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Anticoagulant properties of dextranmethylcarboxylate benzylamide sulfate (DMCBSu); a new generation of bioactive functionalized dextran

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Abstract

Dextranmethylcarboxylate benzylamide sulfate (DMCBSu), a functionalized dextran, exhibits anticoagulant properties. Its synthesis involves three steps: a carboxymethylation with monochloroacetic acid in alkaline water–isopropanol, a benzylamidification of some of the methylcarboxylate groups with benzylamine in the presence of a water soluble carbodiimide and a partial sulfation of the remaining hydroxyl groups with SO_3–pyridine in dimethylformamide. This procedure yields reproducibly DMCBSu with degrees of substitution in methylcarboxylate (MC), benzylamide (B) and sulfate (Su) groups, respectively, up to 1.61, 0.35 and 1.5, each obtained in one step. For a degree of substitution of methylcarboxylate ca. 1, the presence of sulfate groups is absolutely necessary to confer anticoagulant activities to the samples. In addition, the anticoagulant ability is higher for derivatives bearing benzylamide groups. The anticoagulant ability of DMCBSu increases with the degree of sulfation, reaching 20% of heparin activity for a degree of substitution of Su groups about 1.3.

Keywords: Anticoagulant activities; Dextran derivatives; Polysaccharides; Random functionalization

1. Introduction

The ability of sulfated polysaccharides to interfere with biological systems has a long-standing record, as illustrated with heparin. This natural glycosaminoglycan, known to strongly interfere with blood coagulation,[1,2] is also endowed with numerous biological activities such as the anticomplementary activity[3] and the inhibition of smooth muscle cells growth.[4] Over the last 20 years, extensive studies have been made on the preparation and characterization of natural or semi-synthetic bioactive polymers exhibiting heparin-like properties. Some of the most widely studied examples are dermatan sulfate,[5,6] pentosan polysulfate,[7] dextran sulfate,[8,9] chitin sulfate,[10,11] carrageenan,[12,13] fucans extracted from seaweed,[14,15] and the first generation of functionalized dextrans named CMDBS.[16,17]

CMDBS (carboxymethyldextran benzylamide sulfonate/sulfate) (Scheme 1) exhibit some of the biological activities of heparin

Abbreviations: DMC, dextranmethylcarboxylate; DMCB, dextranmethylcarboxylate benzylamide; CMDBS, carboxymethyldextran benzylamide sulfonate/sulfate; DMCBSu, dextranmethylcarboxylate benzylamide sulfate; DMCSu, dextranmethylcarboxylate sulfate.

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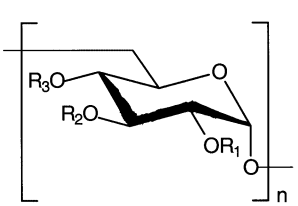
such as the anticomplementary activity,[18] the modulation of cell proliferation,[19,20] and the anticoagulant activity.[16,21] These compounds catalyze the inhibition of thrombin via antithrombin (AT) and heparin cofactor II (HC II).[22,23] These properties closely depend on the overall degree of substitution of the various substituents (carboxymethyl, benzylamide, sulfonate and sulfate). In particular, the anticoagulant activity increased with the degree of substitution in carboxymethyl and sulfonate/sulfate groups[16,21,24] at molecular weights up to 40 kD[18] and was enhanced by the presence of benzylamide groups.[21,25]

We postulate that CMDBS are statistic copolymers exhibiting specific binding sequences which are closely related to the overall degree of substitution of functional groups and to the distribution of these groups inside the glucose units and along the polymer chain.[26] In the case of dextran only substituted by methylcarboxylate groups, the distribution of such sequences was investigated by Monte Carlo simulations and their probabilities of formation were estimated.[27] The exact nature of the active sites is still under investigation. To increase the anticoagulant ability of CMDBS, syntheses were performed involving at least three carboxymethylations, two benzylamidifications and two sulfonations/sulfations.[21,24] However, these highly substituted CMDBS exhibiting an anticoagulant activity up to 1/10th of heparin were revealed to be heterogeneous mixtures of different macromolecular species in terms of molecular weight and chemical composition (data not shown). Thus, it was important to prepare highly substituted functionalized dextran so that each overall chemical composition corresponds to one macromolecular species. By the way, the anticoagulant ability and certainly many other biological properties would be enhanced. Finally, the control of the synthesis of such derivatives will enable efficient studies about their structure–biological activity relationships allowing their industrial development.



R_1, R_2, R_3 = H; CH_2COONa (**CM**); $CH_2CONHCH_2$-C_6H_5(**B**); $CH_2CONHCH_2$-C_6H_4-pSO_3Na or SO_3Na (**S**)

Scheme 1. Structure of CMDBS (containing both sulfonate and sulfate groups: due to the specific sulfation of hydroxyl groups, DMCBSu do not bear sulfonate groups).

In this paper, we report a procedure for the preparation of functionalized dextran leading to a new generation of ionic polysaccharides named dextranmethylcarboxylate benzylamide sulfate (DMCBSu). DMCBSu differ from CMDBS mainly in the conditions of their preparation and in the absence of sulfonate groups. Indeed, they have been synthesized in a reproducible and simple three steps sequence involving successive carboxymethylation, benzylamidification and sulfation resulting in high degrees in substitution of methylcarboxylate (MC), benzylamide (B) and sulfate (Su) groups. The new procedure allowed a good control of all the parameters of the syntheses leading to well-defined compounds. The anticoagulant activity of DMCBSu has been studied with regard to the degree of substitution of different chemical groups.

2. Results and discussion

The chemical characterizations and anticoagulant activities of the dextran derivatives are summarized in Tables 1–4. The three-step reaction, led to a wide range of degrees of substitution up to DS(MC) = 1.1, DS(B) = 0.4 and DS(Su) = 1.6.[†]

Preparation and characterization of dextran derivatives.—Various dextranmethylcarboxylate (DMC) were prepared with DS(MC) ranging from 0.4 to 2 in 15:85 NaOH–isopropanol at 50 °C. For samples with DS(MC) > 1.1 the carboxymethylation reaction was performed once more as previously described.[28]

[†] Degrees of substitution are reported as subscript numbers with the names of the compounds. For example: $DMC_{0.92}B_{0.20}Su_{1.3}$ means DS(MC) = 0.92, DS(B) = 0.20 and DS(Su) = 1.3.

Table 1
Effect of the substitution on the molecular-weight peak (M_p) and on the polydispersity (I) [a]

Products [b]	Charged groups per unit	M_p [c] (g/mol)	$I = \overline{M_n}/\overline{M_w}$
Dextran T40	0	31,000	1.5
$DMC_{0.49}$	0.49	50,000	1.5
$DMC_{0.74}$	0.74	57,000	1.6
$DMC_{1.10}$	1.10	80,000	1.7
$DMC_{1.61}$	1.61	98,000	1.7
$DMC_{1.95}$	1.95	106,000	1.7
$DMC_{1.04}Su_{0.40}$	1.44	87,000	1.7
$DMC_{1.04}Su_{0.70}$	1.74	91,000	1.8
$DMC_{0.90}B_{0.21}$	0.90	67,000	1.6
$DMC_{0.92}B_{0.19}Su_{0.03}$	0.95	69,000	1.6
$DMC_{0.92}B_{0.19}Su_{0.07}$	0.99	67,000	1.6
$DMC_{0.92}B_{0.20}Su_{0.15}$	1.07	78,000	1.6
$DMC_{0.92}B_{0.20}Su_{0.46}$	1.38	83,000	1.7
$DMC_{0.92}B_{0.20}Su_{0.51}$	1.43	83,000	1.7
$DMC_{0.92}B_{0.20}Su_{0.65}$	1.57	79,000	1.7
$DMC_{0.92}B_{0.20}Su_{0.74}$	1.66	83,000	1.6
$DMC_{0.92}B_{0.20}Su_{1.18}$	2.10	88,000	1.7
$DMC_{0.92}B_{0.20}Su_{1.30}$	2.22	92,000	1.6
$DMC_{0.92}B_{0.20}Su_{1.40}$	2.32	83,000	1.8
$DMC_{0.92}B_{0.20}Su_{1.50}$	2.42	86,000	1.8

[a] Both were determined with the CHROMSTAR® software. The charged groups per units represent the average charges born by the average substituted glucosidic unit. M_p reflects the size of the macromolecules assumed as random coils in the buffered aqueous eluent used for experiments. I is defined as $\overline{M_n}/\overline{M_w}$ where $\overline{M_n}$ is the average number molecular weight and $\overline{M_w}$ is the average weight molecular weight.

[b] Degrees of substitution (subscript numbers) are given with the following standard errors: MC $\pm$ 0.01; B $\pm$ 0.03; Su $\pm$ 0.03.

[c] $\pm$ 3000.

Benzylamide derivatives were prepared in two steps. A water-soluble carbodiimide (CMC) was first added to an aqueous solution of DMC at room temperature and at pH 4.75. Then benzylamine was added. The pH increased up to 10 and the reaction was completed in 30 min. With a 1:1 ratio of CMC versus benzylamine, the benzylamidification of $DMC_{1.0}$ was completed in 30 min at room temperature leading to a wide range of DMCB with DS(B) up to 0.35.

The sulfation of dextran derivatives (either DMC or DMCB) took place by treatment of their pyridinium salt with the SO_3–pyridine complex in DMF in 45 min under homogeneous conditions,[21,29] on the contrary to CMDBS which were obtained by treatment of CMDB under heterogeneous conditions.[21,24] We noticed that, as summarized in Table 1, in the same experimental conditions, the degree of sulfation was increased by increasing the B groups content. This could be due to a better solvation of the derivatives bearing B groups by DMF. By the way, the hydroxyls were more easily sulfated. All compounds reported in Tables 1–4 belong to the same series. Other series have been prepared and we found a good fit between the chemical composition and the conditions of synthesis allowing to state a good reproducibility.

FTIR spectra of derivatives have been recorded in the 650–4000 cm^{-1} region. The same patterns have been observed for all series (DMC, DMCB, DMCBSu) as shown in Fig. 1. Arrows point out to some characteristic vibrations:[21,30,31] 3400 cm^{-1} (ν O–H), 2925 cm^{-1} (ν C–H), 1651 cm^{-1} (bound water), 1604 cm^{-1}, (ν C=O carboxylate), 1496 cm^{-1} (ν C–C aromatic ring), 1010 cm^{-1} (ν C–O ether), 1240 cm^{-1} (ν S=O sulfate), 822 cm^{-1} (δ C–O–S sulfate).

The molecular weights of the derivatives have been examined by HPSEC experiments. The molecular-weight peak (M_p) and the polydispersity (I) of some samples have been estimated (Table 2). As shown in Fig. 2, a Gaussian distribution of molecular weights

Table 2
Anticoagulant activity of DMCBSu with different DS(MC)

Products [a]	M_p [b] (g/mol)	a [c] (IU/mg)	C_d [d] (μg/mL)
$DMC_{0.45}B_{0.30}Su_{0.80}$	68,000	15 $\pm$ 1	16 $\pm$ 1
$DMC_{1.22}B_{0.34}Su_{0.80}$	97,000	14 $\pm$ 1	16 $\pm$ 1
$DMC_{1.31}B_{0.34}Su_{0.80}$	95,000	14 $\pm$ 1	17 $\pm$ 1
$DMC_{0.75}B_{0.35}Su_{0.65}$	85,000	10 $\pm$ 1	25 $\pm$ 2
$DMC_{1.28}B_{0.30}Su_{0.65}$	98,000	10 $\pm$ 1	23 $\pm$ 2
$DMC_{1.61}B_{0.35}Su_{0.60}$	98,000	10 $\pm$ 1	25 $\pm$ 2

[a] Degrees of substitution (subscript numbers) are given with the following standard errors: MC $\pm$ 0.01; B $\pm$ 0.03; Su $\pm$ 0.03.

[b] $\pm$ 3000.

[c] Specific anticoagulant activity by comparison with a standard heparin (a = 170 IU/mg).

[d] Concentration of polymer which doubles the control clotting time by comparison with a standard heparin ($C_d = 1.2 \pm 0.1$ μg/mL).

Table 3
Synergistic effect of B groups on the anticoagulant activity of DMCBSu as compared to that of DMCSu [a]

Products [b]	M_p [c] (g/mol)	DS(MC)+DS(B)	a [d] (IU/mg)	C_d [e] (μg/mL)
$DMC_{1.04}Su_{0.40}$	87,000	1.04	1.4 ± 0.5	160 ± 10
$DMC_{0.92}B_{0.20}Su_{0.46}$	83,000	1.12	6 ± 1	25 ± 3
$DMC_{1.04}Su_{0.70}$	91,000	1.04	5 ± 1	35 ± 5
$DMC_{0.92}B_{0.20}Su_{0.74}$	83,000	1.12	12 ± 2	12 ± 1

[a] The comparison was made with DMCSu having a DS(MC) in DMCSu equivalent to the sum [DS(MC)+DS(B)] in the corresponding DMCBSu.
[b] Degrees of substitution (subscript numbers) are given with the following standard errors: MC ± 0.01; B ± 0.03; Su ± 0.03.
[c] ± 3000.
[d] Specific anticoagulant activity by comparison with a standard heparin ($a = 170$ IU/mg).
[e] Concentration of polymer which doubles the control clotting time by comparison with a standard heparin ($C_d = 1.2 \pm 0.1$ μg/mL).

was observed and no degradation of the polymer was detected along the syntheses as reflected by the polydispersity which did not vary significantly. However, some discrepancies were noted. First, M_p and I were obtained from the CHROMSTAR® software through a calibration curve build with standard pullulans. Pullulan is a neutral polysaccharide and the molecular weights of the charged modified dextrans obtained with such standards have to be carefully compared. The increase of M_p with the overall substitution degree of derivatives could be attributed both to the molecular weight increase and to the repulsion between the charged groups (see DMC and DMCSu). However the decrease observed between DMC and DMCB could not be ascribed only to the loss of negative charges, but also to an effect of benzylamide substituents. Indeed, B groups would induce conformational changes of the macromolecules through hydrophobic interactions between aromatic rings, which would reduce the random coil size. More generally, inside the DMCSu and DMCBSu series the molecular weight increased with the sulfate content. However, by examining the variation of the molecular weight with the average number of charged groups per unit, it was observed that the $DMC_{0.92}B_{0.20}Su_{0.74}$ (1.66 charged groups per unit, $M_p = 83{,}000$ g/mol) had a M_p similar to that of the $DMC_{1.1}$ precursor (1.1 charged groups per unit, $M_p =$ 80,000 g/mol). All DMCBSu samples with lower charge content exhibited molecular weights below than that of $DMC_{0.92}B_{0.20}Su_{0.74}$. Moreover $DMC_{1.04}Su_{0.70}$ and $DMC_{0.92}B_{0.20}Su_{1.30}$ had similar M_p (respectively, 91,000 and 92,000 g/mol) but the latter was more highly charged than the former. This means that interactions between B groups were able to deeply change the behavior of the macromolecules in the eluent. Light scattering experiments will have now to be performed to highlight these results.

Anticoagulant ability.—The effect of the different chemical groups on the anticoagulant

Table 4
Effect of sulfate groups on the anticoagulant activity of DMCBSu

Products [a]	M_p [b] (g/mol)	a [c] (IU/mg)	C_d [d] (μg/mL)
$DMC_{0.92}B_{0.19}Su_{0.03}$	69,000	0.4 ± 0.5	> 500
$DMC_{0.92}B_{0.19}Su_{0.07}$	67,000	0.6 ± 0.5	> 500
$DMC_{0.92}B_{0.20}Su_{0.15}$	78,000	1.5 ± 0.5	350 ± 20
$DMC_{0.92}B_{0.20}Su_{0.46}$	83,000	6 ± 1	25 ± 2
$DMC_{0.92}B_{0.20}Su_{0.51}$	80,000	7 ± 1	23 ± 2
$DMC_{0.92}B_{0.20}Su_{0.65}$	79,000	11 ± 1	14 ± 1
$DMC_{0.92}B_{0.20}Su_{0.74}$	83,000	12 ± 2	12 ± 1
$DMC_{0.92}B_{0.20}Su_{1.18}$	88,000	29 ± 2	6.4 ± 0.2
$DMC_{0.92}B_{0.20}Su_{1.30}$	92,000	29 ± 2	5.5 ± 0.2
$DMC_{0.89}B_{0.19}Su_{1.39}$	83,000	31 ± 2	6.5 ± 0.2
$DMC_{0.90}B_{0.20}Su_{1.48}$	86,000	32 ± 3	6.6 ± 0.2

[a] Degrees of substitution (subscript numbers) are given with the following standard errors: MC ± 0.01; B ± 0.03; Su ± 0.03.
[b] ± 3000.
[c] Specific anticoagulant activity by comparison with a standard heparin ($a = 170$ IU/mg).
[d] Concentration of polymer which doubles the control clotting time by comparison with a standard heparin ($C_d = 1.2 \pm 0.1$ μg/mL).

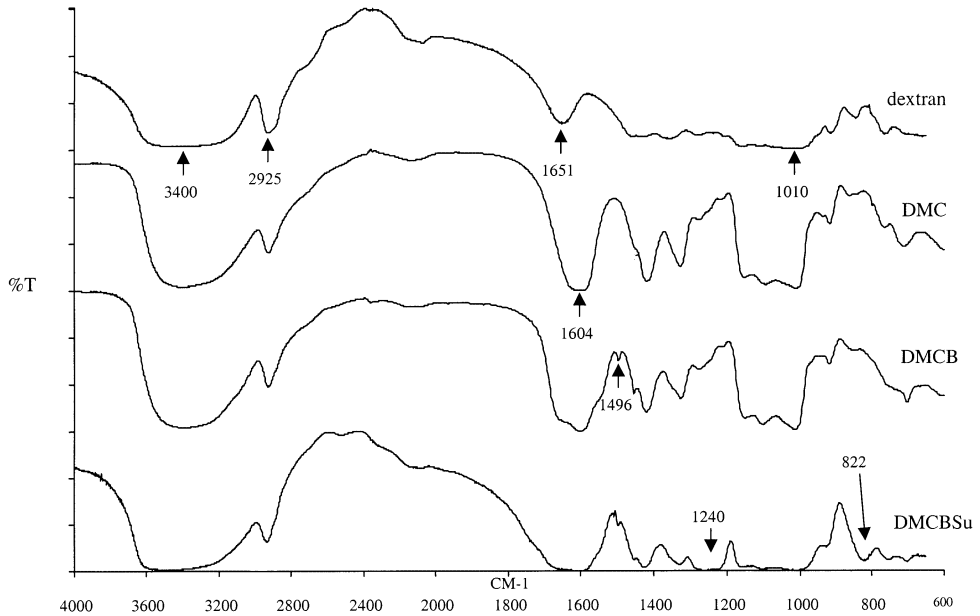


Fig. 1. FTIR spectra of DMCBSu compared with dextrans. Arrows point out some characteristic vibrations (see text).

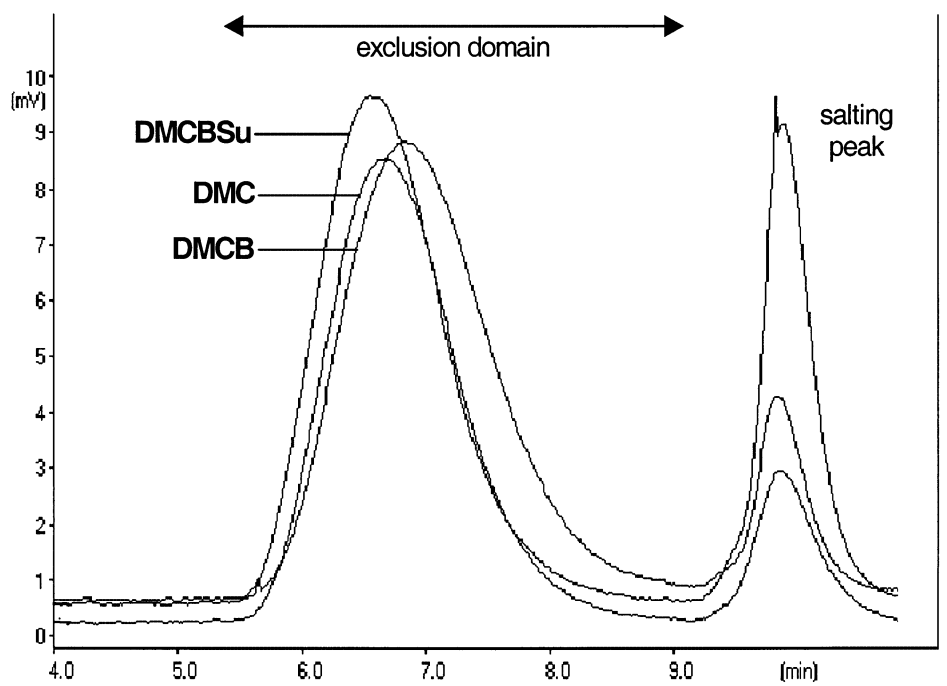


Fig. 2. HPSEC chromatograms of a series of derivatives on two columns (25 × 0.46 cm ID) connected in series (respectively, Licrospher Si 300 diol and Lichrospher Si100 diol). Flow-rate 0.5 mL/min; eluents: 0.15 M NaCl, 0.05 M NaH_2PO_4, buffered at pH 7. Monitoring with differential refractometry.

ability of dextran derivatives is reported in Tables 2–4. In accordance with previous studies on CMDBS,[18] the anticoagulant activity was independent of the molecular weight of the samples over the examined range, i.e., 69,000–92,000 g/mol (data not shown).

As expected, DMC and DMCB did not show any anticoagulant ability. Previous studies on derivatives from the first generation (CMDBS) demonstrated that, for a given degree of sulfation, the anticoagulant activity was greatly enhanced when the degree of car-

boxymethylation was greater than 0.4.[16] Moreover, as described by Chaubet et al.,[32] DMCBSu with carboxymethyl groups reduced into hydroxyethyl groups lost two thirds of their anticoagulant activity while molecular weights remained practically unchanged (data not shown). DMCBSu only varying by their carboxylate groups have been prepared and their chemical composition (and anticoagulant activity) is shown in Table 2. From DS(MC) = 0.45 up to DS(MC) = 1.6, the activity did not vary with the CM content whereas, as expected, the anticoagulant activity was effective and more important for compounds with the highest degree of sulfation. Table 3 shows that B groups enhanced significantly the anticoagulant ability. We can explain this observation by considering a steric role and an orientation effect of B units. On one hand, the spatial arrangement of sulfate groups along a polymer backbone being important for anticoagulant activity, B units could induce conformations promoting a better interaction with blood proteins. On the other hand, the position of B units would promote the sulfation on given hydroxyls.

Table 4 demonstrates clearly that the specific anticoagulant activity expressed as *a* (IU/mg) and as C_d (μg/mL) increased with the sulfate content. The C_d values as a function of the degrees of sulfation are plotted in Fig. 3. Even by taking into account standard errors, a maximum anticoagulant activity was observed for DS(Su) around 1.3. This activity was about 20% of the anticoagulant activity of heparin. Similar results have been obtained with resulfated laminarin by Franz et al.[33] with a maximum for DS(Su) = 1.5.

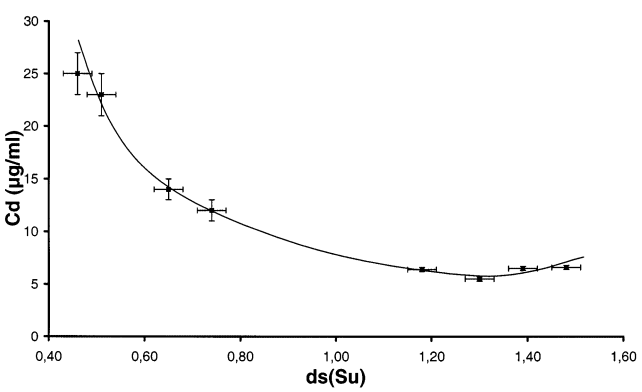


Fig. 3. C_d (μg/mL) vs. the degree of sulfation of DMCBSu which precursor is $DMC_{0.90}B_{0.20}$.

It is obvious that *O*-sulfate groups are required for the anticoagulant activity of functionalized dextrans. These groups have been shown to be essential for biological activities in many sulfated polysaccharides. In particular, the overall sulfate content and the distribution of these groups along the polymer backbone are both very important for the anticoagulant ability of sulfated polysaccharides. On one hand, a high level of sulfate groups produces a strong charge density enabling strong interactions between the polysaccharides and some positively charged peptidic sequences of proteins implied in the coagulation process. It is known that Lys and Arg residues in antithrombin belong to the heparin-binding site.[2] We can also note that the sulfation of heparan sulfate or dermatan sulfate increased their anticoagulant activity in vitro.[5,23,33] In both cases, sulfate groups induce a particular spatial conformation of the polymer leading to a high affinity interaction with specific proteins of blood coagulation.[34] On the other hand, the position of sulfate groups inside the glucosyl unit is also very important for the anticoagulant activity. Indeed, 6-*O*-sulfate groups[35] and 3-*O*-sulfate groups[36] are essential in the pentasaccharide sequence involved in the catalysis of the inhibition of antithrombin by heparin. In the same way, 4-*O*-sulfate of 2-acetamido-2-deoxy-D-galactose residues and 2-*O*-sulfate groups of α-L-iduronic acid units are required on the dermatan sulfate from acidian for the inhibition of the heparin cofactor II.[37] For some of synthetic sulfated polysaccharides as cellulose sulfate or curdlan sulfate, it has been shown an increase of the anticoagulant activity with the amount of sulfate groups at C-2 of the glucose unit.[38,39]

As previously reported with low substituted CMDBS (see Section 1), we postulate that the maximum anticoagulant activity of DMCBSu is directly correlated with the overall chemical composition in MC, B and Su groups through their statistical distribution along the polymer backbone. Some bioactive sequences should be generated with an optimum of appearance, only depending on the degrees of substitution in the three substituents. The isolation of such bioactive sequences from low-molecular

weight species by affinity chromatography is now under investigation.

3. Conclusions

The synthesis of a new generation of bioactive derivatized dextran named DMCBSu has been performed in a more efficient way than for previous dextran derivatives (CMDBS). A reproducible and simple procedure has been established with one carboxymethylation, benzylamidification and sulfation sequence, well-defined DMCBSu with degrees of substitution in MC groups, B groups and Su groups up to, respectively, 1.1, 0.35 and 1.5 were prepared. Based on these results, further studies for an industrial production are under investigation. The anticoagulant activity of DMCBSu was found much higher than that of CMDBS of the same average DS. This activity closely depends on the chemical composition, mainly on the sulfate content, a fact which is often observed with natural sulfated polysaccharides, but also on the amount of benzylamide groups. A notable anticoagulant activity of about 20% of heparin was observed for a DS(Su) around 1.3 with DS(MC) = 0.90 and DS(B) = 0.20. By the way, it should be possible to emphasize this maximum anticoagulant activity by adequately choosing a chemical composition of MC, B and Su groups. Isolation of active sequences by oxidative depolymerization followed by affinity chromatography with plasmatic inhibitors AT or HC II is currently under investigation in order to clarify the effect of this new derivatized dextran on blood coagulation.

4. Experimental

Materials and methods.—Dextran T40 ($\bar{M}_n = 28{,}800$ g/mol, $\bar{M}_w = 40{,}400$ g/mol, batch 228,608) was supplied by Pharmacia (St. Quentin-en-Yveline, France). All 'analytical grade' chemicals and solvents were purchased from Aldrich (St. Quentin Fallavier, France), Acros (Noisy-le-Grand, France), Interchim (Montluçon, France) and Sigma (St. Quentin Fallavier, France).

The MC content was determined on dried aliquots of dextran derivatives (25–45 mg) by acidimetric titration in 1:1 water–acetone mixture acidified with 2 N HNO_3. The proportion of B and Su groups was assessed from N and S elemental analysis performed by the 'Service Central de Microanalyse du CNRS' (Gif/Yvette, France).

FTIR spectra were measured on KBr pellets (150 mg of KBr and 1 mg of sample). Data were computed with the Perkin–Elmer software (IRDM) (Perkin–Elmer, St Quentin-en-Yveline, France).

The chromatographic molecular weight of dextran derivatives samples was determined by high-performance steric exclusion chromatography in 0.15 M NaCl, 0.05 M NaH_2PO_4 buffered at pH 7, using two columns connected in series (respectively, Licrospher Si 300 diol and Lichrospher Si100 diol, Merck-Clevenot, Nogent-sur-Marne, France) and a 510 model pump (Waters, St. Quentin-en-Yveline, France) with an injection loop of 100 μL. The effluent was monitored with a high-pressure differential refractometer (Jobin–Yvon, Longjumeau, France). The flow rate was 0.5 mL/min. The columns were calibrated with standard polysaccharides of narrow-molecular weight pullulans (853,000–5800 g/mol, Polymer Laboratories, Interchim, Montluçon, France); dextran (1500 g/mol), melezitose (522 g/mol), sucrose (342 g/mol) and glucose (180 g/mol) from Sigma (St. Quentin Fallavier, France). The molecular-weight peak (M_p) was determined with CHROMSTAR® software (Bruker, Merck-Clevenot, Nogent-sur-Marne, France).

Clotting assays.—The dextran derivatives were used to supplement normal human platelet poor plasma purchased from Bio Media (Boussens, France). The clotting assays were performed according to the specifications of manufacturers (Diagnostica Stago, Asnières, France). Activated partial thromboplastin time (APTT), thrombin time (TT) (5 NIH U/mL human thrombin, Diagnostica Stago, Asnières, France). Specific anticoagulant activity denoted a in IU/mg or C_d in μg/mL was expressed by comparison with a standard heparin ($a = 170$ IU/mg, $C_d = 1.2$ μg/mL) obtained from Choay–Sanofi (Paris,

France). C_d is the concentration of dextran derivatives which doubles the control clotting-time by comparison with heparin meaning that the lower the value is, the higher is the activity.

Synthesis of dextran derivatives.—As an example the preparation of a DMCBSu with DS(MC) = 0.75, DS(B) = 0.34 and DS(B) = 0.75 is described. Three successive steps are involved: carboxymethylation, benzylamidification and sulfation. All derivatives were similarly prepared.

Dextran T40 (2 g) and NaOH (2.4 g) were dispersed in 42.5 mL of 85:15 isopropanol–water mixture under vigorous stirring and treated at 60 °C with monochloroacetic acid (3 g) for 1.5 h as previously described.[28] After removing the organic phase, the concentrated solution of DMC was neutralized with glacial AcOH and precipitated in cold MeOH. Successive precipitations led to 2.1 g of pure DMC with DS(MC) = 1.00 ± 0.02 (yield: 68%).

The previous DMC (1 g, 4.3 equiv carboxylate/g) was dissolved in 30 mL of bidistilled water ([MC] = 0.10 M). The pH of the solution was adjusted to 4.75. 1-Cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-*p*-toluene sulfonate (CMC, 1.92 g, 4.3 equiv) was added under stirring (Rc = [CMC]/[COOH] = 2). The pH was maintained at 4.75 during the addition. Then 0.470 mL of benzylamine (4.3 mmol) was added dropwise and the mixture was stirred for 30 min. The solution was then successively ultrafiltrated with 2 M NaCl and bidistilled water using an ultrafiltration device (cut off membrane: 5000 D) (Filtron, St Quentin-en-Yveline, France). After freeze drying, 960 mg of DMCB was obtained with DS(MC) = 0.76 ± 0.02 and DS(B) = 0.38 ± 0.04 (yield: 86%).

A sample (1 g) was dissolved in 5 mL of water. The solution was quickly eluted through a cation-exchange column (Amberlite IR120H +). The pH of the effluent was immediately adjusted to 6.5–7 by the careful addition of triethylamine. The solution was then lyophilized to give the triethylammonium salt of DMCB. After drying overnight under vacuum, the compound (1.36 g) was dissolved in 48 mL of anhyd DMF under a stream of Ar. A solution of 0.96 g (6 mmol) of pyridine–sulfur trioxide complex in 12 mL of anhyd DMF was added dropwise. The mixture was stirred for 30 min at rt. The reaction was quenched by the addition of 50 mL of cold water and the pH was adjusted to 9.5 with an NaOH solution. The final solution was then diluted in bidistilled water in order to get a concentration of DMF around 1% (v/v) and purified using an ultrafiltration device cut off membrane: 5000 D) (Filtron). After freeze drying, 1.15 g of DMCBSu was obtained with DS(MC) = 0.75 ± 0.02, DS(B) = 0.34 ± 0.03 and DS(Su) = 0.75 ± 0.05 (yield: 92%).

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