

Influence of Sulfation on Platelet Aggregation and Activation with Differentially Sulfated Hyaluronic Acids

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Abstract. A number of sulfated hyaluronic acid derivatives (HyalS_{2.5}, HyalS₃, and HyalS₄) were prepared by sulfation of the -OH groups present on hyaluronic acid and were generically termed HyalS_x. The anticoagulant properties of this series of compounds has previously been shown to be good in terms of their whole blood clotting inhibition and factor Xa and thrombin inactivation. The purpose of the present study was to investigate whether the use of these compounds would be beneficial to patients who would normally be given heparin, and to perform some preliminary investigations into their effects on platelets. The three compounds were thus studied by investigating their ability to inhibit von Willebrand factor-dependent platelet agglutination in comparison with unfractionated heparin. Agglutination was determined turbidometrically after the addition of ristocetin to stirred formaldehyde-fixed platelets and was demonstrated to be dependent on the presence of sulfate groups on the polysaccharide chain and correlated with the degree of HyalS_x sulfation. Interactions possibly important in low shear environments were investigated by measuring the pharmacological action of the HyalS_x on spontaneous platelet activation and aggregate formation by flow cytometry. The data indicate that platelet activation is not correlated with the number of sulfate or hydroxyl groups on HyalS_x, suggesting that activation occurs not via electrostatic interactions or H bonding, but via some other mechanism. A differentiation between low and high glycosaminoglycan sulfation densities is observed with respect to platelet aggregation, which is correlated with the number of sulfated groups per disaccharide unit. The ability of HyalS_x to inhibit platelet aggregation induced by ADP and thrombin was measured by aggregometry. HyalS₄ resisted thrombin stimulation to a similar extent as heparin. All Hyal derivatives, however, were better at inhibiting ADP-induced aggregation than was heparin. We conclude, therefore, that clinical use of HyalS_x in place of heparin may be beneficial because ristocetin-dependent agglutination, and therefore resistance to platelet aggregation in high shear environments, in addition to resistance to stimulation by ADP, has been shown to be superior to heparin. Spontaneous platelet activation and aggregation are induced at an overall low level, even at high HyalS_x concentrations, and are comparable with that of heparin.

Key Words. sulfated hyaluronic acid derivatives, platelet aggregation, platelet activation, heparin-like

Heparin is the most biologically potent member of the glycosaminoglycans family and is well known for its nonthrombogenic and anticoagulant activity [1]. Indeed, it is extensively used in the management of cardiovascular diseases and contributes to the success of open heart surgery [2]. However, the materials utilized for blood contact in commercially available extracorporeal circuits cannot be used without the administration of a permanent adjuvant anticoagulant therapy such as heparin [3]. Unfortunately, this in turn can lead to long-term medical complications such as hemorrhage [4-6], possibly due to platelet function alteration or thrombocytopenia [7]. The picture is further complicated during cardiopulmonary bypass (CPB), when blood is subjected to high shear rates in the hollow fibers of the blood oxygenator. Many studies have demonstrated that such exposure leads to the formation of platelet aggregates [8,9]. Surgical procedures involving CPB also have an increased risk of adverse cerebral outcome (memory loss, stupor, seizure, or coma) [10-13]. Indeed, the acute changes in neuropsychological function after elective coronary artery bypass surgery are so serious that a recent article in the *New England Journal of Medicine* stated that "new diagnostic and therapeutic strategies must be developed to lessen such injuries" [14]. Further complications related to platelet function alteration after CPB, including gastrointestinal bleeding [8], retinopathy [15], and pulmonary dysfunction syndrome [16], have also been reported.

In order to avoid the complications of heparin therapy and to reduce the risk associated with procedures

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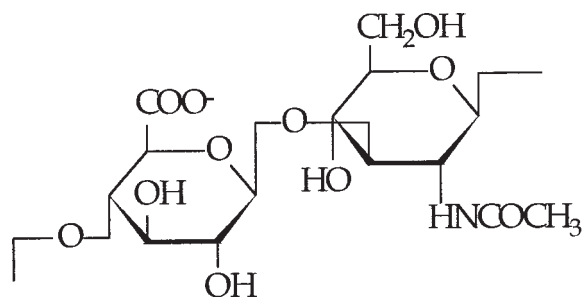
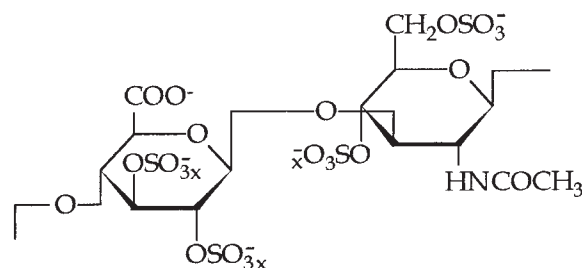


Fig. 1. Chemical structure of hyaluronic acid disaccharide unit.

involving CPB, new anticoagulant molecules were synthesized that could potentially be used as replacement anticoagulants. The linear macromolecule, hyaluronic acid (Hyal; Figure 1), was recently modified by sulfation, obtaining sulfated hyaluronic acid derivatives with different degrees of sulfation (HyalS_x) [17] (Figure 2). We have already demonstrated that the compounds with the highest degrees of sulfation provide good anticoagulant activity [18] in comparison with unfractionated heparin (U.H.). In the present study, we wished to investigate whether these molecules were in fact superior to heparin in their ability to inhibit platelet aggregate formation in simulated conditions of high shear. It was also important to establish if spontaneous platelet activation and aggregate formation in the presence of these compounds were at a physiologically insignificant level and were comparable with that of heparin. A final goal was to define the ability of HyalS_x to resist the stimulation of platelets by the physiological agonists ADP and thrombin in comparison with heparin. Investigation of these interactions would additionally allow assessment of the importance of the degree of sulfation of HyalS_x in platelet reactions.



$0 < x \leq 1$ otherwise -OH groups

Fig. 2. Chemical structure of sulfated hyaluronic acid disaccharide unit. The sulfation involves the C-6 and C-4 positions of glucosamine and the C-2 and C-3 positions of glucuronic acid.

The ristocetin-dependent aggregation method is classically utilized when studying the effects of von Willebrand factor (vWF) on platelet aggregation [19]. This cofactor interacts with vWF in such a way as to mimic its behavior in high shear environments, whereby platelet membrane receptor glycoprotein Ib/IX (GPIb/IX) can interact directly with non-RGD sequences in the vWF molecule [20]. This induces “outside-in” signal transduction in the platelet and a conformational change in the membrane receptor $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa), which allows its binding to RGD sequences in vWF, a precursor to platelet aggregation [21]. vWF also plays a crucial role in platelet hemostasis: at sites of vascular injury it anchors platelets to sections of exposed vascular matrix [22,23].

The static assays, on the other hand, were designed to allow platelets to exhibit interactions with HyalS_x, which may occur in low shear environments. Generation of platelet aggregates under these conditions is unlikely to produce large complexes containing many cells because platelets do not come into such close contact in whole blood as they do in stirred platelet-rich plasma due to steric hindrance by red blood cells. However, the microaggregates thus formed are probably more physiological than the macroprecipitates formed in conventional platelet aggregometry [24]. Various assays have been utilized for studying platelet activation and aggregation in the past, but they have generally been concerned with adherent platelets through either the study of their morphology by scanning electron microscopy [25] or quantification of the presence of platelet activation markers within blood or plasma (such as β -thromboglobulin, 5-hydroxytryptamine, platelet factor 4, or thromboxane B₂) [26–28]. In this study individual cells were analyzed for platelet secretion and aggregate formation by flow cytometry [29,30].

Materials and Methods

Materials

Unfractionated heparin (MW ~10,000) for platelet activation and aggregation was purchased from Roche (Germany). Pharmaceutical grade unfractionated heparin (MW ~14000) for the study of ristocetin-induced agglutination was obtained from Celsus (Cincinnati, OH). Hyal (MW 150,000–200,000) was kindly provided by F.A.B. (Fidia Advanced Biopolymers, Abano Terme, Padova, Italy). HyalS_x with different degrees of sulfation was synthesized as previously described [18]. Anti-CD62P monoclonal antibody, directly conjugated with fluorescein isothiocyanate (FITC), was purchased from Serotec (Oxford, U.K.). Ristocetin was purchased from Bio Data Corp. (Hatboro, PA).

Blood collection

Selected donors were normal, healthy people who had fasted for more than 8 hours and had not received any

medication for at least 14 days. Blood samples were collected into 3.8% (w/v) tri-sodium citrate as anticoagulant at a volume ratio of 9 parts blood to 1 part citrate. Platelet-rich plasma (PRP) was generated by spinning whole blood at 150 g for 15 minutes at room temperature. Formaldehyde-fixed platelets (FP) were prepared by incubation of PRP with an equal volume of 2% buffered formaldehyde for 1 hour at 37°C; the platelets were washed twice in Tris-buffered saline (TBS: 0.05 M Tris, 0.1 M NaCl, pH 7.4) and were resuspended in the same buffer at a concentration of 400,000/ μ L. For aggregometry the FP were diluted 1:1 with normal pooled plasma. Pooled citrated plasma was prepared from 15–20 normal drug-free volunteers and stored in aliquots at -70°C .

Platelet aggregation induced by ristocetin

Incubation of platelets with samples. The response of a platelet-rich plasma to stirring in the presence of ristocetin is normally termed *aggregation*, but because formaldehyde-fixed platelets were tested, the term *agglutination* will be used. The ability of HyalS_{2.5}, HyalS₃, and HyalS₄ to inhibit the agglutination of FP in plasma in response to ristocetin was determined. Having demonstrated that the inhibitory effects of heparin were the same using both fresh and fixed platelets in plasma (data not shown), formaldehyde-fixed platelets were prepared in batches for consistency and were used as previously described [31]. Fixed platelets were suspended in 0.15 M Tris-buffered saline at a final concentration of 200,000/ μ L. For each batch of platelets a standard amount of normal pooled, citrated plasma (the source of vWF) was chosen, based on titrations to the lowest concentration needed to achieve maximal agglutination to ristocetin. Platelets were preincubated with plasma and with different concentrations of the different compounds for 10 minutes at 37°C prior to testing for agglutination with ristocetin.

Inhibition of ristocetin-induced agglutination.

Agglutination in response to ristocetin (1 mg/mL final concentration) was measured with a Scienco aggregometer (Morrison, CO) in standardized aggregation units. The inhibition was expressed as a reduction in the percent of the maximal agglutination in response to ristocetin alone.

Platelet activation and aggregation

Incubation of platelets with samples. The different compounds were reconstituted to a concentration of 20 mg/mL with a filtered (to 0.2 μ m), buffered, and physiological saline solution (FACSFlow, Becton Dickinson, Oxford, UK). A range of HyalS_x and U.H. concentrations (0.5–10 mg/mL) was formulated by adding an appropriate volume of sample solution to 500 μ L

PRP, plus an additional volume of FACSFlow to bring the total volume to 1 mL. The samples were incubated with PRP for 1 hour at 37°C without shaking. After this time the platelets were fixed in phosphate-buffered paraformaldehyde at a final concentration of 1.0% (w/v) for 15 minutes at room temperature.

Analysis of platelet activation. P-selectin, a granule membrane protein expressed on the surface of platelets during α -granule secretion, was used as a measure of platelet activation induced by the polysaccharide samples, a parameter now firmly established in diagnostic and research practice [30,32,33]. The rise in fluorescence over resting platelets due to increased anti-CD62-FITC binding was compared for the different compounds. Then 10 μ L of fixed PRP was incubated with 2 μ L of anti-CD62P for 20 minutes at 4°C. Fluorescence was measured by flow cytometry (Becton Dickinson FACSort, San Jose, CA). Cellular debris and electronic noise were removed from acquisition by setting a threshold on forward light scatter. Platelets were gated on a plot of forward (a measure of volume) versus 90° light scatter (a measure of granularity). Specific (immuno) identification of platelets was not necessary because no other cell types were present in the plasma. A total of 30,000 platelets were acquired per sample. The flow cytometer was calibrated on a daily basis using fluorescent beads of uniform diameter and fluorescence (CaliBRITE beads, Becton Dickinson, Oxford, UK) and a negative control antibody of specificity irrelevant to platelets and of the same isotype and F:P ratio as the test antibody.

Analysis of platelet aggregation. The degree of platelet aggregation was assessed by measuring the distributions of forward and 90° light scatter of platelet populations by flow cytometry, as described previously [30]. The distributions of resting platelets were taken as a baseline and termed the *zero percent aggregated population*. After incubation with test samples, any change in aggregation was observed as an increase in both forward and 90° light scatter for each particle (cell or aggregate of cells) passing through the flow cytometer. The percentage aggregation was determined by measuring the number of particles that were greater in either forward or 90° light scatter than the limits of the control population.

Inhibition of agonist-induced platelet aggregation

A range of HyalS_x concentrations (0.25–10 mg/mL) in PRP was prepared as described earlier in *Platelet Activation and Aggregation*. Prior to analysis, samples were incubated at 37°C for 10 minutes without stirring. To test for HyalS_x resistance to agonist-induced platelet stimulation, the physiological agonists ADP and thrombin were added at final concentrations of 3.9 μ M and 0.17 U/mL, respectively, to the mixtures of PRP

and HyalS_x while stirring at 37°C. A control sample showing the response of non-inhibited platelet aggregation was obtained by preparing a 1:1 mixture of PRP and PBS. Differences in the inhibition of thrombin activation were measured by the minimum glycosaminoglycan concentration needed to effect a shape change in the platelet suspensions. The effect on ADP-induced aggregation of inhibitor incubation was measured by calculating the maximum rate of aggregation.

Results

Platelet inhibition of ristocetin-induced agglutination

Figure 3 shows the relative inhibitory effects of HyalS_x compared with U.H. Unsulfated Hyal has negligible inhibitory activity compared with heparin. HyalS_{2.5} completely inhibits platelet agglutination at 0.1 mg/mL, and both HyalS₃ and HyalS₄ induce the same effect at a lower concentration (0.01 mg/mL). It appears evident that the ability of HyalS_x to inhibit ristocetin-induced platelet agglutination depends on the presence of sulfate groups on the polysaccharide chain and roughly increases with increasing degree of sulfation. Moreover, the agglutination decreases with increasing HyalS_x concentration. The inhibitory response of all the HyalS_x compounds is more intense than heparin, such that more than one order of magnitude greater heparin concentration is required to effect the same inhibition response.

Platelet aggregation and activation

The trend of platelet aggregation induced by U.H. Hyal, HyalS_{2.5}, HyalS₃, and HyalS₄ is shown in Figure 4. We can observe from the graph that for Hyal, the percentage of aggregation increases with increasing concentration. HyalS_{2.5} and HyalS₃ did not induce aggregation at a concentration of 2.5 mg/mL or less.

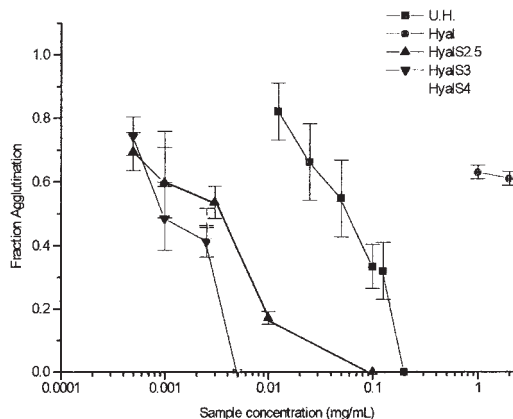


Fig. 3. In vitro inhibition of ristocetin-induced vWF-dependent platelet agglutination by unfractionated heparin, Hyal, HyalS_{2.5}, HyalS₃, and HyalS₄.

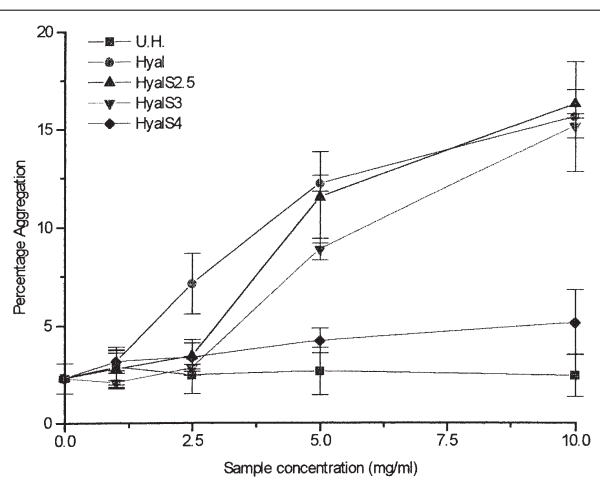


Fig. 4. Platelet aggregation as measured by flow cytometry induced statically by different concentrations of U.H. Hyal, HyalS_{2.5}, HyalS₃, and HyalS₄ ($n = 5$; results expressed as mean \pm SD).

Above this, platelet aggregation increased with increasing HyalS_x concentration up to the same level as that observed with Hyal, at a concentration of 10 mg/mL. U.H. and HyalS₄ did not show platelet aggregation significantly higher than the baseline value, even at the highest concentration analyzed (10 mg/mL).

Platelet activation induced by incubation with different concentrations of U.H. Hyal, HyalS_{2.5}, HyalS₃, and HyalS₄ are shown in Figure 5. There were significant differences between the different HyalS_x, especially at the highest concentration. Hyal induced a similar degree of platelet activation to HyalS₄, with

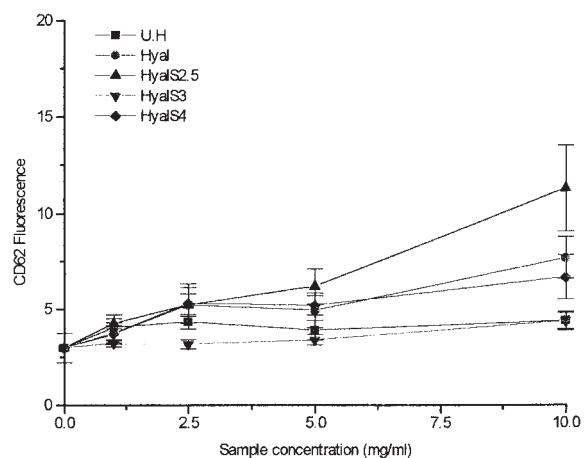


Fig. 5. Platelet activation as measured by flow cytometry induced statically by different concentrations of Hyal, U.H. HyalS_{2.5}, HyalS₃, and HyalS₄ ($n = 5$; results expressed as mean \pm SD).

Table 1. Effect of thrombin (0.17 U/mL final concentration) on platelets incubated with different concentrations of U.H., HyalS_{2.5}, and HyalS₄ as observed by aggregometry

Concentration (mg/mL)	Heparin	HyalS _{2.5}	HyalS ₄
0.01	(✓)	✓	(✓)
0.05	×	✓	×
0.10	×	✓	×
0.25	×	✓	×
0.5	×	(✓)	×
1	×	×	×
2.5	×	×	×
5	×	×	×
10	×	×	×

× indicates that no aggregation was observed;

(✓) indicates that a very slight shape change was observed, with no aggregation;

✓ indicates that a shape change was observed, no aggregation.

HyalS_{2.5} inducing the greatest response. HyalS₃ produced secretion, which was almost indistinguishable from the control at all concentrations tested. This was also true for U.H. at the higher concentrations. The values of CD62P expression for even the most stimulating HyalS_x were very much lower than induced by physiological agonists. The comparative figures for ADP (in PRP) and thrombin (in platelets washed in Krebs-Ringer solution) are 24 and 6.3, measured on the same arbitrary scale as the data in Figure 5.

Inhibition of agonist-induced platelet aggregation

The effects of different concentrations of U.H., HyalS_{2.5}, and HyalS₄ on the inhibition of thrombin-induced platelet aggregation are shown in Table 1. Even at the smallest concentrations tested, all of the samples prevented aggregation and clotting. The responses of HyalS₄ and U.H. were very similar, both requiring only 10 µg/mL final concentration to resist aggregation and all but a very slight shape change. Shape change was observed with HyalS_{2.5} at concentrations less than 500 µg/mL.

U.H. reduced the maximum rate of aggregation induced by ADP from a baseline figure of 3.5 cm/20 s to a minimum of 2.3 cm/20 s (Figure 6). Final concentrations of U.H. less than 1 mg/mL produced aggregation curves that were effectively indistinguishable from the control sample. All the Hyal derivatives tested (Hyal, HyalS_{2.5}, and HyalS₄) resisted aggregation better than U.H. Indeed, at the lowest concentration tested (0.25 mg/mL) all three compounds were as effective as the most efficacious heparin concentration (5 mg/mL), and were much more effective at higher concentrations. Interestingly, there was little difference between any of the Hyal derivatives in their ability to inhibit ADP-induced platelet aggregation.

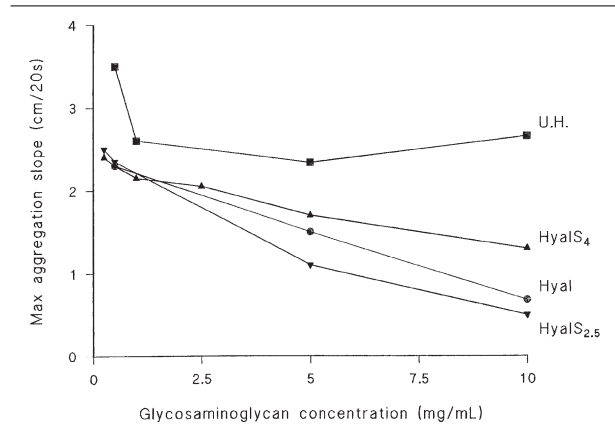


Fig. 6. Inhibition of platelet aggregation by varying concentrations of unfractionated heparin, Hyal, HyalS_{2.5}, and HyalS₄ induced by 3.9 µM ADP. Graph shows the maximum slope of the aggregation curve in cm/20 s. PRP incubated as a 1:1 ratio with PBS gave a figure of 3.5 cm/20 s.

Discussion

Exposure of blood to high levels of shear stress results in platelet aggregation [8,9] and ultimately platelet loss, and is implicated in a number of serious clinical outcomes [8,10–13,15,16]. A reduction in shear stress is achieved in CPB by lowering the temperature of the patient and thus the required rate of gas exchange, but these clinical problems still persist [14]. Our results suggest that whereas heparin inhibits ristocetin-induced vWF-dependent platelet agglutination, HyalS_x has a greater ability to inhibit platelet agglutination under these conditions.

The mechanism of HyalS_x action on vWF-dependent platelet agglutination could be affected in a number of ways. Firstly, it could interact with the heparin-binding domains on the platelet surface at or near vWF-specific sites and block the binding of vWF to platelet receptor GPIIb, the first step in platelet aggregation. Alternatively, the polysaccharides could bind directly to the vWF molecule and inhibit receptor–ligand interaction by steric hindrance or conformational disturbance.

We have recently established that heparin almost certainly does not affect inhibition of vWF-dependent aggregation by binding to platelets, but rather by direct binding of the vWF molecule [31,34]. Thus, a specific interaction between HyalS_x and vWF may similarly occur by interaction of the sulfate groups distributed along the polysaccharide chain and specific protein domains containing cationic residues in vWF. Sulfation is certainly an extremely important criterion in this inhibition, as witnessed by the inability of Hyal to potentiate agglutination inhibition. However, at low HyalS_x concentrations there is little difference in the concentrations required to produce the same degree of

inhibition between any of the polysaccharides in this series.

These results suggest that inhibition of platelet agglutination might accrue from contributions of more than one mechanism. Inhibition to below 50% agglutination by HyalS_{2.5} requires a vast increase in glycosaminoglycan concentration, whereas inhibition by HyalS₃ and HyalS₄ appears to be almost dose dependent. Nevertheless, three $-\text{SO}_3^-$ groups per disaccharide unit appear sufficient to completely saturate all of the interacting groups when one considers that the presence of four $-\text{SO}_3^-$ groups does not alter the inhibition profile.

In previous work we demonstrated that the inhibitory effects of U.H. (pharmaceutical grade) toward platelet agglutination are greater than those of low molecular weight heparin and that the inhibitory potency is related to polysaccharide chain length [31]. The greater effectiveness of HyalS_x in inhibiting vWF-mediated platelet aggregation compared with U.H. may also be due to their larger molecular weights and chain lengths.

Spontaneous aggregation is probably promoted by the simultaneous electrostatic interaction of two platelets and a HyalS_x molecule. It is well known that heparin can induce aggregation via this mechanism, whereby negative charges on the platelet membranes interact with positive residues on the macromolecule. The same scheme can be proposed for HyalS_x. If one considers the chemical structure of HyalS_x it can be seen that Hyal presents $-\text{COO}^-$ groups as negative charges, which would be expected to electrostatically repulse the negatively charged platelet membrane. However, there are enough other groups that can directly interact with the cellular membrane to reverse this trend.

The ability of the HyalS_x to cause aggregation is, therefore, an equilibrium between negative and positive charges. The response of HyalS_x is therefore consistent with this thesis, whereby increasing numbers of $-\text{SO}_3^-$ moieties introduced into the polysaccharide chain in place of $-\text{OH}$ groups would reduce overall electrostatic attraction by inducing ever stronger repulsion: Hyal induced the greatest spontaneous platelet aggregation, with HyalS₄ inducing a response over a large range of concentrations that was almost indistinguishable from the control. By analyzing the structures of the systematic series of synthesized macromolecules, we observe that the $-\text{OH}$ groups may be responsible for the interaction between HyalS_x and platelet membranes such that they are able to induce platelet aggregation above a particular concentration. The absence of $-\text{OH}$ groups, such as in HyalS₄, excludes any mechanism of aggregation, and the presence of four sulfate groups per disaccharide unit considerably increases the HyalS₄-platelet electrostatic repulsion.

The spontaneous platelet activation results suggest that α -granule release is not mediated by the same electrostatic mechanism. One can see that HyalS_{2.5} in-

duces a secretory response that is greater than any of the other molecules in the HyalS_x series. If electrostatic interaction were solely responsible for this response, then one would expect Hyal to induce the largest response and HyalS₄ the smallest. However, whilst electrostatic considerations will control how bulk quantities of the macromolecule will interact with the platelet surface, this denies the possibility of *specific* interactions between the HyalS_x and membrane receptors. Platelets are extremely versatile cells, with large numbers of a wide variety of receptors that can be responsible for both "inside-out" and "outside-in" signal transduction. In our model, Brownian motion would allow a degree of contact between platelets and HyalS_x molecules such that specific charge orientation within the receptor could promote strong ligand binding, even if the overall bulk effect was electrostatic repulsion.

From the results we can assume that the charge density and orientation within HyalS_{2.5} is appropriately arranged to allow strong ligand-receptor interaction, promoting a significant secretory signal within platelets. A smaller or larger density of negative charges within the HyalS_x repeating unit appears to reduce receptor binding. The specificity of receptor-ligand interaction is evidenced by the large difference in secretory response between HyalS_{2.5} and HyalS₃. It still remains for the exact mechanism of interaction to be elucidated and the signal transduction pathway ascertained, but it is clear that HyalS₃ and HyalS₄ induce a negligible secretory response.

Conclusions

This study of interaction towards platelets was performed with a homogeneous series of compounds that are different only in the number of sulfate groups that have substituted $-\text{OH}$ groups. Considering that the chain length is the same for all the polysaccharides studied, the influence of the negative charge density, and consequently the number of $-\text{OH}$ groups, can be assessed.

Platelet activation is not controlled in a dose-dependent manner, but probably by the exact charge density and orientation, which implies a specific receptor-ligand interaction, rather than via electrostatic interactions or H bonding. Differentiation between low and high sulfation densities is observed with respect to platelet aggregation and platelet agglutination mediated by vWF, demonstrating that both these phenomena are influenced by the effects of electrostatic interaction, mediated by hydrogen bonds via $-\text{OH}$ groups.

Low molecular weight fractions of heparin have previously been shown to be less effective than unfractionated heparin at inhibiting agglutination of platelets, but this study demonstrates that HyalS₃ and HyalS₄ are between one and two orders of magnitude better at inhibiting this response than even unfractionated heparin. Because their anticoagulant properties are

adequate and resistance by these two HyalS_x to platelet aggregation by other physiological agonists, such as ADP, is also superior to heparin, we conclude that the postoperative neurological and hemorrhagic complications currently experienced with procedures involving CPB may be significantly reduced if anticoagulation therapy were to be performed using HyalS₃ or HyalS₅.

Acknowledgments

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