Synthesis of *N*-benzyl-O-carboxymethylchitosan and Application in the Solubilization Enhancement of a Poorly Water-Soluble Drug (Triamcinolone)

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ABSTRACT: *N*-Benzyl-*O*-carboxymethyl chitosan (OCChB) was synthesized through a reaction of *O*-carboxymethylchitosan (OCCh) and benzaldehyde by the reductive amination method. The chemical structures and physical properties of the derivatives were confirmed by Fourier transform infrared spectroscopy and ^{1}H-NMR. The cytotoxicity of the polymers was tested by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at concentrations ranging from 0.01 to 1000 μg/mL. The substitution degrees of the derivatives, calculated by ^{1}H-NMR, were 12 and 53% for OCChB1 and OCChB2, respectively. The results show that the derivatives were not toxic at 1000 μg/mL and could decrease the surface tension by concentration on the system surface compared with OCCh. Because of this property, OCChB was applied as a solubility enhancer for triamcinolone (TC), a poorly water-soluble drug. The polymer solutions at 1.0 mg/mL increased the TC solubility up to 3.5 times for OCChB1 and 5.0 times for OCChB2 compared with its solubility in water. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 4206–4212, 2012

Key words: biopolymers; drug delivery systems; water-soluble polymers

INTRODUCTION

Chitosan is derived from the *N*-deacetylation of chitin, the second most abundant natural polysaccharide. Chitosan is poorly soluble in neutral water and soluble in diluted organic acid; these properties result in a gelled solution under neutral physiological pH. *O*-Carboxymethylchitosan (OCCh) is a water-soluble chitosan derivative in which the OH groups are substituted by carboxymethyl groups. OCCh proven to be biocompatible[1] presents antifungal activity[2,3] and very low toxicity.[4] This polymer is used for many purposes, such as controlled release drug carriers,[5–7] carriers for hydrophobic drugs,[8] and burn dressings.[9]

In recent years, natural and biocompatible polymers have attracted considerable attention in the preparation of pharmaceutical dosage forms. Amphiphilic polymers, particularly, hydrophobically modified polysaccharides,[10,11] have been used as vehicles for poorly water-soluble drugs, such as cyclosporine, paclitaxel, vancomycin, and adriamycin, because of their biocompatibility and low toxicity. OCCh functionalized with linoleic acid has been used to encapsulate adriamycin and bromelain[7,12] and, with hexanoyl groups, to encapsulate ibuprofen.[13] Another carboxymethylated derivative of chitosan synthesized for drug incorporation was carboxymethylchitosan modified with 2-hydroxyethylmethacrylate for the controlled release of carbamazepine.[14] Physically crosslinked hydrogels were also prepared with carboxymethyl chitosan and cellulose ethers for controlled drug release.[15] Moreover, Yao et al.[16] synthesized and characterized nanoparticles with carboxymethylchitosan grafted with monomethoxypoly(ethylene glycol) and crosslinked with poly(ethylene glycol).

The purpose of this work was to synthesize OCCh hydrophobically modified with benzyl groups [*N*-benzyl-*O*-carboxymethylchitosan (OCChB)] and to evaluate its cytotoxicity and solubilizing properties with the poorly-soluble drug triamcinolone (TC) as a model drug.

EXPERIMENTAL

Materials

Chitosan (deacetylation degree = 80%, average molecular weight = 265 kD) was obtained from Puripharma, Ltd. (Brazil). Monochloroacetic acid

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and benzaldehyde were purchased from Vetec Co., (Rio de Janeiro, Brazil). TC was purchased from Galena, Ltd. (Campinas, Brazil). L929 fibroblast cells were obtained from RJCB Collection (Rio de Janeiro, Brazil). All of the other reagents were analytical grade and were used as received.

Methods

Synthesis of OCCh

OCCh was prepared by a modification of the method described by Chen and Park.[17] Briefly, 10 g of chitosan was suspended in 100 mL of a solution containing 13.5 g of sodium hydroxide, 80 mL of water, and 20 mL of 2-propanol at a controlled temperature of 5°C. This mixture was stirred for 2 h and maintained at 0°C for 24 h. Monochloroacetic acid (15 g) was dissolved in 2-propanol (25 mL) and added dropwise to the alkalized chitosan for 60 min. The reaction was conducted for 4 h at 5°C, and the suspension was maintained at 0°C for 48 h. At the end, the reaction was stopped by the addition of 70% (v/v) ethyl alcohol (200 mL). The resulting solid was filtered, washed with 70–90% (v/v) ethyl alcohol until the chloride test was negative for $AgNO_3$, and vacuum-dried at room temperature.

Synthesis of OCChB

The OCChB was prepared according to a procedure reported by Guo et al.[3] OCCh (3 g) was dispersed in H_2O (100 mL), and benzaldehyde (1.0 and 1.5 equiv for OCChB1 and OCChB2, respectively) was added under stirring at room temperature. After 24 h of stirring, the pH of the solution was adjusted to 4.5 with sodium hydroxide. An aqueous $NaBH_4$ solution (1.5 equiv of benzaldehyde) was then added, and the solution was stirred for 1.5 h. The OCChB1 and OCChB2 derivatives were precipitated with acetone and filtered. The unreacted benzaldehyde and the other inorganic products were extracted with a Soxhlet extractor with acetone and ethyl alcohol for 24 h.

Characterization of the polymers

The carboxymethylation degree of OCCh was determined by conductometric titration. The polymer (50 mg) in its acidic form was totally dispersed in 50 mL of water, and the conductometric titration was carried out with a standard solution of 0.100*M* NaOH with an Digimed model DM-31 (São Paulo, Brazil) Orion model DM-31 conductivimeter. The experiment was done in triplicate, and the values of the inflexion points corresponding to the initial and final points of neutralization of the carboxymethyl group were applied to calculate the carboxymethylation degree, which was found to be 23%.

The Fourier transform infrared (FTIR) spectra of OCChB1 and OCChB2 were obtained with a Bomem BM100 FTIR spectrometer (Quebec, Canada) with a KBr disk.

^{1}H-NMR spectra were recorded on a Bruker AC 300 spectrometer (Karlsruhe, Germany) at 25°C. The sample was dissolved in a 1.0% v/v CD_3COOD/D_2O solution to give a concentration of 20 mg/mL. The substitution the substitution degree (SD) was calculated by comparing the ratio of benzyl protons (δ 7.0 ppm) and sugar protons (δ 2.9 ppm).[7]

Evaluation of cytotoxicity

L929 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 m*M* L-glutamine, 1% nonessential amino acids, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37°C, 5% CO_2, and 95% relative humidity. The cytotoxicity values of OCCh and OCChB2 were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay at different concentrations of polymers ranging from 0.01 to 1000 μg/mL. The polymers were dispersed in DMEM and sterilized by filtration (0.22 μm). L929 cells were seeded in a 96-well plate at a concentration of 20,000 cells/well and incubated for 24 h. Afterward, the cell culture medium was replaced by the medium containing the polymer solution without FBS. The cells were incubated for 24 h at 37°C, and the medium was replaced by DMEM without FBS containing 0.5 mg/mL of MTT. After 4 h at 37°C in the dark, the medium was removed, and 200 μL/well dimethyl sulfoxide was added. The measurement was performed with an Enzyme-Linked Immunoabsorbent Assay (ELISA) plate reader at 570 nm. The positive control (PC) group test was performed in dimethyl sulfoxide, and the negative control (NC) group test was performed in the culture medium. The experiments were performed in octaplicate, and the results were evaluated by student's *t* test/analysis of variance (ANOVA) with $p < 0.05$.[18]

Drug solubilization

The polymer stock was prepared by the dissolution of 1.0 g of the polymer (OCCh, OCChB1, and OCChB2) in 100 mL of water. The pH was adjusted to 8.5 with a 1*M* hydrochloric acid solution. The solutions were stirred at room temperature for 24 h. Before the experiments, they were diluted to obtain samples with concentrations ranging from 0.1 to 10 mg/mL. The polymer solution (5 mL) was stirred for 24 h at room temperature with 10 mg of TC. The remaining TC in the solution was removed by centrifugation. The amount of solubilized drug was determined by UV spectroscopy (260 nm) with an ultraviolet–visible model DB1201 spectrophotometer, (Chine).

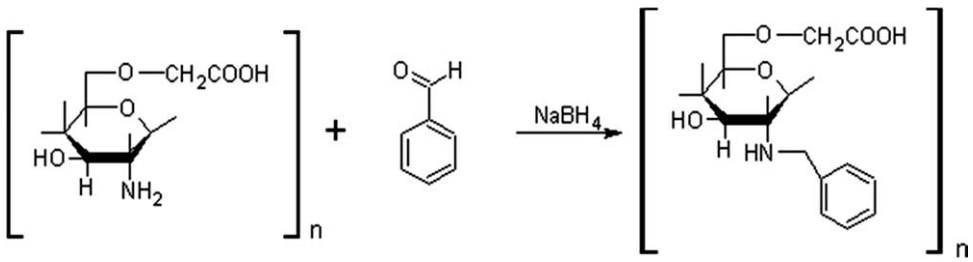

Figure 1 Synthetic route of OCChB.

Surface tension measurements

The interfacial behavior of the polymer solution was studied as a function of the polymer concentration. The surface tension was measured with a Dataphysics model OCA 15 Plus tensiometer (Filderstadt, Germany). The surface tensions were recorded and analyzed with SCA20 software (Filderstadt, Germany). The recorded surface tensions were determined as the mean values of at least three measurements.

Dialysis study

Three dialysis bags prepared from dialysis tubing (molecular weight cutoff (MWC) = 12.000–14.000 and diameter = 2.4 cm) were filled with the following contents: 10 mL of an aqueous solution containing 25 mg of OCChB2 and 10 mg of TC, 10 mL of an aqueous solution containing 25 mg of OCCh and 4 mg of TC, and 4 mg of TC in 10 mL of water at pH 8.5. The bags were individually placed in a beaker containing 250 mL of phosphate buffered saline (PBS; pH 7.4, 0.01*M*). A constant temperature of 37.5°C was maintained, and the receptor medium was constantly stirred to maintain sink conditions. At appropriate time intervals, samples (3 mL each) were taken from the receiver solution and analyzed by UV spectroscopy (260 nm) to determine the amount of TC released through the membrane. Fresh PBS solution (3 mL) was added to replace the sample that was removed to maintain a constant volume. The release experiments were performed in triplicate.

Thermal analysis

Differential scanning calorimetry (DSC) analysis was carried out in a Netzsch STA 449 F3 Jupiter thermal analyzer (Bavária de Selb, Germany) with alumina crucibles with lids containing about 10 mg of sample (pure TC, the OCChB2/TC physical mixture, and the OCChB2/TC films) under a dynamic nitrogen atmosphere (50 mL/min) heated from 25 to 400°C at 10°C/min. The DSC cell was calibrated with indium (mp = 156°C) and zinc (mp = 419.4°C) standards.

RESULTS AND DISCUSSION

Synthesis and characterization of the OCChB derivatives

The OCChB derivatives were synthesized by reductive amination of OCCh with benzaldehyde with sodium borohydride as a reducing agent, as illustrated in Figure 1. In summary, the procedure involved the reaction of benzaldehyde with the amine function of OCCh in neutral solution to form OCCh iminium, followed by its reduction with sodium borohydride.

As shown in Figure 2, the FTIR spectra of OCCh, OCChB1, and OCChB2 showed peaks assigned to the chitosan: 3455 cm^{-1} (O—H stretching), 2860 cm^{-1} (C—H stretching), and 1500 cm^{-1} (N—H bending). We also observed peaks attributed to the carboxymethyl group: 1075 cm^{-1} (C—O stretching) and a broad peak at 1610 cm^{-1}. The peak at 1610 cm^{-1} was explained by the overlapping of the peaks of NH_2 at 1710 cm^{-1} and COONa near 1600 cm^{-1}.[19] The amphiphilic derivatives presented peaks at about 1570, 1500, 1450, and 750 cm^{-1}, corresponding to the benzyl groups.[20]

Figure 3 shows the ^{1}H-NMR spectrum of OCChB1. The chemical shifts at 1.9 and 2.9 ppm were probably due to the protons of the acetyl group and C-2 position (H-2), respectively. In addition,

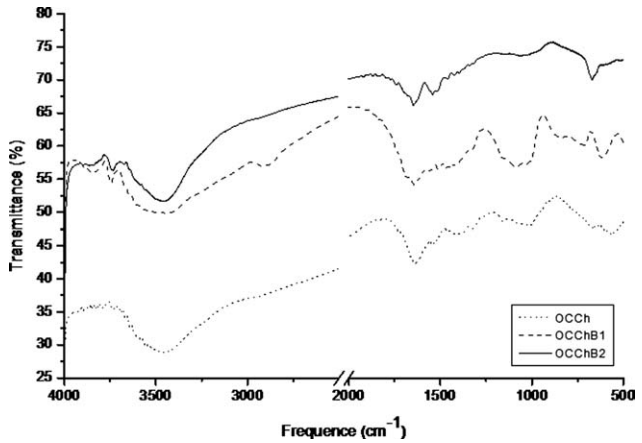

Figure 2 FTIR spectra of OCCh, OCChB1, and OCChB2.

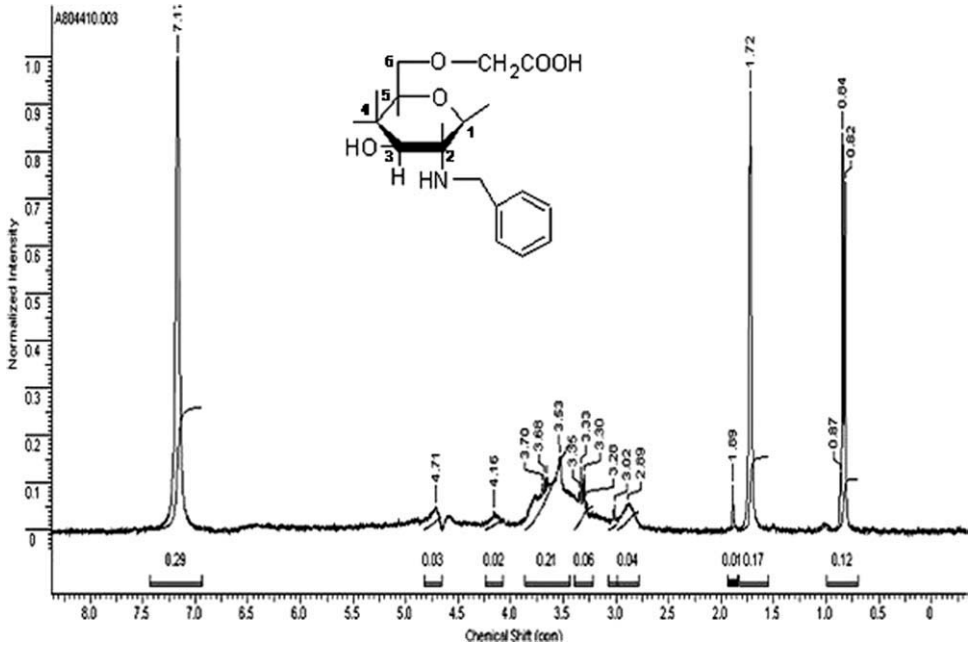

Figure 3 Typical ^{1}H-NMR spectrum of OCChB.

ring protons (H-3 to H-6) are considered to be reasonable at 3.6–4.0 ppm. The chemical shift at 4.2 ppm was assigned to the proton in the —OCH_2COONa group and the sinal at chemical shift at 7.1 ppm was assigned to the proton of the benzyl group. The degree of benzyl substitution was determined by eq. (1):

$$SD = \{[\text{Integrated area at 7.1 ppm(benzyl group)}] / [\text{Integrated area at 2.9 ppm}[H-2]\} \times 1/5 \quad (1)$$

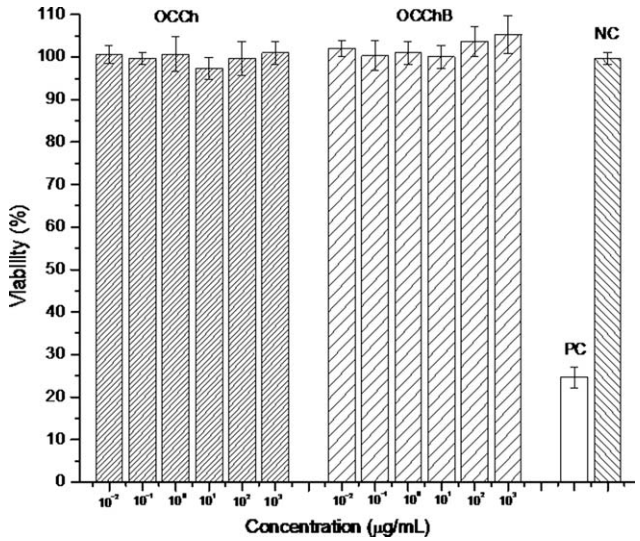

Figure 4 Cytotoxicity of OCCh and OCChB2 measured by MTT assay. Each column represents the mean plus or minus the standard deviation of eight experiments.

The SDs of OCChB1 and OCChB2 were 12 and 53%, respectively.

Evaluation of the cytotoxicity

The MTT assay is based on the cleavage of a yellow tetrazolium salt to insoluble purple formazan crystals by the mitochondrial dehydrogenase of viable cells. The percentage of viability is calculated with consideration of the absorbance of the cells treated with sample and the absorbance of the untreated cells (NC). Figure 4 shows the cytotoxicity values of OCCh and OCChB2 at the tested concentrations, as determined by MTT assay. It was observed that the polymers were not toxic at the evaluated

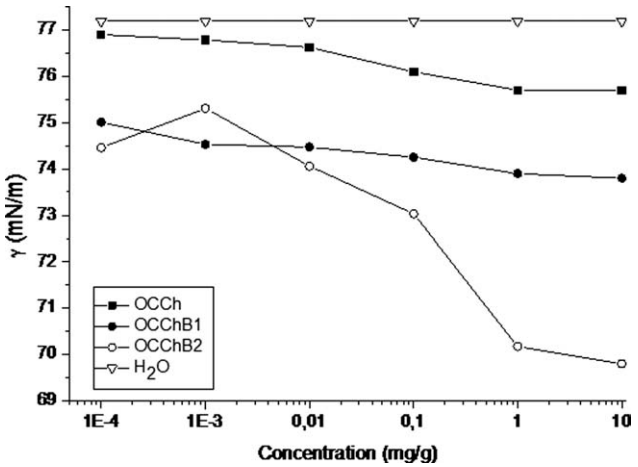

Figure 5 Equilibrium surface tension of water, OCCh, OCChB1, and OCChB2 versus the polymer concentration.

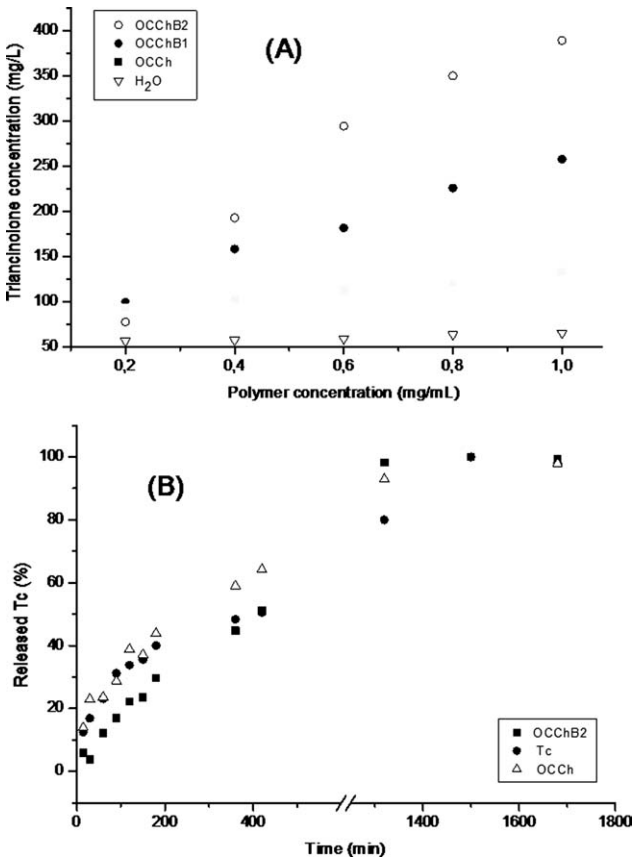

Figure 6 (A) Apparent solubility of TC in water, OCCh, OCChB1, and OCChB2 solutions versus the polymer concentration. (B) Release behavior of TC from the phosphate buffer (pH 7.4), OCCh/phosphate solution, and OCChB2: phosphate solution. The receiver solution was phosphate buffer (pH 7.4) at 37°C.

concentrations ($p < 0.05$). Previous works have reported the biocompatible characteristic of OCCh,[21,22] and the results of our MTT assay suggest that the benzyl group had no influence in this regard.

Surface tension

The amphiphilic characteristics of OCChB1 and OCChB2 were evaluated by tensiometric studies in aqueous solution. The surface tension of the hydrophobically modified polymer solution was measured as a function of the polymer concentration. The results are presented in Figure 5, which shows that OCCh had few surface activity properties, as compared with the results obtained by Zhu et al.[19] for OCCh with a carboxymethylation degree of 100%. After being modified with the benzyl group, the resulting derivatives were converted to an amphiphilic polymer by the introduction of a hydrophobic group. They adsorbed on the surface with the hydrophilic backbone in the solution, whereas the hydrophobic groups pointed upward toward the air to reduce the water surface tension. Figure 5 also shows that with increasing SD of the hydrophobic groups, the surface tension decreased at the same concentration as the derivative, as there were more hydrophobic groups on the surface.

Solubilization of TC

The solubility profile of TC is reported in Figure 6(A), which demonstrates the influence of SD of OCChB on its solubilizing proprieties. The interaction of the drug

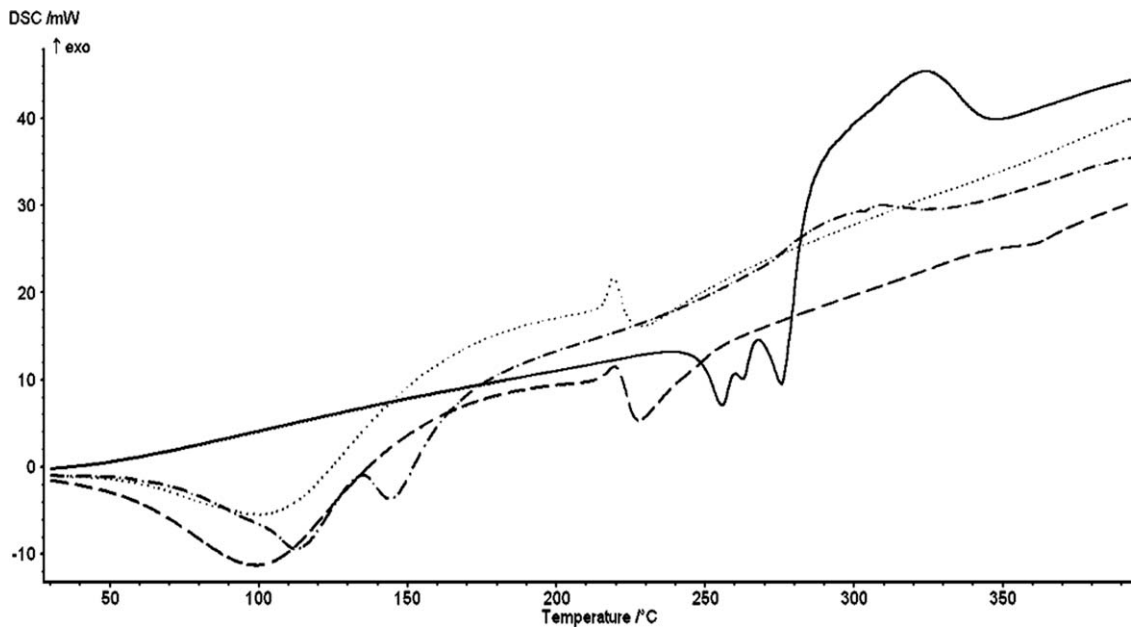

Figure 7 DSC thermoanalysis of the (—) pure TC, (. . .) OCChB2 film, (- - - -) OCChB2/TC physical mixture, and (-.-.-) OCChB2/TC film.

with the polymer enabled the apparent solubility of TC to increase. The curve was nonlinear at high polymer concentration; this demonstrated the limited solubilizing properties of the polymer. Although the solubility of TC in the OCCh solution was equivalent to that of water (65 mg/L), it was significantly enhanced in the amphiphilic polymer solutions. Increases in the solubility were observed up to 3.5 and 5.0 times for OCChB1 and OCChB2, respectively, when the concentration of both polymers was 1.0 mg/mL.

The difference in the solubility of TC in polymer solution was in agreement accordance with the results obtained from the surface tension experiment. The higher solubility of TC could have been directly related to the polymer SD. This behavior for the OCChB1 and OCChB2 solutions may have been due to the enhancement of the surface area and the highly dispersed state of the drug. These findings were in agreement with previous reports on the solubilization of docetaxel by hydrophobically modified polysaccharide,[23] amphotericin B by polymeric micelles,[24] and cyclosporine A by *N*-trimethylchitosan.[25]

Release of TC across the membrane

The effect of the solubilization of TC on the release profile was evaluated with the dialysis method. A membrane that was permeable to TC but impermeable to the polymers was used as the barrier. The rate of release across the membrane was carried out in 0.01*M* PBS (pH 7.4), as shown in Figure 6(B). In the presence of OCChB2 (2.5 mg/g), 100% of the TC was released across the membrane in 24 h; in the presence of OCCh (2.5 mg/g), 90% of the TC was released; and in the absence of polymer, 75% of the TC was released during the same period. In general, the release rate of a drug incorporated into a hydrophobic domain depends on the solubility and diffusivity of the drug. The increase in the dissolution rate of TC incorporated into OCCh and OCChB2 was probably attributable to the following factors: (1) a reduction in the drug particle size to molecular level, (2) the solubilizing effect on the drug by the water-soluble carrier, and (3) the enhanced wettability and dispersability of the drug by the carrier material.[26] A similar result was also reported for cyclosporine A solubilized *N*-trimethylchitosan.[25]

DSC

The DSC thermograms of the curves of pure TC, the physical mixture OCChB2/TC, OCChB2, and OCChB2 film containing TC are shown in Figure 7. The DSC curve of pure TC showed three endothermic peaks characteristic of polymorph B.[27] The first occurred at 254.4°C, change in enthalpy (ΔH), ($\Delta H = -15.4$ J/g) and was due to the characteristic polymorphic transition to form A. This was immediately followed by two more peaks at 262.6°C (ΔH = −3.5 J/g) and 275°C ($\Delta H = -68.7$ J/g), which may have been due to the melting of TC.[28]

The DSC thermograms of the physical mixture showed a melting endothermic peak range of about 220–250°C ($\Delta H = -73.4$ J/g), corresponding to TC in the heating phase. This behavior was recently reported for the physical mixture of *N*-octyl-*N*,*O*-carboxymethylchitosan and paclitaxel.[8]

The DSC thermograms of the OCChB2 film containing TC indicated the absence of a melting endothermic peak corresponding to TC. The absence of the endothermic peak of TC in the OCChB2 films clearly demonstrated the formation of a solid solution within the polymer matrix. Therefore, the absence of the TC melting peak in the thermogram of the film may have been due either to the drug being in an amorphous solid solution or merely to the solubilization of the crystalline drug in the melted OCChB2 during the heating cycle of the DSC study. Similar results were obtained for chitosan films containing salicylic acid[29] and an indomethacin–chitosan composite.[30]

The OCChB2 film and the physical mixture OCChB2/TC showed a broad transition at 100°C and an endothermic peak at 228°C. In the case of the OCChB2/TC film, the transition occurred at 118°C, and a new endothermic peak appeared at 144°C.

CONCLUSIONS

In this study, a new amphiphilic derivative, OCChB, was successfully synthesized through reductive amination with a carboxymethylation degree of 23% and SDs of 12 and 53% for OCChB1 and OCChB2, respectively. The polymers OCCh and OCChB2 were not cytotoxic between 0.01 and 1000 μg/mL for L929 cells. The presence of the aromatic groups in the carboxymethylated derivatives decreased the surface tension of water and enhanced the solubility of TC in aqueous solution at pH 8.5. Finally, the dialysis experiments showed the release of a higher amount of TC in the presence of the polymers. The results indicate that the amphiphilic derivatives could act as solubility enhancers for hydrophobic drugs such as TC.

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