Fabrication and Characterization of Marine Polysaccharides Hemostatic Dressing

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Abstract. Marine polysaccharides composite hemostatic dressing was fabricated with calcium chloride as the crosslinking agent and carboxymethyl chitosan /sodium alginate as raw materials. On the basis of the pre-experiment, the fabrication process of marine polysaccharides hemostatic dressing was optimized by response surface methodology. The dressing prepared under the optimum conditions was characterized by scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR), and then its hemostatic performance was evaluated. The results showed that the optimum conditions for the preparation of hemostatic dressing were as follows: concentration of CMCS is 0.5%, mass ratio of CMCS and SA is 1: 2.5, concentration of CaCl₂ is 1.5%. And the swelling ratio of the dressing reached 8020%. FTIR showed that the crosslinking between carboxymethyl chitosan and sodium alginate was successful. Morphology observation found that the appearance was smooth, the pores were large and uniform. Furthermore, the dressing had good coagulation performance.

1. Introduction
Uncontrollable haemorrhage has always been an accidental accident and an urgent problem to be solved in surgery. It is also a major cause of death [1]. Rapid hemostasis has attracted extensive attention [2]. Currently, the hemostatic dressings mainly consist of gelatin [3], collagens [4], oxidized celluloses [5], chitosan [6], alginic acids [7]. The ideal hemostatic materials should be able to absorb quickly a large amount of exudate, protect the wound from bacterial infection, not cause wound re-wound, and be non-toxic [8]. As for the hemostasis of large wounds and arterial hemorrhages, the traditional hemostatic materials are not effective, therefore, it is urgent to develop a highly effective and safe hemostatic material.

Marine polysaccharide is a kind of polysaccharide extracted and purified from marine organisms. Because of the special growth environment of marine, there are obvious differences in structure and composition between marine polysaccharide and terrestrial polysaccharide. Therefore, marine polysaccharides have a lot of unique biological activities. Chitosan is a product obtained by deacetylation of chitin which is extracted from shrimp and crab shells. It is a natural, basic polysaccharide with many excellent properties, such as hemostasis, antibacterial and promoting wound healing. Additionally, due to its good biocompatibility, low cost, readily availability, it is extensively developed as a wound dressing [9]. Carboxymethyl chitosan (CMCS) is a product obtained by carboxymethylation of chitosan. Compared with chitosan, it has better water-solubility, antibacterial
and hemostatic effect [10]. Meanwhile, carboxymethyl chitosan is biodegradable and non-toxic [11] and has been widely applied in medicine, food and chemical fields [12]. Sodium alginate (SA) is a water-soluble colloidal polysaccharide extracted from marine brown algae, such as Laminaria japonica. It is widely used in medicine and food due to its good biocompatibility, histocompatibility and nontoxicity [13].

The dressing, made by cross-linking CMCS with SA, forms a spatial network structure and combines the characteristics of both marine polysaccharides to make it have a better bioactivity. Therefore, in this paper, the preparation optimization of the dressing was carried out by response surface methodology and the dressing was characterized and evaluated in order to develop a new efficient hemostatic material.

2. Materials and methods

2.1. Materials
Carboxymethyl chitosan, sodium alginate and anhydrous calcium chloride were all purchased from Sinopharm Chemical Reagent Co., Ltd (China). Unless otherwise specified, all the reagents were of analytical grade.

2.2. Fabrication of marine polysaccharide hemostatic dressing
The solutions of carboxymethyl chitosan and sodium alginate were prepared respectively and mixed evenly in different proportions. 2 ml of a certain concentration of calcium chloride solution was added, stirred well, poured into petri dishes, placed at -20 °C overnight and dried in a freeze dryer to obtain the dressing.

2.3. Determination of swelling ratio
Dry dressing was accurately weighed, soaked in 50 mL distilled water, immersed for 5 min, taken slowly out of the water and weighed again. Each group of tests is parallel 3 times and takes the average. The formula for the swelling ratio is as follows:

$$SR = \frac{(m_2-m_1)}{m_1} \times 100\%$$  \hspace{1cm} (1)

Where, SR is the swelling ratio, %; m_1 and m_2 are the mass of dressing before and after soaking, respectively, g.

2.4. Appearance observation
The dressing was observed with a digital camera and a scanning electron microscope. When using the S4800 scanning electron microscope to analyze the dressing, a black plastic was first applied to the copper table, and then a small amount of the sample was placed on the black plastic for gold spray treatment. The working voltage was 3 KV and the working distance was 8.5 mm.

2.5. Measurement of FTIR
Samples and potassium bromide were taken at the mass ratio of 1/100, ground under irradiation of an infrared lamp and compressed. The infrared spectrum was recorded with wave numbers ranged from 4000 to 500 cm^{-1}.

2.6. Dynamic coagulation assay
Samples (10 mg) were weighed and cut into 7 beakers and marked with 0 min, 5 min, 10 min, 15 min, 20 min, 25 min and 30 min. The beaker was placed in a 37 °C water bath for 5 min. 2 mL of anticoagulant rabbit blood was taken and added 100 ul of 0.2 mol/L CaCl_2 solution. And then 0.02 mL of the above blood was taken to the samples. Add 25 mL of distilled water to the beaker at the designated time, gently shake, and stand for 5 min to collect. The solution was measured for absorbance at 545 nm using UV spectrophotometry.
2.7. Determination of blood coagulation index (BCI)

Samples were cut into pieces with 0.5 cm * 0.5 cm size and put in 37 °C water bath. 0.1 mL of anticoagulant rabbit blood was taken and added 20 ul of 0.2mol/L CaCl₂ solution. After 5 min, 25 mL of distilled water was added, and the solution was shaken at 50 rpm for 5 min. The solution was measured for absorbance at 545 nm using UV spectrophotometry. The absorbance of 0.1 mL of whole blood in 25 mL of distilled water was used as a reference (Abs of blank). At the same time, the gelatin sponge available from the market was set as a positive control group. BCI was calculated as follows:

\[
\text{BCI} \% = \frac{A_{\text{sample}}}{A_{\text{blank}}} \times 100\% 
\]

Where \( A_{\text{sample}} \) and \( A_{\text{blank}} \) were for the absorbance of sample and blank, respectively.

3. Results and discussion

3.1. Response surface optimization analysis

On the basis of the pre-experiment of single factor, the fabrication process of hemostatic dressing was designed on Box-Behnken's central combinatorial experimental design principle and optimized by response surface methodology. The experimental design was listed in Table 1.

| Number | A: Concentration of CMCS (%) | B: Mass ratio of CMCS and SA | C: Concentration of CaCl₂ (%) | Y: Swelling rate (%) |
|--------|-----------------------------|-----------------------------|-------------------------------|---------------------|
| 1      | -1 (0.5)                    | -1 (1:1)                    | 0 (2)                         | 5294.93             |
| 2      | -1 (0.5)                    | 1 (1:3)                     | 0 (2)                         | 5594.81             |
| 3      | 1 (1.5)                     | -1 (1:1)                    | 0 (2)                         | 5044.08             |
| 4      | 0 (1)                       | 0 (1:2)                     | 0 (2)                         | 5263.58             |
| 5      | 1 (1.5)                     | 0 (1:2)                     | 1 (2.5)                       | 5090.34             |
| 6      | 0 (1)                       | -1 (1:1)                    | 1 (2.5)                       | 4606.76             |
| 7      | 0 (1)                       | 0 (1:2)                     | 0 (2)                         | 6602.69             |
| 8      | 0 (1)                       | 0 (1:2)                     | 1 (2.5)                       | 4686.20             |
| 9      | -1 (0.5)                    | 0 (1:2)                     | -1 (1.5)                      | 8328.61             |
| 10     | 0 (1)                       | 0 (1:2)                     | 0 (2)                         | 6303.36             |
| 11     | -1 (0.5)                    | 0 (1:2)                     | 1 (2.5)                       | 4696.09             |
| 12     | 0 (1)                       | -1 (1:1)                    | -1 (1.5)                      | 6404.85             |
| 13     | 1 (1.5)                     | 0 (1:2)                     | -1 (1.5)                      | 7529.96             |
| 14     | 0 (1)                       | 0 (1:2)                     | 0 (2)                         | 5933.1              |
| 15     | 0 (1)                       | 1 (1:3)                     | -1 (1.5)                      | 8087.85             |
| 16     | 0 (1)                       | 0 (1:2)                     | 0 (2)                         | 6453.02             |
| 17     | 1 (1.5)                     | 1 (1:3)                     | 0 (2)                         | 6210.53             |

The swelling ratio was taken as the assessment index (Y) and the results were presented in Table 1. A regression equation (3) was established by using response surface software and the analysis of variance for regression was also obtained in Table 2.

\[
Y=6111.15-4.85A+403.50B-1408.99C+216.46AB+298.23AC-0.89BC-55.02A^2-519.86B^2+355.12C^2
\]

As shown in the Table 2, the \( R^2 \) value of the model is 0.9378, indicating that the experimental factor is not a simple linear relationship to the response value, and the adjustment coefficient (\( R^2_{\text{adj}} \)) of the regression model was determined to be 0.8578, which indicates that the model can explain the change of 85.78% response surface. The model's \( p \) value is 0.0019 (\( p <0.01 \)), which shows that the model is
highly significant. And the lack of fit is 0.8849 (\(p > 0.05\)), indicating that the fitting equation is reliable. From the regression equation of the model, the \(F\)-value of the first-order coefficient in the equation is \(C > B > A\). Therefore, calcium chloride concentration is the primary factor that has a significant effect on the swelling ratio.

Table 2. Analysis of variance for regression

| Source of variance | Sum of square | Degree of freedom | Mean square | \(F\)-value | \(P\)-value |
|--------------------|---------------|------------------|-------------|-------------|------------|
| Model              | 1.998E+007    | 9                | 2.220E+006  | 11.72       | 0.0019**   |
| \(A\)              | 188.08        | 1                | 188.08      | 9.931E-004  | 0.9757     |
| \(B\)              | 1.303E+006    | 1                | 1.303E+006  | 6.88        | 0.0343*    |
| \(C\)              | 1.588E+007    | 1                | 1.588E+007  | 83.86       | \(<0.0001**|
| \(AB\)             | 1.874E+005    | 1                | 1.874E+005  | 0.99        | 0.3530     |
| \(AC\)             | 3.558E+005    | 1                | 3.558E+005  | 1.88        | 0.2129     |
| \(BC\)             | 6.429E+005    | 1                | 6.429E+005  | 3.39        | 0.1080     |
| \(A^2\)            | 12746.69      | 1                | 12746.69    | 0.0067      | 0.8028     |
| \(B^2\)            | 1.138E+005    | 1                | 1.138E+005  | 6.01        | 0.0440*    |
| \(C^2\)            | 5.310E+005    | 1                | 5.310E+005  | 2.80        | 0.1380     |
| Residual           | 1.326E+006    | 7                | 1.894E+005  | —           | —          |
| Lack of fit        | 1.803E+005    | 3                | 60088.21    | 0.21        | 0.8849     |
| Pure error         | 1.146E+005    | 4                | 2.864E+005  | —           | —          |
| Cor. total         | 2.131E+007    | 16               | —           | —           | —          |

Notes: \(R^2= 0.9378, R^2_{adj}=0.8578\); * Significant, \(p<0.05\); ** Highly significant, \(p<0.01\)

The optimum process conditions were obtained by using the regression model as follows: concentration of CMCS 0.5%, mass ratio of CMCS and SA 1:2.56, and concentration of \(\text{CaCl}_2\) 1.5%. With the above conditions, the calculated swelling ratio was 8288%. In view of the practical operability, the modified conditions were concentration of CMCS 0.5%, mass ratio of CMCS and SA 1:2.5, and concentration of \(\text{CaCl}_2\) 1.5%. The verification experiments were carried out and the swelling ratio of the dressing was 8020%, which was close to the predicted value. The result demonstrated that the model of regression equation was of high accuracy and was adequate for the optimization.

3.2. Morphology observation

As shown in Figure 1(a), it can be found that the hemostatic dressing had a white spongy appearance and a smooth surface. From Figure 1(b), the dressing possessed a porous sheet-like structure with large and uniform pores, which was conducive to a large number of liquid absorption so as to achieve rapid hemostasis.
3.3. Infrared spectrum analysis
From the IR spectrum of CMCS (Figure 2a), the peak at 3417 cm⁻¹ was the stretching vibrations of hydroxyl and amino groups, 2916 cm⁻¹ belonged to -CH stretching vibration. The absorption peaks of the asymmetric and symmetrical stretching vibration of carboxyl group (-COOH) appeared at 1604 cm⁻¹ and 1426 cm⁻¹, respectively. From the IR spectrum of SA (Figure 2b), it could be found that the OH stretching vibration was at 3422 cm⁻¹, the -CH stretching vibration was at 2927 cm⁻¹, and the asymmetric and symmetrical stretching vibration peaks of carboxyl group were at 1613 cm⁻¹ and 1417 cm⁻¹, respectively. Compared the spectrum of the raw materials with that of dressing (Figure 2c), it was found that the peaks of carboxyl shifted to low wave numbers, demonstrating the strong intermolecular interactions between CMCS and SA.

3.4. Dynamic coagulation assay
The curve of the relationship between the time required to form solid blood clots after the hemostatic materials contact with blood and the absorbance of hemoglobin was presented in Figure 3. Generally, the greater the slope of the curve was, the faster the coagulation rate would be. From the curve in Figure 3, it could be found that the absorbance decreased significantly within 5 min, indicating that the hemostatic dressing could quickly absorb blood and play a vital coagulation effect. After 10 minutes, the absorbance curve tended to be stable, indicating that the blood coagulation process was basically
completed and solid blood clots were formed. This may be due to the formation of blood clot containing a large number of red blood cells, thereby reducing the absorbance of the liquid.

![Dynamic clotting curve](image.png)

**Figure 3. Dynamic clotting curve**

### 3.5. BCI analysis

BCI can be used to characterize the anticoagulant properties of materials, which was determined by the absorbance of hemoglobin solution at 545 nm. The smaller absorbance was, the better blood clotting was. Figure 4 presented the blood clotting performance of the marine polysaccharide dressing in vitro. Compared to the positive control (gelatin sponge), the marine polysaccharide dressing exhibited a better procoagulant effect. This was due to the excellent porosity and water absorption of the dressing.

![Blood clotting index (BCI, %)](image.png)

**Figure 4. The effects of the marine polysaccharide dressing on the BCI in vitro.**

### 4. Conclusions

In this study, a novel composite hemostatic dressing was successfully fabricated by using marine polysaccharides as the matrix materials. The preparation process was optimized by response surface methodology and the optimum conditions were obtained. The marine polysaccharide dressing was
characterized with smooth surface and large pores. Dynamic clotting assay demonstrated the dressing possessed excellent hemostatic performance. Therefore, the marine polysaccharide dressing was beneficial to the development and application of high-absorption hemostatic materials.

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