ELSEVIER

0144-8617(95)00084-4

Carbohydrate Polymers 28 (1995) 145–152

0144-8617/95/$09.50+.00

Synthesis and structure–anticoagulant property relationships of functionalized dextrans: CMDBS

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(Received 19 January 1995; accepted 18 July 1995)

The CMDBS compounds (carboxymethyl dextran benzylamide sulfonate) are prepared from native dextran (average molecular weight of 40,000 g/mol) after three consecutive reactions: carboxymethylation of hydroxyl groups on random D-glucose units (CM), conversion of carboxylate groups to benzylamide structures (B), and sulfonation of the phenyl ring (S). These polymers exhibit a heparin-like anticoagulant capacity which is closely related to their CM/S ratio. Evidence is presented to show that the distribution of S-containing groups along the polymer backbone can be optimized to get the best anticoagulant activity. Indeed, the anticoagulant activity reached a maximum after a sulfonation time of 1 h.

INTRODUCTION

The functionalized dextrans termed CMDBS (carboxymethyl, dextran, benzylamide, sulfonate) show many biological heparin-like activities (Chaubet *et al.*, 1994; Letourneur *et al.*, 1993*a,b*; Jozefonvicz & Jozefowicz, 1990; Crépon *et al.*, 1987; Fischer *et al.*, 1985; Mauzac *et al.*, 1985). For example, these polysaccharides delay the coagulation of plasma by catalysing the inactivation of thrombin, a procoagulant enzyme, by natural inhibitors such as antithrombin (AT) (Fischer *et al.*, 1985) or heparin cofactor II (HCII). The CMDBS family (Fig. 1) is schematically described as composed of some D-glucosyl units (D) bearing methylcarboxylate groups (CM), some of the latter derivatized as benzylamides (B) which are then sulfonated to give sulfonated B groups (S) (Mauzac & Jozefonvicz, 1984). By analogy with heparin, a natural polysaccharide composed mainly of alternating units of sulfated uronic acid and glucosamine derivatives (Casu, 1985), it is possible to postulate that the CMDBS compounds possess binding sites which depend on the nature of the interacting proteins (Mauzac & Jozefonvicz, 1984). Evidence has been provided that the anticoagulant activity of these polysaccharides is explained by their high carboxylic and sulfonate groups content (Fischer *et al.*, 1985; Mauzac & Jozefonvicz, 1984). More generally, in efforts to increase their anticoagulant capacity, it is possible to vary the overall ratio and the random distribution of the differently substituted base units, and the molecular weight of the polymer. Consequently, CMDBS can be considered as models carrying active sites which conform to some recognized structural features found in some biological constituents implied in blood coagulation (Fischer *et al.*, 1985; Jozefonvicz & Jozefowicz, 1990). From a theoretical point of view, it is possible to consider that CMDBS represent bioactive sequences whose proportions vary with the overall substitution of the macromolecule (Jozefonvicz & Jozefowicz, 1992). However, the mechanism of the catalytic activity and the exact chemical nature of the active sites of these macromolecules have yet to be determined but are now under investigation.

In this paper, we describe the synthesis and characterization of some CMDBS exhibiting anticoagulant activity. Synthetic steps are optimized and each compound presents a well defined and reproducible structure. Finally, we discuss the effect of the overall composition of the polymers correlated with their anticoagulant capacity.

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MATERIALS AND METHODS

General methods

Dextran T40 (batch 32202; $M_w = 43{,}900$ g/mol; $M_n = 26{,}200$ g/mol) from *Leuconostoc mesenteroides* was purchased from Pharmacia (St Quentin-en-Yv., France). All reagents were of analytical grade and purchased from

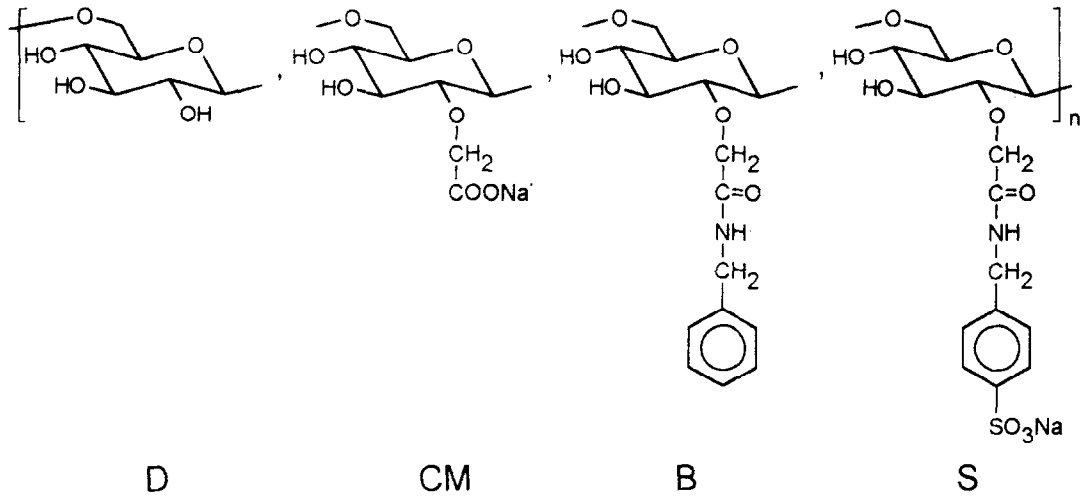


Fig. 1. Schematic structure of the dextran derivatives.

Fluka (Nogent sur Marne, France), Merck (St Quentin Fallavier, France), Sigma (St Quentin Fallavier, France) and Carlo Erba (Nauterre, France). Dichoromethane (CH_2Cl_2) was distilled over P_2O_5 just before use. Hog intestine heparin (H410) with specific anticoagulant activity of 170 UI/mg was obtained from Sanofi Recherche (Montrouge, France). Elemental analyses of nitrogen and sulfur were performed by the 'Service de Microanalyse du CNRS' (Gif sur Yvette, France). The chromatographic molecular weight (M_c) of functionalized dextrans and heparin were determined by high performance steric exclusion chromatography in 0.15 M sodium chloride buffered at pH 7 with 0.05 M NaH_2PO_4 using a Licrospher Si300 Diol column (Merck, Nogent sur Marne, France) connected with a HEMA Sec Bio40 column (Interchim, Montlucon, France). The columns were calibrated with pullulan standards (Touzard et Matignou, Vitry sur Seine, France). For native dextran, a M_c of 27000 g/mol was obtained, in good agreement with the M_w and M_n values reported above. The M_c of H410 heparin obtained under the same conditions was 20000 g/mol.

Preparation of CMDBS

The CMDBS were prepared as previously described for T10 dextran (Mauzac & Jozefonvicz, 1984). They were optimized for T40 dextran in order to obtain as far as possible, good reproducibility, allowing correlations with biological activity. As an example, the synthesis of CMDBS 'JCk' is described below.

Carboxymethylation

50.0 g of T40 dextran (50/162 = 0.308 mol of monomer units) were dissolved under stirring at room temperature in 250 ml of distilled water. In addition 98.4 g (2.46 mol) of sodium hydroxide were dissolved in 170 ml of distilled water. Both solutions were cooled in an ice bath. The NaOH solution was added dropwise over 20 min to the dextran solution at low temperature (4°C) (final NaOH concentration: 6 N). Then, 72.8 g (0.77 mol) of solid chloroacetic acid were added over 20 min (molar ratio: chloroacetic acid/dextran unit = 2.50). The mixture was placed in a temperature controlled reactor at 60°C. After 1 h of heating, the mixture was cooled and neutralized with glacial acetic acid. The carboxymethyldextran (CMD) was precipitated as a white powder by slow addition of the solution in 3 l of iced methanol. After filtration and washing twice with 500 ml of methanol and once with 500 ml of absolute ethanol, the CMD was dried for 18 h at 50°C under oil pump vacuum. The yield of CMD was 60.0 g. An aliquot (200 mg) was purified with an ultrafiltration device with four PTGC 10000 Da cut-off membranes (Millipore S.A., St Quentin en Yv., France). Purification was followed by control of the conductivity of the eliminated water (up to 4×10^{-6} S). Finally, the CMD solution was concentrated, frozen at −80°C and lyophilized. Titration of the carboxylic groups was performed in a water:acetone mixture 50:50 (v/v) with NaOH 0.100 M (TTP2, Tacussel, Nogent sur Marne, France). The results are expressed in milliequivalent of sodium carboxylate per gram of dry polysaccharide (meq/g). Three successive carboxymethylation reactions were performed. The two subsequent reactions were performed as the first treating CMD in the same way as dextran. The overall yield was 77.0 g.

Benzylamine coupling

In the second step, benzylamine was coupled to carboxylic groups of the CMD to form benzylamide units (B). Three successive coupling reactions were undertaken as follows: First, 60.0 g of CMD (0.24 mol) were dissolved in 360 ml of distilled water and the pH was decreased to 4 with HCl (3 M). Next, 59.4 g (0.24 mol) of the coupling agent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Merck, Nogent

sur Marne, France) were dissolved in 400 ml of absolute ethanol at 40°C (water bath). The EEDQ solution was then carefully added to the CMD solution at room temperature under stirring. The apparent pH was maintained at 4 by the addition of HCl (3 M). The mixture was stirred for 30 min at room temperature. Following this, 26.3 ml of benzylamine (0.240 mol) were added. The apparent pH was increased to 9 with NaOH (6 M) and maintained at this value for 1 h with NaOH (1 M). After concentration to 200 ml, the mixture was extracted with diethyl ether (3 × 50 ml) in order to eliminate the excess of benzylamine and the byproducts of the coupling reaction. The aqueous phase was concentrated under primary vacuum to 100 ml and the carboxymethyldextran benzylamide (CMDB) was obtained as a white powder after precipitation in 3 l of iced methanol followed by filtration. The CMDB was washed twice with 250 ml of absolute ethanol and dried for 18 h at 40°C under oil pump vacuum. The yield was 45.0 g. An aliquot (200 mg) was purified as described above. Nitrogen analysis and acidimetric titration were performed as described above to obtain the B and CM contents. The following two B couplings were carried out as for the first by calculating the amounts of reactants and solvants with reference to the CM and B contents. After three steps, the yield was 37.5 g.

Sulfonation of the phenyl rings

12.0 g of dried CMDB containing 1.42 g/100 g of nitrogen (12.2 mmol) were dispersed in 1 l of freshly distilled anhydrous CH_2Cl_2. The suspension was vigorously stirred under a stream of argon. A solution of 2.42 ml (36.6 mmol) of chlorosulfonic acid in 3 ml of CH_2Cl_2 was quickly added (final concentration of chlorosulfonic acid: 0.15 M). After 18 h of reaction, the suspension was filtered under primary vacuum, washed with 150 ml of CH_2Cl_2, 150 ml of a 50:50 mixture of CH_2Cl_2: dioxan and finally with 150 ml of pure dioxan. The crude acidic CMDBS was obtained as a light yellow powder which was dissolved in 250 ml of distilled water. The pH was raised to 9 with an aqueous solution of NaOH (1 M) and maintained at this value until stabilization (1–3 h under stirring). The solution was then neutralized with an aqueous solution of HCl (1 M). After freezing and lyophilization, the crude neutral CMDBS was obtained as a white powder with a yield of 15.0 g. An aliquot (200 mg) was purified as described above. Nitrogen and sulfur analysis, and acidimetric titration were performed to obtain the final composition.

Sulfonated CMDB samples

A sulfonation reaction was performed on a CMDB polymer with 2.90 ± 0.15 meq/g of CM and $2.01 \pm 0.02\%$ of nitrogen, as described above. Samples were taken at 3 min, 30 min, 45 min, 60 min, 6 h and 23 h and, following purification, their S content was determined by elemental analysis.

Coagulation assay

Anticoagulant activities of functionalized dextrans and heparin were assessed by measuring the thrombin clotting time (TT) as follows: 100 µl of polysaccharide solutions in Owren Koller buffer were incubated for 2 min at 37°C with 200 µl of freshly prepared human, citrate-rich, platelet-poor plasma and 100 µl of human thrombin solution (5 NIHu/ml) (Stago, Asuière/seine, France). The specific activities were expressed as previously described in IU/mg of polysaccharide (Crépon *et al.*, 1987).

RESULTS AND DISCUSSION

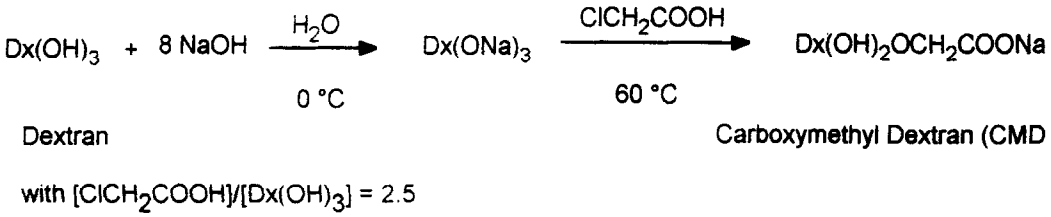
Syntheses and composition of the functionalized dextrans

Various dextran derivatives bearing CM, B and S groups have been synthesised. Data from three series of compounds are presented in Table 1. Figure 2 describes the three main reactions (I–III) leading to the derivatized dextrans (Mauzac & Jozefonvicz, 1984; Antonini *et al.*, 1964).

The CM content of the sample is modulated by the number of carboxymethylation reactions (I) which determine the overall substitution pattern of the final polymer. To obtain compounds exhibiting anticoagulant activity, it is necessary to perform three or more CM reactions followed by two or three B couplings (II) and at least with one S (III). The dextran derivatives are identified by their content of the four basic glucosyl units per hundred units (Mauzac & Jozefonvicz, 1984). However, the degree of substitution (DS) of CM is often greater than 1 CM per glucosyl unit. Based on this result, it is clear that functionalized dextrans with a high CM content contained di- and trisubstituted glucosyl units. Although the carboxymethylation occurred mainly on C2 (Ukai *et al.*, 1992; Virnik *et al.*, 1973; Roberts & Rowland, 1969), preliminary reaction studies have shown that, from the beginning of the reaction, polysubstituted units appear while there are still underivatized glucosyl units (Chaubet *et al.*, 1994). To describe a CMDBS, we have considered the average DS of an arbitrary unit, bearing a single substituted group. The free hydroxyl residues, respectively substituted by CM, B and S, become OCM, OB and OS (Fig. 3). Thus, the CMDBS sample compositions are given in terms of the DS of arbitrary units, i.e. the average number of substituents per arbitrary unit.

This method of calculation is based on the availability of 300 OH residues over 100 glucosyl units. It avoids structural hypotheses about polysubstitutions on the glucosyl unit. Recalculated results are collated in Table 1. As shown in Fig. 4, the overall DS of arbitrary units OCM, OB and OS are correlated with the number of

I



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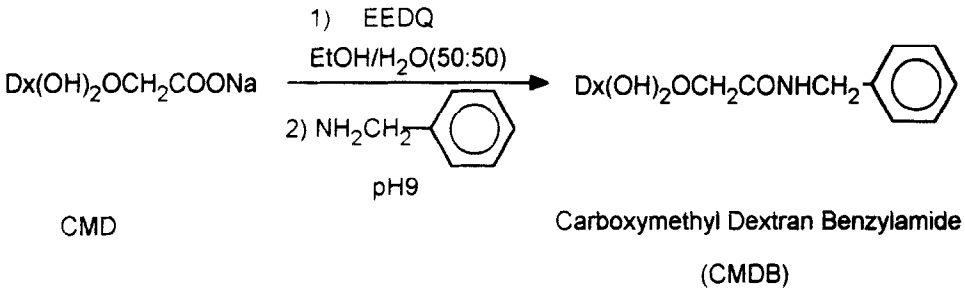

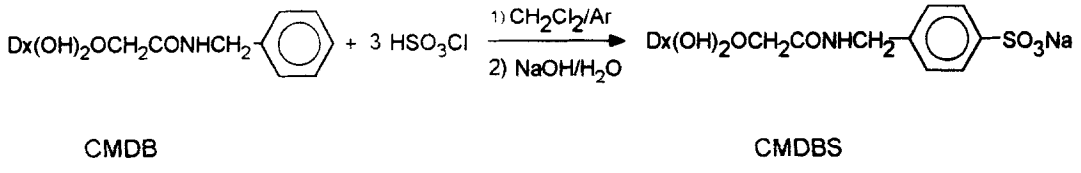

III

Fig. 2. Steps in the synthesis of CMDBS.

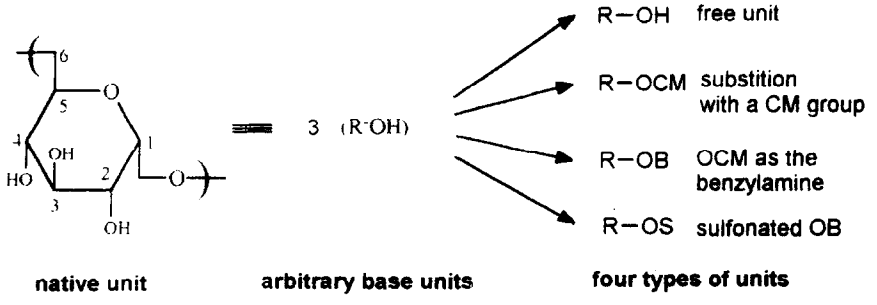

Fig. 3. The four types of arbitrary units.

Table 1. Composition of the functionalized dextrans

		Analyses			Composition											
					No of residues per 100 glucosyl units[a]				No of residues per 300 arbitrary units				Ds of arbitrary units			Chromatographic molecular weight[j] (M_c) (g/mol)
		COONa[b] (meq/g)	N[c] (wt%)	S[c] (w%)	D[d]	CM[d]	B[e]	S[d]	OH[f]	OCM[f]	OB[g]	OS[f]	OCM[h]	OB[i]	OS[h]	
Dextran	T40	0	0	0	100	0	0	0	300	0	0	0	0	0	0	38,000
CMD	CM1D	2.24	0	0	48	52	0	0	248	52	0	0	0.17	0	0	60,000
	CM2D	3.44	0	0	23	77	0	0	223	77	0	0	0.26	0	0	70,000
	CM3D	4.18	0	0	0	102	0	0	198	102	0	0	0.34	0	0	84,000
	CM4D	4.40	0	0	0	110	0	0	190	110	0	0	0.37	0	0	86,000
	CM5D	4.96	0	0	0	133	0	0	167	133	0	0	0.44	0	0	90,000
CMDB	B1	3.61	1.44	0	0	102	29	0	169	102	29	0	0.34	0.10	0	100,000
	B2	3.08	2.14	0	0	95	54	0	151	95	54	0	0.32	0.18	0	80,000
CMDBS	B2S1	2.96	2.25	0.70	0	90	42	7	161	90	42	7	0.30	0.14	0.02	76,000[k]
CMD	CM1D	2.62	0	0	46	54	0	0	246	54	0	0	0.18	0	0	57,900
	CM2D	4.05	0	0	3	97	0	0	200	97	0	0	0.32	0	0	77,500
	CM3D	4.76	0	0	0	124	0	0	176	124	0	0	0.41	0	0	95,200
CMDB	B1	4.29	0.51	0	0	114	10	0	176	114	10	0	0.38	0.03	0	nd
	B2	3.93	0.95	0	0	107	18	0	175	107	18	0	0.36	0.06	0	102,000
	B3	3.81	1.42	0	0	110	29	0	161	110	29	0	0.37	0.10	0	109,000
CMDBS	JCk	3.51	1.25	2.23	0	109	6	22	163	109	6	22	0.36	0.02	0.07	40,000
CMDBS	JC29	2.63	2.30	3.40	0	96	22	39	143	96	22	39	0.32	0.07	0.13	57,000

[a]Referred as previous papers. [b] SD, 0.01 meq/g; [c]SD, 0.03 g/100 g; [d]SSD, 2%; [e] SD, 3%; [f]SD, 2; [g]SD, 3; [h]SD, 0.01; [i]SD, 0.02; [j]SD, 1000 g/mol; [k]peak with a shoulder corresponding to a M_c of 10,000 g/mol; nd, not done.

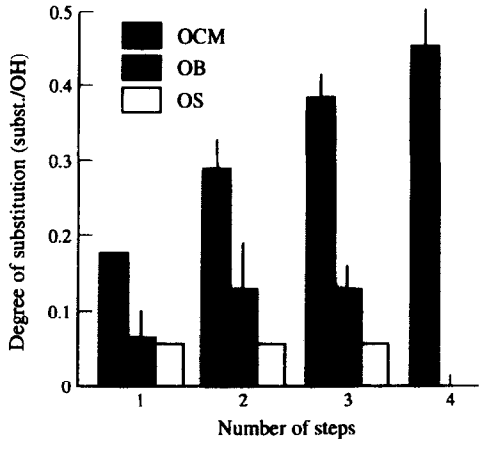

Fig. 4. DS of OCM, OB and OS vs the number of synthesis steps.

synthesis steps. In addition, the overall DS of OCM of a CMD derivative is equal to the overall DS of OCM and OB of the corresponding CMDB (OCM + OB units) and CMDBS (OCM + OB + OS).

Anticoagulant activity

The CMDBS samples prepared from T40 dextran exhibit an anticoagulant capacity. As proposed by Mauzac's group on CMDBS prepared from T10 dextran (Fischer *et al.*, 1985; Mauzac & Jozefonvicz, 1984), this activity implies both CM and S groups and is the result of a co-operative effect between these groups. In addition, the DS of OCM in the final CMDBS must be greater than 0.13 in order to obtain an anticoagulant compound. The anticoagulant activities of native T40 dextran, of dextran derivatives and of heparin standard are shown in Table 2. As previously shown, neither CMD nor CMDB derivatives exhibit anticoagulant activity.

In order to understand the role of the overall composition of the compounds on their anticoagulant capacity, we have compared anticoagulant activity as a function of sulfonation reaction time (Table 3). We observed that, as shown in Fig. 5, the specific anticoagulant activity increases to a maximum for the sample sulfonated for 60–120 min, but then decreases with longer reaction times.

This result clearly demonstrates that the random distribution of the CM and S substitutions on glucosyl units is more favourable after 1 h of sulfonation reaction. We can explain this result in terms of the distribution of glucosyl units bearing either CM or S groups.

CONCLUSIONS

The dextran derivatives CMDBS catalyse the AT–thrombin reaction (Fischer *et al.*, 1985). This catalytic

Table 2. Specific anticoagulant activity (thrombin time) of the different dextran derivatives and of heparin and the sulfur content of each polymer

	Specific anticoagulant activity[a] (UI/mg)	S[b] (wt %)
Heparin H410	170	11.2
Dextran T40	0	0
CMD	0	0
CMDB	0	0
CMDBS		
B2S1	0.6	0.70
JCk	1.0	2.23
JC29	3.5	3.40

[a] SD, 0.5 UI/mg; [b] SD, 0.02 g/100 g.

Table 3. Specific anticoagulant activity of the products as a function of sulfonation reaction time

Sulfonation reaction time	Specific anticoagulant activity[a] (UI/mg)	S[b] (wt%)
3 min	1.5	2.23
30 min	2.6	2.78
45 min	2.3	3.29
60 min	3.5	3.39
6 h	2.3	3.70
23 h	1.2	>3.70

[a] SD, 0.5 UI/mg; [b] SD, 0.02 g/100 g.

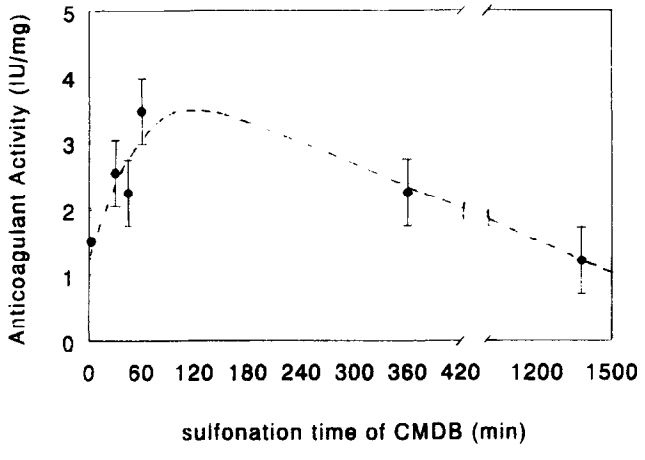

Fig. 5. Specific anticoagulant activity vs the reaction time of sulfonation.

effect depends on the nature of the attached CM and S groups and can be directly related to sulfur content. This dependence for anticoagulant activity is often observed for naturally sulfated polysaccharides. In addition, where known, the mechanisms of interaction with proteins and proteases of blood coagulation are different. For example, if heparin is active mainly via

the AT pathway (Bourrin & Lindahl, 1993), pentosan polysulfate and dermatan sulfate are active via HCII, interactions (Pratt *et al.*, 1989; Wagenvoord *et al.*, 1988; Tollefsen, 1984). Furthermore pentosan polysulfate exerts a strong effect on the main serine proteases in the absence of AT (Fischer *et al.*, 1982). More recently, fucans, sulfated polysaccharides extracted from brown seaweeds, have been demonstrated to exhibit anticoagulant activity by thrombin inhibition mainly via the HCII pathway (Colliec *et al.*, 1991; Nishino *et al.*, 1991). In some cases, there are close relationships between anticoagulant activity and carboxylic acid and sulfate contents. The CM and S distributions in the CMDBS derivatives affect their anticoagulant activity in a similar way. These groups are randomly distributed on the dextran backbone by chemical syntheses. For a given OCM with a DS greater than 0.13, the best distribution is obtained after 1 h of sulfonation rather than overnight. Although the mechanism of anticoagulant activity of CMDBS was shown to involve partly AT (Fischer *et al.*, 1985), their overall mechanism of interaction with serine proteases is now being investigated (Maaroufi *et al.*, 1993). As for heparin (Boneu *et al.*, 1982), the CMDBS do not induce platelet aggregation at the concentrations used for biological experiments. This condition is necessary in order to use these polymers as antithrombotic drugs.

As regards the structure and composition of the CMDBS, the use of arbitrary units made from a single hydroxyl group of a glucosyl unit had two aims: (i) to describe a CMDBS by showing the non-substituted hydroxyl group; and (ii) to avoid hypotheses on di- and trisubstituted units which appear with high CM contents. However, these units are produced at the earlier steps of carboxymethylation (Chaubet *et al.*, 1994). The influence of polysubstituted glucosyl units remains to be established. However, we plan to study their respective proportions and roles in relationship to the anticoagulant activity of the CMDBS.

In conclusion, with suitable conditions, it is possible to prepare functionalized dextrans which are able to mimic a part of the anticoagulant activity of heparin by choosing the best ratio between chemical groups simulating the active chemical functions of the natural products. Among the natural and synthesised anticoagulant sulfated polysaccharides, CMDBS compounds appear to be soft, heparin-like, anticoagulant compounds. However, such dextran derivatives could be efficient antithrombotic agents in the treatment of thrombosis.

ACKNOWLEDGEMENTS

This work was supported by the Centre National de la Recherche Scientifique (CNRS), France.

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