Published in final edited form as:

Biochem Pharmacol. 2009 August 1; 78(3): 292–300. doi:10.1016/j.bcp.2009.04.012.

Oversulfated chondroitin sulfate interaction with heparin-binding proteins: New insights into adverse reactions from contaminated heparins

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Abstract

An oversulfated chondroitin sulfate (OSCS) was identified as a contaminant to pharmaceutical heparin and severe anaphylactoid reactions were ascribed to this contaminant. An examination of the biochemistry underlying both the anticoagulant activity and the toxic effects of oversulfated chondroitin sulfate was undertaken. This study demonstrates that the anticoagulant activity of this oversulfated chondroitin sulfate is primarily dependent on heparin cofactor II mediated inhibition of thrombin. Heparin and oversulfated chondroitin sulfate binding to coagulation, kinin–kallikrein and complement proteins were studied by surface plasmon resonance. While oversulfated chondroitin sulfate binds tightly to antithrombin III, unlike heparin, OSCS does not induce antithrombin III to undergo the conformational change required for its inactivation of thrombin and factor Xa. In contrast to heparin, oversulfated chondroitin sulfate tightly binds factor XIIa suggesting a biochemical mechanism for the factor XIIa-based enhancement of vasoactive bradykinin production.

Keywords

oversulfated chondroitin sulfate; heparin; antithrombin III; factor XIIa; surface plasmon resonance

1. Introduction

Heparin is a polydisperse mixture of linear polysaccharides that has been used clinically as an anticoagulant for over 75 years (Figure 1A) [1–3]. Heparin is unique as one of the oldest drugs currently still in widespread clinical use, predating the US-FDA [4,5] and is one of the first biopolymeric drugs, and one of the few carbohydrate drugs [3]. Heparin is isolated by extraction from animal tissues rich in mast cells, such as porcine intestines [6]. The chondroitin sulfates

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(CSs) are a second, widely distributed and closely related glycosaminoglycan (GAG) family consisting of CS-A (Figure 1B), CS-B, CS-C, CS-D and CS-E [7]. Medicinal and natural products chemists have actively investigated chemically modified GAGs having improved or unique pharmacological properties, including the widely used low molecular weight heparins [2, 6]. One such semi-synthetic GAG, prepared by sulfonation of CS-A, is oversulfated CS (OSCS) [8] (Figure 1C). OSCS displays structural similarity to heparin and enhanced anticoagulant activity when compared with CS [8].

A rapid onset, acute side effect, caused by an anaphylactoid response [9], resulted in a spike in adverse events associated with contaminated lots of heparin. Analysis of these lots revealed the presence of OSCS [10]. Both the isolated contaminant and independently synthesized OSCS activated the kinin–kallikrein pathway in human plasma, leading to bradykinin formation [9]. OSCS also induced the generation of complement (C) proteins C3a and C5a. The activation of the kinin–kallikrein and complement pathways is linked through fluid-phase activation of factor XII (FXII) in the coagulation cascade. The functional activity of OSCS on FXIIa was demonstrated using FXIIa-depleted plasma [9]. The kallikrein-kinin pathway starts with human plasma FXII. When exposed to a negatively charge surface such as damaged endothelial cells, FXII, PK and high molecular weight kininogen (HMWK) assemble into a ternary complex capable of forming bradykinin (Fig. 2A). FXII is activated to FXIIa, which is able to cleave prekallikrein (PK) into kallikrein (KK). This results in the production of the potent vasoactive mediator bradykinin and the complement-derived anaphylatoxins. Thus, the kinin–kallikrein, complement and coagulation pathways suggested an explanation for the anaphylactoid response observed in patients intravenously administered OSCS contaminated heparin. Pigs and humans are sensitive to the effects of OSCS in a similar manner [9].

Heparin is known to be involved in the regulation of the coagulation cascade through its binding and activation of the serine protease inhibitors (serpins) antithrombin III(ATIII) and heparin cofactor II (HCII) [11]. OSCS can also affect the fibrinolytic system, activating plasminogen, possibly explaining the bleeding effects associated with OSCS contaminated heparin [12]. Serpins, activated by heparin, can rapidly inhibit activated blood coagulation enzymes including thrombin (FIIa), FXa and FXIIa [11] and the serine protease kallikrein [13]. OSCS was originally introduced as a potential anticoagulant having primarily anti-FIIa activity [8], but the biochemical mechanism for this anticoagulant activity had not been explored. Heparin [15,16] and CS [4,14,17] also regulate the complement cascade. The activation of complement by endogenous, highly sulfated chondroitin, released by thrombin receptor-activated platelets, was recently reported [14]. Although GAGs are known to affect the action of KK or kininogen hydrolysis [18] and control bradykinin production [19], less is known about the mechanism by which heparin or OSCS functions in kallikrein-kinin pathway. It should be noted that both heparin and OSCS are pharmacological agents and are not normally circulating in the bloodstream so that both are viewed as interfering with rather than regulating the coagulation cascade.

Heparin and OSCS interaction are examined with purified proteins from the blood coagulation cascade, the kallikrein-kinin pathway and complement system. These interactions were measured by surface plasmon resonance (SPR). The physiological importance of these interactions was assessed using blood coagulation assays, amidolytic assays and identifying the plasma proteins binding to OSCS.

2. Materials and methods

2.1. Materials

Sodium porcine intestinal heparin and bovine tracheal CS-A were obtained from Celsius Laboratories (Cincinnati, OH). Human or recombinant human proteins were from different

suppliers: ATIII was from Aniara (Mason, OH); platelet factor 4 (PF4), and HCII were from Haematologic Technologies (Essex Junction, VT); FXII, α -FXIIa, α FIIa (10 U/mL), FXa (bovine) and prekallikrein were from Enzyme Research Laboratories (South Bend, IN); complement proteins C1, C2, and C3 were from Calbiochem (La Jolla, CA), C4, C5, C6, C7, C8, and C9 were from QUIDEL (San Diego, CA); two-chain HMWK were from Sigma-Aldrich (St Louis, MO), Chromogenic substrates, spectrozyme TH, and spectrozyme FXa were from American Diagnostica, (Stamford, CT). Thromboplastin C was from Dade-Behring (Miami, FL); platelin reagents were from BioMeriux (Durham, NC); amine-PEO3-Biotin was from Pierce (Rockford, IL); Heptest reagents were from Haematologic Technologies (Essex Junction, VT). Other chemicals, if not specifically mentioned, were obtained from Sigma-Aldrich (St Louis, MO).

2.2. Chemical sulfonation of CS

Oversulfated chondroitin sulfate was prepared according to a modified procedure from Maruyama *et al.* [8] CS-A (100 mg) was converted to its tributylamine (TBA) salt by strong cation-exchange chromatography and lyophilized. CS-A TBA salt was dissolved in 0.8 mL of *N,N*-dimethylformamide. Pyridine-sulfur trioxide complex (15 mol/equiv. of available hydroxyl group in CS-A) was added. The reaction mixture was then stirred at 40 °C for 1 h then 1.6 mL of water was added and the product was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate. OSCS was centrifuged for collection and then dissolved in water, dialyzed (2,000 MWCO) and lyophilized. The structure of synthesized OSCS were confirmed by ¹H- and ¹³C-nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC), [20] and compared to OSCS isolated from contaminated heparin activate pharmaceutical ingredient (API) [10].

2.3. Bioassay and anticoagulant activity of heparin, OSCS and CS-A

Heparin, CS-A and OSCS were supplemented in pooled normal human plasma (NHP) and tested in a concentration range of 0–10 µg/mL. PT, APTT, Heptest and thrombin time (TT) (with 10 U calcium) were performed to determine the anticoagulant activity of these agents. Amidolytic anti-FXa and anti-FIIa activity was also measured to determine the antiprotease profile of these agents. For the clotting assays plasma samples supplemented with heparin, CS-A or OSCS were incubated with the specified reagent for each clotting assay at 37°C. The time to clot was measured using an ACL 300Plus. Anti-FXa activity was measured by incubating the supplemented plasma samples for 2 min at 37°C with FXa (5.0 µg/mL) and a chromogenic substrate specific for FXa, Spectrozyme Xa (2.5 µM) was added and the optical density at 405 nm/min was determined. Similarly, the anti-FIIa was measured by incubating the supplemented plasma samples for 1 min at 37°C with FIIa (5 U/mL) and a chromogenic substrate specific for thrombin, Spectrozyme TH (1.0 µM) was added and the optical density at 405 nm/min was determined. APTT, anti-FIIa and anti-FXa assays were performed in NHP and ATIII-depleted plasma on OSCS in a range of 0–10 µg/mL.

In the purified assay system (containing no plasma), affinity purified ATIII was diluted in saline to obtain a 1 U/mL solution. Heparin 0–10µg/mL and heparin supplemented with 10µg/mL OSCS was assayed using an amidolytic method the anti-FXa and anti-FIIa activities were measured. All results were expressed in % inhibition.

2.4. Biotinylation of GAGs

Heparin, OSCS or CS-A (2 mg) and amine-PEO3-Biotin (2 mg) were dissolved in 200 µl H₂O, then 10 mg NaCNBH₃ was added. The reaction mixture was heated at 70 °C for 24 h, after that a further 10 mg NaCNBH₃ was added and the reaction was heated at 70 °C for another 24 h. After cooling to room temperature, the mixture was desalted with the spin column (3,000 MWCO). Biotinylated GAGs were collected, freeze-dried and used for SA chip preparation.

2.5. SPR kinetic measurements of protein binding to immobilized heparin, OSCS, and CS-A

SPR was performed on a BIAcore3000 (GE Healthcare, Uppsala, Sweden). Buffers were filtered (0.22 μ M) and degassed. The biotinylated heparin, OSCS and CS-A were immobilized to flow cells in a streptavidin chip. A flow cell was treated with saturated biotin and served as a control. The successful immobilization of GAG was confirmed by the observation of a ~100 resonance unit (RU) increase in the sensor chip.

The protein sample was diluted in PBS (0.05 M sodium phosphate at pH 7.2, 0.15 M NaCl) or HBS-EP buffer (0.01 M HEPES at pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) (GE Healthcare, Uppsala, Sweden). Different dilutions of protein samples in buffer were injected at a flow rate of 30 μ L/min. At the end of the sample injection (180 s), the same running buffer was passed over the sensor surface to facilitate dissociation for 180 s. After dissociation, the sensor surface was regenerated by injecting 2 M NaCl to remove all the binding proteins. The response was monitored as a function of time (sensorgram) at 25 °C. SPR experiments were run in duplicated or triplicate at each concentration to confirm the bindings were repeatable. Multi-concentration data were globally fit and residuals were calculated and used to assess the goodness of the fit.

2.6. Solution competition SPR study

A solution competition SPR study was conducted to test whether OSCS competed with heparin for binding to ATIII. ATIII (500 nM) pre-mixed with different concentrations of heparin, or OSCS in HBS-EP buffer were injected over a heparin chip at a flow rate of 30 μ L/min. After each run, dissociation and the regeneration were performed as described above. For each set of competition experiments on SPR, a control experiment (only protein without added heparin or OSCS) was performed to make certain the surface was completely regenerated and that the results obtained between runs were comparable.

3. Results

3.1. Characterization of heparin, OSCS and CS-A

Porcine intestinal heparin API, CS-A from bovine trachea, and OSCS synthesized from CS-A were characterized by 13 C- and 1 H-NMR spectroscopy. The 1 H-NMR spectra of each (Figure 1A-C) and the 13 C-NMR spectra (not shown) were consistent with the structures presented in Figure 1. OSCS shows a relatively simple and easily assignable 1 H-NMR spectrum (Figure 1C) consistent with its persulfonation (4 sulfo groups/disaccharide). The average molecular weight of OSCS determined by GPC [20] (data not shown) was 18 kDa. This OSCS was indistinguishable from the OSCS recovered from contaminated heparin API [10].

3.2. Bioassay and anticoagulant activity of heparin, OSCS and CS-A

Assays were performed to measure the anticoagulant/antiprotease activities of heparin, OSCS, and CS-A in a concentration range of 0–10 μ g/mL (Table 1). In APTT and TT assays, heparin produced a strong response, OSCS produced a moderate prolongation, and CS-A showed no prolongation of clotting time. Only high concentrations of heparin produced a significant effect on the PT. The Heptest assay is a clot-based assay that is prolonged by an agent with strong anti-FXa effects. CS-A did not produce a measurable effect, OSCS showed a modest concentration dependent increase, and heparin produced a strong anticoagulant effect in the Heptest. Activity was next assessed by amidolytic assays. In the anti-FXa assays, heparin produced a concentration dependent anti-FXa activity, OSCS and CS-A showed negligible activities. In the anti-FIIa amidolytic assays both heparin and OSCS produced concentration dependent effects with IC₅₀ values of ~1 μ g/mL and ~10 μ g/mL, respectively, and CS-A showed no activity. Next, plasma depleted of ATIII was used to assess the APTT, and anti-

FXa and anti-FIIa amidolytic activities of OSCS. ATIII depleted plasma and NHP showed identical OSCS response curves for APTT and anti-FIIa activity (Figure 3). No OSCS activity towards FXa was observed in either NHP or ATIII depleted plasma (not shown). The amidolytic anti-FXa and anti-FIIa activity of heparin alone and heparin supplemented with OSCS was next measured in plasma free assays (Table 2). While added OSCS had no impact on heparin's ATIII-mediated activity towards FXa, it enhanced the ATIII-mediated anti-FIIa activity of heparin.

3.3. SPR measurements of the interaction of proteins with heparin, OSCS and CS-A

Heparin, OSCS, CS-A were immobilized in an SPR chip. Proteins were flowed over the chip and binding kinetics determined by SPR (Table 3). Differences in binding kinetics and affinity of ATIII and HCII for heparin and OSCS were observed (Figure 4). The sensorgrams of heparin-ATIII interaction fit well with biphasic-binding model (Figure 4A) suggesting that ATIII undergoes a conformational change [3]. Sensorgrams of OSCS-ATIII interaction fit well to the 1:1 Langmuir binding model suggesting monophasic-binding (Figure 4B). For HCII binding, while OSCS binds HCII ~50-fold tighter than heparin (Table 3), the sensorgrams of HCII binding to OSCS and heparin show similar shapes (Figure 4C, D). FXII showed comparable binding for both heparin and OSCS, while FXIIa only showed measurable binding ($< 10 \mu\text{M}$) to OSCS. In the presence of plasma, FXII and FXIIa both bind to heparinized surfaces, but the avidity of this binding and whether or not binding depends on the presence of other plasma proteins was not reported [21]. Kallikrein-kinin pathway proteins, HMWK and PK, bound to heparin and OSCS with similar affinity. A survey of the complement proteins shows little differences except that heparin binds C6 tighter than the more highly sulfated OSCS. All proteins show negligible binding to CS-A (Figure 4E, F). Solution competition SPR study (Figure 4G, H) showed that heparin in solution inhibit the ATIII binding to the surface heparin on the chip with a dose dependent fashion, but OSCS did not show any inhibition to ATIII binding, suggesting the OSCS did not compete with heparin for binding to ATIII.

4. Discussion

The principal reason why heparin is administered as a drug is because it is a potent anticoagulant. The reason why semi-synthetic OSCS found its way into pharmaceutical heparin is because it displays some anticoagulant activity in global assays, such as the clotting-based USP assay, and OSCS (4 sulfo groups/disaccharides, average molecular weight 18 kDa) has similar structural and physiochemical to heparin (2–3 sulfo groups/disaccharides, average molecular weight 12–20 kDa). These similarities appear to have motivated its use, decreasing the chance of its discovery and enhancing the chance of co-purification with heparin. Thus, we decided to investigate the biochemical mechanisms for the anticoagulant activity of OSCS to help devise new ways of discriminating between OSCS and heparin in bioassays.

OSCS and heparin both show anticoagulant activity by global coagulation assays such as the APTT, TT (Table 1) and the USP assay [22]. Other coagulation assays such as the Heptest and the PT could begin to distinguish these agents. Specific assays, such as anti-FIIa and anti-FXa amidolytic assays, clearly separate the activities of these two agents pointing to a prominent HCII-mediated inhibition for OSCS and a mixed ATIII- and HCII-mediated inhibition for heparin, consistent with the observations from the clotting assays. Pure OSCS shows no ATIII-mediated anti-FXa or anti-FIIa activity, suggesting such bioassays might be used to detect OSCS. Surprisingly, OSCS can enhance heparin's ATIII-mediated anti-FIIa activity (but not anti-FXa activity). This enhancement of heparin's activity probably results from the stabilization of the ATIII•heparin•FIIa ternary complex through the non-competitive binding of OSCS to the ATIII and the FIIa in this complex.

Compared to heparin, OSCS exhibited modest anticoagulant activity in the APTT, Heptest and TT assays. In contrast, CS-A exhibits no anticoagulant activity in these assays. In the PT assay, none of these three agents produced sizable anticoagulant effects. However, heparin at a high concentration produced a modest prolongation of this assay. The results of these global anticoagulant assays indicate that heparin is a much stronger anticoagulant than OSCS. Neither the CS-A nor OSCS exhibited any anti-FXa activity, however, OSCS produced significant anti-FIIa activity again suggesting that it mediates its' effects through HCII. Heparin produced both the inhibition of FXa and FIIa consistent with its interaction with both HCII and ATIII.

Next, binding of heparin and OSCS to ATIII and HCII was measured using SPR. Heparin and OSCS bind to ATIII with similar high nM affinity but the binding mode is clearly different (Figure 4A, B), based on the shapes of association curves observed on SPR. OSCS shows monophasic-binding to ATIII and fits to an ideal 1:1 Langmuir binding model; while heparin shows biphasic-binding suggesting that ATIII undergoes a change in conformation (Figure 4A). The conformation change in ATIII on heparin binding is well-established and important in ATIII mediated inhibition of many serine proteases; furthermore, biphasic-binding to heparin had been observed by SPR and kinetically associated with the ATIII conformational change [23]. This explains why heparin but not OSCS mediates the ATIII inhibition of FXa. SPR competition studies demonstrate that OSCS does not compete with heparin for binding to ATIII. This suggests that OSCS does not bind to the same site on ATIII as does heparin and explains why OSCS fails to induce a conformational change in ATIII and shown no ATIII-mediated anticoagulant activity. It is also interesting to note that ATIII inhibits FXIIa and kallikrein and that this inhibition is enhanced by heparin [11]. OSCS in contaminated heparin can compete with heparin to bind, but OSCS•ATIII complex does not undergo a conformational change that may be required to inhibit FXIIa and kallikrein. This represents a potential new mechanism explaining the increase in plasma bradykinin observed in animals treated with OSCS. A decrease in the inhibition of FXIIa and kallikrein in the presence of OSCS would cause the kallikrein-kinin pathway to generate more bradykinin. While SPR demonstrates that OSCS binds HCII ~50-fold tighter than heparin (Table 3), the binding curves show similar shapes (Figure 4C, D). HCII is a selective FIIa inhibitor, thus, explaining OSCS's prominent anti-FIIa activity as measured in both clotting and amidolytic assays (Tables 1 & 2). Iduronic acid residues and sulfo groups play an important role in the modulation of antithrombotic and anticoagulant pathways by heparins. A higher level of these functionalities in GAG chains increase anticoagulant functions, based on studies of both heparin and dermatan sulfate having different sulfation levels [2, 4, 20, 24]. A higher level of sulfo group substitution enhances the binding of basic heparin-binding proteins and the conformational flexibility of iduronic acid promotes tight interactions with heparin binding proteins [1]. Thus, the high sulfation level and the conformational similarity of the 2,3-disulfated glucuronic acid in OSCS with the 2-sulfo iduronic acid of heparin observed by ¹H-nuclear magnetic resonance spectroscopy [20] (Figure 1A,C) help explain the activities of OSCS.

Both heparin and OSCS bind to human factor FXII, PK and HMWK, but only OSCS binds with FXIIa (Table 3). Negatively charged surfaces, such as the injured surface of endothelial cells (Figure 2A) and dextran sulfate, reportedly can promote the activation of FXII [25]. When the negatively charged surfaces bind with FXII in the presence of PK, FXII is activated into FXIIa, which stimulates the hydrolysis of PK into KK, which stimulates the hydrolysis of kininogen (in this specific case, HMWK) to generate bradykinin (Figure 2). Considering the structural homology of PK and FXII [26] and heparin's highly negative charge, it is likely that heparin also acts as a cofactor for the FXII•PK•HMWK assembly (Figure 2B), consistent with a previous study showing that heparin induces FXII activation [27]. We hypothesize here that OSCS might function in a similar way to promote the formation of FXII•PK•HMWK complex and the activation of FXIIa. However, heparin does not promote the generation of bradykinin. Research has shown that polyanions actually protect HMWK from being hydrolyzed by plasma

proteases [18,19]. *In vivo* studies confirm that GAGs reduce the release of BK and decrease the inflammatory response. This is consistent with the *in vitro* studies on the activation of these factors by GAGs. Two systems operate *in vivo*, the “mobile” system in the plasma and the “anchored” system on the surface of the endothelium. The GAGs and proteins in one system are potential competitors of the same components in the other system. There are thousands of components present in the plasma and on the endothelium in addition to the GAGs and factors (Figure 5), making the interpretation of the overall biology complicated since none of these GAGs and factors function in an isolated environment. We propose a mechanism for the involvement of heparin and OSCS in bradykinin generation. After the *intravenous* injection of heparin and OSCS, the negatively charged surfaces of these molecules allow both to bind with FXII and PK. These GAGs can both compete with HMWK binding to heparan sulfate on the surface of endothelial cells with heparin and OSCS acting as templates for the assembly of FXII, PK and HMWK in place of the endothelium in the contact pathway (Figure 2A,B). After the FXII•PK•HMWK complex is formed and activation occurs, the initial FXII•PK•HMWK complex is converted to the FXIIa•KK•HMWK complex. Since heparin does not bind with FXIIa (see Table 3), FXIIa can detach from the complex, become exposed to plasma proteases and inactivated. Thus, in the presence of heparin, FXIIa is unable to convert PK to KK and the remaining leftover PK•HMWK complex on heparin is protected and the hydrolysis of HMWK to bradykinin fails to take place. In contrast, FXIIa tightly binds with OSCS (Table 3), the FXIIa•KK•HMWK complex is successfully formed, resulting in bradykinin generation (Figure 2B).

Previous work in our laboratory examined heparin binding and regulation of the complement cascade [15,16]. Here we demonstrate both heparin and OSCS bind to complement proteins with the similar affinity (Table 3) and while not directly regulating the complement system, instead, indirectly cause FXIIa and kallikrein to influence complement system, triggering an immune response. When FXIIa is stabilized by OSCS, the amount of kallikrein synthesized increases. Previous studies show that kallikrein can replace factor D to cleave factor B [28]. Thus, active C3 convertase is generated and alternative pathway C3 convertase of the human complement cascade is activated to give rise to immune response symptoms.

Acknowledgments

This work was supported by National Institutes of health in the form of grants HL62244 and GM38060 (to RJL). The authors thank Michel Weiwer and Sayaka Masuko prepared the OSCS.

Abbreviations

CS	chondroitin sulfate
GAG	glycosaminoglycan
OSCS	oversulfated chondroitin sulfate
AT III	antithrombin III
HC II	heparin cofactor II
PK	prekallikrein

KK	kallikrein
SPR	surface plasmon resonance
APTT	activated partial thromboplastin time
PT	prothrombin time
TT	thrombin time
NHP	normal human plasma
PF4	platelet factor 4
HMWK	high molecular weight kininogen

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Figure 1. Structures and conformations of the major repeating units and the 600 MHz ^1H -NMR spectra of (A) heparin, (B) CS-A and (C) OSCS.

Figure 2.
A. Physiological role of FXIIa on bradykinin production at the surface of a membrane. **B.** Proposed mechanism of OSCS enhancement of bradykinin production on contact activation of FXII, PK & HMWK. Heparin fails to enhance bradykinin production.

Figure 3.

Activity of OSCS in ATIII-depleted plasma. APTT activity (solid lines) and anti-FIIa activity (dot lines) is shown as a function of OSCS concentration in NHP (▲) and ATIII-depleted plasma (○). No anti-FXa activity over this range of OSCS concentrations could be observed in either plasma sample.

Figure 4.

SPR sensorgrams. ATIII interaction with (A) heparin and (B) OSCS: ATIII in the concentrations of 250 nM (azure), 500 nM (purple), 1000 nM (green), 2000 nM (blue), 4000 nM (red), respectively. HCII Interaction with (C) heparin and (D) OSCS: HCII in the concentrations of 100 nM (azure), 250 nM (purple), 500 nM (green), 1000 nM (blue), 2000 nM (red), respectively. The black curved in (A, B, C and D) are the fitting curves using models from BIAevaluate 4.0.1. (E) Comparison of the biphasic interaction of ATIII (2000 nM) with heparin (green), the monophasic interaction of ATIII with OSCS (red) and the failure of ATIII to interact with CS-A (blue). (F) Comparison of the monophasic interaction of HCII (2000 nM) with heparin (green), the monophasic interaction of HCII with OSCS (red) and the failure

of HCII to interact with CS-A (blue). (G) and (H) Heparin and OSCS solution competition SPR study with ATIII, concentrations of heparin or OSCS: 0 nM (red), 500 nM (blue), 1000 nM (green), 2000 nM (purple), and 5000 nM (azure).

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Table 1
Anticoagulant activity of heparin, OSCS and CS-A in plasma-containing assay systems.^a

μg/mL	APTT (s)	TT (10U Ca++) (s)	PT (s)	Hep test (s)	Anti-FXa %Inhibition	Anti-FIIa % Inhibition
CS-A						
10	29.9	10.9	11.3	35	0.0	0.0
5	29.2	10.3	11.1	32.7	0.0	0.0
2.5	28.2	10.4	10.9	35	0.0	0.0
1.25	28.2	10.8	10.9	33.5	0.0	0.0
0.62	28.2	10.3	10.9	33.7	0.0	0.0
0	25.4	9.0	10.8	32.5	0.0	0.0
OSCS						
10	70.7	61.9	10.2	61.0	0.0	55.8
5	44.7	21.4	10.6	49.2	0.0	32.0
2.5	35.7	13.8	11.3	45.7	0.0	12.9
1.25	31.2	11.4	11.4	42.7	0.0	7.1
0.62	28.7	11.0	10.9	38.2	0.0	5.5
0	25.4	9.0	10.8	32.5	0.0	0.0
Heparin						
10	300	300	16.8	300	95.2	100.0
5	300	300	12.9	247	89.8	90.8
2.5	93.2	300	11.7	136	70.7	85.5
1.25	53.4	76.5	11.4	83.9	47.7	62.9
0.62	34.9	17.6	10.9	66.4	22.1	32.0
0	25.4	9.0	10.8	32.5	0.0	0.0

^a All assays were performed in triplicate with errors of <5%.

Table 2

Anti-Xa and anti-IIa activity of heparin and heparin containing added OSCS in plasma-free amidolytic assay system.^a

Concentration ($\mu\text{g/mL}$)	Heparin alone		Heparin plus OSCS ^b	
	anti-FXa	anti-FIIa	anti-FXa	anti-FIIa
10	66 $\pm$ 4	36 $\pm$ 3	69 $\pm$ 5	56 $\pm$ 4
5	40 $\pm$ 3	11 $\pm$ 2	42 $\pm$ 4	22 $\pm$ 3
2.5	18 $\pm$ 2	3 $\pm$ 2	27 $\pm$ 4	16 $\pm$ 2
1.25	8 $\pm$ 2	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
0.62	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
0.31	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0

^a All results represent a mean $\pm$ SD of three replicates.

^b OSCS was supplemented at 10 $\mu\text{g/mL}$.

Table 3Kinetic data of heparin, OSCS-protein interactions.^a

Protein	GAG	$k_{on}(1/MS)$	$k_{off}(1/S)$	$K_D (M)$
AT III	Heparin (conformational change)	$k_{on1} = 4.5 \times 10^4 (\pm 2.0 \times 10^3) (1/MS)$, $k_{off1} = 0.119 (\pm 5.2 \times 10^{-3}) (1/S)$, $k_{on1} = 4.9 \times 10^{-3} (\pm 1.8 \times 10^{-3}) (1/S)$, $k_{off2} = 2.6 \times 10^{-3} (\pm 1.2 \times 10^{-4}) (1/S)$		
	OSCS	$1.5 \times 10^3 (\pm 20)$	$1.1 \times 10^{-3} (\pm 3 \times 10^{-5})$	7.3×10^{-7}
HC II	Heparin	$834 (\pm 57)$	$1.0 \times 10^{-3} (\pm 4.8 \times 10^{-5})$	1.2×10^{-6}
	OSCS	$76.7 (\pm 2.5)$	$5.1 \times 10^{-6} (\pm 1.5 \times 10^{-6})$	6.6×10^{-8}
Factor XII	Heparin	$3.3 \times 10^4 (\pm 222)$	$1.1 \times 10^{-3} (\pm 1.4 \times 10^{-5})$	3.1×10^{-8}
	OSCS	$3.1 \times 10^4 (\pm 234)$	$9.1 \times 10^{-4} (\pm 1.4 \times 10^{-5})$	2.4×10^{-8}
Factor XIIa	Heparin	N.B. ^b	N.B.	N.B.
	OSCS	$2.4 \times 10^5 (\pm 4 \times 10^3)$	$0.034 (\pm 3.8 \times 10^{-4})$	1.4×10^{-7}
PF4	Heparin	$1.1 \times 10^5 (\pm 1.6 \times 10^3)$	$5.1 \times 10^{-4} (\pm 3 \times 10^{-5})$	4.8×10^{-9}
	OSCS	$1.2 \times 10^5 (\pm 1.5 \times 10^3)$	$3.3 \times 10^{-4} (\pm 6 \times 10^{-5})$	2.8×10^{-9}
HMWK	Heparin	$3.0 \times 10^4 (\pm 193)$	$3.7 \times 10^{-4} (\pm 1.2 \times 10^{-5})$	1.3×10^{-8}
	OSCS	$5.5 \times 10^4 (\pm 529)$	$5.7 \times 10^{-4} (\pm 2.5 \times 10^{-5})$	1.3×10^{-8}
PK	Heparin	$2.9 \times 10^5 (\pm 1.4 \times 10^4)$	$0.19 (\pm 4 \times 10^{-3})$	6.5×10^{-7}
	OSCS	$5.6 \times 10^5 (\pm 3.8 \times 10^4)$	$0.30 (\pm 0.0102)$	5.3×10^{-7}
C1	Heparin	$2.2 \times 10^4 (\pm 909)$	$4.8 \times 10^{-3} (\pm 1.5 \times 10^{-4})$	2.2×10^{-7}
	OSCS	$4.2 \times 10^4 (\pm 1.2 \times 10^3)$	$0.018 (\pm 3.4 \times 10^{-4})$	4.2×10^{-7}
C2	Heparin	$2.5 \times 10^3 (\pm 146)$	$8.4 \times 10^{-4} (\pm 2.7 \times 10^{-5})$	3.3×10^{-7}
	OSCS	$1.3 \times 10^3 (\pm 69)$	$1.0 \times 10^{-3} (\pm 2.9 \times 10^{-5})$	7.6×10^{-7}
C3	Heparin	$1.5 \times 10^4 (\pm 1.3 \times 10^3)$	$2.0 \times 10^{-3} (\pm 6 \times 10^{-5})$	1.3×10^{-7}
	OSCS	$1.5 \times 10^3 (\pm 915)$	$1.8 \times 10^{-4} (\pm 3 \times 10^{-5})$	1.2×10^{-7}
C4	Heparin	$2.8 \times 10^4 (\pm 252)$	$2.7 \times 10^{-4} (\pm 1.3 \times 10^{-5})$	1.0×10^{-8}
	OSCS	$2.9 \times 10^4 (\pm 286)$	$4.5 \times 10^{-4} (\pm 1.3 \times 10^{-5})$	1.5×10^{-8}
C5	Heparin	$3.8 \times 10^4 (\pm 1.1 \times 10^3)$	$2.8 \times 10^{-4} (\pm 1.8 \times 10^{-5})$	7.3×10^{-9}
	OSCS	$2.4 \times 10^4 (\pm 1.6 \times 10^3)$	$2.1 \times 10^{-4} (\pm 2.5 \times 10^{-5})$	8.7×10^{-9}
C6	Heparin	$1.1 \times 10^3 (\pm 249)$	$3.1 \times 10^{-4} (\pm 3 \times 10^{-5})$	7.8×10^{-7}
	OSCS	$84 (\pm 5)$	$4.0 \times 10^{-4} (\pm 2.2 \times 10^{-5})$	4.7×10^{-6}
C7	Heparin	$1.0 \times 10^5 (\pm 894)$	$1.7 \times 10^{-4} (\pm 1.9 \times 10^{-5})$	1.7×10^{-9}

Protein	GAG	$k_{on}(I/MS)$	$k_{off}(I/S)$	$K_D(M)$
	OSCS	$1.1 \times 10^5 (\pm 720)$	$1.5 \times 10^{-4} (\pm 1.5 \times 10^{-5})$	1.4×10^{-9}
C8	Heparin	$5.1 \times 10^4 (\pm 266)$	$2.0 \times 10^{-3} (\pm 1.7 \times 10^{-5})$	3.9×10^{-8}
	OSCS	$7.0 \times 10^4 (\pm 268)$	$1.8 \times 10^{-3} (\pm 1.4 \times 10^{-5})$	2.6×10^{-8}
C9	Heparin	$4.8 \times 10^3 (\pm 62)$	$7.1 \times 10^{-4} (\pm 2.3 \times 10^{-5})$	1.5×10^{-7}
	OSCS	$1.2 \times 10^3 (\pm 84)$	$5.4 \times 10^{-4} (\pm 2.5 \times 10^{-5})$	4.7×10^{-8}

^aData in bold face are significantly different for heparin and OSCS, the data with ($\pm$) in parentheses are the standard errors (SE) from the global fitting.

b. N.B.: negligible binding.