New Functional Tools for Antithrombogenic Activity Assessment of Live Surface Glycocalyx


Objective—It is widely accepted that the presence of a glycosaminoglycan-rich glycocalyx is essential for endothelialized vasculature health; in fact, a damaged or impaired glycocalyx has been demonstrated in many vascular diseases. Currently, there are no methods that characterize glycocalyx functionality, thus limiting investigators’ ability to assess the role of the glycocalyx in vascular health.

Approach and Results—We have developed novel, easy-to-use, in vitro assays that directly quantify live endothelialized surface’s functional heparin weights and their anticoagulant capacity to inactivate Factor Xa and thrombin. Using our assays, we characterized 2 commonly used vascular models: native rat aorta and cultured human umbilical vein endothelial cell monolayer. We determined heparin contents to be ≈10,000 ng/cm² on the native aorta and ≈10-fold lower on cultured human umbilical vein endothelial cells. Interestingly, human umbilical vein endothelial cells demonstrated a 5-fold lower anticoagulation capacity in inactivating both Factor Xa and thrombin relative to native aortas. We verified the validity and accuracy of the novel assays developed in this work using liquid chromatography–mass spectrometry analysis.

Conclusions—Our assays are of high relevance in the vascular community because they can be used to establish the antithrombogenic capacity of many different types of surfaces such as vascular grafts and transplants. This work will also advance the capacity for glycocalyx-targeting therapeutics development to treat damaged vasculatures. (Arterioscler Thromb Vasc Biol. 2016;36:1847-1853. DOI: 10.1161/ATVBAHA.116.308023.)

Key Words: anesthesia ■ endothelial cells ■ glycocalyx ■ heparin ■ thrombosis

There is mounting evidence to suggest that the presence of an intact glycocalyx is required to achieve a healthy vascular endothelial cell (EC) lining in blood vessels. In fact, the presence of damaged glycocalyx has been demonstrated in many vascular pathologies: hyperglycemia, trauma, sepsis, and systemic inflammatory states such as diabetes mellitus. The glycocalyx systematic destruction in such disease states has been well documented; unfortunately, no therapeutic option for the restoration of the glycocalyx has emerged.

The endothelial glycocalyx serves several functions, of which the coagulation cascade modulation is of particular interest to this work. Regulated by the glycocalyx constituents, namely, the glycosaminoglycans, they extend from several hundred nanometers up to a few microns. Of the various glycosaminoglycans, heparan sulfate (HS) making up 60% to 90% of the total amount of the vascular glycocalyx is arguably the most important functional glycosaminoglycan. HS serves as a potent negative regulator of coagulation and a key component of vascular hemostasis. The ability of HS to inhibit coagulation has been extensively characterized, where mediation of antithrombin (AT, previously called antithrombin III, also known as heparin cofactor I) is one of HS’s best-recognized roles in the coagulation cascade. Specifically, HS side-chain pentasaccharide sequence binding activates AT via a conformational change, allowing activated AT to inhibit the key coagulation protease Factor Xa (FXa) or thrombin by forming equimolar complexes. For inhibition of thrombin, however, heparin must bind to both AT (via the pentasaccharide sequence) and thrombin (via a minimum of 13 additional saccharide units); thus, HS containing <18 saccharide units does not have the ability to inhibit thrombin. Thus, compared with unfractionated heparin, low-molecular-weight heparin has less ability to inactivate thrombin, but there is no difference for FXa inhibition between unfractionated heparin and low-molecular-weight heparin. To be noted, endothelialized surfaces such as native rat aorta or HUVEC monolayer

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generate 2 different standard curves (Figure 1B and 1C). The first standard curve relates the heparin weight to absorbance slope (Figure 1B). As expected, decreasing slopes were generated with increasing heparin weights, as heparin inactivates FXa, resulting in a reduction of color development of unbound FXa (Figure 1B). The second standard curve was generated based on the assumptions that: (1) the assay was at steady state at 16 minutes and (2) the remaining FXa unit is linearly proportional to the measured absorbance at the steady state. For example, 2 U of FXa activity remains in the absence of heparin (0 ng heparin), meaning that absorbance at 16 minutes represents 2 U of residual FXa. At 10 ng heparin, the absorbance is 0.21, 70% of that for 0 ng heparin (2 U of residual FXa), representing 1.4 U of residual FXa (Table I in the online-only Data Supplement). The second standard curve relates the absorbance at the end of assay (16 minutes) with the inactivated FXa enzymatic units (Figure 1C). For the thrombin activity assay, we recorded the capacity of logarithmically increasing heparin concentrations to inactivate 0.5 U of thrombin (Figure 2A). Similar to FXa assay, we chose the initial slope (blue box, 2–6 minutes) and the absorbance at the end of the assay (11 minutes) to generate 2 standard curves (Figure 2B and 2C). The first standard curve relates the heparin weight to thrombin absorbance slopes (Figure 2B). As expected, decreasing slopes were generated with increasing heparin weights, because heparin inactivates thrombin, resulting in a reduction of color development of unbound thrombin (Figure 2B). The second standard curve was similarly generated based on the assumptions that: (1) the assay was at steady state at 11 minutes and (2) the remaining thrombin unit is linearly proportional to the measured absorbance at the steady state.

Results

Generation of Standard Curves Using Heparin Standard

We first measured the potential of logarithmically increasing heparin concentrations to inactivate 2 U of FXa by adding excess AT. AT binds available heparin (which activates AT) to form the activated AT–FXa complex. Using FXa-specific chromogenic substrate, we then measured the color developed by free FXa over time (Figure 1A). The absorbance curve had 2 areas of interest: initial slope (green box, 2–6 minutes) and absorbance at 16 minutes (end of assay; Figure 1A), which were used to generate 2 different standard curves (Figure 1B and 1C). The first standard curve relates the heparin weight to absorbance slope (Figure 1B). As expected, decreasing slopes were generated with increasing heparin weights, as heparin inactivates FXa, resulting in a reduction of color development of unbound FXa (Figure 1B). The second standard curve was generated based on the assumptions that: (1) the assay was at steady state at 16 minutes and (2) the remaining FXa unit is linearly proportional to the measured absorbance at the steady state.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Figure 1. Standard curves of Factor Xa (FXa) inactivation induced by heparin. A, Representative data showing absorbance readings of residual FXa at logarithmically increasing heparin weights. Slopes of curve between 2 and 6 min (green area) were determined. B, Standard curve showing linear correlation between the slope of curve (generated from the green area in A) and log [heparin weights]. C, Standard curve showing linear correlation between the absorbance at 16 min (end point in A) and the inactivated FXa units. The results are mean ± SEM of 6 independent assays using new heparin batches for each assay run.
For example, 0.5 U of thrombin activity remains in the absence of heparin (0 ng heparin), meaning that absorbance at 11 minutes represents 0.5 U of residual thrombin. At 10 ng heparin, the absorbance is 0.13, 86.7% of that for 0 ng heparin (0.5 U of residual thrombin), representing 0.42 U of residual thrombin (Table II in the online-only Data Supplement). The second standard curve relates the absorbance at the end of detection (11 minutes) with the inactivated thrombin enzymatic units (Figure 2C).

**FXa and Thrombin Inactivation by Native Rat Aorta and EC Culture**

We then proceeded to measure the FXa and thrombin inactivation potentials for the following biological substrates: HUVEC monolayer, native rat aorta, and heparinase-treated rat aorta. Using the standard curves (Figures 1B, 1C, 2B, and 2C), we calculated the biological substrates’ HS contents. The absorbance curves on the biological surfaces generated by residual FXa and thrombin are shown in Figures 3A and 4A, respectively. For both FXa and thrombin, the slope at 2 to 6 minutes for native aorta with intact glycocalyx was almost flat, whereas that for heparinase-treated aorta (essentially all heparin was removed) was steep. When these slopes are applied back to the FXa standard curve (Figure 1B), we obtain per cm²: 10,500 ng heparin on native aorta, 920 ng heparin on HUVECs, and heparin absence on heparinase-treated aorta (Figure 3B). Similarly, the thrombin inactivation slopes applied back to the standard thrombin curve (Figure 2B) result in comparable heparin equivalents: 10,100 ng heparin on native aorta, 890 ng heparin on HUVECs, and heparin absence on heparinase-treated aorta (Figure 4B).

When the absorbance at the end of assay (16 minutes for FXa) was applied back to the standard curve (Figure 1C), we obtained the biological surface’s capacity to inactivate FXa as shown in Figure 3C: native aorta inactivates 1.85 U of FXa, an inactivation capacity it loses after heparinase treatment; and cultured HUVEC monolayer inactivated 0.4 U of FXa, which was 5-fold lower than rat aorta’s FXa inactivation. A similar trend was seen with the biological surface’s capacity to inactivate thrombin (Figure 4C) where the native aorta inactivates 0.45 U of thrombin but loses thrombin inactivation capacity.
after the heparinase treatment. Cultured HUVEC monolayer inactivated 0.1 U of thrombin, which was 5-fold lower than rat aorta’s thrombin inactivation.

**Liquid Chromatography–Mass Spectrometry Quantification of HS in Rat Aorta and EC Monolayer Surfaces**

Using liquid chromatography–mass spectrometry (LC-MS), the HS component disaccharides were identified and quantified per cm² surface area, for native aorta and cultured HUVECs. Resulting extracted ion chromatograms and complete disaccharide analyses are presented in Figure 5. The primary disaccharide components in the native aorta were found to be unsulfated (0S) and singly N-sulfated (NS) disaccharides, accounting for 61% and 27% of the bulk HS structures, respectively. The remaining 12% of the HS bulk was diverse in structure with only 8% of the aortic HS belonging to the most highly sulfated disaccharide, the trisulfated (TriS) disaccharide. In contrast to that in the native aorta, HS in cultured HUVEC monolayer was mainly composed of TriS disaccharide (48%). Of higher relevance to our study, the cumulative sum of all disaccharides amounted to 12,560 ng of HS per cm² of aorta and 573 ng of HS per cm² of confluent HUVECs (Figure 5). These results are consistent with the heparin weights determined using the FXa and thrombin assays (Figures 3 and 4). The strong correlation between the newly developed assays and the established LC-MS quantification serves as verification supporting the newly developed assays’ accuracy in quantifying high and low

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**Figure 4.** Measurement of 1 cm² biological surface’s capacity to form procoagulant complexes and heparin equivalent estimation with the thrombin assay. A, Absorbance readings of residual thrombin generated from: native aorta, heparinase-treated aorta, and human umbilical vein endothelial cell (HUVEC) monolayer of 1 cm² surface area. The results are means±SEM of 5 independent assays using 5 different rats and HUVEC culture (n=5). B, Heparin equivalent weight was estimated via the slope of curve at 2 to 6 min (blue box in A) against the standard curve in Figure 2B. C, Inactivated thrombin was estimated via the absorbance at 11 minutes (end point in A) against the standard curve in Figure 2C. *P<0.05.

**Figure 5.** Liquid chromatography–mass spectrometry quantification of heparan sulfate (HS) content on rat aorta and human umbilical vein endothelial cell (HUVEC) monolayer surfaces. A, Representative extracted ion chromatogram of HS disaccharide standard, HUVEC monolayer (1 cm² surface area), and native rat aorta (1 cm² surface area). B, Quantification of HS disaccharide composition. The results are mean of 5 independent assays using 5 different rats and HUVEC culture (n=5).
heparin content on live surfaces within the ranges tested herein (Figure I in the online-only Data Supplement).

When the anticoagulant properties of the biological structures are normalized to milligrams of heparin, cultured HUVECs resulted in a 5-fold higher FXa and thrombin inactivation ratio relative to native aorta and a 6-fold higher ratio of TriS to total HS disaccharides relative to native aorta (Table 1). These results indicate a high anticoagulant heparin-like structure for HUVECs and low anticoagulant properties for the aortal HS.

Visual Verification of Glycocalyx Removal in Rat Aorta
HS removal by heparinase digestion on the rat aorta was visually verified at an ultrastructural level using transmission electron microscopy (TEM) imaging (Figure 6). Control aorta revealed luminal fuzzy structures, typical of the glycocalyx architecture, extending ≤50 nm in thickness (Figure 6C and 6E). After heparinase digestion, the plasma membrane of ECs was exposed, with little or no remaining glycocalyx structures (Figure 6D and 6F). The preservation of ECs post heparinase treatment was verified by hematoxylin and eosin staining (Figure 6B).

Glycocalyx Imaging With AT Binding and Immunofluorescent Staining
We probed HS chains’ capacity to bind and retain AT, to provide a visual confirmation for glycocalyx HS functionality in native aorta but loss of functionality in heparinase-treated aorta. As expected, fluorescein-labeled AT binding revealed a continuous HS chain layer on native aortas (Figure 7A), which was lost after heparinase treatment (Figure 7B). HS chain removal was further verified using a standard HS proteoglycan antibody, which also demonstrated a significant reduction in HS staining after heparinase treatment (Figure 7C and 7D). Using 3-dimensional reconstructed images, we estimated the height of the glycocalyx on the native aorta to be 60 nm thick (Figure 7E and 7G). After heparinase treatment, the green-stained layer was mostly lost, and the cell nuclei are surface exposed (Figure 7F and 7H). Using AT binding and anti-HS antibody, we also detected the glycocalyx in a monolayer of ECs that was lost after heparinase treatment (Figure II in the online-only Data Supplement), indicating the applicability of our imaging approaches.

Table 1. Comparison of Anti-FXa and Antithrombin Activity on Live Surfaces

<table>
<thead>
<tr>
<th>Live Endothelialized Surface</th>
<th>Anti-FXa Activity, U/mg</th>
<th>Antithrombin Activity, U/mg</th>
<th>TriS, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native aorta</td>
<td>154±12</td>
<td>38±7</td>
<td>8</td>
</tr>
<tr>
<td>HUVECs</td>
<td>800±21</td>
<td>200±9</td>
<td>48</td>
</tr>
<tr>
<td>HUVECs/native aorta (ratio)</td>
<td>5.2</td>
<td>5.3</td>
<td>6</td>
</tr>
</tbody>
</table>

FXa and thrombin assays were used for anti-FXa and antithrombin activity assessment, and TriS percentage was measured by established LC-MS. The results are mean±SD of 5 independent assays using 5 different rats and HUVEC cultures (n=5). FXa indicates Factor Xa; HUVEC, human umbilical vein endothelial cell; LC-MS, liquid chromatography–mass spectrometry; and TriS, trisulfated.

Discussion
The glycocalyx is thought to contribute to the ability of healthy vessels to remain antithrombogenic. Indeed, a damaged and reduced glycocalyx has been linked to various vascular pathologies.2,3,5,7,10,14,23,24 However, studies exploring the role and structure of the glycocalyx have yet to directly quantify the antithrombogenic properties of this important endothelial coating.

The primary goal of this study was to develop quantification tools to measure the antithrombogenic capacity of the glycocalyx on live endothelialized surfaces. Our newly developed assays measure a given surface’s HS content and the surface’s capacity to inactivate FXa and thrombin, 2 key enzymes in the coagulation cascade. To the best of our knowledge, the assay described in this work is the reported measurement of live endothelialized surface’s HS content and capacity to inactivate coagulant enzymes. These measurements are important to the vascular biology community, as they set the boundary conditions for heparin equivalent activity for 2 common human glycocalyx experimental models—the in vivo rat vasculature and in vitro cultured ECs.

To correlate our results with other published studies, we quantified the glycocalyx HS content on native aorta and cultured HUVECs using standard LC-MS analytic techniques. LC-MS quantification confirmed the heparin masses that were functionally determined by FXa and thrombin assays for both the aorta and HUVECs. In other words, the accepted standard LC-MS disaccharide quantification technique validated the assays’ capacity to accurately predict the HS weight content per surface area of live biological structures.

Although the primary focus of our study is the validation of the newly developed assays, it is worth mentioning that the LC-MS quantification revealed 2 different HS
glycocalyx compositions of the in vitro and in vivo endothelialized surfaces. Only 8% of the bulk aortic HS belonged to TriS, whereas the bulk of HS on HUVECs was composed of TriS. This suggests that in vitro cells preferentially initiate production of the highest functional HS chains, resulting in a 5-fold higher anti-FXa and antithrombin activity per milligram HS on cultured cells compared with native aorta. Despite the low heparin-like mass (TriS content), the aortic surface demonstrated higher anticoagulant activity per surface area because of higher total bulk HS. Therefore, our studies suggest that the functional difference observed between in vitro and in vivo glycocalyx is based on overall HS density: a denser glycocalyx is more antithrombogenic, even if it displays lower percentages of highly sulfated HS structures. These results are all the more important because there has been speculation that cultured glycocalyx is less efficacious (per area) than native vascular glycocalyx, but this functional difference has not been previously assessed.

One potential limitation of our analysis is the incomplete or partial digestion of the surfaces, resulting in inaccurate LC-MS quantification of HS amounts. To confirm full digestion of the surface glycocalyx, we visually verified the removal of HS after heparinase digestion using TEM and AT binding. Although TEM is an accepted glycocalyx visualization tool, we postulated that the HS–AT binding interaction would allow direct visualization of heparin-like domains in the glycocalyx, because heparinase-digested glycocalyx is known to have reduced AT-binding capacity. Our TEM and confocal imaging confirmed the complete HS removal after heparinase digestion treatment.

Another potential limitation of our assay is an inaccurate measure of the glycocalyx HS chains because of the bound form while the functional assay standard was soluble unfractionated heparin. One advantage of fully soluble heparin is that all of heparin chains are accessible to AT binding and FXa/thrombin inactivation. However, in assessing the anticoagulant activity of surface-bound HS, it is possible that there is an underestimation of activity, because there may be sections of the HS chains that are buried or inaccessible to AT binding for subsequent FXa/thrombin inhibition.

Finally, it should be noted that our assay is limited in scale, by only measuring the inactivation of 2 enzymes (FXa and thrombin). Because the coagulation cascade is more complex than just these 2 proteases, these assays performed together and independently may not be representative of a surface’s complete antithrombogenic capacity. However, because FXa and thrombin are prominent proteases of the coagulation cascade, and the final common enzymes of the intrinsic and extrinsic pathways, their inactivation represents a reasonable approximation of the coagulation cascade activation potential leading to fibrin formation and blood clots. In fact, the inactivation of FXa and thrombin is routinely used to measure the anticoagulant property of medical heparin and heparin-like materials.

In this work, we developed novel assays to examine the capacity of live surfaces to inactivate coagulant enzymes. Our measurements of anticoagulant activity, HS structural composition, and quantification of HS amounts will help set a baseline of structure and function of healthy endothelium and endothelial surfaces. This assay is a powerful yet simple-to-use tool for studying the functional anticoagulant capacity of live endothelialized surfaces. Furthermore, this assay can be used to characterize the antithrombogenic capacity of many different types of surfaces, such as vascular grafts and transplants, which commonly fail because of blood clotting (thrombosis). Better characterization of live surfaces’ coagulant capacity will also enable us to develop therapeutic treatments for glycocalyx restoration.

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**Disclosures**

L.E. Niklason is a founder and shareholder in Humacyte, Inc, which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. L.E. Niklason’s spouse has equity in Humacyte, and L.E.
Niklason serves on Humacyte’s Board of Directors. L.E. Niklason is an inventor on patents that are licensed to Humacyte and that produce royalties for L.E. Niklason. L.E. Niklason has received an unrestricted research gift to support research in her laboratory at Yale. The other authors report no conflicts.

References

Highlights
- New in vitro assays were developed to quantify live vascular structures' and cultured endothelial cells' glycocalyx capacity to inactivate procoagulant enzymes.
- The assays provide easy and accurate evaluation of live endothelialized surface antithrombogenic functions as verified by liquid chromatography–mass spectrometry disaccharide analysis.
- Cultured endothelial cells initiate glycocalyx production with high anticoagulant structures but produce less overall glycocalyx compared with native aortas.
New Functional Tools for Antithrombogenic Activity Assessment of Live Surface Glycocalyx


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New functional tools for anti-thrombogenic activity assessment of live surface glycocalyx

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Materials and methods

Rat aorta harvest for functional assessment
All experimental animal work was approved by the Yale University Institutional Animal Care and Use Committee. Thoracic aortas were harvested from young adult (3 month-old) male Sprague Dawley rats. After induction of anesthesia with sodium pentobarbital, a transverse incision was made below the costal margin, entering the abdominal cavity. The heart and aorta were exposed after resecting the ribs. The portion of the aorta at the level of the renal arteries was partially incised to allow blood to be removed. The aorta was perfused through the right ventricle with 5 mL PBS, after which the thoracic aorta from the aortic valve to the diaphragm was excised and placed in Dulbecco’s Modified Eagle Medium. The aorta was incised along its longitudinal axis to provide luminal surface exposure of the ECs and used without further treatment unless indicated otherwise.

HS surface digestion of rat aorta
Digestion of HS on the luminal EC surface was carried out in vivo in three rats. The renal portion of the aorta was incised to allow blood to be removed and the aorta was gently flushed with PBS. Heparinase digestion was restricted to the aorta by ligating the main aortic branches using Prolene 6.0 suture under magnification. Following the isolation of the aorta from its large branches, a 5 mL mixture of heparinase I, II and III (1000 mU each) was injected into the arch using a 28 gauge needle syringe. Following a 30 min incubation, the heparinase solution was aspirated and reserved for further LC-MS analysis. The aorta was excised for further analysis.
Culture of HUVEC monolayer and heparinase digestion

HUVECs were isolated from umbilical veins by treatment with collagenase. HUVECs were serially passaged 2 times in gelatin-coated tissue culture plastic using M199 medium supplemented with 20% FBS, 2% L-glutamine, 1% penicillin and streptomycin, 50 μg/mL endothelial cell growth supplement at 37 °C in a 5% CO₂ humidified air incubator. HUVECs were seeded onto 48 well plates until confluence (2-3 days). For heparinase digestion, cell monolayer was treated for 30 min with a heparinase I, II, and III mixture (1000 mU each) and the heparinase solution was aspirated and saved for further LC-MS analysis.

Functional analyses of aorta and cell culture monolayer’s capacity to inactivate FXa and thrombin

Native vasculature and EC’s ability to inhibit FXa and thrombin was determined per unit surface area (cm²) on native rat aorta (freshly harvested and untreated), heparinase-treated rat aortas (heparin was completely removed while preserving ECs), and HUVEC monolayer. Native rat aortas were cut to 1 cm² surface area and placed in a 48 well plates with the luminal EC surface facing upwards. Both the FXa and thrombin assays were limited to 16 min, thereby preserving cellular viability for the duration of the experiment.

The FXa assay measures a surface’s ability to inactivate FXa, via the formation of the AT-FXa complex. Aortas or cells were incubated with 100 μl AT (25 mU/mL) in 50 mM Tris buffer (pH 8.4) for 5 min at 37°C. This allowed AT to bind surface-exposed HS and become activated. Thereafter, FXa (20 U/mL in 1 mg/mL BSA/PBS) was added and incubated for 2 min at 37°C, allowing activated AT to bind to FXa and inactivate FXa. The supernatants were then transferred into a 96-well plate where 25 μL of 1 mM chromogenic substrate S-2222 (Chromogenix) was added, mixed, and incubated for 10 min at 37°C and color change recorded every minute. Hence, the amount of FXa that was unbound to the cell or tissue surface is measured by the chromogenic reaction. Each sample group had three biological replicates, and each biological replicates was analyzed twice to provide technical duplicates.

The thrombin colorimetric assay measures a surface’s ability to inactivate thrombin through the formation of AT-thrombin complex. Aortas or cells were incubated with 100 μl AT (25 mU/mL) in 50 mM Tris buffer (pH 8.4) for 5 min at 37°C. Thereafter thrombin (5 U/mL in PBS) was added and incubated for 2 min at 37°C. The supernatants were then transferred into a 96 well plate where 25 μL of 1 mM chromogenic substrate S-2238 was added, mixed, and incubated for 16 min at 37°C, and the color change recorded. Similar to the FXa assay, it is the amount of residual, non-complexed thrombin that is measured in this assay. Each sample group had three biological replicates, and each biological replicates was analyzed twice to provide technical duplicates.

Both FXa and thrombin assays used logarithmically increasing heparin (heparin sodium salt from porcine intestinal mucosa, MW 6000-30000 D, Sigma) dissolved in 100 μl 50 mM Tris buffer as controls. Briefly, 100 μL AT (25 mU/mL) in 50 mM Tris buffer (pH 8.4) was added to the heparin solution and incubated for 5 min at 37°C. Thereafter, FXa (20 U/mL in 1 mg/mL BSA/PBS) or thrombin (5 U/mL in PBS) was added and incubated for 2 min at 37°C, allowing activated AT to bind and inactivate FXa
or thrombin, respectively. The mixture was then transferred into a 96-well plate where 25 μL of 1 mM chromogenic substrate S-2222 (for FXa assay) or S-2238 (for thrombin assay) was added, mixed, and incubated for 10 min at 37°C and color change recorded every minute.

**Liquid chromatography mass spectrometry**

The weight of HS, in both the aortic wall and in cell culture monolayer, was measured by liquid chromatography mass spectrometry (LC-MS) analysis. Briefly, as explained in the previous section a mixture of heparinase I, II, and III (a 5 mL mixture of heparinase I, II and III (1000 mU each) was added to either the aortas of three Sprague Dawley rats or three independent HUVEC monolayers grown in 48 well plates, and incubated for 30 min at 37°C. HS disaccharides were recovered by syringe aspiration, followed by centrifugal filtration on an YM-10 spin column, and lyophilizing the resulting retentate overnight. To ensure equal surface area of 1 cm², the length and diameter of perfused aortas were measured and the recovered retentate volume was normalized to 1 cm² surface area. The entire recovered retentate from HUVEC culture was used, as each 48 well represents 1 cm² surface area. Recovered unsaturated disaccharides were then labeled using 2-aminoacridine (AMAC) by adding 10 μL of 0.1 M AMAC solution onto HS-derived disaccharides or to a mixture of the following 8 HS disaccharide standards: 0S, 2S, 6S, 2S6S, NS, NS2S, NS6S and TrisS (Iduron Ltd). Next, 10 μL of 1 M NaBH₃CN was added in the reaction mixture and incubated at 45°C for 4 h. Finally, the AMAC-tagged disaccharides were diluted to different concentrations (0.5-100 ng) using 50% (v/v) aqueous DMSO. LC-MS analyses were performed on Agilent 1200 LC/MSD (Agilent Technologies) with a 6300 ion-trap and a binary pump and poroshell C18 column. Eluent A was 80 mM ammonium acetate and eluent B was methanol. The electrospray interface was negative ionization with a skimmer potential of -40.0V, a capillary exit of 40V, and a source temperature of 350°C.

**Transmission electron microscopy (TEM)**

The heparinase efficacy for removing glycocalyx was visually verified using TEM by comparing native and heparinase-digested aortas. Both native and heparinase-digested aortas were perfusion-fixed through the right ventricle by direct injection of 4% phosphate-buffered paraformaldehyde, pH 7.3, over 5 min. Aortas were then excised and immersed for 1 hour in 2.0% paraformaldehyde and 2.5% glutaraldehyde containing 0.075% ruthenium red, 75 mM lysine, and 0.1 M cacodylate pH 7.2 and then immersed overnight in the same solution. Following a rinse with 0.1 M cacodylate, tissues were post-fixed with 1% osmium tetroxide and dehydrated through a graded series of ethanol solutions, and embedded in LX112 Resin. All sections were viewed with a Zeiss EM-900, and imaged with a Mega View III Soft Imaging System.

**Visualization of HS-specific proteoglycan**

We developed a method for detection of HS chains directly through AT binding and validated it by direct visualization using immunofluorescent staining with a well-characterized HS-proteoglycan specific antibody. Fluorescein-labeled AT was prepared by mixing 1 mL of AT (25 UG/mg, purified from human plasma) with N-
hydroxysuccinimide-fluorescein (10 μL, 830 nmol, Pierce) in 50 mM (pH 8.5) sodium borate. The reaction was mixed, incubated for 1 h, and dialyzed against distilled water. Heparinase-treated and untreated control rat aortas and cell monolayer cultures were fixed in 4% paraformaldehyde for 2 h, washed with PBS, and blocked in 1% FBS for 30 min. The samples were then incubated with fluorescein-AT (1:100) or anti-HS antibody (1:100, HepSS-1, Amsbio) for 1 h, washed with PBS, and incubated with Alexa Fluor 488 secondary antibody (Invitrogen) for 1 h. For nuclear staining, the samples were incubated with DAPI (Invitrogen) for 5 min, mounted with Vectashield medium (VectorLabs) and imaged using a Leica microscope.

**Statistics**

Each sample group had three biological replicates, and each biological replicates was analyzed twice to provide technical duplicates. Data were expressed as mean ± standard error of means (SEM). Unpaired, two-tailed Student's t-tests were performed to evaluate whether the different groups were significantly different from each other. A value of p ≤ 0.05 was considered statistically significant.
Supplemental Material

New functional tools for anti-thrombogenic activity assessment of live surface glycocalyx

Authors

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Supplemental Figure I. Comparison between different assays for detecting heparin weight on live surfaces. Total weight of heparin equivalent per cm² live surfaces (native aorta and HUVEC monolayer) was measured by LC-MS, FXa assay and thrombin assay. The results are mean of five independent assays using five different rats and HUVEC cultures (n=5).
Supplemental Figure II: Confocal images of the glycocalyx layer on rat fat pad endothelial cell (RFPEC) monolayer. (A, B, E, F) HS is visualized by fluorescein-labeled AT (green). (C, D, G, H) HS is visualized by anti-HS antibody (green). Also shown is DAPI costaining (blue). The X-Z plane side view showing the depth of the glycocalyx in RFPEC monolayer (E, G) and heparinase-treated RFPECs (G, H).
Supplemental Table I. Calculation of inactivated FXa using heparin standard

<table>
<thead>
<tr>
<th>Heparin weight (ng)</th>
<th>Absorbance at 16 min</th>
<th>Residual FXa (Unit)</th>
<th>Inactivated FXa (Unit)</th>
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<tr>
<td>0</td>
<td>0.3</td>
<td>2</td>
<td>0</td>
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Absorbance from FXa assay using heparin standard shown in Figure 1A
## Supplemental Table II. Calculation of inactivated thrombin using heparin standard

<table>
<thead>
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<th>Heparin weight (ng)</th>
<th>Absorbance at 11 min</th>
<th>Residual thrombin (Unit)</th>
<th>Inactivated thrombin (Unit)</th>
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<tr>
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<tr>
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Absorbance from thrombin assay using heparin standard shown in Figure 2A