Optimized Preparation of Carboxymethyl Chitosan/Sodium Alginate Hemostatic Microcapsule

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Optimized Preparation of Carboxymethyl Chitosan/Sodium Alginate Hemostatic Microcapsule

Z Hu*, J Guang, S T Lu, Y Chen, S D Li and Y Cai

Department of Applied Chemistry, Faculty of Chemistry and Environmental Science, Guangdong Ocean University, Zhanjiang 524088, China

*E-mail: (Zhang HU)huzhangqyx@126.com.

Abstract. In order to prepare and optimize a novel and efficient hemostatic material, a hemostatic microcapsule was prepared by piercing granulation technique with carboxymethyl chitosan and sodium alginate as substrate materials. Three factors in the process (mass percentage of carboxymethyl chitosan, concentration of calcium chloride and pH value of cross-linking system) were investigated through single-factor experiment to explore the effect of experimental factors on water absorbance of hemostatic microcapsule. On the basis of single-factor experiment, orthogonal experiment was conducted to optimize the preparation process of hemostatic microcapsule. Optimized process conditions were obtained as following: mass percentage of carboxymethyl chitosan 25%, concentration of calcium chloride 3.0% and cross-linking system pH=9.0. Microcapsule with normal morphology, uniform particle size, good dispersibility and porous structure was prepared under these conditions. And its water absorbance ratio reached 16.17. In vitro procoagulant test showed that the microcapsule had satisfactory procoagulant effect and was in a dose dependent manner.

1. Introduction

In wars, traffic accidents and other accidents, traumatic hemorrhage might cause severe results such as wound infection, shock, coagulation disorder, sepsis, multi-organ failure or even death [1, 2]. Therefore, quick and effective hemostasis has become one of the research focuses in medicine today. With the development of biomedical engineering, more and more attention has been attracted to the research and application of various biological hemostatic materials. There are many kinds of local biological hemostatic materials commonly used in clinic, such as collagen, oxidized cellulose and fibrin glue. Though with advantages, each type of these traditional materials has its disadvantages too. For example, collagen materials, which are made from the skin of pig or cow, have species difference from human and are likely to cause allergy when used for human hemastasis. While oxidized cellulose dressing has coarse surface and poor adhesiveness and is likely to cause wound affection. The clinic application of these traditional hemostatic dressings is greatly limited by their disadvantages [3, 4]. The ideal hemostatic material should have the following combination characteristics: rapid hemostasis, biocompatibility, biodegradability, non-toxic side effects and cheap costs. However, there are no ideal materials to be found today. So, it is of great practical value to develop novel, safe and effective hemastatic materials.

Chitosan is the only basic polysaccharide found in nature at present. It is safe, non-toxic, biocompatible and biodegradable and has broad-spectrum antibacterial function. However, the

application fields of chistan are greatly limited by its poor water solubility [5, 6]. Carboxymethyl chitosan (CMCS), a carboxymethylation product of chitosan, is a safe and non-toxic material with satisfactory water solubility, film forming capacity and moisture absorption and retention. It is widely used in agriculture, food, chemical industry and medicine [7, 8].

Sodium alginate (SA) is a sodium salt of polyanionic polysaccharides extracted from natural brown alga and composed of mannuronic acid and guluronic acid units. For its unique physical and chemical properties and good biocompatibility, sodium alginate is widely applied in pharmaceutical preparation, tissue engineering, clinic therapy, cell culture and food processing [9, 10].

The clinical application of composite functional materials has been one of the most attractive research fields at present. This paper optimized the preparation of carboxymethyl chitosan/sodium alginate composite hemostatic microcapsule by piercing granulation technique and provides a reference for industrialized production.

2. Materials and methods

2.1. Materials

Carboxymethyl chitosan (degree of substitution, 63%) was donated by Lianjiang Taixing Marine Biotechnology Co., Ltd. Sodium alginate was purchased from Sinopharm Chemical Reagent Co., Ltd. Unless otherwise specified, the other reagents are analytical reagents.

2.2. Preparation of microcapsule

Hemostatic microcapsule was prepared by piercing granulation technique with carboxymethyl chitosan and sodium alginate as the substrate materials. Carboxymethyl chitosan solution and sodium alginate solution (2%, w/v) were prepared with distilled water, respectively. The two solutions were mixed and stirred well in specified proportions of carboxymethyl chitosan in the substrate materials. Anhydrous $CaCl_2$ powder was dissolved in distilled water to prepare $CaCl_2$ solution with certain mass concentration and its pH was adjusted with dilute acid and alkali solutions. Then $CaCl_2$ solution was placed on a magnetic stirrer and stirred at constant speed. The mixture solution of carboxymethyl chitosan and sodium alginate was slowly injected into $CaCl_2$ solution with a syringe. After continuously stirred for 30 min, the solution was allowed to stand, centrifuged, washed and freeze-dried to obtain microcapsule.

2.3. Water absorbance property of microcapsule

A certain amount of microcapsule was weighed, soaked in distilled water, allowed to swell for 2h, filtered with 150-mesh filters and the surface water was absorbed with filter paper and then weighed. Water absorption ratio was calculated with the following equation:

$$\text{Water absorption ratio} = (W_1\text{-}W_0)/W_0 \quad (1)$$

where W_0 and W_1 are the mass (g) of microcapsule before and after soaking in distilled water, respectively.

2.4. Observation of microcapsule morphology

Microcapsule was fixed on the specimen table with conductive adhesive and treated for conductive coating with vacuum ion sputter. Afterwards, the surface of the microcapsule was observed with a scanning electron microscope (SEM, Hitachi S-4800).

2.5. In vitro procoagulant effect of microcapsule

The in vitro procoagulant effect of microcapsule with different mass was investigated as described below. 80, 60, 40 and 20 mg of microcapsules were weighed and put into 4 test tubes, respectively, and another tube was used as blank control. The five tubes were warmed in water bath at 37 ^{0}C for 1h. One mL of fresh anticoagulant rabbit blood was added into each of the above five tubes, shaken well

and continuously warmed in water bath at 37 ^{O}C. The tubes were taken out to check per 30s until coagulation of blood. The coagulation time was recorded.

2.6. Statistical analysis
All the tests were parallel performed for three times and the mean values were given. All statistical analyses were performed using Statistica 7.0 (StatSoft Inc., USA)..

3. Results and discussion

3.1. Single- factor tests

3.1.1. Mass percentage of carboxymethyl chitosan. Carboxymethyl chitosan and sodium alginate mixed solutions were prepared with varied carboxymethyl chitosan percentage in the substrate material (0%, 25%, 50%, 75%, and 100% respectively) and were slowly dropped into 2% CaCl2 solution (pH=7.0) to obtain microcapsule. The water absorbance ratio of the microcapsule was measured and showed in Figure 1. As illustrated in Figure 1, water absorbance of microcapsule was significantly influenced by carboxymethyl chitosan mass percentage. The water absorbance ratio of microcapsule increased first and then decreased as the carboxymethyl chitosan mass percentage increased. It peaked at 11.53 when the carboxymethyl chitosan mass percentage increased to 25%. Sodium alginate, containing a great number of –OH, -COOH and -COO^-, can directly or indirectly form hydrogen bonding with water and thus has good water absorbance. Microcapsule prepared with sodium alginate alone could absorb water quickly and showed high water absorbance, but was partially cracked later. These showed that microcapsule prepared with sodium alginate alone had poor stability and addition of carboxymethyl chitosan may increase the stability of microcapsule. When the mass fraction of carboxymethyl chitosan reached 50% or even higher, the water absorption ratio decreased. It might be due to the formation of a denser structure by cross-linking with carboxyl and amino groups in carboxymethyl chitosan with calcium chloride.

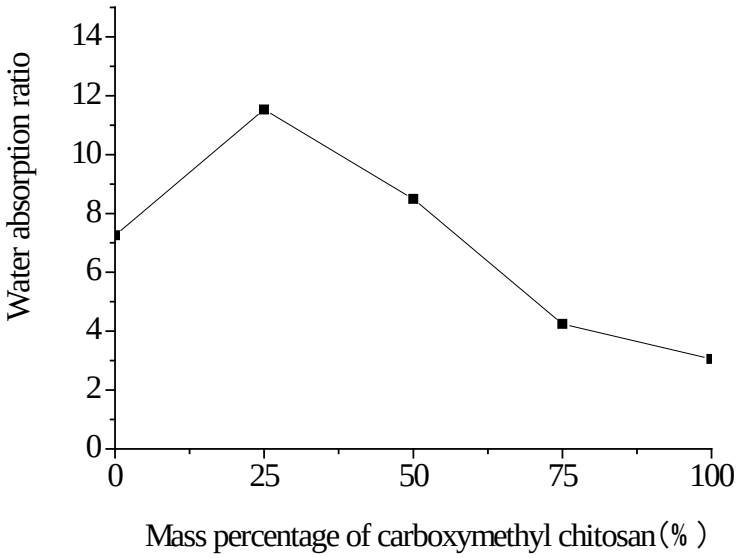


Figure 1. Effect of mass percentage of carboxymethyl chitosan on water absorption of microcapsule

3.1.2. Concentration of $CaCl_2$. Carboxymethyl chitosan/sodium alginate mixed solution was prepared with carboxymethyl chitosan in mass percentage 50% and then added dropwise to $CaCl_2$ solutions

(pH=7.0) with mass concentration of 1%, 2%, 3.0%, 4.5%, 6.0% and 7.5%, respectively to obtain microcapsules. As shown in Figure 2, the influence of $CaCl_2$ concentration on the water absorption of microcapsules increased first and then decreased, and finally tended to be stable. At a low concentration of $CaCl_2$, the water absorbance was low due to coacervation of a portion of microcapsules caused by association of hydrogen bonds between carboxylate and amino groups in molecule chains of polysaccharide when the speed of complexation of calcium ion and carboxylate ion was slow. As the $CaCl_2$ concentration increased, microcapsules were cross-linked with good dispersibility and loose mesh structure, which provided large and effective free space for water molecules [11]. However, with the concentration of $CaCl_2$ further increasing, the cross-linking density between molecular chains also increased, resulting in inhibiting movement of macromolecules, reducing mesh space, and constricting effective pathway for water molecules to permeate microcapsules [12]. When the concentration of $CaCl_2$ was further increased thereafter, as the quantity of free carboxylate ions that can be contacted was decreased dramatically, there was no significant change in the spatial structure of polymers, and thus the water absorbance ratio showed a flat trend.

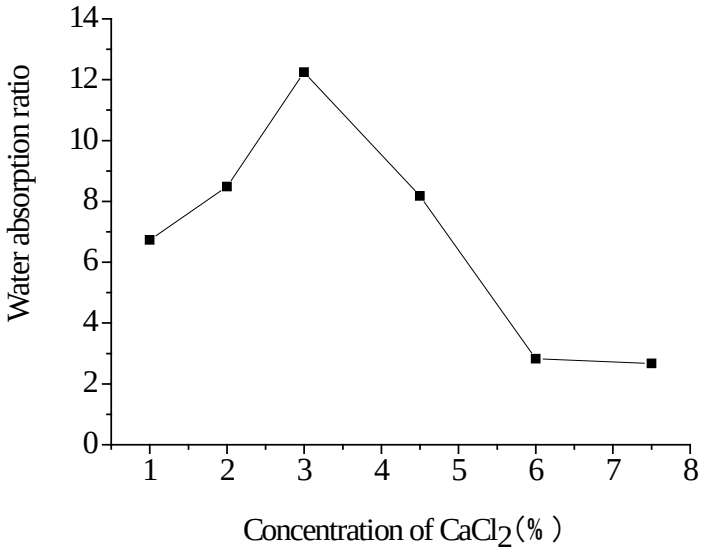


Figure 2. Effect of concentration of $CaCl_2$ on water absorption of microcapsule

3.1.3. Cross-linking system pH. Carboxymethyl chitosan/sodium alginate mixed solution was prepared with carboxymethyl chitosan in mass percentage 50% and then slowly added dropwise to 2% $CaCl_2$ solutions with cross-linking system pH values of 6.0, 7.0, 8.0, 9.0 and 10.0, respectively, to obtain microcapsules. As shown in Figure 3, the change of pH values of the cross-linking system had significant influence on water absorbance of the microcapsules. The water absorbance ratio was low under acid conditions, gradually increased from neutral to basic conditions, peaked at pH=9.0 and decreased as the pH values further increased. The change of water absorbance of microcapsule is closely related with its structure. When the pH values of the cross-linking system changed, due to the different degree of dissociation of sodium alginate and carboxymethyl chitosan, the amino groups and carboxyl groups on carboxymethyl chitosan molecular chains and the carboxyl groups on sodium alginate molecular chains will significantly change and crosslink with $CaCl_2$ to different extent to form microcapsules of different spatial structure. Carboxyl groups in carboxymethyl chitosan and sodium alginate existed completely in the form of $-COO^-$ when pH reached 10.0, and they cross-linked with calcium chloride entirely, resulting in the low water absorption ratio.

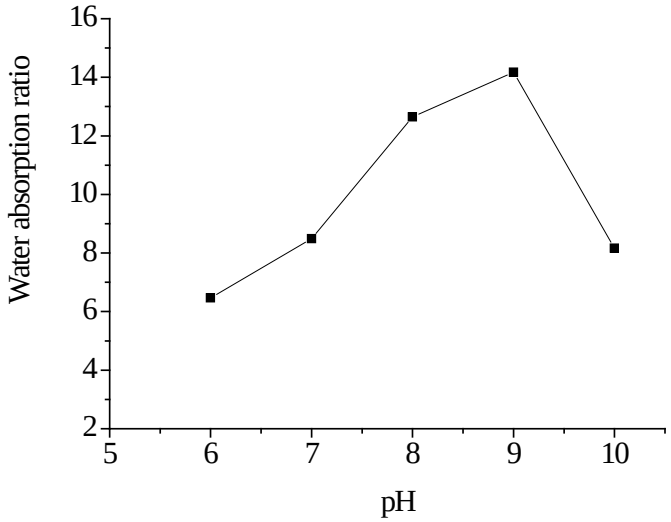


Figure 3. Effect of cross-linking system pH on water absorption of microcapsule

3.2. Preparation optimization of hemostatic microcapsule

On the basis of single-factor experiment, $L_9(3^4)$ orthogonal experiment was designed to optimize the preparation process of hemostatic microcapsule. The water absorbance ratio of hemostatic microcapsule was used as the investigation index for the optimization.

Table 1. L_9 (3^4) orthogonal experimental design and results

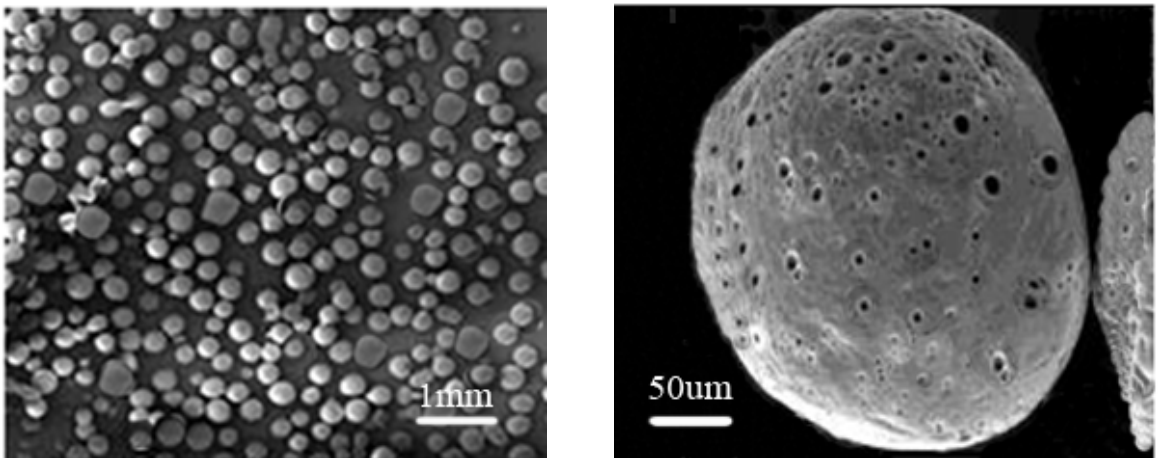
Run	Factors			Water absorption ratio
	A: Mass percentage of carboxymethyl chitosan (%)	B: Concentration of $CaCl_2$ (%)	C: Cross-linking system pH	
1	25	2.0	7	11.13
2	25	3.0	8	15.03
3	25	4.5	9	14.11
4	50	2.0	8	11.63
5	50	3.0	9	14.82
6	50	4.5	7	13.19
7	75	2.0	9	12.90
8	75	3.0	7	13.82
9	75	4.5	8	12.34
K1	40.27	35.66	38.14	
K2	39.64	43.67	39.00	
K3	39.06	39.64	41.83	
R	1.21	8.01	3.69	

The design process and results of the orthogonal experiment were shown in table 1. Range analysis revealed that the extent of three factors impact on microcapsule water absorbance descended in the order of B>C>A. The concentration of $CaCl_2$ has the maximum influence, while mass percentage of

carboxymethyl chitosan has the minimum influence. Analysis of K values showed that the optimum condition for preparation of microcapsule is $A_1B_2C_3$, i.e., mass percentage of carboxymethyl chitosan 25%, concentration of $CaCl_2$ 3%, and cross-linking system pH=9.0. As verified by parallel experiments, the average water absorbance ratio of microcapsule prepared under these conditions was 16.17.

3.3. Morphology of hemostatic microcapsule

The surface morphology of microcapsule was observed by SEM (Figure 4). As shown in Figure 4(a), the microcapsule prepared has a regular near-spherical shape, uniform and appropriate particle size, good dispersibility and no coacervation. From Figure 4 (b), it is observed that there are numerous small pores of different diameter on the surface of the microcapsule, indicating a porous structure of microcapsule, making for easy hemostasis by water absorbing and swelling.



(a) (b)

Figure 4. The surface morphology of microcapsule observed by SEM

3.4. Procoagulant property of microcapsule in vitro

Traditional hemostasis research is mostly carried out through clinical practice, but there are some shortcomings such as long time consuming and high cost. Screening of potential hemostatic medicine by in vitro hemostatic experiment is the trend for developing new hemostatic medicines. Using in vitro hemostasis, microcapsule was added into fresh anticoagulant rabbit blood, the coagulation effect of microcapsule was investigated by measuring the blood coagulation time. The results were shown in Table 2. It was observed that the coagulation time of the blank control group exceeded 30min, which means no coagulation. After addition of microcapsule the anticoagulant blood rapidly coagulated, indicating strong in vitro procoagulant effect of microcapsule. Besides, with the dose of microcapsule increasing, the coagulation time shortened, indicating that the procoagulant effect of microcapsule was dose dependent to certain extent.

Table 2. Procoagulant effect of the microcapsule in vitro.

Amount of microcapsule (mg)	0	20	40	60	80
Coagulation time (mins)	>30 (not coagulation)	8.7	6.2	4.8	4.3

4. Conclusions

The composite hemostatic microcapsule was successfully prepared by piercing granulation technique with carboxymethyl chitosan and sodium alginate as substrate materials. The effects of experimental

factors in the preparation process on water absorbance of hemostatic microcapsule were explored by single-factor experiment. An orthogonal experiment was conducted to optimize the preparation process of hemostatic microcapsule. In vitro procoagulant tests showed that the microcapsule had significant procoagulant effects. Further researches are needed to develop a clinical medical hemostatic material.

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Author contributions
Zhang Hu designed the experiments and wrote the paper; Jie Guang and Si Tong Lu performed the experiments; Yu Chen and Si Dong Li analyzed the data; Ying Cai contributed reagents, materials and analysis tools.

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