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Overcoming Chitosan Hydrogen Bond Network: Another Aspect of Chitosan Nanomaterial

Rangrong YOKSAN[‡], Juthathip FANGKANGWANWONG and Suwabun CHIRACHANCHAI*[†]

[‡]Division of Physico-Chemical Processing Technology, Kasetsart University, Bangkok 10900, Thailand
The Petroleum and Petrochemical College, Chulalongkorn University, Bangkok 10330, Thailand
[†]Center of Chitin-Chitosan Biomaterials, Chulalongkorn University, Bangkok 10330, Thailand
**To whom correspondence should be addressed.*

Abstract

A series of chitosan materials, i.e., (i) chitosan nanospheres, and (ii) chitosan scaffold gel based on the concept of overcoming the hydrogen bonds at the molecular level are proposed. For (i), the introduction of hydrophobic groups and hydrophilic chains onto chitosan to give *N*-phthaloylchitosan-mPEG shows the sphere particles in nanometer sizes. In the case of (ii), the functionalization of epichlorohydrin onto *N*-phthaloylchitosan proves to be a simple way to obtain scaffold gel without using dialdehyde crosslinkers. In addition, the scaffold gel shows the significant biomineralization with hydroxyapatite.

Keywords: Chitosan Nanospheres, Chitosan Scaffold Gel, Chitosan Nanocomposites, Cosmetics, Drug and Gene Delivery Systems, Bone Tissue Engineering, Bone Glue, Biomineralization, Organo-clay

Introduction

Chitin-chitosan is the second most abundant natural occurring copolymer of β-(1-4)-2-acetamido-2-dedoxy-β-D-glucose and β-(1-4)-2-amino-2-dedoxy-β-D-glucose existing in crustacean shells. For decades, the fundamental studies on chitin-chitosan bring us an understanding about its unique amino polysaccharide properties, such as, significant complexation with metal ions (Covas *et al.*, 1992), antibacterial and antimicrobial activities (Sudarchan *et al.*, 1992), tissue and cell compatibility (Cima *et al.*, 1991) and biodegradability by the enzymes such as chitinase (Hung *et al.*, 2002), chitosanase (Muzzarelli, 1977), and lysozyme (Hirano *et al.*, 1989), etc. In order to apply those properties for practical products, the materialization have been proposed for years. The fabrication, for example, from flakes to fibers (Agboh and Qin, 1997), films (Hosakawa *et al.*, 1990) and beads Juang *et al.*, 2002) is reported as successful approaches. Chemical modification with some functional groups as seen in the case of organic solvent soluble chitosan (Nishimura *et al.*, 1991), water soluble chitin and chitosan, (CM chitin and CM chitosan), (Lingyun *et al.*, 2002) colloidal nanosphere chitosan (Yoksan *et al.*, 2004) etc., is another plausible materialization. However, similar to chitosan, most chitosan derivatives are difficult to dissolve in most common organic solvents and this limits the practical applications. At present, chitosan related products, mostly, are in powder or flake forms whereas the gels, beads, and membranes are the products with safety awareness from the acids and aldehydes used in the production. With this viewpoint, it is our challenge to materialize chitin-chitosan material based on the minimal hydrogen bonds chitosan network. Here, we summarize our current research, i.e., (i) chitosan nanospheres, and (ii) chitosan scaffold gel.

E-mail: csuwabun@chula.ac.th

In the case of (i), as nanospheres are accepted as an ideal material for controlled release cosmetics and drugs, various nanosphere production methods are proposed. In our case, we stand on the key point of a micelle-like structure, which consists of hydrophobic and hydrophilic segments in a single molecule. The present work, thus, focuses on the modification of chitosan with hydrophobic and hydrophilic groups including the nanosphere formation phenomena.

For (ii), it should be noted that the μ-(1-4)-2-acetamido-2-deoxy-μ-D-glucose unit is reported for the function of osteoconductivity (Lahiji *et al.*, 1999), whereas the μ-(1-4)-2-amino-2-deoxy-μ-D-glucose unit is known for the hybridization with inorganic compounds (Zhang and Gonsalves, 1995). Thus, it is a challenging theme to develop chitin-chitosan as a material for bone tissue engineering. In the past, Tokura *et al.* showed that chitin and its derivatives, *O*-carboxymethyl-chitin, accelerate the bone tissue formation (Tokura and Tamura, 2001). In order to apply chitin-chitosan in this field, it is necessary to start the material in gel form. We, then, focus on chitosan gel via an epoxy group conjugated on a chitosan chain.

Experimental Section

Materials

Chitosans (degree of deacetylation (DD) = 0.9, $M_v = 1.7 \times 10^5$ Dalton for chitosan nanospheres studies, and DD = 0.89, $M_v = 1.6 \times 10^6$ Dalton for chitosan scaffold gel) were supplied from the Seafresh Chitosan (Lab) Company Limited, Thailand. Phthalic and succinic anhydrides were from Fluka Chemika, Switzerland. Poly (ethylene glycol) methyl ether (mPEG) was from Aldrich Chemical Company, Inc., USA. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride and hydrazine monohydrate were purchased from TCI Kyoto and Nacalai Tesque Inc., Japan, respectively. 1-Hydroxy-1H-benzotriazole, monohydrate (HOBt) was from BDH Laboratory Supplies, England. *N,N*-Dimethylformamide (DMF), sodium acetate, isopropanol and potassium hydroxide were the products of UNIVAR, Australia. Acetic acid, hydrochloric acid, calcium chloride, disodium hydrogenphosphate, methanol and tris(hydroxymethyl)aminomethane (Tris) were purchased from Carlo Erba Regenti, Italy. Epichlorohydrin was from Acros Organics, Belgium.

Instruments and Equipment

FT IR spectra were recorded on a VECTOR 3.0 BRUKER spectrometer with 64 scans at a resolution of 4 cm^{-1}. ^{13}C CP/MAS NMR spectra were taken at 300 MHz with a BRUKER DPX-300 at 23 ±1 °C. ^1H NMR spectra were obtained from a JEOL GSX 400 (400 MHz) at 70 ± 1°C for chitosan and 25 ± 1 °C for its derivatives. Elemental analysis (EA) was done by a YANAKO CHN CORDER MT-3, MT-5 Analyzer with a combustion temperature at 950 °C under air with O_2 as a combustion gas. A Dupont thermogravimetric analyzer 2950 was used with an N_2 flow rate of 20 mL/min and a heating rate of 20 °C/min from 30 °C to 600 °C. Scanning electron microscopy (SEM) analysis was obtained from a JEOL JSM-5200, operating voltage of 25 kV. Transmission electron microscopy (TEM) analysis was carried out by a Hitachi H700, accelerate voltage 200 kV. Electron spectroscopy for chemical analysis (ESCA) was done by a Shimadzu ESCA1000 with Mg X-ray source (1253.6 eV), pass energy 31.5 eV, operating at 8 kV and 20 mA. Dynamic Light Scattering (DLS) measurement was carried on a COULTER® model N4SD at 20 °C with a scattering angle of 90 °C.

Preparation of Chitosan Nanosphere, Compound 4

Chitosan nanosphere or *N*-phthaloyl-chitosan-mPEG, **4**, was prepared as reported previously. In brief, **4** was obtained from the conjugating of mPEG-COOH, **3**, onto *N*-phthaloylchitosan, **2**. Compound **3** was prepared from the reaction of poly(ethylene glycol) methyl ether (mPEG, M_n = 5,000 Dalton, 3.00 g, 6×10^{-4} moles) and succinic anhydride (0.06 g, 1 mole equiv to mPEG) in DMF (2 mL) at 60 °C for overnight with a catalytic amount of pyridine. The purification of **3** was obtained from the extraction of the crude product with diethyl ether. Compound **3** (7.58 g, 0.40 moles equiv (40%) to **2**) was stirred with **2** (1.00 g, 3.71×10^{-3} moles) in DMF (20 mL) solution containing a catalytic amount of 1-hydroxy-1H-benzotriazole,

monohydrate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (WSCI, 0.85 g, 3 moles equiv to **3**) at 4 °C for 30 min and then room temperature for overnight. The mixture was dialyzed against water to obtain white particles, **4**.

Preparation of Chitosan Scaffold Gel, Compound **6**

Chitosan-epoxy gel, **6**, was obtained as follows. *N*-phthaloylchitosan, **2**, (1.00 g) was dissolved in DMF (20 mL) and heated to 60 °C *in vacuo* (Nishimura *et al.*, 1991; and Yoksan *et al.*, 2001). After 30 min, a catalytic amount of potassium hydroxide-isopropanol solution and epichlorohydrin (3.50 g, 10 moles equiv to pyranose ring) were added. The reaction was carried out at 60 °C for 5 h. The solution was concentrated and reprecipitated in cold water. The precipitate was collected and washed with methanol several times, followed by drying *in vacuo* to obtain epoxy-*N*-phthaloylchitosan, **5**. Compound **5** (1.00 g) and hydrazine monohydrate were mixed in water (20 mL). The optimal reaction condition was identified under the variables of the reactant molar ratio, reaction time, and temperature. The product was washed thoroughly with water, methanol and ethanol for several times to obtain gel, **6**, for the use in further steps.

An alternate soaking to form hydroxya-patite (HA) as reported by Tachaboonyakiat *et al.* (2001) was done. A piece of gel, **6** (1.00 g), was immersed in $CaCl_2$ (200 mM)/ Tris-HCl (pH 7.4) aqueous solution (20 mL) at 37 °C for 2 hours, followed by rinsing thoroughly several times with deionized water. The product was immersed in Na_2HPO_4 (120 mM) aqueous solution (20 mL) at 37 °C for 2 hours and washed thoroughly with water.

Results and discussion

Chitosan Nanosphere

Figure 1 shows FT IR spectra of mPEG and chitosan including their derivatives to clarify the success of reactions in each step. Compound **2** (Figure 1d) shows new peaks of phthalimido group at 1776 and 1714 cm^{-1} referring to carbonyl anhydride and 721 cm^{-1} belonging to an aromatic ring. In addition, a new peak at 7.6 ppm referring to aromatic protons of phthalimido group in **2**. Compound **3** (Figure 1b) gives a significant peak at 1736 cm^{-1} belonging to a carbonyl group and shows additional peak at 2.4 ppm for methylene protons in succinic anhydride. Compound **4** (Figure 1e) gives an increase in peak intensity at 2882 cm^{-1} belonging to methylene groups as compared to **2** (Figure 1d) implying the successful introduction of mPEG on **2**. ^1H NMR was also applied to confirm the structure of **4**, the peaks at 2.4, 3.2, and 3.5 ppm referring to methylene protons of succinic anhydride, methoxy protons of mPEG terminal chain, and methylene protons of mPEG, respectively, indicate the hydrophilic part, whereas the peaks in the range of 2.8-4.7 and 7.6-7.8 ppm belonging to protons in pyranose ring and phenyl ring of phthalimido group, respectively, imply to hydrophobic part.

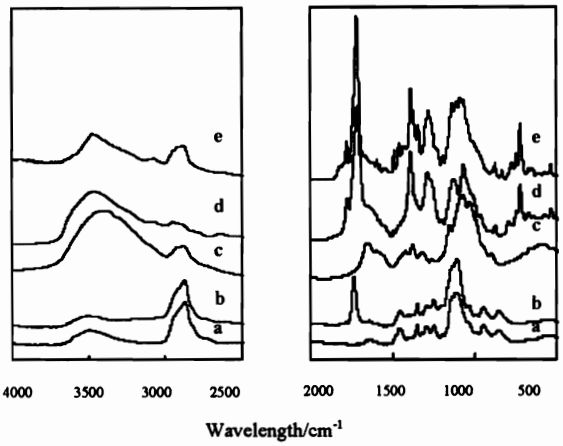

Figure 1. FT IR spectra of mPEG, chitosan and derivatives.

Figure 2 shows the colloidal solution of **4** in protic and aprotic solvents. In the cases of protic solvents (i.e., water, 1% acetic acid, methanol, ethanol, and iso-propanol), the turbidity was clearly observed (Figure 2c-g). It is important to note that the milky solution is stable for more than a week at ambient. For aprotic solvents (i.e., DMF, DMSO, toluene, n-hexane, chloroform), **4** is dissolved in DMF and DMSO (Figure 2i-j), whereas soon precipitated out in toluene and n-hexane (Figure 2k-l). However, it gives colloidal solution in chloroform

(Figure 2h). This might be related to the dielectric constant and dipole moment of each solvent.

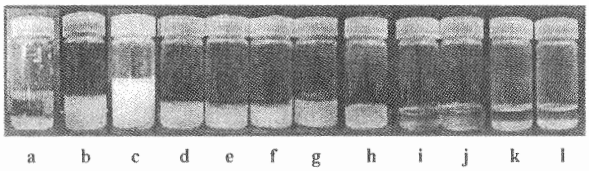

Figure 2. Appearance of **1** in water (a), **2** in water (b), and **4** in water (c), 1% aqueous acetic (d), methanol (e), ethanol (f), iso-propanol (g), chloroform (h), DMSO (i), DMF (j), toluene (k), and n-hexane (l).

Figure 3 illutrates SEM photographs of chitosan and its derivatives. It was found that **1** shows irregular flake (Figure 3a), while **2** performs partially round in shape (Figure 3b). Figure 3c confirms that by balancing of hydrophobicity and hydrophilicity of chitosan chain, the well-defined spheres are obtained. Here, the sizes of chitosan spheres, **4**, were averagely examined to be 200 nm (Figure 3c). Figure 4 illustrates TEM photographs of **4**, the images of **4** confirm the round shape particle with different sizes. It was found that the sizes of **4** obtained from mPEG with M_n of 550 (Figure 4a) and 5,000 (Figure 4b) are 400 and 85 nm, respectively.

Figure 3. SEM photographs at 25 kV of **1** at 15,000× (a), **2** at 20,000× (b), and **4** at 50,000× (c).

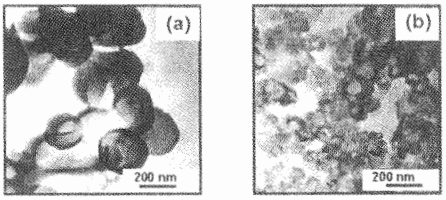

Figure 4. TEM photographs at 30,000× of **4** obtained from mPEG with M_n of 550 (a) and 5,000 (b).

Chitosan Scaffold Gel

The characterization of epoxy-*N*-phthaloylchitosan, **5**, is as follows. The reaction of epichlorohydrin with *N*-phthaloylchitosan was done in DMF *in vacuo* at 60 °C for 5 hours with a catalytic amount of potassium hydroxide in isopropanol. The product obtained shows the oxirane ring peak at 907 cm^{-1} (Figure 5c). The thermogravimetry analysis of **5** reveals the significant weight loss during 300-400 °C. The low ash content (~10%) might come from the continuous chain degradation of highly amorphous structure of chitosan with bulky groups of phthalamido and oxirane rings.

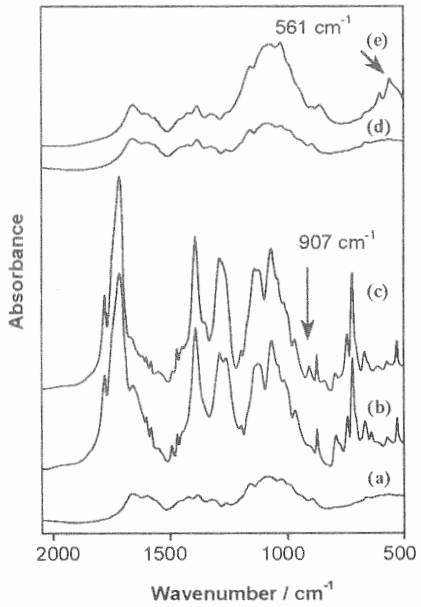

Figure 5. FT IR spectra of compounds (a) Chitosan, (b) **2**, (c) **5**, (d) **6**, and (e) **6** after alternate soaking in calcium and phosphate solutions.

When the hydrazine was added for 20 moles and the reaction was carried out at 60 °C, the gel was obtained after a few minutes to obtain chitosan-epoxy gel, **6**. Figure 5d shows the disappearance of 1776 and 1714 cm^{-1} and the appearing of the characteristic peaks of amide I and II implying the successful deprotection. It should be noted that the oxirane ring peak at 907 cm^{-1} also disappeared. This confirms that hydrazine functions

not only for the deprotection of the amino group for chitosan but also crosslinking after the ring opening reaction of the epoxy group.

For some particular applications, such as bone glue, it is important to let the gel form at room temperature. Here, the gelation was quantified by clarifying the decrease of oxirane ring peak (907 cm^{-1}) for the reaction times 4, 12, 20, and 28 minutes. By using the C-O-C in a pyranose ring (1026 cm^{-1}) as an internal standard peak, a kinetic reaction scheme was determined to find that the reaction was almost completed after 15 minutes.

It is important to note that the attractive points to use chitosan-epoxy scaffold is not only related to the gel formation but also the biocompatibility and the inorganic compound hybridization. Compound **6** was immersed into calcium chloride solution for two hours before immersing in Na_2HPO_4 solution. FT IR was used to trace the HA formation (561 cm^{-1} phosphate group). It was found that after soaking for five cycles, the phosphate group was identified (Figure 5e). The observation by WAXD declared that **6** after alternate soaking in calcium and phosphate solutions gave two little sharp peaks at 26° and 32° 2θ belonging to the HA on the wide range of the broad peak of chitosan.

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