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Full Length Research Paper

Synthesis and characterization of hydrophobically modified chitosan

Qin He[1,2], Jing Guan[2*], Miaolei Jing[1], Shujie Huang[2], Cheng Xu[2], Zhihong Li[2], Jimin Wu[2] and Xizheng Zhang[2]

[1]School of Textiles, Tianjin Polytechnic University, Tianjin (300387), China.
[2]Institute of Medical Equipment, Academy of Military Medical Sciences, Tianjin (300161), China.

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The aim of this paper was to prepare and evaluate the feasibility of hydrophobically modified chitosan for hemostatic use. Chitosan was grafted with different branched chains by schiff reaction with salicylaldehyde and dodecyl aldehyde and followed by reductive amination with $NaBH_4$. Elemental analysis was carried out to calculate the degree of substitution (DS) for NH_2. Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) tests were used to confirm the composition and structure of modified chitosan products. Rheological experiments were performed to evaluate the ability of fluids including modified chitosan and its mixtures with heparinized blood to form gels. Dynamic blood coagulation experiments were tested to compare the hemostatic property of chitosan with its derivatives *in vitro*. The results demonstrated that orhto-hydroxytoluene and dodecyl were grafted into the backbone of chitosan successfully and the crystallity of chitosan was changed sligtly. Although the DS of ortho-hydroxytoluene chitosan was higher than that of dodecyl chitosan, the later had better gel-forming ability and lower absorbency at specific wavelength of haemoglobin. Dodecyl chitosan had great potential as an ideal candidate for local hemostasis.

Key words: Ortho-hydroxytoluene chitosan, dodecyl chitosan, rheological property, dynamic blood coagulation.

INTRODUCTION

Hemorrhage is the second leading cause of death in civilian trauma and the leading cause of death from battlefield trauma in the military (Acosta et al., 1998; Bellamy, 1984; Brown et al., 2009; Sauaia et al., 1995). So, hemostatic agents that could staunch the bleeding from serious wounds are pressing needs both in civilian trauma centers as well as for military personnel. A fairly diverse range of hemostatic agents have been developed that could arrest bleeding and stabilize the casualty before evacuation to definitive care (Ong et al., 2008). But most of these agents are highly expensive and scarce in supply.

Chitosan, the second most abundant natural polymer next to cellulose, is an interesting polysaccharide because of the presence of amino functionality on the C_2 position (Hall and Holme, 1986; Yalpani and Hall, 1984). Chitosan has been used as a local hemosatic agent attributed to its cationic, antimicrobial nature (Malette et al., 1983; Rao and Sharma, 1997; Whang et al., 2005), biocompatibility and low immunogenicity (Hirano, 1999; Kean and Thanou, 2010; Kurita, 2006; Mi et al., 2001; Mourya and Inamdar, 2008; Yi et al., 2005), however, its efficacy in dealing with severe wounds has been questioned (Kheirabadi et al., 2005). Alkylated chitosan has been prepared by using aldehydes with different alkyl chains, but seldom studies of the effects of hydrophobic modified chitosan on hemostatic property were performed so far.

Recently, Dowling et al. (2011) grafted chitosan with 4-octadecylbenzaldehyde and proposed a hypothesis that hydrophobically modified chitosan could anchor hydrophobes from the polysaccharide into the hydrophobic interiors of blood cell membranes, and thereby, blood cells would become connected by biopolymer chains into a sample-spanning gel network, which could potentially

*Corresponding author. E-mail: janeamms@yahoo.com.cn. Tel: 86 22 84656260. Fax: 86 22 84656717.

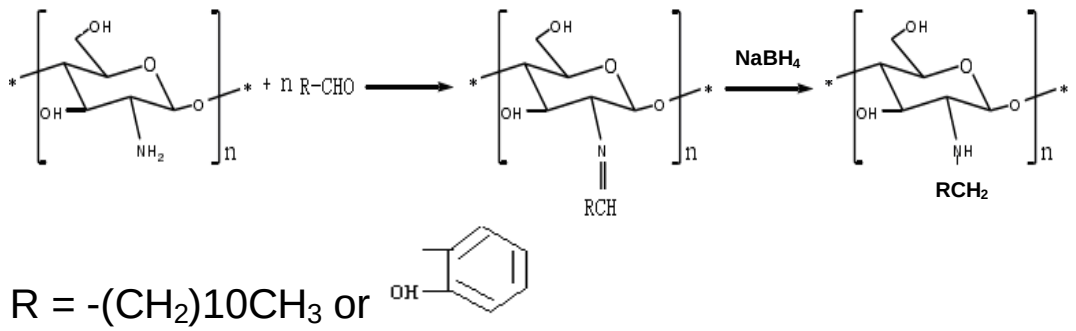

Figure 1. Reaction scheme between the amino on chitosan and aldehyde.

halt the flow of blood. However, the effects of hydrophobically chitosan modified by grafting with other branched chains, including both aromatic and aliphatic groups, have never been studied as far as we know. Thus, alkylated chitosan by introducing another two kinds of aldehydes (salicylaldehyde and dodecyl aldehyde) were studied in this paper, the DS under the same molar ratio of NH_2 to aldehyde were tested by elemental analysis, and its structure and crystal property were analyzed by FTIR and XRD, respectively. The rheological experiments were performed to evaluate the ability of fluids including grafted chitosan and its mixtures with heparinized blood to form gels (Shi et al., 2009). Dynamic blood coagulation experiments were also tested to compare the hemostatic property of chitosan with its derivatives *in vitro*.

MATERIALS AND METHODS

Chitosan of medium molecular weight (about 270 k) was purchased from AK biotech Limited (Shandong, China). Salicylaldehyde was purchased from Shanghai reagent factory, and dodecyl aldehyde was purchased from Sigma-Aldrich.

Synthesis of hydrophobic modified chitosan (hm-CS)

For synthesis of grafting copolymers, chitosan was dissolved in 1% (v/v) acetic acid to form a 1% (w/v) solution, 2 h later, the pH of the solution was adjusted to 4.5 by adding sodium hydroxide solution, then a given weight of aldehyde (the molar ratio of amino group to aldehyde group was fixed at 1:1) was added into the polysaccharide solution under stirring until complete dissolution. An excess of reducing agent ($NaBH_4$) was added into the mixture to reduce the intermediate imine. Then, the pH was adjusted to 10 until the deposition appeared. The precipitation was filtrated and washed in distilled water; the supernatant was purified 24 h in presence of ethanol and acetone to eliminate the remaining aldehyde and mineral. The outcome was dried in 60°C (Muzzarelli et al., 1985). The reaction mechanism was shown in Figure 1.

Obtaining blood

Two mature male New Zealand white rabbits (each approximately 2.5 kg in weight) were obtained from the center of experimental animal of the Academy of Military Medical Science, anesthesia was carried out by an intramuscular injection of 2% pentobarbital sodium salt (40 mg/kg intramuscularly). Then, 20 ml intervals of blood were drawn into syringe containing 2 ml 3.8% (w/v) citrate sodium.

Characterizations of chitosan and its derivatives

Elemental analysis

Elemental analysis allowed us to determine the carbon and nitrogen content yielded from the copolymers in comparison with those yielded from initial chitosan, further, the degree of deacetylation (DD) of original chitosan and DS of grafted branched chains can be calculated according to formula (1) and (2), respectively.

$$DD = \frac{[(1-x)\times 8 + x\times 6]\times 12}{14\times 100\%} = \frac{C}{N} \quad (1)$$

$$DS = \frac{[(1-x)\times 8 + x\times 6 + DS\times n]\times 12}{14\times 100\%} = \frac{C}{N} \quad (2)$$

Where, x represents the DD of chitosan C, N represents the percentage of carbon and nitrogen content in the material, respectively and n represents the number of carbon introduced to the amino group.

FITR spectroscopy

Composition of chitosan and its derivatives were performed using FTIR. Tensor37 spectrometer (Germany) was used to record the infrared spectra of chitosan and copolymers.

XRD

X-ray diffraction (XRD) measurement was performed with a D8 Discover with Gadds diffractometer (Germany). CuKα radiation (λ = 0.15406 nm) was used, and scanning rate was 2°/min with a generator voltage of 40 kV and a generator current of 30 mA. XRD peaks were recorded from 2θ = 5° to 40°.

Rheological experiments

Dynamic rheological experiments were performed on a Rheometrics AR1500 from TA Instruments (USA) stress-controlled

Table 1. Elemental analysis of the chitosan and its derivatives.

Samples	C (%)	N (%)	C/N	DS (%)
Chitosan	39.57	7.17	5.52	77.84
Copolymer 1	46.72	5.59	8.36	47.36
Copolymer 2	50.01	5.56	8.99	33.75

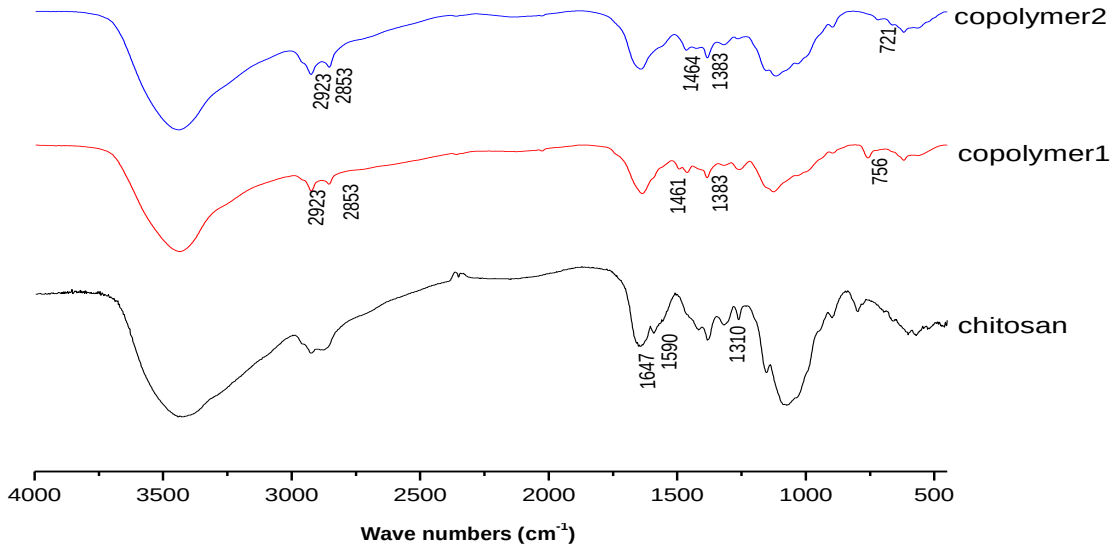

Figure 2. FITR spectra of chitosan, copolymer 1 and copolymer 2.

rheometer. A cone and plate geometry of 3.59° angle and 40 mm diameter was used, and samples were run at the physiological temperature of 37°C. For unmodified polysaccharides and their derivatives in solution, dynamic experiments were performed in the linear viscoelastic region where G' (storage modulus) and G" (loss modulus) are independent on the stress applied. Dynamic modulus were determined as a function of the angular frequency (ω) expressed in Hz. Temperature variation of 3°/min is imposed when necessary and controlled by a plate (Rinaudo, 2011).

Dynamic blood clotting

Materials were placed into polypropylene beaker, 100 µL of citrated sodium blood was then dispensed onto the surface of measuring materials, and 25 µL of 0.2 M calcium chloride solution added, 50 ml of distilled water was slowly poured to each sample at the interval of 5 min except the interval was kept as 10 min between 20 and 30 min. Ten minutes later, the suspension was collected and tested by ultraviolet spectrophotometer. The absorbency of dispersed haemoglobin at the wavelength of 540 nm was recorded at different time point.

RESULTS and DISCUSSION

Elemental analysis

The degree of grafting was expressed in terms of aldehyde chains grafted per a given number of monomeric units (usually 100 acetylation and deacetylation units). The results of DS are shown in Table 1. The DS value of chitosan equals to the DD datum of original chitosan, which indicates that 77.84% of total monomeric units were deacetylation units in chitosan and under the same reaction condition, the degree of grafting of salicylic aldehyde onto chitosan (copolymer 1) was larger than that of dodecyl aldehyde (copolymer 2). The salicylic aldehyde had a benzene ring with a short alkyl chain and smaller molecule of 122, while the dodecyl aldehyde had a long alkyl chain and a larger average molecule of 184, all of which might be attributed to the discrepancy in DS value.

FITR

Figure 2 shows the IR spectra of chitosan, orhto-hydroxytoluene chitosan (copolymer 1) and dodecyl chitosan (copolymer 2). The significant characteristic bands of CS at 1647 and 1590 cm^{-1} were the carbonyl (C=O) stretching vibration (amide I) and N–H bending vibration (amide II), respectively (Almdal et al., 1993; Li et al., 2011). For copolymers 1 and 2, after modification, the broad band in the region of 3200 to 3500 cm^{-1} of N-H and O-H stretching vibrations became narrow, the vibrational band of amino group at 1590 cm^{-1} disappeared and both

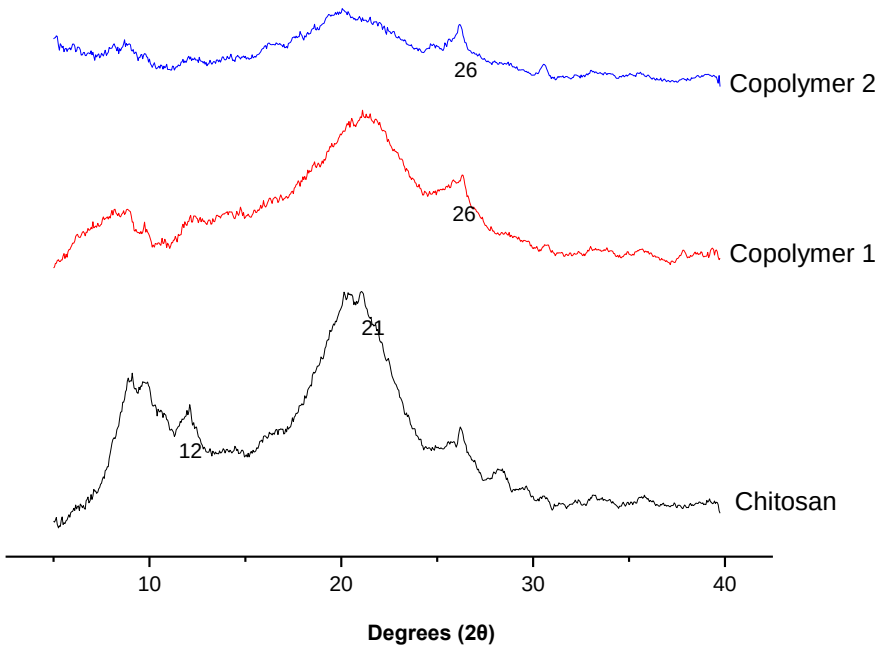

Figure 3. X-ray diffraction spectrum of chitosan and copolymers.

showed the substituted reaction on the amino group of CS. Also, the peaks intensity of both copolymer at 2923, 2853 and 1383 cm^{-1} increased, proving the introduction of $-CH_2$, while the corresponding peak intensity of copolymer 2 stronger than that of copolymer 1 implied that much more $-CH_2$ groups had been grafted into the chitosan backbone in copolymer 2.

The absorptions in copolymer 1 at 1416 and 756 cm^{-1} were assigned to C=C stretching vibration and C-H distortion vibration of benzene ring, respectively. For copolymer 2, the absorptions at 1464 and 721 cm^{-1} were assigned to the $–CH_2$ distortion vibration and $(CH_2)n$ ($n \geq 4$) backbone vibrations of alkyl groups, respectively, indicating the introduction of long alkyl groups. The FTIR analysis demonstrated that orhto-hydroxytoluene chitosan and dodecyl chitosan have been successfully prepared, respectively under desired experimental method.

XRD

The main feature of the X-ray diffraction spectrum for chitosan power is the obvious high intensity peaks in the range from 5° to 40° of 2θ, what could be seen from the Figure 3a. After modification, the absorbance peaks at 12° of 2θ disappeared, and the intensity at 26° improved both in Figure 3b and c. After the introducing of benzene ring and dodecyl into chitosan, the regulation of polysaccharide along the backbone changed and thus the intensity of hydrogen bond was disturbed, which led to the peaks of 12° disappearance and peaks of 26° highlighted. The degree of crystalline of copolymers changed slightly after hydrophobically modification of chitosan no matter what the introduced group was.

Rheological behavior of chitosan solution (CS) and its derivations versus temperature

Original chitosan

The modulus obtained from the dynamic rheology tests performed on chitosan solution from 25 to 67°C at a constant rate of 3°C/min showed that all the solutions are characterized by G" (loss modulus) > G' (storage modulus) independence of the temperature and the concentration of solution. The modulus changed a little when the temperature increased, and there was a slight difference among the different concentrations except that the loss modulus of chitosan at 1 g/L decreased quickly at the initial temperature. All the storage modulus and its corresponding loss modulus had the same trend along the temperature (Figure 4). The results indicate that chitosan acetic solution with concentration range from 1 to 10 g/L under temperature from 25 to 67°C is a viscous liquid.

Hydrophobic modified chitosan copolymers

The modulus of the hydrophobic modified chitosan copolymers at the concentration of 10 g/L related to temperature were also obtained from the rheological analysis. After modification, both the storage modulus

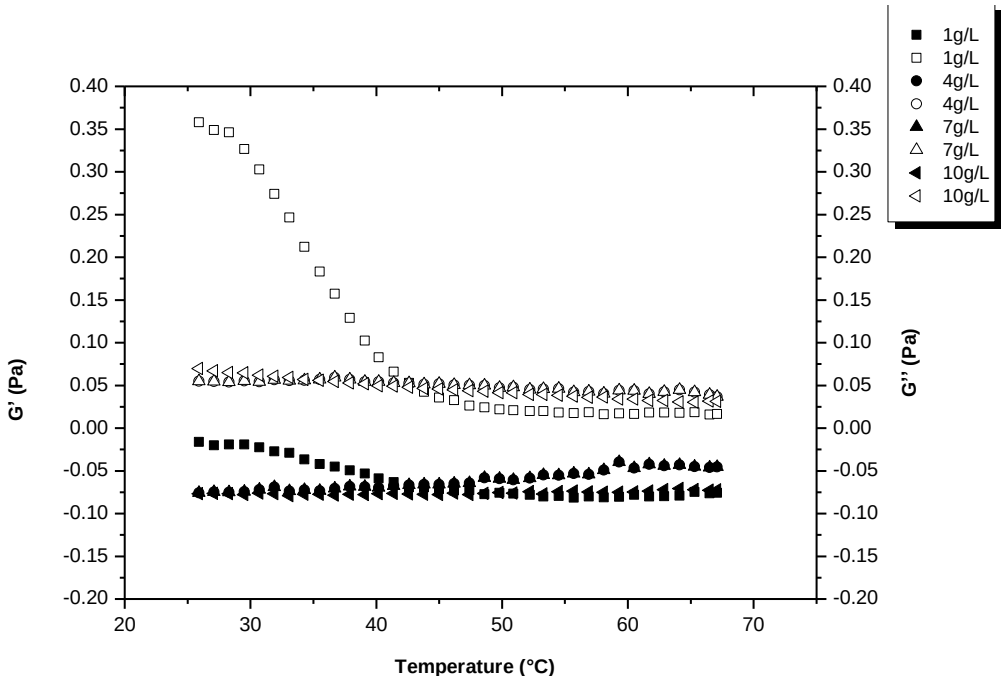

Figure 4. Storage (G' in Pa, filled symbols) and loss (G" in Pa, open symbols) modulus as a function of temperature for chitosan in 0.3 M acetic acid at different concentrations (1 -10 g/L).

and the loss modulus of the copolymers were larger than that of initial chitosan at the temperature from 25 to 40°C, and all the curves tend to be parallel in the range from 40 to 67°C. Although the DS of copolymer 2 was lower than that of copolymer 1, the modulus of copolymer 2 was larger than that of copolymer 1 at the atmosphere ambient which may be caused by stronger intermolecular forces of copolymer 2 due to grafting long alkyl chains. In the next period temperature, the modulus became the same as that of the three kinds of material and changed slightly. There was no doubt all the polymers were characterized by G" > G' over entire temperature range and kept viscous liquid status.

Rheological of mixtures of chitosan/derivatives and blood

The modulus of the citrated sodium whole blood, chitosan and copolymers mixed with citrated sodium blood in dependence of temperature were described in Figure 6. It showed that the G' > G" is in most range of temperature for all the four kinds of material, which is quite different from those of chitosan and copolymers. In the whole blood curve, the modulus values were low and nearly not changed after a thermal treatment but larger than that of mixture of chitosan at the concentration of 10 g/L with whole blood. The modulus values of the other two curves had a quantitative increase in Pa, especially for copolymer 2 solution, which attributed to both the larger molecular weight and longer alkyl chains on the backbone of the polysaccharide. The G' > G" over the most temperature range implies that a largely elastic response typical of a physical gel formed in mixtures of both copolymers and blood. The results indicate that although the DS of copolymer 2 was lower than that of copolymer 1, the gel-forming ability of copolymer 2 was better than that of copolymer 1 when contacted with whole blood.

Rheological behavior of mixtures of CS/derivations with blood versus frequency

According to Almdal et al. (1993), the systems characterized by specific mechanical properties in which the storage modulus (G') presents a plateau with frequency and the loss modulus (G") smaller than G', will be considered as gels. From the rheological behavior of the polysaccharides related to frequency (Figure 7), the gel-like structure could be seen from all the polysaccharide in the entire range of the frequency and in independence of the frequency, the extent was the question among the material. In the modulus obtained from the rheological of citrated sodium whole blood and chitosan, G' exceeded G" slightly in the entire range of the frequency but there was no linear increase in both modulus. The storage modulus of mixture of chitosan and whole blood was near that of whole blood and tended to be the same with the increase of frequency, the slightly

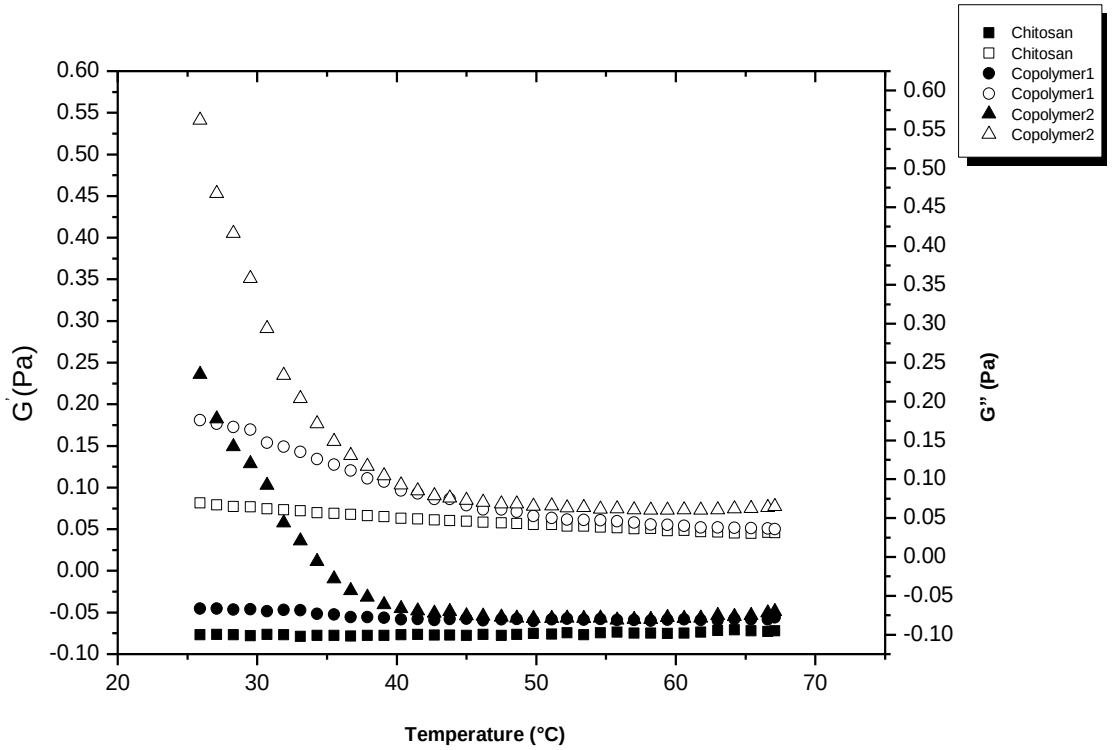

Figure 5. Storage (G' in Pa, filled symbols) and loss (G" in Pa, open symbols) modulus as a function of temperature for Chitosan, copolymer 1 and copolymer 2 at the concentration of 10 g/L.

gel-like structure attributed to the amino on the backbone of chitosan which formed cationic in acetic acid could react with the anion of erythrocyte membrane, thus forming electrostatic attraction.

There was no doubt the copolymers mixed with blood could transform the solutions to gels quickly in independence of frequency compared to that of copolymer solutions alone as shown in Figure 5. Our results were consistent with the theory proposed by Dowling et al. (2011) and further proved that the hydrophobic modified chitosan could be a useful hemostasis.

Dynamic blood clotting

During dynamic blood clotting experiment, the greater the materials influenced the blood coagulation factorial and the blood platelet adhesive, the lower absorbency at specific wavelength of haemoglobin, the better the blood coagulation property. The data in Table 2 showed that copolymer 2 could clot blood in a quick and short time, the absorbance of copolymer 2 was least at 5 min compared with that of two samples, and in the whole range of time, the values of the absorbance decrease quickly and tended to be zero at 30 min, the absorbance of copolymer 1 had the same tendency with that of copolymer 2 but higher than that of copolymer 2. As for chitosan, the values in the entire range of time had only a slight difference, proving that chitosan had poor hemostatic property compared with the other two hydrophobically modified chitosan.

Conclusion

The amphiphilic biopolymer, hydrophobically modified chitosan could act as an effective hemostatic agent. From the rheological tests conducted on chitosan and copolymers, the modulus both in storage and loss were rather little and the loss modulus was larger than that of storage, no matter the temperature and the frequency change before adding the whole blood; while the modulus changed dramatically, G' exceeded G" in the whole range of temperature and frequency after adding blood and showed that the mixture of copolymers and blood could form gel-network quickly and stanch the flow of blood. As for dynamic blood clotting test, copolymers could clot blood in an effective way and could be used as hemostatic agents.

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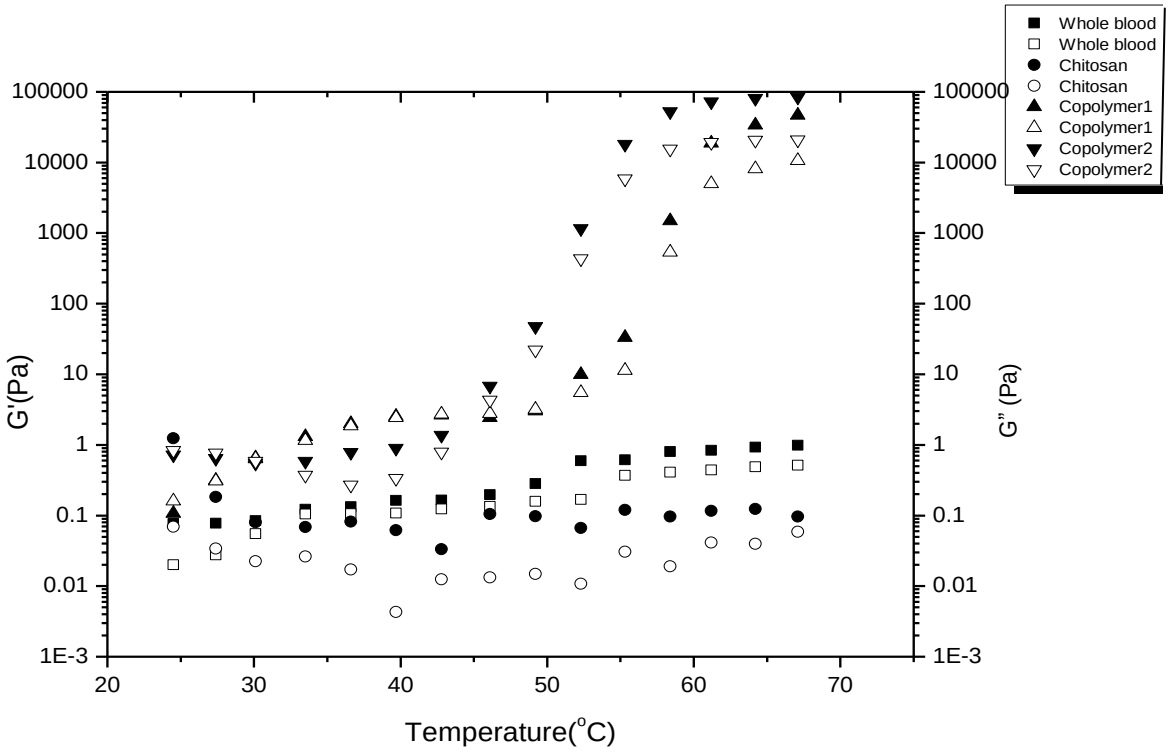

Figure 6. Storage (G' in Pa, filled symbols) and loss (G" in Pa, open symbols) modulus as functions of temperature for whole blood, chitosan, copolymer 1 and copolymer 2 mixed with citrated sodium whole blood at the concentration of 10 g/L and the frequency was fixed at 1 Hz.

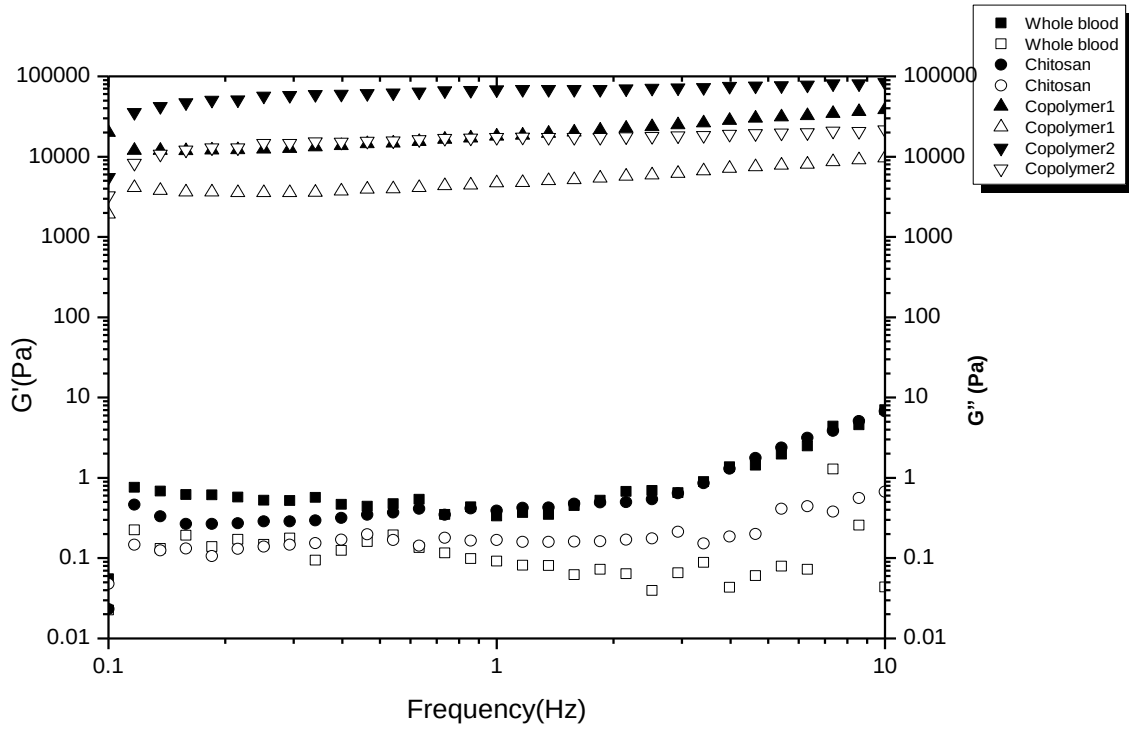

Figure 7. Storage (G' in Pa, filled symbols) and loss (G" in Pa, open symbols) modulus as functions of frequency for whole blood, chitosan, copolymer 1 and copolymer 2 mixed with citrated sodium whole blood at the concentration of 10 g/L and the temperature was fixed at 37°C.

Table 2. Absorbency at 540 nm of mixtures of chitosan/copolymers and blood.

Samples	5 min	10 min	15 min	20 min	30 min
Chitosan	0.055	0.056	0.055	0.054	0.05
Copolymer 1	0.048	0.045	0.02	0.009	0.006
Copolymer 2	0.032	0.031	0.006	0.003	0.001

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