Optimization of preparation process and characterization of carboxymethyl chitosan/sodium alginate hemostatic sponge

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Abstract. Composite hemostatic sponge was prepared by vacuum freeze-drying using carboxymethyl chitosan and sodium alginate as the main materials and CaCl\textsubscript{2} as a crosslinking agent. On the basis of single factor experiments, an orthogonal experiment was carried out to optimize the preparation process of hemostatic sponge. The appearance, water absorption, porosity ratio, and in vitro hemostasis of the sponge were evaluated. The optimum conditions to prepare hemostatic sponge were obtained as follows: mass ratio of sodium alginate to carboxymethyl chitosan 4:1, mass fraction of CaCl\textsubscript{2} 2\%, and crosslinking temperature 30\textdegree C. The hemostatic sponge prepared under such conditions was off-white and porous. Its water absorption and porosity ratio were 3050\% and 67.23\%, respectively. Meanwhile, the hemostatic sponges had significant in vitro procoagulant activity. Therefore, the hemostatic sponge is expected to be developed as a novel medical material.

1. Introduction
With a special porous structure which facilitates the discharge of wound secretions, sponge is widely used in surgical hemostasis in many aspects, such as neurosurgery, dentistry, otorhinolaryngology and gynecology [1-4]. Many polymer materials can be used to prepare sponge. Particularly, the development of biomedical sponge with natural biopolymer has attracted much attention from researchers.

Chitosan, a linear polysaccharide consisting of 2-acetamido-2-deoxy-D-glucose linked by β-1, 4-glycosidic bonds, is the only natural alkaline polysaccharides up to day. Owing to its excellent biocompatibility, biodegradability, antibacterial and accelerating wound healing properties, chitosan has been widely used in delivery system for drugs, wound dressing and scaffolds for tissue engineering [5-7]. Carboxymethyl chitosan (CMCS) is a modified derivative of chitosan with high water solubility, and has great potential for application in the biomedical field [8-9].

Sodium alginate (SA), a seaweed polysaccharide extracted from brown algae, has good biocompatibility and hemostatic function and has been widely applied in tissue engineering [10-11]. Additionally, SA is particularly suitable for the treatment of burns and mechanical trauma due to its strong water absorptivity and gelation ability, which can relieve pain and prevent secondary bleeding from the wound by forming gels on the wound surface.
In this study, the composite sponge was prepared by freeze-drying using CMCS and SA as the matrix materials. The preparation conditions were optimized with water absorption as an evaluation index, and the related properties were examined to develop a new medical sponge material with excellent hemostasis.

2. Materials and methods

2.1. Materials
CMCS (degree of substitution, 63.5%) was purchased from Zhejiang Aoxing Biotechnology Co., Ltd (China). SA (chemically pure) was purchased from Zhengzhou Jianda Chemical Products Co., Ltd (China). The other reagents were of analytical grade.

2.2. Preparation of sponge
3% CMCS and 3% SA solutions were prepared, respectively, and then mixed at a certain mass ratio. CaCl$_2$ solution was slowly added at a 1/5 (v/w) ratio under stirring. Then, 10 g of the mixture was dispensed into a 5-cm glass culture dish and cross-linked at a constant temperature for 2 h. The dishes were then frozen for 24 h in a refrigerator at -20°C and dried in a vacuum freeze dryer for 18 h to obtain porous composite sponges.

2.3. Determination of water absorption
The sponge was placed in a desiccator containing anhydrous CaCl$_2$ and dried at room temperature to constant weight ($W_0$), and then immersed in distilled water to fully swell for 10 min. Afterwards the sponge was taken out from the water and weighed ($W_1$) after surface water was removed with filter paper. The water absorption was calculated as follows:

$$\text{Water absorption (\%)} = \frac{(W_1 - W_0)}{W_0} \times 100\%$$

where $W_0$ and $W_1$ are the mass of the sponge before and after water absorption, respectively, in g.

2.4. Morphological observation
The appearance and pore structure of the sponge were observed by a DSC-TX10 digital camera (SONY Corporation, Tokyo, Japan) and a scanning electron microscope (SEM) JSM-6330F (JEOL Corporation, Tokyo, Japan).

2.5. Porosity ratio determination
A 50-mL volumetric flask was filled with ethanol to the calibration line, and weighed as $W_1$. A piece of sponge was weighed as $W_0$. The sponge was placed in the flask containing 2/3 volumetric ethanol and degassed to allow ethanol to penetrate into the pores of the sponge. And then ethanol was again added to the calibration line. The flask containing ethanol and sponge was weighed as $W_2$. Afterwards, the sponge soaked with ethanol was taken out and the flask containing the remaining ethanol was weighed as $W_3$. The porosity ratio of the sponge was calculated as equation (2):

$$\text{Porosity ratio (\%)} = \frac{(W_2 - W_3 - W_0)}{(W_1 - W_3)} \times 100\%$$

2.6. Procoagulant activity in vitro
The in vitro procoagulant activity of the sponge was determined according to the literature method [12]. The specific operations are as follows: 0, 20, 60, 100, 140, 180 mg of sponge fragments were placed in clean test tubes, respectively. The tubes were then placed in a water bath at a constant temperature of 37°C for 1 h. The anticoagulated rabbit blood (1 mL) was added to each of the above test tubes in turn and kept in the water bath. The tubes were tilted at 30° once every 30 seconds until the blood in the tubes did not flow. The coagulation times of blood in different tubes were recorded.

2.7. Statistical analysis
All the experiments were parallel performed in triplicate and the mean values were presented. All statistical analyses were performed using Statistica 7.0 (StatSoft Inc., USA).

3. Results and discussion

3.1. Single factor experiments

3.1.1. Effect of SA/CMCS mass ratio on water absorption of the sponge. The SA and CMCS were mixed at a mass ratio of 1: 1, 2: 1, 3: 1, 4: 1, and 5: 1, respectively. CaCl$_2$ solution with a mass fraction of 1.0% was slowly added at a 1/5 (v/w) ratio under stirring. Next, 10 g of the mixture was dispensed into a 5-cm glass culture dish and cross-linked at 25°C for 2 h. The dishes were then incubated for 24 h in a refrigerator at -20°C and dried in a vacuum freeze dryer for 18 h to obtain the sponge. The water absorption of the sponge was measured and the results were shown in Figure 1. The water absorption increased with the increase of the SA proportion, and peaked at a SA/CMCS mass ratio of 4: 1. Afterwards the water absorption decreased with further increase of the SA proportion. Therefore, three levels of SA/CMCS mass ratio of 3: 1, 4: 1, and 5: 1 were used for optimization of sponge preparation by orthogonal experiment.

![Figure 1](image1.png)

**Figure 1.** Effect of SA/CMCS mass ratio on water absorption of the hemostatic sponge.

3.1.2. Effect of mass fraction of CaCl$_2$ on water absorption of the sponge. The sponge was prepared at a SA/CMCS mass ratio of 3: 1 and 25°C with mass fraction of CaCl$_2$ 1.0%, 2.0%, 3.0%, 4.0% and 5.0%, respectively. The water absorption was measured and the results were shown in Figure 2. The water absorption of the sponge peaked at mass fraction of CaCl$_2$ 2.0%, and then decreased sharply with further increase of the mass fraction of CaCl$_2$. This may be because the too high calcium chloride concentration led to a high degree of cross-linking in the sponge, thus resulting in tightness of the sponge and consequently decrease in water absorption. Therefore, the hemostatic sponge preparation was optimized by orthogonal experiment with mass fraction of CaCl$_2$ of 1.0%, 2.0% and 3.0%.
3.1.3. Effect of cross-linking temperature on water absorption of the sponge. The sponge was prepared by cross-linking for 2 h on standing at 25, 30, 35, 40, 45, and 50°C, respectively, with SA/CMCS mass ratio of 3:1 and 1.0% CaCl$_2$ solution. The water absorption was measured and the results were shown in Figure 3. The water absorption of the sponge peaked at 35°C. It is because too high temperature will accelerate cross-linking and result in an excessive cross-linking degree, thus narrowing the pores in the sponge. Therefore, optimization of the hemostatic sponge preparation was carried out at 30, 35 and 40°C.

3.2. Optimization of the hemostatic sponge preparation
Based on the results of the single factor experiments, an orthogonal experiment was designed with three factors and three levels by using water absorption as an evaluation index to optimize the
hemostatic sponge preparation. The factors and levels are listed in Table 1 and the experiment design and results are shown in Table 2.

**Table 1.** Factors and levels for orthogonal experiment.

|    | 1      | 2      | 3      |
|----|--------|--------|--------|
| A  | SA/CMCS mass ratio | 3:1    | 4:1    | 5:1    |
| B  | Mass fraction of CaCl₂ (%) | 1.0    | 2.0    | 3.0    |
| C  | Cross-linking temperature (°C) | 30     | 35     | 40     |

**Table 2.** Design, results and range analysis of L₉(3⁴) orthogonal experiment.

| Run | Factors | Water absorption (%) |
|-----|---------|----------------------|
|     | A       | B        | C        |                     |
| 1   | 1       | 1        | 1        | 1907                |
| 2   | 1       | 2        | 2        | 2052                |
| 3   | 1       | 3        | 3        | 2019                |
| 4   | 2       | 1        | 2        | 2087                |
| 5   | 2       | 2        | 3        | 2991                |
| 6   | 2       | 3        | 1        | 2166                |
| 7   | 3       | 1        | 3        | 1531                |
| 8   | 3       | 2        | 1        | 2644                |
| 9   | 3       | 3        | 2        | 2020                |
| k1  | 1993    | 1842    | 2239    |
| k2  | 2415    | 2562    | 2053    |
| k3  | 2065    | 2068    | 2180    |
| R   | 422     | 720     | 186     |                      |

Range analysis revealed that the ranking order of the factors influencing the water absorption of hemostatic sponge was B＞A＞C. In order words, mass fraction of CaCl₂ had the greatest influence, followed by SA/CMCS mass ratio and cross-linking temperature. According to the results of orthogonal experiment, the optimum preparation conditions were A₂B₂C₁, which was that SA/CMCS mass ratio 4: 1, mass fraction of CaCl₂ 2% and cross-linking temperature 30°C. As confirmed by three parallel experiments, the average water absorption of hemostatic sponge prepared under the optimum conditions was 3050%, representing strong water absorption capacity.

**3.3. Morphology Studies**

Macroscopic observation showed that the hemostatic sponge was off-white, tough and porous, meanwhile, it became swollen and soft after being soaked in water. SEM showed a clear reticular structure, pores with a uniform size and appropriate density (Figure 4).
Figure 4. The optical microscopy (a) and SEM micrograph (b) of the hemostatic sponge.

3.4. Sponge porosity ratio
The porosity ratio of the hemostatic sponge was investigated and the results were shown in Table 3. The average porosity ratio was 67.23% and the relative average deviation was 1.55%. The hemostatic sponge with a high porosity ratio provides enough pores and reticular space to absorb more blood. Moreover, it provides great contact surfaces between the sponge and the wound, which is conducive to the interaction between hemostatic materials and coagulation factors resulting in rapid hemostasis [13].

|                | 1         | 2         | 3         |
|----------------|-----------|-----------|-----------|
| $W_0$ (g)      | 0.013     | 0.018     | 0.016     |
| $W_1$ (g)      | 56.52     | 56.52     | 56.52     |
| $W_2$ (g)      | 56.43     | 56.42     | 56.43     |
| $W_3$ (g)      | 56.22     | 56.15     | 56.19     |
| Porosity ratio (%) | 65.67  | 68.11     | 67.91     |
| Average porosity ratio (%) | 67.23 |  |  |
| Relative average deviation (%) | 1.55 | | | |

3.5. In vitro procoagulant effect of the hemostatic sponge
Coagulation time is the time required for blood to clot outside of the body, which is an important indicator of procoagulant activity of the hemostatic materials. As shown in Table 4, the addition of sponge decreased the coagulation time of anticoagulated rabbit blood, indicating that the hemostatic sponge had significant procoagulant activity. Moreover, the coagulation time was significantly decreased with the increase of the sponge, demonstrating a dose-dependent relationship.

| Mass of sponge (mg) | 0 | 40 | 60 | 100 | 140 | 180 |
|---------------------|---|----|----|-----|-----|-----|
4. Conclusions
The composite porous sponge was prepared by freeze-drying using CMCS and SA as the matrix. Based on the results of single factor experiments, an orthogonal experiment was performed to optimize the preparation conditions to develop the hemostatic sponge with excellent performance as a novel medical biomaterial.

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