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The influence of various structural parameters of semisynthetic sulfated polysaccharides on the P-selectin inhibitory capacity

Juliane Fritzsche[a], *Susanne Alban*[b], *Ralf J. Ludwig*[c], *Simone Rubant*[c], *Wolf-Henning Boehncke*[c], *Gabriele Schumacher*[a], *Gerd Bendas*[a,*]

[a] *Institute of Pharmaceutical Chemistry, Rheinische Friedrich Wilhelms University Bonn, Germany*
[b] *Pharmaceutical Institute, Christian Albrechts University Kiel, Germany*
[c] *Department of Dermatology, Johann Wolfgang Goethe University Frankfurt, Germany*

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Abbreviations:
BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein-acetoxymethyl ester
CurS, curdlan sulfate
DP, degree of polymerisation
DS, degree of sulfation
ESI-MS, electrospray ionisation mass spectrometry
LMWH, low molecular weight heparin
mAb, monoclonal antibody

ABSTRACT

Selectin-mediated leukocyte rolling along the endothelium is of key importance for maintaining the cellular immune response. The anti-inflammatory activities of heparin have partly been related to inhibition of P-selectin binding. Heparin, however, suffers from its heterogeneous variable structure, the animal origin and multiple *in vivo* effects. As P-selectin is a promising target for anti-inflammatory approaches, we focused on P-selectin inhibition by other sulfated polysaccharides and compared them with six heparins. We examined 15 structurally defined semisynthetic sulfated glucans, non-animal-derived from the linear glucans phycarin, curdlan or pullulan. The derivatives gradually differ in their degree of sulfation, molecular weight, and glycosidic linkage. The inhibitory capacity was analysed in a parallel plate flow chamber, detecting the rolling of U937 cells on P-selectin layers.

Unfractionated heparins displayed variabilities between different preparations. Considering fractionated heparins, exceeding of a minimal mass is essential for activity. Comparing the glucan sulfates, charge density is the most important parameter for P-selectin binding. Highly sulfated derivatives are excellent inhibitors, the reduced cell binding up to $16.2 \pm 6.4\%$ strongly exceeded the heparin activities. Molecular weight is of minor effects, while glycosidic backbone linkage holds certain importance.

To check the P-selectin inhibition *in vivo*, heparin and one phycarin sulfate were tested using intravital microscopy of microvasculature in mice. Both compounds significantly reduced the rolling fractions of activated platelets on endothelium as effective as a blocking P-selectin antibody.

Our study indicates that semisynthetic glucan sulfates with optimal structures block P-selectin excellently and might become promising candidates for anti-inflammatory drugs to replace heparin for certain applications.

* *Corresponding author at*: Department of Pharmacy, Institute of Pharmaceutical Chemistry, Rheinische Friedrich Wilhelms University Bonn, An der Immenburg 4, 53121 Bonn, Germany. Tel.: +49 228 735250; fax: +49 228 734692.
E-mail address: gbendas@uni-bonn.de (G. Bendas).

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MMWH, medium molecular weight heparin
MW, molecular weight
PhyS, phycarin sulfate
PGE_1, prostaglandin E_1
PSGL-1, P-selectin glycoprotein ligand-1
PulS, pullulan sulfate
RT, room temperature
sLex, sialyl $Lewis^X$
TRAP, thrombin receptor agonist peptide
U937, human monocytic cell line
UFH, unfractionated heparin
VLMWH, very low molecular weight heparin

1. Introduction

The recruitment of leukocytes from the blood stream into tissues is a highly orchestrated process, which proceeds in postcapillary venules of most organs in a cascade-like fashion [1]. It is initiated by capturing of flowing leukocytes (tethering), which then roll along the endothelial surface as a functional prerequisite for the subsequent firm adhesion and transmigration. Tethering and rolling is mediated by selectins, a family of three carbohydrate-binding receptors on both endothelium and leukocytes [2]. They are transmembrane glycoproteins which share a highly conserved N-terminal lectin domain [3]. According to their different kinetics of expression, the three selectins have various, although overlapping functions in the inflammatory reaction. P-selectin is rapidly mobilised to the surface of endothelial cells and platelets upon a variety of stimuli and is thus important for the early phase of leukocyte extravasation [4].

However, dysregulation, i.e. responding to autogenous or non-threatening factors, may lead to an uncontrolled excessive infiltration of leukocytes into healthy tissue. Numerous model experiments using selectin-deficient animals have clearly proven that selectins are implicated in the development of pathological inflammations such as rheumatoid arthritis, asthma, inflammatory bowel disease and psoriasis [5,6]. Furthermore, selectins are important in atherosclerosis and cancer metastasis [7,8]. Consequently, the blocking of selectins has attracted much attention during the last decade as a promising strategy for therapeutic interventions.

Since the minimal binding structure recognised by all selectins, the tetrasaccharide sialyl $Lewis^X$ (sLex) [9] showed to display certain anti-inflammatory activity [10,11], sLex was regarded as lead structure for extensive drug research [12]. However, the very low binding affinities of sLex (K_d 0.1–5.0 mM) [13,14] could only be slightly improved by structural modifications. Presently, only few compounds are investigated in advanced preclinical or clinical trials [15].

Insight into the molecular binding characteristics was gained by analysing the crystal structure of P-selectin complexed with its natural ligand PSGL-1 [16]. In addition to a certain carbohydrate pattern, sulfated tyrosines revealed to dominantly contribute to the binding. The importance of electrostatic interactions for P- and L-selectin binding is in line with former results by Skinner et al. [17,18], showing that sulfated polysaccharides such as fucoidan, dextran sulfate or heparin bind to P-selectin.

Heparin belongs to the group of vertebrate glycosaminoglycans and is a complex, highly sulfated polysaccharide mixture with a molecular weight (MW) between 3000 and 30,000. Heparin has been used as antithrombotic drug for more than 65 years, but it also displays many other biological activities, amongst others anti-inflammatory effects [19,20]. The anti-inflammatory efficacy of heparin-derivatives are currently investigated in clinical trials [21]. Both the anti-inflammatory and anti-metastatic effects of heparin are assumed to be at least partly due to its P-selectin blocking capacity [7,22]. Initial studies indicated that heparins can act as ligands for P- and L-selectin and thus interfere with sLex-related structures [17,18,23,24].

A detailed understanding of the molecular mechanisms of heparin–selectin interactions is an important prerequisite for therapeutic approaches. As heparin exhibits strong antithrombin-mediated anticoagulant activities, the risk of bleeding may limit its therapeutic use in inflammation or cancer treatment. A major challenge is therefore to find heparin-like structures binding to selectins, but exhibiting less anticoagulant activity. Meanwhile, several studies show that the antithrombin- and selectin-binding properties of heparins can be controlled by structural modifications [24]. Xie et al. and Gao et al. used carboxy-reduced heparins and demonstrated a loss in anticoagulant activity while retaining P-selectin binding due to sulfation [25,26]. Wang et al. and Wei et al. focused on the influence of the sulfation pattern of heparin on its P-selectin binding. They proved 6-*O* sulfation of heparin to be essential, while 2,3-*O* desulfation retains selectin binding activity but strongly reduce anticoagulation [27,28]. But the fact that the natural heparin represents a highly variable complex mixture might complicate the specification of structure activity relationships.

The heparin findings focus a strong interest on other sulfated polysaccharides as potential selectin inhibitors. We recently compared the influence of heparin on selectin-

mediated cell rolling with that of five well-defined sulfated polysaccharides [29]. These semisynthetic glucan sulfates displayed considerably stronger inhibitory activity than heparin, but the compounds did not allow for comprehensive structural discussions.

To derive structure–activity relationships regarding the inhibitory potency on P-selectin-mediated cell rolling, we performed the present study including three series of defined glucan sulfates that display strongly reduced anticoagulant activity [30] and compared these to different heparins. The variety of polysaccharides allows for the first time to clearly examine the dependency of P-selectin binding on the MW, the degree of sulfation (DS) and the carbohydrate backbone. Referring to static cell binding studies, where we found similar binding behaviour to P- and L-selectin (Alban et al., in preparation), we choose here the investigation of the P-selectin-dependent cell rolling in a dynamic flow chamber system. To check the validity of the *in vitro* results, we compared the influence of a selected glucan sulfate with that of heparin and a P-selectin blocking antibody on the P-selectin-dependent rolling of activated platelets along endothelial surface in murine skin vasculature using intravital microscopy.

2. Materials and methods

2.1. Materials

Recombinant human P-selectin-Fc chimera (P-sel-Fc) was purchased from R&D Systems (Wiesbaden, Germany), anti-human P-selectin (clone AK-4), anti-PSGL-1 (clone KPL-1) antibodies were from BD Biosciences (Heidelberg, Germany). Anti-mouse IgG_1 (FITC-labeled), BSA and cyanuric chloride were from Sigma (Deisenhofen, Germany).

2.2. Preparation of the endothelial model surface

For immobilising P-selectin to simulate the endothelial surface, microscope slides (glass, diameter of 18 mm, thickness of 0.2 mm) were used as transparent supports. To achieve a highly homogeneous surface the slides were treated as follows: after incubation in conc. H_2SO_4/H_2O_2-mixture (7/3, v/v) for 30 min at 80 °C under ultrasonic conditions they were rinsed with ultra-pure water for 30 min at RT. To increase the density of silanole groups on the surface, a cleaning procedure with $NH_3/H_2O_2/H_2O$ (1/1/5, v/v/v) was performed, followed by a final rinse with ultra-pure water and drying of the slides.

For the covalent immobilisation of selectins, cyanuric chloride was used as cross-linker between glass and proteins. In a first step, a solution of cyanuric chloride in chloroform reacted with the glass under ultrasonication. After drying, the slides were incubated with a mixture of 3.75 μg/mL P-sel-Fc and 0.5% (m/v) BSA in borate buffer (pH 8.8) for 2 h at RT. This mixture is sensitively balanced to achieve a cell rolling and avoid unspecific firm adhesion. Immediately before the rolling experiment, the slides were thoroughly rinsed with ultra-pure water. Routinely, the slides were analysed by immune fluorescence investigations.

2.3. Sulfated polysaccharides

Low molecular weight fucoidan (average MW 8.2 kDa, Kraeber, Ellerbek, Germany) was extracted from brown seaweed (mainly *Ascophyllum nodosum*).

Unfractionated heparin (UFH 1) and the fractionated ones were kind gifts from Novartis (Nürnberg, Germany): UFH 1 (from porcine intestinal mucosa, average MW 15 kDa), MMWH (medium molecular weight heparin), average MW 10.5 kDa with a narrow molecular weight range from 9.5 to 11.5 kDa, LMWH (low molecular weight heparin/certoparin), average MW 6.0 kDa (4.2–6.2 kDa) and VLMWH (very low molecular weight heparin mainly consisting of decasaccharides, average MW 3.4 kDa). The three heparins with lower MW were produced from the same UFH 1 by isoamylnitrite degradation. MMWH and VLMWH were additionally fractioned by gel permeation chromatography.

Two further, commercially available UFHs were used as comparison to UFH 1. Liquemin® N (Hoffman-La-Roche AG, Grenzach-Wyhlen, Germany; 5000 IU/500 μL) and Heparin ratiopharm® (Ratiopharm GmbH, Ulm, Germany; 5000 IU/200 μL) were compared to UFH 1 on mass basis according to the relation 1 mg ≅ 150 IU.

Semisynthetic linear glucan sulfates were produced by sulfation of the natural polysaccharides phycarin (PhyS) (Goemar Laboratories, St. Malo, France) a β-1,3-glucan (degree of polymerisation (DP) 23–25), curdlan (CurS), a β-1,3-glucan with high MW (Wako Pure Chemical Industries, Osaka, Japan), and thermally degraded and fractionated pullulan (PulS), an α-1,4/1,6-glucan (Wacker Chemie, München, Germany) with SO_3/pyridine in dimethylformamide. To consider the different molecular weights, the basic sulfation procedure was modified regarding the reaction time and temperature and the concentration of the sulfation reagent [30,31].

As previously described [31], the degree of sulfation (DS, sulfate groups per glucose unit) of the test compounds was determined by ion chromatography on a HPLC system and their molecular weight (MW_{HD}), i.e. their hydrodynamic volume, by gel permeation chromatography using neutral pullulans of defined MW as standards. Both the PhyS and the PulS have an average MW_{HD} of about 18–19 kDa, which corresponds to an average MW of 10 kDa (as confirmed by ESI-MS). The CurS are only characterised by their MW_{HD}, which is correspondingly higher than their real average MW. The sulfation pattern of the glucan sulfates was established by methylation–ethylation analysis with subsequent gas–liquid chromatography–mass spectrometry of the resulting partially methylated, ethylated alditol acetates [32].

2.4. Cell cultivation

U937 cells, a human monocytic cell line expressing the P-selectin ligand PSGL-1, were cultivated at 37 °C and 5% CO_2 in RPMI 1640 medium containing 10% fetal calf serum and 1% penicillin–streptomycin solution (Sigma, Deisenhofen, Germany). PSGL-1 expression was routinely analysed by FACS. The cells were used for the rolling experiments within 4 h after separation, centrifugation and resuspension in serum free medium.

2.5. Laminar flow experiments

The parallel plate flow chamber used in these studies has been described in detail in our previous investigations [29,33]. The flow apparatus was mounted onto an inverted fluorescence microscope Axiovert 200 (Carl Zeiss, Germany). PBS (pH 7.4) containing 1 mM $CaCl_2$ and $MgCl_2$ was used as flow medium at a shear rate of about 200 s^{-1}, driven by either hydrostatic pressure or a pump system, respectively. For the flow experiments, 1×10^6 U937 cells in 100 μL pure medium (control experiment) or containing the indicated amount of test compound were injected into the streaming medium, thereby dynamically diluted and analysed immediately. As even polysaccharides with low polydispersity do not have an exactly defined MW, the effects of the test compounds were not compared on a molar, but on weight basis. A mass was chosen which corresponds to 5, 50 or 500 μg/mL, respectively, after dynamic dilution in the flow chamber.

Alternatively, the flow was stopped for 5 min to allow interaction of the cells with the supported membrane. After this period shear force was applied and the adhesion behaviour of the cells was monitored for 10 s capturing 25 frames/s with a CCD-camera (CSC 795) using a long distance objective of 20× magnification. The video sequences were saved and analysed by application of a specific software (Imagoquant Multitrack-AVI-2, Mediquant, Halle, Germany) resulting in detailed and automated analysis of the experimental data. All cells (about 150) per image were analysed for the evaluations. To quantify a reduction in cell adhesion, cell number at each second was counted (from $t = 0$ to 4 s) and divided by the number on the start image ($t = 0$ s). The results were expressed as percentage adhesion and related to the control experiment.

Rolling velocity is defined as the distance covered by a cell during a certain time interval and determined by analysing the video sequences with the software. The program is tracking the movement of each single cell over a variable period of time and calculates the velocity. Cells rolling slower than 5 μm/s were not evaluated in order to exclude artefacts due to deformation of strongly adhered cells in the flow.

2.6. Intravital microscopy

Platelet isolation and intravital microscopy were performed as previously described [34]. Platelets were isolated from blood, drawn from healthy volunteers through a 21 gauge needle after informed consent had been obtained. Blood was collected in 3 mL tubes containing sodium-citrate (Sarstedt, Nümbrecht, Germany) and immediately centrifuged at 110 × g for 15 min at 24 °C to gain platelet-rich plasma. Platelets were separated from plasma proteins by gel filtration through a Sepharose 2B (Pharmacia, Uppsala, Sweden) column. Platelet isolation did not change P-selectin expression as determined by FACS. After isolation, platelets were stained using BCECF-AM (0.2 mM, 30 min at RT, Molecular Probes, Eugene, USA). Platelets were separated from excess dye by gel filtration as mentioned above. Thrombin receptor agonist peptide (TRAP) was used for platelet activation (2.5 mM, for 5 min), which leads to an over 30-fold increase in platelet P-selectin expression as confirmed by flow cytometry. Before use for intravital microscopy, the platelet suspension (1×10^8 mL^{-1}) was washed and resuspended in PBS containing Ca^{2+} and Mg^{2+}. To prevent platelet aggregation, PGE_1 (Sigma, Deisenhofen, Germany) was added before centrifugation.

For intravital microscopy, mice were anesthetised with an intraperitoneal injection of Ketamin® (Schwabe-Curamed, Karlsruhe, Germany) and Rompun® (Bayer, Leverkusen, Germany) and placed on a homothermic blanket. The right carotid artery was prepared microsurgically and a catheter was inserted for injection of cells. The left ear of the mouse was gently placed on a microscope slide, covered with glycerin and a coverslip. Vascular architecture and labelled cells were visualised during their passage through vessels under fluorescent epi-illumination using a multiband-filter system (XF 53, Omega Optical, Brattleboro, VT). The microcirculation was continuously recorded by video recordings from a 1/3″ DSP 3-CCD camera (DXC-390, Sony, Köln, Germany) mounted on a modified Zeiss microscope (Axiotech Vario 100 HD, Zeiss, Germany) equipped with a 10× saltwater immersion objective (Nikon, Düsseldorf, Germany). Images were digitally stored using Media Studio Pro 7.0 (Ulead, Kaarst, Germany). Subsequently, platelets or platelets with Liquemin® N or PhyS-4, respectively (25 mg/kg body weight) or P-selectin antibody (2 mg/kg) were injected. Cell behaviour in individual vessel segments was determined where cells were considered non-interacting when they moved at the velocity of the mean blood flow, whereas detectable lower velocities were defined as rolling.

2.7. Statistics

Data are represented as means of at least three identical experiments ± standard deviation (S.D.). Statistical comparisons were performed with the unpaired Student's t-test.

3. Results

3.1. Inhibition of cell binding by heparins

The dynamic inhibition experiments were performed in a parallel plate flow chamber by microscopically analysing the rolling of PSGL-1-carrying U937 cells along a surface of immobilised P-selectin-Fc chimera. The selectin–BSA-mixture was optimised to result in a cell rolling. The velocity of 8–15 μm/s corresponds to the physiological rolling of leukocytes. Rolling experiments without inhibitors representing the control values (100% adhesion) were always performed in parallel. Inhibitory effects on cell rolling were analysed either directly after injection of the agents, or cells were allowed to incubate for a short time with the selectins in presence of the inhibitors before applying shear force again. In principle, both methods provided comparable results, but the latter procedure records a higher cell number and detects cell detachment. It was therefore chosen for the presented studies.

In order to investigate to which extent heparin inhibits P-selectin cell binding in this assay, and to establish the dependency on the chain length, four heparins (UFH 1, MMWH, LMWH and VLMWH) with different MW were examined (Table 1). Since all the three degraded heparins were produced by gradual isoamylnitrite degradation of the

Table 1 – Structural characteristics of the heparins used in this study

Heparins		
Substance	Degree of sulfation (DS)	Molecular weight (MW) (kDa)
Unfractionated heparin (UFH 1)	1.20	15.0
Medium molecular weight heparin (MMWH)	1.20	10.5
Low molecular weight heparin (LMWH)	1.20	6.0
Very low molecular weight heparin (VLMWH)	1.20	3.4

The three fractionated heparins were produced from the same UFH 1 by isoamylnitrite degradation. MMWH and VLMWH were additionally fractionated resulting in a low polydispersity. Since the determined DS was identical for the four heparins, they differ exclusively in their molecular weight.

same UFH 1, they mostly match in all other structural parameters, such as end groups. The determined DS after fractionation was identical at 1.2, corresponding to a DS per heparin disaccharide unit of 2.4. Furthermore, two commercially available UFHs (Heparin ratiopharm® and Liquemin® N) were included. For comparison, a branched fucoidan with a MW of 8.2 kDa and a DS of about 1.6 was examined as established inhibitor of sulfated polysaccharide structure [18].

Fig. 1A illustrates the reduction in cell binding by the different heparins compared to fucoidan at a concentration of 50 μg/mL. Because several artificial factors influence the cell rolling in an *in vitro* assay, inhibitor concentrations can hardly be compared with those used in other models or even heparin plasma levels in patients. Due to the variable specific anticoagulant activity of heparins, they are clinically applied in international units. Since however their P-selectin binding potency is a feature structurally independent of their anticoagulant activity, all the heparins used in these study were compared on their weight basis. However, 50 μg/mL corresponds to about 7.5 IU/mL (referring to about 150 IU/mg) and thus represents moderately increased clinical levels.

It is evident that fucoidan reduced cell binding to 56.0 ± 8.1% and exceeds the activity the heparins, which is in agreement to earlier findings [18]. The two commercial products Liquemin® N and Heparin ratiopharm® reduced cell binding to 77.5 ± 17.8 and 68.2 ± 6.4%, respectively. They are superior to UFH 1, which reduced cell binding to 86.0 ± 9.8%, roughly comparable to LMWH and MMWH, both with lower MW than UFH. Since only the VLMWH is completely ineffective, it can be assumed that exceeding a critical threshold of a minimal MW is essential for activity. The observed differences between the three UFH preparations exemplify that heparins are not suited to derive definite structure–activity relationships.

These findings were confirmed by testing various concentrations. Ten-fold higher amount of the heparins did not significantly increase the activities and approve the relations in Fig. 1A (data not shown); a reduction to a concentration of 5 μg/mL abolished all activities. This non-linear dependency

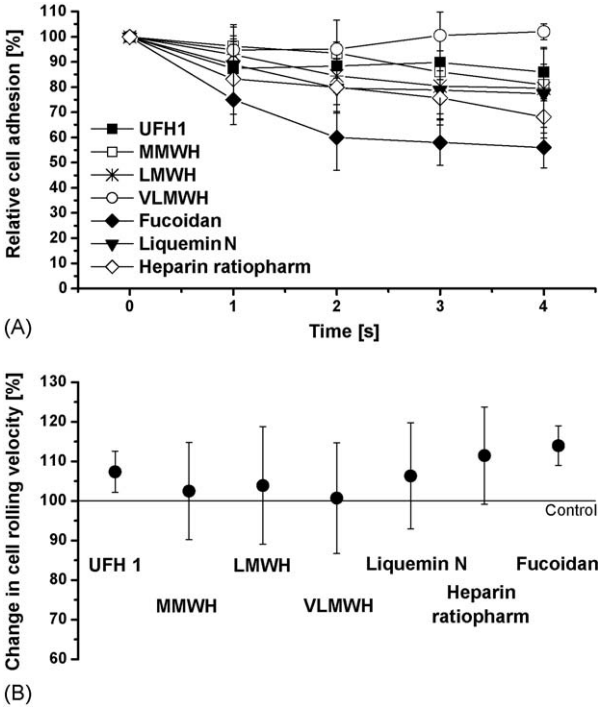
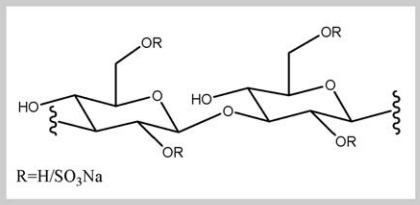


Fig. 1 – Capacity of different heparins to inhibit P-selectin induced cell rolling in comparison to fucoidan. (A) The number of adhering and rolling U937 cells at a P-selectin layer (expressed as percentage compared to the blank at each time) in presence of different heparins and fucoidan at a concentration of 50 μg/mL/1 Mio cells. The data of Liquemin® N, Heparin ratiopharm®, MMWH, LMWH, and fucoidan are significantly different from the blank ($P \leq 0.05$) after 4 s. (B) Increase in cell rolling velocity within the first second related to the inhibitor free system. The rolling velocity is expressed as percentage compared to the blank. Data represent means of at least three independent experiments ± S.D.

Table 2 – Structure and characteristics of phycarin sulfates

Phycarin sulfates



Substance	Degree of sulfation (DS)
PhyS-1	0.00
PhyS-2	0.75
PhyS-3	1.48
PhyS-4	1.80
PhyS-5	2.21
PhyS-6	2.80

Phycarin sulfates (β-1,3-glucans) display an average MW of about 10 kDa and differ in their DS from 0.00 to 2.80.

of selectin binding on concentration is in agreement with our earlier findings [29], obviously related to a multivalent binding of the polymeric structures.

The inhibitory capacities of the compounds were also reflected by the increase in rolling velocity in Fig. 1B. While the heparins overall only slightly increased the rolling velocity, the two most active compounds Heparin ratiopharm® and fucoidan caused an increase to 111.5 ± 12.3 and 114.0 ± 5.1%, respectively.

The inactivity of VLMWH and the interpretation that a certain minimum chain length is required for P-selectin inhibition is in agreement with earlier findings [24]. But regarding the superiority of the fucoidan with a MW of about 8 kDa over LMWH (6.0 kDa) and MMWH (10.5 kDa), the relevance of the MW for the activity compared to other structural parameters like the polysaccharide basic structure or the DS, becomes questionable.

Since heparins with their variable and complex structure are unsuitable to definitely determine structure activity relationships, we used well-defined semisynthetic glucan sulfates to further elucidate the influence of various structural parameters on the P-selectin inhibitory activity.

3.2. *Influence of the degree of sulfation on inhibitory capacity*

The natural selectin ligand PSGL-1 is known to contain sulfated tyrosines, arranged in negatively charged clusters [35]. Accordingly, a higher DS may imply better inhibitory properties. To examine this hypothesis, a series of six PhyS only differing in their DS, which ranges from 0.0 for the starting polymer up to 2.80 (Table 2), were tested. With a DP of 23–25 monosaccharides, the chain length of the PhyS is between that of the active heparins LMWH and MMWH.

The inhibitory data, illustrated in Fig. 2A give a clear correlation between DS and activity. While the non-sulfated PhyS-1 and the low-sulfated PhyS-2 (DS 0.75) showed to be unable to inhibit P-selectin, compounds with a DS ≥ 1.50 exhibit much stronger activity than LMWH and MMWH. PhyS-4 with a DS of 1.80 reduced cell adhesion to 32.6 ± 7.6%. However, further increase in DS up to 2.80 did not improve the inhibition.

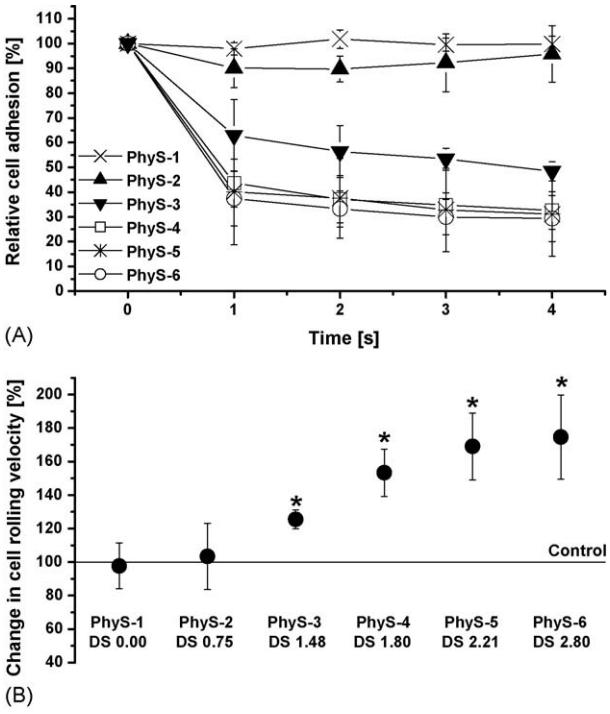
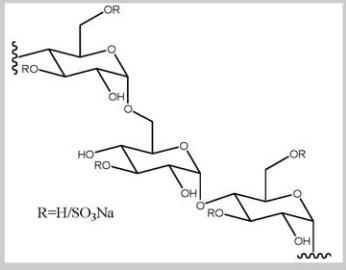


Fig. 2 – Capacity of phycarin sulfates to inhibit P-selectin induced cell rolling. (A) The capacity of the different PhyS (50 μg/mL/1 Mio cells) to inhibit the P-selectin-dependent rolling of U937 cells in the plate flow chamber within the indicated time range. The data of PhyS-3 to PhyS-6 are significantly different from the blank ($P \leq 0.01$) after 4 s. (B) Increase in cell rolling velocity within the first second related to the inhibitor free system. The rolling velocity is expressed as percentage compared to the blank. Data represent means of at least three independent experiments ± S.D., *indicates significance at $P \leq 0.05$.

Table 3 – Pullulan sulfates used in this study

Pullulan sulfates	

Substance	Degree of sulfation (DS)
PulS-1	0.23
PulS-2	0.58
PulS-3	1.50
PulS-4	2.00

The pullulan sulfates have a comparable MW like the phycarin sulfates of about 10 kDa, range in their DS from 0.23 to 2.00, and possess a glycosidic backbone consisting of α-1,4/1,6-glucans.

The clear correlation between DS and activity becomes also evident considering the cell rolling velocity in Fig. 2B. The velocity increase correlates well with the effects on cell adhesion in Fig. 2A. The data for PhyS-3 to PhyS-6 are significantly different from that in the presence of PhyS-1 and strongly exceed the fucoidan and Heparin ratiopharm® effects (Fig. 1B).

The crucial role of the charge density for the activity was further confirmed by the effects of pullulan sulfates, another series of sulfated polysaccharides of identical molecular size with DS ranging from 0.23 up to 2.00 (Table 3).

As illustrated in Fig. 3, PulS-1 and PulS-2 with DS < 1.0 turned out to be inactive. Similar to the PhyS (Fig. 2) the PulS have to exceed a DS of about 1.5 (PulS-3) to efficiently inhibit cell binding.

Summarising, a certain minimum charge density is critical for any activity of sulfated polysaccharides with MW similar to heparins (MW < 15 kDa). Higher DS lead to improved activity, a maximum seems to be reached with a DS of about 1.8–2.0. Fucoidan, which displays a DS of about 1.6 matches perfectly

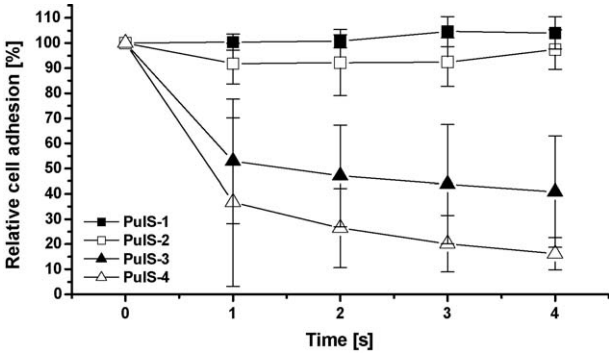


Fig. 3 – P-selectin blocking capacity of different pullulan sulfates. The diagram illustrates the capacity of different PulS (50 μg/mL/1 Mio cells) to reduce the P-selectin-dependent rolling of U937 cells in the plate flow chamber assay. Data represent means of at least three independent experiments ± S.D. The data of PulS-3 and PulS-4 are significantly different from the blank ($P \leq 0.01$) after 4 s.

with these findings and showed similar activity to PhyS-3 (DS 1.48) and PulS-3 (DS 1.50).

Although the DS is not directly comparable to the charge density in heparins due to the carboxy moiety, and the influence of the position of sulfation will not be considered in this study, charge might contribute to the higher activity of fucoidan and the glucan sulfates over heparins.

3.3. *Influence of the molecular weight on inhibitory capacity*

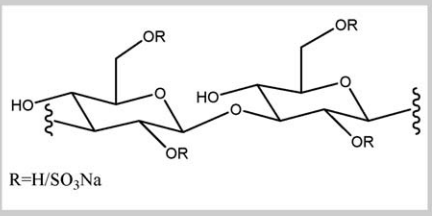
The results of the UFH 1-derived heparins suggest a non-linear influence of the MW on the selectin binding capacity. To focus on the influence of size on inhibition, we compared the activity of β-1,3-glucan sulfates with similar DS of about 1.8, but different MW_{HD} by choosing two members of the curdlan sulfate series (Table 4), CurS-4 (MW_{HD} 170 kDa) and CurS-5 (MW_{HD} 97 kDa), compared to PhyS-4 (MW_{HD} 19 kDa) in Fig. 4A. In contrast to UFH 1 (MW_{HD} 28 kDa), all three compounds strongly inhibited cell adhesion to a range between 20 and 35% and no significant differences were observed between their activities.

This implies that MW, if it exceeds a certain minimum as shown by the inactive oligosaccharide VLMWH (Fig. 1), has only a minor influence on the selectin binding of compounds with an optimal DS.

The lower activity of the three UFHs compared to the glucan sulfates cannot be explained with MW and should be related to backbone effects or to charge characteristics. In order to check the interference between MW and DS, we investigated the complete CurS series (Table 4) which gradually increase from DS 0.16 to 1.78 and compared to the effects of the PhyS and PulS (Figs. 2 and 3). The inhibitory capacities of the different CurS are illustrated in Fig. 4B, which again confirms the DS dependency. Only the very low-sulfated CurS-1 (DS 0.16) turned out to be unable to inhibit cell adhesion, while all the other CurS are equally effective. However, compared to PhyS and PulS, the critical DS threshold for activity showed to be much lower (DS 0.64) for these large molecules.

Table 4 – Structural characteristics of curdlan sulfates used in this study

Curdlan sulfates



Substance	Degree of sulfation (DS)
CurS-1	0.16
CurS-2	0.64
CurS-3	1.35
CurS-4	1.74
CurS-5	1.78

Curdlan sulfates are linear polymers with a β-1,3-glucan backbone identical to phycarin sulfates, they display a similar range in DS, but they represent a much higher molecular weight expressed as hydrodynamic MW_{HD} (CurS-1–4 about 170 kDa, CurS-5 97 kDa).

This could be explained with a certain balance of the local charge density and the net charge. The PhyS should have a more ordered molecular organisation on the P-selectin layer than the much bigger CurS. Their more randomly distributed orientation will lead to a higher local charge density, even at lower DS per monomer. Consequently, at least for glucan sulfates, a higher MW seems to compensate a lower DS. This leads to the assumption that the charge density seems to be less important than the total number of negative charges presented to P-selectin, whereby there is an upper limit of negative charges for improving the interaction with P-selectin.

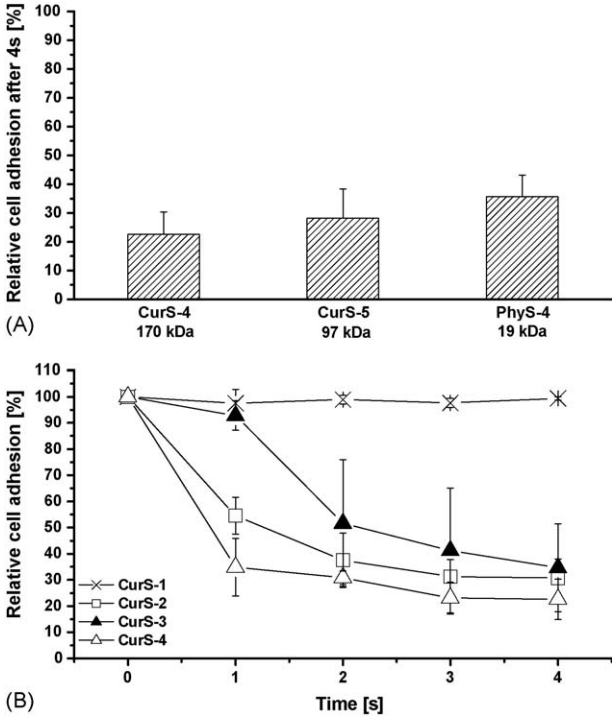


Fig. 4 – Capacity of curdlan sulfates to inhibit P-selectin induced cell rolling. (A) In order to focus on the influence of the MW of β-1,3-glucan sulfates on P-selectin inhibition, three derivatives with similar DS of about 1.8, that cover a MW_{HD} range from 19 to 170 kDa were investigated at a concentration of 50 μg/mL/1 Mio cells with respect to blocking P-selectin induced cell rolling. (B) The capacity of CurS to inhibit P-selectin. Data represent means of at least three independent experiments ± S.D. The data of CurS-2 to CurS-4 are significantly different from the blank ($P \leq 0.01$) after 4 s.

3.4. *Influence of the glycosidic backbone on the inhibitory capacity*

But these findings concerning the role of molecular size and the charge density of sulfated polysaccharides cannot completely explain the lower activity of the heparins than several glucan sulfates. Additional structural parameters may play a role for the optimal presentation of the negatively charged sulfate groups. To prove this assumption, we compared the activity of the PhyS and PulS series, which have the same MW and cover a comparable DS range, but differ in the type of glycosidic linkages of their glucose units. PhyS consist of β-1,3-linked glucose units, whereas PulS are α-1,4/1,6-glucans consisting of 1,6-linked maltotriose units. In Fig. 5, the inhibition of cell adhesion by PhyS and PulS are presented in dependence on the DS. As the PhyS series, PulS with DS lower than 1.0 are also inactive. However, PulS-4 is significantly more active than PhyS-4 despite comparable DS (indicated with +). These results demonstrate the importance of the type of the polysaccharide backbone for the ability to interact with P-selectin.

This may also contribute to the results of MMWH, which is significantly less active compared to PhyS-3 and PulS-3 despite of identical size and a comparable DS (indicated with *). This should be discussed with respect to backbone influences on flexibility and the resulting local presentation of charges moieties.

3.5. *The in vivo impact of a selected phycarin sulfate on P-selectin-dependent interaction using intravital microscopy*

To investigate whether the capacity for P-selectin inhibition in our *in vitro* assay also applies to *in vivo* conditions, PhyS-4 was

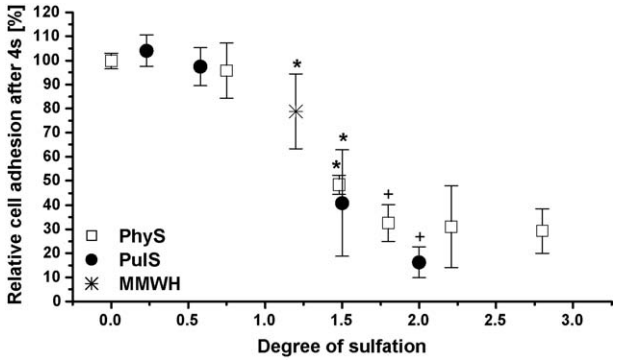


Fig. 5 – P-selectin inhibitory capacities of PhyS and PulS series in comparison to MMWH (all 50 μg/mL/1 Mio cells) related to DS. The figure focuses on the importance of the backbone structure for the P-selectin blockade. All derivatives have a mean MW of about 10 kDa. The effect of PulS-4 is significantly different from that of PhyS-4 (marked with +, $P \leq 0.05$). Further, both PhyS-3 and PulS-3 are significantly more effective than MMWH despite comparable DS and MW (marked with *, $P \leq 0.05$). Data represent means of at least three independent experiments ± S.D.

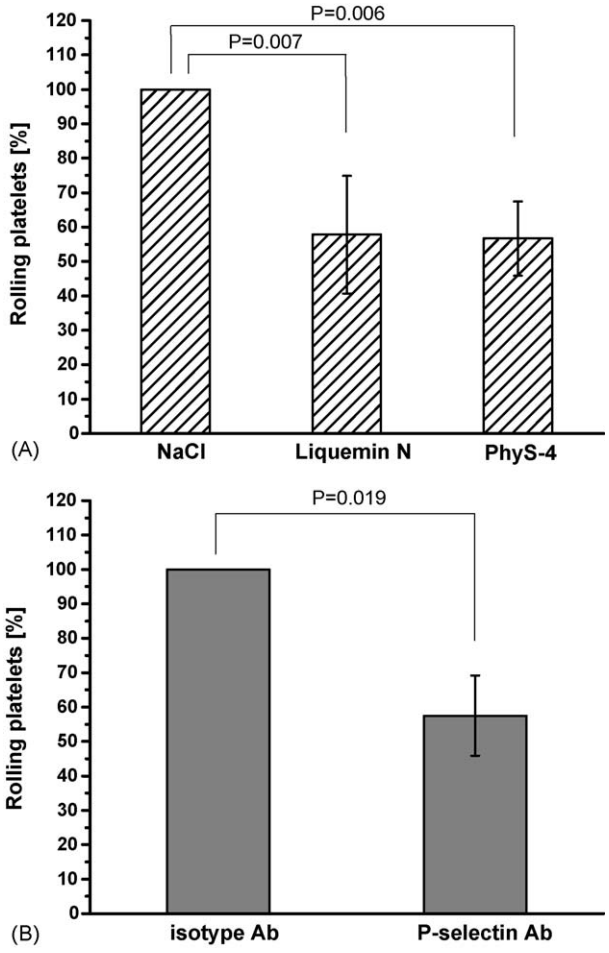


Fig. 6 – Intravital microscopic investigation of the P-selectin-dependent interaction of fluorescently labelled platelets with endothelium. (A) The interaction of TRAP-activated platelets with the skin microvasculature of C57Bl/6 mice was observed in presence of Liquemin® N or PhyS-4 vs. NaCl (set as 100%, respectively) or (B) a P-selectin blocking antibody vs. an isotype antibody (set as 100%). Data represent means ± S.E. from at least six vessels from at least three mice. Comparisons were performed using the paired *t*-test.

tested for its influence on P-selectin-dependent rolling of injected activated human platelets in the skin microvasculature of mice compared to Liquemin® N using intravital microscopy. In contrast to the investigation of leukocyte rolling *in vivo*, where all three types of selectins are involved, this model allows to solely focus on P-selectin function *in vivo*. Since P-selectin is constitutively expressed by normal mouse skin vessels [36] and murine P-selectin ligands efficiently interact with human selectins and vice versa [37], an inhibition of P-selectin would reduce the interactions of platelet or platelet/leukocyte with the vascular endothelium. As illustrated in Fig. 6B, a blocking mAb against P-selectin significantly decreased the percentage of rolling platelets from 32.9 ± 10.6 to 17.2 ± 9.3% after injection of the mAb. Although this confirms P-selectin dependency of this interaction, the partial inhibition suggests that other adhesion receptors contributes to that process. For instance, glycoprotein IIb/IIIa of the integrin family significantly influences these interactions [38].

In identical setups (Fig. 6A), activated platelets and subsequently PhyS-4 or Liquemin® N were injected and platelet interactions with the endothelium were recorded. PhyS-4 reduced the fractions of rolling activated platelets clearly from 46.9 ± 20.9 to 23.4 ± 12.9%, which is identical to the activity of the blocking antibody. Surprisingly, Liquemin® N displays a comparable activity in this assay by reducing the fraction of rolling platelets from 35.1 ± 9.6 to 16.0 ± 10.1%, which does not reflect the differences found in the *in vitro* cell rolling assay. We assume that the overall sensitivity of the *in vivo* assay is relatively low due to contribution and partial overlapping function of different adhesion receptors. Although this assay is specific with respect to P-selectin, obviously its inhibition intensity does not correlate very sensitively with reduced platelet contacts. This could be the reason that compounds with different *in vitro* activities exhibit similar *in vivo* effects. Since Liquemin® N and PhyS-4 display an identical activity to a total P-selectin blockade by the mAb, it might suggest that already a certain P-selectin inhibitory capacity is sufficient to cause a maximal effect on the P-selectin contribution to platelet interaction. However, these data demonstrate that both heparin and glucan sulfates are active P-selectin inhibitors and that the activity of the novel glucan sulfates is relevant under *in vivo* conditions.

The relevance of PhyS-4 activity in selectin inhibition *in vivo* becomes further evident, since PhyS-4 has a similar influence on rolling interactions of leukocytes in the skin microvasculature in mice (Alban and Ludwig et al., in preparation), which might be related due to interferences with both P- and L-selectin.

4. Discussion

In addition to the clinically established application of heparin as an anticoagulant drug, heparin and its derivatives display a variety of biological effects. The potential anti-inflammatory efficiency of heparin has been related to its ability to interfere with P- and L-selectin bindings in course of the leukocyte adhesion cascade [23,25–27]. The inhibition of P-selectin binding might also be the reason for the effects of heparin in reducing cancer cell metastasis, since P-selectin is regarded as critical factor in that process [8,20,22].

Several studies considered P-selectin binding characteristics of heparins and related these to structural parameters. Carboxy-reduced heparin was shown to have lower anticoagulant activity while retaining P-selectin binding [25,26]. The 6-*O* sulfation of heparin was proved to be essential for P-selectin binding, while 2,3-*O* desulfation retains selectin binding activity but strongly reduce anticoagulation [27,28]. These findings provide an interesting insight into the heparin effects and strongly increase the plausibility to use heparin-derivatives clinically as selectin-blocking therapeutics.

However, the natural glycosaminoglucan heparin has the disadvantage of a heterogeneous and variable structural composition and the characteristic to exhibit multiple effects *in vivo*. Two obscurities are arising from this. On the one hand, the anti-inflammatory activities cannot solely be related to selectin inhibition. Although heparin reduced leukocyte extravasation in several experimental setups [39–41], the input of selectin blocking and thus leukocyte rolling inhibition has been discussed controversy. On the other hand, due to the heterogeneity in structure, it is difficult to derive a clear correlation between heparin structure and selectin binding characteristics.

In this study we focused on the second point. The aim was to consider whether and how a structural simplification of heparin and emphasis of certain structural parameters influence selectin binding. This should lead to heparin-like structures with well controlled biological functions and potent selectin inhibition as potential candidates for anti-inflammatory therapeutic approaches. To avoid the problems related to the multiple pharmacological activities of heparin, we used less anticoagulant semisynthetic sulfated polysaccharides [30] to structurally optimise selectin binding. Three series of glucan sulfates gradually differing in their structural parameters were investigated. Consequently, the influence of molecular size, DS, and the impact of the glycosidic backbone on the activity should be detectable. Furthermore, the comparison of the glucan sulfates with two commercial UFHs as well as four heparins differing in their chain length should allow estimate objectively the selectin binding activities of heparin and their *in vivo* relevance.

Two of the fractionated heparins and the UFH 1 behave nearly similar and appeared as relatively weak P-selectin blockers in this study. The smallest heparin fraction with a MW of 3.4 kDa showed to be completely inactive. The importance of a certain minimum size corresponds to the findings of Koenig et al. [24]. The two commercial UFHs display a moderate activity. This emphasises the importance of heparin batch variability with respect to selectin binding and the inability to derive valide structure–activity relationships. In contrast, several of the glucan sulfates strongly exceed these activities, which can clearly be related to their structural attributes.

The heparin fractions used in this study were derived from the same UFH 1 and thus displayed identical parameters and only differed in size. The comparable activities of UFH 1, LMWH and MMWH advise that molecular weight is of minor importance for P-selectin binding. This is strongly confirmed by the effects of the semisynthetic polysaccharides. As polysaccharides such as phycarin sulfates and curdlan sulfates, which differ in size by factor 10 are equally effective, MW cannot explain the much higher activity of glucan sulfates over heparin, especially when those have a size similar to heparin.

The charge density could be derived as a dominant factor, since DS directly correlates with inhibitory efficiency of the glucan sulfates. MW has a modulating effect on the charge, i.e. high MW structures need lower DS for activity, obviously due to increased flexibility of high MW structures and resulting higher degree of freedom for local charge arrangement. A DS of >1.5 was found as threshold level of efficiency for molecules in the heparin size range. The heparins possess a DS (monosaccharide) of 1.20, which appeared to be less optimal for the charge restrictions, but it has to be considered that their charge density is additionally increased by their carboxy groups (i.e. one per disaccharide) and thus exceeds the critical charge threshold for selectin binding. Consequently, charge density cannot explain the much lower capacity of heparins for inhibition compared to the efficiency of the most active polysaccharides in this study.

The glycosidic linkage of the monomers, which influences the flexibility of the polymers, has also a strong impact on P-selectin binding. Flexibly presented polymers of defined identical monomer subunits are obviously advantageous, as in the case of the flexible PulS. Flexibility could achieve higher local charge densities, which is in agreement with findings for PSGL-1 binding to P-selectin [35]. This could explain the slightly better activity of PulS over PhyS.

However, to what extent the monomer units, their linkage and resulting flexibility of heparin contribute to the minor activity compared with the higher inhibitory capacity of the glucans and fucoidan (fucose) cannot be elucidated here.

Concluding the structural interpretations, heparin shows weaker P-selectin inhibition compared to selected glucan sulfates, which is not only due to its molecular size and charge density. Possibly, a combination of all structural parameters, emphasising backbone flexibility, is finally responsible for this behaviour. The glucan sulfates allow clear structural correlations and exhibit excellent P-selectin inhibition. Considering that the glucan sulfates are well manageable with respect to biological functions [30], they appear as more promising drug candidates instead of heparin for selectin inhibition.

We could not find a higher activity of the selected PhyS than the commercial heparin in our *in vivo* assay focusing on P-selectin-mediated platelet adhesion. We relate this to the peculiarities of this assay. The contribution of different adhesion receptors to platelet interaction may decrease the sensitivity to correlate reduced P-selectin function and decreased platelet interaction. This is supported by the fact that both, PhyS-4 and Liquemin® N are as effective as a

blocking antibody, which does not reflect the more sensitive evaluation in the *in vitro* assay. Finally, the data illustrate on one hand that glucan sulfates inhibit P-selectin not only *in vitro* but also *in vivo*, and on the other hand, that despite its moderate effect in the chosen *in vitro* assay heparin interferes with P-selectin *in vivo*.

However, the lower activity of heparin than optimised glucan sulfates *in vitro* could lead to a hypothesis that alternative heparin activities besides P-selectin inhibition should partially be assumed *in vivo*. This agrees with earlier results on heparin [39]. Jones et al. could show that although UFH exhibited a spectrum of anti-inflammatory activities with respect to reducing plasma exudation or leukocyte accumulation in a rabbit skin model, the effects were different from the activities of fucoidan as a defined selectin antagonist [40]. They postulated that heparin generally acts by inhibiting inflammatory-related polycationic molecules. Xie et al. did not find a clear correlation between the reduction of leukocyte rolling and firm adhesion by heparin. They postulated that heparin has a stronger influence on adhesion [41] than on rolling, possibly by interference with leukocyte integrins [42].

The structural similarities of heparin to cell surface glycosaminoglycans are evident. Wang et al. presented an excellent insight into the differential roles of endothelial and leukocyte heparan sulfates in neutrophil trafficking during inflammation [43]. It can be assumed that heparin competes with endothelial heparan sulfates for chemokine bindings and thus influences leukocyte emigration, which could partly explain the anti-inflammatory activities [44,45].

Referring to potency and the ability for structure–activity relationships, the semisynthetic sulfated polysaccharides can be regarded as attractive drug candidates, which could replace heparin for certain applications.

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