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A Novel Chitosan-based Sponge Coated with Self-assembled Thrombin/Tannic Acid Multilayer Films as a Hemostatic Dressing*

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Abstract In order to prepare a novel hemostatic dressing for uncontrolled hemorrhage, a porous chitosan sponge was coated with self-assembled (thrombin/tannic acid)$_n$ films, which were based on hydrogen bonding interactions between thrombin and tannic acid at physiologic pH. According to the whole blood clotting test, the coated chitosan sponges showed a significantly high rate of blood clotting due to the addition of thrombin. On the other hand, the storable half-life of immobilized thrombin is extended to 66.9 days at room temperature, which is 8.5 times longer than unfixed thrombin. It is because of the immobilization effect of, not only the porous structure of chitosan sponge but also the interactions between thrombin and tannic acid. In addition, the tannic acid has similar antibacterial effect to chitosan. Therefore, it is an excellent combination of chitosan, thrombin and tannic acid. Besides, all of materials in this research have been approved by the United States Food and Drug Administration (FDA). So the chitosan-based sponge is a promising candidate dressing for uncontrolled hemorrhage due to its storable, bio-safe and highly effective hemostatic properties.

Keywords: Chitosan; Thrombin; Tannic acid; Layer-by-Layer assembly; Hemostatic dressing.

INTRODUCTION

Uncontrolled hemorrhage is the main cause of death due to military trauma and in the civilian setting[1, 2]. For prehospital emergency treatment, an ideal hemostatic dressing should be stable, flexible, inexpensive, none adverse reaction and effectively control hemorrhage[3, 4]. A number of hemostatic agents have been developed including fibrin dressings, chitosan bandages, zeolite powders and so on[5]. But none of them can meet all requirements although they have shown efficacy in animal and human studies[1, 4, 6]. For example, zeolite powders may cause severe burns[6, 7].

Chitosan (CS), as a kind of natural polymer, has been considered as an ideal candidate for a hemostatic agent due to its excellent biocompatibility, biodegradable properties and inherent wound healing characteristics[8–10]. However, the clinically available porous and absorbent chitosan sponge demonstrates limited efficacy for severe hemorrhage, if without chemical modification or physical blending[11]. The addition of blood clotting factors to chitosan is a feasible and simple way to enhance the hemostatic efficacy, such as thrombin. But as an efficient protein-based hemostatic agent in surgical, thrombin should be store in freeze after vacuum

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dehydration, which restricts the application of thrombin-containing hemostatic dressing in prehospital environment[12].

Enzyme immobilization is a technique to protect the enzyme properties with broad prospects[13]. As one of potential immobilization carrier, chitosan sponge may improve the thermo stability of thrombin. But the porous structure of sponges is just able to support limited effect of immobilization compared to the microsphere that is the most common carrier structure[14, 15]. Therefore, thrombin still needs to be immobilized by other matter through effective chemical or physical bounding.

It has just been confirmed hydrogen bonding interactions exist at physiologic pH between thrombin and tannic acid that has an abundance of hydrogen bond donating phenols[16]. The layer-by-layer (LBL) assembly appears to be an excellent solution for not only the aforementioned problem but also the potential problem. On one hand，the thrombin can be immobilized with tannic acid by hydrogen bonding interactions, which would not destroy thrombin activity as a secondary bound. On the other hand, LBL assembly can load a large and controlled amount of thrombin into sponges. In addition, the tannic acid may enhance the antibacterial and antioxidant activity of the sponges[16]. Therefore, it is an excellent combination of chitosan, thrombin and tannic acid. Besides, all of materials in this research have been approved by FDA.

In consequence, the $(\text{thrombin/tannic acid})_n$ films were coated onto the chitosan sponges to create a storable, bio-safe and highly effective hemostatic dressing. The results of whole blood clotting supported the coated sponge is a promising storable, bio-safe and high effective candidate dressing for uncontrolled hemorrhage.

EXPERIMENTAL

Materials

Chitosan ($M_v = 5.01 \times 10^5$) was supplied by the Qingdao Haihui Bioengineering Co. Ltd (Qingdao, China) with 90.6% degree of deacetylation (D.D). Lyophilizing bovine thrombin powder was obtained from Hangkang Pharmaceutical Co., Ltd (Hangzhou, China). Tannic acid, branched polyethyleneimine (BPEI, M_n = 50−100 kDa) and dulbecco's phosphate buffered saline (PBS, 0.1 mol/L) were obtained from Sigma-Aldrich (St. Louis, MO). Lyophilizing fibrinogen powder (59% protein) was supplied by Beijing JCKY Institute of Chemical Technology (Beijing, China) as a kind of reference material. All other materials were obtained from the Chinese Pharmacy Group Shanghai Reagent Company (Shanghai, China) unless otherwise noted. All other chemicals were commercially available analytical grade reagents.

Preparation of Thrombin/Tannic Acid Films on Flat Substrates

In order to characterize film properties, the $(\text{thrombin/tannic acid})_n$ films (n represents the number of bilayers) were assembled on silicon wafers and gold coated quartz crystals. First, the substrates should be washed with 0.01 mol/L PBS (pH 7.4) and dried under nitrogen, then soaked in BPEI solution (2 mg/mL, pH 7.4, in 0.01 mol/L PBS) for 20 min, and rinsed with PBS for 5 min[16]. Then, the substrates were soaked in thrombin solution (1 mg/mL, pH 7.4, in 0.01 mol/L PBS) for 15 min and subsequently rinsed with PBS for 5 min. After each rinsing, the samples were dried by nitrogen. Substrates were then soaked in tannic acid solution (2 mg/mL, pH 7.4, in 0.01 mol/L PBS) for 15 min, followed by the same rinsing and drying procedures.

Characterization of Thrombin/Tannic Acid Film Thickness

The LBL assembly progress was monitored by quartz crystal microbalance (Q-Sense E4 system, Q-Sense AB, Sweden). QCM-D was used to measure the frequency decrease after each alternate assembly of thrombin and tannic acid.

An ellipsometer (M-2000, Woollam, USA) was used to measure the thickness of multilayer films with different bilayer numbers. The measurements were performed by using continuous wavelength ranging from 200 to 1500 nm and incidence angle of 75°. The DafIBM program supplied by Rudolph Technologies was employed to determine the thickness.

Preparation of Chitosan-based Sponge Coated with Thrombin/Tannic Acid Film

First, the porous chitosan sponges were prepared by vacuum freeze-drying as the matrix for the dressing coating. 2 g of chitosan was dissolved in 2% (*V*/*V*) aqueous acetic acid (60 mL) and then added 2% (*V*/*V*) NaOH solution till a homogeneous gel suspension was obtained. The chitosan gel with neutral pH was obtained by rinsing thoroughly with tri-distilled[17, 18]. Then the gel was made into sponge by vacuum freeze-drying at −50 °C overnight. The process for the sponges with bilayer films (n = 5, 10, and 15) was similar to that for the flat substrates.

Preparation of Chitosan-based Sponge Immobilized with Thrombin

The chitosan sponge was prepared according to the above method. Then the sponge was soaked in thrombin solution (1 mg/mL, pH 7.4, in 0.01 mol/L PBS) for 15 min and dried by vacuum freeze-drying at −50 °C overnight.

Scanning Electron Microscope (SEM) Observations

The morphology of sponges was observed by field emission scanning electron microscopy (FE- SEM, FEI-SIRION).

Testing of Thrombin Activity of Coated Sponges

Thrombin activity, *i.e*. its efficiency in forming fibrin from fibrinogen, can be determined by comparing the time of first fibrin clot formation which is eventually converted to fibrin *via* the initial activity of thrombin. First of all, a standard curve of log thrombin activity versus log clotting time should be obtained by exposing known activity thrombin solution (2 mL) to 0.2% (*W*/*V*) fibrinogen solution (1 mL) at 37 °C. Then, the thrombin activity of coated sponge was tested by monitoring fibrin clot formation upon soaking coated sponges (0.02 g) in PBS solution (2 mL) and exposing to the same fibrinogen solution. The time of first fibrin clot formation was used to determine thrombin activity from comparison to a standard curve. By using this method, the relationship between bilayer and thrombin activity of coated sponges (n = 5, 10, and 15) was demonstrated.

Storage Stability Test

The coated sponges (n = 5) and thrombin powder were stored at room temperature (25 ± 5 °C) for 0, 7 and 31 days. By testing the thrombin activity, the storage stability of the thrombin within and without the sponge was gained.

Whole Blood Clotting Test

The blood clotting test was adapted from Shih *et al*[19]. First, citrated whole blood (0.2 mL) was soaked respectively onto the uncoated chitosan sponge, coated sponges (n = 5, 10 and 15) and coated sponge (n = 5, stored at room temperature for 31 days) in a tube. Then, 20 μL of 0.2 mol/L $CaCl_2$ solution was added to start coagulation. The tubes were shaken at 30 r/min for 0.5 min, 5 min and 15 min respectively. Immediately, the red blood cells (RBCs) without trapping in the clot were rinsed by water (25 mL). Ultraviolet detector was used to measure the absorbance of the resulting hemoglobin solution at 540 nm[20]. It should be noted that all of materials were prewarmed to 37 °C.

RESULTS AND DISCUSSION

Film Properties on Flat Substrates

The frequency decreased after each alternate assembly of thrombin and tannic acid was measured by QCM-D as Fig. 1(a). The results revealed the formation of thrombin/tannic acid multilayer films, as the ΔF reflected the absorbed mass. It indicated the LBL assembly was achievable at physiologic pH.

The thickness of multilayer films (n = 5, 10 and 15) and thickness per bilayer was measured by ellipsometer, which is shown in Fig. 1(b). The result indicated the thickness of films nearly linearly increased with number of bilayers. It means a large amount of thrombin can be added into sponges by increasing the number of bilayers. And the film thickness per bilayer shows little change, which indicated the stable assembly of films.

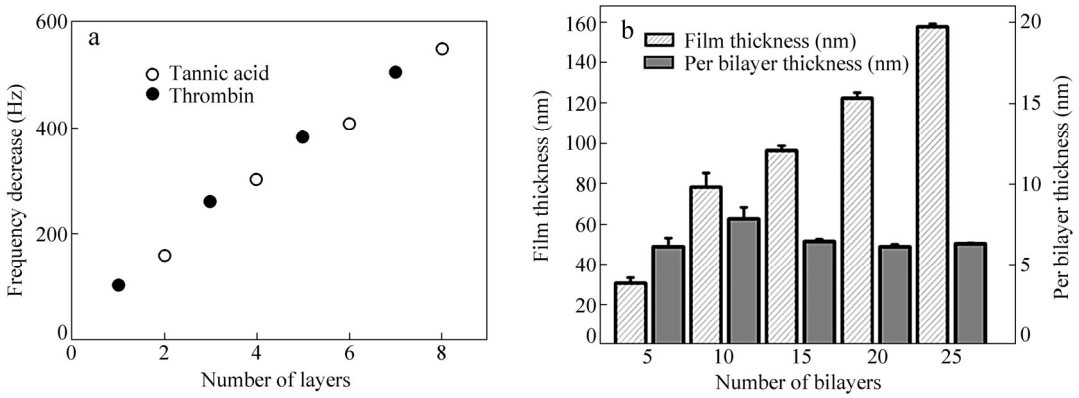

Fig. 1 (a) QCM frequency shifts as a function of layer number for the alternate assembly of thrombin and tannic acid; (b) the film thickness and thickness per bilayer with different bilayers: 5, 10, 15, 20, 25

The Morphology of Sponges

SEM photographs of uncoated chitosan sponge and coated sponge (n = 5) are shown in Fig. 2. By comparing the photographs, two significant results can be gained. On one hand, large deposits were observed at high magnification on coated sponge (Fig. 2a) while the uncoated sponge (Fig. 2b) was scrupulously clean. It indicated the assembly contributed significantly to attach large amount of thrombin to sponges**.** On the other hand, the porous structure of coated sponge (Fig. 2c) and uncoated sponge (Fig. 2d) showed a remarkable consistence at low magnification. It means the LBL coating wouldn't influence the porous structure of sponges, so the coated sponge still can facilitate hemostasis by absorbing water in the blood and immobilize thrombin by the structure.

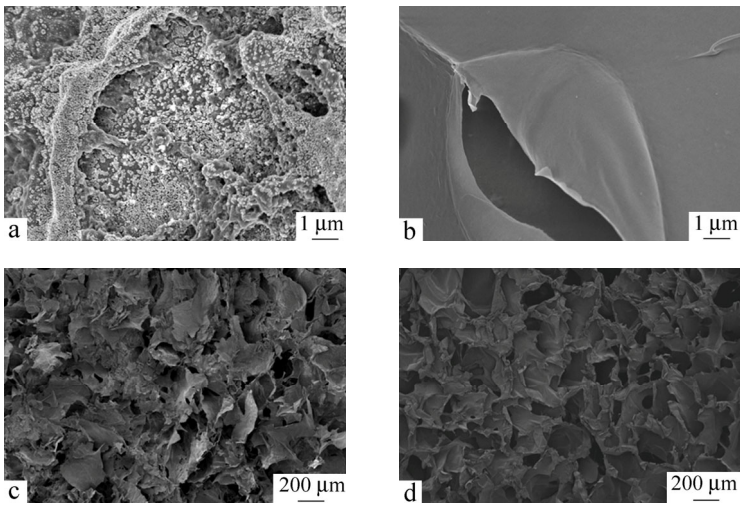

Fig. 2 SEM photographs for (a, c) the coated sponge (n = 5) and (b, d) the uncoated sponge

Thrombin Activity of Coated Sponges

By exposing the known activity thrombin to fibrinogen, the standard curve of log thrombin activity ($\ln U$) versus log clotting time ($\ln t$) is shown in Fig. 3(a). The figure indicated a significant linear correlation between $\ln t$ and $\ln U$ with a linear correlation coefficient of 0.99. It means the fibrin clotting time can convert to the thrombin activity of coated sponge successively by the standard curve equation:

$$\ln U = -2.6144 \times \ln t + 11.028$$

where U is the thrombin activity and its unit is IU, t is the clotting time and its unit is second.

By this way, the thrombin activity per mg with different bilayers was gained and shown in Fig. 3(b). Comparing to the film thickness with different bilayers, the thrombin activity reveals a relatively consistent trend: the activity strengthens significantly as the increasing of bilayers. It predicts the potential to adjust the activity of the coated sponges by changing the number of bilayers.

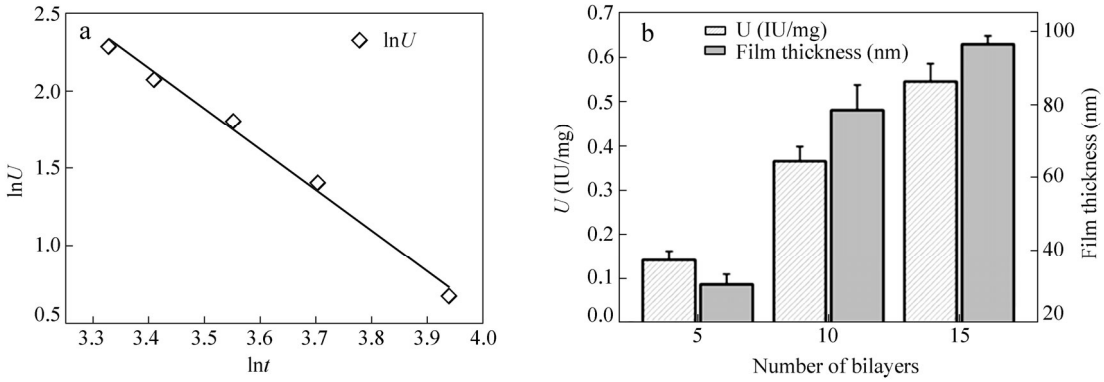

Fig. 3 (a) The standard curve of log thrombin activity versus log clotting time; (b) the thrombin activity per mg and film thickness with different bilayers: 5, 10 and 15

Storage Stability of Coated Sponges

The activity of thrombin within coated sponge and thrombin powder which were stored at room temperature for 0, 7 and 31 days was measured. The initial value of thrombin activity refers to those that was stored for 0 day, and is set to 100%. The activity recycle ratio of thrombin refers to the ratio of stored thrombin activity to the initial activity. Table 1 shows the activity recycle ratio of thrombin within coated sponge and thrombin powder with different storage time at room temperature. It is obviously that the storage stability of the thrombin within the sponge is much better than the thrombin powder. By a formula, the storage stability can be indicated by the storage half-life:

$$t_{1/2} = \frac{0.693 \times t}{2.303 \times \lg\left(\frac{E_0}{E}\right)}$$

where $t_{1/2}$ and t are the half-life and the storage time (unit is day) respectively, E_0 and E are the initial activity and the stored activity respectively[21, 22]. According to the formula, the half-life of coated sponge was 66.9 days by plugging t = 7, while the half-life of thrombin powder was just 7.9 days. It indicates the storage half-life of thrombin within sponge is extended by 8.5 times, so the coated sponges are pretty stable for first-aid hemostatic agent at room temperature.

Table 1. The activity recycle ratio of thrombin within coated sponge and thrombin powder with different storage time at room temperature

Storage time	Coated sponge	Thrombin powder
0 day	100%	100%
7 days	93%	54%
31 days	71%	0%

Whole Blood Clotting Results

The absorbance of hemoglobin solution at 540 nm indicated the concentration of the RBCs that didn't convert to clotting. Therefore, the decrease of absorbance means a high efficiency of blood clotting. There are five significant conclusions in the Fig. 4. First of all, the chitosan sponge showed a limited coagulated efficiency of hemostasis comparing to the blank. Due to the addition of thrombin, the coated sponges caused remarkably higher rate of blood clotting than the uncoated sponge within the same time. The chitosan sponge immobilized with thrombin showed lower hemostatic efficiency than LBL coated sponge, which indicated LBL assembly has

advantage over immersion method of loading a larger amount of thrombin. In addition, the rate of blood clotting for coated sponges enlarged as the bilayers increased. The trend was consistent to the relation between thrombin activity and bilayers. Comparing with LBL 5-1, LBL 5-2 indicated a small gap in the rate of clotting even if it has been store at room temperature for 31 days. In order to confirm these conclusions visually, Figure 5 shows these dressings with blood clotting and uncoagulated hemoglobin solutions. The lighter color of hemoglobin solutions and heaver color of dressing means a higher efficiency of blood clotting[23].

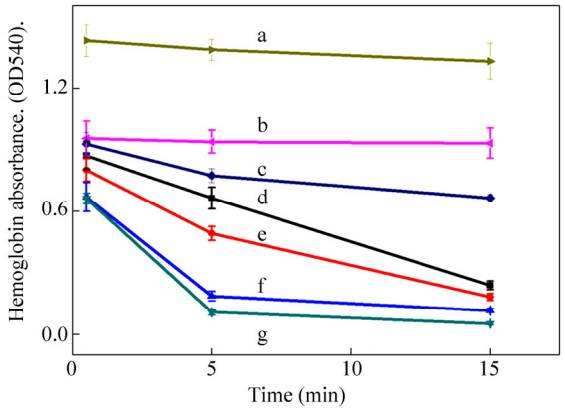

Fig. 4 The hemoglobin absorbance at 540 nm of different dressings within different time: (a) blank control group; (b) uncoated chitosan sponge; (c) chitosan sponge immobilized with thrombin; (d) LBL 5-2 coated sponge (n = 5) stored for 31 days at room temperature; (e) LBL 5-1 coated sponge (n = 5); (f) LBL 10 coated sponge (n = 10); (g) LBL 15 coated sponge (n = 15)

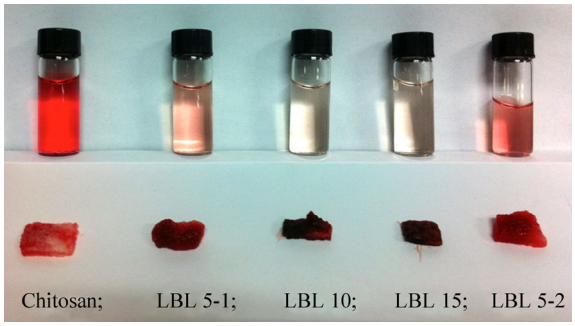

Fig. 5 Different dressings for blood clotting and uncoagulated hemoglobin solutions within 15 min

CONCLUSIONS

In order to prepare a novel hemostatic dressing for uncontrolled hemorrhage, a porous chitosan sponge was coated with self-assembly (thrombin/tannic acid)$_n$ films, which were based on hydrogen bonding interactions between thrombin and tannic acid at physiologic pH. The experimental results indicated three advantages of the novel dressing. First, the coated sponges showed a significantly high rate of blood clotting due to the addition of thrombin. Secondly, the hemostatic activity strengthened as the increasing of bilayers, which predicted the potential to adjust the hemostatic efficiency of the coated sponges by the number of bilayers. Thirdly, the half-life of coated sponge (n = 5) was 66.9 days, about 8.5 times longer than the unfixed thrombin. It means coated sponges are pretty stable as first-aid hemostatic agent. Besides, all materials in this research have been approved by the FDA. Consequently, the chitosan-based sponge is a promising candidate dressing for uncontrolled hemorrhage due to its storable, bio-safe and highly effective hemostatic properties.

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