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Poly(*N*-ethyl-4-vinylpyridinium) bromide as a potential probe to select heparin-like anticoagulant polyanions

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Abstract

Carboxymethyl dextran benzylamide sulfates compounds are polyanionic synthetic dextran derivatives with a random distribution of glucosyl units substituted with carboxymethyl, benzylamide, and sulfate groups. Two samples of derivatized dextrans with similar carboxymethyl and benzylamide contents, and with different sulfate content and one sample without benzylamide groups were prepared. The anticoagulant activity of the samples was estimated by the capacity to inhibit procoagulant activity of thrombin, following which the inhibition of this activity was monitored by addition of the polycation poly(*N*-ethyl-4-vinylpyridinium) bromide (PEVP). The potent inhibition by PEVP strongly suggests that the anticoagulant activity of the derivatives is attributable to a small portion of their overall negative charges. The comparison study of the samples implies that specific sites capable to bind thrombin are composed of rather small number of sulfate groups recognized and blocked by PEVP. These findings demonstrate high potential of PEVP as a probe for screening of heparin-like anticoagulant polyanions.

Keywords: Anticoagulant activity; Modified dextrans; Interpolyelectrolyte complex

1. Introduction

Polysaccharides termed carboxymethyl dextran benzylamide sulfates (CMDBS) (Fig. 1) have been suggested to mimic some biological properties of heparin (Crépon, Maillet, Kazatchkine, & Jozefonvicz, 1987; Letourneur, Champion, Slaoui, & Jozefonvicz, 1993a; Logeart-Avramoglou, & Jozefonvicz, 1999; Logeart, Avramoglou, & Jozefonvicz, 1994). They are endowed with anticomplementary activity in vitro (Crépon et al., 1987) and in vivo (Thomas, Maillet, Letourneur, Jozefonvicz, & Kzatchkine, 1995) and can modulate the proliferation of vascular cells (Letourneur et al., 1993a; Letourneur, Logeart, Avramoglou, & Jozefonvicz, 1993b; Logeart et al., 1994). In particular, they delay plasma coagulation by catalyzing the inactivation of thrombin, a blood coagulation proteinase, by primary physiological inhibitors, especially antithrombin (AT) or heparin cofactor II (HCII) (Maaroufi, Jozefonvicz, Tapon-Bretaudière, Jozefonvicz, & Fischer, 1997; Mauzac & Jozefonvicz, 1984) and also by a direct inhibition of the proteinase (de Raucourt, Mauray, Chaubet, Maïga-Revel, Jozefowicz, & Fischer, 1998). De Raucourt et al. demonstrated that, unlike bivalent inhibitors such as hirudin, CMDBS do not interact with the active site of the enzyme (de Raucourt et al., 1998). These derivatives were suggested to bind both anion binding exosites (ABE I and ABE II) (de Raucourt et al., 1998). ABE I and ABE II are located at the surface of the thrombin molecule and are probably involved in the recognition of fibrinogen and in the interaction with heparin, respectively (Stubbs & Bode, 1995; Bode, Turk, & Karshikov, 1992). These properties of CMDBS depend on the various anionic groups in the molecule and on their distribution along the macromolecular chain (Jozefowicz & Jozefonvicz, 1997). In particular, the anticoagulant activity increased with the number of carboxymethyl (CM) and sulfate (S) groups (Chaubet, Champion, Maïga-Revel, Mauray, & Jozefonvicz, 1995; Mauzac & Jozefonvicz, 1984) with the increase of the molecular weight up to 40,000 g/mol (Crépon et al., 1987), and with the presence of benzylamide (B) groups (Crépon et al., 1987;

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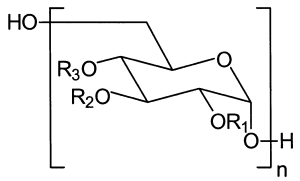

R1, R2, R3 = H (**D**), CH_2COONa (**CM**), $CH_2CONHCH_2$-C_6H_5 (**B**), SO_3Na (**S**).

Fig. 1. Schematic structure of CMDBS.

Maïga-Revel, Chaubet, & Jozefonvicz, 1997; Mauzac & Jozefonvicz, 1984).

Although the anticoagulant activity of CMDBS has been intensively studied regarding their mechanisms of action, little data is available. Nevertheless it is well established that the presence of sulfate groups in the chain is a prerequisite for the modified dextran to prevent coagulation, since neither CMD nor CMDB precursors of CMDBS exhibit anticoagulant activity. Depending on the relative content of the anionic groups in the final product, these dextran derivatives display a broad spectrum of affinity to proteins, ranging from absence of specificity to highly specific binding. The structure-function correlation of the modified dextrans needs to be established to select the binding sites responsible for the anticoagulant activity. This approach requires creating a library of CMDBS samples with various degrees of substitution, and monitoring their anticoagulant activity, which is time consuming.

Prescreening the CMDBS library by use of macromolecular probes capable of recognizing and blocking the binding sites would greatly simplify the task. The efficiency of prescreening would depend upon the choice of a probe (i) of high binding affinity and (ii) of suitable chain length. A step forward in this direction was made recently with the use of poly(*N*-ethyl-4-vinylpyridinium) bromide, PEVP (Fig. 2) (Izumrudov, Chaubet, Clairbois, & Jozefonvicz, 1998) with reduced chain length, which was able to (i) recognize segments of the dextran chain enriched with negatively charged groups (e.g. sulfate groups) and (ii) block the latter efficiently through forming a stable polyelectrolyte complex. This recognition was accomplished by the trial-and-error method via competitive interpolyelectrolyte reactions that were monitored by fluorescence quenching technique, using the ability of PEVP to quench the fluorescence of pyrenyl-labeled CMDBS against sodium poly(methacrylate) which competed for binding with the polycation. The results show that this polycation represents a powerful tool for the structure mapping of the heparin-like polyanions. By analogy with heparin, binding sites responsible for the anticoagulant activity of some dextran derivatives appear to be the highly charged anionic segments of the chains selectively blocked by the polycation. Accordingly, PEVP should inhibit the anticoagulant activity of these derivatives efficiently.

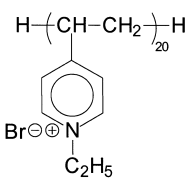

Fig. 2. Structure of poly(*N*-ethyl-4-vinylpyridinium) bromide.

In the present work we aimed to prove this assumption by monitoring the activity of thrombin inhibited by various derivatized dextrans and then restored by addition of PEVP. The established correlation between the neutralizing concentration of the polycation and anticoagulant activity of the samples implies that PEVP is a promising probe for screening of the heparin-like anticoagulant polyanions.

2. Experimental section

Sample of poly(*N*-ethyl-4-vinylpyridinium) bromide with degree of polymerization ≈ 20 was prepared as described elsewhere (Izumrudov et al., 1998). Dextran derivatives were prepared from dextran T40 (Pharmacia, St Quentin-en-Yv., France) and characterized as described elsewhere (Izumrudov et al., 1998; Maïga-Revel et al., 1997). In brief, three to five carboxymethylations of dextran were performed with monochloroacetic acid followed by coupling of benzylamine to the carboxylic groups using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoleine as coupling agent. Finally, sulfation of free OH was conducted in one step by action of SO_3-pyridin complex on the precursor (CMD or CMDB) prepared as the tributylammonium salts (Huynh, Chaubet, & Jozefonvicz, 2001).

Specific anticoagulant activity was estimated as the concentration (Cd, μg/ml) of the dextran derivative needed to double standard clotting time. The time was 16 ± 1 s corresponding to 30 IU of thrombin/ml. Cd values were compared with Cd $= 1.2 \pm 0.1$ μg/ml of heparin (Choay-Sanofi, Paris, France) measured using the same protocol with the assumption that the lower the Cd, the higher the activity of the sample (de Raucourt et al., 1998).

3. Results and discussion

Chemical composition of the three derivatized dextrans and their anticoagulant activity are listed in Table 1.

Table 1
Chemical composition of the derivatized dextran samples, concentration Cd of the samples doubling the standard clotting time, concentration Cp of PEVP needed for complete restoration of the thrombin time, and concentrations of negative $Q-$ and positive $Q+$ charges and their ratio at complete restoration of the thrombin time

	Chemical composition					Cd[a] (μg/ml)[b]	Cp (μg/ml)[b]	Concentration of the charges[c] (mmol/ml)		
	ds(CM)[d]	ds(B)[d]	ds(S)[d]	COO^- (meq/g)[e]	OSO_3^- (meq/g)[f]			$Q-$	$Q+$	$Q+/Q-$
CMDBS1	0.73	0.22	0.11	2.77	0.42	150	22	0.48	0.10	0.21
CMDBS2	0.70	0.20	0.35	2.43	1.22	52	10	0.19	0.05	0.26
CMDS	1.35	–	0.36	3.10	2.48	82	40	0.46	0.20	0.43

[a] Cd of heparin: 1.2 ± 0.1 μg/ml.
[b] ± 2 μg/ml.
[c] The concentrations are given in charged units per ml (mmol/ml).
[d] The degrees of substitution (subscript numbers) are given with the following standard errors: CM ± 0.01; B ± 0.03; S ± 0.03.
[e] ± 3%.
[f] ± 3–10%.

CMDBS1 and CMDBS2 differed mainly in their sulfate contents. CMDS did not contain benzylamide groups but bore the same amount of sulfate groups as CMDBS2. Molecular weight of the samples were comparable, being in the range 61,000–67,000 g/mol, i.e. with chain length that provided maximum anticoagulant activity for the dextrans of given chemical compositions (Crépon et al., 1987).

Fig. 3 gives the correlation between thrombin time and concentration of the dextran derivatives. The concentration required to double the standard clotting time have been derived from the curves and listed in Table 1 as Cd values (μg/ml). The three presented compounds are much less anticoagulant than standard heparin (see Table 1).

As would be expected, Cd of CMDBS2 (52 μg/ml) is lower than that of CMDBS1 (150 μg/ml). The improved anticoagulant activity of CMDBS2 is evidently provided by higher amount of sulfate groups since the content of CM and B groups in both samples is the same. Nevertheless, in spite of the same content of sulfate groups the activity of CMDBS2 is higher than that of CMDS (82 μg/ml). This confirms the results of Maïga-Revel et al. (Maïga-Revel et al., 1997) suggesting a synergistic role of B groups in the anticoagulant activity of CMDBS.

We have performed clotting assays in the presence of the modified dextrans taken at concentration Cd and increasing amounts of the added PEVP polycation. The neutralizing concentrations Cp of PEVP needed for complete restoration of the thrombin time are listed in Table 1. In each case, the concentration $Q-$ of all negative charges of the polyanion corresponding to the concentration Cd and concentration $Q+$ of positive charges of PEVP corresponding to the concentration Cp have been calculated. These values as well as values of charge ratio $Q+/Q-$ reflecting efficiency of the neutralization by PEVP are summarized in Table 1. It is obvious that the smaller $Q+/Q-$ ratio, the higher the efficiency of the neutralization.

In all samples studied, the $Q+/Q-$ ratios are much smaller than 1. This strongly suggests that only a small part of negative charges of the polyanions are responsible for their anticoagulant activity. $Q+/Q-$ values are not determined by overall negative charges of the modified dextrans and might even increase with the decrease of $Q-$ as shown in Table 1. These findings support the view that anticoagulant activity of modified dextrans cannot be explained only by electrostatic interactions, but is also conditioned by formation of specific sites on the chains capable to bind thrombin. Most likely, sulfate groups are of crucial importance in the formation of specific sites since $Q+/Q-$ value increases with increase of their content in CMDBS, as shown by the results related to CMDBS1 and CMDBS2. However, an increase of 24% ((0.26 − 0.21)/0.21) of $Q+/Q-$ observed on substitution of CMDBS1 for CMDBS2 is not proportional to ca. 218% ((0.35 − 0.11)/0.11) increase of sulfate substitution. The significant growth of the neutralization efficiency in the latter case shows that a relatively small portion of added PEVP proved to be sufficient to block the formed specific sites. It indicates that sulfate sequence forming the sites is rather small and/or the sulfation gives rise to non-uniform distribution of sulfate groups along CMDBS chains.

These assumptions are supported by data obtained by Izumrudov et al. (Izumrudov et al., 1998). The number of sulfate groups as well as their density on CMDBS chains was shown to control equilibrium of the interpolyelectrolyte reactions. In particular, several sulfate groups located close to each other on the chain provided convincing recognition of these sequences by added PEVP and their blocking due to the electrostatic binding. Accordingly, the same PEVP-delivery and blocking mechanism realized by the trial-and-error method appears to be the reason for the highly efficient inhibition of anticoagulant activity of the dextran. The lower efficiency of the inhibition of anticoagulant activity of CMDS, $Q+/Q- = 0.43$ as compared with $Q+/Q- = 0.26$ of CMDBS2 containing the same amount of sulfate is an argument in favor of the assumption of non-uniform distribution of these groups along CMDBS chains. It is not unconceivable that the synergistic role of B groups in the anticoagulant activity of CMDBS suggested by

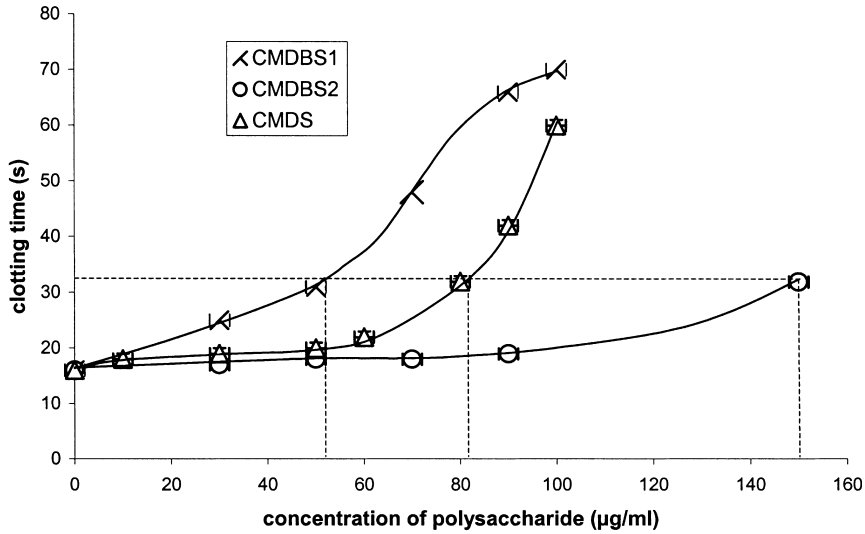

Fig. 3. Clotting time vs concentration of dextrans derivatives. Dotted lines refer to concentrations of polysaccharide which double the standard clotting time (Cd).

Maïga-Revel et al. (Maïga-Revel et al., 1997) reduces to a control of CMDB sulfation resulting in better distribution of these groups on the backbone with respect to their anticoagulant activity.

The data obtained represent an important step toward the development of screening of heparin-like anticoagulant polyanions of different nature and structure. The exhibited properties of PEVP as a molecular probe for CMDBS samples increases the hope that using this approach it is possible to select potential anticoagulant polyanions by mapping their structure. This, in turn, can be readily performed by mere fluorescence measurement using the fluorescence quenching technique (Izumrudov et al., 1998), which is currently underway. Preliminary results on establishing the correspondence between the inhibition of the activity of different CMDBS by PEVP and their mappings are extremely encouraging.

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