ONE POT PREPARATION OF CHITOSAN NANOSCAFFOLD AND ITS EFFECTIVE FUNCTIONALIZATION WITH SUGAR FOR SOFT AND COTTON-LIKE MATERIAL

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Abstract

Chitosan nanoscaffold is obtained directly from chitin whiskers via one pot preparation.[1] The structural analysis by a nuclear magnetic resonance (^{1}H NMR) confirms that the alkaline treatment changes chitin whisker to chitosan nanoscaffold with as high as 98% degree of deacetylation. The micrographs from a scanning electron microscope (SEM) and a transmission electron microscope (TEM) confirm that the short fiber of chitin whiskers develop itself to be a network in nano-scale of chitosan or chitosan nanoscaffold. The increases in surface area, pore volume, and pore size as studied by the Brunauer-Emmett-Teller (BET) gas adsorption inform the porous chitosan. The well dispersion of the nanoscaffold in solution is easily controlled by the solvent polarity and salt concentration, and enables an effective surface functionalization of the chitosan nanoscaffold with sugar unit, i.e. lactose and maltose, under mild and water-based reaction to obtain a novel type of chitosan nanoscaffold with a soft and cotton-like appearance chitosan containing mesopores.[2] As all steps are organic solvent free, the chitosan nanoscaffold and chitosan-sugar nanoscaffold are promising materials for biopolymer-supported tissue engineering.

Introduction

Chitin-chitosan is a natural occurring copolymer of β-(1-4)-2-acetamido-2-deoxy-β-D-glucose and β-(1-4)-2-amino-2-deoxy-β-D-glucose (Figure 1) obtained from shells of crustaceans, cuticles of insects, and cell-walls of fungi and yeasts. The copolymer exhibits specific properties, especially, bioactivity,[3] biocompatibility,[4] biodegradability,[5] and non-toxicity[6] to show the potential applications in pharmaceutical and biomedical fields. In the past, simple materializations, such as solvent casting for films[7] spray drying for beads or spheres[8] and cross-linking for gels or membranes[9] were reported.

In recent years, scaffolds are recognized as an alternative material with porous structure for the advanced applications, such as bioactive molecules delivery, medical implants, cultured artificial organs, and tissue engineering.[10-14] Up to now, the techniques known for preparing scaffold are, such as polymer assembly,[15] phase separation,[16] and electrospinning.[17]

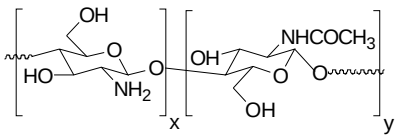


Figure 1 : Chemical structure of chitin-chitosan copolymer.

For chitosan, the various scaffold preparations were reported and in most cases chitosan solutions or the crosslinked chitosan gels were used. Scaffold modifications are also another step to achieve the materials for specific applications. Park *et al*. and Li *et al*. demonstrated that the conjugation with bioactive molecules, such as galactose and fructose, onto chitosan scaffold increased the hepatocytes adhesion.[18-19] Herein, we report for the first time that chitosan nanoscaffold can be directly prepared from the chitin whisker. The nanoscaffold provides us a simple but effective model reaction to obtain a novel soft and cotton-liked chitosan nanoscaffold as soon as the disaccharide molecules were conjugated. As all preparation steps are in aqueous, they might be developed for promising biomaterials.

Material and Methods

Chemicals

Chitin flakes from shrimp shells were provided by Seafresh Chitosan (Lab) Company Limited, Thailand. Lactose monohydrate, maltose monohydrate, and sodiumcyanoborohydride ($NaBH_3CN$) were purchased from Wako, Japan. Sodium hydroxide, sodium chloride, hydrochloric acid, dimethylsulfoxide (DMSO), and acetic acid were provided by Lab-Scan, Ireland. Hexafluoro-2-propanol (HFP) was purchased from Central Glass Co., Ltd., Japan. Sodium trifluoroacetate (CF_3COONa) was obtained from TCI-EP, Japan. Sodium acetate was purchased from Univar, Australia. All chemicals were analytical grade and were used without further purification.

Instruments and Equipment

Qualitative and quantitative Fourier transform infrared spectra were obtained from a Thermo Nicolet Nexus 670 with 32 scans at a resolution of 2 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed using a deuterated triglycinesulfate detector with a specific detectivity, D*, of 1 × 109 cm Hz½ w^{-1}. Wide X-ray diffraction (WAXD) patterns were recorded over 2º-60º 2θ by a RIGAKU RINT 2000 using CuKα as an X-ray source equipped with Ni filter with operating conditions of 40 kV and 30 mA. TG-DTA thermogravimetric analyses were carried out using a Perkin Elmer Pyris Diamond with N_2 flow rate of 20 mL/min and a heating rate of 10ºC/min starting from 30 to 650ºC. ^1H NMR spectrum was recorded for the samples dissolved in CD_3COOD/D_2O on a JNM-A500 500 MHz spectrometer at 70°C. The morphology of whisker was investigated by a JEOL JEM-1230 transmission electron microscope (TEM) at 80 kV, and a JEOL JSM-5200 scanning electron microscope (SEM) at 15 kV. The turbidity was determined by a Shimadzu UV-2550 UV-Vis spectrophotometer. Surface area was measured by a Quantachrome Corporation Autusorb-1 gas sorption analyzer via the Brunauer-Emmett-Teller (BET) method. Nitrogen gas was applied to calibrate the analyzer and as the adsorbate at liquid nitrogen temperature. The samples were degassed at 100ºC for overnight before measurement. Molecular weight was measured by a Tohso HLC-8220 gel permeation chromatography (GPC), equipped with KI detector and Tohso TSK-gel super H-RC and HM-N columns and the operating temperature was 40°C. HFP containing 10 mM CF_3COONa was used as an eluent with the flow rate of 0.2 mL/min and polymethyl methacrylate was used as a standard. Relative viscosity [η_r] was measured by a calibrated viscometer Cannon-Ubbelohde (No. 2, A149) in 0.2 M CH_3COOH/0.1 M CH_3COONa aqueous solution at 30 ± 0.05°C.

Procedures

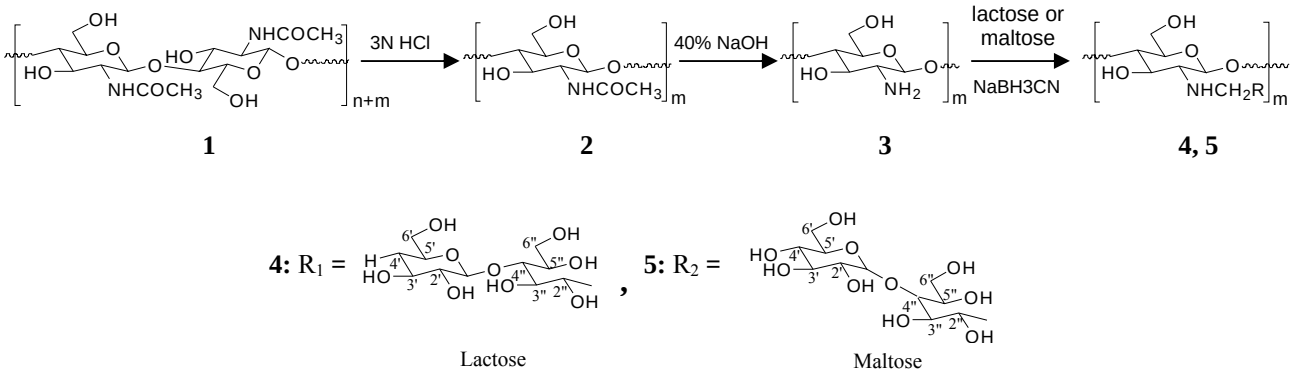


Figure 2 : Preparations of chitosan nanoscaffold, **3**, and chitosan nanoscaffold conjugated with lactose, **4**, and maltose, **5**.

Chitin Whiskers, 2

Chitin flakes, **1** (1.00 g), were treated in 3N hydrochloric acid (HCl) (100 mL) and stirred at reflux for 3 h before centrifugation. The treatment with 3N HCl was repeated three times. Finally, the residues were collected and dialyzed in distilled water until neutral to obtain chitin whiskers, **2**, for 86% yield (Figure 2).

FT-IR (KBr, cm^{-1}): 1661, 1624, and 1557 cm^{-1} (amide I, and amide II).

Chitosan Nanoscaffold, 3

Compound **2** (20 mL) was stirred in NaOH aq. (40% w/v, 100 mL) at reflux for 7 h before leaving at room temperature for overnight. The treatment with 40% NaOH aq. was repeated three times. The crude product was dialyzed till neutral in distilled water to obtain **3** for 92% yield (Figure 2).

FT-IR (KBr, cm^{-1}): 1595 cm^{-1} ($-NH_2-$). ^1H NMR (δ, ppm): 2.367 (NHAc), 3.435 (H2 of GluN unit in chitosan), 4.023-4.255 (H2 of GluNAc, and H3-H6 of pyranose ring), 4.9 (H1 of GluNAc), 5.138 (H1 of GluN).

Chitosan Nanoscaffold Conjugated Lactose, 4

Compound **3** (0.03 g in 5.0 mL) was suspended in NaCl solution (1.0 M, 10 mL). Lactose monohydrate (0.224 g, 3 moles equivalent to pyranose rings) and $NaBH_3CN$ (0.039 g, 3 moles equivalent to pyranose rings) were added with a catalytic amount of acetic acid to the suspension of **3**. The reaction was carried out at room temperature for 3 days. The crude product was purified by dialyzing in distilled water and lyophilized to obtain **4** in 87.6 % yield (Figure 2).

FT-IR (KBr, cm^{-1}): 1530 cm^{-1} ($-NH-CH_2-$). ^1H NMR (δ, ppm): 2.414 (NHAc), 3.527 (H2 of GluN unit in chitosan), 3.554-4.431 ($-NH-CH_2-$, H2 of GluNAc, H3-H6 of pyranose ring, and H2', H3', H4', H5', H6', H2", H3", H4", H5", H6" substituted), 4.907-4.932 (H1 of GluNAc), 5.203-5.230 (H1 of GluN).

Chitosan Nanoscaffold Conjugated Maltose, 5

Compound **5** was prepared similar to **4** but using maltose monohydrate and obtained 72.1 % yield (Figure 2).

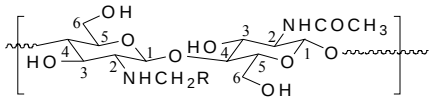
FT-IR (KBr, cm^{-1}): 1530 cm^{-1} ($-NH-CH_2-$). ^1H NMR (δ, ppm): 2.410 (NHAc), 3.519 (H2 of GluN unit in chitosan), 3.723-4.442 ($-NH-CH_2-$, H2 of GluNAc, H3-H6 of pyranose ring, and H2', H3', H4', H5', H6', H2", H3", H4", H5", H6" substituted), 4.902-4.927 (H1 of GluNAc), 5.197-5.224 (H1 of GluN).

Results and Discussion

Chemical Structure Analysis

The FTIR spectrum of **2** demonstrated a typical type of chitin with the characteristic peaks at 1661, 1624, and 1557 cm^{-1} for amide I, and amide II, respectively. Obviously, **2** showed the sharp characteristic peaks as compared to the starting chitin flake, which might be due to the highly crystallinity. The morphology of **2** was further studied by TEM to find that **2** was in the whiskers form as reported by Nair *et al.*[20] (see Morphological Studies). The overall of FTIR pattern of **3** was

similar to that of chitosan flakes confirming the successful deacetylation. Although **3** was expected to show the sharp peak as it was derived from highly crystalline chitin of **2**, we found that **3** showed the broad bands suggesting the increase in amorphous fraction (see Morphological Studies). The ^{1}H NMR spectrum exhibits the chitosan peaks, of which the degree of deacetylation is 98%. Here, the effects of solvent, especially based on the polarity and ionic strength, to the colloidal solution of **3** in water was studied. The interaction of **3** with water, methanol, DMSO, and *iso*-propanol, having the δ values for 23.4, 14.5, 12, and 11.5 $(cal.cm^{-3})^{0.5}$ as reported in Polymer Hanbook,[21] respectively, was extensively studied. The particle size of **3** was found to be increased when the solubility parameter of the solvent increased. The particle size was as high as 2000 nm when **3** was in water suggesting the good swelling of **3** in aqueous. A certain concentration of sodium chloride solution (0.05, 0.1, 1.0, and 5.0 M) was added to observe how ionic strength in the solution affected the colloidal formation of **3**. The well dispersion of **3** in sodium chloride solution was useful for the functionalization of the **3** with sugar unit, i.e. lactose and maltose. Successful conjugations of lactose, and maltose onto **3** to obtain **4** and **5** were confirmed by disappearing of the peak at 1595 cm^{-1} (-NH_2, -N-H- in plane bending) and appearing of the new peak at 1530 cm^{-1} (-NH-CH_2-, -N-H- in plane bending). Based on ^{1}H NMR technique, the degrees of substitution of **4** and **5** were found to be 9.5 % and 7.3 %, respectively (Figure 3).[22-23]



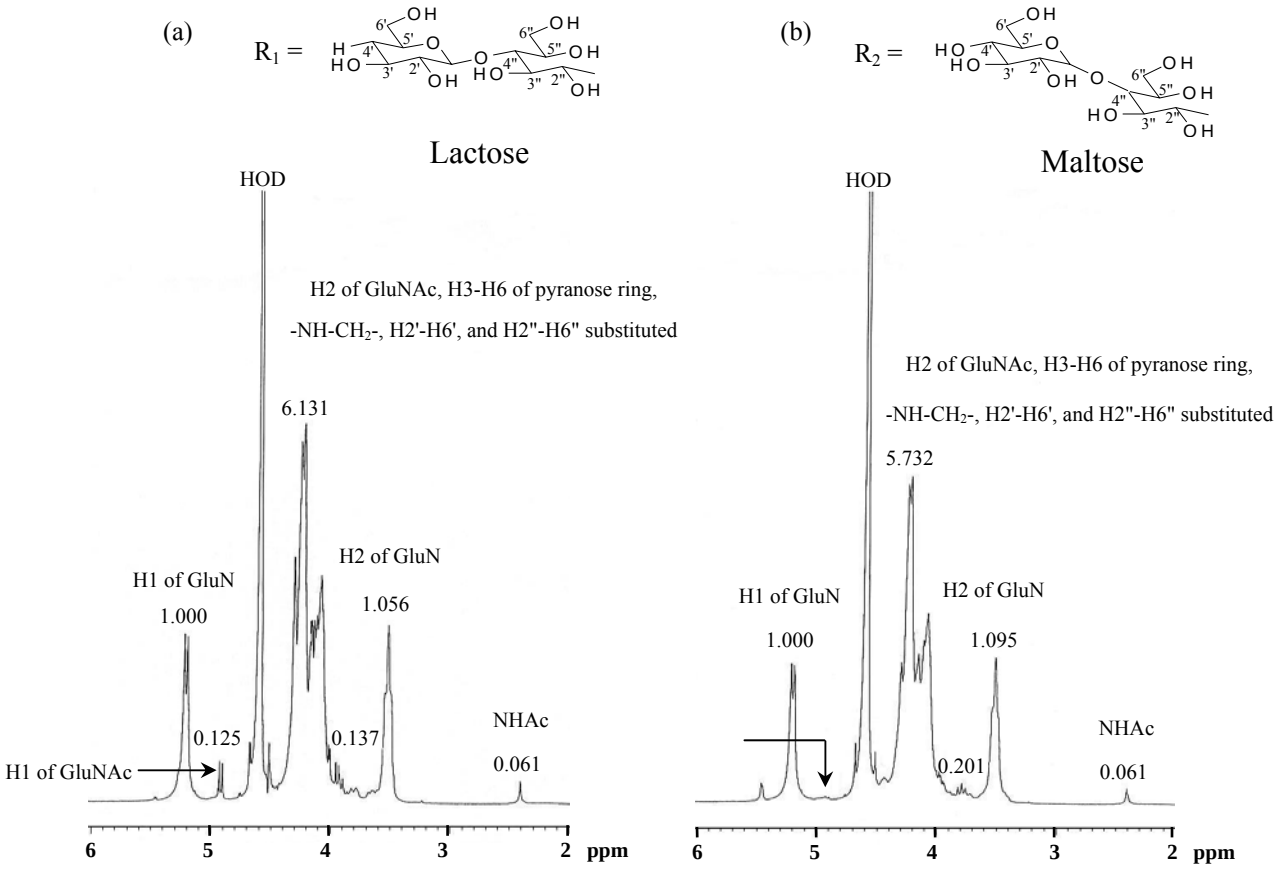
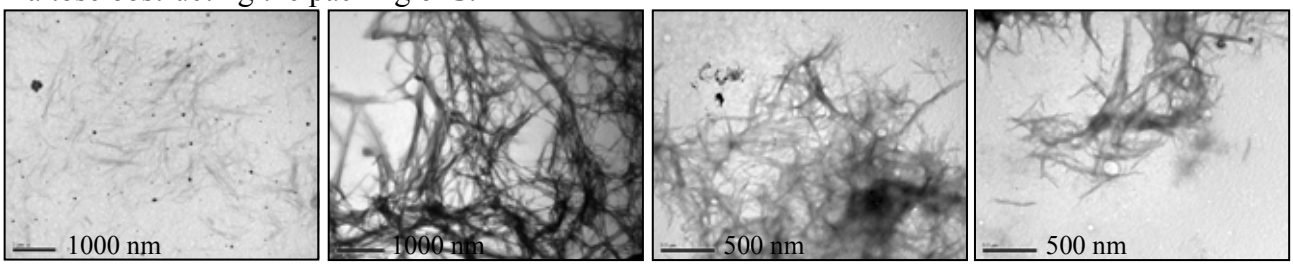


Figure 3 : ^{1}H NMR spectra of: (a) **4** and (b) **5**.

Morphological Studies

Transmission electron microscopy (TEM) gives us the information to determine the morphology in details. Compound **2** gives a uniform needle, so-called whisker, with a length of 200-560 nm, a

width of 18-40 nm, and an average aspect ratio of 18 (Figure 4(a)). The observation of **3** by TEM shows that **3** is in fibrous form and each fiber is branching and networking (Figure 4(b)). Taking this result with those from IR into our considerations, it can be concluded that the alkaline treatment changes chitin nano-whisker to chitosan nanoscaffold. Figure 4 ((c) and (d)) shows that **4** and **5** are in nano-scaled scaffold as similar to **3**. The average thickness of each fiber in nanoscaffold was 10-15 nm which was smaller than that of **3**, implying that the modification with sugar initiates the separation of the original fibrous network of **3**. This might be due to the introduction of lactose and maltose obstructing the packing of **3**.



(a) (b) (c) (d)

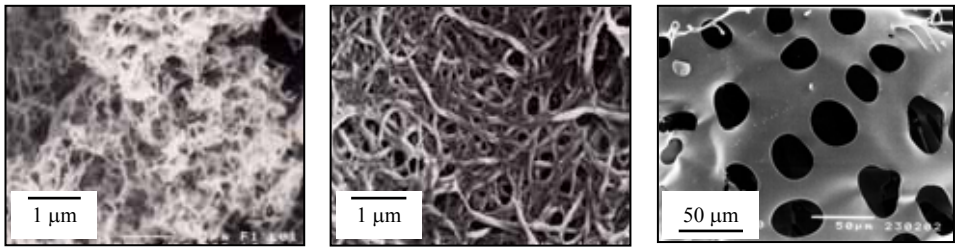
Figure 4 : TEM micrographs of: (a) **2**, (b) **3**, (c) **4**, and (d) **5**.

(a) (b)

Figure 5 : Appearances of: (a) **3** and (b) **4**, after lyophilization.

It is important to mention that after **4** and **5** were lyophilized, the morphology was changed totally. Both exhibit themselves a glossy soft and cotton-like appearance (Figure 5 (b)) consisting of tiny fibers whereas **3** shows a flat white soft porous sheet (Figure 5 (a)).

The scaffold structure was further confirmed by SEM. After **2**, **3**, **4**, and **5** were lyophilized, the observation by SEM was carried out. For **2**, the SEM micrograph shows the aggregation of whiskers (Figure 6 (a)), implying the packing of whisker after the lyophilization. When it comes to **3**, the packing was induced significantly to obtain a regular matrix structure as shown in Figure 6 (b). The fibrous network forms a porous structure with an average pore diameter of ~ 200 nm. The observation by SEM clearly shows that **4** and **5** are in mesoporous network. Figure 6 (c) shows the flat mesoporous networks of **4** with an average pore diameter of 27 ± 3 μm. For **5**, the similar morphology as **4** was observed.



(a) (b) (c)

Figure 6 : SEM micrographs at 15 kV of: (b) **2** (× 20,000), (d) **3** (× 20,000), and (f) **4** (× 500).

Conclusions

Chitosan in nanoscaffold structure was successfully prepared via a one pot deacetylation of chitin whisker. As a consequence of deacetylation, the branching of each chitin whisker initiated the scaffold formation as observed by TEM. The lyophilization induced the packing of nanoscaffold to be a regular network as evidenced by SEM. The aggregation of the scaffold could also be controlled by the high ionic strength solvent such as NaCl. The system offered an effective reaction to conjugate the maltose and lactose in heterogeneous system at room temperature. After maltose or lactose was successfully conjugated, the material exhibited itself as beautiful glossy tiny cotton ball fibers. The SEM and TEM micrographs revealed that the materials obtained were in nanoscaffold structure.

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[22] Degree of substitution (%) = {[(6.131 + 0.137 + 1.056) - 6] / 14} × 100 = 9.46%.

[23] Degree of substitution (%) = {[(5.732 + 0.201 + 1.095) - 6] / 14} × 100 = 7.34%.