Anticoagulation Activity of Crosslinked *N*-Sulfofurfuryl Chitosan Membranes

Wenguang Liu, Jianrong Zhang, Nan Cheng, Zhiqiang Cao, Kangde Yao

Research Institute of Polymeric Materials, Tianjin University, Tianjin 300072, People's Republic of China

Received 29 October 2003; accepted 10 March 2004
DOI 10.1002/app.20711
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: *N*-Sulfofurfuryl chitosan (SuCS) was prepared through the coupling of 5-formyl-2-furansulfonic acid (FFSA) with chitosan via Schiff's base reaction in an aqueous solution of acetic acid. Fourier transform infrared spectra confirmed the formation of SuCS, and elemental analysis indicated that the concentration of the sulfonic acid groups in modified chitosan increased as the feeding ratio of FFSA increased. A protein adsorption experiment revealed that at higher concentrations of the sulfonic acid groups, glucose aldehyde crosslinked SuCS membranes were inclined to adsorb bovine serum albumin, but the adsorption of fibrinogen was hindered to some extent. Circular dichroism demonstrated that SuCS significantly altered the conformation of thrombin, whereas no obvious variation in the conformation of thrombin was observed with the addition of chitosan. The anticoagulation activity of glucose aldehyde crosslinked SuCS and chitosan membranes was evaluated through assays of the prothrombin time (PT), thrombin time (TT), and activated partial thromboplastin time (APTT). APTT of an SuCS membrane was prolonged in comparison with that of its chitosan counterpart and showed a rising trend with an increasing concentration of the sulfonic acid moieties. However, PT and TT were not markedly affected. The anticoagulant mechanism of the SuCS membranes supposedly originated from an intrinsic pathway to the inhibition of coagulation enzymes. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 94: 53–56, 2004

Key words: adsorption; biomaterials; membranes

INTRODUCTION

Heparin, a mucopolysaccharide consisting of repeating disaccharide units of D-glucosamine and uronic acid, is widely used as an anticoagulant drug because of its ability to accelerate the rate at which antithrombin inhibits serine proteases in the blood coagulation cascade.[1] However, its side effects, such as thrombocytopenia,[2] hemorrhaging,[3] and hyperkalemia,[4] limit its clinical applications to a certain degree. To develop an alternative to heparin, researchers have prepared heparin-like sulfated polysaccharide derivatives, including hyaluronic acid, chondroitin, xylan, amylose, cellulose, curdlan, and D-galactan sulfates.[5,6]

In the case of chitosan, work has also been focused on enhancing its structural similarity to heparin through sulfated modifications. An improved anticoagulant activity of chitosan sulfates has been reported.[7] A frequently used route is to add sulfate groups to chitosan via a chlorosulfuric acid reaction in a medium of MeOH and *N*,*N*-dimethylformamide.[8] However, this method involves a series of complicated steps; moreover, the fuming and corrosive characteristics of chlorosulfuric acid cause a lot of trouble for practical manufacturing. Recently, Amiji[9] synthesized *N*-sulfofurfuryl chitosan (SuCS) by Schiff's base reaction between 5-formyl-2-furansulfonic acid (FFSA) sodium salt and chitosan on the basis of Muzzarelli's method. The blood compatibility of the modified chitosan was initially evaluated through the measurement of the adhesion and activation of platelets.

In this study, SuCS was prepared according to the synthetic method proposed by Amiji.[9] To explore the anticoagulation mechanism, we investigated the adsorption of bovine serum albumin (BSA) and fibrinogen onto crosslinked chitosan and SuCS membranes by UV spectroscopy. The interaction between thrombin and SuCS was elucidated with circular dichroism (CD), and the anticoagulant activities of crosslinked membranes were evaluated with assays of the prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (APTT).

EXPERIMENTAL

Materials

Chitosan (degree of deacetylation = 80%, weight-average molecular weight = 100,000) was supplied by Haihui Bioengineering Co. (Qing Dao, China). FFSA sodium salt was purchased from Sigma Co., St. Louis,

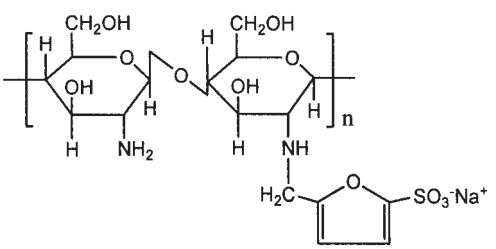
Correspondence to: W. Liu (wgliu@tju.edu.cn).
Contract grant sponsor: Tianjin Municipal Natural Science Foundation; contract grant number: 023604411.

Journal of Applied Polymer Science, Vol. 94, 53–56 (2004)
© 2004 Wiley Periodicals, Inc.

MO, USA. Thrombin was provided by Aldrich Co., Milwaukee, WI, USA. PT, TT, and APTT kits were purchased from Dade Behring Co., Deerfield, IL, USA. Glucose aldehyde was prepared according to a method previously reported.[10]

Synthesis of SuCS

Briefly, 1 g of chitosan was dissolved in 0.1*M* acetic acid to produce a 2% (w/v) solution, to which was added a certain amount of FFSA. Then, the mixture was magnetically stirred at room temperature for 24 h. The Schiff's base that formed was reduced by the dropwise addition of sodium borohydride. The resultant product was purified with a dialysis tube (molecular weight cutoff = 8000) against distilled water for 5 days, and this was followed by lyophilization. The chemical structure of the SuCS can be illustrated as follows:



In this experiment, three types of SuCS were prepared through variations in the feeding ratios of FFSA. Elemental analysis confirmed that the final concentrations of sulfur in the three SuCS types were 3.74, 2.24, and 0.79%, which were coded SuCS1, SuCS2, and SuCS3, respectively.

Preparation of the crosslinked chitosan and SuCS membranes

Purified chitosan (200 mg) or SuCS was dissolved in 20 mL of double-distilled water to form a homogeneous solution, and this was followed by the addition of 10 mg of glucose aldehyde. The mixture was poured onto a freshly cleaned plastic plate and kept *in vacuo* at 50°C overnight for membrane formation. The obtained membranes were rinsed with a 0.9% sodium chloride solution.

Fourier transform infrared (FTIR) analysis

FTIR spectra of the samples were measured on a Bio-Rad FTS 135 spectrophotometer (Hercules, CA, USA).

Protein adsorption

For a typical adsorption experiment, the initial concentration of BSA or fibrinogen in a phosphate-buffered saline (PBS) buffer was 0.5 mg/mL. A piece of a membrane was placed in the protein solution, and then it was incubated at 37°C for 8 h. Subsequently, the membrane was taken out of the solution, and the surface of the membrane was washed gently with double-distilled water. The washing liquid was mixed with the remaining solution. The concentration of the protein in the solution was determined by the Bradford method,[11] which is based on the shift in the absorption maximum of Brilliant Blue G from 465 to 595 nm after the formation of the dye–protein complex, and the absorption intensity at 595 nm is proportional to the concentration of protein in the solution. The adsorbed amount of the protein was calculated with the following formula:

$$Q = [(C_0 - C_A)V]/A$$

where C_0 and C_A are the concentrations of the protein in the solution before and after the adsorption, respectively (μg/mL); V is the total volume of the solution (mL); and A is the surface area of the membrane (cm^2).

CD

CD spectra were collected in a 1-cm-pathlength cuvette with a J-7150 spectropolarimeter in a range of wavelengths of 320–180 nm at 25°C. The measurements were performed at a speed of 20 nm/min and a resolution of 0.5 nm. The spectra were corrected by subtraction of the PBS buffer spectra, and three spectra were accumulated and averaged for each sample. The final concentration of thrombin in the solution was $1.11 \times 10^{-5}M$. The CD signal was converted into the molar ellipticity ([θ]; $^\circ \cdot$ cm^2/dmol).

In vitro anticoagulation assay

The crosslinked SuCS of chitosan membranes was placed in test tubes containing 100 μL of platelet-poor plasma for anticoagulation testing.

PT assay

The aforementioned test tubes were water-bashed at 37°C for 1 min, and 200 μL of Thromborel S reagent was added. Meanwhile, the starting time and the time at which the fibrous substance appeared were recorded, and the clotting time was calculated.

TT assay

In a similar way, the test tubes were water-bashed at 37°C for 1 min, and this was followed by the addition of 200 μL of a Thromborel testing reagent. The clotting time was recorded.

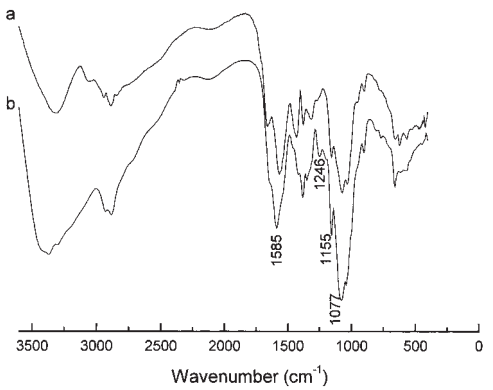


Figure 1 FTIR spectra of (a) chitosan and (b) SuCS1.

APTT assay

An actin-activated cephaloplastin reagent (100 μL) was added to the test tube, and it was incubated at 37°C for 5 min. Then, a 0.025*M* $CaCl_2$ solution (100 μL) was added, and the clotting time was recorded.

RESULTS AND DISCUSSION

Figure 1 shows the FTIR spectra of chitosan and SuCS1. In comparison with chitosan, apart from the characteristic absorptions of the asymmetric carbonyl stretching vibration at 1585 cm^{-1} and the C—O stretching vibrations of the pyranose ring at 1155 and 1077 cm^{-1}, a new peak around 1246 cm^{-1}, assigned to S═O bond stretching, appears for SuCS1, indicating the coupling of FFSA to chitosan.[12]

Protein adsorption

Proteins are adsorbed onto the surfaces of materials instantly upon contact with foreign materials, and they react further with blood or tissue components. Therefore, protein adsorption might regulate all the subsequent body–material interactions. Among a number of proteins in blood, fibrinogen and gamma globulin have been shown to adsorb more strongly onto surfaces believed to be thrombogenic.[13,14] Albumin tends to adsorb onto more antithrombogenic surfaces.[15] Figure 2 shows the amounts of BSA and fibrinogen adsorbed onto the surfaces of chitosan and SuCS. On the surfaces of chitosan and SuCS3, no distinct difference in the adsorption between albumin and fibrinogen can be observed. For SuCS2 and SuCS1, the adsorption of the two proteins is significantly different. Furthermore, the adsorbed amount of BSA markedly increases, and the adsorption of fibrinogen remains nearly unchanged; this suggests that the modified chitosan promotes the adsorption of albumin and suppresses fibrinogen adsorption to some extent. The isoelectric points of fibrinogen and BSA are 5.8 and 4.7, respectively.[16] Therefore, in a PBS buffer, these two proteins are negatively charged. As a weak base with a pK_a value of 6.5, chitosan is in the neutral state because of the deprotonation of amino groups in PBS, whereas SuCS always carries positive charges. Thus, it seems rational to imagine that the electrostatic attractive force facilitates the adsorption of BSA at higher contents of sulfonic moieties. The reason for the different adsorption behavior of fibrinogen is still unclear because of the complex process of protein adsorption. A probable reason is that the materials inhibit the self-aggregation of fibrinogen, preventing adsorption onto the surfaces.

CD

Thrombin is a multifunctional serine protease that plays a central role in thrombosis and hemostasis by regulating the blood coagulation cascade and platelet activation processes. One anticoagulant is capable of specially recognizing and binding to thrombin and consequently inhibits the occurrence of coagulation. This interaction is reflected by variations in the conformation of thrombin. Figure 3 presents the CD spectra of thrombin and chitosan–thrombin and SuCS1–thrombin mixtures in PBS. For pristine thrombin, a negative CD signal around 226 nm can be observed, an indication of β-sheet conformation. The band is only slightly altered with the addition of chitosan, and this implies a weak interaction between chitosan and thrombin. In comparison, an evident alteration of the CD spectrum occurs after the mixing of SuCS1 with thrombin. The conformation has been transformed from a β sheet to a random coil. This implies that there exists a strong interaction between modified chitosan and thrombin.

In vitro anticoagulation assay

Here we are concerned about the anticoagulation activity of an *N*-sulfofurfuryl membrane crosslinked

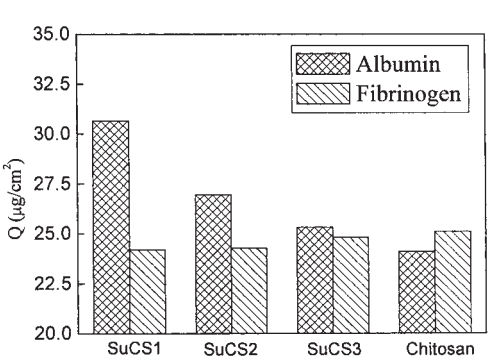


Figure 2 Adsorbed amounts of BSA and fibrinogen on chitosan and SuCS membranes.

with glucose aldehyde, and we take into account the noncytotoxicity of this crosslinker.[10] The anticoagulation activity of chitosan and SuCS is given in Table I. With an increasing concentration of sulfonic acid groups, APTT increases and reaches a maximum value, about 60.0 s. TT and PT of modified chitosan display an irregular change and remain at the same level as that of chitosan. The prolongation of APTT suggests that SuCS prevents blood coagulation by inhibiting all intrinsic pathway coagulation enzymes. No evident variation of PT and TT implies that SuCS does not inhibit an extrinsic pathway of coagulation.[17] The effect of the chitosan molecular weight on the anticoagulation activity is being studied in our laboratory.

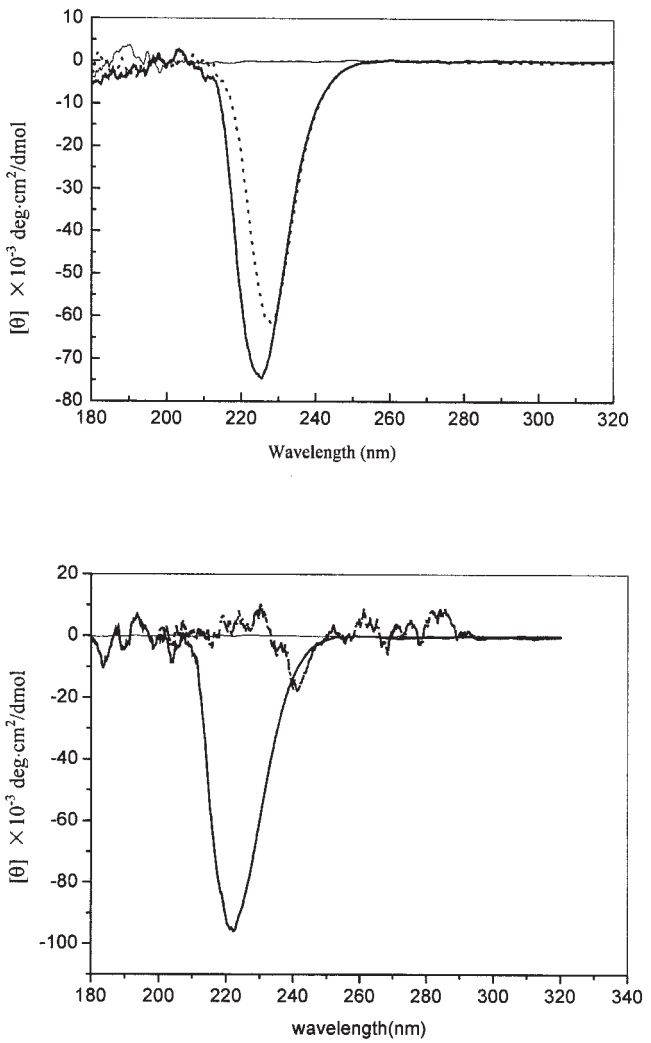


Figure 3 CD spectra of (—) thrombin, (· · ·) a mixture of thrombin and chitosan, and (– · –) a mixture of SuCS1 and thrombin in a PBS solution.

TABLE I
Anticoagulation Activity of Chitosan and SuCS

Sample	TT (s)	PT (s)	APTT (s)
SuCS1	22.8 ± 2.1	15.8 ± 1.6	59.1 ± 3.1
SuCS2	19.8 ± 1.7	15.6 ± 1.4	48.2 ± 2.8
SuCS3	17.9 ± 0.9	16 ± 1.3	42.4 ± 2.9
Chitosan	20.9 ± 1.1	15.2 ± 1.5	34.9 ± 2.6

The values are the results of three measurements, and they are given as the mean values plus or minus the standard deviation.

CONCLUSIONS

A glucose aldehyde crosslinked SuCS membrane is capable of restraining the adsorption of fibrinogen and of promoting the adsorption of bovine serum protein. Upon contact with unmodified chitosan, the conformation of thrombin remains nearly unchanged, whereas it is significantly altered through interaction with SuCS; this indicates that there exists a strong interaction between thrombin and SuCS. APTT increases as the concentration of sulfonic acid moieties increases, and this suggests the anticoagulant mechanism of an intrinsic pathway. SuCS has potential as an anticoagulant biomaterial.

References

1. Capila, I.; Linhardt, R. J. Angew Chem Int Ed Engl 2002, 41, 390.
2. Warkentin, T. E.; Chong, B. H.; Greinacher, A. Thromb Haemostasis 1998, 79, 1.
3. Thomas, D. P. Thromb Haemostasis 1998, 78, 1422.
4. Orlando, M. P.; Dillion, M. E.; O'Dell, M. W. Am J Phys Med Rehab 2000, 79, 93.
5. Magnani A.; Albanese, A.; Lamponi, S.; Barbucci, R. Thromb Res 1996, 81, 383.
6. Chaidedgumjorn, A.; Toyoda, H.; Woo, E. R.; Lee, K. B.; Kim, Y. S.; Toida, T.; Imanari, T. Carbohydr Res 2002, 337, 925.
7. Warayuth, S.; Dumrat, S.; Prachya, K. Carbohydr Res 2002, 337, 1233.
8. Gamzazade, A.; Sklyar, A.; Nasibov, S.; Sushkov, I.; Shashkov, A.; Knirel, Y. Carbohydr Polym 1997, 34, 113.
9. Amiji, M. M. Colloids Surf B 1998, 10, 263.
10. Li, F.; Liu, W. G.; Yao, K. D. Biomaterials 2002, 23, 343.
11. Gan, D. J.; Lyon, L. A. Macromolecules 2002, 35, 9634.
12. Vongchan, P.; Sajomsang, W.; Subyen, D.; Kongtawelert, P. Carbohydr Res 2002, 337, 1239.
13. Han, D. K.; Park, K. D.; Ryu, G. H.; Kim, U. N.; Min, B. G.; Kim, Y. H. J Biomed Mater Res 1996, 30, 23.
14. Martins, M. C. L.; Wang, D.; Ji, J.; Feng, L.; Barbosa, M. A. Biomaterials 2003, 24, 2067.
15. Kim, Y. H.; Han, D. K.; Park, K. D.; Kim, S. H. Biomaterials 2003, 24, 2213.
16. Yang, M. C.; Lin, W. C. Polym Adv Technol 2003, 14, 103.
17. Matsubara, K.; Matsuura, Y.; Bacic, A.; Liao, M. L.; Hori, K.; Miyazawa, K. Int J Biol Macromol 2001, 28, 395.