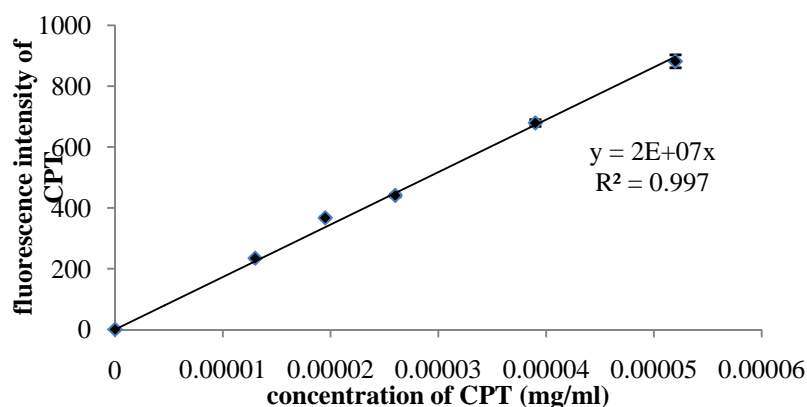


Fig. 7. Calibration curve of camptothecin solution ($y = 2E+07x$, $R^2 = 0.997$).

Linearity of calibration curve

Linearity of the method was evaluated with a five-point calibration curve, spanning a concentration range of 0.000013 to 0.000052 mg/mL of drug substance in phosphate buffered solution (pH 7.4) with 2% DMSO. Three independent determinations were performed for each concentration per day and the test was repeated in three different consecutive days. Linearity curves were plotted for this medium (Fig 7), considering mean absorbance and standard deviation (SD) for each concentration. These data indicated that the absorbance was linear over the concentration range of 0.000013 to 0.000052 mg/mL of drug substance. The correlation coefficient (R²) value for the regression line was 0.997, with a slope of 2×10^7 and y-intercept of zero. These results were considered acceptable and the linearity curves were used to calculate in vitro drug release studies.

In vitro dissociation study

Polymeric micelle drug delivery systems are advantageous because of their wide applicability in delivering hydrophobic drugs. Micelle stability, long-circulation properties, and control drug release, are critical factors for achieving highly selective delivery to tumor target sites. Although conjugation of CPT to polymers could solve water solubility, control release was not addressed as well in these systems. Conjugation of CPT is limited to esterification which is not stable in aqueous medium and can cause dissociation of micelles and premature release into the blood stream (31, 32). Our results indicated that the release of CPT from CPT-SUCC grafted chitosan micelles is very slow. As shown, only about 0.831% of the drug was released after 52 h at pH 7.4 and 37°C and 0.691% of the drug was released during 26 h at pH 5.5 and 37°C (Fig. 8). This indicates that the esterification bonds between CPT-20-OH and SUCC-COOH are stable at 37°C and reduction at pH degree (from 7.4 to 5.5) has no effect on integrity of micelles. This unexpected stability of ester bond can be related to core-shell structure of micelle. Micelle formation by CPT-CHO conjugate creates a greater hindrance for the

hydrolysis of ester bonds by reducing the water content in the core of the micelle.

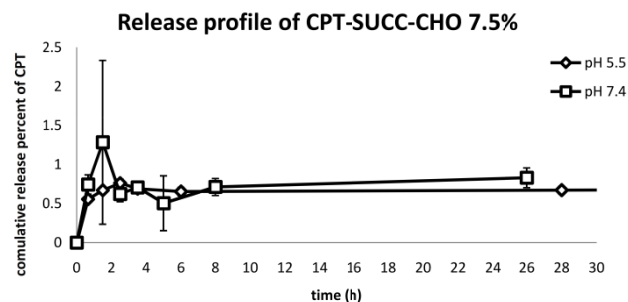


Fig. 8. Dissociation of CPT-SUCC-CHO 7.5% micelle; evaluation of release as a function of dissociation in two medium of different pHs.

In this manner, premature release by acidic hydrolysis will be reduced.

Conclusion

Camptothecin was conjugated to chitosan oligosaccharide as a novel derivative of chitosan using succinic acid as a linker. The self-aggregation process created nano-sized polymeric micelles in an aqueous medium, and the resulting micelles at the optimized conduction had CMC of 6.1×10^{-3} g/L and a size of 197.8 ± 66 nm. The dissociation study indicates that the integrity of the micelle is not affected by pH variations and almost none of the drug as hydrophobic segment of CPT-CHO co-polymer was released into the medium, which can reduce premature drug release into the blood stream. Altogether, these phenomena make CPT-CHO nano-micelle a potent drug delivery system which is capable of delivering hydrophobic drugs.

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Conflict of interest statement

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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