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Preparation and anticoagulation activity of sodium cellulose sulfate

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Abstract

Semi-synthesis of cellulose sulfate sodium (Na-MCS) was carried out by sulfation of microcrystalline cellulose (MCC) with chlorosulfonic acid-dimethylformamide complex as sulfating agent. As shown by FT-IR, NMR spectroscopy, and elemental analysis, the sulfation occurred mainly at C_6, partially at C_2, and no substitution at C_3. The substitution degree ranged from 1.10 to 1.70 and the average molecular weight is between 1.1 and 3.5×10^4 Da. The anticoagulant efficacy and its possible mechanism were investigated using *in vitro*, *in vivo* coagulation assays and amidolytic tests in comparison with heparin. Results indicated that Na-MCS exhibited higher anticoagulation activity based on activated partial thromboplastin time (APTT) assay and prolonged the thrombin time (TT) to a lesser extent than heparin. No effect was detected on the prothrombin time (PT). Subcutaneous administration of Na-MCS to mice increased the clotting time (CT) in a moderate dose-dependent manner with a longer duration. Na-MCS exhibited anticoagulation activity mainly by accelerating the inhibition of antithrombin III (AT-III) on coagulation factors FIIa and FXa in plasma.

Keywords: Sodium cellulose sulfate; Anticoagulation activity; Mechanism of action

1. Introduction

Anticoagulants have been widely used both in therapeutic processes and *in vitro* medical treatments. It has been estimated that 2–4 persons per 1000 require anticoagulant therapy each year for symptomatic deep-vein thrombosis and/or pulmonary embolism [1]. Natural heparin is one of the widely used anticoagulants in clinical application. Unfortunately, heparin shows some side effects, such as bleeding and other disadvantages (heterogeneity and variability of anticoagulation activity [2]). Moreover, heparin is isolated from animal materials, which tends to cause the risk of contamination of animal-derived pathogens, such as BSE and AIV etc. [3]. Therefore, development of heparin alternatives is an important field of research. The structurally well defined and nonmammalian source of heparin derivative is one of the best choices, among which polysaccharide sulfates are of special interest. Many polysaccharide sulfates with anticoagulation activity have been developed, such as pullulan sulfate, sulfated galactan, sulfated flavonoids [4–6].

Cellulose sulfates (CS) are the analogies of polysaccharide sulfates with β-1,4-glucan as the main chain and sulfate groups substituted to possible hydroxyl groups (position 2, 3 and 6) in cellulose anhydroglucose unit (AGU). In 2001, Groth et al. reported that CS obtained by regioselective sulfation showed an anticoagulation activity. The results indicated that the anticoagulation activity was varied with the raw material and the sulfation method [7], which had effect on the molecular weight (M_w) and the degree of substitution (DS) of CS. It was pointed out that medium M_w and relatively high DS were important for increasing the anticoagulation activity of some heparinoids [8–10].

In this study, microcrystalline cellulose (MCC) was used as starting polymers to prepare sodium CS with high anticoagulation activity. MCC is a cheap, readily available, chemically well-defined natural polysaccharide with proper M_w, which is suitable for the requirements of heparin substitute. We focused on the anticoagulation activity evaluation and the action mechanism investigation of the preparation.

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2. Experimental

2.1. Materials

MCC with an average degree of polymerization 250 was supplied by Shanghai forth reagent factory (Shanghai, China). Heparin (no less than 150 IU/mg) and antithrombin III (AT-III) were obtained from Bo'ao company (Shanghai, China). Activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) assay reagents and calcium chloride (0.025 mol/l) were purchased from Biochem science group of USA. Bovine factor FXa (20 nkat/ml), human factor FIIa (8.4 IU/mg), and chromozym P were purchased from Dade-Behring Marburg, Germany. CBS31.39 was from Diagnostica stago, France. Standard plasma was from Sun bio-tech company, Shanghai, China. All other chemicals used were of reagent grade quality.

2.2. Animals

In vivo anticoagulation experiments were performed on Sprague–Dawley mice weighing 200–250 g, 50% being male, and obtained from the animal research center, Guangdong province, China. The animals were kept on commercial standard pellet diet, allowed free access to water and received humane care according to Chinese version of guide for the care and use of laboratory animals.

2.3. Preparation of Na-MCS

Dried MCC (2.0 g) was suspended in anhydrous dimethylformamide (DMF) (10 ml) and stirred overnight at room temperature for activation. The sulfation agent, complex of chlorosulfonic acid ($ClSO_3H$) and DMF was prepared by slowly adding 40 ml of $ClSO_3H$ into 360 ml of DMF during a period of 30–45 min, with continuous stirring and cooling in an ice bath by keeping the temperature at 5–10 °C. Sulfation was carried out by continuous addition of sulfation agent to MCC with vigorous stirring. After 3 h at 30 °C, the reaction was interrupted by pouring the reaction mixture into three volumes of saturated ethanolic solution of anhydrous sodium acetate. The precipitate was collected by centrifugation and washing with anhydrous alcohol to obtain raw products. The raw products were then dissolved in water and neutralized with 0.5 mol/l NaOH to get the sodium salt form. The neutralized products were dialyzed against distilled water for 12 h and dried under vacuum. The final product of sodium CS was coded as Na-MCS.

2.4. Gel filtration chromatography

Molecular weights and the molecular size distribution analysis were performed on a Waters HPLC instrument with TST5000 and TSK3000 gel column and a RI detector. TOSOH pullulans with molecular weight ranging from 2.7 to 78 kDa were used as a calibration standard. Tris–HCl buffer (pH 7.4) was used as eluent, at a flow rate of 0.8 ml/min.

2.5. NMR spectroscopy

The sulfation pattern was determined by ^{13}C NMR spectrometry on a Bruker DRX-400 NMR spectrometer (100.6 MHz) at room temperature. The samples were dissolved in D_2O. DDS was used as internal standard.

2.6. Infrared spectra (IR) spectroscopy

The IR spectra were obtained with KBr pellets on a Bruker Vector-33 IR spectrometer.

2.7. Sulfur content and DS

Sulfur content was analyzed by the turbidimetric method of Dodgson and Price [11]. DS of each product indicating molar ratio to AGU was determined by elemental analyzer CHNOS (Vario El, Foss Heraeus, Germany).

2.8. Anticoagulation activity assays in the in vitro system

2.8.1. Coagulation assay

In vitro anticoagulation assay were performed with citrated human platelet poor plasma (PPP) as test system. PPP was prepared according to the guidelines for preparing citrated plasma for hemostaseological analyses [12]. Briefly, human blood was pooled from individual healthy donors and mixed immediately with 3.8% trisodium citrate in volume ratio 9:1. Then the mixture was centrifuged at $2500 \times g$ for 15 min to obtain PPP.

The *in vitro* anticoagulant activity of Na-MCS was evaluated by the classical coagulation assay of APTT, TT, and PT with heparin (150 IU/mg) as reference. APTT assay was carried out as follows: 50 μl of test sample was mixed with 500 μl of PPP and incubated at 37 °C for 1 min. Then the APTT assay reagent 100 μl cephatin was added to the mixture and incubated at 37 °C for 3 min. Thereafter, 100 μl of aqueous $CaCl_2$ (0.025 mol/l) was added and the clotting time (CT), that is, APTT was recorded with CA-1500 automatic blood coagulation instrument (Diagnostica Stago, France). TT and PT were determined as for APTT, using their relative assay reagents thrombin containing calcium and thrombin respectively instead of cephatin.

2.8.2. Coagulation factors activity assay

Activities of coagulation factor FIIa or FXa were analyzed on the base of the absorbance at 405 nm (A_{405}) of their specific chromogenic substrates released *p*-nitroaniline. A_{405} was positively related to FIIa and FXa activities and analyzed as follows: chromogenic FXa substrate CBS31.39 or chromogenic FIIa substrate chromozym P was added into 100 μl of the system to be detected. After mixing and incubating at 37 °C for 4 min, 50 μl of interrupting agent was added to stop the reaction. A_{405} was measured on an ELX800 micro plate reader. The inhibition on FIIa or FXa by Na-MCS was investigated by comparing the residual FIIa or FXa activities in PPP after the addition of

Na-MCS, which were calculated according to the formula:

$$\text{Residual activity}(\%) = \frac{A_{405}}{A'_{405}} \times 100\%$$

where, A'_{405} and A_{405} were the absorbance value at 405 nm before and after the addition of Na-MCS.

2.9. In vivo anticoagulation activity assays

CT in mice was assayed to evaluate the *in vivo* anticoagulation activity by Na-MCS in comparison with that of heparin. The test samples were dissolved at saline and given by *sc*. with an administration volume of 5 ml/kg. The mice were divided randomly into 26 groups. Each group contained eight mice. Experiment was designed as follows: (i) 11 groups of mice received Na-MCS or heparin at doses of 0.3, 0.6, 0.9, 1.2, 1.5 mg/kg or saline, respectively. After 2 h, blood was collected; (ii) 15 groups of mice received Na-MCS or heparin at a dose of 1.2 mg/kg. Then blood was collected at 0, 1.0, 1.5, 2.0, 2.5, 3.0, and 6.0 h after 12.0 h administration of the samples. Blood sample was obtained from heart under an anesthesia by ketamine (10 mg/kg) muscle injected. Each blood sample from the heart was drawn directly into a plastic test tube to record the CT.

2.10. Anticoagulant mechanism investigation

2.10.1. Direct inhibition on FIIa or FXa by Na-MCS

A model system consisting of 0.2 ml FIIa, 0.5 ml FXa and 0.3 ml tris–HCl buffer (pH 7.4) was established to exclude the participation of any antithrombin during the anticoagulation process. The residual FIIa or FXa activities in this system with addition of Na-MCS at various concentrations were analyzed to investigate direct inhibition on FIIa or FXa by Na-MCS.

2.10.2. Inhibition on FIIa or FXa mediated by AT-III

Three systems, including the diluted plasma system, AT-III-in-presence and AT-III-in-absence systems were established for investigating mechanism of the inhibition by Na-MCS. Diluted plasma system was obtained by mixing 0.1 ml of standard plasma and 2.0 ml of buffer. AT-III-in-presence system contained 40 μl AT-III, 15 μl FIIa and 45 μl FXa. AT-III-in-absence system was established according to the methods by Alban [12], by adding 2.0 ml AT-III antibody serum into 0.1 ml standard plasma to neutralize AT-III in plasma. Inhibition on FIIa or FXa mediated by AT-III which was investigated by comparing the residual FIIa or FXa activities in these systems with addition of Na-MCS at various concentrations.

2.11. Statistical analysis

All *in vitro* tests were made in triplicate and *in vivo* experimental results are expressed in terms of mean ± standard deviation (S.D.). Statistical evaluations were performed by Duncan's multiple-range *t*-test. Significant differences were considered at $^*p < 0.01$.

3. Results

3.1. Preparation and characterization of Na-MCS

Sulfation of MCC was performed at different conditions and resulted in Na-MCS having varied levels of sulfation. Structure parameters of the products were summarized in Table 1.The DS was calculated by elemental analysis. The value of DS was found between 0.6 and 1.7, and it increased with increasing concentration of sulfation agent. The average M_w ranged from 1.2 to 2.7×10^4 Da. Wide molecular mass distributions in most products were observed. The polydispersity in most products were observed may be due to the hydrolysis of main chain in acid medium. Previous reports suggested that the anticoagulant activity of polysaccharide sulfates always increased with the increasing of their DS [10]. Therefore, samples of Na-MCS with the highest DS of 1.70 were chosen for further structural and activity assays.

The presence of sulfate groups in Na-MCS was confirmed by the IR as shown in Fig. 1. Comparing with MCC, the −OH stretching vibration bands of Na-MCS at 3400 cm^{-1} appeared wider and shifted to higher wave number, suggesting that the original intermolecular hydrogen bonding in MCC was broken during sulfation. Similarly, the intensity of the bands at 2900 cm^{-1}, attributed to the stretching and/or deformation vibration of C–O–H bonds, was decreased in the spectrum of Na-MCS. In the spectrum of Na-MCS, new absorption bands at 1240 cm^{-1} were assigned to the stretching of S=O and –$COSO_3$. The absorptions at about 800–820 cm^{-1} were tentatively ascribed to sulfate half-esters. In addition, the absorption bands at 1040 cm^{-1} (C–OH) were decreased due to sulfation substitution. These IR spectroscopy data indicated that sulfation derivatives Na-MCS were successfully prepared from MCC.

Table 1
Sulfation of microcrystalline cellulose

Reaction conditions					Results			
Products	MCC (g)	Sulfation agent (mol mol^{-1} AGU)	Time (h)	Temperature (°C)	Yield (g)	M_w ($\times 10^4$)	S content (%)	DS
$Na\text{-}MCS_1$	2.0	4.5	2.0	25	2.2	2.1	15.81	1.70
$Na\text{-}MCS_2$	2.0	6.0	3.0	30	1.9	2.7	13.07	1.45
$Na\text{-}MCS_3$	2.0	1.5	2.0	30	2.1	1.4	12.61	1.19
$Na\text{-}MCS_4$	2.0	3.0	1.0	25	2.1	1.2	12.07	1.10
$Na\text{-}MCS_5$	2.0	1.0	3.0	30	1.8	1.5	10.87	0.81
$Na\text{-}MCS_6$	2.0	0.8	3.5	25	1.3	1.3	8.13	0.59

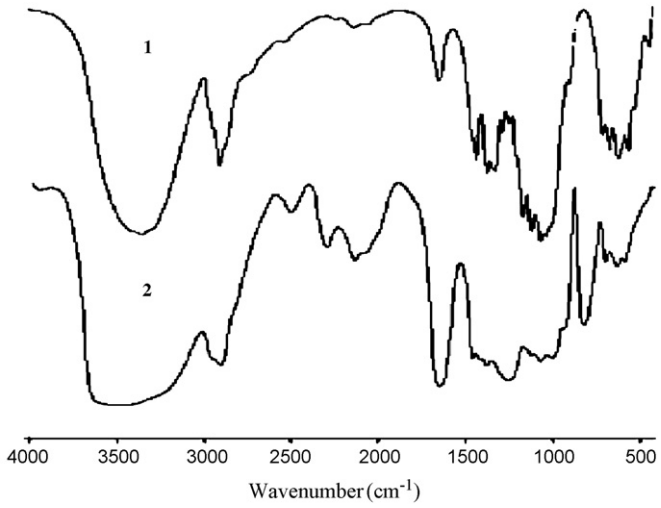

Fig. 1. IR spectra of Na-MCS and MCC: (1)MCC, (2)Na-MCS.

The positions and distributions of the sulfate groups in Na-MCS were investigated by ^{13}C NMR spectroscopy in D_2O. As shown in Fig. 2, the assignment of the peaks was determined by reference to the relative work [13]. The chemical shifts of C_1–C_6 were at 102, 74.1, 73.8, 79.5, 77.4, and 60.1 ppm, respectively. The C_6 absorption of MCC, which appeared at 60.1 ppm, shifted completely to 66.3 ppm after sulfation. All the hydroxyl groups of MCC at C_6 position were sulfated. The C_2 absorption of MCC shifted partially from 74.1 to 81.5 ppm, indicating that the −OH groups at C_2 position were partially sulfated. Sulfated −OH groups at C_3 position were negligibly small. Thus, there were sulfate groups at all C_6 and some C_2 position for Na-MCS$_1$.

3.2. In vitro anticoagulation activity of Na-MCS

3.2.1. Prolongation on coagulation time

The anticoagulant properties of Na-MCS were assessed by determining the coagulation time APTT, TT, and PT using human PPP. The normal values of APTT, TT, and PT for healthy human plasma were 26.3, 10.6, and 11.5 s, respectively. As shown in Table 2, Na-MCS prolonged APTT and reached 50.5 s at 0.6 μg/ml and 119.4 s at 1.4 μg/ml, which was approximately 2.0- and 4.0- fold compared with the saline group (the control), respectively. In comparison, heparin (150 IU/mg) prolonged APTT only above 1.0 μg/ml and reached 100.7 s at 1.4 μg/ml. Thus, Na-MCS showed higher anticoagulant activity than that of heparin at the concentration range from 0.6 to 1.4 μg/ml based on APTT assay. Significant prolongation on TT was observed by Na-MCS at 1.0 μg/ml. Compared with heparin, the prolongation on TT was weaker by Na-MCS in the experimental concentration range. On the contrary, Na-MCS showed no prolongation effect on PT at the test concentration range.

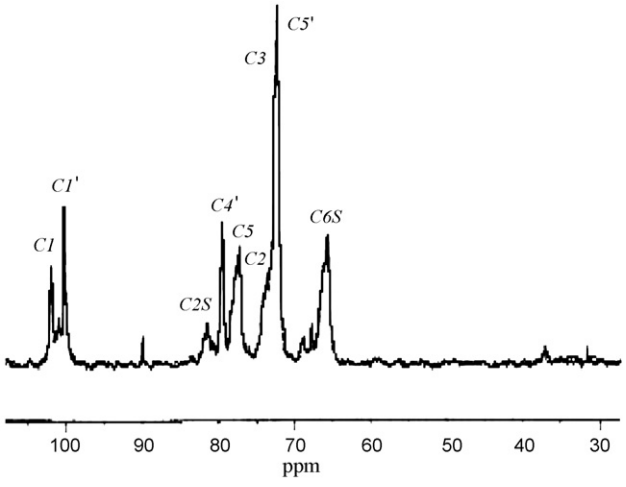

Fig. 2. ^{13}C NMR spectroscopy of Na-MCS.

3.2.2. Inhibition on coagulation factors activity

In our experimental conditions, Na-MCS showed an important *in vitro* anticoagulant action, evidenced by the increase of APTT and TT times. To further investigate *in vitro* anticoagulant potential, coagulation factors levels were assayed in samples of normal human plasma incubated with Na-MCS. As shown in Fig. 3, the residual activity of coagulation factor IIa (FIIa or thrombin) and FXa decreased immediately with the addition of Na-MCS in PPP. The residual activity of FIIa decreased to 73% at 1 μg/ml, while that of FXa remained nearly 90% at the same concentration. A sharp decrease in the residual activity of FXa from 90 to 35% was observed when test concentration was raised from 1 to 100 μg/ml, while the change of FIIa was relatively mild at the same concentration range. It indicated that most part of FIIa and FXa (approximately 70%) was inhibited by Na-MCS. The results showed that inhibition on FIIa by Na-MCS can be seen at low concentration and FXa was inactivated by Na-MCS at a higher concentration.

3.3. In vivo anticoagulation activity of Na-MCS

3.3.1. Dose-dependent anticoagulation activity of Na-MCS in mice

Na-MCS was administered to mice by *sc*. in various doses and its anticoagulant activity was measured by CT assay with heparin as positive control. As shown in Fig. 4, CT was prolonged significantly by Na-MCS at 0.6 mg/kg and heparin at 0.3 mg/kg according to statistic analysis compared with saline group (control). The prolongation on CT by Na-MCS was weaker than that of heparin in the test concentration range. It demonstrated that Na-MCS exhibited a little lower *in vivo* anticoagulation activity than heparin. A dose-dependent increase in CT was caused by Na-MCS in a lesser extent than by heparin. The result demonstrated that Na-MCS showed relatively mild *in vivo* anticoagulation activity in a slow dose-dependent manner.

3.3.2. Time-dependent anticoagulation activity of Na-MCS in mice

Effect of the administration time on the anticoagulant activity of Na-MCS was studied by determining CT a certain period after subcutaneous injection of samples to mice. As shown in

Table 2
Anticoagulation activity of Na-MCS in the *in vitro* test compared with heparin(n = 3)

Groups	Concentration (μg ml^{-1})	APTT (s)	TT (s)	PT (s)
Saline	0.0	26.3 ± 1.3	10.6 ± 0.8	11.5 ± 0.2
Na-MCS	0.2	33.2 ± 1.1	13.5 ± 1.2	11.6 ± 0.2
	0.6	50.5 ± 2.3	22.9 ± 1.5	11.8 ± 0.2
	1.0	83.1 ± 5.6	40.2 ± 3.2	11.9 ± 0.1
	1.4	119.4 ± 8.3	78.9 ± 6.8	12.1 ± 0.2
	1.8	157.5 ± 10.4	141.7 ± 11.5	12.4 ± 0.3
	2.2	200.8 ± 13.3	218.8 ± 16.2	13.6 ± 0.2
Heparin	0.6	39.5 ± 1.8	29.6 ± 1.5	12.2 ± 0.4
	1.0	49.6 ± 2.5	80.7 ± 4.9	12.5 ± 0.2
	1.4	100.7 ± 7.3	167.3 ± 8.9	12.9 ± 0.2

Fig. 5, CT was prolonged to four-fold by Na-MCS and five-fold by heparin 1 h after administration. The action continued with the increasing of the administration time and reached the highest peak at 2.0 h after injection. Then CT decreased with the longer administration time. The increase in CT induced by Na-MCS continued to exist till 12 h after administration. In contrast, prolongation of CT promoted by heparin was restored to a normal level only 3 h after administration. This demonstrated that the *in vivo* anticoagulant efficacy of Na-MCS was maintained longer than by heparin.

3.4. The mechanism of anticoagulant activity of Na-MCS

3.4.1. Direct inhibition on FIIa or FXa activities by Na-MCS

From the *in vitro* and *in vivo* tests, Na-MCS demonstrated a marked prolongation on coagulation time and an obvious inhibition on FIIa and FXa. Therefore, further investigation was devoted to disclosing the action mechanism of anticoagulant activity by Na-MCS. Firstly, a test system including FIIa and FXa without any other coagulation-leading factor was established to evaluate whether Na-MCS conduct a direct inhibition on FIIa or FXa. The residual FIIa and FXa activities in the test system after the addition of Na-MCS with various concentrations were shown in Fig. 6. It remained as high as above 96% even at the concentration of Na-MCS up to 1000 μg/ml, showing that there was no significant direct inhibition on the FIIa or FXa activities by Na-MCS. Thus, Na-MCS did not exhibit anticoagulant activity through a direct inhibition pathway. Other possible anticoagulation pathways by Na-MCS, such as antithrombin-mediated ways, were worth further studying.

3.4.2. AT-III-mediated inhibition on FIIa or FXa activities by Na-MCS

AT-III is an important antithrombin in plasma and was reported to participate in anticoagulation triggered by many kinds of anticoagulants [2,14,15]. The experiment was designed to investigate whether Na-MCS could enhance the AT-III-mediated inhibition on FIIa and FXa by comparing the residual activities in AT-III-in-presence system and AT-III-in-absence system. The diluted plasma system was used as a control. As shown in Table 3, Na-MCS exhibited a strong concentration-dependent inhibition on FIIa and FXa activities in the AT-III-in-presence system with the residual activity of FIIa 33.7% and FXa 32.7%, respectively at 1000 μg/ml. In con-

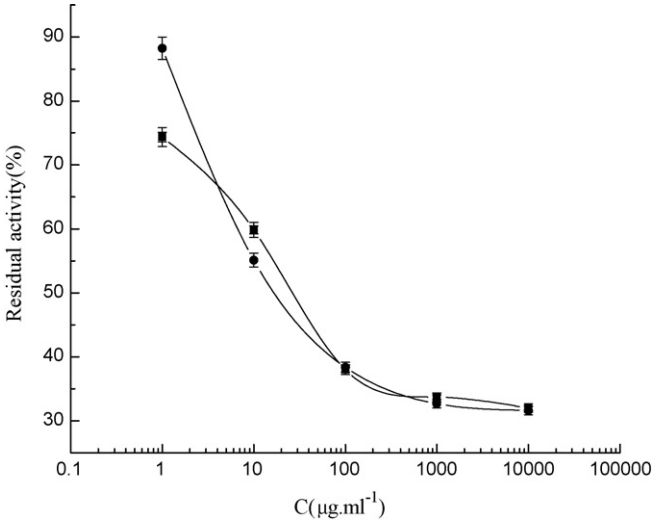

Fig. 3. Inhibition on the coagulation factor FIIa, (■)) and FXa, (●) by Na-MCS in PPP ($n = 3$).

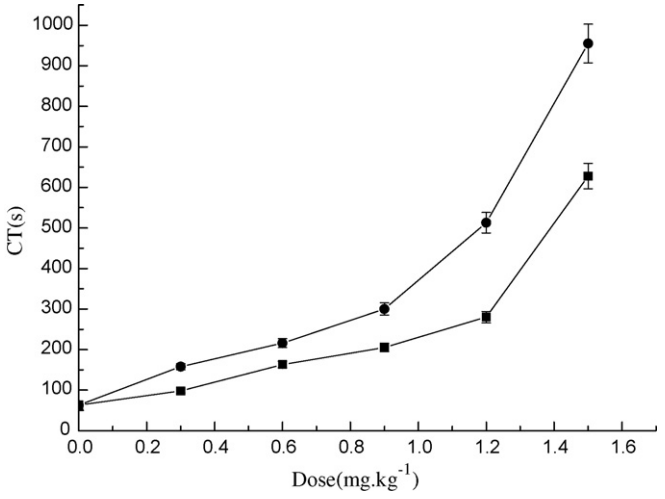

Fig. 4. Effect on clotting time (CT) in mice by Na-MCS, (■)) or heparin, (●) at different doses ($n = 8$).

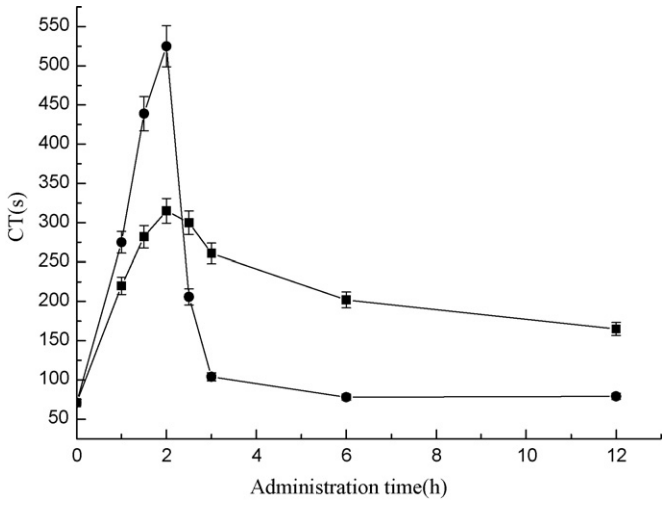

Fig. 5. Effect on clotting time (CT) in mice by Na-MCS, (■)) or heparin, (●) at different administration time ($n = 8$).

trast to this, the residual activity of FIIa and FXa remained at a high level (72.1 and 69.0%, respectively) even at a concentration of 10,000 μg/ml. It demonstrated that Na-MCS inhibited FIIa and FXa activities in the presence of AT-III and it was much less effective in the absence of AT-III. AT-III-in-presence system was designed to have a higher amount of AT-III than that in the diluted plasma system with other components remaining the same. The residual activity of FIIa and FXa was found to be lower in the AT-III-in-presence system compared with that in the diluted plasma system. It indicated that more AT-III contributed to an even stronger inhibition on FIIa and FXa, showing that AT-III was further evidenced to be participant in the anticoagulation process promoted by Na-MCS. Thus, it can be safely concluded that Na-MCS exerted anticoagulation mainly by AT-III-mediated inhibition on the activities of coagulation factors FIIa and FXa.

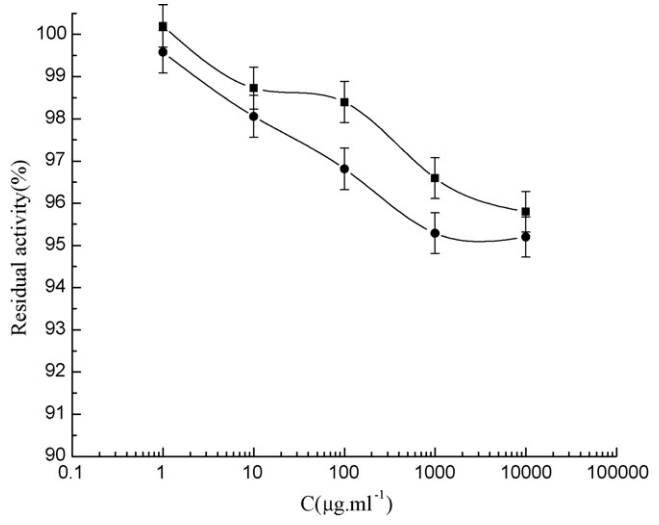

Fig. 6. Direct inhibition on coagulation factor FIIa, (■)) and FXa, (●) by Na-MCS ($n = 3$).

4. Discussion

Anticoagulants have a wide application in various fields as mentioned [1]. A wide variety of applied fields or specific medical conditions require anticoagulants with distinct properties. For example, prevention of venous thromboembolism requires an anticoagulant with a durable action and pre- and postsurgery application needs an agent with the property to avoid bleeding, which was found mainly due to the extreme high activity and a sharp dose-dependent effect [16]. As proven in clinics, the commonly used anticoagulant heparin can induce bleeding and thrombocytopenia along with other disadvantages. Thus, appropriate heparin alternatives for clinics purpose are in great demand. According to the experiment in mice, Na-MCS continued to prolong CT to three times that of the control till 12 h after subcutaneous administration, while heparin was found not to exist in blood 3 h later. Therefore, Na-MCS has a longer half-life than heparin, which met the requirement of venous thromboembolism prevention (Fig. 5). Moreover, Na-MCS showed lower activity based on TT assay (Table 2) and CT determination (Fig. 4) in mice compared with heparin. A mild dose-response activity trigged by Na-MCS, not a sharp effect as conducted by heparin was observed in *in vivo* test. Thus, Na-MCS seemed to have the potential to take place of heparin in clinic application.

Blood clotting is the result of a complex process initiated by the intrinsic system or the extrinsic system and/or a common pathway. As the various coagulation assays indicated the interactions with different stages of the coagulation, they provided basic information about the mode of action of anticoagulants. Na-MCS can prolong APTT and TT, but not PT even at a high concentration, which agreed with the published data about anticoagulation activities for sulfated pullulans, fucans, and other sulfated polysaccharides [1,5]. The prolonging of APTT suggests the inhibition of an intrinsic and/or common pathway, whereas the prolongation of TT indicates inhibition of thrombin activity or fibrin polymerization [17]. No effect of Na-MCS on PT shows that it does not inhibit extrinsic pathway of coagulation. Therefore, it can be deduced that Na-MCS interfered with the intrinsic coagulation process or common pathway, not with the extrinsic process. Coagulation consists of a series of zymogens that can be converted by limited proteolysis to active enzymes leading to the generation of thrombin, which in turn converts fibrinogen into fibrin [18]. FIIa and FXa are two important serine proteasegen in plasma. They play a vital role in coagulation process via the intrinsic and/or common pathway. FIIa and FXa were trigged by two serpin proteases AT-III and HC-II. Na-MCS was found to conduct anticoagulation activity by AT-III-mediated way. In this way, Na-MCS initiated its interaction with AT-III to cause the conformation change of AT-III. The specific interaction between Na-MCS and AT-III was due to the electrostatic interaction of positively charged amino acids in the binding pocket of AT-III with negatively charged sulfate residues [19]. Thus, a catalytic surface to which FIIa or FXa and AT-III bind was formed. Then, a complex of Na-MCS, AT-III, and FIIa or FXa was formed, which caused the inhibition of FIIa or FXa activity. Finally, Na-MCS disassociated from the com-

Table 3
Inhibition on coagulation factor FIIa and FXa by Na-MCS in the test model systems ($n = 3$)

Test system		Concentration($\mu g\ ml^{-1}$)				
		1	10	100	1000	10,000
Diluted plasma system	FIIa	74.4 ± 3.0	59.8 ± 1.9	38.0 ± 1.3	33.7 ± 1.3	32.0 ± 1.7
	FXa	88.2 ± 3.9	55.1 ± 1.6	38.4 ± 1.5	32.7 ± 1.0	31.6 ± 1.3
AT-III-in-present-system	FIIa	70.0 ± 4.2	59.5 ± 2.3	40.6 ± 1.6	35.6 ± 1.6	32.8 ± 1.4
	FXa	81.6 ± 4.0	49.3 ± 1.9	32.1 ± 1.5	27.8 ± 1.0	26.2 ± 1.0
AT-III-in-absent-system	FIIa	94.4 ± 4.3	89.8 ± 4.5	78.0 ± 3.5	73.7 ± 3.7	72.1 ± 3.1
	FXa	97.2 ± 5.2	92.7 ± 6.4	83.9 ± 4.8	71.9 ± 4.3	69.0 ± 2.9

plex once FIIa or FXa lost its activity and was ready to participate the next inhibition process.

The participation of AT-III in the inhibition of FIIa and FXa by Na-MCS has been disclosed in this experiment. Except for AT-III, there is another thrombin inhibitor heparin cofactor II(HC-II) physiologically presenting in plasma. HC-II is one of the members of the serpin superfaminly as AT-III and they share a similar mechanism of action to inactivate thrombin. Heparin is known to exert its anticoagulant effect through activation of two pathways involving both AT-III and HC-II [20]. Na-MCS was found to exert anticoagulation activity mainly by AT-III-mediated pathway in this paper, whereas FIIa and FXa activities were not inhibited completely by this pathway according to amidolytic analysis. As indicated in Table 3, there was residual activity with 32.8% for FIIa and 26.2% for FXa left without inhibition by Na-MCS in the AT-III-in-presence system, which was designed to contain more AT-III than in plasma. It demonstrated that FIIa and FXa were not inhibited completely by Na-MCS in AT-III-mediated pathway. Therefore, it is desirable to investigate the contribution of HC-II-mediated pathway in the anticoagulation process promoted by Na-MCS.

5. Conclusion

Na-MCS can be developed as a new anticoagulant prepared by means of sulfonic modification of MCC using $ClSO_3H$-DMF as sulfation agent. Compared to heparin, Na-MCS can prolong APTT to a greater extent, and to a lesser degree, increase TT. There was no discernable change on PT in the test concentration range according to *in vitro* coagulation assays. Na-MCS exhibited a weaker anticoagulation activity in mice in a mild dose-dependent manner and seemed to maintain a longer action time than heparin. It had a potent anticoagulation activity mainly due to its ability to support the inactivation of FIIa and FXa activities by the mediation of AT-III, but not in a direct manner.

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