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Preparation and evaluation of squid ink polysaccharide-chitosan as a wound-healing sponge

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ABSTRACT

A new type of wound healing agent was developed using two marine biomaterials (squid ink polysaccharide and chitosan) as carriers and calcium chloride as an initiator for coagulation. Based on central composite design-response surface methodology, comprehensive evaluation of appearance quality for composite sponges and water absorbency were used as evaluation indices to identify the optimized preparation conditions and further evaluate the performance of the squid ink polysaccharide-chitosan sponge (SIP-CS). The optimized formulation of SIP-CS was as follows: chitosan concentration, 2.29%; squid ink polysaccharide concentration, 0.55%; and calcium chloride concentration, 2.82%, at a volume ratio of 15:5:2. SIP-CS was conducive to sticking on the wound, characterized by the spongy property, strong absorptivity, and tackiness. Rabbit ear arterial, hepatic, and femoral artery hemorrhage experiments indicated that, compared with chitosan dressings and absorbable gelatin, the hemostatic times were shorter and the bleeding volume was smaller. Furthermore, SIP-CS absorbed a large amount of hemocytes, leading to rapid hemostasis. The healing areas and wound pathological sections in scalded New Zealand rabbits indicated that SIP-CS promoted wound healing more rapidly than chitosan and better than commercially available burn cream. Thus, SIP-CS is a good wound healing agent for rapid hemostasis, promoting burn/scalded skin healing, and protecting from wound infection.

1. Introduction

Uncontrolled aggressive bleeding is the main cause of death during wars, traffic accidents, and other accidents [1,2]. Safe and efficient hemostatic agents can effectively decrease mortality caused by hemorrhage. Thus, the production of wound healing agents has rapidly increased recently [3,4], and scholars recently have developed some antibacterial polymers material that resistant to multiple drug resistant strains [5,6]; however, the prices of high-quality sterile absorbable hemostatic agents [7] and medical materials that can promote wound-healing are very expensive [8,9]. In addition, large amounts of high molecular materials are generated during marine product processing, and their utilization is simple with low additional value, limiting marine economic development. To take full advantage of these abandoned marine resources, squid ink polysaccharide (SIP) and chitosan have been successfully extracted from squid ink and shrimp shells [10,11].

Squid ink has exhibited efficacy for the treatment of cardiodynia caused by bleeding in a medical book of ancient China, *Compendium of Materia Medica*. Squid ink has had good efficacy in various hemorrhagic symptoms in the clinic, including in gynecology, surgery, and internal medicine, without toxicity and side effects. Our preliminary data indicated that SIP could shorten the blood coagulation time and hemostatic time in mice and also activate coagulation factor FXII, which possessed significant pro-coagulant effect.

In order to inhibit microbial growth for preventing infections, antibacterial agent like chitosan is considered [12]. As a high molecular material generated during the processing of marine products, chitosan has good biocompatibility and antibacterial activity, and is also low cost [13,14]. The pro-coagulant activity of chitosan was first reported by Malette et al. [15]. Some current commercial hemostatic agents use chitosan as the main component, such as Celox powder [16] and HemCon ligature [17]; these agents are mainly used for emergency hemostasis in combat and civil accidents. However, it was reported that the hemostatic effects of chitosan dressing are limited when severe injury occurs, which may be related to the inhibitory effect of excessive

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positive charges in chitosan on coagulation [18]. Therefore, the hemostatic effects of repair agents based on chitosan still need further improvement.

The current study combined SIP and chitosan to decrease the negative influences of positive charge on chitosan and initiate the coagulation cascade, which could promote blood coagulation via synergistic effects. The composite sponges were obtained by lyophilization and the central composite design (CCD) response surface method was used to optimize the preparation. SIP-CS with a promising application and excellent performance was obtained. To evaluate the effects of the agent, hemorrhage animal models were generated and the hemostatic effects were compared with commercial chitosan dressing (CS) and absorbable gelatin sponge (GS). Meanwhile, a scald model was used to compare the pro-would healing effects of SIP-CS, CS, and burn cream [19].

2. Materials and methods

2.1. Materials

SIP and chitosan were made in our laboratory [20,21], and the deacetylation degree of chitosan was higher than 90%. CS was purchased from Keji Medical Equipment Co., Ltd. (Guangzhou, Guangdong, China). GS was obtained from Xiangen Medical Technology Development Co., Ltd. (Nanchang, Jiangxi, China). Medical gauze was purchased from Zhende Medical Co., Ltd. (Shaoxing, Zhejiang, China). Burn cream (abbreviated MEBO) was purchased from MEBO Pharmaceuticals Co., Ltd. (Shantou, Guangdong, China). Hematoxylin, eosin Y, and neutral balsam were obtained from Shanghai Yuanye Biotechnology Co., Ltd. Section paraffin was purchased from Hualingkangfu (Shanghai, China).

2.2. Preparation of SIP-CS

To make solution A, chitosan was dissolved at the designated concentrations in 1% acetic acid. For solution B, SIP was dissolved at the designated concentrations in DI water. Fifteen milliliters of solution A were added into a 50-mL beaker, and then 5 mL of solution B was added slowly with stirring. Two milliliters of calcium chloride was added to the beaker, and was further stirred to make a homogenous solution. The solution was then poured into a culture dish (diameter, 6 cm) until a gel formed. Then, the culture dish was transferred to − 20 °C for 12 h, and lyophilized for another 16 h to obtain a porous spongy solid (SIP-CS).

2.3. Evaluation standard for SIP-CS

We first investigated the influences of chitosan molecular weight, chitosan concentration, SIP concentration, the ratio of solution A to solution B, and calcium chloride concentration on SIP-CS preparation. The evaluation standards included two parts.

Part one was the appearance quality score of the sponge (Y_1), including gross appearance, surface features, and morphology, with a maximum total score of 50. 1) Gross appearance had a maximum score of 20, with 10 points each for the shape and color of the sponge. A smooth circular shape was scored 10; if the shape was not circular, showing depressions or bumps, points were deducted from the score, with the minimum score being 1. A uniform and white color was scored 10. If the color was yellowish or non-uniform, points were deducted from the score; more points were deducted for the more yellowish sponge, with the minimum score being 1. 2) Surface features had a maximum score of 10. A surface with a consistent density, spongy shape, light texture, high elasticity, and good handle was scored 10. Any obvious cracks, uneven pore thickness on the surface, and poor elasticity led to a deduction of points, with the minimum score being 1. 3) Morphology, as observed by a biological microscope (B203LED, Chongqing Optec Instrument Co., Ltd), had a maximum score of 20. The surface structure of a thin layer from the surface of sponge was observed. A network structure with uniformly distributed open pores was scored20. If the network structure was not evident or the pores were not uniformly distributed, points were deducted from the score, with the minimum score being 1.

Part two was water absorbency (Y_2). A piece of sponge (1 cm × 1 cm) was weighed (W_1) and then immersed in water (20 °C). After fully absorbing water, the sponge was lifted out of the water using tweezers, and weighed after draining naturally for 30 s (W_2). Water absorbency was calculated as $(W_2 - W_1)/W_1$.

Table 1
Central composite design and results.

Serial number	*A*/%	*B*%	*C*%	Y_1	Y_2
1	2.25 (0)	0.60 (0)	5.50 (0)	44	8.83
2	2.25 (0)	0.60 (0)	5.50 (0)	45	9.68
3	3.29 (1)	0.84 (1)	8.18 (1)	41	10.1
4	2.25 (0)	0.60 (0)	5.50 (0)	44	10.5
5	2.25 (0)	1.00 (1.682)	5.50 (0)	39	6.03
6	2.25 (0)	0.60 (0)	5.50 (0)	38	11.5
7	2.25 (0)	0.20 (− 1.682)	5.50 (0)	35	9.99
8	1.21 (− 1)	0.84 (1)	2.82 (− 1)	35	30.0
9	4.00 (1.682)	0.60 (0)	5.50 (0)	40	9.25
10	2.25 (0)	0.60 (0)	5.50 (0)	38	12.2
11	1.21 (− 1)	0.36 (− 1)	8.18 (1)	31	10.1
12	2.25 (0)	0.60 (0)	1.00 (− 1.682)	45	62.6
13	3.29 (1)	0.36 (− 1)	8.18 (1)	33	9.50
14	1.21 (− 1)	0.84 (1)	8.18 (1)	32	11.9
15	3.29 (1)	0.36 (− 1)	2.82 (− 1)	36	18.1
16	0.50 (− 1.682)	0.60 (0)	5.50 (0)	28	7.38
17	3.29 (1)	0.84 (1)	2.82 (− 1)	37	14.8
18	2.25 (0)	0.60 (0)	10.00 (− 1.682)	43	6.24
19	2.25 (0)	0.60 (0)	5.50 (0)	40	10.5
20	1.21 (− 1)	0.36	2.82 (− 1)	36	34.3

2.4. Optimizing the sponge preparation by CCD

Next, chitosan concentration (Factor A), SIP concentration (Factor B), and calcium chloride concentration (Factor C) were investigated to further optimize the preparation conditions. A total of 20 combinations (an orthogonal experiment of three factors and five levels) were generated by Design-Expert 8.0, using Y_1 and Y_2 as evaluation indices (Table 1).

2.5. Animal experiments

One hundred and ten New Zealand rabbits (half male and half female, conventional grade) weighing 1.5–1.8 kg before modeling were provided by Guangdong Medical Laboratory Animal Center. The rabbits were individually housed with free access to water and food under the following conditions: 20–26 °C temperature, 40%–70% humidity, and 10 h light/14 h dark cycles. All the rabbits were anesthetized with 3% pentobarbital sodium (30 mg/kg) via the ear vein. The operation was performed in accordance with the ARRIVE guidelines [22].

2.6. Evaluating hemostasis

Thirty New Zealand rabbits were used in an ear artery hemorrhage model and a hepatic hemorrhage model, and were divided into SIP-CS, CS, and GS groups (n = 10/group). Twenty rabbits were used to build the femoral artery hemorrhage model, and were randomly divided into the SIP-CS group and the CS group (n = 10/group). SIP-CS, CS, and GS were cut into pieces sized 2 cm × 2 cm × 0.4 cm, weighed on sterile medical gauze, and recorded as m_1.

2.6.1. Ear artery hemorrhage model

After anesthesia, the ears were de-haired and sterilized. A wound