

A Study on Hemostatic Properties of Polyphenols Crosslinked Gelatin Microspheres Based on Michael Addition Reaction

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Abstract: Massive blood loss caused by severe injury is one of the important challenges in the present research of hemostatic materials. In this study, we first prepared polyphenols crosslinked gelatin through Michael addition reaction at 60 °C, and pH = 9.5 for 30 min, herein the mass ratio of polyphenols to gelatin was 1:50. Then emulsion crosslinking method of water-in-oil was used to obtain polyphenols crosslinked gelatin microspheres, including dopamine crosslinked gelatin microspheres (DOPA-GMs) and gallic acid crosslinked gelatin microspheres (GA-GMs). The results showed that the hemostatic microspheres with polyphenols crosslinked gelatin could effectively enhance the hemostatic properties of single-component gelatin microspheres (GMs), and this study provided a new method and strategy for rapid hemostatic microspheres.

1. Introduction

The mortality rate for severe post-traumatic hemorrhage is approximately 30-40%, and about half of these patients lost their lives on the way to hospital^[1, 2]. Therefore, it is necessary and urgent to conduct sufficient and widespread research on materials for rapid hemostasis. Gelatin is an excellent hemostatic material that can activate the coagulation pathway to promote blood clotting^[3]. Gelatin-based microspheres (GMs) have a wide range of applications in several biomedical fields, such as drug delivery systems and tissue engineering^[4, 5]. However, GMs obtained by emulsion crosslinking method have low water absorption, which limits the hemostatic effect of GMs.

In recent years, catechol-based mechanisms of bio-adhesion inspired by marine mussels have been widely applied in biomedical functional materials, including gelatin-based adhesives^[6]. Catechol can be readily oxidized under alkaline conditions to form reactive benzoquinone, which subsequently reacts with amino groups to form covalent bonds through Michael addition reaction^[7-9]. Polyphenols have been confirmed to improve the adhesion properties and hemostatic effect of gelatin-based adhesives by Michael addition reaction under slightly alkaline conditions^[10]. In addition, polyphenols can inhibit the dissolution of fibrin in the blood and promote blood coagulation^[11].

In the present study, we prepared polyphenols crosslinked gelatin based on Michael addition reaction under slightly alkaline conditions, including dopamine containing catechol crosslinked gelatin and gallic acid

containing pyrogallol crosslinked gelatin. Subsequently, dopamine crosslinked gelatin microspheres (DOPA-GMs) and gallic acid crosslinked gelatin microspheres (GA-GMs) were prepared by the emulsion crosslinking method of water-in-oil. DOPA-GMs and GA-GMs had micrometer particle sizes and were effective in improving the hemostatic efficacy of the single-component gelatin microspheres. These microspheres can penetrate deep into narrow and deep bleeding wounds due to their micron size distribution, making them suitable for irregular bleeding wounds. In addition, the hemostatic microspheres can be more conveniently used by medical personnel for emergency and massive bleeding caused by traffic accidents or war injuries. In this study, we provided an effective strategy for the preparation of hemostatic microspheres.

2. Materials and methods

2.1. Materials

Gelatin (gel strength ~250g Bloom), dopamine hydrochloride, gallic acid, and sodium citrate dihydrate were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. Paraffin liquid, span-80, glutaraldehyde 25% aqueous solution, hydrochloric acid, and sodium hydroxide were obtained from Sinopharm Chemical Reagent Co., Ltd. Calcium chloride anhydrous (CaCl₂) and acetone were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China).

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The blood was obtained from the arteries of New Zealand rabbits. Then the fresh blood was mixed with 3.8% sodium citrate solution to obtain anticoagulant whole blood.

2.2. Methods

2.2.1 Preparation of dopamine crosslinked gelatin and gallic acid crosslinked gelatin

The dopamine crosslinked gelatin solution was prepared by a one-step Michael addition reaction under slightly alkaline conditions. In short, gelatin was first dissolved in ultra-pure water to obtain the 20% gelatin solution. The NaOH solution was added to 20% gelatin solution to adjust pH value to 9.5. Dopamine was dissolved in 2 mL ultra-pure water to obtain 20 mg/mL dopamine solution. The dopamine solution was added drop by drop to the continuously agitated gelatin solution at 60 °C, and the mixture was kept in contact with air. After the mixed solution was continuously stirred at 60 °C for 30 min, its pH value was adjusted to 7.4 by HCl solution to obtain dopamine crosslinked gelatin.

The gallic acid crosslinked gelatin was obtained with same method. The gallic acid crosslinked gelatin solution could be obtained by replacing 20 mg/mL dopamine solution with 20 mg/mL gallic acid solution in the above steps.

2.2.2 Preparation of dopamine crosslinked gelatin microspheres (DOPA-GMs) and gallic acid crosslinked gelatin microspheres (GA-GMs)

The preparation of the microspheres was referred to previous study^[12]. The dopamine crosslinked gelatin microspheres (DOPA-GMs) were obtained by the emulsion crosslinking method of water-in-oil. In brief, liquid paraffin containing 1% span-80 was first heated to 60 °C to obtain the mixed oil phase. The dopamine crosslinked gelatin solution was added drop by drop to the mixed oil phase, the volume ratio of the dopamine crosslinked gelatin solution to the mixed oil phase was 3:10, and the water-oil mixture was continuously stirring at 60 °C for 20 min. Then the water-oil mixture was transferred to the ice water bath, meanwhile, 2 mL glutaraldehyde 25% aqueous solution was added to the mixture for stirring 60 min. The acetone solution was used to remove excess the oil phase and separate the microspheres. After removing the liquid paraffin, glutaraldehyde, and acetone from the water-oil mixture, the microspheres were dried for 24 h to obtain dry dopamine crosslinked gelatin microspheres (DOPA-GMs).

The gallic acid crosslinked gelatin microspheres (GA-GMs) was obtained with same method. GA-GMs could be obtained by replacing the dopamine crosslinked gelatin solution with the gallic acid crosslinked gelatin solution in the above steps. The gelatin microspheres (GMs) could be

obtained by replacing the dopamine crosslinked gelatin solution with the gelatin solution in the above steps.

2.2.3 The surface morphology observation of DOPA-GMs and GA-GMs

The surface morphology of DOPA-GMs and GA-GMs was observed by a field emission scanning electron microscopy (FE-SEM, Ultra Plus, Carl Zeiss, Germany).

2.2.4 The particle size statistics of DOPA-GMs and GA-GMs

The particle size of DOPA-GMs and GA-GMs was calculated by Image J, and the particle size distribution was plotted by Origin 2018 Pro.

2.2.5 The whole blood coagulation test *in vitro*

The whole blood coagulation was used to evaluate the hemostatic effect of microspheres *in vitro*. 5 mg microspheres and 200 μ L of anticoagulated whole blood were mixed in a tube. Then 20 μ L of 0.1 M calcium chloride solution was added to the centrifuge tube and thoroughly mixed. The tube was incubated at constant 37 °C, and blood clotting was observed every 10 seconds. When the blood could not flow, the whole blood coagulation time was recorded. The experiment was repeated three times for each group and the results were obtained from the average.

3. Results and discussion

3.1. Preparation of DOPA-GMs and GA-GMs

As shown in Fig. 1, dopamine and gallic acid were oxidized under pH = 9.5 to form benzoquinone, and then benzoquinone underwent the Michael addition reaction with the amino group from the gelatin to obtain dopamine crosslinked gelatin and gallic acid crosslinked gelatin.

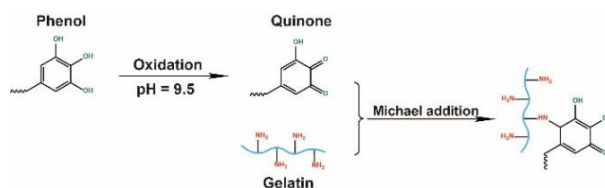


Fig. 1. The scheme graph of Michael addition reaction.

DOPA-GMs and GA-GMs were obtained by emulsion crosslinking method. The digital photos of GMs, DOPA-GMs, and GA-GMs were shown in Fig. 2, GMs presented light yellow powders, DOPA-GMs presented grey powders and GA-GMs presented brown powders. The different colour of the microsphere powders indicated the difference in the source of their composition.

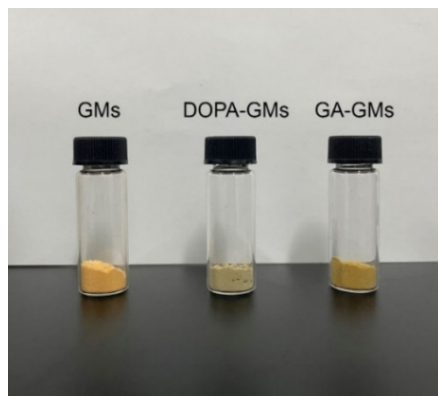


Fig. 2. The digital photos of GMs, DOPA-GMs, and GA-GMs.

3.2. The surface morphology observation and particle size distribution of DOPA-GMs and GA-GMs

The scanning electron microscopes of DOPA-GMs and GA-GMs are shown in Fig.3 and Fig.4. DOPA-GMs and GA-GMs had a complete spherical structure and a particle size of micrometers. DOPA-GMs and GA-GMs have a minimum particle size of a few microns and a maximum of tens of microns. DOPA-GMs had a smooth surface structure when observed by scanning electron microscopy at a magnification of 1500x. Under scanning electron microscopy observation at a magnification of 2000x, several smaller microspheres were adhered to the surface of GA-GMs, which could be attributed to the crosslinking of GA and gelatin leading to the increased adhesion on the surface of microspheres.

DOPA-GMs

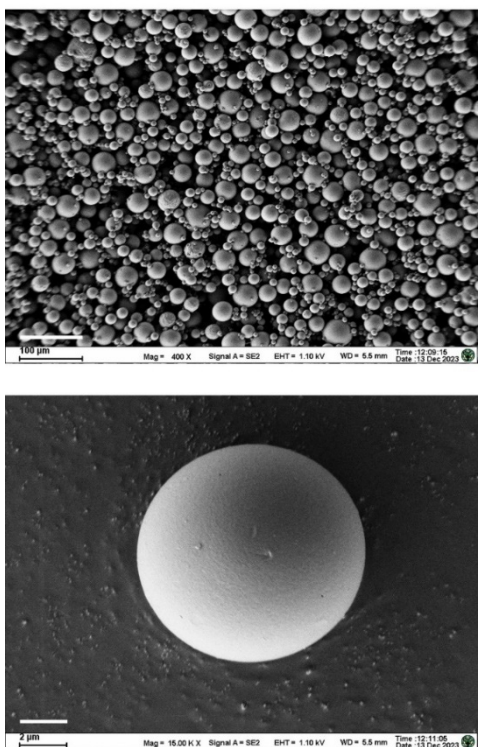


Fig. 3. The SEM images of DOPA-GMs, magnification 400x,

scale: 100 μm and magnification 1500x, scale: 2 μm .

The particle size distributions of DOPA-GMs and GA-GMs was shown in Fig.5 and Fig.6. The particle size distributions of DOPA-GMs was distributed from the range of 5-70 μm , while the particle size distributions of GA-GMs was distributed from the range of 5-60 μm . The average particle size of DOPA-GMs was 28.9 μm , while the average particle size of GA-GMs was 22.1 μm . These results were also consistent with the scanning electron micrographs.

GA-GMs

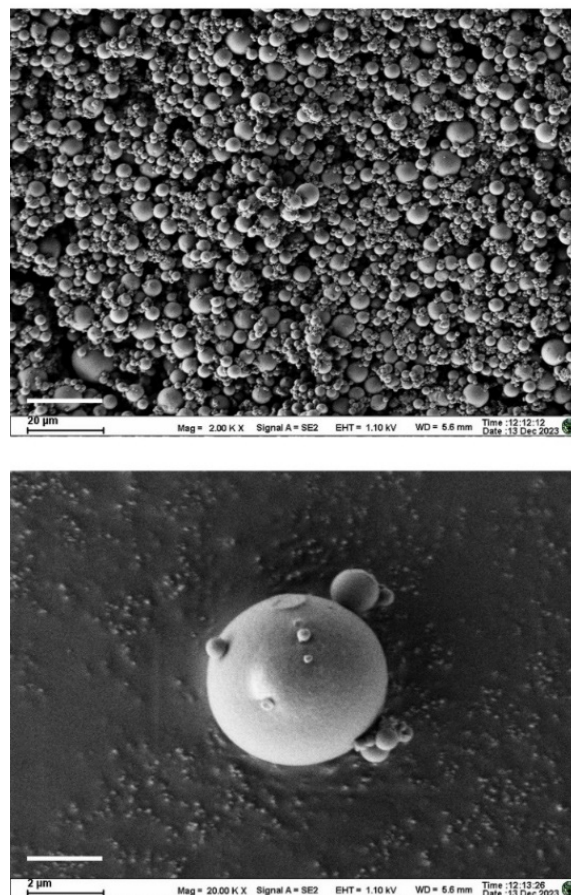


Fig. 4. The SEM images of GA-GMs, magnification 200x, scale: 20 μm and magnification 2000x, scale: 2 μm .

DOPA-GMs

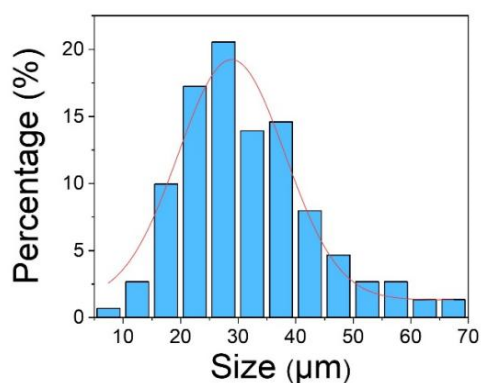


Fig. 5. The particle size distribution of DOPA-GMs.

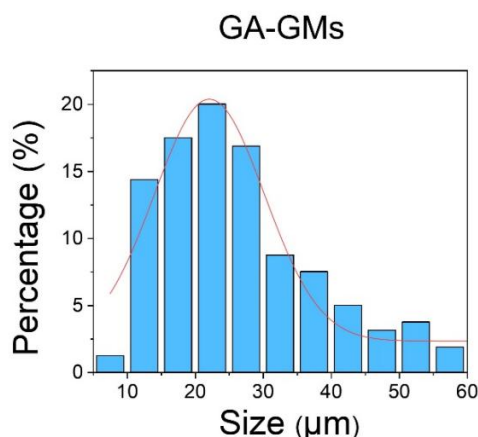


Fig. 6. The particle size distribution of GA-GMs.

3.3. The whole blood coagulation time of DOPA-GMs and GA-GMs

To evaluate the blood coagulation properties *in vitro* of DOPA-GMs, and GA-GMs, the microspheres and blood were mixed at 37 °C. The blank control group was the time of natural coagulation of blood without any treatment. In Fig. 7, the blood clotting time of blood without any treatment was 154 seconds and GMs group had a blood clotting time of 150 seconds. After the Michael addition reaction between polyphenols and gelatin, the coagulation time of blood *in vitro* was further reduced. GA-GMs had a reduced blood clotting time of 146 seconds, whereas DOPA-GMs had a reduced blood clotting time of 126 seconds.

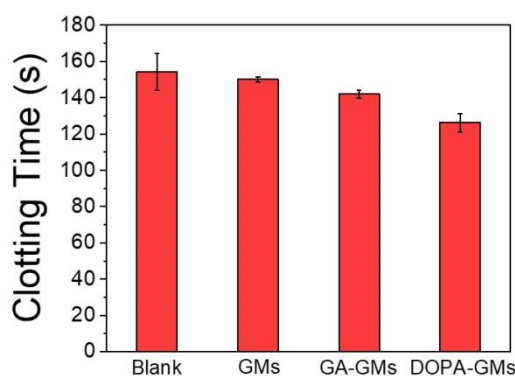


Fig. 7. The whole blood coagulation time of GMs, DOPA-GMs and GA-GMs.

Fig. 8 demonstrated blood coagulation in blank control group, GMs, GA-GMs and DOPA-GMs. The blood mixed with GA-GMs and DOPA-GMs had completely coagulated, while the blood of blank control group and GMs was still flowing without coagulation. These results indicated that microspheres obtained from the Michael addition reaction of the polyphenols such as dopamine and gallic acid with gelatin could improve the hemostatic properties *in vitro* of single-component gelatin microspheres.

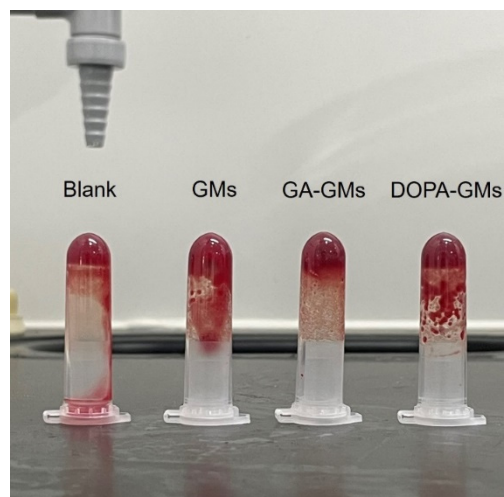


Fig. 8. The digital photos of whole blood coagulation of GMs, DOPA-GMs and GA-GMs

4. Conclusion

In the present study, we first prepared dopamine crosslinked gelatin and gallic acid crosslinked gelatin by Michael addition reaction under slightly alkaline conditions. Then, DOPA-GMs and GA-GMs were prepared by water-in-oil emulsion cross-linking method. DOPA-GMs and GA-GMs with intact spherical structure and micron size distribution could effectively improve the hemostatic properties of single-component gelatin microspheres. An effective strategy was provided for the preparation of hemostatic microspheres. In the future, for non-compressible, uncontrollable and irregular bleeding wounds, composite hemostatic materials with high water absorption, great biocompatibility and rapid hemostasis will always be the focus of research.

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