Binding between heparin and the integrin VLA-4

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Summary
Heparin possesses antimetastatic effects that were related to various molecular mechanisms beyond anticoagulant activities. The ability of heparin to interfere with the function of adhesion receptors in the metastatic course appears as a promising therapeutic approach. This refers to numerous findings that heparin attenuates metastasis in a selectin-dependent manner. We recently demonstrated that heparin interferes with the integrin VLA-4 on murine melanoma cells binding to VCAM-1. To confirm this activity and to obtain further insight into molecular recognition of heparin by VLA-4, we investigated the inhibition of VLA-4 mediated binding of human melanoma MV3 cells to immobilised VCAM-1 by different heparins. The size of heparin has an important impact on inhibition. Unfractionated heparin (UFH) and tinzaparin, a low-molecular-weight heparin (LMWH) representing a mean of about 18–20 monomers, displayed high inhibitory activity. Fractionating tinzaparin to 14–18 monomers reduced inhibition slightly, while the pentasaccharide fondaparinux was without effects. To confirm molecular recognition of tinzaparin by VLA-4, a surface acoustic wave-biosensor was applied. AVLA-4 containing membrane preparation of MV3 cells was immobilised at the sensors to allow for detection of kinetic binding constants of tinzaparin compared to VCAM-1. Tinzaparin binds to VLA-4 with affinity in the low micromolar range (4.61×10⁻⁶ M), which clearly indicates specific molecular recognition. Furthermore, tinzaparin displays a nearly identical kₐ compared to VCAM-1 (5.13×10⁻³ s⁻¹ versus 3.44×10⁻³ s⁻¹) which is evident for interference with the ligand binding. The data provide evidence for a direct confirmation of heparin binding to VLA-4 and thus, contribute to understand the antimetastatic activity of heparin.

Keywords
Heparin, melanoma cell adhesion, VLA-4, antimetastatic activity

Introduction
Tumor cell metastasis may lead to serious complications in cancer. Thus, its effective treatment is required to inhibit tumor growth or to improve survival of cancer patients. The mechanisms of metastasis are complex and versatile including multiple steps that allow tumor cells to disseminate and to proliferate. Cell adhesion molecules (CAMs) are crucially involved in the metastatic cascade promoting cell-cell, cell-endothelium, cell-platelet, and cell-extracellular matrix (ECM) interactions. Thus, anti-adhesion strategies appear as promising approaches in oncology (1). The selectins, a class of carbohydrate-binding proteins, recognise several structures on tumor cell surfaces, leukocytes, platelets, and endothelial cells, thereby creating multiple binding possibilities. This leads to the formation of microemboli enabling the tumor cells to survive and to arrest in the microvasculature (2). Beside selectins, integrins and their ligands of the immunoglobulin superfamily participate in adhesive interactions between tumor cells and the endothelium, such as the integrin α4β1 (very late activation antigen-4, VLA-4) and vascular cell adhesion molecule-1 (VCAM-1). Both, the selectin and the VLA-4 mediated binding pathway contribute to experimental metastasis of tumor cells bearing the respective ligands/receptors, such as malignant melanoma cells (3, 4), whereas their activities are even of clinical relevance (5). We and others were able to show that especially melanoma cells utilise the VLA-4/VCAM-1 binding pathway to directly adhere to the endothelium, whereas selectins are involved in forcing platelet-cell interactions and in polymorphnuclear neutrophils (PMNs)-facilitated extravasation (6, 7).

For prophylaxis and treatment of thrombosis, heparin has been the drug of choice for several decades. With the findings that – in addition to the prevention of thromboembolism – heparin prolongs the survival of cancer patients (8), research is going
on to identify the molecular mechanisms. Currently assumed heparin actions beside anticoagulation are (i) inhibition of P- and L-selectin, (ii) inhibition of angiogenesis via stimulation of tissue factor pathway inhibitor (TFPI)-release, (iii) modulation of growth factor binding, and (iv) inhibition of heparanase enzymatic activity (9–12). Taking into account that VLA-4 plays a dominant role in melanoma cell adhesion, its inhibition seems to be essential to decrease metastasis. In a recent study, we showed for the first time that the adhesion of murine B16F10 melanoma cells to VCAM-1 could be inhibited by heparin in vitro and suggest VLA-4 as a novel heparin target (6). Although a combination of different techniques was used, the final proof of a direct binding between heparin and VLA-4 was lacking. Indeed, there are observations of others, who refer to the binding ability of heparin to the integrins αIIbβ3, αMβ2 (Mac-1), αxβ2 (gp 150,95) (13–15) and to specific integrins on a transfected erythroleukemic cell line in dependence on the integrin β-subunit (16). However, none of these studies was associated with the antimetastatic properties of heparin.

To strengthen our hypothesis of heparin/VLA-4 interactions, the present study investigates the inhibitory effects of different heparins on human MV3 melanoma cell adhesion to VCAM-1 under flow conditions. Unfractionated heparin (UFH), low-molecular-weight heparin (LMWH) tinzaparin, a fraction of tinzaparin representing a proportion of 14, 16, and 18 monosaccharides as well as fondaparinux were included. The results show a clear dependence of the inhibitory capacity on the molecular weight of heparin.

To detect heparin/VLA-4 binding on a molecular level, a surface acoustic wave (SAW) biosensor was applied. On basis of the piezoelectric properties, mass changes on the surface of sensors can be detected as phase shift of an applied acoustic wave. For detailed principles of measurement, the underlying physical mechanisms, and the mathematical bases for determination of kinetic binding constants the reader is referred to (17) and for several applications to (18–20). SAW-based cell experiments have been applied mainly using bacteria, bacteriophages, and yeasts (21–23), but also mammalian cell interactions (24, 25).

In the present study, the SAW method was applied for the first time at adhesion receptor mediated interactions, i.e. between VLA-4 expressing MV3 cells and VCAM-1. By immobilizing a membrane preparation of MV3 cells on the sensor surface we were able to determine binding kinetics of VCAM-1 and tinzaparin to VLA-4 and thus, confirmed heparin binding to VLA-4.

Therefore, VLA-4 is coming into light as an attractive target for the antimetastatic effects of heparin leading to a decrease of human melanoma cell adhesion.

Materials and methods

Cell culture

All cell lines were cultured at 37°C in humidified atmosphere containing 5% CO2.

The human melanoma cell line MV3 was grown in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (FCS). For subcultivation, MV3 were detached at 80–90% confluency using EDTA-solution (0.2 g/l EDTA × 4 Na) for 3 min at 37°C.

reagents were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Human umbilical vein endothelial cells (HUVECs) were cultured in Endothelial Cell Growth Medium. Subcultivation was performed at 60–80% confluency using DetachKit in accordance with the provided protocol. HUVECs and all reagents were obtained from PromoCell GmbH (Heidelberg, Germany).

Antibodies and adhesion molecules

The humanised anti-VLA-4 (α-chain) mAb natalizumab was kindly provided from Biogen Idec GmbH (Ismaning, Germany). Goat anti-human IgG-horseradish peroxidase (HRP) was purchased from Antibody Online (Aachen, Germany).

Function-blocking anti-human P-selectin mAb and recombinant human VCAM-1Fc (human IgG1) chimera were obtained from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany).

Sulfated polysaccharides

UFH 1 was kindly provided by Dr Annamaria Naggi (“G Ronzoni” Institute for Chemical and Biochemical Research, Milan, Italy). UFH 2 (Heparin-Natrium 5000 ratiopharm®) was purchased from Ratiopharm GmbH (Neu-Isenburg, Germany), tinzaparin was from LEO Pharma GmbH (Neu-Isenburg, Germany) and fondaparinux from Sanofi-Aventis GmbH (Frankfurt/M, Germany). The fraction of tinzaparin consisting of 14, 16, and 18 monosaccharides was isolated by size-exclusion chromatography on Biogel-P10 as described by Bisio et al. in the present issue of T.H.

Flow chamber assay

Adhesive interactions of MV3 cells with VCAM-1-Fc chimera or HUVECs were determined under physiological flow conditions. Glass slides containing 0.2 µg VCAM-1 or confluent HUVECs, stimulated with 50 ng/mL human tumor necrosis factor (TNF)-α (Sigma-Aldrich GmbH, Taufkirchen, Germany) for 4 to 6 hours, were incorporated into a parallel plate flow chamber as described in detail in our previous investigations (6, 26). Briefly, the flow chamber was mounted onto an inverted microscope. PBS (pH 7.4) as flow medium was driven by hydrostatic pressure at a shear rate of about 200 s⁻¹. Experiments were performed after treatment of 1×10⁶ MV3 cells suspended in FCS-free RPMI 1640 with 1 mM Mn²⁺ for 5 min at 37°C (stimulated cells) in comparison to untreated cells. To determine the contribution of P-selectin on MV3 cell interaction with HUVECs, 10 µg of a function-blocking anti-human P-selectin mAb were mixed with 1×10⁶/100 µL MV3 cell suspension and put into the flow chamber.

For inhibition experiments, heparin preparations and natalizumab were used at 500 µg (according to 150 IU = 1 mg) and 5 µg, resp., and compared with the inhibitor-free control experiment (cells in FCS-free medium only). The substances were added to 100 µL stimulated MV3 cells and incubated for 5 min at 37°C prior to injection into the flow system.

Adhesion behavior of the MV3 cells was monitored over a period of 5 s. Video sequences were captured with the camera CSC-795 (Pacific Corporation, Tokyo, Japan) and analysed with the software Imagoquant MultiTrack-AVI-2 (Mediquant GmbH, Lützen, Germany).
SAW biosensor measurements
Biosensor investigations were performed using a S-Sens™ K5 surface acoustic wave biosensor supplied by Biosensor GmbH, Bonn, Germany.

Immobilisation of VCAM-1 and natalizumab on the SAW sensor
Gold-coated sensors (Biosensor) were incubated in a solution of 11-mercaptoundecanoic acid (10 mM) in ethanol abs. forming a self-assembled monolayer by binding of the sulfur headgroups to the gold surface. The carboxyl groups were activated with 200 mM N-ethyl-N-(dimethylaminopropyl)-carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS) to bind 50 µg/mL natalizumab and VCAM-1, respectively, forming carboxylamides. An excess of activated unreacted NHS esters was blocked by 1 M ethanolamine (pH 8.5). The amount of bound protein was determined using the protocol described in (17).

Cell binding experiments to VCAM-1 and natalizumab
After immobilisation of VCAM-1 and natalizumab, respectively, a baseline equilibration in PBS containing 1 mM Ca²⁺ and Mg²⁺ was performed for 30 min. In that time, 5×10⁶ MV3 cells were suspended in 1 mL running buffer containing 1 mM Mn²⁺. The number of injected cells was optimised based on the findings that 1×10⁶ cells was the minimum to detect binding events, while numbers >1×10⁶ did not lead to increased binding. The flow rate was ensured sufficient time for cell-ligand interaction and to prevent unspecific binding due to sedimentation was 100 µL/min.

For inhibition experiments, 500 µg tinzaparin in 10 µL buffer were added to 1×10⁶ cells, injected into the biosensor system and rinsed either over immobilised VCAM-1 or natalizumab. Binding of cells with or without inhibition was measured as shift of phase. Since the penetration depth of a love wave sensor is restricted to the near sensor surface, only mass changes caused by ligand-receptor/antibody interactions close to the sensor surface were detected without balancing the whole cell.

Preparation of membrane vesicles
MV3 cells were harvested from 80% confluently grown cell dishes. Cell membrane vesicles were prepared according to the protocol described in detail in (27). All steps were performed at 4°C. Membrane vesicles were homogenised using a Potter-Elvehjem homogeniser. The protein yield was determined applying a SDS-page under non-reducing conditions. Proteins from the gel were plotted on a polyvinylidifluoride membrane at 100 V, 350 mA for 60 min. Unspecific binding sites on the membrane were blocked using a solution of skim-milk protein (5%) in TBS with 0.2% Tween. VLA-4 was detected on the membrane at 100 V, 350 mA for 60 min. Unspecific binding due to sedimentation was 100 µL/min.

For inhibition experiments, 500 µg tinzaparin in 10 µL buffer were added to 1×10⁶ cells, injected into the biosensor system and rinsed either over immobilised VCAM-1 or natalizumab. Binding of cells with or without inhibition was measured as shift of phase. Since the penetration depth of a love wave sensor is restricted to the near sensor surface, only mass changes caused by ligand-receptor/antibody interactions close to the sensor surface were detected without balancing the whole cell.

Immobilisation of membrane vesicles on SAW sensors
Gold-coated SAW sensors were inserted into an aqueous solution of 1-β-thiogluconate (10 mM) over night to form an adhesion layer for the membrane vesicles. After rinsing briefly with ethanol and drying under a stream of nitrogen, the sensor chips were inserted into the flow chamber of the SAW biosensor and a baseline equilibration was performed for 30 min in PBS containing 1 mM Ca²⁺ and Mg²⁺. The amount of injected membrane vesicles was defined by a dilution series of the membrane vesicles. A 1:10-dilution of the membrane vesicles in running buffer was assumed to be appropriate after detection experiments using VLA-4 binding antibody natalizumab.

Determination of kinetic binding constants of VCAM-1, tinzaparin, and fondaparinux
VCAM-1, tinzaparin, and fondaparinux, resp., were injected in dilution series between 10⁻¹² M and 10⁻³ M to interact with the immobilised membrane vesicles. Association and dissociation events were detected as phase shifts indicating binding and unbinding events at the membrane vesicle surface. Kinetic binding constants were calculated from the slopes of the obtained curves applying a 1:1-binding model.

Statistics
Data represent means ± standard deviations of at least three independent experiments. Comparisons were performed by the unpaired Student’s t-test (*p<0.05, **p<0.01, ***p<0.001).

Results and discussion
VLA-4-mediated binding of MV3 melanoma cells to VCAM-1 can be blocked by different heparins – Insight into structural requirements
In order to simulate the situation of tumor cell adhesion to the endothelium, the binding of MV3 cells to confluent, TNF-α-treated HUVECs was determined under physiological flow conditions. TNF-α induces VCAM-1 expression on HUVECs in a time-dependent manner with near maximal expression being seen after 4 to 6 hours of stimulation (29). It can be noticed that Mn²⁺-treatment enhanced cell adhesion in a statistically significant extent from 76.5% ± 6.9% up to 87.3% ± 5.8% at 4 s (Fig. 1A). Beside chemokines, the treatment with Mn²⁺ is an experimentally accepted way of activation of almost all integrins. Thus, the Mn²⁺-induced increased cell adhesion points to a contribution of integrin-mediated binding pathways. Since the VLA-4-blocking mAb natalizumab was able to decrease cell binding (76.0% ± 4.2%) to the level of unstimulated cells, an explicit VLA-4 dependency can be assumed. VLA-4 positivity of MV3 was ensured by flow cytometry (data not shown).

In addition to VCAM-1, a variety of other cell adhesion molecules are expressed/induced on HUVECs, such as P-selectin. MV3 cells possess binding ability to P-selectin Fc chimera as investigated by flow cytometry (data not shown). To determine the contribution of P-selectin expressed at the cellular level to MV3 cell binding, experiments on HUVECs were carried out using a function-blocking P-selectin mAb. A slight, statistically not significant decrease in cell adhesion could be observed upon P-selectin blockade, which indicates that P-selectin is of minor relevance regarding the MV3/HUVEC interaction. This fact is of special importance for our inhibition studies on heparins, since endothelial P-selectin is another target of heparin action (3).

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However, to rule out any P-selectin-mediated cell adhesion, inhibition studies on HUVECs were performed at concomitant application of the P-selectin mAb. The effects of different heparin preparations were analysed after a preincubation period of 5 min with stimulated cells according to our previous investigations (6). Figure 1A illustrates that both UFH 2 and tinzaparin were able to reduce cell adhesion (74.3% ± 11.6% and 72.5% ± 9.7%, resp.) to the same extent as natalizumab in the presence of the P-selectin mAb. In contrast, fondaparinux failed to further decrease cell adhesion. These results reveal that heparin blocks VLA-4-mediated MV3 cell binding under physiological conditions using an endothelial cell layer. However, the observed inhibitory effects are relatively weak and probably hindered by unspecific binding events, which impede the determination of clear structure-activity relationships.

Thus, further inhibition studies were performed on immobilised VCAM-1 Fc chimera allowing to focus more closely on the involvement of VLA-4 (Fig. 1B). As observed for the adhesion experiments on HUVECs, Mn²⁺-treatment led to an increase of percentaged cell adhesion, which is much more pronounced on the isolated ligand. We initially used tinzaparin, which also displayed a high inhibition capacity regarding P- and L-selectin (30). Tinzaparin reduced the MV3 cell adhesion to isolated VCAM-1 in a statistically significant manner. It is known that tinzaparin contains more high molecular weight heparin fragments than other LMWHs. In order to investigate whether molecular weight of heparin has an impact on the blocking capacity of VLA-4/VCAM-1 interaction, two UFHs and the penta-saccharide fondaparinux have been applied. Preincubation of cells with each of the UFHs decreased cell adhesion stronger than tinzaparin, while fondaparinux revealed no inhibitory effect. Although it is not clear, which saccharide component is responsible for VLA-4 inhibition, a correlation between molecular size and inhibitory efficiency might be assumed. Moreover, exceeding a critical size appears as essential for inhibition. Since a smaller tinzaparin fraction (MW 5,600 Da compared to 6,500 Da of tinzaparin) representing only fragments of 14, 16, and 18 monosaccharides displayed significant activity (slightly reduced compared to tinzaparin), the critical threshold of heparin size seems to be between five and 14 monosaccharides. In further investigations, even smaller heparin fractions should be applied to further focus on this aspect, as well as UFH derivatives differing in sulfation or N-acetylation to obtain more information on the heparin binding epitope to VLA-4.

Our findings on size dependency correspond to others, who were able to show that only certain heparin fractions inhibit the
This appears to be due to synergistic effects with SDF-1α-induced phosphorylation of kinases implicated in integrin function (31). Consequently, inhibition of this process by heparin might result in opposed effects leading to impaired VLA-4 regulation. Additionally, the functional heparin binding domain of heparanase was shown to facilitate cell adhesion and spreading by clustering of cell surface syndecan-1 and syndecan-4 on human tumor cells as a consequence of initiating different signaling pathways (32). However, the influence of these heparanase-dependent actions by heparin might be excluded for our discrete experimental conditions using the recombinant immobilised ligand. Moreover, the integrin activation performed in the present study was chemokine-independent. Thus, our data point to a direct interaction between heparin and VLA-4.

**Biosensor-based investigation of MV3 binding**

To get a deeper insight into the molecular mechanisms of MV3 binding, we applied a SAW biosensor. The principle of measurement is schematically illustrated in Figure 2. VCAM-1 Fc chimera were immobilised on the sensor surface to investigate the binding of MV3 cells. Binding of 1×10⁶ stimulated MV3 cells was measured versus binding of 1×10⁶ MV3 cells under preincubation with 500 µg tinzaparin. Figure 3A shows that tinzaparin effectively suppressed MV3 binding to VCAM-1 in agreement with the microscopic assay.

Next, we immobilised the VLA-4 antibody natalizumab to the SAW sensor surface and detected the binding of 1×10⁶ stimulated MV3 cells as well as inhibition by tinzaparin (Fig. 3B). Assuming that an interaction between cellular VLA-4 and natalizumab is based on binding of the α-subunit of VLA-4, further insight into heparin binding activity can be obtained. Thus, tinzaparin may bind directly to the α-subunit of VLA-4 and cause a steric hindrance for natalizumab binding. Another possibility is the conformational change of VLA-4 after tinzaparin binding leading to a prevented binding to the α-subunit allosterically. However, the data strengthen our hypothesis of direct heparin/VLA-4 interaction, since we could also exclude (i) binding of natalizumab and of tinzaparin to immobilised VCAM-1 and (ii) binding of VCAM-1 and of tinzaparin to immobilised natalizumab. For concentrations between 1×10⁻⁸ M and 1×10⁻⁴ M neither interaction nor even unspecific binding could be detected (data not shown).

**Detection of kinetic binding constants of tinzaparin and VCAM-1 using a VLA-4 membrane preparation**

In order to investigate the heparin/VLA-4 binding on a molecular level, a membrane preparation of MV3 cells was performed to obtain VLA-4-containing membrane fractions. The presence of VLA-4 in the membrane vesicles was confirmed by SDS-PAGE and Western Blot leading to bands at 280 kDa for the VLA-4 heterodimer, at 150 kDa for the α-subunit and at 80 kDa, P AGE and Western Blot leading to bands at 280 kDa for the α-subunit and at 80 kDa, P AGE and Western Blot leading to bands at 280 kDa for the α-subunit and at 80 kDa.
Inhibition of MV3 cell binding by tinzaparin in a SAW biosensor assay. A) VCAM-1 was immobilised to gold-coated SAW sensors by EDC/NHS chemistry. 1×10^6 MV3 cells bound to VCAM-1 after stimulation by 1 mM Mn^{2+} indicated by the phase shift due to a mass load at the sensor surface. Addition of 500 µg tinzaparin inhibited binding of MV3 cells inducing a significant lower phase shift. B) In an identical experimental setting, natalizumab instead of VCAM-1 was immobilised. Natalizumab binds highly specific to the α-subunit of VLA-4. Tinzaparin induced a significant inhibition of MV3 cell binding to natalizumab, which might indicate a specific interaction of tinzaparin with the α-subunit of VLA-4. Data are presented as means ± SD (n>3; *p<0.05, **p<0.01). SAW, surface acoustic wave; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.

Table 1: Kinetic binding constants of VCAM-1 and tinzaparin binding to VLA-4 in an immobilised membrane preparation.

<table>
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<tr>
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<th>K_D [M]</th>
<th>k_on [M^{-1}s^{-1}]</th>
<th>k_off [s^{-1}]</th>
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<tbody>
<tr>
<td>VCAM-1</td>
<td>1.01×10^{-8}</td>
<td>3.53×10^{5}</td>
<td>3.44×10^{-3}</td>
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<tr>
<td></td>
<td>± 0.90×10^{-8}</td>
<td>± 0.57×10^{5}</td>
<td>± 2.95×10^{-3}</td>
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<tr>
<td>Tinzaparin</td>
<td>4.61×10^{-4}</td>
<td>1.27×10^{3}</td>
<td>5.13×10^{-3}</td>
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<td></td>
<td>± 2.68×10^{-4}</td>
<td>± 0.58×10^{1}</td>
<td>± 1.56×10^{-3}</td>
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<tr>
<td>Fondaparinux</td>
<td>n.d.</td>
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Key: n.d., not detectable.
cell rolling assay nor binding could be detected in the SAW assay, the specific recognition of tinzaparin by VLA-4 is confirmed.

In summary, following a recent hypothesis of inhibiting VLA-4-mediated cell binding by heparin, this study provides the first clear indication that a LMWH binds directly to VLA-4 with an affinity in a low micromolar range. Although a clear size-dependency could be obtained, further experiments using modified heparins are necessary to evaluate structural requirements of this recognition process. These results focus the interest on VLA-4 as a novel target for heparin in antimetastatic approaches and should be considered as worthwhile to continue research in this field.

Acknowledgements

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References


Abbreviations

ECM, extracellular matrix; EDC, N-ethyl-N-(dimethylaminopropyl)-carbodiimide; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cells; Kdis, equilibrium dissociation constant; keq, dissociation rate constant (off-rate); Kass, association rate constant (on-rate); LMWH, low molecular weight heparin; MS, monosaccharide; MW, molecular weight; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; PMNs, polymorphonuclear neutrophils; QCM, quartz crystal microbalance; SAW, surface acoustic wave; SDF-1, stromal cell derived factor-1; SD, sodium-dodecylsulfate; TBS, tris-buffered saline; TFPI, tissue factor pathway inhibitor; UFH, unfractionated heparin; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen-4.