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# Preparation and blood coagulation evaluation of chitosan microspheres

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**Abstract** Cross-linked chitosan microspheres (40–100 μm) with smooth surface were prepared by the methods of emulsification and ethanol coagulant. FTIR results showed that the cross-linking reaction occurred on the amino groups of chitosan molecules. The swelling characteristic of chitosan microspheres was influenced by the environment pH, being generally greater at low rather than higher pH values. The coagulation properties of chitosan microspheres were evaluated by dynamic blood clotting, platelet adhesion and activation, erythrocyte adhesion, hemolysis, and protein absorption assays. Chitosan microspheres can shorten the clotting time and induce the adhesion and activation of platelets. But the shortening of clotting time by chitosan microspheres may be related to not only platelet aggregation, but also erythrocyte aggregation. Take together, chitosan microspheres may be potential use as thrombospheres.

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## 1 Introduction

Polymeric microspheres are used in medical diagnostics, therapy, and support for enzymes and proteins in biotechnology. These particles have also been found application in fundamental studies in the life sciences as useful tools for separation of the living cells and subcellular fragments. Various materials are used for preparing these microspheres, including chitosan, alginate, poly(lactic-co-glycolic acid), and cellulose. Among these materials, chitosan has been attracted great attention recently because of its biodegradable and biocompatible properties [1, 2]. Chitosan, a linear polysaccharide composed of $\beta$-(1 → 4)-linked 2-amino-2-deoxy-D-glucose (GlcN) and 2-acetamido-2-deoxy-D-glucose (GlcNAc), is obtained on an industrial scale by the alkaline deacetylation of chitin.

Chitosan microspheres were found to have great utility in drug carrier and delivery systems [3]. The more recent research published in the major biomaterial journals focused on the area of implantable applications of chitosan including orthopedic/periodontal, tissue engineering and wound healing [4]. Chitosan microspheres are potentially useful as bone and periodontal filling materials [5, 6]. Li reported that chitosan–alginate microspheres had excellent short- and long-term effects on renal arterial embolization [7]. The preceding section highlighted the principal implantable application of chitosan microspheres, among which chitosan microspheres have the opportunity to contact with blood. Therefore, the blood coagulation property seems to be important for the safely using of chitosan microspheres. Previously, reports indicated that chitosan solutions can induce the adhesion and aggregation of platelets. Chitosan may induce the adhesion of erythrocytes with its amino groups or forming a three-dimensional network structure in blood that captured erythrocytes and

then aggregated [8]. However, no further research has concerned about whether the amino groups or the three-dimensional network structure of chitosan takes charge of the adhesion of erythrocytes. Further more, the coagulation property of chitosan microspheres has not been reported.

In this article, a smooth-surface chitosan microsphere was prepared by emulsion and cross-linked method. The characteristics and blood coagulation properties of the chitosan microspheres were investigated.

## 2 Materials and methods

### 2.1 Materials

Chitosan (MW 1,380,000 Da, DD 80%) was made from crab-shell and obtained from Biochemical Medicine Plant of Qingdao (Qingdao, China). Ethanol, Tween-80, and span-80 were purchased from the Sigma Co., St. Louis, USA. Glutaraldehyde and formaldehyde was provided by Shanghai Chemical Reagent Co. of Chinese Medical Group. Blood plasma was supplied by the Affiliated Hospital of Medical College of Qingdao University (Qingdao, China).

### 2.2 Preparation of chitosan microspheres

Chitosan microspheres were prepared by following a patented procedure [9]. Chitosan was dissolved in acetic acid aqueous solution (1%, v/v) and dropped into toluene (oil phases) containing 1% (v/v) Tween-80 and 1% span-80 through a nozzle. The mixture was stirred vigorously for 30 min and formaldehyde (15 mL) was added into the reaction system for 1 h, then chitosan microspheres were separated, washed with deionized water, and further cross-linked chemically with glutaraldehyde (0.025%) for 2 h. After that, chitosan microspheres were treaded with $H_2O_2$, washed repeatedly and dehydrated successively with ethanol (30, 50, 80, 95, and 100%) and finally vacuum dried over night.

### 2.3 Characterization of chitosan microspheres

The morphology of the chitosan microspheres was determined by observation of the samples on a scanning electron microscope (SEM) (XL 40, Philips, Eindhoven, The Netherlands). The size was measured with Laser Diffraction Particle Size Analyzer SALD-3101 (Shimadzu, Japan). The IR spectra of chitosan and chitosan microspheres were recorded on an FT/IR-430 Fourier Transform Infrared Spectrometer (Jasco Co., Tokyo, Japan) at room temperature based on the method of Shigemasa [10]. Swelling ratio of chitosan microspheres was measured in various pH buffer solutions. At given times, the diameter of microspheres was measured using microscopes. The swelling ratio was calculated from the following equation:

$$\text{Swelling ratio } (\%) = \frac{D_t - D_0}{D_0} \times 100$$

where $D_t$ is the diameter of the microspheres at a given time and $D_0$ is the diameter of the dry state of microspheres.

### 2.4 Blood pro-coagulation activity

#### *2.4.1 Dynamic blood clotting test*

For clotting time measurement, a kinetic method similar to the work described by Huang was used [11]. Microspheres were put into beakers and placed in a thermostat at 37 °C for 5 min, then dropped ACD whole blood (0.25 mL) onto the surface of these microspheres, followed by adding 0.02 mL $CaCl_2$ solution (0.2 L $mol^{-1}$). The blood clotting test was carried out by spectrophotometric measurement at 540 nm. The relative absorbency of 0.25 mL ACD whole blood diluted by 50 mL distilled water was assumed to be 100. The blood clotting index (BCI) of biomaterial can be quantified by the following equation.

$$\text{BCI}\,(\%) = \frac{\text{Absorbency of blood contanted with sample}}{\text{Absobency of solution of distilled water and ACD blood}} \times 100$$

#### *2.4.2 Platelet adhesion and aggregation*

The platelets were separated from a freshly drawn citrated (trisodium citrate 3.8%, w/v, solution) rabbit blood (at the ratio of 1:9) by the procedure described [12]. In brief, the blood was centrifuged at 140*g* for 10 min to remove leukocytes and erythrocytes. Then the supernatant was again centrifuged at 560*g* for 10 min. The platelet button was washed three times with tyrode solution. The washed platelet suspensions (0.5 mL) spread over the microspheres and incubated at 37 °C. After the specified incubation period, the platelet suspensions were taken count of using a cytometer.

$$\text{Adhesion radio } (\%) = \frac{\text{Total platelets} - \text{Suspension platets}}{\text{Total platelets}} \times 100$$

The microspheres after incubation were gently and uniformly washed with 0.1 M phosphate buffer saline (pH

7.4), fixed with 2.5% glutaraldehyde solution in saline 4 °C for 2 days, washed with saline and dehydrated with series of graded ethanol–water solutions (0, 30, 50, 70, 90, and 100%), and dried under vacuum condition over night. The dried microspheres after gold coating were examined with scanning electron microscope.

#### 2.4.3 Erythrocyte adhesion

The erythrocyte adhesive studies were done by using the procedure described elsewhere. In short, the erythrocyte was separated from the citrated calf blood by centrifuging at 70*g*. The supernatant was discarded. The sedimented erythrocyte was washed three times with the 0.1 M phosphate buffer pH 7.4, and suspended in phosphate buffer and studied similarly as mentioned above.

#### 2.4.4 Hemolysis test

The hemolytic activity of the microspheres was investigated according to Singhal's method [13]. Fresh rabbit blood obtained by the same procedure described above was diluted using saline water (1:1.25, v/v). Microspheres with different concentrations were suspended in 10 mL 0.9% NaCl solution and incubated for 1 h at 37 °C in a shaking water bath. Diluted blood (0.2 mL) was added into microsphere suspensions and incubated for 1 h. The release of hemoglobin was determined after centrifugation (700*g* for 10 min) by photometric analysis of the supernatant at 545 nm. Positive and negative controls were produced by adding 0.2 mL of diluted blood to 10 mL of distilled water and saline water. The percent hemolysis was calculated as follows:

$$\text{Hemolysis } (\%) = \frac{\text{AS} - \text{AN}}{\text{AP} - \text{AN}} \times 100$$

where AS, AN, and AP are the absorbency of sample, positive control and negative control. Less than 10% hemolysis was regarded as non-toxic effect level in our experiments.

#### 2.4.5 SDS-PAGE

SDS-PAGE was performed on vertical slab gels (18 cm × 13 cm × 0.1 cm). Resolving and stacking gels conditions were 8 and 5% acrylamine, respectively. Protein extract samples were mixed in a 1:1 ratio with sample buffer, heated at 100 °C for 5 min and the applied 20 μL protein-buffer solution to the gel. The protein was visualized by staining with Coomassie Brilliant Blue R-250. Molecular weights were estimated using the linear relationship between the lag of the molecular weight of the standards and relative mobility.

### 2.5 Statistical analysis

Statistical data analysis was performed using the one-paired Student's *t*-test with $p < 0.05$ as the minimal level of significance. Calculations were done using the software SPSS10.0.

## 3 Results

### 3.1 Characteristics of chitosan microspheres

Chitosan microspheres were prepared using the O/W emulsion method under similar reaction conditions. All microspheres were of a well spherical shape with a rather smooth surface (Fig. 1). Chitosan microspheres were obtained in the size range 40–100 μm with a rather narrow size distribution and had a mean diameter about 60 μm. The infrared spectra of chitosan and chitosan microspheres are similar to previous reports [14]. The broad band at 3433 $cm^{-1}$ was –OH stretching, which overlapped the NH stretching in the same region. The shifts at 1660 and 1597 $cm^{-1}$ were the bands of amide I and II, respectively. For chitosan microspheres, a new peak at 1635 $cm^{-1}$ can be observed (Fig. 2B), which corresponded to stretching vibrations of C = N bond. It indicated that the reactions were occurred between carbonyl groups of cross-linking agent and amine groups of chitosan molecules.

The swelling behavior of chitosan microspheres was determined by immersing microspheres in buffer solution with different pH values (3.14, 4.69, 5.78, 7.32, and 9.07). At pHs of 3.14, 4.69, 5.78, and 9.07, chitosan microspheres

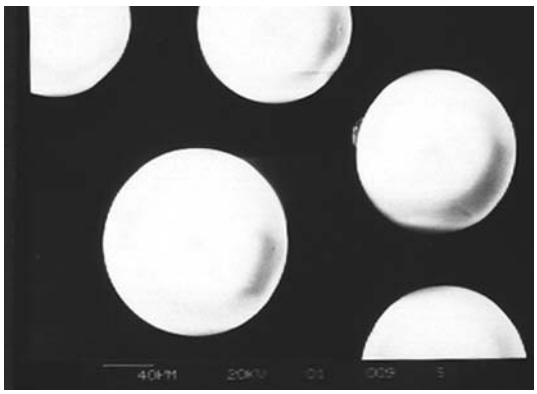

**Fig. 1** The scanning electron micrographs of the chitosan microspheres

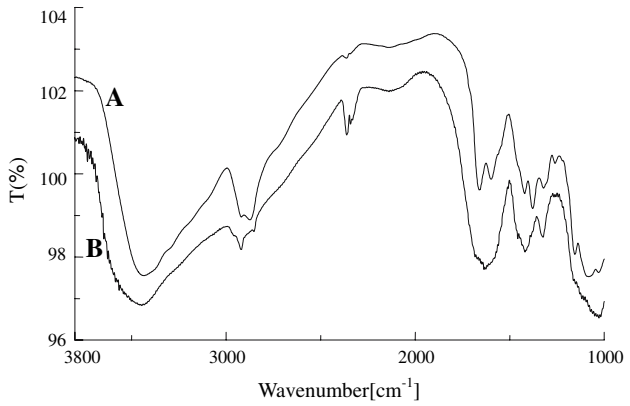


**Fig. 2** The FTIR spectra of chitosan and the chitosan microspheres. (**A**) Chitosan and (**B**) chitosan microcarriers

showed rapid swelling at the first 1 h and reached a relatively stable level at 3 h, whereas at pH 7.32 it took only 10 min (Fig. 3). The swelling ratio was influenced by the environment pH, being generally greater at low rather than higher pH values. The swelling of chitosan microspheres was significantly higher in low pHs (3.14, 4.69, and 5.78) than that of high pHs (7.32 and 9.07).

## 3.2 Blood pro-coagulation activity

### *3.2.1 Dynamic blood clotting test*

In order to evaluate the effectiveness of chitosan microspheres on blood pro-coagulation activation, preliminary dynamic blood clotting test was conducted in vitro. Figure 4 shows the dynamic blood clotting profiles for chitosan microspheres. The absorbance of the hemolyzed hemoglobin solution varied with time. Chitosan microspheres showed rapid decrease of BCI at 5 min, while the control exhibited the same phenomenon at 10 min. The time at which the absorbance equals 0.01 was generally defined as the clotting time, and the slower the BCI value decreased with the time, the longer the clotting time was [15]. The clotting time for chitosan microspheres was 30 min compared to 50 min for the control, indicating that chitosan microspheres can induce the blood coagulation ($p > 0.05$, $n = 3$, Students' $t$-test, data not shown).

### *3.2.2 Platelet adhesion*

Figure 5 shows the platelet adhesion on microspheres in platelet suspensions. Chitosan microspheres caused quick and stable platelet adhesion at 10 min. With time elapsing, the number of platelets adhered did not significantly increase. The morphology of the aggregated platelets in each microsphere was investigated using SEM (Fig. 6A). Platelets adhered strongly on the surface with pseudopodia. Further more, platelets were bound to each other and formed the aggregated mass.

### *3.2.3 Erythrocyte adhesion*

The erythrocyte suspension after adhering by chitosan microspheres shown lighter color (red) compared with the control (date not showed). This phenomenon can be further conformed by the SEM photomicrographs (Fig. 6B). It demonstrated that erythrocytes considerably adhered upon surface of chitosan microspheres. The surface thus tended to enhance erythrocytes agglutination or agglomeration; such an effect was similar to platelets adhesion upon the same surface. The adhering ratio of erythrocytes on the chitosan microspheres was similar to that of the platelets (Fig. 5).

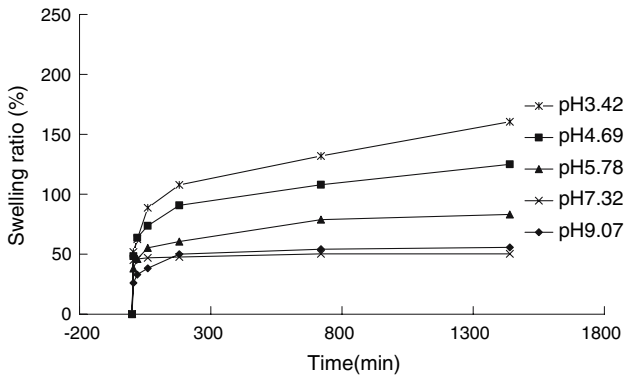


**Fig. 3** Swelling ratio of chitosan microspheres in different pH values solution buffer

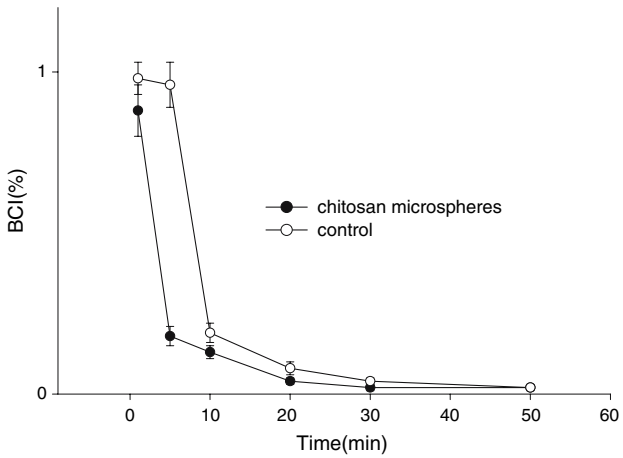


**Fig. 4** The blood clotting index (BCI) of chitosan microspheres

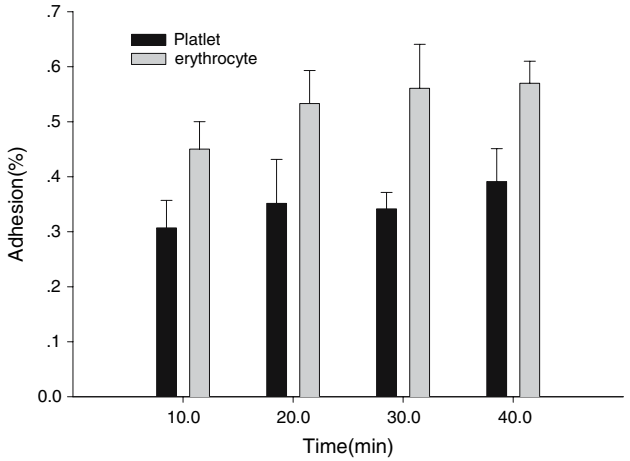


**Fig. 5** Platelet and erythrocyte adhesion studies on chitosan microspheres with different time

### 3.2.4 *Hemolysis*

Results obtained for hemolysis of rabbit blood with chitosan microspheres are shown in Table 1. It was observed that hemolysis was less than 5% at the concentration of 1 mg/mL chitosan microspheres, which came well within permissible limit [16]. When the concentration of microspheres changed to 100 mg $mL^{-1}$, chitosan showed a little hemolysis, with the value 7.99%.

### 3.2.5 *SDS-PAGE*

To examine the specific interactions of protein with the microspheres, adsorption for plasma was studied using SDS-PAGE. Figure 7 shows gels of proteins adsorbed to microspheres from 4% plasma. It can be seen by comparing the microspheres gels (lane 3) to the gel of the plasma (lane 2) that the surface of chitosan microspheres bound large amount of plasma proteins, a fact that may explain the observed coagulation behavior of chitosan microspheres.

**Table 1** Hemolytic activity of chitosan microspheres with different concentration

| Sample | Optical density at 545 nm | Hemolysis (%) |
|---|---|---|
| Distilled water | 0.886 ± 0.041 | +ve control |
| Normal saline | 0.035 ± 0.010 | –ve control |
| Chitosan microspheres (500 mg $mL^{-1}$) | 0.103 ± 0.020 | 7.99% |
| Chitosan microspheres (5 mg $mL^{-1}$) | 0.044 ± 0.009 | 1.20% |

## 4 Discussion

In the paper, we prepared chitosan microspheres with a rather smooth surface. It had small swelling ratio at high pH compared with low pH. It may be attributed to the absence of amine group ionization. There are two main networks in chitosan microspheres. One is formed by chitosan molecules themselves, and another is formed by chitosan and cross-linking agent. $H_2O$ diffusion from water to microspheres and the repulsion between $-NH_3^+$ groups can attribute to swelling, while, the cross-linking force, hydrogen bond, and hydrophobic interaction will restrict the swelling property of microspheres. In acid solution, amino groups on chitosan present with the form $-NH_3^+$, it did not directly promote swelling by the electric resistance but also can induce $H_2O$ into the internal of microspheres for its hydrophilic. When modified the $-NH_3^+$ with $CH_3COO^-$, all microspheres had the same swelling ratio when the pH varied from 3.42 to 9.07 (date not shown).

When blood is in contact with a foreign material surface, firstly, occurred the adsorption of plasma proteins and followed by platelet adhesion and deformation. These platelets release substrates that start the coagulation process, resulting in thrombosis. Blood compatibility is reached when there is not too much interaction of platelet with the material surface. Therefore, platelet adhesion on the microspheres from blood is an important test for the evaluation of blood compatibility of microspheres. The platelet adhesion and activation are the two important steps

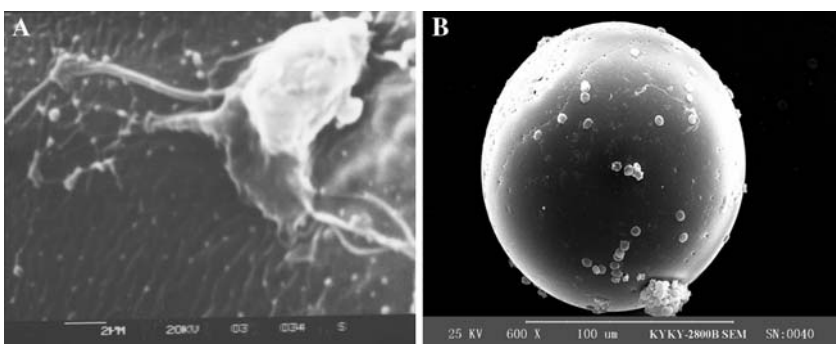


**Fig. 6** The scanning electron micrographs of platelet and erythrocyte on chitosan microsphere surfaces. (**A**) Platelets with pseudopodia and (**B**) erythrocyte aggregation

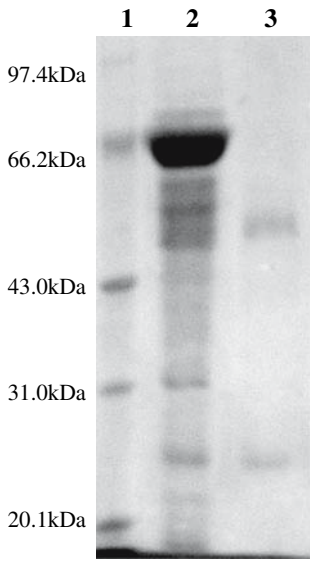


**Fig. 7** SDS-PAGE electrophoresis gel stained by Coomessie Blue. Lane 1, maker; lane 2, 4% of the plasma before adsorption; lane 3, supernatant after adsorption of chitosan

which regulate the formation of the thrombus and medical device rejection. During the initial stage of surface activation, the change in conformation of the adsorbed proteins exposes RGD sequences which are sensitive to the platelet GPIIb/IIIa receptor. When platelets are surface activated they progress through a sequence of morphological changes. The surface activation contributes to the change in the organization of the cytoskeleton, and thus increases the surface area of the platelets by the formation of pseudopods. The platelets are thus, adhered and activated, go through a sequence of cytoskeletal events, and rise in endoplasmic $Ca^{2+}$ concentration, polymerization of action filaments, thrombin activation and release of cytoskeletal granule contents, as well as platelet aggregation. The extent of the shape change and the spread area has been related to the surface energetics of the polymer materials [17]. Our studies show that large amount of platelets adhere to the chitosan microspheres and make them activated. Chou has demonstrated that chitosan is an effective inducer for rabbit platelet adhesion and aggregation, and explained that the mechanisms of action by chitosan may be associated with the increasing of $Ca^{2+}$ mobilization and the enhancing expression of GPIIb/IIIa complex [18]. It has also been reported that chitosan and chitin enhance platelet aggregation due to their amino residues [19]. In acidic solution, amine groups of chitosan are protonated to $-NH_3^+$, which makes chitosan a cationic nature, allowing for electrostatic interactions with the negatively charged biological molecules on the platelet surface [20].

However, the shortening of clotting time by chitosan may be related to not only platelet aggregation, but also erythrocyte aggregation [19]. Earlier it has been observed that pro-coagulation properties of chitosan were partly due to the erythrocyte [21]. Chitosan may induce the adhesion of erythrocytes with its amino groups or forming a three-dimensional network structure in blood that captures the erythrocytes and then make them aggregated [8]. In our study, chitosan microspheres were directly immersed into erythrocyte suspension without any blood proteins. The surface of chitosan microspheres greatly enhanced erythrocytes agglutination or agglomeration, which indicated that chitosan can directly induce erythrocyte adhesion without forming any dimensional network structure or adsorbing and plasma proteins at first. The reasons for the promotion of erythrocyte aggregation may be due to its cationic nature as above mentioned. Further more, it could also adsorb various plasma proteins, which may enhance the pro-coagulation.

Hemolysis of the blood is the problem associated with *bio*-incompatibility [22]. Previous studies indicated that chitosan promoted surface induced hemolysis, which can be attributed in part to the electrostatic interactions [20, 23]. In this article, when the concentration of microspheres add to 100 mg $mL^{-1}$, chitosan showed a little hemolysis. However, the largest hemolytic activity was lower than 10% which indicated a wide safety margin in blood contacting applications and suitability for intravenous administration [24, 25]. Further more, by comparing the lowly hemolytic activity with the highly erythrocyte agglutination, it showed that chitosan only induce the adhesion of erythrocytes but not seriously damage the cell membrane.

The pro-coagulation properties of chitosan microspheres give them the potential to use as thrombospheres. Most thrombospheres (Hemosphere, Irvine, CA) are composed of cross-linked human albumin with human fibrinogen bound to the surface. A similar product, Synthocytes (Andaris Group Ltd., Nottingham, UK), has just entered into clinical trials in Europe [26].

## 5 Conclusion

In the present study, smooth surface chitosan microspheres with good shape were prepared by the emulsion and cross-linked method. The cross-linking reaction occurred on the amino groups of chitosan molecules and the swelling property was greatly influenced by the environment pH values. Dynamic blood clotting test showed that chitosan microspheres can shorten the clotting time. It may be related to not only platelet aggregation, but also erythrocyte agglutination, and also it may be due to the large amount of plasma proteins absorbed on the chitosan microsphere surfaces. Chitosan microspheres can induce the adhesion and activation of platelets. It also can directly

promote the aggregation of erythrocytes without forming any dimensional network structure or adsorbing any plasma protein at first, but they did not greatly damage the cell membranes of erythrocytes. Chitosan microspheres may be potential use as thrombospheres.

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