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The efficient hemostatic effect of Antarctic krill chitosan is related to its hydration property

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ABSTRACT

Antarctic krill chitosan (A-Chitosan) was first evaluated in its hemostatic effect in this study. The prepared A-Chitosan powder showed low level of crystallinity and significantly high water binding capacity as 1293% (w/w). By mice tail amputation model and blood coagulation timing experiment, it is showed that this chitosan accelerated the tail hemostasis by 55% and shortened the blood clotting time by 38%. This efficacy was better than two other commercial chitosans investigated and was corresponding to their water binding capacities. Through examining the effect of chitosan on blood components, it could be found that platelets adhesion was mainly affected by the water binding capacity, and red blood cells aggregation was dependent on their deacetylation degree. The physicochemical properties resulted in better hydration property of chitosan would improve its hemostatic effect. These results suggested that Antarctic krill chitosan is a good candidate for hemostatic application.

1. Introduction

Antarctic krill (*Euphausia superb*) as the dominant prey in the Southern Ocean has been widely studied (Atkinson, Siegel, Pakhomov, & Rothery, 2004; Trivelpiece et al., 2011). Its astonishing standing biomass approximately reached hundreds of million tons (Atkinson, Siegel, Pakhomov, Jessopp, & Loeb, 2009). Its protein and lipid have been commercially developed. The discarded krill shell has been determined as a source of chitosan (Gigliotti, Davenport, Beamer, Tou, & Jaczynski, 2011; Gigliotti, Jaczynski, & Tou, 2008). Chitosan, a polysaccharide, derived from naturally occurred chitin by partial deacetylation existed in a wide range of sources including the exoskeleton of crustaceans, cuticle of insects and cell wall of fungi. It is well known for its fascinating biological properties, which recommend it for plenty of biomedical applications (Albanna, Bou-Akl, Blowytsky, Walters Iii, & Matthew, 2013; Bhattarai, Gunn, & Zhang, 2010; Dash, Chiellini, Ottenbrite, & Chiellini, 2011).

Different species as sources for chitosan preparation could bring the differences in physicochemical properties such as crystalline structure and molecular weight (Rhazi, Desbrieres, Tolaimate, Alagui, & Vottero, 2000). The chitin of Antarctic krill belongs to α crystalline form with molecules aligned in antiparallel fashion, which is by far the most abundant form (Rinaudo, 2006; Wang et al.,

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2013). Antarctic krill chitin contains more free OH groups in the chemical structure and smaller crystallite size comparing with the crab chitin (Wang et al., 2013). It also showed higher water-holding capacity and stronger water–polymer interaction, which would result in the difference in hydration properties (Kittur, Prashanth, Sankar, & Tharanathan, 2002). These properties of chitin would further influence the physicochemical properties of its derived chitosan and give rise to the difference in the biological function. However, the biological properties of Antarctic krill chitosan have not been investigated yet.

The hemostatic effect of chitosan has been demonstrated in many studies, which gives rise to many hemostatic products, such as TraumaStat, Celox, HemCon and so on (Dowling et al., 2011; Gu et al., 2010). It has been suggested that the hemostatic effect of chitosan was realized by activating the platelets through absorbing the serum proteins or directly contact (Fischer et al., 2005; Lord, Cheng, McCarthy, Jung, & Whitelock, 2011). However, the conflicting results of its hemostatic effect could also be found in the literatures, revealed the challenge in the development of effective hemostatic agent (Achneck et al., 2010; Jeweleivicz, Cohn, Crookes, & Proctor, 2003; Pusateri et al., 2003; Whang, Kirsch, Zhu, Yang, & Hudson, 2005). It is well known that hemostatic responses of chitosan are highly dependent on its physicochemical properties such as molecular weight, deacetylation degree, intrinsic viscosity, hydration and crystallinity (Yang et al., 2008). Therefore, the structure properties of strong water–polymer interaction found in Antarctic krill chitin as mentioned above may benefit the hemostatic effect of its derived chitosan. In addition, the astonishing standing biomass of Antarctic krill is another advantage for chitosan preparation in terms of the adequate supply.

In this study, Antarctic krill chitosan was first evaluated in the hemostatic effect by both *in vitro* and *in vivo* experiments using mice tail amputation model and blood coagulation timing experiment. Since the molecular weight and deacetylation degree have been considered at the major properties influencing the chitosan's hemostatic effect, the commercial chitosans with the similar molecular weight or similar deacetylation degree were selected as references. The mechanism of chitosan's hemostatic effect was explored by investigating the platelets adhesion and red blood cells aggregation.

2. Material and methods

2.1. Materials

The Antarctic krill was provided by Shanghai Deepsea Fisheries Co. Ltd. (China) and was preserved at −20 °C in refrigerator before use. It was unshelled after thawing under 4 °C and the shell was dried at 60 °C in a vacuum drying oven. Medium molecular weight chitosan with 75–85% deacetylation degree was obtained from Sigma-Aldrich. Chitosan with 90% deacetylation degree was provided by Shandong AoKang Biological Technology Co. Ltd. (China) in pharmaceutical grade. Calcein AM was obtained from AAT Bioquest. Deuterium chloride and deuterium oxide were purchased from Sigma. C57BL/6 mice and Sprague-Dawley (SD) rats were obtained from Medical Laboratory Animal Center (China). All other chemicals were of analytical grade.

2.2. Preparation of chitosan

Chitosan preparation from Antarctic krill shell was carried out by a common chemical method using alkali and acid treatment for the deproteinization, demineralization and deacetylation of the raw materials (No & Meyers, 1995). Specifically, the shell was deproteinized with 1 M NaOH at 100 °C for an hour at a solid/solvent ratio of 1:20 (w/v) and washed to neutrality in deionized water. Demineralization was carried out in 2 M HCl solution at room temperature for an hour at a solid/solvent ratio of 1:20 (w/v) and washed to neutrality in deionized water. The deproteinization and demineralization were performed twice to get chitin, which was further dried at 60 °C in a vacuum drying oven. Deacetylation was performed at 110 °C in 50% NaOH solution with refluxing for an hour at a solid/solvent ratio of 1:50 (w/v). After repeating the deacetylation, the obtained product was collected and washed to neutrality. The crude chitosan was purified by dissolving in 1% acetic acid solution, filtering, and then precipitating from the solution by adjusting the solution's pH to 8.5 with 1 M NaOH. The obtained product was washed and dried by lyophilization to get the chitosan powder. The chitosan obtained from Sigma (S-Chitosan) and the pharmaceutical grade chitosan from domestic company (P-Chitosan) were also purified by precipitation for comparison. The chitosan powder was evaluated in the hemostatic effect in mice tail amputation model and blood coagulation timing experiment. The chitosan film was used for mechanism investigation to observe its effect on platelets adhesion and red blood cells aggregation. Chitosan thin film was prepared on the round cover slice by the spin-coating (Chemat Technology spin-coater), and dried at 60 °C in a vacuum oven for 12 h. Films were then sterilized by ultraviolet light.

2.3. Chitosan characterization

The molecular structure of the extracted chitosan was characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and nuclear magnetic resonance (NMR) spectroscopy. The FTIR spectrum was obtained using a Bruker Vertex 70 FTIR spectrometer. The crystalline structure of chitosan was recorded on an X-ray diffractometer (XRD; model D/max 2550V, Rigaku, Tokyo, Japan) using Cu$K\alpha$ radiation (k = 0.15406 nm) at a scan rate of 10°/min, in the range 10–80° 2θ. ^{1}H NMR spectrum was performed on an Avance III 400 NMR spectrometer in 2 wt% DCl/D_2O. The degree of deacetylation was calculated by analyzing the integrals of the representative proton peaks in chitosan's NMR spectrum.

The molecular weight of chitosan was determined by the capillary viscometry method using an Ubbelohde viscometer at 25 °C. Five concentrations were prepared by diluting the chitosan in 0.3 M acetic acid/0.2 M sodium acetate solution. The specific viscosity η_{sp} of chitosan solution was measured at different concentrations. The intrinsic viscosity $[\eta]$ was obtained by extrapolating the linear plot of η_{sp}/C *versus* C (concentration) to the zero concentration. The viscosity average molecular weight (M) was then calculated using Mark–Houwink equation $[\eta] = KM^{\alpha}$, in which the constants K and α depend on the degree of deacetylation according to the literature (Brugnerotto, Desbrieres, Roberts, & Rinaudo, 2001). The moisture and ash contents were determined by the thermogravimetric analyzer SDT Q600 (TGA) with a heating ramp of 20 °C/min to 800 °C (20 min isothermally).

Equilibrium water binding capacity (WBC) of chitosan was measured using a modified method of Wang and Kinsella (Wang & Kinsella, 1976; Kucukgulmez et al., 2011). Water absorption was initially carried out by weighing a centrifuge tube containing 0.1 g of sample, adding 2 mL of deionized water, and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 24 h with shaking for 5 s every 10 min and centrifuged at 3500 rpm for 25 min. After the supernatant was decanted, the tube was weighed again. WBC was calculated as follows: WBC (%) = [water bound (g)/sample weight (g)] × 100. To monitor the relationship of WBC with the incubation time, 0.1 g samples of chitosan powder were placed in small bags of nonwoven materials (Bidgoli, Zamani, & Taherzadeh, 2010). Then, the bags