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Porous chitosan microspheres containing zinc ion for enhanced thrombosis and hemostasis

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ABSTRACT

Quick hemostats for non-lethal massive traumatic bleeding in battlefield and civilian accidents are important for reducing mortality and medical costs. Chitosan (CS) has been widely used as a clinic hemostat. To enhance its hemostatic efficiency, Zn^{2+} in the form of zinc alginate (ZnAlg) was introduced to CS to make porous CS@ZnAlg microspheres with ZnAlg component on the surface. Such microspheres were prepared by successive steps of micro-emulsion, polyelectrolyte adhesion, and thermally induced phase separation. Their structure and hemostatic performance were analyzed by SEM, FT-IR, XPS and a series of *in vitro* hemostatic experiments including thromboelastography analysis. The composite microspheres had an outer and internal interconnected porous structure. Their size, surface area, and water absorption ratio were *ca.* 70 μm, 48 m^2/g, and 1850%, respectively. Compared to the neat chitosan microspheres, the CS@ZnAlg microspheres showed shorter onset of clot formation, much faster *in vitro* and *in vivo* whole blood clotting, bigger clot, less blood loss, and shorter hemostatic time in the rat liver laceration and tail amputation models. The synergetic hemostatic effects from (1) the electrostatic attraction between chitosan component and red blood cells, (2) the activation of coagulation factor XII by Zn^{2+} of zinc alginate component, and (3) physical blocking by microsphere matrix, contributed to the enhanced hemostatic performance of CS@ZnAlg microspheres.

1. Introduction

Traumatic bleeding is a major cause of military and civilian casualty and disability. After massive hemorrhage, blood transfusion may cause possible complications such as coagulation disorders, infection and multiple organ failure, in addition to the high medical costs. Therefore, a timely hemostasis treatment of the bleeding trauma is extremely important for reducing blood loss, increasing survival ratio, and embracing optimal recovery [1]. The autopsy report of Iraqi and Afghan wars indicated that nearly 24% of the war deaths can be avoided by effective hemostatic treatments [2]. Therefore, the development of efficient hemostats in various forms such as sponge, fabric, hydrogel, particle, and adhesive has attracted many research interests [3–5].

Many topical hemostats such as protein and gelatin (Floseal®) [6], inorganic molecular sieves (QuikClot®) [7], and oxidized cellulose (Surgicel®) [8] have been developed and commercialized. Each has pros and cons. For example, protein and gelatin have strong hemostatic ability, but they are expensive, and are prone to immune and antigenic reactions. QuikClot zeolite powder is effective for external use in massive bleeding trauma, but it can cause severe burns to wounded tissues due to strong exothermic hydration [9]. Among many hemostatic materials reported so far, chitosan (CS), a cationic naturally-occurring polysaccharide, appears to be the most promising hemostat in terms of price, cell and tissue compatibility, toxicity, biodegradability, antimicrobial activity, and hemostatic performance [10]. Therefore, many chitosan-based hemostats have been clinically used in brand-names of HemCon and Celox *etc.* The main hemostatic mechanism of chitosan is that it carries a certain amount of positive charge, which promotes erythrocyte aggregation and increase platelet adhesion [11]. However, the hemostatic efficiency of chitosan has not yet met the need for severe bleeding wounds [12]. Hence how to enhance its hemostatic potential has gotten much research attention. Blending chitosan with other clotting-active polymers such as alginate (SA) is a conventional method [13–15].

SA is an anionic natural polymer containing blocks of β-(1,4)-D-mannuronate (M) and α-L-guluronate (G) units. Its complex compound

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with Ca^{2+} is a well-studied biodegradable and non-cytotoxic *in vitro* and *in vivo* hemostat [16–18]. But in fact, (i) next to iron, Zn^{2+} is the second most abundant metal in blood; (ii) In the process of clotting, activated platelets release Zn^{2+}; and (iii) Zn^{2+} concentration increases in the vicinity of thrombus [19]. These evidences suggest that Zn^{2+} is essential to blood coagulation and thrombosis.

In a previous work we successfully improved the hemostatic efficacy of chitosan by introducing pore structure into chitosan particles [20]. Since blood clotting is a complex process including biological, chemical and physical reactions, functioning together of two or more hemostatic mechanisms instead of a merely morphological change would substantially improve the hemostatic efficacy of chitosan [21]. Here we aim to improve chitosan's hemostatic potential through introducing Zn^{2+} (in the form of zinc alginate (ZnAlg)) into chitosan microspheres. We combine the oppositely charged chitosan and zinc alginate into one porous microsphere matrix through electrostatic attraction. Both raw materials are abundantly available, economical, biocompatible, biodegradable, and non-toxic. The two components in the composite hemostat are supposed to play synergistic clotting ability to accelerate thrombosis and hemostasis.

2. Materials and methods

2.1. Material

Chitosan (degree of deacetylation ≥ 95%, viscosity 100–200 mpa·s) was purchased from Aladdin Co. China. Sodium alginate, zinc chloride (AR), acetic acid (AR), Span 80 (CP), Tween 60 (CP), petroleum ether (AR), anhydrous ethanol (AR), sodium hydroxide (AR), glutaraldehyde (GA, 25% aq. solution, AR), and xylene (AR) were bought from Sinopharm Chemical Reagent Co. Ltd. Sprague-Dawley (SD) rats with weight of *ca.* 200–250 g were purchased from Shanghai Slack Experimental Animal Co., Ltd., China (License number: SCXK (Shanghai) 2007–0005). The mouse embryonic fibroblast (MEF) cells were derived from Sprague-Dawley (SD) rats embryos in our laboratory. The animal care and study followed the rules administered by the Research Animal Ethics Committee of Fujian Normal University.

2.2. Preparation of chitosan microsphere (CSMS) and CS/ZnAlg composite microsphere (CS@ZnAlg)

The fabrication of CSMS was carried out according to our previous work [20]. Briefly, 1 w/v% chitosan solution was prepared by dissolving pre-calculated amount of chitosan in 1 v/v% aq. acetic acid solution. It was used as a disperse phase. 1 g of NaOH was dissolved in 150 mL of water/ethanol (1:14, v/v) to make an inverse solution. It was pre-cooled at − 20 °C. An emulsifier mixture of Tween60/Span80 (0.2/2.4, w/w) was added to petroleum ether to form a continuous phase solution with a total emulsifier content of 5 w/v%. The disperse phase was added dropwise to the continuous phase under magnetic stirring (1200 rpm) and at 40 °C for 3 h to obtain a w/o emulsion. It is then poured into liquid nitrogen to let the microemulsion droplets harden through phase separation. Then the pre-cooled inverse solution was added to regenerate chitosan microspheres. The microspheres were washed successively with water and ethanol several times and dried in a vacuum oven at 50 °C, and were labeled as CSMS.

For preparation of CS@ZnAlg microspheres, 1 w/v% SA and 2 w/v % $ZnCl_2$ aq. solutions were prepared separately. 1 w/v% CS solution was prepared by dissolving CS in 0.34 w/v% aq. acetic acid solution. It was used as a disperse phase. The coagulant of 1 w/v% NaOH/water-ethanol solution (water:ethanol = 1:14, v/v) was pre-cooled to − 20 °C. Petroleum ether with 5 w/v % Tween 60/Span 80 (0.2:2.4, v/v) was used as a continuous phase. The composite microspheres were prepared according to the scheme shown in Fig. 1. The CS solution was added dropwise to the continuous phase at 30 °C and stirred at 1200 rpm for 1 h to obtain a w/o emulsion. Into it the SA solution was dropwise added and stirred at 30 °C for 1 h. Then 4 mL of $ZnCl_2$ solution was dripped to the emulsion and stirred for another 1 h. The emulsion was frozen at − 20 °C for 2.5 h, followed by addition of the pre-cooled coagulant. The microspheres were washed successively with water and ethanol several times and dried in a vacuum oven at 50 °C. The white powder product was labeled as CS@ZnAlg.

2.3. Characterization

The surface and cross-section morphology was observed on a scanning electron microscopy (SEM) (S-3400N, Hitachi, Japan). All samples were sputter-coated with platinum at 3 mA for 150 s. The cross-sectional morphology was obtained by cutting the microsphere with a surgical knife. The images were analyzed with Smileview (v. 2.0) software. The statistical average values were reported for measurement of at least 100 microspheres at different locations. The energy dispersive X-ray spectroscopy analysis (EDS) was performed on Oxford INCA 250 Energy dispersive X-ray spectrometer (UK).

The porosity was determined *via* a liquid displacement method with ethanol as the displacement liquid [22]. Into a pycnometer filled with absolute ethanol (M_1), about 0.01 g (M_s) of sample was added. The bottle was vacuumed slowly until the air in the microsphere was fully removed and replaced with ethanol. The bottle was refilled with ethanol and was weighed as M_2. Then the microsphere saturated with ethanol were collected by carefully discarding the ethanol, the remaining ethanol on the microsphere surface was sucked by filter paper. The pycnometer with wet microsphere was weighed as M_3. The volume of the microsphere was $V_s = (M_1 - M_2 + M_3 - M)/\rho$ (ethanol). The density (ρ) and porosity (P) was calculated by Eqs. (1) and (2). Three parallel measurements were run for each sample.

$$\rho = \frac{M_s \rho_{C_2H_5OH}}{M_1 + M_3 - M - M_2} \quad (1)$$

$$P = \frac{V_p}{V_p + V_s} = \frac{M_3 - M - M_s}{2(M_3 - M) - M_s + M_1 - M_2} \times 100\% \quad (2)$$

A certain amount of dry microsphere (M_d) was immersed into phosphate buffer saline (PBS, pH 7.4) solution. The glass beaker containing the mixture was evacuated for 3 min to allow solution fill into the microsphere pores, then stood for 24 h. Subsequently, the PBS solution was discarded and the excess PBS was sucked by filter paper. The mass of the wet microsphere was measured (M_w). Triplets were run for each sample. The water absorption ratio (A) was calculated by Eq. (3):

$$A = \frac{M_w - M_d}{M_d} \times 100\% \quad (3)$$

The specific surface area was determined from N_2 adsorption-desorption isotherm at 77 K on a Belsorp-Max analyzer (Japan) by the Brunauer Emmett Teller (BET) method. The samples were degassed at 105 °C for 12 h before measurement.

Thermogravimetric analysis was performed on Mettler Toledo TGA 50 (Switzerland) by heating from 30 °C to 600 °C under nitrogen at 10 °C/min.

FTIR spectra in wavelength range of 400–5000 cm^{-1} were recorded on a Thermo Nicolet's Model 5700 (USA) Fourier Infrared Spectrometer (FTIR) using the KBr pellet method.

XPS analysis was run on an ESCALB 250 spectrometer (USA) by using a monochromatic Al Ka source with energy of 1486.6 eV and working power of 150 W. Energy correction was performed on the surface contamination of C1s (284.6 eV). Peaks were analyzed with nonlinear fitting software XPSPEAK 4.1.

2.4. In vitro and in vivo hemostasis analysis

2.4.1. In vitro pro-coagulant activity

A 2 mL disposable plastic tube with 10 mg of samples was placed in