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Synthesis of O,O'-dipalmitoyl chitosan and its amphiphilic properties and capability of cholesterol absorption

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Abstract

The regioselective synthesis of an amphiphilic chitosan derivative, O,O'-dipalmitoyl chitosan (DPCT) was achieved by the selective protection of amino groups in $MeSO_3H$. The amphiphilic behavior and miscibility with cholesterol of DPCT were studied through Langmuir–Blodgett (LB) technique. The condensed monolayer formed at water–air interface had a collapse pressure of 40 mN m^{-1} and could be transferred onto various substrates by Langmuir–Blodgett technique. Furthermore, this amphiphilic chitosan with free amino groups was cross-linked with glutaraldehyde. The adsorption behavior of the cross-linked amphiphilic chitosan to cholesterol was also studied. The results showed that this cross-linked amphiphilic chitosan could effectively adsorb cholesterol both in polar and in non-polar solvents.

Keywords: O,O'-diacrylchitosan; Amphiphilic property; Langmuir–Blodgett technique; Cholesterol absorption

1. Introduction

Chitosan is a natural aminopolysaccharide obtained by hydrolysis of chitin, which is the second most plentiful natural biopolymer. Chitosan and chitin are currently receiving a great deal of interest for medical and pharmaceutical applications (Jung, Kim, Choi, Lee, & Kim, 1999; Khor, 2001; Kurita, 2003; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). For example, chitosan is a high antimicrobial and biocompatible natural material, which has an analogous structure to human body constituents. Moreover, it can be metabolized by certain human enzymes, especially lysozyme, and is considered to be biodegradable. Chitosan and its derivatives have also attracted much attention to use as cholesterol lowering materials (Kim & Chun, 1999) introduced chitosan and its derivative as a bile salt absorbent, which was believed to be effective to reduce cholesterol level. Chiu and his co-workers (Chiu, Chung, Giridhar, & Wu, 2004) have immobilized β-cyclodextrin in chitosan beads for separation of cholesterol from egg yolk. However, till now, almost all the efforts on lowering cholesterol by chitosan and its derivatives are indirect, because chitosan is insoluble in most common solvents and has little adsorption to cholesterol.

On the other hand, certain naturally occurring amphiphilic polysaccarides have been found to remarkably enhance not only the stability, but also the cell specificity of liposomes (Sunamoto, Sato, & Hamazakj, 1992). Similar to many other structural modifications of chitosan, introduction of hydrophobic chain into chitosan significantly improves the solubility of chitosan (Hirano, Yamaguchi, & Kamiya, 2002). Chitosan amphiphiles have possessed increasing interest for its potential use as a matrix for drug delivery system (Martin et al., 2002; Noble, Gray, Sadiq, & Uchegbu, 1999) and as a matrix for hydrophobic interaction chromatography (Wang, Guo, & Jiang, 2002). In addition, Langmuir–Blodgett (LB) films from amphiphilic chitosan have been prepared for mimicing biological membranes (Li, Xin, & Miyashita, 2002).

Although attempts have been made to prepare amphiphilic polysaccharide derivatives from chitosan, only limited work has been done with selective modification, especially selective O-derivation of chitosan (Goosen, 1999; Kohgo,

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Ishii, Nishimura, & Kurita, 1992). In this work, DPCT with free amine group was prepared from chitosan and palmitoyl chloride. Amphiphilic assembly and miscibility properties of this chitosan derivative were also investigated through Langumir–Blodgett trough. Finally, based on its amphiphilic properties, it was cross-linked with glutaraldehyde and directly used as cholesterol absorbent.

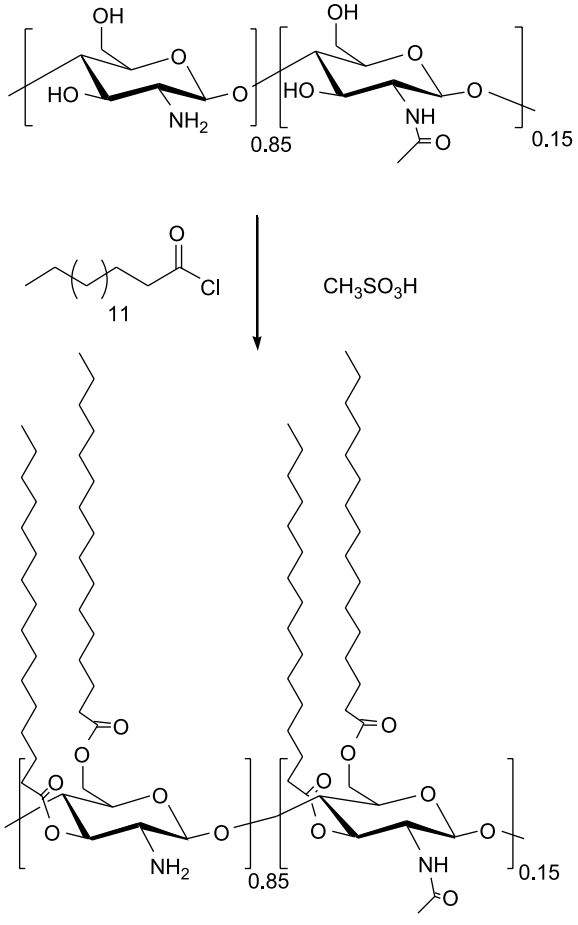
2. Experiments

2.1. Materials

Glutaric dialdehyde (50 wt% solution in water), methanesulfonic acid ($MeSO_3H$, 99.5%) and chitosan (with a degree of deacetylation of 85%) were supplied by Aldrich Chemical Co., Inc. Palmitoyl chloride was purchased from Merck (Darmstadt, Germany). All other commercial chemicals were used as received.

2.2. Preparation of O,O'-dipalmitoyl chitosan (DPCT)

The synthetic route leading to DPCT is shown in Scheme 1. The typical synthesis procedure for DPCT is as follows: 2.56 g of palmitoyl chloride was added dropwise to the mixture of 1 g of chitosan in 25 ml $MeSO_3H$ over 30 min with vigorous stirring. The resulting mixture was continued to stir for overnight, and then the mixture was poured into ice water. The suspension was centrifuged and $MeSO_3H$ was removed. The product was washed with water thoroughly, followed by saturated sodium bicarbonate and water. The crude product was collected by filtration, and then washed with water and methanol. The crude product was dissolved in chloroform as little as possible and precipitate in methanol. The solid was filtered and then extensively washed with water followed by extracting with methanol in Soxhelt apparatus overnight. Finally, it was dried in vacuum oven at room temperature and collected as a pale white solid. ^{1}H NMR ($CHCl_3$): 0.90 (*t*, 3H), 1.10–1.45 (*m*, 27.6H), 1.61 (*s*, 2.6H), 1.8–2.1 (*br*, 0.3H), 2.35 (*m*, 2.6H), 2.7–2.9 (*br*, 0.5H), and 3.2–5.2 (*m*, 4.3H) ppm. The degree of substitution (DS) of each functional group was estimated by ^{1}H NMR spectra. DS (NHAc) was estimated from δ 1.9–2.1 vs. 3.2–5.2. DS (NHCOR + OCOR) was estimated from δ 0.90 (Me) vs. 2.7–5.2. DS (NH_2) was estimated from δ 2.7–2.9 vs. 3.2–5.2. DS (NHCOR) = 1 − DS (NH_2) − DS (NHAc)/3; DS (OCOR) = DS (NHCOR + OCOR) − DS (NHCOR). Palmitoyl chain: chitosan repeating unit = 1.7:1, free amino: 83%. The molecular weight and polydispersity of DPCT was estimated by GPC test in $CHCl_3$: M_w 3799; M_n 2611; M_z 5684 and PD 1.43.



Scheme 1. Synthesis of O,O'-dipalmitoyl chitosan.

2.3. Preparation of cross-linked O,O'-dipalmitoyl chitosan (CDPCT)

One gram DPCT was dissolved in 20 ml THF and 4 ml of 50% glutaraldehyde solution were added. This solution was stirred overnight at room temperature. The solid was filtered and then extensively washed with water and methanol. The resulting polymer was dried, ground and passed through a 200-mesh sieve.

2.4. Formation and transfer of monolayers

DPCT was dissolved in $CHCl_3$ with a concentration of around 1 mg/ml and was dispersed on the aqueous subphase surface held in a NIMA 632D1D2 LB trough to get the isotherms for investigating spreading behavior or deposition condition. Purified water from a Milli-Q II-Millipore system was used as the subphase and surface pressure of the ultrapure water was around 72.8 mN m^{-1}. After spreading solution on the subphase and the evaporation of the solvent, the surface pressure (π)—area (A) isotherms were measured at the room temperature. The films were compressed at a rate of 10–100 cm^2/min up to the selected target deposition pressures based on the individual isotherm. The transfer of the monolayer onto the substrates was carried out through the air/water interface at a rate of 5 mm/min by vertical dipping. The amount of spreading solution was used in

the range of 10–100 μl. The isotherms were reproducible within an error of ± 1.5 Å^2 mol^{-1}. Each isotherm was obtained by averaging three runs.

2.5. Measurement of absorption amount

To a required mass of CDPCT (10–40 mg), a solution of cholesterol in hexane or a THF–water mixture (3:2 v/v) was added, and the vial was sealed and shaken overnight at room temperature to allow the equilibrium absorbing to be established. Polymer suspensions were rapidly transferred and filtered directly into HPLC vials to remove all polymer particles. The amount of cholesterol present in the supernatant was then quantified by HPLC and the amount of cholesterol uptake by the polymer beads was thus calculated. HPLC analyses were performed on a Water 2690 system using a Luna silica column (250×4.6 mm) in conjunction with acetonitrile as mobile phase (with a flow rate of 1 ml/min) and a Water 996 UV detector. The data reported here were the average of two replicate experiments.

2.6. Characterization

Fourier transform infrared (FTIR) spectra (transmission) were measured on a Perkin–Elmer FTIR spectrophotometer 2000 in the range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1}. FTIR measurements (reflection) were conducted with a Bruker EQ-55 FTIR spectrometer with *p*-polarized light and grazing incident reflection (incident angle: 85°). ^{1}H NMR spectra were recorded on a Bruker DRX-400 spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard. GPC measurements were performed on a Waters GPC system containing Waters 2690 separation module and Waters 2487 UV detector. Polystyrene samples were used as a calibration standard and the mobile phase used was THF.

3. Results and discussion

3.1. Selective *O*,*O*′-acylation of chitosan

Chitosan can be thoroughly acylated with long chain aliphatic carboxylic acid chlorides such as hexanoyl, dedecanoyl, and tetradecanoyl chlorides in pyridine/chloroform to give derivatives with a degree of acylation of 4 (Fujii, Kumagai, & Noda, 1980). Under similar conditions, the lauroylation of chitosan with various deacetylation degrees was also examined (Grant, Blair, & McKay, 1990). It is of great interest and importance that selective acylation can be achieved either for hydroxyl or for amine groups in chitosan, especially leaving free amine groups for further modification. The challenge for the latter lies in the higher reactivity of amine group than hydroxyl group. For the selective *O*-acylation of chitosan, the preparation of *O*,*O*′-didecanoylchitosan was reported through protected *N*-phthaloylchitosan as an intermediate (Nishimura et al., 1992). However, this method needs several steps for the protection and deprotection of *N*-phthaloyl groups. Recently Sashiwa et al. (Sashiwa et al., 2002) have reported a novel one-pot synthesis method for the *O*-acylation of chitosan in $MeSO_3H$. It was believed that the formation of salt between primary amino group and $MeSO_3H$ provided the protecting effect of amino group. Following a similar strategy, we obtained an *O*,*O*′-dipalmitoyl chitosan with a degree of acylation 1.7 and the protecting degree of NH_2 of 83%.

3.2. Monolayer behavior of DPCT

The Langmuir–Blodgett (LB) technique is known as one of the best ways to study molecular amphipathy and prepare ultrathin films with well-defined molecular orientation (Petty, 1996). The introduction of palmitoyl groups into hydrophiphlic backbone remarkably improves the solubility of chitosan and makes it possible to examine its capacities to form LB monolayer. Therefore, the DPCT amphiphile in chloroform solution was spread onto a water surface to measure surface pressure—area isotherm. Fig. 1 shows that the isotherms have a steep increase in surface pressure and a high collapse pressure of about 40 mN m^{-1}, which indicates that condensed monolayer has formed on the water surface. Surface pressure–area isotherms of DPCT extrapolate to molecular areas of approximately 55 Å^2 per repeating unit at the collapse pressure (ca. 40 mN m^{-1}). This suggests that the long acyl chains may align along the molecular axis with a slight increase in diameter. This value is consistent with the size of the glucosamine unit, suggesting the formation of a monolayer with highly condensed packing.

Monolayer behavior can be demonstrated with the film balance by comparing the surface pressure versus area per molecule curves for different initial surface concentrations on the fully expanded surface. If the insoluble surface material forms a true monolayer, the surface pressure versus area per molecule curves should be superimposable at

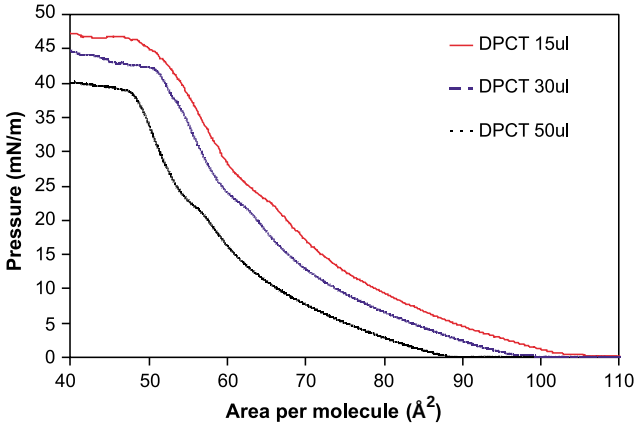


Fig. 1. Surface pressure–area isotherm of monolayers of DPCT with different concentrations.

different loading concentrations. By contrast, systems that form small insoluble floating aggregates, so-called expanded liquid structures or 'lakes', will demonstrate pressure–molecular area curves that vary with concentration. The more surface-loaded samples should give curves shifted to smaller surface area per molecule because a substantial fraction of the molecules are not in contact with the surface and have no effect on the surface energy. Fig. 1 demonstrates the surface concentration dependence of DPCT monolayers by surface pressure versus molecular areas isotherms. The position in area per molecule of first rise decreases as the loading increase. This suggests that the spreading behaviour of DPCT is a group phenomenon.

Two-component monolayers were also prepared from DPCT and cholesterol by Langmuir method. When there are two major components, the question of miscibility arises in the same sort of way as with bulk liquids and solids. The collapse pressure for mixed monolayer is often a useful guide to miscibility. It is assumed that a true mixture would give only a single collapse, probably at a pressure different from that of either component. As shown in Fig. 2, all binary monolayers have a single and well-defined collapse pressure, which is different from that of an individual component, indicating the miscible property. We also investigated the miscibility of mixed monolayers by the deviation of the area per molecule. The excess area per molecule of the mixture, A_E at constant surface pressure can be evaluated by following equation (Gzyl & Paluch, 2001)

$$A_E = A_{12} - (x_1A_1 + x_2A_2) \quad (1)$$

where A_1 and A_2 are here the area per molecule of each of component (A_1-CHOL and A_2-DPCT) at a constant surface pressure (π), x_1 and x_2 are their corresponding mole fraction ($X_1+X_2=1$, X_1-CHOL and X_2-DPCT) and A_{12} is the mean area of mixed film at the same π. For a mixed monolayer, if A_E deviates from zero, either positive or negative, it indicates the miscibility and a non-ideal mixing behavior. Further from Fig. 3, the excess area per molecule of

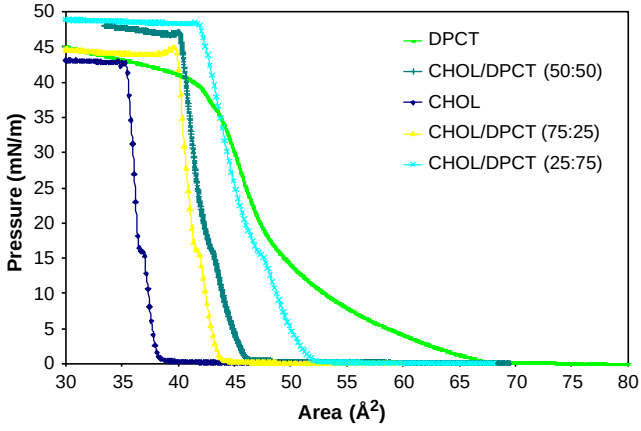


Fig. 2. Surface pressure–area isotherm of binary monolayers of DPCT/cholesterol mixtures.

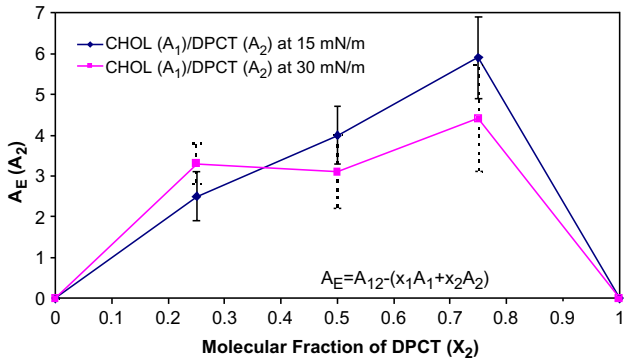


Fig. 3. Plot of the deviation of the area per molecule for binary monolayers.

the mixture, A_E at constant surface pressure depends on the compress pressure. This means that the miscibility is affected by compress conditions.

Optimum conditions of preparing Langmuir–Blodgett film were mostly determined by surface pressure–area isotherms. It was found that the proper surface pressure of the film deposition was about 32 mN m^{-1} for DPCT.

We also succeeded in the transformation of DPCT monolayers to different substrates by conventional vertical dipping at a rate of 5 mm/min. The molecular structure and coverage of DPCT monolayer on gold substrate were confirmed by grazing angle FTIR (Fig. 4).

3.3. Adsorption properties of CDPCT

The extent of adsorption of cholesterol as a function of the polymer concentration at room temperature is shown in Fig. 5. The amount of cholesterol adsorbed on cross-linked DPCT increased with increasing the polymer bead concentration both in polar and in non-polar solution. As little as 20 mg/ml of CDPCT was sufficient to adsorb 33 or 29% of CHOL initially presented in hexane and water–THF (2/3 v/v) solution, respectively.

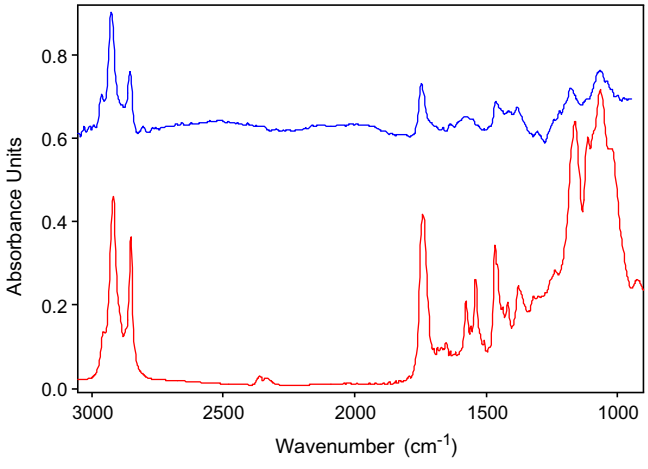


Fig. 4. Comparison of FT-IR spectra of DPCT (top for monolayer and bottom for thin film).

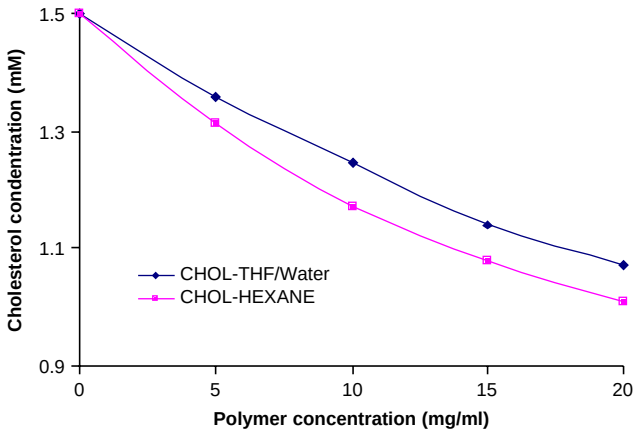


Fig. 5. Batch binding experiments: the concentration of cholesterol measured in the supernatant at equilibrium as a function of the mass of added polymer of cholesterol in hexane or a THF–water mixture (3:2 v/v).

We have examined that chitosan and chitin had no absorption to cholesterol in hexane or water–THF (2/3 v/v) solution. The introduction of hydrophobic chain into hydrophilic chitosan backbone resulted in the high absorbing capability for cholesterol at different solvents. As little as 20 mg/ml of CDPCT was sufficient to adsorb almost one-third of the cholesterol initially presented. This result implies that hydrophobic interaction between CDPCT and cholesterol plays the key role for the absorption of cholesterol. We also consider the degree of acylation in chitosan. However, low degree of acylation leads to poor absorption of cholesterol, while high degree of acylation leaves small fraction of free amino groups and makes the polymer hard to be crosslinked. In our experiment, the degree of acylation 1.7 is considered to be the proper one.

4. Conclusions

O,*O*′-dipalmitoyl chitosan has been successfully synthesized by introducing long hydrophobic alkyl chains and protecting amine groups in $MeSO_3H$. The amphiphilic polymer has good compatibility with cholesterol and the free amine groups can be used as functional group for further modification and/or cross-linking. The monolayers of DPCT have been prepared and transferred onto a solid support using the LB technique. Cross-linked *O*,*O*′-dipalmitoyl chitosan polymer has been further developed and demonstrated that it could be directly used as a cholesterol acceptor both in polar or in non-polar solvents. The adsorption of cholesterol to CDPCT may mainly depend on the hydrophobic interaction. The present study also suggests the potential application of CDPCT adsorbents to remove cholesterol in food industry.

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