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Amino Groups Are Crucial for Chitosan to Stop Bleeding

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Abstract: Marine organisms adhere themselves onto wet surfaces by adhesive proteins containing L-3,4-dihydroxyphenylalanine (DOPA). Several research groups have incorporated DOPA, or other compounds that contain 3,4-dihydroxybenzene, into backbone of polymeric materials, and have found the improved adhesive properties. Although current positively charged chitosan hemostatic agents have limited adhesive property, especially to wet surfaces underneath blood pool, there is no report about modifying chitosan with 3,4-dihydroxybenzene to achieve improved adhesive property so far, and the exact mechanism of chitosan's positive charging is still unknown. Using two methods, we modified chitosan with 3,4-dihydroxybenzene. One is modifying with 3,4-dihydroxybenzaldehyde (DHBH), the other is with DOPA. The chemical structures of chitosan, Celox, DHBH, DMCTS, DOPA and DOPAMCTS were characterized with Fourier transform infrared (FTIR) spectroscopy. The coagulation test was performed to compare the hemostatic property of DMCTS and DOPAMCTS to that of chitin, chitosan and Celox. FTIR results revealed extreme similarity of chemical structures of chitosan and Celox, especially in presence of N-H bending vibration of primary amines, the incorporation of 3,4-dihydroxybenzene from DMCTS and DOPA into backbone of chitosan. The coagulation time of chitosan, Celox and DOPAMCTS was significantly shorter than that of chitin and DMCTS. The blood drops touching Celox, chitosan and DOPAMCTS particles appeared significant surface-tension phenomenon. Amino groups are crucial for chitosan to stop bleeding. Modification with 3,4-dihydroxybenzene does not impair the hemostatic property of chitosan as long as the free or protonated amino groups does not be modified. It is feasible to modify chitosan with 3,4-dihydroxybenzene to develop a novel hemostatic dressing.

Keywords: 3,4-dihydroxybenzaldehyde, L-3,4-dihydroxyphenylalanine, Chitosan, 3,4-dihydroxybenzene Modified Chitosan, L-3,4-dihydroxyphenylalanine Modified Chitosan, Celox, Hemostatic Agent, FTIR

1. Introduction

Chitosan-based hemostatic agents, such as Celox and Hem Con, because of their good histocompatibility, low toxicity, no danger of thrombosis or heat relieving, are superior to other kind of hemostatic agents, such as Quick Clot and Wound Stat, and have been approved by FDA and widely used to deal with uncontrolled bleeding. [1-6] The reason of these chitosan-based hemostatic agents to stop bleeding is that, they are positively charged particles that attract to negatively charged red blood cells and subsequently undergo chemical and mechanical linkages to form a barrier at the site of injury that works independently of clotting factors. [7, 8] Nevertheless, why chitosan being positively charged is still unknown, and because of these hemostatic agents have limited

adhesive strength to tissue surfaces, especially to wet surfaces underneath blood pool, although they can effectively control the venous bleeding, they can't effectively stop the uncontrolled bleeding from arteries with high pressure [9]. Therefore, it's significant to reveal the exact mechanism of chitosan's positive charging and find a new way to enhance the adhesive property of these chitosan-based agents to further improve the hemostatic effect.

It is well known that marine organisms, such as barnacles and mollusks, adhere themselves onto wet surfaces, such as rocks, stones and others. Extensive scientific researches have been conducted to understand and explain the mechanism and the molecular interactions that take effect behind this adhesiveness. [10-12] These researches led to the very interesting discovery that some proteins with specific chemical groups that were extracted by these organisms were responsible for this adhesive ability of the marine creatures to stick on various surfaces underwater. [13] Accordingly, a biomimetic system that would contain the structural moieties present in the amino acids that confer adhesiveness to the mollusk proteins could be a key aspect towards the goal of the formulation of a biocompatible, biodegradable, non-toxic and versatile glue that could provide adhesiveness on a diversity of wet surfaces. One such approach would be to attach the key molecular features present in the proteins to the backbone of a biodegradable, biocompatible and nontoxic polymeric backbone.

The researches in this field have developed a theory that the curing of the marine adhesive proteins occurs possibly through the quinone equivalent of the polyphenolic groups that they contain. [14] Most studies have indicated that one of the characteristics of these marine adhesive proteins is the presence of the amino acid L-3,4-dihydroxyphenylalanine (DOPA), which is believed to play an important role in the bonding of these creatures onto wet surfaces. The exact mechanism behind this adhesiveness remains a point of study. [12] Following the polyphenolic theory, several research groups have incorporated DOPA, or other compounds that contain the same functional groups, into backbone of polymeric materials, and have found that the adhesive properties of the obtained materials have improved. [15-18] Moreover, other researchers have studied the rheological properties of chitosan solutions upon addition of diphenolic compounds and enzymes, which promote the oxidation to quinone structures, and found this modification could lead to water-resistant adhesives. [19] However, the coagulation promoting is the most important property for chitosan as a hemostatic dressing, whether the coagulation promoting property of chitosan can be affected by incorporation of the polyphenolic groups is still unknown.

In present study, we used two different methods to incorporate 3,4-dihydroxybenzene into backbone of chitosan: First, following the principle of Schiff's base formation (Figure 1), we incorporated 3,4-dihydroxybenzene, which was provided by 3,4-Dihydroxybenzaldehyde (DHBH), into backbone of chitosan. Second, using a condensation reaction, we incorporated 3,4-dihydroxybenzene of DOPA, into backbone of chitosan (Figure 2). Afterwards, we characterized the synthesized materials, 3,4-dihydroxybenzene modified chitosan (DMCTS) and DOPA modified chitosan (DOPAMCTS), by FTIR spectroscopy, and compared the coagulation promoting property of chitosan, chitin, Celox, DMCTS and DOPAMCTS using coagulation test.

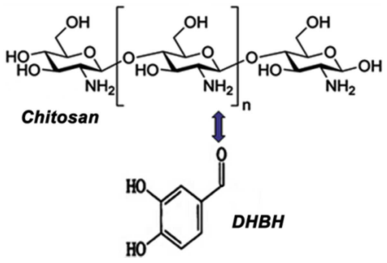

Figure 1. *The principle of Schiff's base formation.*

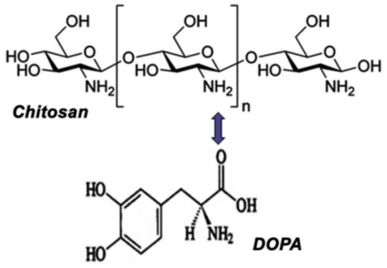

Figure 2. *Incorporating 3,4-dihydroxybenzene of DOPA to chitosan.*

2. Materials and Methods

2.1. Synthesis of DMCTS

0.4 g of chitosan (practical grade, Sigma-Aldrich) was dissolved in 20 ml of 1 wt% acetic acid in H_2O (*sol* A) in a 50ml single-neck round bottom flask. 2.0 g of DHBH (synthesis grade, Sigma-Aldrich) were then dissolved in another vial, which contained 10 ml of 100% ethanol, and stirred for 5 minutes until full dissolution (*sol* B). *Sol B* was then added drop-wise into *sol A,* and the obtained mixture was stored for 4 hours at 70°C in a water bath. Then, DMCTS was precipitated with the addition of a large amount of 0.1mol/L NaOH. After being filtrated and washed with copious amounts of ethanol, DMCTS was extracted in a Soxhlet apparatus with 100% ethanol at 80°C for 24 hours to further remove potential impurities and/or any unreacted compounds. Afterwards, DMCTS was stored overnight at room temperature in a fuming cupboard and then frozen at -40°C for 2 hours. Finally, DMCTS was lyophilized at -60°C for 48 hours.

2.2. Synthesis of DOPAMCTS

2850 mg Boc-L-DOPA (practical grade, Sigma-Aldrich), 338 mg chitosan and 920 mg TBTu, were dissolved into the solution of 20 ml DMF, 1.52 ml (2M) HOBt and 1.04 ml DIEA. The mixture was stirred at room temperature for 12 hours. Afterwards, 4 ml of 50% TFA-DCM solution was added to remove the Boc-protecting group of Boc-L-DOPA and stirred at 4°C for 2 hours. Then, DOPAMCTS was precipitated with the addition of a large amount of iced ether. After being filtrated and washed with copious amounts of

ether, DOPAMCTS was stored overnight at room temperature in a fuming cupboard and then frozen at -40°C for 2 hours. Finally, DMCTS was lyophilized at -60°C for 48 hours.

2.3. Characterization of Chitosan, Celox, DHBH, DMCTS, and DOPAMCTS by FTIR Spectroscopy

A Vertex 70 Fourier transform infrared spectrometer (Bruker, Germany) equipped with a KBr beam splitter and a DLaTGS detector was employed for all the IR spectral measurements with a nominal resolution of 4 cm^{-1} and accumulating 16 scans.

The particles weighted 1.50 mg of chitosan, Celox (Medtrade Biopolymers, Crewe, UK, distributed in the United States by SAM Products, Portland, OR), DHBH and DMCTS were respectively added to 150.0 mg KBr powder (particle diameter small than 2 micrometer). The mixtures were ground and homogenized for 3 min in a dryagate mortar. Then they were pressed at 20 tones in a 13mm diameter evacuable pellet die and the FTIR spectrum of the resultant KBr disk recorded in the range from 4000 cm^{-1} to 400 cm^{-1}, at 4cm^{-1} resolution and accumulating 16 scans for spectrum.

2.4. Coagulation Test

Before testing, all of the agents were ground for 1 minute in a dry mortar to ensure the diameters of the particles were consistent. All of the testing particles were divided into 5 groups: Group A, Chitin Group, with chitin particles; Group B, Chitosan Group, with chitosan particles; Group C, Celox Group, with Celox particles; Group D, DMCTS Group, with DMCTS particles; and Group E, DOPAMCTS Group, with DOPAMCTS particles. Each group included 3 samples. Each sample comprised 0.02g of particles according to different groups. The total number of samples was 15.

2ml of fresh basilica venous blood was harvested from a healthy, 32-year old male volunteer. Before the test, the volunteer didn't receive any anticoagulant therapy. After blood harvesting, Heparin dose of 100usp per 1 mL blood were added. And immediately, each sample was added respectively to single well of a sterilized 12-well cultivation board. As soon as one drop of the heparinized fresh blood was touching the surface of the particles in each well, the chronograph began to record. The time when the blood drop was clotted was recorded. If the blood drop in a sample did not clot within 180 seconds then its time was still recorded as 180 seconds. After the times of all the samples were recorded, the cultivation board was photographed.

3. Results

3.1. Characterization of Chitosan, Celox, DHBH, DMCTS, DOPA and DOPAMCTS by FTIR Spectroscopy

FTIR spectra of chitosan and Celox can be seen in Figure 3. Chitosan was found to exhibit characteristic absorptions at 1655 cm^{-1}, which was attributed to the N-H bending vibration of primary amines. The other characteristic peaks of the saccharide structure of chitosan appeared at 1157 cm^{-1}, 1076 cm^{-1} and 1022 cm^{-1}. Celox was found to exhibit characteristic absorptions at 1645 cm^{-1}, which was attributed to the N-H bending vibration of primary amines. The other characteristic peaks of the saccharide structure of Celox appeared at 1155 cm^{-1} and 1071 cm^{-1}. This figure shows extreme similarity of chemical structures of chitosan and Celox Dressing.

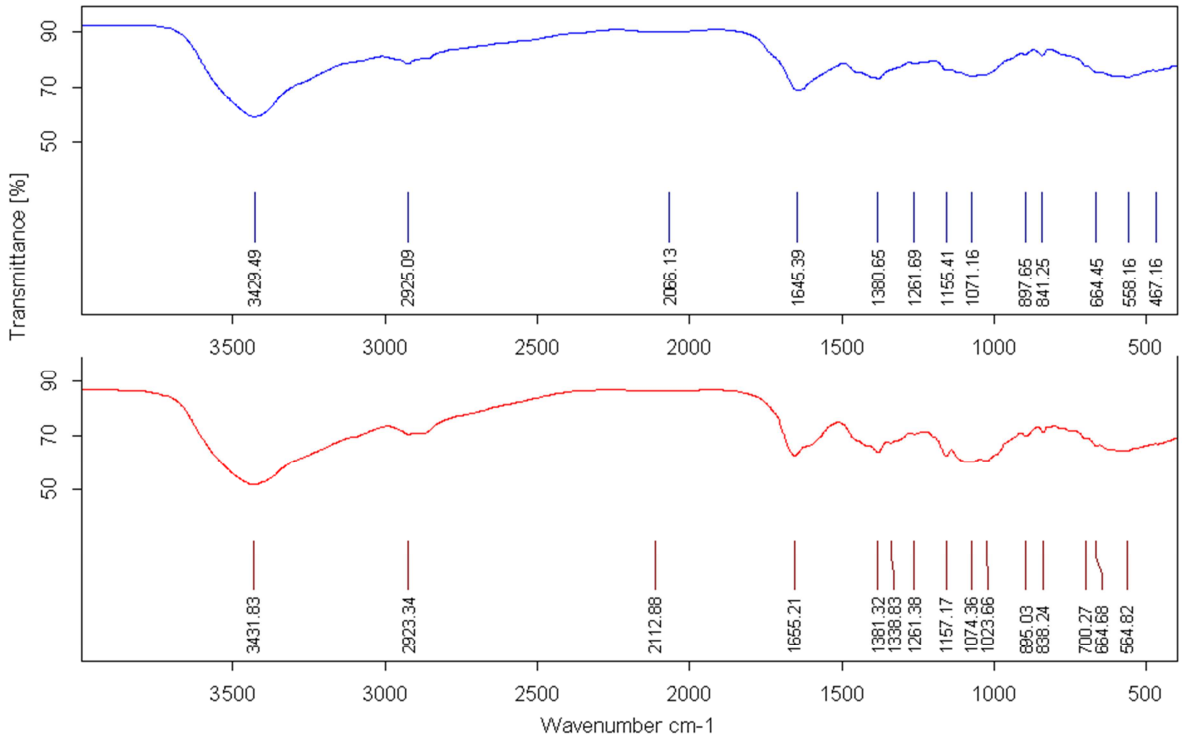

Figure 3. *FTIR spectra of chitosan and Celox. Blue: Celox; Red: chitosan.*

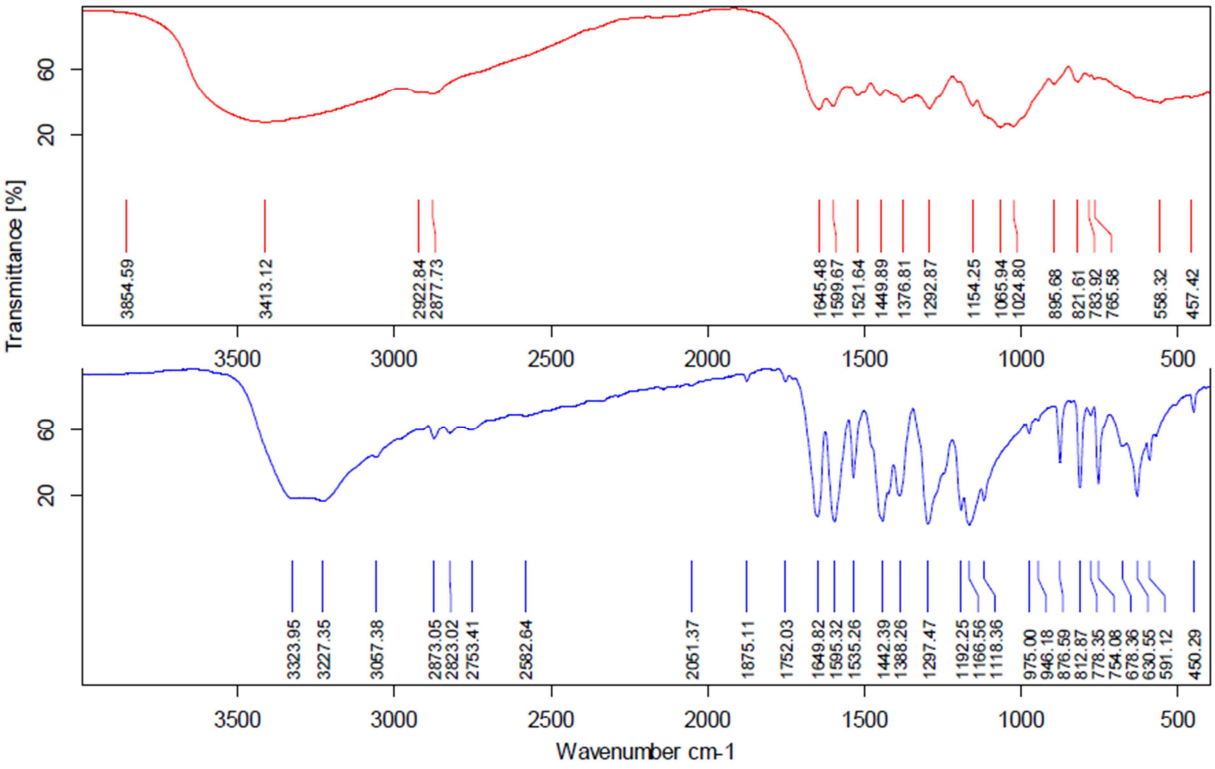

Figure 4. *FTIR spectra of DHBH and DMCTS. Red: DMCTS; Blue: DHBH.*

FTIR spectra of DHBH and DMCTS can be seen in Figure 4. The spectrum of DHBH revealed the characteristic carboxyl peak at 1650 cm^{-1}, whereas in the spectrum of DMCTS there was no peak corresponding to the carboxyl group of the DHBH due to the amide bond formation between DHBH and the amine group of chitosan (Schiff's base). The prominent peaks at 1645 cm^{-1}, which was observed in the IR of the DMCTS were assigned to the C=N stretching vibration of amides. The other characteristic peaks of the benzene ring appeared at 1600cm^{-1} and 1521 cm^{-1}. This figure shows, in DMCTS molecule, the incorporation of dihydroxy benzene group into backbone of chitosan.

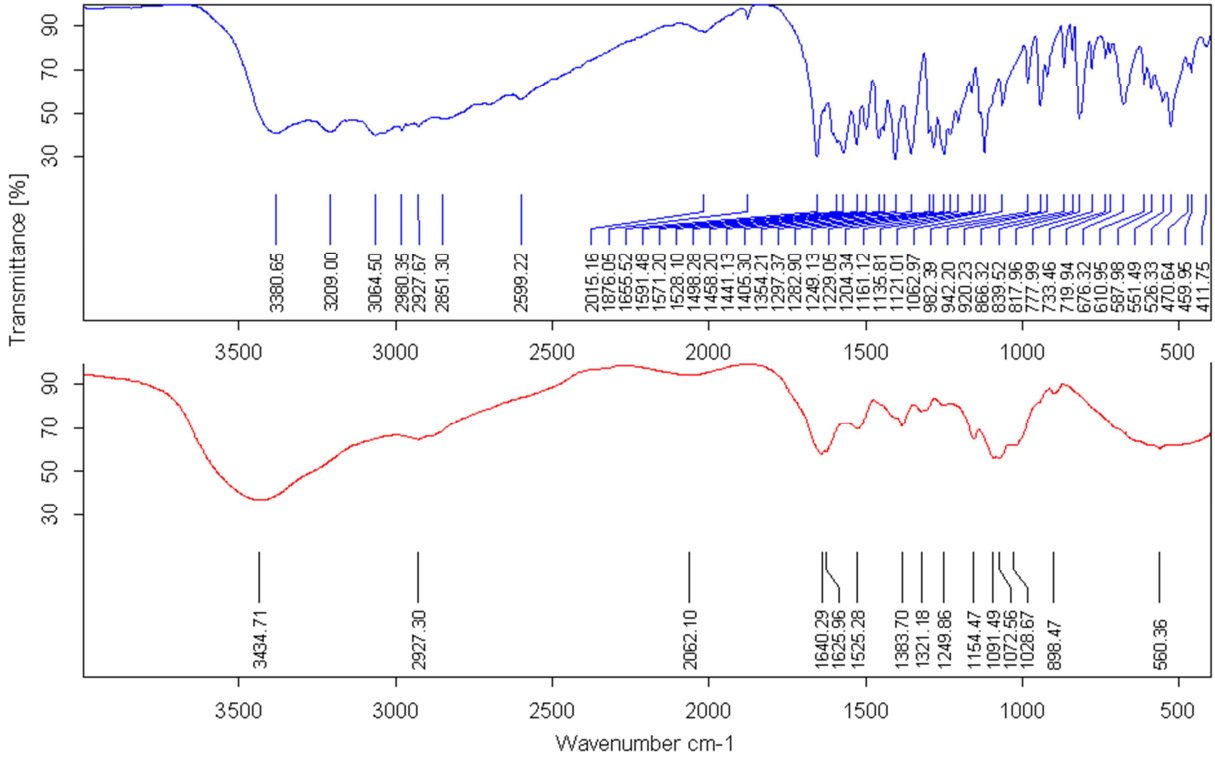

Figure 5. *FTIR spectra of DOPA and DOPAMCTS. Blue: DOPA; Red: DOPAMCTS.*

FTIR spectra of DOPA and DOPAMCTS can be seen in Figure 5. The spectrum of DOPA revealed the characteristic peaks at 3380 cm^{-1}, 3209 cm^{-1}, and 3064 cm^{-1}, which was attributed to the -OH bending vibration. The characteristic peaks of the benzene ring of DOPA appeared at 1571 cm^{-1}, 1528 cm^{-1}, 1121cm^{-1}, 942 cm^{-1}, 810 cm^{-1} and 616 cm^{-1}. DOPAMCTS was found to exhibit characteristic absorptions at 3434 cm^{-1}, which was attributed to the -NH and -OH bending vibration. The other characteristic peaks of the alkane structure of DOPAMCTS appeared at 2927 cm^{-1} and 1383 cm^{-1}. This figure shows, in DOPAMCTS molecule, the incorporation of dihydroxy benzene and amino groups into backbone of chitosan.

3.2. Coagulation Test

Figure 6 shows the different appearance of the heparinized fresh blood touching chitin (A), chitosan (B), Celox (C), DMCTS (D) and DOPAMCTS (E) particles. The blood drop coagulated as soon as they touched the chitosan, Celox and DOPAMCTS particles while there was no any sign of coagulation 180 seconds after the blood drops touched the chitin and DMCTS particles. Furthermore, the blood drop touching the chitosan, Celox and DOPAMCTS particles appeared significant surface-tension phenomenon.

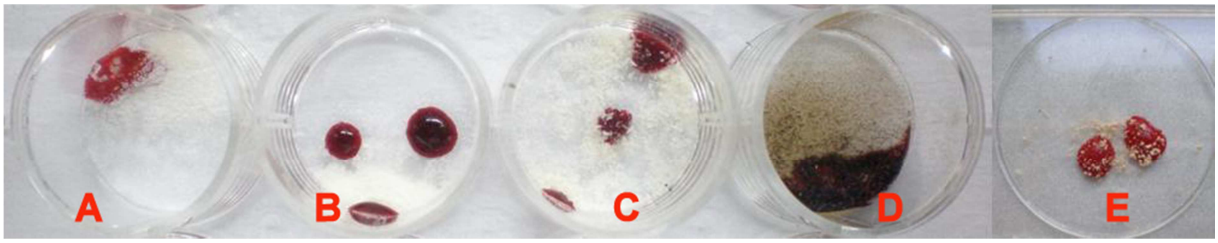

Figure 6. *The different appearance of the theheparinized fresh blood touching different hemostaticagent particles. (A: chitin; B: chitosan; C: Celox Dressing; D: DMCTS; E: DOPAMCTS.).*

Figure 7 shows the different coagulation time of blood drops touching different hemostatic agents. The coagulation time of blood drops touching the chitosan, Celox and DOPAMCTS particles was significantly shorter than that of blood drops touching the chitin and DMCTS particles.

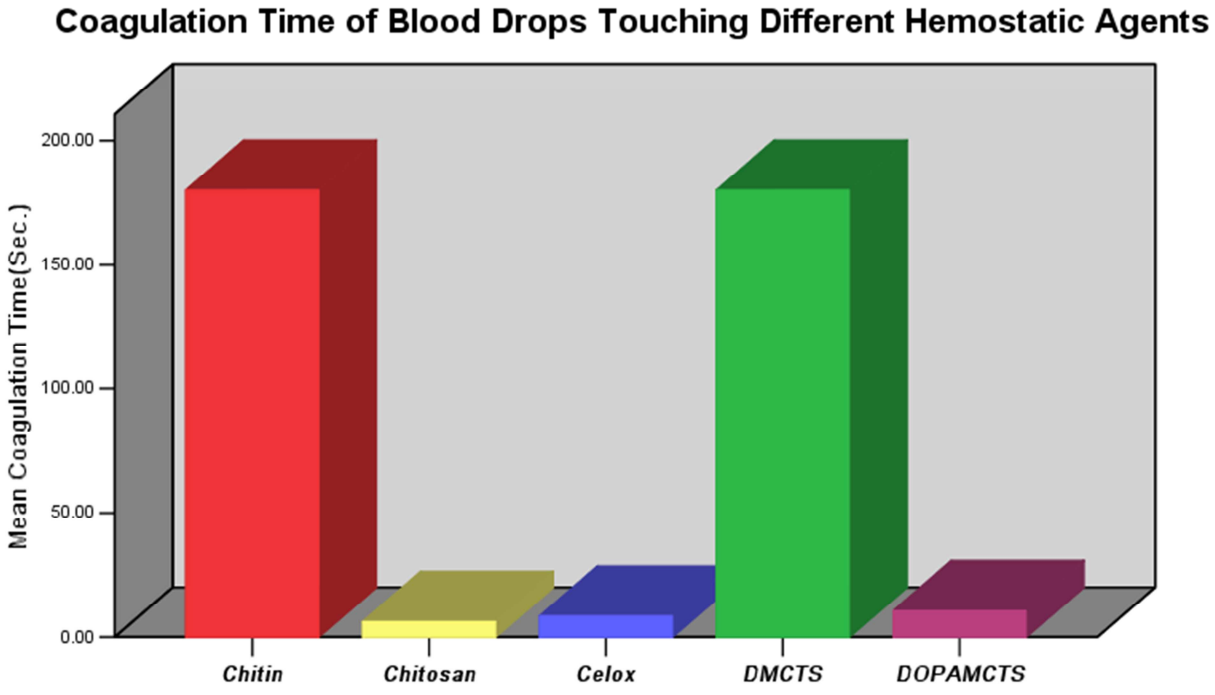

Figure 7. *The different coagulation time of blood drops touching different hemostatic agents.*

4. Discussion

In this study, we used two methods to modify chitosan with 3,4-dihydroxybenzene. The first modification method is that, we modified chitosan with DHBH and synthesized a new polysaccharide, DMCTS. The results of FTIR spectroscopy have verified the incorporation of 3,4-dihydroxybenzene into backbone of chitosan. But it's unexpected that, this modification compromised the initial hemostatic property of chitosan. We speculate the reason might as follows.

As is known, Chitosan is a natural polysaccharide that is derived from chitin by deacetylation. After deacetylation,

the -$COCH_3$ groups are replaced by a positively charged -H, and this makes chitosan possess protonated amino groups. Our coagulation test has revealed the significant difference of hemostatic property between chitosan and chitin. This difference hint that there might be some relationship between chitosan's amino groups and chitosan's hemostatic property.

After being modified by 3,4-Dihydroxybenzaldehyde, the 2 positively charged -H of each protonated amino group were replaced by =CRH (R represents 3,4-dihydroxybenzene). And our coagulation test also revealed the loss of hemostatic property of DMCTS. This finding made us presume that, amino groups might be crucial for chitosan to possess hemostatic property.

To verifiy the presumption above, we attempted the second method to modify chitosan: using a condensation reaction to connect DOPA and chitosan. The main difference of DOPA and DHBH is that, DOPA possesses a free amino group. And we used Boc-protection groups to prevent the amino group being modified during the reaction. After the condensation reaction and removel of the Boc-protection groups, DOPAMCTS still has protonated amino groups. And our coagulation test revealed the significant hemostatic property of DOPAMCTS.

The results of the second modification methods verified our presumption, that possessing amino groups is a necessary and sufficient condition for chitosan to have hemostatic property. Moreover, it is reasonable to believe that, in electrolyte solution, free amino groups of chitosan can be easily changed into ammonium salt and this can therefore make chitosan positively charged.

Significant surface-tension phenomenon occurred when blood drops touched chitosan, Celox and DOPAMCTS particles. We explain the reason as follows. The surface-tension of water molecule is determined by the distance of two adjacent molecules on the surface of water. As soon as the distance increases, the surface-tension increased accordingly. When the blood drops touched chitosan, Celox and DOPAMCTS particles, because of the effect of electrostatic attraction, the positively charged particles absorbed and assembled the negatively charged erythrocytes on the surface of the blood drops and relatively increased the distance between water molecules and therefore induced significant surface-tension phenomenon. The occurrence of surface-tension phenomenon is an evidence of electrostatic interaction between erythrocytes and chitosan / Celox/ DOPAMCTS particles.

Our study has two aspects of significance. First, we firstly revealed the crucial role of amino groups for chitosan as a hemostatic agent. Second, we firstly verified the feasibility of modification with 3,4-dihydroxybenzene to develop a novel chitosan-based hemostatic agent. In our future study, we will eveluate the adhesive strength of DOPAMCTS in vitro and in vivo.

5. Conclusion

Amino groups are crucial for chitosan to stop bleeding. Modification with 3,4-dihydroxybenzene does not impair the hemostatic property of chitosan as long as the amino groups within molecular are not be modified. It is feasible to modify chitosan with 3,4-dihydroxybenzene to develop a novel hemostatic dressing.

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