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Preparation and anticoagulation activity of cellulose sulfate sodium

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ABSTRACT

In order to develop new anticoagulants as the potential alternatives of heparin, microcrystalline cellulose (MCC) was used as a starting polymer and chlorosulfonic acid in N,N-dimethylformamide ($ClSO_3H$-DMF) was used as a sulfation agent for the semi-synthesis of cellulose sulfate sodium, coded as Na-MCS. IR spectra showed that the sulfate group was introduced by the reaction. The sulfation degree (D_S) was estimated by means of elemental analysis. ^{13}CNMR indicated that sulfation occurred mainly at C_6, partially at C_2 and no substitution at C_3. Na-MCS with D_S of 1.70 was selected for anticoagulant activity investigation. The anticoagulant efficacy was evaluated by coagulation assays and the anticoagulant mechanism was further disclosed by inhibitory analysis of the coagulation factors using chromogenic substrates. Results showed that 1mmol/L Na-MCS could significantly prolong APTT and TT, but less effect on PT. It represented potent anticoagulant in vitro reaching the efficacy of heparin (150IU/mg) in a certain range of concentration. Further investigation indicated that Na-MCS had a potent anticoagulant activity due to the inhibition of II_a & X_a activities by antithrombin AT III.

KEYWORDS

Cellulose sulfate;
Anticoagulation;
Action mechanism;
Microcrystalline cellulose.

INTRODUCTION

Anticoagulants are the drugs of choice in the prevention and treatment of thromboembolic disorders, and clinical pre-surgical ? post-surgical prophylaxis of thrombotic events. Heparin is one of the widely-used anticoagulants in clinical application. However, heparin shows wide variation in its structural parameters and consequently in its anticoagulation activity[1]. 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[2]. Therefore, the development of alternatives to substitute heparin is attracting more and more focus. The structurally well-defined and no animal-derived heparin substitute is one of the best choice, among which polysaccharide sulfates are of special interest. Many polysaccharide sulfates with anticoagulation activity have been developed, such as pullulan sulfate, sulfated fucans, sulphated flavonoids, etc[3-5].

Cellulose sulfates(CS) are the analogies of polysaccharide sulfates with β-1,4-glucan as the main chain and sulfation substitution at some hydroxyl. In 2001, Groth T. et.al. reported that CS obtained by regiose lective sulfation showed anticoagulation activity. The results revealed that their anticoagulation activity was varied with the raw material and the sulfation methods[6], which affected the molecular weight (MW) and the sulfation degree (Ds) of CS. It was pointed out that medium MW and relatively high Ds were essential for the potentiation of anticoagulation activity of some heparinoids[6-8].

In this study, microcrystalline cellulose (MCC) was used as starting polymers to prepare for cellulose sulfates with high anticoagulation activity. MCC is a cheap, readily available, chemically well-defined natural polysaccharide with proper MW, which meets the requirement of a heparin substitute. Further, the anticoagulation mechanism was investigated for a new anticoagulant agent development.

MATERIALS AND METHODS

Materials

Heparin (no less than 150 U/mg) and AT III were obtained from Bo'ao Company (Shanghai, China). APTT, PT and TT assay reagents and calcium chloride (0.025 mol/L) were purchased from Biochem Science Group of USA. Bovine Factor Xa (20nkat/mL), human Factor IIa, thrombin(8.4IU/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 analytical grade.

Preparation of microcrystalline cellulose sulfate sodium

Dried MCC(2.0g) was suspended in anhydrous DMF(10mL) and stirred over night at room temperature for activation. The sulfation agent $ClSO_3H$-DMF was prepared by slowly adding 40mL of $ClSO_3H$ into 360mL of DMF during a period of 30~45min, with continuous stirring and cooling in an ice bath to maintain the temperature at 5~10^{0}C. Sulfation was carried out by continuous addition of sulfation agent and vigorous stirring. After 3 h at 30^{0}C, the reaction was interrupted by pouring the reaction mixture into 3 volumes of cold ethanol saturated with anhydrous sodium acetate. The precipitate was collected by contrifuging and washed with anhydrous alcohol to obtain raw products. The raw products were then dissolved in water and neutralized with 0.5mol/L NaOH to get the sodium salt form. The neutralized products were dialyzed against distilled water for 12h and dried under vacuum. The final product cellulose sulfate sodium was coded as Na-MCS.

Structural characterization methods.

Ds of each product was determined by Vario El CHNOS elemental analyzer based on the carbon to sulfur ratio. The sulfation pattern was determined by ^{13}C NMR spectra. The samples were dissolved in D_2O. DDS was used as internal standard. ^{13}C NMR spectra (100.6MHz) were recorded on a Bruker DRX-400 NMR spectrometer at ambient temperature. IR spectra were recorded with KBr pellets on a Bruker Vector-33 IR instrument. The number average molecular weight(Mn) were measured by a gel permeation chromatography(GPC) system incorporated a TSP P100 instrument, using polyethylene glycol standards of known molecular weight for calibration.

Coagulation assays

The coagulation assays were performed using citrated human platelet poor plasma (PPP). All parts of blood collected from individual healthy donors were drawn into one part of 3.8% trisodium citrate. The ratio of blood to trisodium citrate was 9:1 by volume. The blood was contrifuged for 20min at 2400×g to obtain PPP and the plasma was stored at 4^{0}C until use.

The anticoagulant activity of Na-MCS in vitro was investigated by the classical coagulation assay of APTT (Activated Partial Thromboplastin Time), TT(Thrombin Time) and PT (Prothrombin Time) with heparin (150IU/mg) as reference. APTT assay was carried out as follows: 50μL of anticoagulant solution with different concentrate was mixed with 500μL of citrated normal plasma and incubated at 37^{0}C for 1min. Then the APTT assay reagent 100μL Cephatin was added to the mixture and incubated at 37^{0}C for 3min. Thereafter, 100μL of aqueous $CaCl_2$(0.025mol/L) was added and the

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clotting time, i.e., APTT was recorded with CA-1500 Automatic Blood Coagulation Instrument (Diagnostica Stago, France). TT and PT were determined as APTT, using their relative assay reagents thrombin containing Calcium and thrombin respectively instead of Cephatin.

Inhibition mechanism investigation

1. IIa & Xa activities assays with chromogenic substrates

IIa & Xa activities assays were carried out on the base of the absorbance at 405nm (A_{405}) of their specific chromogenic substrates released p-nitroaniline. A_{405} was positively related to IIa & Xa activities. A_{405} was analyzed as follows: Chromogenic Xa substrate CBS31.39 or Chromogenic IIa substrate Chromozym P was added into 100?L of the system to be detected. After mixing and incubating at 37^0C for 4min, 50?L of interrupting agent was added to stop the reaction. A_{405} was measured on an ELX800 microplate reader. To investigate the inhibition of IIa & Xa activities of Na-MCS, the residual IIa & Xa activities after the addition of Na-MCS were calculated according to the formula: Residual activity (%)=100%×(A_{405}/$A_{405'}$), where $A_{405'}$ and A_{405} were the absorbance at 405nm before and after the addition of Na-MCS in the system to be detected.

Four systems, including the diluted plasma system, IIa+Xa pure system, AT III-in-presence and AT III-in absence systems were applied for the inhibition mechanism investigation of Na-MCS.

2. Inhibition of IIa & Xa activities in diluted plasma system

0.1mL of standard plasma was diluted by 2.0mL of Tris-HCl buffer (pH7.4) to obtain diluted plasma. IIa & Xa activities were recorded as A_{405IIa} and A_{405Xa}. 0.1mL of Na-MCS with various concentrates was mixed with 0.9mL of the diluted plasma system to form the diluted plasma system to be detected. The residual IIa & Xa activities at various Na-MCS concentrations were measured according to 1.5.1.

3. Inhibition of IIa & Xa activities in the IIa+Xa pure system

The IIa+Xa pure system included 0.2mL IIa, 0.5mL Xa and 0.3mL buffer. Direct inhibitions of Na-MCS on IIa and Xa activities were determined by employing the same assay as 1.5.2, only incorporating IIa+Xa pure system in place of the diluted plasma.

4. Inhibition of IIa & Xa activities in AT III-in-presence and AT III-in absence systems

AT III-mediated inhibition of IIa and Xa activities were investigated in AT III-in-presence and AT III-in absence systems. AT III-in-presence system contained 40μL AT III, 15μL IIa and 45μL Xa. AT III-in-absence system was established according to the methods by S. Alban[9], by adding 2.0mL AT III antibody serum into 0.1mL standard plasma.

RESULTS

Preparation and characterization of Na-MCS

Sulfation of MCC was performed at different conditions and resulted in Na-MCS having different levels of sulfation. The sulfation degree (D_S) of the preparations was shown in TABLE 1, presented in molar ratio to an anhydroglucose unit (AGU). The degree of the sulfations estimated by elemental analysis was in the range of 0.6 to 1.70. The Ds of the preparations increased with increasing the amount of sulfation agent. Na-MCS with Ds of 1.70 was selected from the four

TABLE 1 : Sulfation of microcrystalline cellulose

	Reaction conditions				Results				
Products	MCC/g	Sulfation agent /mol.mol^{-1}AGU	Time/h	Temperature/°C	Yield /g	Mn (×10^4)	C content /%	S content/%	Ds
Na-MCS_1	2.0	4.5	2.0	25	2.2	2.5	18.98	15.81	1.70
Na-MCS_2	2.0	6.0	3.0	20	1.9	-	20.66	13.07	1.45
Na-MCS_3	2.0	1.5	2.0	30	2.1	-	23.92	12.61	1.19
Na-MCS_4	2.0	3.0	1.0	25	2.1	-	24.22	12.07	1.10

"-"no detection

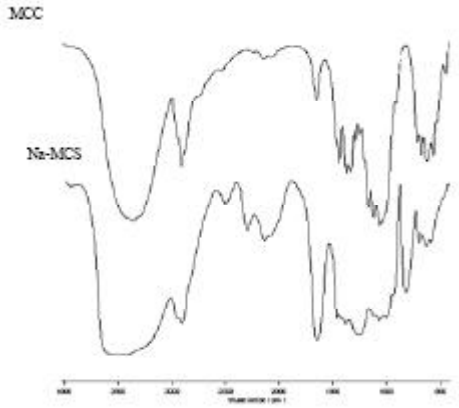

Figure 1 : IR spectra of Na-MCS and MCC

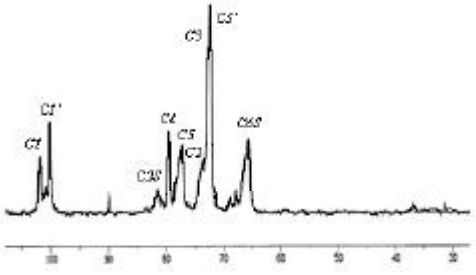

Figure 2 : ^{13}CNMR spectra of Na-MCS

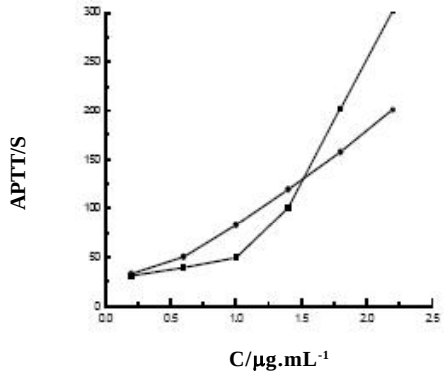

Figure 3: Effect of Na-MCS(●) and heparin (■)on the prolongation of APTT

TABLE 2: ^{13}CNMR chemical shifts of Na-MCS

	C_1	C_1	C_2	C_{2S}	C_3	C_4	C_5	C_5	C_6	C_{6S}
Na-MCS	102.0	100.3	74.1	81.5	73.8	79.5	77.4	72.8	-	66.3

products for structural assay.

The occurring of the sulfate group in Na-MCS was confirmed by the infrared spectra (IR) as shown in figure 1. Compared with MCC, the –OH stretching vibration bands of Na-MCS at 3400cm^{-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 2900cm^{-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 1240cm^{-1} were assigned to the S=O stretching of and -$COSO_3$. The absorptions at about 800-820cm^{-1} were tentatively ascribed to sulfate half-esters. In addition, the absorption bands at 1040cm^{-1}(C-OH) significantly decreased due to sulfation substitution. This result indicated that sulfation derivative Na-MCS was successfully prepared from MCC.

The position and distribution of the sulfate group in Na-MCS was investigated by ^{13}C NMR spectroscopy in D_2O. As shown in figure 2 and TABLE 2, the assignment of the peaks was determined by reference to the relative works[10]. The chemical shifts of C_1 to C_6 were at 102, 74.1, 73.8, 79.5, 77.4 and 60.1ppm respectively. The C_6 absorption of MCC, which appeared at 60.1ppm, shifted completely to 66.3ppm after sulfation. Thus, all the hydroxyl group of MCC at C_6 position were sulfated. The C_2 absorption of MCC shifted partially from 74.1ppm to 81.5ppm, indicating that the C_2 position was partially sulfated. Sulfation at C_3 position was negligibly small. Thus, the selected product Na-MCS with Ds of 1.70 has sulfate groups at all C_6 and some C_2 position.

Anticoagulation activity of Na-MCS

The anticoagulant properties were assessed by APTT, TT and PT assays using normal human plasma as shown in figure 3~figure 5. In the positive control,

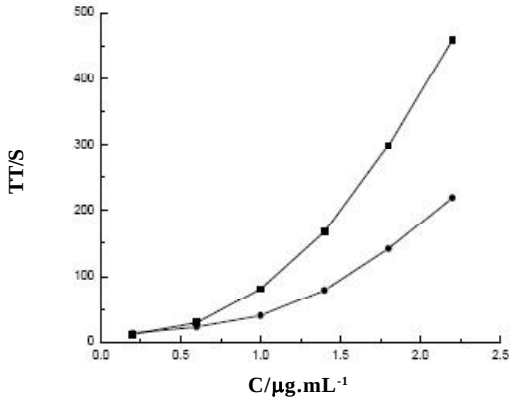

Figure 4: Effect of Na-MCS(●) and heparin (■)on the prolongation of TT

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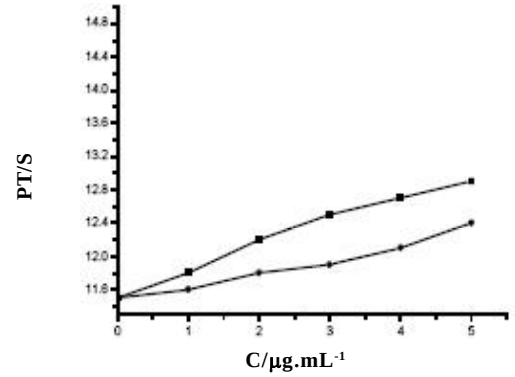

Figure 5 : Effect of Na-MCS(●) and heparin (■)on the prolongation of PT

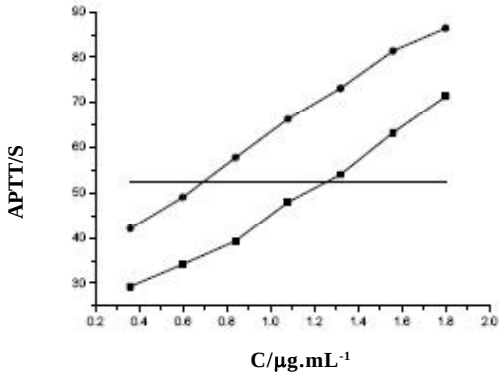

Figure 6: The double APTT concentrate value of Na-MCS (●) and heparin (■)

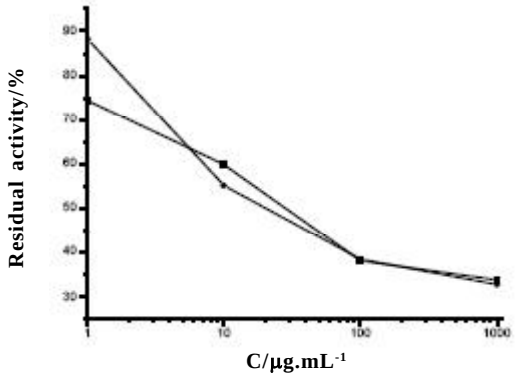

Figure 7 : Effect of Na-MCS on IIa (■)and Xa(●) residual activity in diluted plasma system

heparin (150IU/mg) at 1.0μg/mL prolonged APTT and TT, whereas no clotting inhibition was observed in PT assay at the same concentrate. Similarly, APTT and TT were significantly prolonged by Na-MCS and there was no significant prolongation of PT at the test concentration range. Compared with heparin, the prolongation on APTT of Na-MCS was stronger at 0.6~1.4?g/mL concentration range and that on TT was weaker.

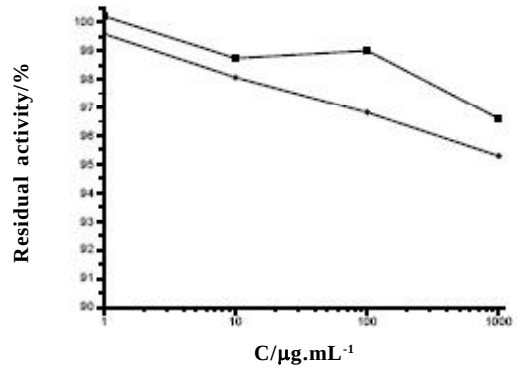

Figure 8 : Effect of Na-MCS on IIa(■) and Xa(●) residual activity in IIa+Xa pure system

The double APTT concentrate value is an indication of the effective dose in clinical application of anticoagulants. It was detected based on the APTT-concentration curve and the double APTT value[11]. As shown in figure 6, the required concentration of Na-MCS to prolong the coagulation time to the doubled baseline value in APTT was 0.7μL/mL, lower than that of heparin(1.3μL/mL) . It indicated that Na-MCS was likely to be more effective than heparin in clinical.

Anticoagulation mechanism of Na-MCS

1. Inhibition of IIa and Xa activities by Na-MCS in diluted plasma

Due to the marked prolongation of APTT and TT, the interference of Na-MCS with some coagulation factors, i.e., IIa and Xa was further examined. Na-MCS was tested at concentration ranging from 1μg/mL to 1000μg/mL in the diluted plasma system. As shown in figure 7, it was apparent that the residual activities of IIa and Xa decreased immediately with the addition of Na-MCS in the diluted plasma. At concentration >100μg/mL, there were about 40% IIa and Xa residual activities left. It can be seen that most part of IIa and Xa were inhibited by Na-MCS. Thus, Na-MCS showed anticoagulation by inhibition of IIa and Xa activity in some extent.

2. Ability of Na-MCS on direct inhibition of IIa & Xa activities

In order to further clarify the inhibition mechanism, IIa+Xa pure system was firstly established for the investigation of the direct inhibition of IIa and Xa activities by Na-MCS. The residual IIa and Xa activities in

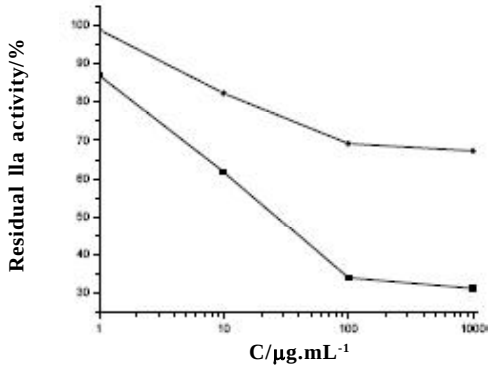

Figure 9 : Effect of Na-MCS on the inactivation of IIa in the presence (■) and in the absence(●) of AT III

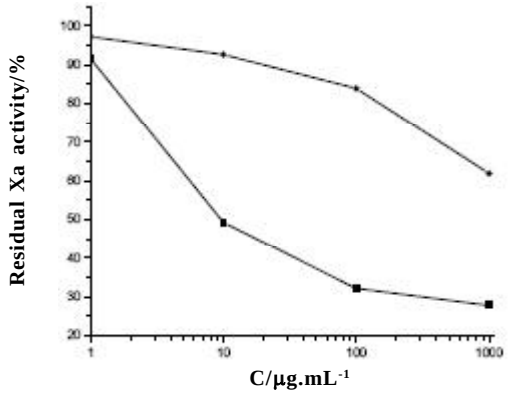

Figure 10 : Effect of Na-MCS on the inactivation of Xa in the presence (■) and in the absence (●) of AT III

the pure system after the addition of Na-MCS with various concentrations were shown in figure 8. It remained as high as above 96% even at the concentration of Na-MCS up to 1000μg/mL. It indicated that there was no significant direct inhibition of IIa and Xa activities by Na-MCS.

3. Ability of Na-MCS on AT-III-mediated inhibition of IIa & Xa activities

Since Na-MCS exerted no inhibition effect on IIa & Xa activities by a direct manner, the next experiment investigated whether Na-MCS could enhance AT-III-mediated inhibition of IIa and Xa by comparing the residual activities in AT-III-in-presence system and AT-III-in-absence system. As shown in figure 9 and figure 10, Na-MCS exhibited a strong concentration-dependent inhibition of IIa and Xa activities in the AT-III-in-presence system. In contrast to this, the residual IIa activity and the residual Xa activity remained high level even at the concentration of Na-MCS above 100μg/mL. It demonstrated that Na-MCS inhibited IIa & Xa activities in the presence of AT-III and it was almost inactive in the absence of AT-III. Thus, it can be safely concluded that Na-MCS exerted anticoagulation mainly by AT-III-mediated inhibition of IIa & Xa activities.

DISCUSSIONS

As the various coagulation assays indicated the interactions with different stages of the coagulation, they provide basic information about the mode of action of anticoagulants. Na-MCS can prolong APTT and TT, but not PT even at a high concentration. Prolongation of APTT suggests the inhibition of intrinsic and/or common pathway, whereas the prolongation of TT indicates inhibition of thrombin activity or fibrin polymerization[12]. No effect of Na-MCS on PT shows that it does not inhibit extrinsic pathway of coagulation.

In order to disclose the anticoagulation pathway in detail, amidolytic analysis was performed for coagulation factors activities investigation. IIa and Xa are the most sensitive thrombin to anticoagulants and its activities are closely related with the blood coagulation. Thus, the change of IIa and Xa activities in diluted plasma was examined firstly to find out the final aim of Na-MCS during anticoagulation. Results indicated that Na-MCS had a strong inhibitory on coagulation factors IIa and Xa activities in diluted plasma, similar to most of the anticoagulants, such as heparin, dextran sulfate, ect[12,13].

To further elucidate the inhibitory mechanism of Na-MCS, three systems were established for the investigation of the change of IIa and Xa activities with the addition of Na-MCS. Examination with IIa+Xa pure system showed that Na-MCS had no direct inhibition on IIa or Xa activities. AT-III is a common antithrombin in plasma and was reported to participate in anticoagulation trigged by many anticoagulants[14-16]. To investigate the participation of AT-III in the inhibition of IIa and Xa activities, the residual IIa and Xa activities was compared in the presence or in the absence of AT-III. It was deduced from the result that AT-III mediated the inhibitory of IIa and Xa activities by Na-MCS.

In conclusion, Na-MCS had a potent anticoagulant activity due to the inhibition of IIa and Xa activities by AT III, not by a direct manner.

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REFERENCES

[1] D.M.Mc Ginnis, A.S.Outschoorn; Pharmacopeial Forum, **5**, 2438-41 **(1991)**.
[2] A.S.Paulo, Mourao, S.P.Mariana; Trends Cardiovasc Med., **9**, 225-232 **(1999)**.
[3] M.S.Pereira, B.Mulloy, P.A.S.Mourao; J.of Biological Chemistry., **274(12)**, 7656-7667 **(1999)**.
[4] H.A.Guglielmone, A.M.Agnese, S.C.N.Montoya et al.; Thromb Res., **105**, 183-188 **(2002)**.
[5] S.Alban, A.Schauerte, G.Franz; Carbohydr Polym., **47(2)**, 267-276 **(2002)**.
[6] T.Groth, W.Wagenknecht; Biomaterials., **22**, 2719-2729 **(2001)**.
[7] S.Alban, G.Franz; Pure.Appl.Chem., **66**, 2403-2406 **(1994)**.
[8] T.Nishino, T.Nagumo; Carbohydr Res., **214**, 193-197 **(1991)**.
[9] S.Alban, G.Franz; Thromb Res., **99**, 377-388 **(2000)**.
[10] I.Nehls, W.Wagenknecht, B.Philipp et al.; Prog. Polym.Sci., **19**, 29-78 **(1994)**.
[11] R.E.Merton, D.P.Thomas; Thromb Haemost, **8**, 839-42 **(1987)**.
[12] S.Beguin, T.Lindhout, H.C.Hemker; Thromb Haemost., **60**, 457-462 **(1988)**.
[13] K.Hatanaka, T.Hirobo, T.Yoshida et al.; Polym J., **22**, 435-441 **(1990)**.
[14] K.Matsubara, Y.Matsuura, A.Bacic et al.; Int.J.of Biol.Macromol., **28(2)**, 395-399 **(2001)**.
[15] R.W.Carrell; Br.J.Haematal., **82**, 189-196 **(1992)**.
[16] P.Sie, F.Ofosu, F.Femandez, et al.; Br.J.Haematol., **64**, 707-712 **(1986)**.