

A microscopic view on the renal endothelial glycocalyx

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Dane MJ, van den Berg BM, Lee DH, Boels MG, Tiemeier GL, Avramut MC, van Zonneveld AJ, van der Vlag J, Vink H, Rabelink TJ. A microscopic view on the renal endothelial glycocalyx. *Am J Physiol Renal Physiol* 308: F956–F966, 2015. First published February 11, 2015; doi:10.1152/ajprenal.00532.2014.—Endothelial cells perform key homeostatic functions such as regulating blood flow, permeability, and aiding immune surveillance for pathogens. While endothelial activation serves normal physiological adaptation, maladaptation of these endothelial functions has been identified as an important effector mechanism in the progression of renal disease as well as the associated development of cardiovascular disease. The primary interface between blood and the endothelium is the glycocalyx. This carbohydrate-rich gel-like structure with its associated proteins mediates most of the regulatory functions of the endothelium. Because the endothelial glycocalyx is a highly dynamic and fragile structure *ex vivo*, and traditional tissue processing for staining and perfusion-fixation usually results in a partial or complete loss of the glycocalyx, studying its dimensions and function has proven to be challenging. In this review, we will outline the core functions of the glycocalyx and focus on different techniques to study structure-function relationships in kidney and vasculature.

renal; endothelial glycocalyx; endothelial surface layer; imaging; glomerular endothelium

THE ENDOTHELIAL GLYCOCALYX, or endothelial surface layer, is a negatively charged gel-like surface structure of proteoglycans with their covalently bound polysaccharide chains called glycosaminoglycans (GAGs), glycoproteins, and glycolipids. Its main carbohydrate constituents are heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronan (HA). The endothelial glycocalyx governs transcapillary fluid exchange and acts as a biomechanical sensor to confer shear to the endothelium (endothelial cells) (48, 49, 52). GAGs within the glycocalyx function as a molecular scaffold that facilitates protein binding, in a very selective manner. In this way, circulating proteins, such as e.g., growth factors and chemokines, are concentrated and spatially organized in gradients at the endothelial surface (64, 90). The proteins that bind to the glycocalyx include proteins involved in cell attachment, migration, differentiation, morphogenesis, blood coagulation, lipid metabolism, and inflammation, thus putting the endothelial surface layer at the very center of the pathophysiology of cardiovascular and renal disease. However, despite its pivotal role in endothelial cell biology, glycocalyx function has proven to be hard to study due to its complex carbohydrate chemistry and the difficulties

in interrogating its function *in vivo* and *in vitro*. Here, we describe the structure and main biological functions of the endothelial glycocalyx in the kidney. In addition, we will discuss our current approaches to study and visualize the glycocalyx.

Biochemical Structure of the Endothelial Glycocalyx

The membrane-bound part of the endothelial glycocalyx consists of proteoglycans, glycoproteins, and glycolipids (Fig. 1). Proteoglycans with bound GAGs are the main contributors to endothelial glycocalyx structure and function (87). Of these GAGs, heparan sulfate and hyaluronan constitute up to 90% (28, 78, 88).

Heparan sulfate is a linear polysaccharide which consists of the repeating disaccharide β 1–4-linked D-glucuronic acid (GlcA) and α 1–4-linked N-acetyl-D-glucosamine (GlcNAc). This polymer is covalently attached to a limited number of core proteins at the cell surface called heparan sulfate proteoglycans (HSPGs). The proteoglycans found on the luminal endothelial side are syndecans 1 and 4, glypican 1, versican, and thrombomodulin. HS chains are processed in the Golgi apparatus, where they undergo a series of modifications in which subsets of glucosamine residues can become N-deacetylated and N-sulfated and where glucuronic acids may undergo epimerization to L-iduronic acid (IdoA). In particular, C2 of uronic acid and

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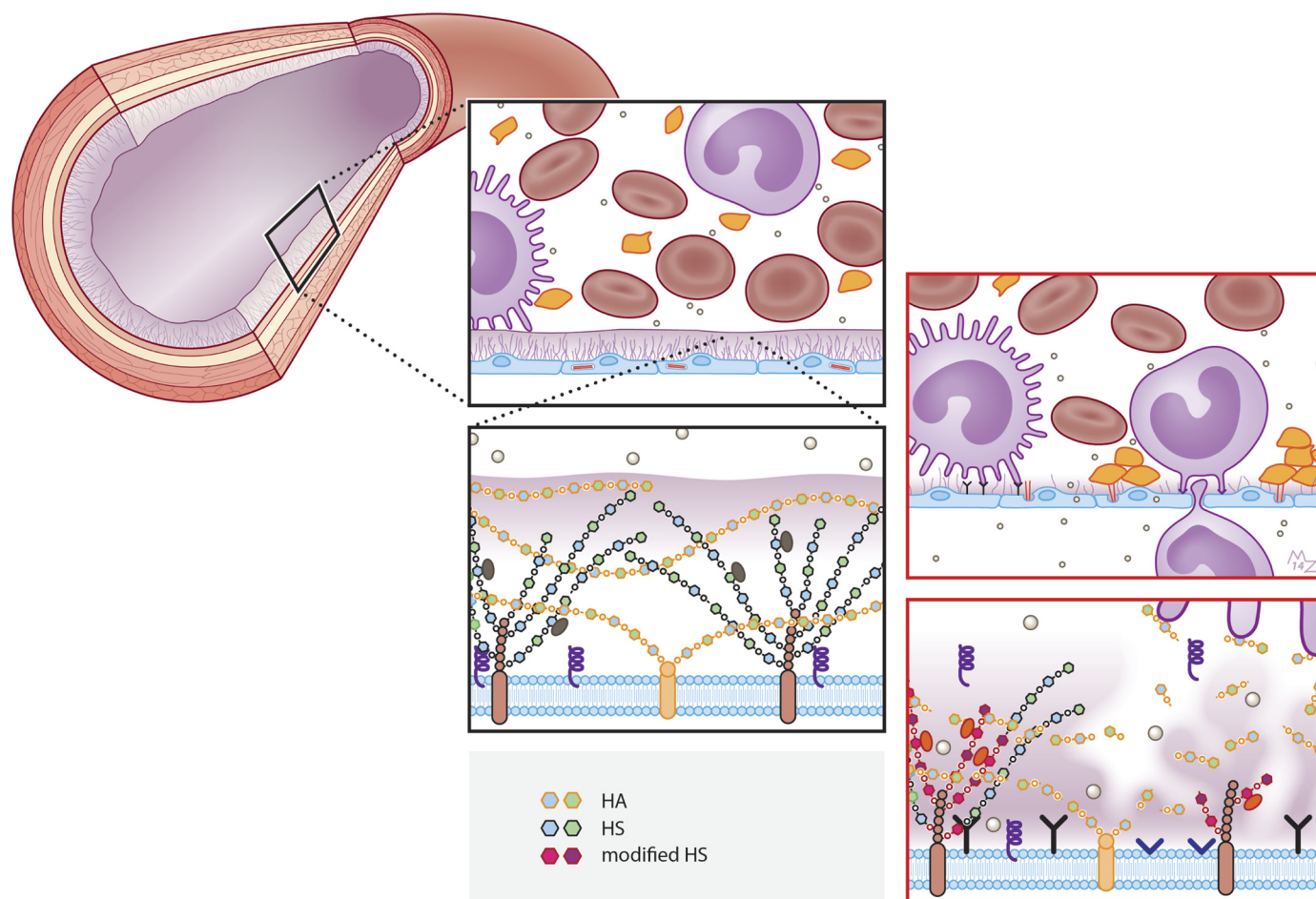


Fig. 1. Schematic overview of the endothelial glycocalyx (EG) under healthy and diseased conditions. *Left*: in a physiological state, the EG protects against protein leakage, inflammation, and coagulation. Heparan sulfates, bound to a heparan sulfate (HS) core protein, and hyaluronan (HA), bound to e.g., CD44, are the main constituents of the endothelial glycocalyx. The order and modification of disaccharide repeats within HS determine the binding site for specific proteins. *Right*: upon endothelial activation, heparan sulfate disaccharide modification occurs, resulting in a change in protein binding sites. During a chronic disease condition, the EG gets damaged, mainly due to upregulation of glycocalyx-degrading enzymes such as hyaluronidase, heparanase, and proteinases. Shed proteoglycans and glycocalyx fragments in the serum can bind and influence circulating leukocytes. Both HS modification and EG degradation result in inflammation, coagulation, and protein leakage.

C6 (and rarely C3) of glucosamine residues may become sulfated (25). After intracellular processing, further extracellular modification can occur. Heparanase (HPSE1) and endosulfatases (SULF1,2) can cleave the HS chain or further modify the sulfate groups within HS, respectively (26). Although they do attribute to a wide variety of specific HS binding sites for protein interactions that determine endothelial cell biology, regulation of these modifications is still not well understood. While the core proteins can function independently of the HS chains they carry, the HS chains predominantly dictate ligand-binding capability and therefore the biological roles of HSPG. Some examples are discussed in the next section. HS is structurally related to heparin, an extremely highly sulfated form of HS that is restricted to mast cells (11, 25, 26).

Hyaluronan lacks the complex chemical editing of HS. It is a nonsulfated GAG composed of repeating polymeric disaccharides D-glucuronic acid and N-acetyl-D-glucosamine linked by a glucuronidic bond (48, 49). Under physiological conditions, hyaluronan is synthesized by membrane-bound synthases (HAS1, 2 and 3) as a macromolecule of 10^5 – 10^7 Da. Following its synthesis, hyaluronan is directed to the cell

surface where it interacts with hyaluronan binding surface proteins (hyaladherins) such as CD44, or is assembled into the pericellular extracellular matrix (52). The repeating nature of the polysaccharide, and hence its protein binding sites, has been suggested to confer structural periodicity to the endothelial surface layer (21). The main modifier of hyaluronan is hyaluronidase, an enzyme that cleaves high-molecular weight (HMW) hyaluronan chains into smaller low molecular weight (LMW) hyaluronan fragments.

Function of the Renal Endothelial Glycocalyx

The glycocalyx and endothelial heterogeneity. Glycocalyx thickness and composition vary from organ to organ, and its differences may well contribute to heterogeneity in endothelial function. For example, at sites with lower or oscillatory shear such as the carotid artery bifurcation, the thickness of the glycocalyx is reduced compared with the high shear in areas such as the common carotid (104). This reduction is associated with increased transcapillary transport of lipoproteins. We recently could demonstrate that the endothelial glycocalyx is

structurally different when comparing fenestrated to nonfenestrated endothelium within the glomerular capillary (19). Also, the chemical composition of the glycocalyx changes from one organ to another and even within an organ (57). Compared with the heart, HS in the kidney has e.g., a lower sulfation grade, and as we will discuss below such changes may translate into different biological behavior (95) and organ specificity of diseases. The highly heterogeneous renal endothelial bed in the kidney, with fenestrated endothelium in the glomerular capillaries, adapted to support a high fluid flux, and the nonfenestrated endothelium in the vasa recta, specialized in uptake of ions, are most likely also different in endothelial glycocalyx composition (2).

The glycocalyx and glomerular filtration. Albumin has a radius of 3.5 nm, and while it readily can pass through the large glomerular fenestrae (~60 nm) from this perspective, it is nevertheless assumed to have a very low sieving coefficient ($\Theta = 0.0021$) across the glomerular filtration barrier (35, 59). Numerous calculations have been made to understand the physical properties of the glomerular barrier and how the glycocalyx may affect the transport of proteins across the glomerular barrier, which have been reviewed earlier (35). It has been postulated that glycocalyx polyanions such as heparan sulfate and sialated proteins on the surface of the endothelium and podocytes, as well as in the glomerular basement membrane, would electrically repel the negatively charged albumin (12, 13). Although this is supported by data with nonmetabolizable negatively charged probes that would support that electric charge would modify filtration of albumin (7, 75), this concept has been challenged by *in vivo* studies of the fractional clearance of negatively charged Ficoll compared with Ficoll where negative charge selectivity did not exist (33, 65).

Irrespective of this debate, it is very plausible that the glycocalyx acts as a size barrier to albumin filtration. The glycocalyx basically functions as a hydrogel where glycosaminoglycans, i.e., heparan, chondroitin sulfate, and hyaluronan, constitute a fiber mesh with pores (4, 16, 50). Plasma proteins bind multivalent to these GAGs, thus creating steric hindrance to protein filtration. This barrier function will be further modified by the fluid drag through the glycocalyx, which allows for dynamic equilibrium with glycocalyx-bound and -free circulating proteins (114). Such a size barrier function of the endothelial glycocalyx has in fact also been suggested from observations in the microcirculation in general, where the hydrostatic and osmotic forces that determine fluid filtration were shown to exist only across the glycocalyx and not across the endothelial cell toward the interstitium (1). It is of interest that elongated molecules, such as bikunin and hyaluronan have >100-fold higher glomerular sieving coefficients than albumin, despite similar molecular weights and charges (58). While such experiments may be challenged by confounding due to metabolism of albumin by the proximal tubulus (15), they can also be interpreted to demonstrate the importance of pore characteristics in relation to the molecular conformation of the protein sieved. The latter is further supported by recent observations that large straight nanotubes (200–300 nm) may be filtered by the glomerulus similarly to small molecules (92).

The relevance of the glomerular endothelial glycocalyx as a first-line barrier against albumin filtration was demonstrated by Ryan and Carnofsky (93). They demonstrated that under phys-

iological conditions hardly any albumin was observed in the GBM or underlying podocytes (93), indicating that the endothelial layer functions as a barrier to proteins. In support, GAG-degrading enzymes such as chondroitinase and heparinase have been shown to alter the permeability of the glomerular filter (43, 44). We recently demonstrated that the fenestrae in the glomerular endothelium are predominantly filled with hyaluronan (19). Quantitative electromicroscopy confirmed that under physiological conditions there is virtually no albumin passage through the glomerular filter, while enzymatic removal of hyaluronan from these fenestrae, and more recently endothelial-specific deletion of the hyaluronan-producing enzyme *hs2*, results in albumin passage into the nephron (102). Even local displacement of only the noncovalently HS-bound proteins has been demonstrated to result in a 12-fold increase in fractional albumin clearance (30). This indicates that besides GAGs, as discussed above, the loosely bound plasma proteins are also essential for the structure of the endothelial glycocalyx and for the barrier function of the glomerulus.

Biomechanical properties. An intact glycocalyx serves as the primary sensor of shear stress on endothelial cells. Hydrodynamic drag arising from plasma flow through the layer transmits fluid shear stress through the thin sublayer containing core proteins and into the endothelial cell cytoskeleton (113). The presence of both hyaluronan, possibly by linking to endothelial sodium channels in the endothelium, and HS appears to be critical for this function (84). For example, enzymatic removal of hyaluronan or HS from the glycocalyx results in reduced nitric oxide (NO) release during shear (66). Heparinase III, a bacterial enzyme that cleaves HS proteoglycans, has a dramatic effect on the endothelial cells' ability to produce NO to shear stress and transfer shear forces to the actin cytoskeleton (28, 101). Interestingly, the enzyme chondroitinase, employed to selectively degrade CS, did not affect shear-induced NO production, underpinning the importance of HS for the biomechanical properties of the endothelial glycocalyx (80).

Inflammation. As illustrated in Fig. 1, the disease process resulting in endothelial glycocalyx perturbation shows that upon endothelial activation, i.e., inflammation, the dense glycocalyx will be modified in amount and composition, mainly due to upregulation of glycocalyx-degrading enzymes such as hyaluronidase, heparanase, endosulfatases, and proteinases. These shed proteoglycans and glycocalyx fragments in the serum can bind and in turn influence circulating leukocytes. The gel-like antiadhesive properties of the endothelial glycocalyx preclude direct leukocyte interaction with adhesion molecules on the endothelial surface. However, during their passage through the capillaries the leukocytes will compress the endothelial glycocalyx, thus allowing for engagement of the microvilli of the leukocyte with the GAGs in the glycocalyx. For example, HS on the endothelium acts as a direct ligand for L-selectin (73, 74). Interestingly, this interaction is modulated by very specific HS sulfation patterning. Endothelial deletion of the biosynthetic enzyme *N*-acetylglucosamine *N*-deacetylase-*N*-sulfotransferase-1 (*Ndst1*), which leads to decreased sulfation of the HS chains in the endothelium, reduces the binding of L-selectin and leads to an increase in neutrophil rolling velocity (112). Inflammatory stimuli such as TNF- α in their turn induce *NDST1*, which results in expression of HS domains on the glomerular endothelium that are associated

with inflammation (89, 91). In agreement, we recently showed that endothelial deletion of NDST1 results in reduced leukocyte extravasation in experimental anti-GBM glomerulonephritis (91).

The migration of leukocytes across the endothelium occurs along chemoattractant gradients composed of chemokines that have bound to the endothelium (64). Nearly all members of the chemokine family bind to HS by way of a positively charged C-terminal domain. Again, this binding may be modified by chemical editing of HS chains. For example, changing the domain structure of HS by inactivation of *Hs2st* (which causes a compensatory increase in glucosamine *N*-sulfation and 6-*O*-sulfation) results in enhanced binding of IL-8 to the endothelium and increases inflammation (5).

Endothelial HS has also been shown to be critical for the function of the complement system. The function of many of the components of the complement system, including C1, C1q, C1 inhibitor, C2, C4, C4b, FactorB, FactorD, FactorH, Properdin, and complement receptors CR3(CD11b/CD18) (24) and CR4 (CD11c/CD18), is dependent upon binding to specific GAG domains (83, 94, 115). The clinical importance and the specificity of these interactions are illustrated by patients with mutations in the short consensus repeats 19 and 20 of the complement inhibitor factor H. These mutations result in impaired binding of factor H to the glomerular endothelium, but not to other endothelial beds. Upon a trigger that activates the complement system, this may result in glomerular endothelial injury and presents itself clinically as atypical hemolytic uremic syndrome (8).

In addition to HS, hyaluronan has also been demonstrated to modulate inflammatory processes. The HMW form of hyaluronan possesses anti-inflammatory, antiangiogenic, and immunosuppressive properties and protects cells from injury and leukocyte adhesion (117). In part this is related to its gel-like physical properties. In addition, binding of TNF-stimulated gene 6 (TSG-6) to hyaluronan inhibits chemokine-stimulated transendothelial migration of neutrophils via a direct interaction between TSG-6 and the GAG-binding site of CXCL8 (23). However, during tissue injury and inflammatory processes hyaluronan can become depolymerized through oxidative stress and enzymatic cleavage by hyaluronidases. This results in the formation of LMW hyaluronan fragments (45). The removal of LMW hyaluronan from sites of injury is dependent on their interaction with CD44, and the associated downstream signaling events have been implicated in cell proliferation, migration, and activation. LMW hyaluronan also has been demonstrated to interact with Toll-like receptors, enabling hyaluronan signaling in inflammatory cells (45, 46).

Anticoagulation. Both the biomechanical properties as well as glyocalyx-protein interactions play a prominent role in preventing blood from clotting. The gel-like properties of the glyocalyx prevent platelets from accessing the endothelium (86). HS in the glyocalyx is required to activate antithrombin, a serine protease that inhibits thrombin and thus the conversion of fibrinogen to fibrin. By acting as a scaffold and simultaneously binding to both enzyme and substrate, HS greatly increases engagement of thrombin with antithrombin (55, 77). This interaction depends upon a specific pentasaccharide, an insight that led to the clinical development of fondaparinux. By binding the plasma protein heparin cofactor II (HCII), heparan sulfate repeats in the glyocalyx further inhibit the procoagu-

lant activity of thrombin. The latter pathway has been shown to counteract thrombus formation in the presence of endothelial injury (36).

A key regulator of the natural anticoagulant system is the endothelial glyocalyx protein thrombomodulin. Upon binding of thrombin, it generates activated protein C (APC). APC regulates blood coagulation by cleaving and inhibiting two cofactors, activated factor V (FVa) and activated factor VIII (FVIIIa), which serve as phospholipid- membrane-bound cofactors to factor Xa (FXa) and factor IXa (FIXa), respectively. APC not only has anticoagulant properties but also through PAR signaling induces a quiescent phenotype in the endothelium (39, 82). Activation of this pathway could prevent the development of diabetic nephropathy, underscoring the importance of APC formation in prevention of kidney disease (39). Finally, thrombomodulin-thrombin binding activates thrombin-activatable fibrinolysis inhibitor (TAFI), thus suppressing fibrinolysis at the same time. This system is intensively modulated by interactions with GAGs. First, thrombomodulin can bind chondroitin sulfate, greatly facilitating the interaction with thrombin and the formation of APC (56, 116). The activity of APC and thrombin in their turn are negatively regulated by protein C inhibitor, which form together with different HS repeats ternary complexes (54). While very complex and still only partially understood, the intricate interaction of the coagulation system with the glyocalyx demonstrates the importance of the latter as a molecular scaffold.

Studying the Endothelial Glyocalyx

Because the endothelial glyocalyx is a highly dynamic and fragile structure, it is unstable when taken out of its *in vivo* environment. Consequently, bound plasma components and GAGs can be lost during fixation, dehydration, sectioning, and staining procedures. As a result, only membrane-bound parts of the glyocalyx will remain. Avoiding this loss of the surface layer is one of the biggest challenges in studying structure-function relationships of the endothelial glyocalyx. In this section, we will discuss the state-of-the-art and recent developments on technology that enables the imaging and characterization of the endothelial glyocalyx.

Electron microscopy. The high polysaccharide content of the endothelial glyocalyx interacts poorly with the commonly used postfixation stains. As a result, the endothelial glyocalyx scatters few electrons and is indistinguishable from its environment in conventionally processed samples for transmission electron microscopy (TEM) (24). Thus, even if the pretreatment of the sample maintains endothelial glyocalyx integrity, an additional staining or preservation is required for the visualization of the glyocalyx in TEM. This explains the absence of visible glyocalyx structures in almost all tissues that are conventionally processed for TEM. The first to succeed in staining the glyocalyx for electron microscopy was Luft in 1966, who used ruthenium red, or ammoniated ruthenium oxychloride, which stains the aldehyde-fixed mucopolysaccharides and generates an electron-dense stain in the presence of osmium tetroxide (63). The presence of the luminal glyocalyx has subsequently been shown using TEM in combination with a variety of staining and preservation techniques such as ruthenium red, ferritin, and lanthanum. Most of these cationic dyes bind to the negative charge of the sulfated GAGs and

form electron-dense contrast together with osmium tetroxide, to enable visualization (6, 10, 14, 41, 111). Using alcian blue 8GX, van den Berg et al. (105) were the first to be able to better stabilize the anionic carbohydrate structures in myocardial capillaries, thereby visualizing an impressive endothelial glycocalyx of up to 500 nm thick (105). The saccharine nature of these visualized structures was confirmed by perfusion of gold-labeled lectins before the staining procedure. Recently, we were able to visualize the endothelial glycocalyx in the renal glomerulus using cupromeronic blue, a chemically more stable cationic dye resembling some alcian blue properties (19). This revealed a staining of matrix polysaccharides on the luminal surface of the glomerular endothelial membranes and a dense staining of polysaccharide matrix within the fenestrae. Examples of the described methods to visualize the endothelial glycocalyx are shown in Fig. 2.

Because optimal preservation of the glycocalyx and its visualization require the use of perfusion-fixation techniques, it has not been possible to reliably image the layer in patient biopsy material. Although not yet shown for this purpose, a combination of rapid freezing and freeze substitution of the tissue might be an interesting way to preserve the glycocalyx. With a combination of instantaneous high pressure and a rapid decrease in temperature, high-pressure freezing preserves the tissue while reducing the formation of ice crystals. In a frozen state, water can be replaced and tissue can be stained simultaneously. In this way, molecules can be fixated in plastic, while their native structure is preserved as much as possible. Results of this procedure are shown in the example of a high-pressure frozen mouse renal sample which is stained during freeze substitution with acridin orange and uranyl acetate (Fig. 3). Although further optimization is necessary, it does appear to be a promising technique that might pave the way for studying changes in the glycocalyx in biopsies of patients.

Fluorescence microscopy. The carbohydrates within the glycocalyx have been usually imaged using specific lectins, mostly in combination with fluorescence microscopy. Lectins are carbohydrate binding proteins that recognize specific sugar moieties and 3D configurations. The most commonly used lectins are *Lycopersicon esculentum* agglutinin (LEA) and wheat germ agglutinin (WGA) (Fig. 4). Although lectin staining of the endothelial glycocalyx can be used to study changes in dimensions, it does not allow determination of changes in the specific composition of different GAGs. Consequently, antibodies are needed to more specifically determine compositional changes within the glycocalyx. Over recent years, several antibodies have been obtained against specific carbohydrate domains within HS (e.g., JM403, 10E4, and HEPSS1), HA, and CS sulfates or against the proteoglycan core proteins such as syndecan, glypican, or perlecan (31, 96–98, 118, 119). CS and HA have been shown to be present on the endothelium, using an anti-CS antibody and by capitalizing on the protein-binding properties of hyaluronan with a fluorescent hyaluronan binding protein (HABP), respectively (29, 62, 118). Although all these techniques have shown the presence of glycocalyx components on the endothelial cells, a considerable part of these studies are done using epifluorescence microscopy, which has major limitations with respect to optical spatial resolution necessary for imaging the luminal endothelial glycocalyx. With the development of confocal and two-photon laser-scanning microscopy (TPLSM), fluorescence microscopy

has been improved in such a way that the resolution is high enough to be used for detailed endothelial glycocalyx imaging *in situ*.

A complex but promising field for studying endothelial surface heparan sulfates is the use of single-chain antibodies. For the selection of these single-chain antibodies, a synthetic single-chain variable fragment library is used. Of these single-chain variable fragments, specific antibodies can be selected. In this way, antibodies against tissue-specific modified HS domains can be produced. This method has been used previously to study heparan sulfate domains present in the basement membrane (106). Although the same principle is used to study the role of heparan sulfates present on glomerular endothelial cells (89), it has never been used to image these heparan sulfates specifically on the luminal endothelial surface. Therefore, the use of single-chain antibodies in conditions that allow for endothelial HS staining might be interesting to obtain more insight into the complexity of the different subcomponents of the glycocalyx.

Circulating markers of the glycocalyx. In response to reactive oxygen species (ROS) or other inflammatory mediators, both single GAGs and proteoglycans can be shed from the endothelium (47, 61, 68). This has been suggested to be mediated by endoglycosidases such as heparanase and hyaluronidase, or proteases such as matrix metalloproteases (60, 61, 69). Consequently, measuring shed glycocalyx components might be considered for use as a marker for endothelial glycocalyx stability. For example, syndecan-1 and heparan sulfate are released from the tissue and can be detected in the circulating blood of patients with perioperative global or regional ischemia (85, 100). In addition, release of the endothelial proteoglycan thrombomodulin in diabetes has been shown to coincide with diabetic nephropathy (40, 76). We demonstrated that patients with renal failure have increased circulating levels of syndecan-1 and thrombomodulin, which was reversed by kidney transplantation (110). Furthermore, renal failure was shown to correlate with increased concentrations of shed hyaluronan (18). Altogether, while several glycocalyx components are shed upon endothelial activation, the exact location, mechanism, and timing of shedding is complex and mostly unknown. Interpreting the results therefore currently remains challenging.

Atomic force microscopy. To determine the elastic properties of the endothelial glycocalyx, atomic force microscopy (AFM), has been shown to be a valuable tool. AFM consists of a cantilever with a spherical tip which can scan the surface of the specimen. Reaching close contact with the specimen, forces between the tip and sample will result in deflection of this cantilever, which can be detected by changes in reflection of a laser spot. This technique has been previously used to measure mechanical stiffness in endothelial cells. However, by comparing endothelial cell layers with and without removal of its surface layer, the presence and stiffness of the glycocalyx could also be measured. Changes in the endothelial surface layer stiffness and thickness were associated with both endothelial activation and shedding of syndecan-1 and hyaluronan in patients with renal failure (79).

Exclusion of macromolecules. Using intravital microscopy, a luminal microdomain that is inaccessible for erythrocytes, leukocytes, and macromolecules (70-kDa dextran; dex70) can be demonstrated. For small molecules, however, this microdo-

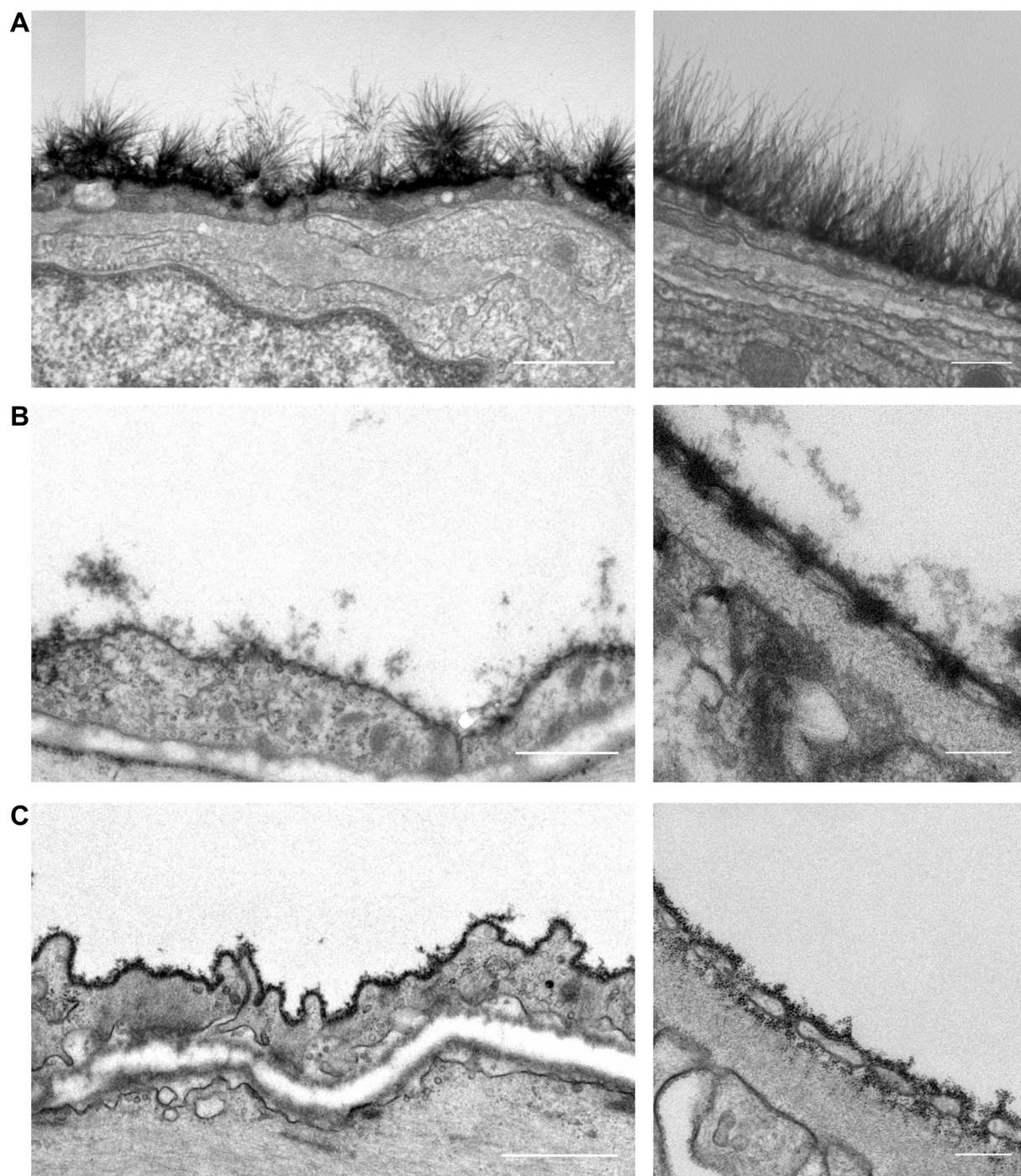
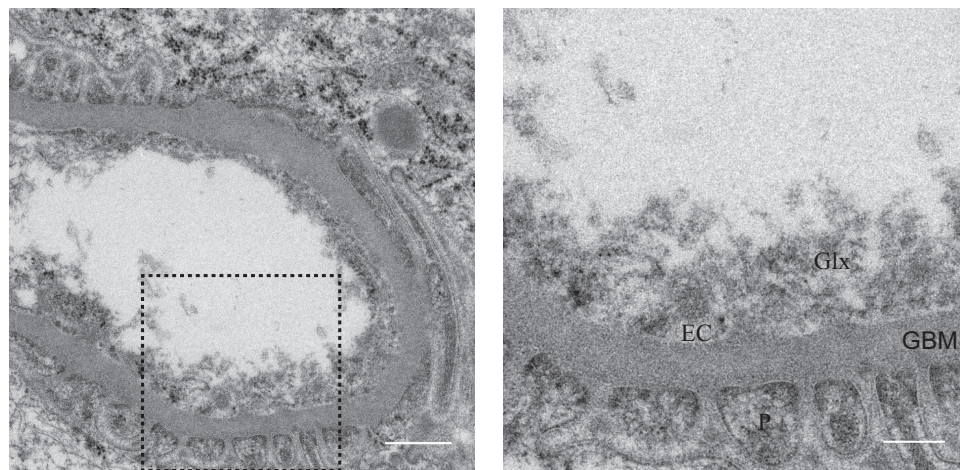


Fig. 2. Three different methods to stabilize and stain the EG visualized with electron microscopy. *A*: artery stained with alcian blue 8GX, which stains acidic polysaccharides in this case glycosaminoglycans within the glycocalyx (*left*). *Right*: detailed image of an alcian blue 8GX-stained myocardial capillary. *B*: vein stained with cupromeronic blue, which mainly stains sulfated proteoglycans (*left*). Detailed image of a glomerular capillary (*right*). *C*: vein stained with cationic ferritin (CF). CF stains the negatively charged components of the endothelial glycocalyx (*left*). *Right*: detailed image of glomerular capillary. Scale bars = 500 nm (*left*) and 200 nm (*right*). *B* and *C* are snapshots of stitches made as described previously (27).

main is still accessible. When damaging this layer, using a 1- to 2-min light-dye treatment, erythrocytes and dex70 are able to enter this microdomain (109). In addition to the demonstration of size exclusion, this technique can also be used to show the charge exclusion properties of the glycocalyx (103). Most of the techniques developed to estimate the changes in the glycocalyx are based on this principle. Hjalmarsson et al. (37)

used intralipid, a chylomicron-like suspension of purified soybean oil, egg yolk phospholipids, glycerol, and water to detect the physical exclusion properties of the glycocalyx and determine the glycocalyx thickness in TEM. After making electron micrographs of capillaries, the location of the lipid drop was identified as central or peripheral (being within 200 nm from the luminal endothelial surface) (37). By comparing the distri-

Fig. 3. EG stained in a glomerular capillary of a high-pressure frozen kidney section. EG was stained afterward with acridin orange and uranyl acetate during the freeze substitution stage. Shown are an overview (left) and detailed image of the glycocalyx on top of the glomerular filtration barrier (right). EC, endothelial cell; GBM, glomerular basement membrane; P, podocytes; Glx, glycocalyx. Scale bars = 500 nm (left) and 200 nm (right).



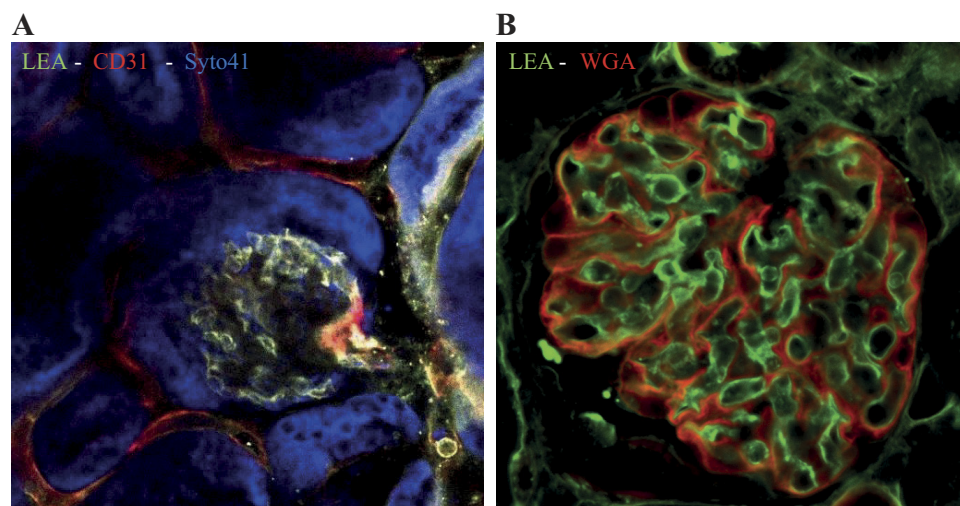
bution of these lipid particles before and after adriamycin treatment, for example, changes in the thickness of the surface layer could be estimated (42). In vivo, a theoretically comparable method was used by Smith et al. (99), who looked into the location of flowing microspheres. Using dual-flash epillumination, the velocity of infused fluorescently labeled microspheres was estimated in the near wall plasma-rich region of venules before and after light dye treatment to degrade the glycocalyx. Using this technique, the theoretically predicted hydraulic resistivity of the surface layer was demonstrated. Also, glycocalyx thickness was estimated to be 0.33–0.44 μm , which confirms the measurements by Vink and Duling (109).

Tracer dilution technique. Based on their in vivo observations in mice, the group of Vink et al. (108) has mainly focused on the development of a method to measure changes in the endothelial surface layer in patients. The tracer dilution technique was the first technique to estimate glycocalyx volume. By comparing circulating blood volume, using a glycocalyx-impermeable tracer, with total intravascular volume, using a glycocalyx-permeable tracer, total glycocalyx volume could be estimated. This concept was first applied when Nieuwdorp et al. (71, 72) showed that both acute hyperglycemia and type 1 diabetes mellitus were associated with a reduction of estimated endothelial glycocalyx volume. Unfortunately, this technique

has the disadvantage that it is very time-consuming and invasive to infuse labeled red blood cells and Dex40.

Red blood cell-endothelial cell gap and sidestream darkfield imaging. Based on previous observations of the red blood cell exclusion zone in animal models, the presence of a red blood cell exclusion zone between the red blood cell column and the endothelium was demonstrated in transilluminated hamster cremaster muscle capillaries (17, 108). More recently, we tested the hypothesis that the endothelial glycocalyx occupies this red blood cell-endothelial cell gap. In a comparison of the red blood cell column with the border of the fluorescent endothelium, a decreased red blood cell-endothelial cell gap was observed after degradation of the glycocalyx with hyaluronidase (18). In addition, Han et al. demonstrated a decrease in this red blood cell-endothelial cell gap when a leukocyte was passing through the capillary. Based on earlier data and a rapid recovery of the red blood cell-endothelial cell gap, it was concluded that the passing leukocyte temporarily compresses the glycocalyx (34). Consequently, local endothelial glycocalyx thickness can be estimated in vivo by measuring the difference in red blood cell column width before and directly after the passing of a leukocyte. This hypothesis was validated when the glycocalyx was degraded by oxidized LDL after

Fig. 4. EG imaged ex vivo with confocal- or multiphoton imaging techniques. A: mouse glomerulus visualized with in situ perfused anti-CD31 antibodies and TRITC-labeled conjugate for endothelium (red), syto41 for nuclei (blue), and lycopersicon esculentum (LEA) for EG (green). B: mouse glomerulus visualized after immersion staining with LEA (green) and wheat-germ agglutinin (WGA; red).



which no change in the red blood cell-endothelial cell gap could be observed before and after leukocyte passage (70).

Using sidestream darkfield (SDF) imaging, the ability of the red blood cell to access the glycocalyx in the microcirculation can be measured. The technique uses polarized light with a wavelength within the absorption spectrum of hemoglobin. A connected video camera visualizes the scattered light from the illuminated tissue (32). Using this wavelength, the red blood cell column of the superficial microvasculature can be imaged *in vivo*. Based on the concept that a red blood cell can penetrate deeper into an unstable or damaged glycocalyx, variations in red blood cell column width in time can be measured and taken as a proxy for endothelial glycocalyx accessibility. This phenomenon has been coined the perfused border region (PBR) and is shown schematically in Fig. 5. This automated technique only images the sublingual microcirculation and therefore cannot differentiate between endothelial glycocalyx perturbations in different capillary beds, such as glomerular or extrarenal capillaries. Nonetheless, using an adapted method, studies have been performed to estimate endothelial glycocalyx changes in renal capillaries during kidney transplantation (100). In addition, since chronic endothelial activation is expected to result in systemic perturbation of the endothelial glycocalyx, the automated SDF technique can be used to estimate systemic changes in the (micro-) vasculature including glomerular and extrarenal capillaries.

PBR was demonstrated to be associated with microvascular perfusion and to be increased in patients with coronary artery disease, sepsis, and renal failure (22, 51, 67, 110). In addition, PBR was also associated with microvascular changes in the retinal vascular bed (9). A recent study, however, failed to demonstrate a distinction between the sublingual microvascular PBR of a pooled group of participants with a variably increased cardiovascular risk profile, stressing the need for a better understanding of PBR profiles in different patient subclasses based on gender and e.g., ethnic background (3). Nonetheless, patient studies and validation studies in animal models suggest that measuring the PBR to estimate microvascular risk is a promising concept.

Conclusion

The endothelial glycocalyx is a highly interactive scaffold that facilitates protein-protein and protein-receptor interactions and thereby serves as a key modifier of endothelial function. Both structural dimensions as well as its biochemical composition determine its properties as a bioactive scaffold. Studying these properties has proven very challenging due to the structural fragility and the complex sugar biochemistry involved. However, modern imaging technology, as discussed in this review, opens up opportunities to take the glycocalyx into account when studying vascular and renal physiology. Unfortunately, technically it is still

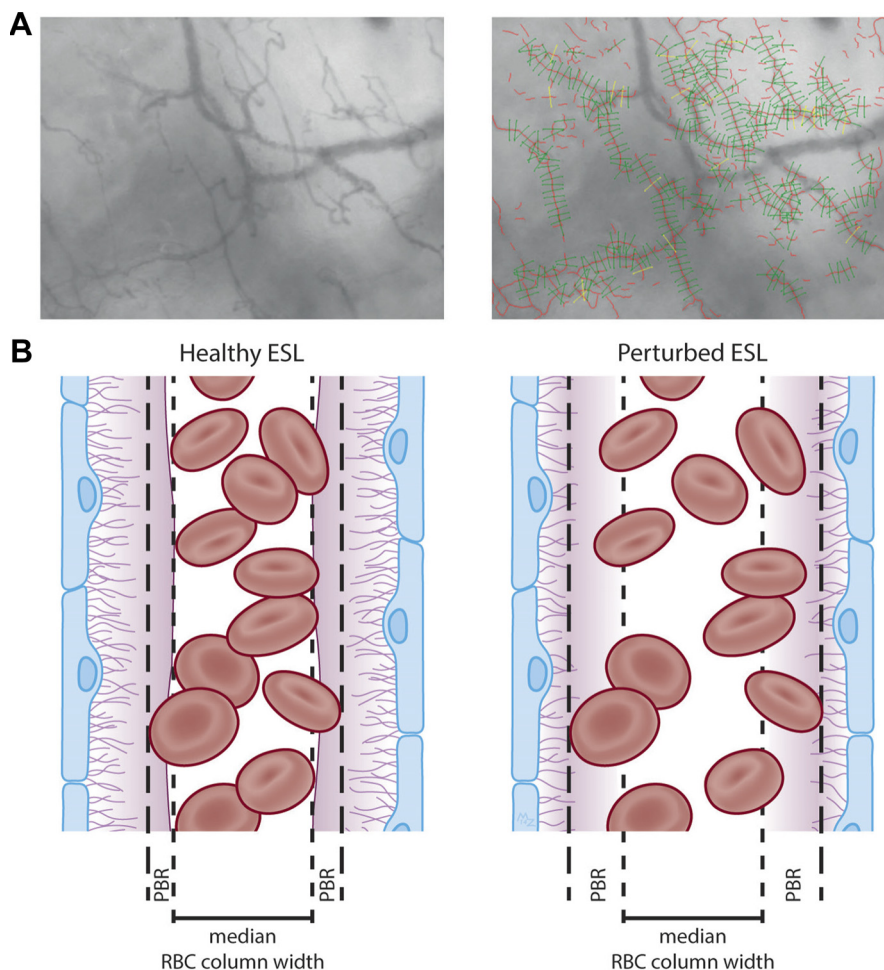


Fig. 5. Sidestream darkfield (SDF) imaging to measure the perfused boundary region (PBR) in the sublingual capillary bed. *A*: recordings from the sublingual capillary bed made with the SDF camera (*left*). Capillaries are automatically recognized and analyzed after various quality checks (*right*). Based on the shift in red blood cell (RBC) column width in time, the PBR can be calculated. *B*: model of a blood vessel showing the PBR in a healthy situation (*left*). The EG prevents the RBC to approach the endothelial cell; thus a small PBR is measured. In a disease situation (*right*) or after enzymatic EG breakdown in an animal model, the damaged EG allows the RBCs to approach the endothelium more often. This results in a higher variation in RBC column width reflected by a high PBR. ESL, endothelial surface layer.

not possible to unequivocally chemically characterize HS and hyaluronan variability. Most of our knowledge on disaccharide modification-function relationships needs to be derived from experiments where enzymes that edit the chemical composition of the glycocalyx are conditionally knocked out.

Further development of insights into structure-function relationships of glycocalyx will create new perspectives in our understanding of physiology and provide opportunities to innovate in medicine. Development of technological innovations that enable further interrogation and modulation of glycocalyx function will prove to be a critical success factor. For example, oligosaccharides or single-chain antibodies, which have now become available, are being screened for their potential to block the HS moieties that initiate inflammation and angiogenesis on the endothelial surface (38, 53, 81, 107). Furthermore, changes in structural dimensions of the glycocalyx are being probed to prospectively identify patients that are at risk for cardiovascular events or loss of renal function (19, 51, 110).

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DISCLOSURES

H. Vink is commercially involved with the SDF acquisition and analysis technique and is Chief Scientific Officer at GlycoCheck (Maastricht, The Netherlands).

AUTHOR CONTRIBUTIONS

Author contributions: M.J.D. and M.C.A. prepared figures; M.J.D. and T.J.R. drafted manuscript; M.J.D., B.M.v.d.B., D.H.L., M.G.B., G.L.T., M.C.A., A.J.v.Z., J.V.d.V., H.V., and T.J.R. edited and revised manuscript; M.J.D., B.M.v.d.B., D.H.L., M.G.B., G.L.T., M.C.A., A.J.v.Z., J.V.d.V., H.V., and T.J.R. approved final version of manuscript; T.J.R. provided conception and design of research.

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