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Chitosan Derivatives as Novel Potential Heparin Reversal Agents

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In emergency cases anticoagulant action of heparin needs to be stopped instantaneously, which is usually achieved by intravenous administration of protamine sulfate (PS). However, PS shows many adverse effects. The objective of the present work was to find out if chitosan (Ch) and a cationically modified chitosan, *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC), may be applied for heparin reversal. For chitosan the efficiency of unfractionated heparin (UFH) binding decreases with increasing pH while for cationically modified chitosan heparin binding is efficient even for high pH values. Complexation of UFH and low-molecular-weight heparin (LMWH) by cationically modified chitosan in the aqueous solution at pH = 7.4 was studied. Complexes of the modified chitosan with UFH are smaller and of lower dispersity than those with PS. Cationically modified chitosan was found to bind both UFH and LMWH. The complex formation capability of cationically modified chitosan is comparable to that of PS.

Introduction

Heparin has been widely used in medicine for more than six decades on account of its anticoagulant and antithrombotic activities which have been known for even a longer time.[1] The mechanism of the anticoagulant action of heparin has been extensively studied and largely explained.[2] It acts by binding to antithrombin III present in blood plasma and by forming a complex that inhibits thrombin and factors IXa, Xa, and XIa. Factors IXa and Xa are relatively inaccessible to antithrombin III when they are bound in a complex with their cofactors. Heparin is effective in blocking thrombin circulating in plasma but is relatively ineffective in blocking platelet or clot-bound thrombin.[3] Heparin is inhibited by acute-phase proteins, such as platelet factor 4 and thrombospondin.

Heparin is also known to show effects independent of its anticoagulant activity. These effects may be both adverse and beneficial. The disadvantageous ones include inhibition of smooth muscle cell proliferation,[4] leukocyte-mediated vascular damage,[5] and complement activation-induced injury,[6] while the beneficial ones include inhibition of metastatic spread of tumor cells.[7] Heparin has been also shown to promote the growth of collateral coronary arteries.[8]

Structure and chemical properties of heparin have been elucidated in detail.[9,10] Heparin is a sulfated glycosaminoglycan composed of macromolecules with molecular weights varying from 3000 to 30000. Heparin shows complex pharmacokinetics. It is administered intravenously, although oral administration of heparin has been also tried.[11,12] Its anticoagulant effect cannot be precisely predicted and differs considerably among patients. The dose−response relationship for heparin is nonlinear. Also, patient's response depends upon age, gender, body weight, smoking status, and renal function.[13] As a consequence, it is very difficult in clinical practice to attain therapeutic anticoagulation as judged by the APTT[a] (activated partial thromboplastin time). That is why cases of heparin overdose are not rare.

Clinical use of heparin use may lead to some side effects although heparin is usually well tolerated. Except for already mentioned adverse effects, they include severe ones such as thrombocytopenia[14] and osteoporosis.[15]

In the mid-1980s low-molecular-weight heparins (LMWH) were introduced to clinical use as antithrombotic drugs, initially to prevent postoperative deep vein thrombosis, with good results.[16] LMWHs have molecular weights ranging from 2000 to 10000, usually in the range of 4000−6000.[17] LMWHs are produced by several procedures: nitrous acid depolymerization (dalteparin, nadroparin, reviparin), enzymatic degradation (tinzaparin), or benzylation followed by alkaline hydrolysis (enoxaparin). Recent studies have shown that LMWHs cause less side effects although, at the same time, they are less powerful anticoagulants than unfractionated heparin (UFH). In a prospective study the incidence of thrombocytopenia during UFH treatment was evaluated to fall between 1% and 5% while for enoxaparin, the corresponding numbers were 0% and 0.9%, respectively.[18] There are also experimental data suggesting that LMWHs may induce less severe osteoporosis in rats than UFH.[19]

However, the most severe side effect is hemorrhage, which is common to both LMWH and UFH. In contrast to the chronic effects of heparin, hemorrhage requires the rapid administration of a heparin antidote. At the moment protamine sulfate (PS) is the drug of choice for heparin reversal. Its complex with

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[a] Abbreviations: APTT, activated partial thromboplastin time; Ch, chitosan; HTCC, *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride; GTMAC, glycidyltrimethylammonium chloride; LMWH, low-molecular-weight heparin; PS, protamine sulfate; UFH, unfractionated heparin.

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heparin is stable and does not have anticoagulant activity.[20] PS, however, induces numerous adverse reactions ranging from mild hypotension to idiosyncratic fatal cardiac arrest. It shows immunogenicity[21,22] and "cross-linking" ability[23,24] which may lead to the formation of antigen−antibody-like large network structures.[25] Another drawback of PS is the fact that it deactivates LMWH only partially. That problem is of particular importance taking into account growing use of LMWHs. Since the administration of PS may cause severe side effects, sometimes even lethal, it is desired to develop a safer method for fast heparin (both UFH and LMWH) neutralization. It can be expected that, in spite of the intensive search for new anticoagulants,[26] both forms of heparin, a drug of well-established clinical use, will still be used for a difficult to predict but certainly considerable period of time.

Recently, we have suggested novel, convenient, and safe methods of both administration[27] and removal of heparin.[28] We have shown that microspheres of cationically modified chitosan cross-linked with genipin are able to quickly remove heparin from the aqueous solution and plasma. This kind of material might be potentially useful for heparin removal in devices such as those used in supporting extracorporeal circulation. Of much greater clinical interest, however, is a safe inhibitor of the anticoagulative action of heparin which could be administered as a water-soluble intravenous drug.

In the current paper we present results of our investigations on the interaction of chitosan (Ch) and *N*-(2-hydroxypropyl)-3-trimethylammonium chitosan chloride (HTCC), a cationically modified chitosan, with both UFH and LMWH in the aqueous solution. Quaternized chitosans were found to be nontoxic and have many beneficial effects; e.g., they enhance adsorption of hydrophilic drugs through mucous membranes.[29,30] HTCC shows antibacterial and antifungal properties,[31] inhibits the proliferation of various cancer cell lines,[32] and improves proliferation of human periodontal ligament cells (HPDLC).[33] We have found that HTCC might be useful for the neutralization of heparin. Our model laboratory studies have also shown that heparin complexing ability of HTCC is comparable to that of PS. What is also of great importance, we have shown that HTCC forms stable complexes with LMWHs suggesting that it may potentially be a more effective reversal agent for LMWHs than PS.

Results and Discussion

Binding of Unfractionated Heparin by the Native Chitosan. We have studied the interactions between UFH and Ch in an aqueous acidic solution. It was observed that the addition of acidic (pH = 5.0) aqueous solution of Ch to the aqueous solution of UFH (pH = 5.0, PBS buffer) resulted in the appearance of the readily visible turbidity. That suggests the formation of insoluble UFH−Ch complexes due to the electrostatic interactions between the sulfate and carboxylate groups of UFH and the amino groups of Ch.

The amount of UFH complexed by Ch was determined as a difference of the analytical concentration of UFH in a solution and the concentration of "free" UFH (UFH which is in equilibrium with the UFH−Ch complex). For that purpose several samples of the same UFH concentration (c_{UFH} = 0.196 mg/mL) containing various concentrations of Ch were prepared, and the systems were allowed to equilibrate at room temperature. To eliminate the interference from light scattering during the UV−vis absorption measurements, the insoluble UFH−Ch complexes were removed by filtration.

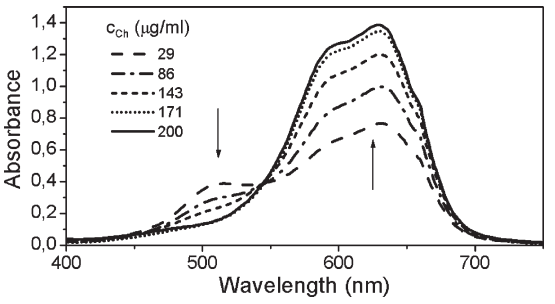


Figure 1. Electronic absorption spectra of Azure A ($c = 4.0 \times 10^{-5}$ M) in the presence of UFH (c_{UFH} = 0.196 mg/mL) at different concentrations of Ch (pH = 5.0, PBS buffer, T = 25 °C).

Figure 1 shows the electronic absorption spectra of Azure A in the presence of constant concentration of UFH and growing concentration of Ch. The absorption band of Azure A at λ = 630 nm is characteristic of the nonassociated dye molecules, while the 513 nm band originates from the absorption of the associated molecules: dimers, trimers, and larger aggregates.[41]

The addition of chitosan to the UFH solution containing Azure A results in the decrease of the absorption intensity of the dye at the wavelength λ = 513 nm and an increase of absorption at λ = 630 nm. These spectral changes can be interpreted as follows. In the solution the attractive interaction between anionic UFH and cationic dye leads to the adsorption of the dye molecules along UFH chains. Proximity of the adsorbed dye molecules and shielding of the repulsive interaction between them (Azure A is a cationic dye) by the oppositely charged polymer promotes formation of Azure A aggregates. Ch, when added to this solution, forms aggregates with UFH which leads to the disruption of Azure A aggregates and the appearance of monomeric dye absorbing at 630 nm. When the concentration of Ch added is equal to about 200 μg/mL, the absorption spectrum of Azure A is identical with that of the dye measured in the absence of UFH. That indicates that at the Ch concentration equal to or higher than 200 μg/mL all UFH molecules are complexed by Ch chains and there is no free UFH present in the solution. It should be pointed out that qualitatively identical spectral changes accompanied addition of PS to UFH solution in the presence of Azure A, which are due to the PS−UFH complex formation. This is another confirmation of the complexation of UFH by Ch.

As expected, the efficiency of UFH binding by Ch in the aqueous solutions is strongly pH-dependent and decreases with the decrease of the solution acidity (see Figure 2). That could be explained considering the higher degree of protonation of amino groups in solutions at lower pH. In acidic solutions Ch chains are positively charged and interact strongly with the anionic UFH molecule due to electrostatic attractions which lead to the complexation of UFH.

As can be seen in Figure 2, at pH = 6 the complete removal of free UFH from the solution can be achieved when the ratio of Ch to UFH mass is about 0.7. The value of this ratio increases to more than 1.7 and more than 5.0 at pH = 7.4 and 8.0, respectively.

Binding of Unfractionated Heparin by the Modified Chitosan. Taking into account the low efficiency of UFH complexation by Ch at neutral and basic pH and in view of decreased Ch solubility with increasing solution pH, one can conclude that native Ch could not be considered for UFH reversal when administered intravenously and should be

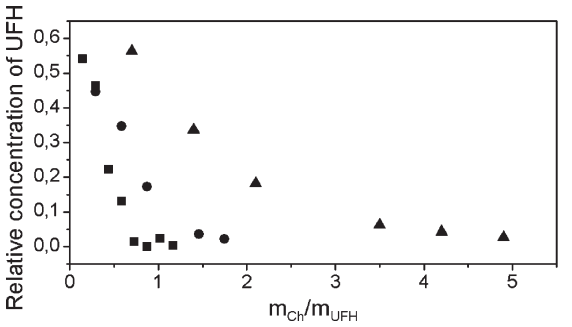
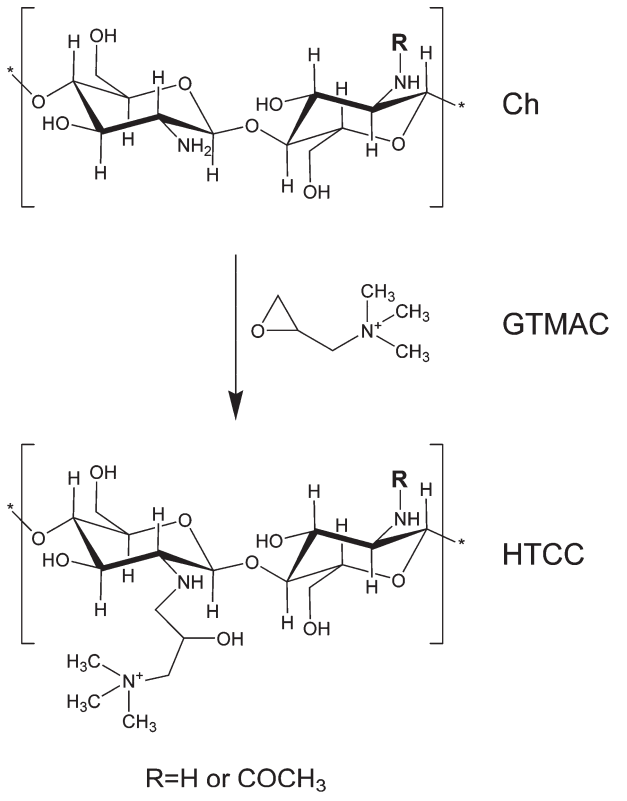


Figure 2. Dependence of relative UFH concentration (c_0 = 0.196 mg/mL) on the mass ratio of Ch and UFH present in the solution at pH 6.0 (■), 7.4 (●), and 8.0 (▲).

Scheme 1. Chitosan Substitution with GTMAC



modified. Considering possible biomedical applications, the improved system should be characterized by good solubility at neutral pH (ideally, at pH = 7.4, which is the value typical of blood) and high efficiency of UFH binding under physiological conditions.

For that purpose Ch was subjected to cationic modification (quaternization) with glycidyltrimethylammonium chloride (GTMAC) (see Scheme 1).

Two polymers, HTCC1 and HTCC2, with a different degree of substitution were synthesized (Table 1). Contrary to chitosan, both of them were found to be very well soluble in water at neutral pH. Polymer modification by covalent attachment of GTMAC was confirmed by FT-IR spectroscopy (see Figure 3).

In the FT-IR spectra of HTCC1 and HTCC2, a band at 1483 cm^{-1} (marked with an arrow) can be seen. That can be attributed to an asymmetric angular bending of the methyl groups of GTMAC. This band is absent in the spectrum of

Table 1. Degrees of Substitution of Ch with GTMAC

	degree of substitution, DS (%)	molar ratio of GTMAC and monosaccharide units of Ch in the reaction mixture
HTCC1	63.6	3:1
HTCC2	90.5	6:1

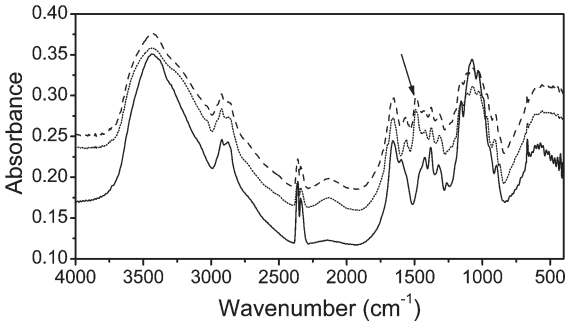


Figure 3. FT-IR spectrum of Ch (solid line), HTCC1 (dashed line), and HTCC2 (dotted line).

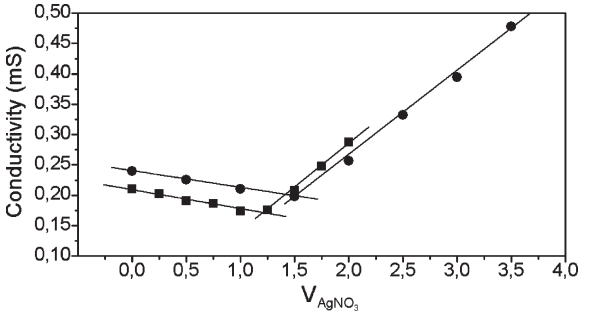


Figure 4. Conductivity of HTCC1 (■) and HTCC2 (●) (c = 1.0 g/L) as a function of $AgNO_3$ volume added ($c = 1.7 \times 10^{-5}$ M, T = 20 °C).

unmodified Ch. Moreover, the band that corresponds to the deformation vibrations of the primary amino group at 1560 cm^{-1} which is present in the Ch spectrum is much weaker in the HTCC1 and HTCC2 spectrum due to the substitution of the amine groups. Similar features of the IR spectra were described in the literature for the quaternized chitosan.[34−36] The structure of the product was confirmed also with NMR spectra (see Experimental Section). In the GPC chromatogram of each product (data not shown) a single peak was found confirming their purity.

The degree of substitution (DS) was calculated using conductometric titration (Figure 4). The degree of substitution was calculated using theg equation:[40]

$$\mathrm{DS}\ (\%) = \frac{(1.7 \times 10^{-3})V^*_{AgNO_3}}{\left(\dfrac{W_{HTCC} - (1.7 \times 10^{-3})V^*_{AgNO_3} m_{GTMAC}}{m_G \mathrm{DDA} + m_{AG}(1 - \mathrm{DDA})}\right)\mathrm{DDA}} \times 100$$

where W_{HTCC} is the weight of either HTCC1 or HTCC2 in 10 mL, m_{GTMAC} is the molecular weight of GTMAC (i.e., 151 g/mol), m_G is the molecular weight of glucosamine (i.e.,161 g/mol), m_{AG} is the molecular weight of acetylated glucosamine (i.e., 203 g/mol), DDA is the degree of deacetylation of chitosan found to be 78.9% from the elemental analysis,[37] $V^*_{AgNO_3}$ is the volume of $AgNO_3$ added at the end point, and 1.7×10^{-3} is the coefficient

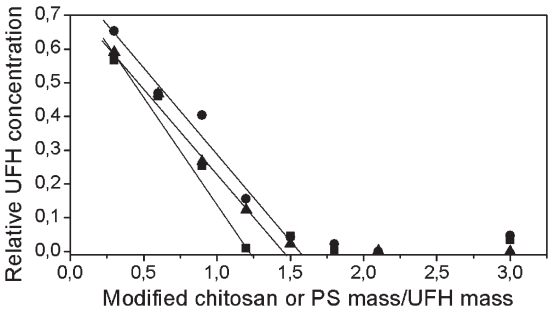


Figure 5. Relationship between relative free UFH concentration (c_0 = 0.200 mg/mL) and the ratio of PS (■), HTCC1 (●), or HTCC2 (▲) mass and UFH mass (pH = 7.4, T = 25 °C).

Table 2. Efficiency of UFH Binding by PS, HTCC1, and HTCC2 (PBS Buffer, pH = 7.4, T = 25 °C)

polymer	mass necessary to bind 1.0 mg of UFH in PBS, pH = 7.4, buffer (mg)
PS	1.3 ± 0.1
HTCC1	1.6 ± 0.2
HTCC2	1.4 ± 0.1

corresponding to the number of $AgNO_3$ moles in 1 mL of the solution.

The degrees of substitution found are high and equal to 63.6% for HTCC1 and 90.5% for HTCC2 (see Table 1), respectively. They correlate well with the molar ratio of GTMAC and Ch monosaccharide units in the reaction mixture.

Interaction between HTCC and UFH at pH = 7.4 (in PBS buffer solution) was then studied (Figure 5). Using the spectrophotometric method it was observed that cationically modified Ch efficiently removes free UFH from the solution by complexation. The binding effectiveness of HTCC2, i.e., the polymer with a higher degree of substitution, measured as the minimum weight ratio of HTCC2 and UFH necessary for complete UFH binding, is only slightly higher than that for HTCC1 in spite of much greater positive charge of its chains. The UFH complexation ability of both polymers was also compared with that of PS. It was found that the masses of both polymers necessary to completely bind free UFH present in the solution are only slightly greater than that of PS (see Table 2). Thus, it was concluded that HTCC polymers show high enough solubility and UFH binding ability to be considered for further studies on heparin complexation.

Binding of Low-Molecular-Weight Heparin by the Modified Chitosan. As discussed in the Introduction, currently low-molecular-weight heparins (LMWHs) are more and more frequently used, and in some clinical applications they replaced unfractionated heparin (UFH). Therefore, it was of great importance to find out if the modified chitosans studied are able to form complexes also with LMWH. Unfortunately, in the case of LMWHs, the heparin deactivation procedure based on PS administration fails quite often. Thus, there is a considerable interest in the development of a new effective antagonist for these anticoagulants. We have tested the possibility of application of substituted chitosan for that purpose. Studies with LMWH were conducted using the same method as in the case of UFH (pH = 7.4, PBS buffer, T = 25 °C).

Data presented in Figure 6 demonstrate clearly that LMWH is complexed with HTCC2 under experimental conditions applied. On the basis of that observation one can expect that HTCC2 is a promising material for potential reversal of heparin, with a broad spectrum of action involving

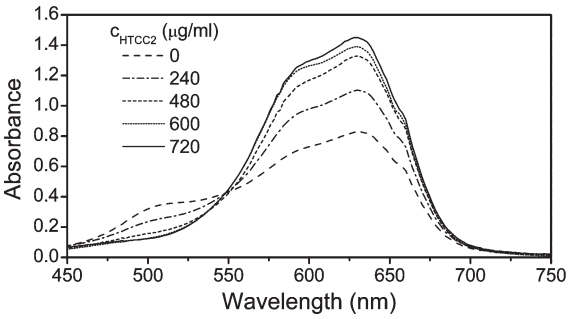


Figure 6. Electronic absorption spectra of Azure A ($c = 4.0 \times 10^{-5}$ M) in the presence of LMWH (nadroparin, 47.5 AXa units/mL) at different concentrations of HTCC2 (pH = 7.4, PBS buffer, T = 25 °C).

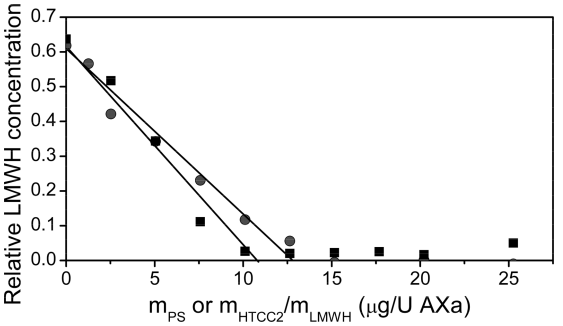


Figure 7. Dependence of the relative concentration of free LMWH (nadroparin, initial concentration 47.5 AXa units) on the ratio of PS (■) and HTCC2 (●) mass and LMWH mass (pH = 7.4, T = 25 °C).

both UFH and LMWH. This is an important finding in view of the reports that PS reverses only 60% of the anti-Xa activity of LMWH[38] and it does not affect the anticoagulant activity of fondaparinux.[39] Efficiency of heparin binding by HTCC2 was determined as equal to 13 μg of HTCC2/AXa units of LMWH (see Figure 7). That means that the deactivation of 100 AXa units of low-molecular-weight heparin applied (nadroparin) can be achieved by the administration of 1.3 mg of modified chitosan. The mass of PS required to bind LMWH is only slightly lower, i.e., about 11 μg of HTCC2/AXa units of LMWH.

Studies on the Heparin Complex with Protamine Sulfate and Cationically Modified Chitosan Using Dynamic Light Scattering (DLS). The dimensions of the objects formed as a result of complexation of UFH with PS and HTCC1 in the aqueous solutions (PBS buffer, pH = 7.4) were determined by the DLS technique (see Figure 8).

One can observe that the UFH−PS aggregates have large polydispersity and their diameter ranges from 100 nm to 10 μm while UFH complexes with modified chitosans do not reach such large sizes and their polydispersity is rather low. Moreover, the size of HTCC2 complexes is about one-half that of HTCC1, i.e., 350 and 700 nm, respectively. More compact organization of HTCC2 aggregates with UFH results probably from the higher positive charge density along their chains compared to those of HTCC1. One can expect that smaller UFH−HTCC1 and UFH−HTCC2 complexes of much lower polydispersity than those formed by UFH with PS could be advantageous in biomedical procedures, such as an intravenous application of HTCC for heparin reversal.

Zeta Potential Study of Heparin Complexes. The driving force of polycation−polyanion complex formation involves the electrostatic interactions, therefore we have studied the

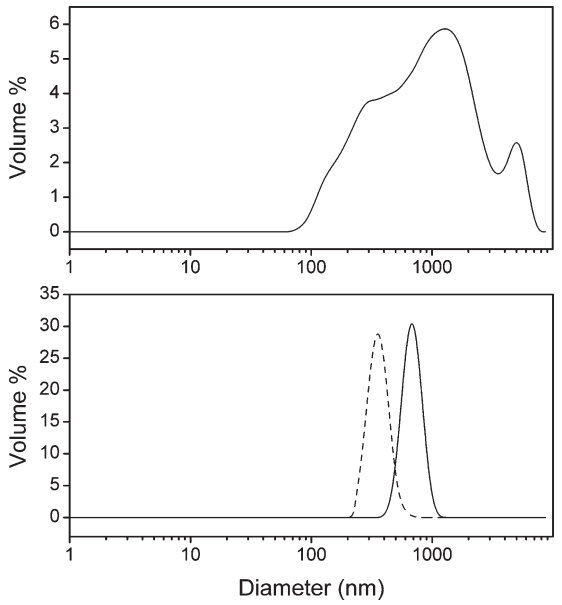


Figure 8. Size distribution of UFH complexes formed with PS (top), HTCC1 (bottom, solid line), and HTCC2 (bottom, dashed line).

Table 3. Zeta Potential of Nanoparticles of Polymers and Complexes

substance	zeta potential at pH 7.4 (mV)
UFH	−23.3 ± 1.53
PS	8.67 ± 3.75
HTCC1	13.28 ± 1.21
HTCC2	24.2 ± 0.83
UFH−PS complex	−19.52 ± 0.60
UFH−HTCC1 complex	1.95 ± 0.53
UFH−HTCC2 complex	−2.52 ± 1.15

zeta potentials of PS, both HTCCs, UFH molecules, and UFH−PS, UFH−HTCC1, and UFH−HTCC2 complexes in PBS buffer at pH = 7.4. The results are presented in Table 3.

As expected, UFH particles have a negative zeta potential which results from the presence of sulfonic groups attached to the main chain of UFH, while the PS and HTCC particles have positive zeta potential resulting from the presence of positively charged groups along the polymer chain. The zeta potential of HTCC1 is considerably higher than that for PS. That reflects the fact that at pH = 7.4 the quaternary amino groups in HTCC1 are fully ionized while the deprotonated amino acid units in PS are in equilibrium with the protonated ones. The zeta potential of HTCC2 is higher than that of HTCC1, which is due to its higher degree of substitution with cationic ammonium groups. Interestingly, the zeta potential of the UFH−PS complex is negatively charged, which is distinctly different from the behavior of the UFH−HTCC1 complex. Zeta potentials for both UFH−HTCC1 and UFH−HTCC2 complexes are close to zero, which could indicate that both UFH and HTCC polymeric chains are present at the complex particle surface. Most likely the heparin and HTCC chains form an interpenetrated network. The relatively high negative surface charge of the UFH−PS complex suggests that the surface of PS chains is coated by the UFH chains.

Conclusions

Quaternized chitosan derivatives (HTCCs) bind the high-molecular-weight (unfractionated) heparin in PBS buffer solution of pH = 7.4 with efficiency comparable to that for the clinically used protein, protamine sulfate. More importantly, HTCC polymers bind also the low-molecular-weight heparin. DLS measurements indicated that while heparin−protamine complexes are large and polydisperse, the heparin−HTCC aggregates are relatively small and their polydispersity is low. On the basis of these data one can suggest that HTCC materials could be considered as good candidates to be used intravenously for reversal of high- and low-molecular-weight heparin in blood. The studies on their toxicity and heparin reversal activity in vivo are currently in progress and will be published in a future paper.

Experimental Section

Materials. Low-molecular-weight chitosan (Ch; Aldrich), glycidyltrimethylammonium chloride (GTMAC; Fluka, 90%), heparin sodium salt from bovine intestinal mucosa (Sigma), nadroparinum calcium, 5700 units of AXa/0.6 mL of solution (LMWH; Fraxiparine, GlaxoSmithKline), protamine sulfate (PS) salt from salmon (Grade X; Sigma), Azure A chloride (Fluka, Fluka standard), potassium chloride (analytical grade, POCh), potassium dihydrogen phosphate (analytical grade, POCh), disodium hydrogen phosphate (analytical grade, POCh), sodium chloride (analytical grade, POCh), acetic acid (reagent grade, POCh), and acetone (analytical grade, CHEMED) were used. Water was distilled twice and deionized using the Millipore SIMPLCITY system.

Apparatus. UV−vis absorption spectra were recorded using an HP8452A diode-array spectrophotometer in 1 cm optical path quartz cuvettes. The dimensions of the aggregates and their zeta potential in aqueous suspension were determined using Malvern Instruments Ltd. Nano ZetaSizer. Elemental analysis was performed using a Vario Micro CHNS elemental analyzer (Elementar). NMR spectra were measured in D_2O/CD_3COOD using a Bruker AMX 500 spectrometer. 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. Parameters of the GPC analysis were as follows: eluent, 0.1 M NaCl; flow rate, 0.8 mL/min; temperature, 30 °C; sample concentration, 5 g/L; sample volume, 50 μL).

Synthesis of *N*-(2-Hydroxypropyl)-3-trimethylammonium Chitosan Chloride (HTCC). Modification of the polymer was performed using the method previously described.[28] Briefly, 2.5 g of chitosan (Ch) was dispersed in 100 mL of distilled water. Ten milliliters of 0.5% acetic acid was added, and the solution was stirred with a magnetic stirrer for 30 min. Then, 6.9 or 13.8 mL of GTMAC was added to obtain the polymers with various degrees of substitution. The mixture was heated and kept at 55 °C for 18 h while stirring. The suspension was subsequently centrifuged at 4000 rpm for 10 min to remove suspended unreacted Ch. The product was extracted from the supernatant via precipitation in acetone and then centrifuged at 4000 rpm for 10 min. The solution was decanted, and the precipitate containing the product was dried in air and dissolved in distilled water. The resulting solution was centrifuged as before, and the polymer dissolved in the supernatant was precipitated with a new portion of acetone. That purification procedure was repeated two more times. The product obtained after last precipitation cycle was dried in vacuum for 24 h. Two polymers with different degrees of substitution, referred to as HTCC1 and HTCC2, were obtained. The polymers were characterized with elemental analysis (HTCC1, C % = 40.98, H % = 7.80, N % = 7.29; HTCC2, C % = 42.75, H % = 7.99, N % = 7.32). The GPC chromatograms (data not shown) revealed single peaks. In the NMR spectra (data not shown) a signal appeared at 3.2 ppm (methyl protons of the trimethylammonium group), confirming the occurrence of the reaction.

Determination of the Degree of Substitution (DS) of HTCC Polymers. The DS of the HTCC was determined using a titration method described in the literature.[40] Briefly, 10 mg of HTCC was dissolved in 10 mL of deionized water, and the conductivity of the solution was measured as a function of the volume of 0.017 M silver nitrate added. The $AgNO_3$ aqueous solution was added in 0.25 mL aliquots, and the solution conductivity was measured after addition of each portion. Initially, when $AgNO_3$ was added to the polymer solution, its conductivity gradually decreased. The reduction of conductivity is caused by the combination of Cl^- and Ag^+ ions which form a precipitate. When all Cl^- ions are consumed (i.e., at the end point of the titration), further addition of $AgNO_3$ results in an increase of the solution conductivity.

Determination of Heparin Concentration. Heparin concentration (both UFH and LMWH) in the solutions was determined by using the Azure A spectrophotometric method.[41] In short, to 0.1 mL of heparin solution were added 0.9 mL of the PBS buffer and 1 mL of 4.0×10^{-5} M Azure A solution. The solution was mixed, and its UV−vis absorption spectrum was measured. Heparin was assayed based on the intensity of the 630 nm band, which corresponds to monomeric Azure A molecules. In the presence of heparin Azure A molecules form dimers absorbing at 513 nm, and the absorption band with maximum at 630 nm decreases due to falling concentration of monomeric Azure A molecules.

The concentration of heparin which was not complexed by a studied polymer (Ch, both HTCCs, or PS) was determined in a similar way, but the obtained mixture was additionally shaken for 10 min, and then the suspension was centrifuged at 3000 rpm for 10 min to separate the insoluble complex of heparin with a respective polymer. The concentration of noncomplexed ("free") heparin was determined in the supernatant. A control experiment was performed to ensure that centrifugation does not influence the concentration of noncomplexed heparin.

Measurements of Dynamic Light Scattering (DLS). Polymeric solutions (0.2−1.2 g/L) were prepared in the pH 7.4 PBS buffer. DLS measurements of UFH−PS and UFH−HTCC complexes were carried out in the systems in which all UFH was complexed. The hydrodynamic diameter, polydispersity, and zeta potential of the particles were determined.

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