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Cationic Derivatives of Dextran and Hydroxypropylcellulose as Novel Potential Heparin Antagonists

Kamil Kamiński,[†] Monika Płonka,[†] Justyna Ciejka,[†] Krzysztof Szczubiałka,[*,†] Maria Nowakowska,[†] Barbara Lorkowska,[‡] Ryszard Korbut,[‡] and Radosław Lach[§]

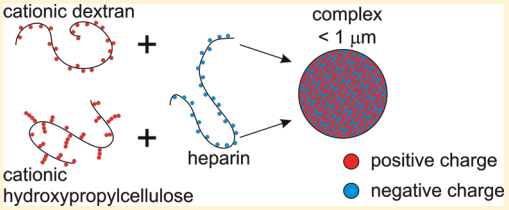
[†]Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Kraków, Poland

[‡]Chair of Pharmacology, Jagiellonian University Medical College, Grzegórzecka 16, 31-531 Kraków, Poland

[§]Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Al.Mickiewicza 30, 30-059 Kraków, Poland

Supporting Information

ABSTRACT: Cationic derivatives of dextran (Dex) and hydroxypropylcellulose (HPC) were studied as potential alternatives of protamine sulfate (PS) used in the reversal of anticoagulant activity of heparin. The modification was performed by the attachment of cationic groups to the Dex main chain or by grafting short side chains of a polycation onto HPC. The cationic derivatives of these polysaccharides were found to bind heparin with the efficiency increasing with growing degree of cationic modification. The degree of cationic modification and consequently the ζ potential of the polymers do not have to be high to achieve effective heparin binding. The size of the complexes of cationic Dex with unfractionated heparin (UFH) is a few micrometers. For complexes of cationic HPC and UFH the size is much below 1 μm, both below and above the lower critical solution temperature of HPC. None of the cationic polysaccharides studied caused hemolysis. The concentrations of the polymers inducing the aggregation of human erythrocytes in vitro were determined.



INTRODUCTION

Cationic polymers are intensively studied primarily as nonviral gene vectors since the first successful clinical gene therapy of adenosine deaminase deficiency in 1989.[1] The list of cationic polymers that were studied as candidates for gene vectors is quite long and includes poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) and poly(L-glutamic acid), the two key polymers being poly(L-lysine) (pLL) and polyethyleneimine (pEI).[2−6] Another clinically important, although much less intensively studied, application of cationic polymers is the neutralization (reversal) of anticoagulative activity of heparin. Unfractionated heparin (UFH), which is its high-molecular-weight form, is widely used as an intravenous anticoagulant of choice during cardiac surgeries,[7] while low-molecular-weight heparin (LMWH) is used mostly in outpatient antithrombotic prophylaxis. Heparin remains the most commonly used anticoagulant during cardiopulmonary bypass (CPB) surgeries.[7] The scale of its demand is reflected by the fact that in 2004 nearly 427 000 procedures of coronary artery bypass grafting (CABG), the most common surgical procedure, were performed in the U.S. alone.[8]

The heparin anticoagulation activity should be stopped within minutes of completion of a cardiosurgery and when a massive hemorrhage occurs. This is achieved by the intravenous administration of protamine,[9] a positively charged protein containing up to 80 mol % of arginine, lysine, and histidine. Protamine forms a complex with heparin that is stabilized by the electrostatic interactions. This complex has no anticoagulant activity[10] and it is removed by the reticuloendothelial system.[11]

There are, however, several problems related to the neutralization of heparin with protamine. UFH shows a very variable and dose-dependent half-life. This is why frequent anticoagulation tests are necessary during CPB[12] and reliable and fast methods of heparin determination are still searched for.[13] The anticoagulative effect of LMWH is more predictable;[14] however, its anticoagulant activity is only partly reversed with protamine.[15]

Because of the complexity of heparin pharmacokinetic and pharmacodynamic properties, the doses of protamine applied have to be established empirically with an unavoidable risk of protamine under- or overdosing, of which underprotamination is considered as a lesser evil.[15] Protamine, when overdosed, shows an effect opposite to that intended, i.e., anticoagulant one[16] due to platelet aggregation and consumption.[15,17] But even when administered in proper doses, protamine may show severe adverse effects. It may induce systemic hypotension due to histamine release from the mast cells,[18] especially after too rapid injections, catastrophic pulmonary hypertension,[19] and anaphylactic reactions,[20,21] frequently encountered in diabetic patients taking injections of neutral protamine Hagedorn insulin.[22] The list of serious side effects of protamine is, however, much longer.

Since both heparin and its antidote have serious drawbacks and taking into account their vast use, the search continues for alternatives of both heparin and protamine.

Many anticoagulants have been developed; however, most of the new anticoagulants are introduced without antidotes.[15] The risk of bleeding due to anticoagulants and the lack of fast acting antidotes is a main reason of the underutilization of these

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important drugs. One class of possible heparin replacements are vitamin K antagonists (VKAs) (warfarin, acenocoumarol, phenoprocoumon). VKAs inhibit vitamin K which is necessary for carboxylation of precursor coagulation proteins (factors II, VII, IX, X, proteins C, S, and Z).[23] The antidotes for VKAs are vitamin K_1,[24] prothrombin complex concentrates (PCC),[25] and recombinant activated factor VII (rFVIIa),[26] which is also used in the treatment of hemophilia.

Pentasaccharides are a class of anticoagulants that act by binding to antithrombin.[27−30] Two pentasaccharides have been investigated in clinical research, idraparinux and fondaparinux. Fondaparinux has a half-life of 18−22 h and is administered subcutaneously once daily while much longer half-life of idraparinux allows for once weekly administration.

Direct thrombin inhibitors (DTI), such as hirudin and its derivatives, are intravenous anticoagulants that offer potential advantages over heparins and VKAs, especially in patients with heparin induced thrombocytopenia (HIT).[31]

A promising novel class of anticoagulants are aptamers, which are small nucleic acid molecules.[32,33] The major advantage of aptamers over conventional anticoagulants is the possibility of their reversal and the ability to neutralize their anticoagulant effect immediately.[15]

However, nonheparin anticoagulants are far from being perfect either. Up to 2.0% of patients require emergency transfusion, hospitalization, or surgery because of bleeding caused by warfarin toxicity.[23] The use of warfarin is responsible for 17% of emergency department visits among patients 65 years of age and older in the U.S. Moreover, for warfarin the reversal takes a long time. The full effect of vitamin K is only achieved 12−24 h after the administration.[15] The volume of plasma required to bring the international normalized ratio (INR) down to 1.5 is about 2 L for most patients.[15] Such large volume cannot be infused quickly. Pentasaccharides have no specific antidotes, while hemofiltration may be necessary to reverse direct thrombin inhibitors in case of their overdose. For aptamers scarce clinical data are available at present.

Our approach to the problems with the anticoagulant therapy is to search for safer protamine alternatives. Until now protamine has no substitute in clinical practice, although alternative substances for heparin reversal have been studied.

Recombinant activated factor (rFVIIa) was found to be effective for LMWH reversal.[34] It was successfully applied in a patient with an overdose of enoxaparin and life-threatening hematoma.

Heparin reversal by a specific heparin-degrading enzyme heparinase I was studied.[35] The enzyme cleaves heparin glycosidic bonds, resulting in the formation of the oligosaccharide fragments that have only very slight anticoagulant activity. It was found, however, that protamine had a better safety profile; patients who were administered heparinase I had hospital stays longer by 1 day, were more likely to experience a serious adverse event, and were less likely to avoid transfusion. It was concluded that although heparinase I reverses heparin anticoagulation after aortocoronary bypass graft surgery, it is not equivalent to protamine because of its inferior safety profile.

An interesting alternative to protamine are peptides containing amino acid sequences that are common to a variety of heparin-binding proteins.[36] The peptides were found, unlike protamine, to neutralize the LMWH in patients treated with enoxaparin. They were also more efficient at neutralizing UFH in vitro than protamine and had little or no hemodynamic toxicity in rats compared with protamine. In a more recent paper a novel PM102 synthetic peptide was studied as a heparin antagonist.[37] In the peptide three identical amino acid helix segments are joined by an organic tether.[38] PM102 administered intravenously produced dose dependent reversal of heparin-induced anticoagulation.

Heparin reversal can be also achieved by platelet factor 4 (PF4), an endogenous inhibitor of heparin, synthesized by megakaryocytes and released from the α granules of the platelets.[39] PF4 was found to reverse the anticoagulant effect of heparin, as measured by activated coagulation time, and no serious adverse effects were observed.

Each of these approaches (maybe except for the most recent one of still unknown applicability) has limitations, and none has been approved for clinical use.

In our view polysaccharides may be considered as yet another class of materials that, after cationic modification, may reverse heparin. They are generally nontoxic, biodegradable, easy to functionalize, biocompatible, and last but not least, inexpensive materials, which should also be considered when comparing them with alternative heparin reversal agents obtained by complicated and costly synthetic methods.

We have synthesized cationic chitosan microspheres composed of *N*-(2-hydroxyl)propyl-3-trimethylammonium chitosan chloride (HTCC), which were found to be useful for heparin removal in vitro[40] while the water-soluble HTCC was found to complex heparin in solution.[41]

The current paper, which is a continuation of these studies, is devoted to the cationic derivatives of other polysaccharides as possible antiheparin agents. It is well-known that cationic polymers are more or less toxic.[42,43] The adverse effects of polycations include hemolysis and hemagglutination. One of the reasons is that the isoelectric point of most of the plasma proteins is low; therefore, at physiological pH they are polyanions and strongly interact electrostatically with polycations. This interaction, and consequently toxicity, is obviously stronger for polycations with greater positive ζ potential which is usually greater for polycations with higher charge density along the main chain. However, the structure of a polycation also significantly influences its interaction with polyanions and therefore its toxicity.[44] In the case of polymers making contact with blood, toxicity relates mainly to the thrombotic response and may differ significantly. Our studies on the cationic polysaccharides may help in finding the relationship between the structure of the polysaccharide, its charge (ζ potential), the ability to bind heparin, and the size of the complex with heparin. This paper is concentrated on dextran and hydroxypropylcellulose, two FDA-approved polysaccharides, which were chosen as the starting materials because they are generally nontoxic, biodegradable, and biocompatible. They were modified by substitution with cationic ammonium groups or by grafting with monomers possessing ammonium groups.

RESULTS AND DISCUSSION

Two polysaccharides, dextran (Dex) and hydroxypropylcellulose (HPC), were cationically modified. Both of them are water-soluble, irrespective of pH. Dextran is frequently used for intravenous supplementation of iron. On the other hand, medical uses other than pharmaceutical formulations are quite rare. It is used in ophthalmic inserts in the treatment of dry eye syndrome[45,46] and to enhance nasal absorption of drugs.[47,48]

Cationically Modified Dextran. Two cationic derivatives of Dex were obtained by the substitution of hydroxyl groups with

GTMAC (Scheme 1). The elemental analysis confirmed the attachment of GTMAC groups to the Dex backbone and allowed calculation of the degrees of substitution of the polymers obtained, which were found to be 0.50 and 0.65 of GTMAC groups per glucose unit (Table 1). These polymers are referred in the text as Dex-GTMAC1 and Dex-GTMAC2, respectively.

The structure of the cationic dextrans synthesized was confirmed using ^{1}H NMR. A new signal at 3.1 ppm was found in the spectrum of the cationic dextrans which was ascribed to the methyl group protons of GTMAC (see Supporting Information). In FTIR spectra of both Dex-GTMAC polymers a weak band at 1480 cm^{-1} appeared that could be attributed to an asymmetric angular binding of the methyl groups of GTMAC. In addition, the bands that correspond to the vibrations of the hydroxyl groups at 1345, 1260, and 1000 cm^{-1} are weaker in the substituted polymers.

Cationically Modified Hydroxypropylcellulose. Cationic derivative of hydroxypropylcellulose (HPC) was obtained by grafting APTMAC from the HPC chain (see Scheme 2).

Scheme 1. Synthesis of the Cationic Derivative of Dex

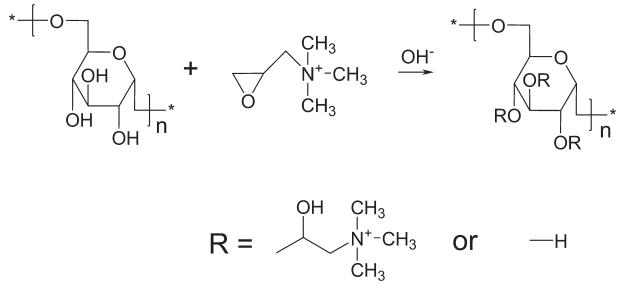


Table 1. Degrees of Cationic Modification of Studied Polymers Expressed as the Average Number of Cationic Charges per Glucose Unit (n^c_g)

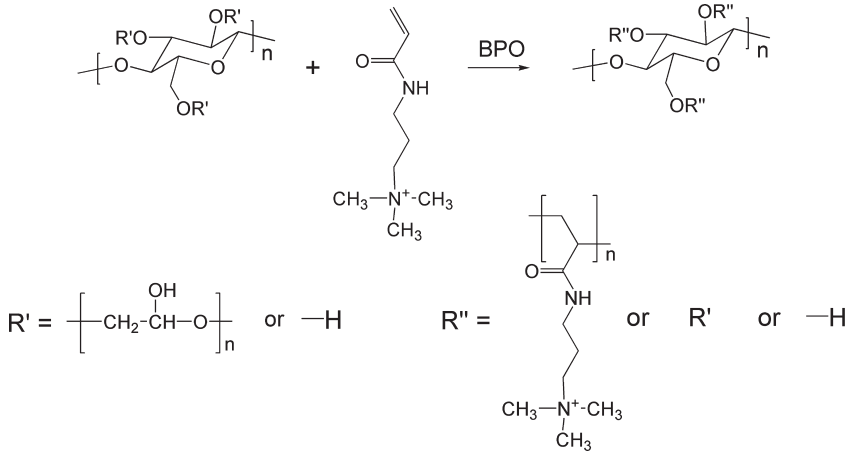
polymer	av no. of cationic charges/glucose unit (n^c_g)
Dex-GTMAC1	0.50
Dex-GTMAC2	0.65
HPC-APTMAC1	0.22
HPC-APTMAC2	1.98
HPC-APTMAC3	4.11

By reaction of HPC with three different concentrations of APTMAC, three cationic graft polymers were obtained with the number of APTMAC units per one glucose unit being 0.22, 1.98, and 4.11, as found from the elemental analysis. The polymers are hereafter denoted as HPC-APTMAC1, HPC-APTMAC2, and HPC-APTMAC3, respectively. The changes in FT-IR spectra of HPC upon grafting were analogical to those seen for Dex-GTMAC, since both APTMAC and GTMAC contain methyl groups attached to quaternary amine nitrogen. Moreover, a band at 1540 cm^{-1} corresponding to deformation vibrations of the −NH bond of the amide group appeared in the spectrum of HPC-APTMAC (see Supporting Information). In the ^{1}H NMR spectrum of HPC-APTMAC a signal at 3.1 ppm appeared coming from the methyl groups of APTMAC and a peak at 2.15 ppm appeared coming from the amide proton (see Supporting Information). By use of GPC, it was verified that one peak was obtained in the chromatogram and its retention times in chromatograms obtained using two different detectors (UV−vis and IR) were identical; therefore, the formation of APTMAC homopolymer could be excluded (data not shown).

Thermosensitivity of HPC-APTMAC Polymers. HPC is a thermosensitive derivative of cellulose; i.e., it shows the lower critical solution temperature (LCST) in the aqueous solution. In a dilute solution (0.1 g/dm^3) its LCST falls around 41 °C.[49] Exceeding the LCST results in breaking the hydrogen bonds stabilizing the HPC chains in the solution. The hydrophobic interactions between polymeric chains then become relatively strong and result in the aggregation of the chains. At temperatures just above the LCST (42−44 °C) this is accompanied by the formation of nanospherical HPC aggregates.[49] Further temperature increase leads to breaking of more hydrogen bonds and consequently to even greater increase of the HPC aggregate size. To find how cationic modification and increased ionic strength influence LCST of HPC, turbidity of the respective polymer solutions (expressed as $1 - T$ where T is solution transmittance at 400 nm) was measured in water and in PBS (Figure 1).

It was found that grafting of HPC with APTMAC increases the LCST while increasing the ionic strength decreases it. These changes are typical of ionically modified thermosensitive polymers showing LCST, both synthetic[50,51] and of natural origin.[52,53] For HPC-APTMAC3 dissolved in PBS the effect of cationic modification and the effect of increased ionic strength cancel each other, so in PBS this polymer shows a LCST value

Scheme 2. Synthesis of the Cationic Derivative of HPC



similar to that of HPC in water. Moreover, the increase in turbidity is more gradual than in the case of the native HPC. The calculated values of LCST are given in Table 2. In view of possible intravenous applications it is important to note that the LCST of the grafted polymer is above normal physiological temperatures. However, since the LCST values found are only slightly higher than the physiological temperature, it was important to investigate what is the size of the objects formed above the LCST in HPC-APTMAC solutions, both alone and in the presence of UFH. This problem is discussed below.

Binding of Heparin. The cationic derivatives of Dex and HPC are expected to form complexes with heparin based on electrostatic interactions between their chains. The relative ability of the studied polymers to bind heparin was assessed with a colorimetric method using Azure A, a cationic dye, as described previously.[40,41] Briefly, the monomeric form of Azure A absorbs at 630 nm. Heparin chains, when present in the solution of Azure A, complex the dye molecules which then form aggregates absorbing at 513 nm. Successive addition of a heparin-binding polymer leads to the disruption of the dye aggregates and an increase of the concentration of monomeric Azure A molecules. Thus, adding a heparin-binding substance to the solution of heparin and Azure A is accompanied by an increase of the 630 nm absorption band intensity and a decrease in 513 nm band intensity. When complete heparin binding by added substance is achieved, the Azure A spectrum is typical of the dye dissolved in the absence of any interacting substances.

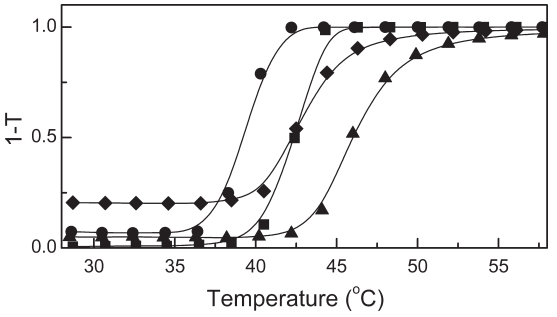


Figure 1. Dependence of turbidity of the polymer solution on temperature: (■) HPC in water; (●) HPC in PBS; (▲) HPC-APTMAC3 in water; (◆) HPC-APTMAC3 in PBS.

Table 2. LCST of Native and Cationically Modified HPC in Water and PBS

	LCST (°C)	
Polymer	water	PBS, pH 7.4
HPC	42	40
HPC-APTMAC3	45	42

Binding of UFH by Cationic Dextrans. The dependence of free UFH (i.e., uncomplexed UFH possessing anticoagulant activity) concentration on the cationic dextran concentration expressed as the mass ratio of cationic dextran and total UFH mass is shown in Figure 2.

The concentration of free UFH in the solution was found to decrease with increasing concentration of both modified dextrans. However, for dextran with lower degree of substitution (Dex-GTMAC1) this decrease was slower than for Dex-GTMAC2; i.e., its greater mass was required to achieve the same decrease in free UFH concentration. In both cases it is difficult to determine the exact amount of polymer necessary to completely remove UFH. Therefore, the mass of the polymer necessary to remove 90% of UFH was calculated by extrapolation of the trend in the region of linear decrease of UFH concentration (for Dex-GTMAC1) or by interpolation (for Dex-GTMAC2). The values

Table 3. Efficiency of UFH Binding by Cationic Polymers Studied and PS (PBS Buffer, pH 7.4, T = 25°C)

polymer	polymer mass required to bind 90% of UFH in PBS buffer, pH 7.4 (mg/mg)	polymer concn required to bind 90% of UFH at c_0 = 0.200 mg/mL (mg/mL)
Dex-GTMAC1	3.0	0.60
Dex-GTMAC2	1.8	0.36
HPC-APTMAC1	8.7	1.74
HPC-APTMAC2	1.7	0.34
HPC-APTMAC3	1.1	0.22
HTCC1[a]	1.5	0.30
HTCC2[a]	1.3	0.26
PS	1.1	0.22

[a] Based on ref 41.

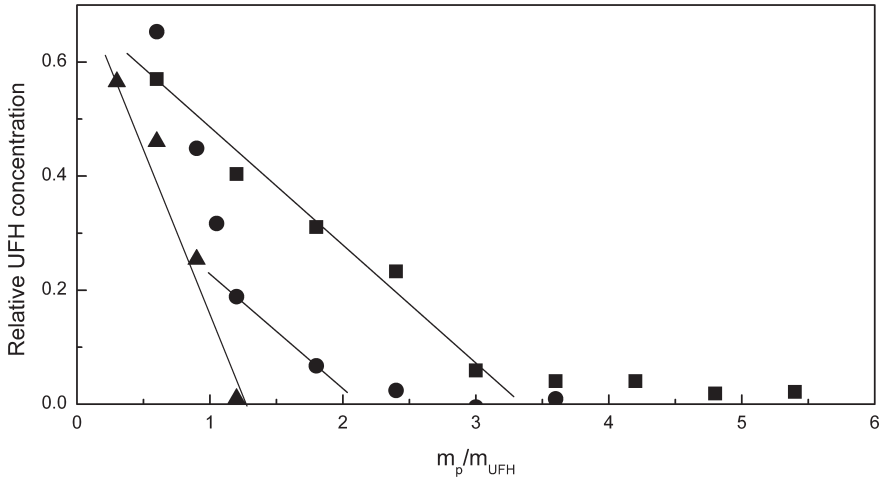


Figure 2. Relationship between relative free UFH concentration (c_0 = 0.200 mg/mL) and the ratio of Dex-GTMAC1 (■), Dex-GTMAC2 (●), and PS (▲) mass and UFH mass (m_p/m_{UFH}) (pH 7.4, T = 25 °C).

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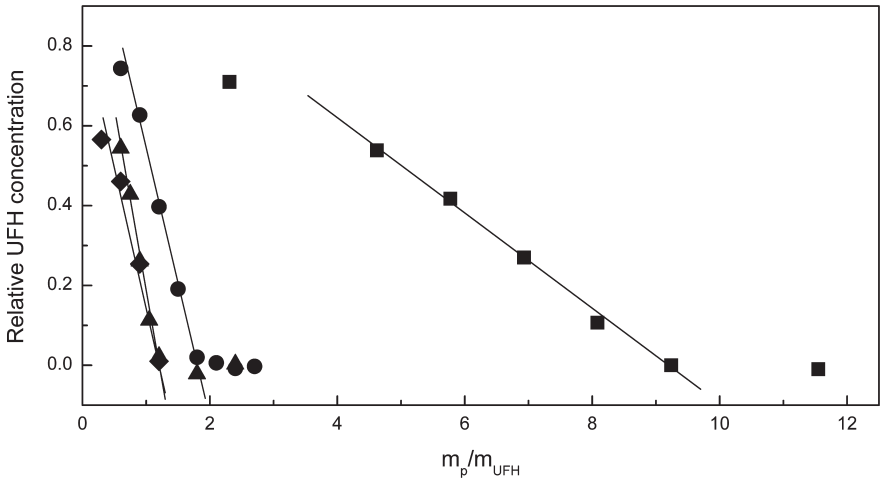

Figure 3. Relationship between relative free UFH concentration (c_0 = 0.2 mg/mL) and the ratio of HPC-APTMAC1 (■), HPC-APTMAC2 (●), HPC-APTMAC3 (▲), and PS (◆) mass and UFH mass (pH 7.4, T = 25 °C).

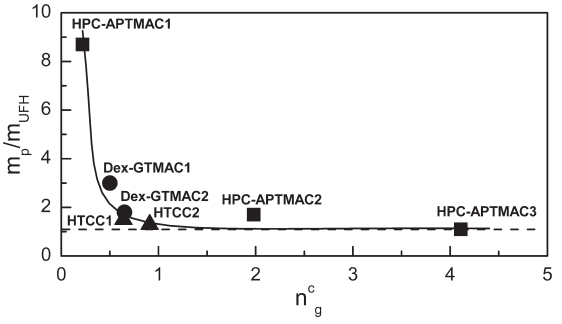

Figure 4. Dependence of the relative mass of the cationic polymer required to bind 90% of UFH (c_0 = 0.200 mg/mL) on the number of cationic charges per glucose unit in the modified polysaccharide. The m_p/m_{UFH} characteristic of PS is marked as a horizontal dashed line.

found were 3.0 and 1.8 mg for Dex-GTMAC1 and Dex-GTMAC2 (see Table 3). Both these values are greater than the respective value for PS.

Binding of UFH by Cationic Hydroxypropylcellulose. The cationic hydroxypropylcellulose derivatives were also found to complex UFH in PBS buffer (Figure 3).

The masses of the polymers required to bind 90% of UFH calculated from plots in Figure 3 are given in Table 3. They indicate that the heparin-binding ability of HPC derivatives studied is very different, reflecting various degrees of grafting. As the data collected in Table 3 show, higher concentrations of all studied polymers except for HPC-APTMAC3 are necessary to bind 90% of UFH compared to PS. This, however, does not mean that they cannot be treated as potential candidates for substitutes of PS as antiheparin agents. They do not induce erythrocyte aggregation and hemolysis (see below), and their general cytotoxicity and blood compatibility parameters such as ability to induce complement and platelet activation and coagulation are still unknown. The same pertains to the cationic dextrans.

It is very informative to consider the dependence of the relative mass of the polymer required to bind UFH on the degree of cationic modification of a polymer, expressed as (n^c_g). The dependence is plotted in Figure 4. The figure also contains data obtained earlier for cationically modified chitosans (HTCC1 and HTCC2) and described elsewhere.[41]

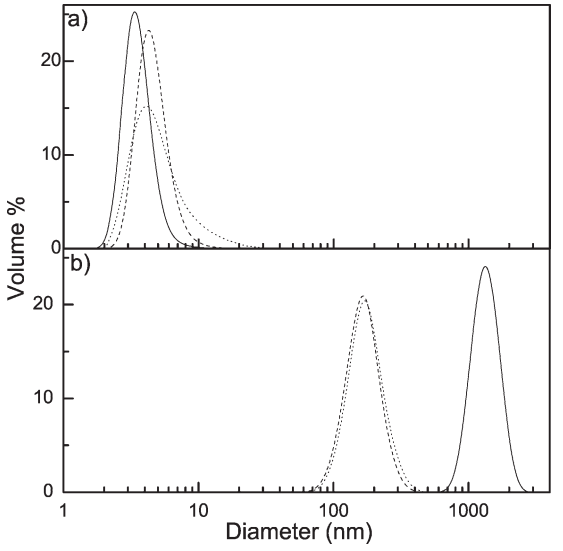

Figure 5. Size distribution of the objects in the solutions of HPC-APTMAC1 (solid line), HPC-APTMAC2 (dashed line), and HPC-APTMAC3 (dotted line) (a) below (at 25 °C) and (b) above LCST (at 45 °C), c_p = 2.4 mg/mL.

It can be concluded that for the polymers with low n^c_g (below 0.5) the dependence of m_p/m_{UFH} on n^c_g is quite strong while for $n^c_g > 0.5$, m_p/m_{UFH} practically does not depend on n^c_g and that even relatively highly charged polymers do not bind more effectively than PS. Therefore, it seems that the polymers with $0.5 < n^c_g < 1$ are optimal for UFH binding considering their high binding ability and low degree of cationic modification. However, the clinical applicability should be estimated only together with blood compatibility and toxicity data. Interestingly, the presence of the primary amine groups except for the cationic ammonium groups in cationically modified chitosans (HTCC) does not improve their efficiency of heparin binding compared to polysaccharides which do not contain amine groups. This can be explained by the fact that at physiological pH the chitosan amine groups are not protonated.

Dynamic Light Scattering Studies. The size of complexes formed by the cationically modified polysaccharides and UFH in

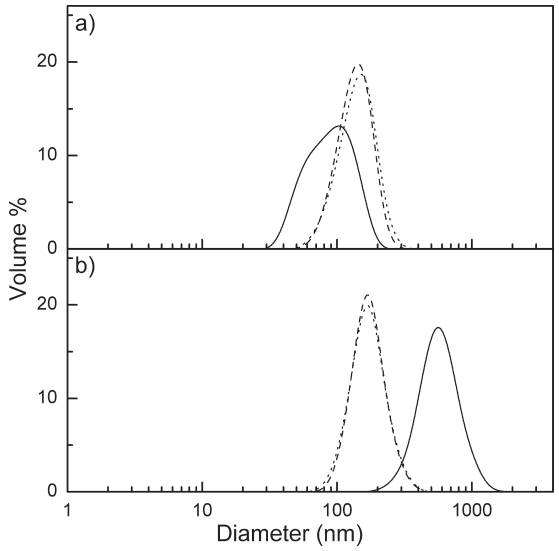

Figure 6. Size distribution of the complexes of HPC-APTMAC1 (solid line), HPC-APTMAC2 (dashed line), and HPC-APTMAC3 (dotted line) with UFH (a) below (at 25 °C) and (b) above LCST (at 45 °C), c_p = 2.4 mg/mL.

Table 4. ζ Potential for Polysaccharides Studied and Their Complexes with UFH in Water and PBS at 25 and 45 °C

	ζ potential		
		PBS, pH 7.4	
polymer	water, pH 6 25 °C	25 °C	45 °C
PS[a]		8.67 ± 3.75	
UFH[a]		−23.3 ± 1.75	
Cationic Polysaccharides			
Dex-GTMAC1	2.97 ± 2.10	1.16 ± 0.441	
Dex-GTMAC2	4.55 ± 4.77	3.22 ± 0.632	
HPC-APTMAC1	16.83 ± 3.23	2.82 ± 0.202	1.79 ± 0.388
HPC-APTMAC2	49.8 ± 6.80	8.09 ± 0.392	10.6 ± 1.94
HPC-APTMAC3	50.17 ± 6.09	8.53 ± 0.362	14.9 ± 2.45
HTCC1[a]		13.28 ± 1.21	
HTCC2[a]		24.2 ± 0.83	
UFH−Cationic Polysaccharide Complex			
UFH-Dex-GTMAC1		0.56 ± 1.00	
UFH-Dex-GTMAC1		0.92 ± 0.81	
UFH-HPC-APTMAC1		−4.02 ± 0.374	−2.74 ± 0.157
UFH-HPC-APTMAC2		−2.85 ± 0.465	−11.3 ± 0.917
UFH-HPC-APTMAC3		−4.17 ± 0.124	−0.353 ± 0.228
UFH-HTCC1[a]		1.95 ± 0.53	
UFH-HTCC2[a]		2.52 ± 1.15	
UFH−PS Complex			
UFH-PS[a]		−19.52 ± 0.60	

[a] From ref 41.

the aqueous solutions (PBS buffer, pH 7.4) was studied with the dynamic light scattering (DLS) technique. All the measurements

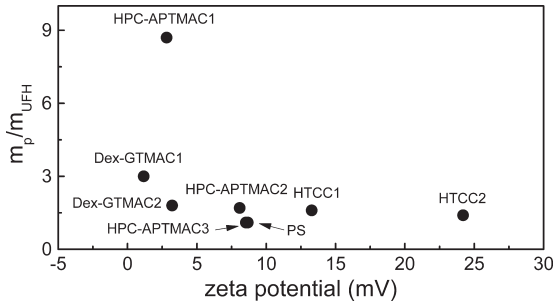

Figure 7. Dependence of the relative mass of the cationic polymer required to bind 90% of UFH on the ζ potential of the polymer in PBS. The markers for PS and HPC-APTMAC3 coincide.

Table 5. Hemolysis Caused by the Cationic Polymers Studied and PBS at 4°C and 37°C after 1 h[a]

	hemolysis (%)	
medium	T = 4 °C	T = 37 °C
PBS (pH 7.4)	0.38	0.51
Dex-GTMAC2	0.43	0.40
HPC-APTMAC3	0.50	0.46
PS	0.37	0.45

[a] The concentration of the polymers corresponded to the lowest polymer concentration resulting in erythrocyte aggregation. The uncertainty of the hemolysis degree was estimated to be 0.04%.

were carried out at polymer concentrations high enough to bind at least 90% of UFH.

For dextrans it was found that the size of both native and cationic polymers is about 3 nm (see Supporting Information) while the size of their complexes with UFH was found to be about a few micrometers, too high to be accurately measured using DLS. The microphotograph taken using an optical microscope confirmed this result, additionally revealing that the aggregates have fibrous morphology (see Supporting Information).

Taking into account that HPC is a thermosensitive polymer and the size of the aggregates it forms may be significantly influenced by the temperature, the measurements were carried out at 25 °C (below LCST) and at 45 °C (above LCST) for the cationic HPC derivatives both in the absence and in the presence of UFH.

Below LCST cationically modified HPC polymers form objects that are 3.8−4.4 nm in diameter (Figure 5a). Their size significantly increases above LCST and is highest for HPC-APTMAC1 (about 1330 nm), while for HPC-APTMAC2 and HPC-APTMAC3 it is much smaller at about 170−200 nm (Figure 5b).

As expected, the size of the objects in the solutions of cationic HPC derivatives increases after addition of UFH to about 110 nm for HPC-APTMAC1 and to about 148 nm for HPC-APTMAC2 and HPC-APTMAC3 (Figure 6a). Quite unexpectedly, however, increasing the temperature above LCST does not significantly increase the size of the complexes of HPC-APTMAC2 and HPC-APTMAC3 with UFH (to about 171 nm) (Figure 6b). In only the case of the complexes of UFH and the least modified polymer, i.e., HPC-APTMAC1, the size increases noticeably, up to about 553 nm.

ζ Potential Measurements. ζ potential was measured for all studied cationic polysaccharides and their complexes with

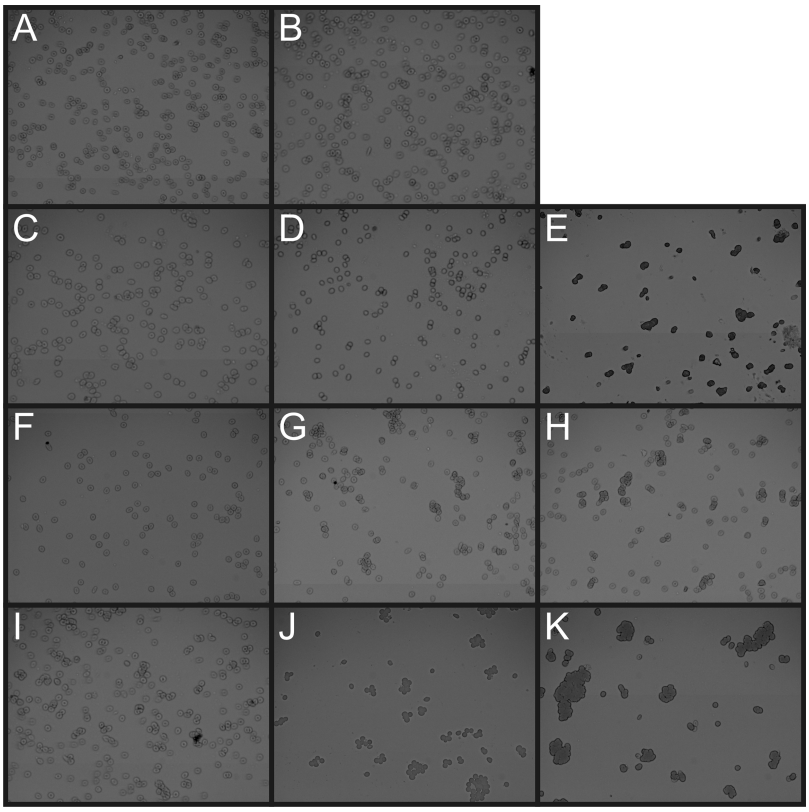

Figure 8. Optical microphotographs of human erythrocytes after addition of cationic polymers at different concentrations to whole blood: (A) PBS measured immediately; (B) PBS measured after 1 h; (C, D, E) protamine at 2.16, 2.52, and 2.88 mg/mL, respectively; (F, G, H) Dex-GTMAC2 at 0.108, 0.144, and 0.18 mg/mL, respectively; (I, J, K) HPC-APTMAC3 at 0.036, 0.072, and 0.144 mg/mL, respectively. All images are taken at 20× magnification.

UFH. Additionally, for cationically modified HPC the ζ potential was measured above the LCST in the absence and in the presence of UFH. The results of the measurements are collected in Table 4.

The ζ potentials of the complexes of the cationic derivatives of HPC with UFH are much less negative than that of UFH-PS but still negative as opposed to the complexes of cationic chitosan with UFH studied previously.[41]

All the values of ζ potential for Dex-GTMAC and HPC-APTMAC are positive and correlate with the number of cationic charges per glucose unit (n^c_g) in the polymer. Also, the efficiency of heparin binding seems to be related to the ζ potential (Figure 7).

From the data in Figure 1 one may conclude that only the values m_p/m_{UFH} and ζ potential obtained for HPC-APTMAC1 do not fit the behavior of the rest of polymers. The measured value of ζ potential for this polymer is, however, quite uncertain due to its very low charge. The value of ζ potential of 3 mV and above is enough to achieve efficient UFH binding. Further increase in the ζ potential of the polymer above this value practically does not improve binding efficiency of UFH. The conclusion from this finding is that the polymer does not have to be strongly positively charged to efficiently bind UFH. This is an important conclusion, since polymers bearing weak positive charge should be less toxic than those bearing a large charge. It could be also found that there is no clear dependence between the ζ potential of a cationic polysaccharide and the size of its complex with UFH.

Hemolysis of Erythrocytes. Hemolysis of the erythrocytes is caused by the destabilization of their membrane, resulting in the release of hemoglobin. The ability of cationic polymers to induce hemolysis is one of their most important and most frequently studied biocompatibility measures.[54−57] It is generally assumed that the molecular weight and the balance between hydrophobic and hydrophilic groups of a cationic polymer are the factors governing its hemolytic activity. For the polymethacrylate derivatives hemolysis increased both with growing polymer hydrophobicity and with rising molecular weight.[58] The absence of hemolytic effect is one of the requirements to be fulfilled by a candidate material for intravenous applications.

The hemolytic activity of the studied cationic polymers was traced by measuring the absorbance of the supernatant at a wavelength of 575 nm from above the settled erythrocytes at the polycation concentrations resulting in complexation of at least 90% of UFH (see Table 3). After addition of water the erythrocytes were considered as 100% hemolyzed. The values of the degree of hemolysis caused by the cationic polysaccharides and by PS after 1 h at 4 and 37 °C are given in Table 5.

In all the systems studied hemolysis was about 0.5% or lower, which may be due to the predominantly hydrophilic character of the studied polysaccharides. These data indicate that the cationic polysaccharides do not cause hemolysis. Increasing the temperature from T = 4 °C to T = 37 °C does not result in statistically significant change of hemolysis degree. The absence of the hemolytic effect of the cationic derivatives of Dex and HPC is an important and promising

Table 6. Concentrations of the Cationic Polysaccharides Studied Causing Erythrocyte Aggregation

polymer	polymer concn inducing erythrocyte aggregation (c_{agg}) (mg/mL)
Dex-GTMAC1	1.08
Dex-GTMAC2	0.18
HPC-APTMAC1	3.24
HPC-APTMAC2	0.108
HPC-APTMAC3	0.072
PS	2.88

finding in view of their potential intravenous applications as heparin antidotes.

Erythrocyte Aggregation. Interaction of cationic polymers with erythrocytes may be especially potent, particularly because the erythrocyte membrane is negatively charged. The cationic polysaccharides were added to the samples of whole blood, and the erythrocytes were investigated for changes in their morphology and aggregability (Figure 8). The blood samples containing only PBS were observed immediately after blending and 1 h later (Figure 8A,B). It was verified that no visible changes in the erythrocyte size and morphology and no aggregation have occurred within this time period. That is why any changes occurring to erythrocytes in PBS polymer solutions could be ascribed to the presence of the polymer solely. Moreover, neither GTMAC nor APTMAC induced the aggregation of the erythrocytes. On the other hand it was found (Figure 8C,D,E) that under the experimental conditions applied, the aggregation occurred with PS at 2.88 mg/mL and greater. In the Figure 8 the microphotographs of blood samples containing different concentrations of Dex-GTMAC2 and HPC-APTMAC3, i.e., polysaccharides with the highest degree of cationic modification among those studied (Dex-GTMAC2 in Figure 8F,G,H and HPC-APTMAC3 in Figure 8I,J,K) are shown. In the presence of Dex-GTMAC2 the first evidence of aggregation occurs when the polymer concentration exceeds 0.18 mg/mL, while for HPC-APTMAC3 aggregation starts below 0.072 mg/mL, which could be ascribed to its higher positive charge. These threshold proaggregatory concentrations (c_{agg}) are of the order of the concentration of each of the respective polymers required to complex 90% of UFH from its 0.200 mg/mL solution (see Table 3). The values of c_{agg} for the other cationic Dex and HPC as well as for HTCC polymers were also determined using the above method (microphotographs not shown). All obtained data are shown in Table 6.

It was found that all c_{agg} values for the cationic polysaccharides are lower than the respective value for PS. Taking into account that these polysaccharides, unlike PS, are known to be of low toxicity and generally nonallergenic, it is tempting to speculate that they may bind heparin safely at higher concentrations than PS. To confirm this assumption, however, further in vivo studies are necessary.

Analysis of the data in Table 6 reveals also that for both cationic Dex and HPC, increasing the degree of cationic modification lowers the value of c_{agg}. Thus, higher efficiency of UFH binding, which is obviously desirable, is accompanied by lower values of c_{agg}, which is an undesired effect. The efficiency of UFH binding of Dex-GTMAC2 is about twice that of Dex-GTMAC1 (see Table 3), while its c_{agg} is 6 times lower (see Table 6). These differences are unexpectedly high taking into account quite small difference in the average number of GTMAC units per glucose unit in these polymers (Table 1). For the most and the least modified HPC the respective values are about 8 and 45. This may indicate that the degree of cationic modification of the polysaccharide should be kept as low as possible, since the increase of UFH binding efficiency is achieved for the price of much greater increase in aggregative activity of the polymer. The concentrations of cationic polymers inducing erythrocyte aggregation were determined in the absence of heparin. One may expect that in the presence of heparin these values would be much higher, which should increase their therapeutic window.

CONCLUSIONS

Cationic derivatives of dextran and hydroxypropylcellulose with different degrees of cationic modification were studied as potential antiheparin agents. They were found to complex unfractionated heparin. The efficiency of binding is related to the degree of cationic modification. The cationic polymers do not cause the hemolysis of the erythrocytes. The concentrations at which the polymers induce erythrocyte aggregation were determined to be lower than that of protamine sulfate.

EXPERIMENTAL SECTION

Materials. Dextran (Dex, M_w = 40 kDa from Leuconostoc spp., Sigma), hydroxypropylcellulose (HPC, M_n = 10 kDa, M_w = 80 kDa, Sigma), glycidyltrimethylammonium chloride (GTMAC, Fluka, 90%), *N*-acrylamidopropyl-*N*,*N*,*N*-trimethylammonium chloride (APTMAC, 75 wt % solution in water, stabilized with 3000 ppm MEHQ, Aldrich), heparin sodium salt from bovine intestinal mucosa (Sigma), protamine sulfate (PS) salt from salmon (Grade X, Sigma), Azure A chloride (Fluka, Fluka standard), benzoyl peroxide (BPO, 75% remainder water Luperox A75, Aldrich), potassium chloride (analytical grade, POCh), potassium dihydrogen phosphate (analytical grade, POCh), disodium hydrogen phosphate (analytical grade, POCh), sodium chloride (analytical grade, POCh), NaOH (analytical grade, POCh), DMF (analytical grade, Sigma-Aldrich) were used as received. The number of hydroxypropyl units per anhydroglucose unit in HPC (MS) was calculated to be 6.6 based on ^{1}H NMR spectrum.[59] Water was distilled twice and deionized using the Millipore Simplicity system. The purity of all synthesized materials was at least 95% as found using elemental analysis, GPC, and ^{1}H NMR spectroscopy.

Apparatus. FTIR spectra were obtained on a Bruker IFS 48 spectrometer. NMR spectra were measured in a 1:1 mixture of D_2O and DMSO-d_6 using a Bruker AMX 500 spectrometer. UV−vis absorption spectra were recorded using an HP8452A diode-array spectrophotometer in 1 cm optical path quartz cuvettes. Elemental analysis was performed using a Vario Micro CHNS elemental analyzer (Elementar). GPC analyses were performed using a Waters GPC system equipped with a bank of three columns (PL Aquagel-OH 30, 40, and 60) and tandem PDA/RI detectors. The eluent was 0.1 M NaCl, flow rate was 0.6 mL/min, sample volume was 150 μL, and concentrations of polymers were 5 g/L for Dex-GTMAC and 10 g/L for HPC-APTMAC. The dimensions of the aggregates and their ζ potential were measured using a Malvern Instruments Zetasizer Nano-ZS. The samples were prepared in a PBS pH 7.4 solution, filtered through a Chromafil filter (0.45 μm) and measured at room temperature. The data were analyzed using the Malvern software. LCST measurements were performed using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a Hewlett-Packard 89090A Peltier temperature control accessory allowing for the precise digital temperature control (±0.1 °C). Temperature was measured with a Hewlett-Packard 89102A temperature sensor immersed in the solution. LCST values were determined from changes

in the sample transmittance at λ = 400 nm. Microscopic images were obtained using a Nikon TE-2000 microscope.

Synthesis of Dex-GTMAC. Two grams of dextran was dissolved in 100 mL of distilled water. Then 400 mg of NaOH was added and the solution was stirred with a magnetic stirrer and heated to 60 °C. In the next step 12 or 24 mL of GTMAC (90%) was added to obtain the polymers with two different degrees of substitution. The mixture was heated and kept at 60 °C for 4 h while being stirred. Then the reaction mixtures were cooled and transferred to dialysis tubes (M_w cutoff value of 12.8 kDa). The dialysis was carried out against distilled water until the conductivity of the polymer solution in the tube decreased to 2 μS. The polymer was separated from the solution using the freeze-drying technique. Yield: ~85%.

Synthesis of HPC-APTMAC. In a three-necked flask 1.5 g (5 mmol of glucose units) of HPC was dissolved in 15 mL of DMF. The solution was degassed by bubbling with nitrogen for 30 min, and a solution of the initiator (1.35 g of BPO dissolved in 7.5 mL of degassed DMF) was added. After 5 min a mixture of 16 mL of DMF and 1.77 or 4.43 or 17.72 g of APTMAC, 75 wt % solution in water, was added. The reaction mixture was heated at 70 °C for 3 h under constant mixing with a magnetic stirrer and under bubbling with nitrogen. Then the mixture was cooled and dialyzed first against DMF and after that against a mixture of DMF and water. The fraction of water was gradually increased, and finally the dialysis was performed in pure water. The dialysis was carried out against water for 2 more weeks. The polymers obtained were isolated from the solution using the freeze-drying technique. Yield: ~65%.

Hemolysis Tests. The samples of 1.80 mL of the polymer solution in PBS with 0.20 mL of blood freshly drawn from anticubital vein of human volunteers were added to a centrifuge tube with a pipet tip wetted previously with 4% citrate. Then the samples were incubated for 1 h at 4 °C and centrifuged for 5 min at 8000 rpm (565g). After centrifugation 1.00 mL of the supernatant was drawn with the pipet and the hemoglobin concentration was measured by light absorbance at the maximum of the 575 nm absorption band. To record the UV spectra of blood added to pure water for 100% hemolysis, a 10-fold sample dilution was necessary before measurement.

Aggregation of Erythrocytes in Whole Human Blood. An amount of 2 μL of venous blood freshly drawn from human volunteers was added to anticoagulant sodium citrate at a concentration of 3.8%. Then the samples were mixed with 18 μL of the solution of a respective cationic polymer dissolved in PBS (pH 7.4). After a subtle mixing procedure the samples were examined instantaneously using transmitted bright field light microscopy (Nikon TE-2000 microscope) for the presence of aggregates containing erythrocytes. The experiments were performed in triplicate. Images were captured using 20× magnification.

■ ASSOCIATED CONTENT

Supporting Information. FTIR and ^{1}H NMR spectra and ζ potentials of the polymers at different pH. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +48 12 6632020. Fax: +48 12 6340515. E-mail: szczubia@chemia.uj.edu.pl.

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■ ABBREVIATIONS USED

APTMAC, *N*-acrylamidopropyl-*N*,*N*,*N*-trimethylammonium chloride; CABG, coronary artery bypass grafting; CPB, cardiopulmonary bypass; Dex, dextran; DLS, dynamic light scattering; DTI, direct thrombin inhibitors; GTMAC, glycidyltrimethylammonium chloride; HIT, heparin induced thrombocytopenia; HTCC, *N*-(2-hydroxyl)propyl-3-trimethylammonium chitosan chloride; HPC, hydroxypropylcellulose; INR, international normalized ratio; LMWH, low molecular weight heparin; PCC, prothrombin complex concentrate; pDMAEMA, poly(2-(dimethylamino)ethyl methacrylate); pEI, polyethyleneimine; PF4, platelet factor 4; pLL, (L-lysine); PS, protamine sulfate; rFVIIa, recombinant activated factor VII; UFH, unfractionated heparin; VKA, vitamin K antagonist

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