Spatial distribution, temporal development and mechanical properties of the endothelial glycocalyx in vitro
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Spatial distribution, temporal development and mechanical properties of the endothelial glycocalyx *in vitro*

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Abstract

The endothelial glycocalyx is a thin layer of macromolecular matrix on the luminal surface of vascular endothelial cells. It determines fluid and solute transport across the vessel wall; affects the mechanotransduction of endothelial cells and contributes to vascular patho-physiology. This thesis investigates the spatiotemporal development of the endothelial glycocalyx \textit{in vitro} using fluorescence confocal microscopy. The Young’s modulus of the glycocalyx is evaluated using AFM indentation.

The glycocalyx on cultured HUVECs shows temporal development: up to day 5 after cell seeding, it covers predominantly the edge of cells and appears on the apical membrane of cells as time progresses. After day 14, the entire cell membrane is covered by the glycocalyx. The thickness of this layer is estimated to be between 300nm and 1μm. AFM indentation result reveals the Young’s modulus of the cell membrane decreases with time. The Young’s modulus of the glycocalyx is deduced from Young’s moduli of cell membranes with and without the glycocalyx layer. The results show the glycocalyx on cultured HUVECs has a Young’s modulus of \(~0.39\)kPa.

The thesis further investigates the distribution of the glycocalyx on the endothelial cell membrane following shear flow stimulation. Both the percentage area of the cell membrane that is covered with the glycocalyx and the
fluorescence intensity ratio between the apical and edge areas on endothelial cells are used to measure the glycocalyx distribution. It is observed that the glycocalyx appears near the edge of the endothelial cell following shear stimulation and develops towards the apical area with time. The speed of the recovery of the glycocalyx layer is faster in the earlier period, i.e. 0hr - 4hrs after shear stimulation, than that in the later period, i.e. 8hrs - 24hrs. Additionally, the recovery of the glycocalyx following neuraminidase degradation is investigated under either the static or the shear flow conditions.
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Abbreviations

Atomic Force Microscopy (AFM)
Scanning electron microscope (SEM)
Cell Tracker Red CMTPX (CTR)
Chondroitin sulfate (CS)
Glycosaminoglycans (GAGs)
Heparan Sulfate (HS)
Heparan sulfate proteoglycans (HSPG)
Heparinase III (Hep III)
Human umbilical vein endothelial cells (HUVECs)
Hyaluronic acid (HA)
Nitric oxide (NO)
Polymethylmethacrylate (PMMA)
Sialic Acid (SA)
Wheat germ agglutinin (WGA-FITC)
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Chapter 1. Introduction

Vascular endothelial cells cover the entire luminal surface of the vascular system. They play vital roles in most vascular events, including blood homeostasis, fibrinolysis and coagulation, blood-tissue exchange, vasotonus regulation, the vascularization in various tissues, blood cell activation and migration during physiological and pathological processes (Reitsma et al., 2007).

The endothelial glycocalyx is a negatively charged brush-like structure with containing proteoglycans, glycosaminoglycans and glycoproteins. This combination of macromolecules is continuously modified and has a dynamic relation with the blood plasma proteins and molecules that affect the shape and size of the glycocalyx layer (Pries et al., 2000). Microenvironmental factors, e.g. the ionic strength and pH level of plasma, also affect this layer (Seog et al., 2005; van Haaren et al., 2005).

The endothelial glycocalyx has been increasingly appreciated as an important factor in vascular patho-physiology. It is not only a barrier between the blood and vascular tissues, but also a mechanotransducer (Michel & Curry, 1999; Levick & Michel, 2010; Oberleithner et al., 2011). The endothelial glycocalyx also plays a significant role in the initiation and development of a number of diseases, such as atherosclerosis, diabetes and inflammation (Mulivor & Lipowsky, 2004; van Golen et al., 2012).
1.1 The composition and structure of the endothelial glycocalyx

The endothelial glycocalyx is made from a variety of macromolecules, including glycoproteins and proteoglycans that have glycosaminoglycans chains (GAGs) attached to their core proteins. These are believed to be the main components of the endothelial glycocalyx structure. Each one of these components will be discussed in details in the following sections.

1.1.1 Proteoglycans

Endothelial glycocalyx is made from a variety of macromolecules, including glycoproteins and proteoglycans that have glycosaminoglycans chains (GAGs) attached to their core proteins.

Proteoglycans are glycoproteins with O-glycosyl linkage and are made of a core protein to which long, unbranched carbohydrate polymers, called glycosaminoglycans, are covalently attached (Pries et al., 2000). They are considered to be able to regulate the cell growth, supported by the findings that proteoglycans bound to the various growth factors such as fibroblast growth factor (Presta et al., 1992), platelet-derived growth factor (Raines & Ross, 1992) and transforming growth factor (Segarini et al., 1989). Proteoglycans’s core proteins act as the pillars for the structure of the glycocalyx, to which sulphated
glycosaminoglycans (GAGs) are attached. The core proteins in proteoglycans contain specific sites for the attachment of the GAGs chains, as seen in Figure 1.1. Different proteoglycans have different core proteins. These core proteins are different in various aspects including their size, number of attached GAGs chains, and whether or not they are bound to the cell membrane. The major core protein families that present in the vascular system consist the transmembrane syndecans, the membrane bound glypicans, the basement matrix associated perlecans, where syndecans and glypicans core protein families are incorporated to the cell membrane, whereas perlecan is not directly incorporated to the cell membrane (Rosenberg et al., 1997).

Figure 1-1 Major core protein families present in human cells. Displayed are the human members of the membrane spanning syndecans, the glypicans, and the matrix localized perlecan (467 kd). GAGs attachment sites are indicated by dotted lines. For
the syndecans, the homologous transmembrane domain is indicated with (black) and intracellular domain with (stipple) and conserved tyrosines with (dots). Adapted from (Rosenberg et al., 1997).

**Syndecans**

Syndecans have a firm connection to the cell membrane via a membrane - spanning domain (syndecans) as all syndecans are type I transmembrane proteins (Carey, 1997). Endothelial cells express three types of syndecans: syndecan-1, syndecan-2 and syndecan-4. All of these syndecans have three glycosaminoglycans (GAG) attachment sites, close to their N terminus and distal to the apical surface, as shown in Figure 1.2. These attachment sites are mainly occupied by heparan sulphates, however they can be occupied by other GAG chains as well (Rosenberg et al., 1997; Tkachenko et al., 2005). The tail of the syndecans which extend inside the cell cytoplasm is associated with the endothelial cytoskeleton. It is suggested that it assist in reorganization of the cytoskeleton, through molecules such as tubulin, dynamin, and α-actinin (Yoneda & Couchman, 2003).
Figure 1-2 Schematic drawing of the syndecan structure. Cytoplasmic domains are shown in red, and glycosaminoglycan chains are shown in green. Adapted from (Tkachenko et al., 2005)

**Glypicans**

The only glypican that is expressed by the endothelial cells is glypican-1 (Rosenberg et al., 1997). Close to the membrane, it has three to four GAG attachment sites which are exclusively for the attachment of heparan sulfate GAG chain (Fransson et al., 2004). Glypican is anchored close to lipid rafts in the membrane, via a glycosylphosphatidylinositol anchor. These lipid rafts are incorporated to the membrane, and are rich in cholesterol- and sphingolipid. Caveolae is a subset of these lipid rafts. Caveolae is composed of protein caveolin-1, a cholesterol carrier
which is incorporated into the plasma membrane, where it forms a cave-like 
structures that is proposed to be linked and supported by the actin cytoskeleton of the 
cell (van Deurs et al., 2003). Glypicans with their associated heparan sulphate GAG 
chains are generally attached in plasma membrane regions where caveolin-1 is 
presented.

*Perlecan*

Perlecan is another core protein expressed by the endothelial cell. It is associated 
with basement matrix. The perlecan core protein contains five separate regions 
which constitute attachment sites for GAG chains, O-linked nonsulfated 
oligosaccharides as well as long chain fatty acids. Perlecan is secreted by endothelial 
cells and interacts with collagens, laminin, and other components within the 
basement membrane (Murdoch et al., 1992).

**1.1.2 Glycosaminoglycan chains (GAGs)**

Glycosaminoglycans chains (GAGs) are polysaccharides in the extracellular matrix 
that attached on specific sites of proteoglycans (Jackson et al., 1991). Five types of 
glycosaminoglycan chains (GAGs) are present in the endothelial glycocalyx: 
heparan sulphate, chondroitin sulphate, dermatan sulphate, keratan sulphate, and 
hyaluronan. These GAG chains have variable lengths that are modified by sulphation 
and/or deacetylation to a variable extent (Reitsma et al., 2007). The representations
of proteoglycans and glycoproteins elements of the endothelial glycocalyx on the luminal surface of endothelial cells was shown in Figure 1.3 (Tarbell & Pahakis, 2006).

Figure 1-3 Schematic representations of proteoglycans and glycoproteins elements of the endothelial glycocalyx on the luminal surface of endothelial cells. Adapted from (Tarbell & Pahakis, 2006)

**Heparan sulphate**

Within the different types of glycosaminoglycan chains (GAGs) presented on the surface of endothelial cells lining the vasculature, heparan sulphate is the most common type. It is estimated that heparan sulphate proteoglycans represent 50–90% of the total amount of proteoglycans present in the endothelial glycocalyx (Ihrcke et al., 1993; Pries et al., 2000).
**Chondroitin sulphate**

Another common type of GAGs present in the endothelial cell glycocalyx is chondroitin sulphate, which represents 15-24% of the proteoglycans present on the cell surface (Rapraeger et al., 1985). The heparan sulphate and chondroitin sulphate GAG chains are attached mainly to the transmembrane syndecans and the membrane-bound glypicans core proteins (Pries et al., 2000).

**Hyaluronan**

Hyaluronan is a large polymer, unlike heparan and chondroitin sulphate it is neither connected to a core protein nor synthesized in the golgi apparatus. It is synthesized in the plasma membrane. Its exact link to the cell membrane is unknown; however it can be bound to the cell membrane receptor CD44 (Nandi et al., 2000). It is also suggested that hyaluronan may be attached to its assembly proteins, the hyaluronan synthases (Weigel & DeAngelis, 2007) which are located at the cytosolic side of the cell membrane.

**Other GAG chains**

The GAG chains dermatan and keratan sulphate are also presented on the endothelial cells though to a lesser extent than chondroitin sulphate and heparin sulphate (Mulivor & Lipowsky, 2004). Dermatan sulphate is often regarded as a separate class of glycosaminoglycans, as some hold the view that it is type B chondroitin
sulfate. Keratan sulphate glycosaminoglycan in vasculature on the other hand is less well understood in terms of its physiological significance and its role in pathophysiology.

**Variation in GAGs chains expression**

The number of GAGs attached to the core proteins depend on various factors including the physiological state and the location of the tissue. Furthermore the expression of proteoglycans by endothelial cells is dependent on various stimuli. For example syndecans expression by the endothelial cells is tightly regulated by various factors, including tumor necrosis factor-α (TNF-α) which upregulate syndecan-2 expression and downregulate syndecan-1 expression in endothelial cells (Halden et al., 2004). Additionally syndecan-4 levels are increased in several disease states, including arterial injury (Geary et al., 1995) and acute myocardial infarction (Li et al., 1997).

**1.1.3 Glycoproteins**

Glycoproteins alongside proteoglycans are regarded as the backbone molecules of endothelial glycocalyx, they connect the glycocalyx to the endothelial cell membrane. These endothelial glycoproteins are characterized by relatively small (2-15 sugar residues) and branched carbohydrate side chains. Major glycoprotein groups in the endothelial glycocalyx include adhesion molecules and components of the
coagulation and fibrinolysis system. The endothelial cell adhesion molecules presented in the endothelial glycocalyx include selectins group, the integrins group, and the immunoglobulins. These adhesion molecules play a vital role in cell recruitment from the bloodstream and in cell signaling.

**Selectins**

Glycoprotein molecule from the selectin group typically has a tail that extends to the cytoplasm of the cell. The extracellular region of each selectin contains an amino-terminal C-type lectin domain, followed by an EGF-like domain, and multiple short consensus repeat units (SCR) (Tedder *et al.*, 1995). These structures facilitate the binding of carbohydrate groups on glycosylated proteins or lipids.

Selectins have two families found on the vascular endothelium: E-selectin and P-selectin, both of which play crucial roles in leukocyte–endothelial cell interactions. The P-selectin is the main receptor interacting with leukocyte on acutely inflamed endothelial cells in vivo (Sperandio, 2006). The epidermal growth factor (EGF)-like domain in P-selectin has a significant role in selectin-ligand recognition. In a study by Kansas *et al* demonstrated that the EGF-like domain on P-selectin is involved in ligand recognition and cell adhesion (Kansas *et al*., 1994). The E-selectin is spontaneously made and expressed on the cell surface. When the endothelial cells are stimulated by cytokines such as interleukin-1, tumor necrosis factor α, and lipopolysaccharide E-selectin expression is increased (Jung & Ley, 1997).
**Integrins**

Integrins are found on many types of cells, including endothelial cells, white blood cells and platelets. In the luminal membrane of these cells, endothelial cells express integrin $\alpha_v\beta_3$ which is an important mediator of platelet–endothelial cell interactions. It is shown that $\alpha_v\beta_3$ integrin interacts with intercellular adhesion molecule (ICAM)-1 (which is an immunoglobulin) to facilitate the adhesion of activated platelets to intact endothelium (Bombeli et al., 1998; Xiong et al., 2003).

**Immunoglobulin group**

The other group of glycoproteins is immunoglobulin. Examples of immunoglobulin include intercellular adhesion molecule 1 and 2 (ICAM-1 and -2), vascular cell adhesion molecule 1 (VCAM-1), and platelet/endothelial cell adhesion molecule 1 (PECAM-1), which all act as ligands for integrins on leukocytes and platelets and are crucial mediators of leukocyte adhesion to the endothelium and its passage to the tissue where that is needed i.e inflammation areas (Muller et al., 2002; Reitsma et al., 2007).

1.3.4 Models for the glycocalyx structure

Though glycocalyx had been visualized using electron microscopy on the luminal surface of the endothelial cells since 1966 (Luft, 1966), very little was known about
its structural organization. Squire et al presented the first structural model for the organization of the endothelial glycocalyx (Squire et al., 2001).

When the endothelial glycocalyx layer undergoes degradation by enzymes, this directly affected its physiological functions such as permeability, when the endothelial glycocalyx was partially digested using pronase, the hydraulic conductivity of the vessels increased (Adamson & Clough, 1992). The evidence suggested an important role for the glycocalyx structural organisation.

Curry and Michel developed the “fibre matrix” theory of vascular permeability in 1980 (Curry & Michel, 1980). More recently, analyzing micrographs from various specimens prepared for electron microscopy using either freeze-fracture or the other staining procedures, Squire et al demonstrated that the various glycocalyx specimens showed spacing of roughly 20 nm, usually in both parallel and perpendicular directions, as shown in Figure 1.4. Therefore it was concluded that this is a fundamental spacing in the glycocalyx and that the glycocalyx is a quasi-periodic three-dimensional meshwork with a spacing of 20nm in all directions (Squire et al., 2001).
The first glycocalyx model presented by Squire et al. and redrawn by Weinbaum et al., as shown in Figure 1.5 proposed that cell membrane layer of the glycocalyx is linked to actin cytoskeleton under the cell membrane this conclusion was based on the description by Satcher et al. of the fine cytoskeletal structure of cultured bovine aortic endothelium (Satcher et al., 1997). It suggested that complex distributed cytoplasmic structural actin network (DCSA) physically connects the apical and basal cell membranes and provide a mechanism for transmitting mechanical forces across cells and a signaling pathway from membrane to nucleus. Squire et al. suggested that the thickness of the endothelial glycocalyx is less than 200 nm and it contains fibrous structures which exhibit periodicities of about 20 nm both parallel to and
perpendicular to the cell surface. There is also evidence for a quasi-hexagonal lattice of larger spacing, approximately 100 nm, between groups of endothelial glycocalyx macromolecules (Squire et al., 2001).

Figure 1-5 A simplified model of endothelial glycocalyx structure. (A) model of Endothelial glycocalyx layer showing core protein arrangement and spacing of scattering centers along core proteins and their relationship to actin cytoskeleton as proposed by squire et al (b) en face view of idealized model for core protein clusters and cluster foci. Adapted from (Weinbaum et al., 2003).

This model provided a valuable description for the way in which the endothelial
glycocalyx macromolecules are organized on the luminal surface of the cell and the way this luminal structure is connected to the underlying endothelial cell cortical cytoskeleton. This study provides a model for the organization of the matrix of the endothelial glycocalyx for the first time. It describes the core proteins of the proteoglycans and their relation to the underlying cortical cytoskeleton and suggests that the core proteins are linked to the underlying cortical cytoskeleton.

1.2 Physiological functions of the endothelial glycocalyx and its response to shear flow

As the interface between the blood flow and the blood vessel, endothelial glycocalyx is not only a permeability barrier but also a mechanotransducer to mechanical signals in the blood vessel. It has been well established that the morphology and function of endothelial cells can be affected by the shear stress generated by the blood flow, which includes affecting the nitric oxide (NO) production caused by the shear stress, the cell morphology changes, proliferation rate, and many other biochemical signaling events (Dewey et al., 1981; Ebong et al., 2008; Lopez-Quintero et al., 2009).

1.2.1 Permeability barrier and the revised starling principle.

*Revised Starling Principle*

The Starling equation explains the role of hydrostatic and colloid osmotic forces in
the movement of fluid across the capillary wall, this filtration includes four forces,

\[ \frac{J_v}{A} = L_p (P_c - P_t) - \sigma (\pi_c - \pi_t) \]

where \( P_c \) is the hydrostatic pressure in the capillary lumen and \( P_t \) is the hydrostatic pressure in the tissue, respectively; \( \pi_c \) is the osmotic pressure in the blood vessel lumen and \( \pi_t \) is tissue osmotic pressure; \( J_v/A \) is the filtration rate per unit area of capillary wall; \( \sigma \) is the reflection coefficient to the plasma proteins; and \( L_p \) is the hydraulic conductivity.

The classical Starling equation suggests that fluid filtration in the blood vessels occur across the capillary endothelium, with the capillary wall as the semi-permeable membrane through which the filtration occurs. The filtration rate across this semi-permeable membrane is determined by the hydraulic and colloid osmotic pressures in the vascular lumen and in the surrounding tissue. This balance has been applied across the entire trans-endothelial barrier, with the different pressures being assessed globally. The widely accepted view of the Starling equation was that there is a net filtration in the capillaries on the arterial side and almost an equal re-absorption on the venous side due to osmotic forces leaving a small net positive filtration that accounts for the lymph flow.

**Classical Starling principle paradox**

In 1987 Michel and Phillips performed an experiment on isolated perfused frog mesentery microvessels, measuring the capillary pressure and analyzing the variation
of filtration rate in respect to changing capillary hydrostatic pressure while maintaining a constant osmotic pressure in the perfusate (Michel & Phillips, 1987). They observed 15–30 seconds of re-absorption after the pressure in the capillary was quickly reduced from its maximum value to some lower value, this re-absorption period quickly stopped and then it was followed by a very low filtration rate in a steady-state behavior that was achieved 2–5 minutes after the capillary pressure was changed and maintained constant. The classical view of the Starling equation was only true for the short period of the re-absorption, however it did not account for the filtration period, these observations provided evidence that that the Starling equation was not properly applied.

Though the classical version of the Starling equation was seriously challenged by Levick, he showed that when the tissue osmotic pressures were measured the generally accepted view of the classical Starling equation was not valid in all the body tissues except the kidney and the intestinal mucosa (tissues whose main function is venous re-absorption), and there was no re-absorption on the venous side of capillaries (Levick, 1991). Without adequate venous re-absorption in most of the body tissues as previously thought, the low quantity of lymph produced by the body could not be properly understood in the context of the classical application of the Starling equation.

*Michel and Weinbaum revised Starling principle*
Michel and Weinbaum separately proposed a revised version of the Starling equation in which the primary molecular sieve for plasma proteins was the endothelial glycocalyx (Michel, 1997; Weinbaum, 1998). Furthermore the model presented by Michel and Weinbaum proposed that the Starling forces were determined, not by the global difference in osmotic pressure between blood vessel lumen and tissue, but by the local difference in protein concentration across the endothelial glycocalyx.

In the Michel and Weinbaum model of the revised Starling equation, the tissue osmotic pressure ($\pi_t$) is replaced by osmotic pressure in the region between the luminal side of the tight junction strand and the distal side of the endothelial glycocalyx layer ($\pi_0$) and the tissue hydrostatic pressure ($P_t$) is replaced by the hydrostatic pressure ($P_0$) in the distal part of the endothelial glycocalyx. This is because the Starling force balance is applied just across the endothelial glycocalyx layer. However applying the revised Michel-Weinbaum model is difficult as the local Starling forces behind the endothelial glycocalyx layer, ($P_0$) and ($\pi_0$), are variable and unknown because of the large gradients in velocity and protein concentration that are produced by the presence of the tight junction strand in the cleft. It is worth mentioning that a tight junction strand in the cleft with infrequent discontinuities can decrease the water back diffusion into the luminal side of the cleft. This water back diffusion into the luminal side of the cleft occur due to the convective flux of water, the decrease due to tight junction strand with infrequent breaks become severe at high filtration rate (Hu & Weinbaum, 1999).
**Three-dimensional model to determine Starling forces**

Hu & Weinbaum proposed the first detailed three-dimensional model to determine the \( P_0 \) and \( \pi_0 \) Starling forces in the revised version of the Starling equation. \( P_0 \) and \( \pi_0 \) Starling forces which are evaluated at the cleft entrance behind the endothelial glycocalyx layer, can vary significantly along the length of the cleft because the both the streamlines for water flow and solute flux lines follow a curved path through the tight junction breaks, as shown in Figure 1.6. Hu & Weinbaum model was based on the carefully measured geometry of the size and frequency of tight junction strand breaks in frog mesenteric microvessels by Adamson and Michel (Adamson *et al.*, 2004), this model predicted that the local protein concentration behind the endothelial glycocalyx layer can be extremely different to the tissue protein concentration, since the convective flux of proteins through the orifice-like pores in the tight junction strand greatly hinder the back diffusion of the proteins into the lumen side of the cleft, when the local Peclet number (Péclet number is a dimensionless number relating the rate of advection of a flow to its rate of diffusion) at the pores in the tight junction is more than 1. They concluded that the net result is that the filtration in the capillaries is far less than realized and there may be no need for venous reabsorption.
area \(a_c\) and perimeter \(p_c\) measurements of individual cells seen in silver-stained whole mounts of venular microvessels were used to calculate cleft length per unit area, \(l_c\); B). a cross-section of an interendothelial cleft covered by endothelial glycocalyx layer; C). oblique view of cleft segment reconstructed from serial sections illustrates the length of tight junction strand gaps, \(2d\), and the mean distance between strand gap centers, equal to the functional unit length, \(2d\); D). idealized mathematical model showing four regions: endothelial surface glycocalyx (egl), cleft with tight junction strand (region a), near-field tissue space <5μm (region b), and far-field tissue space >5μm from cleft exit. Adapted from (Adamson et al., 2004)

The revised Starling hypothesis was tested by Hu et al (Hu et al., 2000). They performed their experiments on frog mesenteric microvessels, the microvessels were
back loaded with albumin (by damaging the mesothelium at a distance of approximately 100 μm from the perfuse microvessel) and a superperfusate was introduced that was isotonic (50 mg/ml) with respect to the lumen. The tissue concentration was carefully measured over a period of time using confocal microscopy. Experimental measurements of $J_v/A$ were not performed until the lumen and tissue were observed to be isotonic. According to the classical Starling equation, there should be no osmotic force across the vessel wall. However, measurements of $\sigma\Delta\pi$ in frog microvessels when $J_v/A = 0$ showed that nearly the full lumen osmotic pressure was present despite the fact that confocal microscopy indicated that the interstitial albumin concentrations were isotonic to within a few microns of the vessel wall.

**Evidence for the revised Starling principle**

The role of the endothelial glycocalyx as molecular sieve through which the filtration and absorption of colloids and water occur had been shown by studies of Rehm et al and Jacob et al; that showed impaired endothelial barrier function after glycocalyx degradation in an isolated, perfused heart model. Infusion of 5% albumin or 6% hydroxethyl starch, a natural and an artificial colloid, led to decreased fluid extravasation. However, after 20 minutes of warm ischemia, only albumin infusion prevented vascular leakage (Rehm et al., 2004; Jacob et al., 2006). This highlight the importance of an intact glycocalyx and the role of plasma-derived proteins for competent glycocalyx functioning. The revised Starling principle has provided more
detailed insight into vascular permeability and stresses the importance of the endothelial glycocalyx as a major determinant.

1.2.2 The endothelial glycocalyx as a mechanotransducer

The morphology of the endothelial cells can be affected by shear stress and the glycocalyx plays a crucial role in the rearrangement of the cytoskeleton (Galbraith et al., 1998). Thiel et al examine the endothelial cells treated with shear fluid, the heparan sulfate dropped significantly after undergoing 5 hours of shear exposure, as shown in Figure 1.7. In the figure, the effect of Hep III was also shown.

Figure 1-7 Confocal microscopy images of the heparan sulphate analysis in the endothelial glycocalyx layer. Image (a) show cells cultured in DMEM with 10% FBS for 5 hours, image (b) cells cultured in DMEM only for 5 hours, image (c) cells
cultured with heparinase iii (hep iii) for 2 hours. In the graph the first three columns from left to right correspond to image a, b and c and the fourth column represent postcultured heparan sulphate in DMEM + 1% BSA for 5 hours. To visualize cell-surface HSPG, cells were stained with primary antibody for HSPG (green) and with cell tracker orange dye. XZ show different degrees of cell surface heparan sulphate proteoglycan distributions. Adapted from (Thi et al., 2004).

The coordinated response of three main actin structures in the endothelial cells to fluid shear stress with or without the presence of endothelial glycocalyx have been described by Thi and coworkers in the so called “Car bumper model” which describes the reorganization and function of the following actin structures: actin cortical web (ACW), stress fibers (SFs) and dense peripheral actin bands (DPABs). The model assumes that dense peripheral actin bands (DPABs) are almost free-floating rim structures that are attached to filamentous actin fibers by stress fibers (SFs) at the basal surface and actin cortical web (ACW), as shown in Figure 1.8. The function of dense peripheral actin bands (DPABs) is described like a rubber bumper on a bumper car that is constantly undergoing small collisions with its neighbours. The dense peripheral actin bands (DPABs) are linked to neighbouring cells by weak CD155-cadherin linkages in the adherens junctions, through this the DPABs in neighbouring endothelial cells are kept lateral, however this is only true under low shear stress, because these linkages are weak and can only prevent collisions between neighboring cells in unprotected regions above and below the
DPABs when they are not disturbed by high shear stress. When shear stress and torques acting on the DPABs and the actin cortical web (ACW) exceed the weak bonds of the CD155-cadherins, these bonds rupture, and the DPAB breaks up into smaller fragments, these small fragments can be used for the formation of new stress fibers (stress fibers attach the dense peripheral actin bands (DPABs) to the basal adhesions are weak compression elements that support tension) in the rest of the cell. During the transient period of the cytoskeleton reorganization to a new steady state, the vinculin migrate to cell borders to establish temporary focal adhesions at the periphery of the cell. These changes stabilize the cell during the transient state of the cytoskeleton reorganization.

The mechanism through which the DPABs break up when the endothelial cells are exposed to fluid shear stresses above a critical stress level is believed to be caused by a drag force on the tips of the core proteins of the endothelial glycocalyx. This drag force cause a torque on the actin cortical web which is linked to the core protein of the endothelial glycocalyx, this torque produces a clockwise rotation. The bending torque on each core protein is small, but the collective behavior of all of the core proteins acts to produce a clockwise rotation of the DPAB. If this torque exceeds a specific threshold it will disrupt the CD155-cadherins in the adherens junction.
Figure 1-8 Schematic representation of the bumper-car model for the structural reorganization of the endothelial cytoskeleton in response to fluid shear stress. Figure 1-8A show endothelial cell with an intact DPAB linked to the adherence junction, and act as the base for the actin cortical web (ACW). Figure 1-8 B show endothelial cell cytoskeletal reorganization in response to shear stress when the
endothelial glycocalyx is intact. Figure 1-8 c show endothelial cell response to fluid shear stress when the endothelial glycocalyx is compromised. The image at the top is an ideal representation of freeze-fracture electron micrographs (Squire et al., 2000) in regions close to the plasmalemma, which exhibit a highly ordered hexagonal lattice with a characteristic spacing of 100 nm between junctional nodes (inset). Adapted from (Thi et al., 2004).

1.2.3 Mechano-signaling transmission mechanisms of the endothelial glycocalyx

A number of mechanotransduction mechanisms of the endothelial glycocalyx were reported in the past. Tarbell & Pahakis hypothesize that partial or complete degradation of endothelial glycocalyx layer change the mechanism through which fluid shear stress is transmitted to the endothelial cells and that the force created by fluid shear stress would not be conveyed through the transmembrane core proteins of the glycocalyx (Tarbell & Pahakis, 2006). However, mechanical equilibrium principles ensure that the stresses delivered to the basal adhesion plaques and the intercellular junctions are the same as they would be with an intact glycocalyx, suggesting that signaling through these structures is independent of the integrity of the glycocalyx. Tarbell & Pahakis suggests that this may provide a clue that degrading GAGs, only block shear-induced nitric oxide (NO) production, and has no effect on shear-induced prostacyclin production. It is worth noting that this conclusion
was partially based on a study by Ponik and Pavalko in which they demonstrate that prostacyclin is transduced by basal adhesion plaques. However Ponik and Pavalko carried their experiments on bone cells (bone cells) and this might not be an accurate comparison to endothelial cells (Ponik & Pavalko, 2004).

**Heparan Sulphate**

Florian and colleagues tested the role of heparan sulphate which is a major glycosaminoglycan component of the surface glycocalyx layer in translating the fluid shear stress sensor on endothelial cells into nitric oxide (NO). They analyzed nitric oxide (NO₂⁻ and NO₃⁻) production in response to fluid shear stress following enzymatic removal of heparan sulfate using the enzyme heparinase III. They reported that the significant nitric oxide (NO) production usually caused by steady shear stress (20 dyne/cm²) or Oscillatory shear stress (10 +/- 15 dyne/cm²) was inhibited completely when heparan sulphate was degraded using heparinase III enzyme (Figure 1.9). Fluorescent imaging with a heparan sulfate antibody revealed that heparinase III treatments removed a significant proportion (46%) of the heparan sulfate bound to the surfaces of endothelial cells (Florian et al., 2003). The heparinase III enzyme did not degrade chondroitin sulphate and it was also demonstrated that addition of bradykinin increased nitric oxide production despite the enzymatic treatment of the endothelial cells, demonstrating that nitric oxide production is not exclusively controlled by the heparan sulphate mechnotransducing properties.
Figure 1-9 Effects of the steady shear stress on nitric oxide (NO) production after heparinase treatment. NO production caused by 20 dyne/cm² steady shear stress was significantly greater than control, control with heparinase exposure, and steady shear with heparinase treatment. Comparing NO production induced by 20 dyne/cm² before and after heparinase exposure show that treatment with heparinase inhibited expected NO production under 20 dyne/cm². Adapted from (Florian et al., 2003).

**Sialic acid**

Previously, sialic acid was also shown to have a role in flow dependent vasodilation of the vessels, Pohl *et al* used the enzyme neuraminidase to remove sialic acid residues from saline-perfused rabbit mesenteric arteries, and it was observed that flow-dependent vasodilation was abolished within 30-minutes of enzymatic treatment of the vessels (Pohl *et al*., 1991). As it was established that vasodilatation in arteries was mediated by nitric oxide production (NO) by the endothelial cells
therefore the results of the study by Pohl et al suggested a role for the sialic acid in the fluid shear stress induced production of the nitric oxide (NO). Using a similar method, Hecker et al investigated the mechanisms through which nitric oxide (NO) and prostacyclin (PGI2) are released from endothelium-intact rabbit femoral arteries under resting conditions and after stimulation by either shear stress or acetylcholine (ACh). They reported that a six- to sevenfold increase in shear stress (from 0.15 to 1 dyn/cm²) caused a five- to sevenfold increase in nitric oxide (NO) release, which was linked to the increasing shear stress. The same increase in shear stress also enhanced the release of prostacyclin (PGI2) from the femoral artery segments by 11- to 12-fold. In arteries pretreated with neuraminidase, the nitric oxide (NO) production was abolished however the shear stress-dependent prostacyclin (PGI2) release was not affected (Hecker et al., 1993). This study illustrate that there are multiple mechanotransducer.

**Hyaluronan**

The role of the hyaluronan glycosaminoglycans in shear-induced endothelium-derived nitric oxide (NO) release was investigated by Mochizuki and co workers using isolated canine femoral arteries. The production of nitric oxide (NO) in response to shear stress was assessed in arteries that were treated with hyaluronidase (enzyme degrading hyaluronan). It was reported that hyaluronidase treatment significantly decreased flow-induced NO production to 19 ± 9% of control values (production of nitric oxide without hyaluronidase treatment) (Figure 1.10).
This outcome indicate that hyaluronan acid glycosaminoglycans within the glycocalyx play an important role in mechanotransducing the shear force of flowing blood that triggers endothelium-derived nitric oxide (NO) production in canine femoral arteries (Mochizuki et al., 2003).

![Figure](image)

Figure 1-10 The NO production rate and perfusion rate relationship. The relation between nitric oxide (NO) production rate from the isolated canine femoral artery and perfusion rate before (○) and after (●) treatment with hyaluronidase. Adapted from (Mochizuki et al., 2003).

**Chondroitin sulphate**

An *in vitro* study using bovine aortic endothelial cells assessed the role of chondroitin sulphate in fluid shear stress induced nitric oxide production, using the enzyme chondroitinase, to degrade chondroitin sulphate; the results demonstrated that despite the degradation of chondroitin sulphate, the characteristic shear-induced nitric oxide production remained the same. However when the samples were treated
with either neuraminidase or hyaluronidase, the nitric oxide (NO) production was highly affected (Pahakis et al., 2007). The result of this study alongside other studies such as the study by Hecker et al or Mochizuki et al showed that only specific GAG chains in the endothelial glycocalyx layer are involved in mechanotransduction, as removal of chondroitin sulphate had no effect, while removal of heparan sulphate, sialic acid and hyaluronan blocked the shear-induced nitric oxide production or at least reduced it (Hecker et al., 1993; Mochizuki et al., 2003). It was also shown that none of the four enzymes used had an inhibitory effect on shear-induced prostacyclin (PGI₂) production, therefore suggesting multiple mechanisms of mechanotransduction.

1.3 Endothelial glycocalyx in vascular pathology

The dysfunction of endothelial glycocalyx is involved in many diseases, for example, atherosclerosis, diabetes, inflammations, to name a few.

1.3.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory response in the layers of the arteries, caused by the accumulation of macrophages due to the subendothelial retention of atherogenic lipoprotein (Libby, 2002; Ross, 1999). The progression of the atherosclerotic lesion is promoted by the accumulation of the low density
lipoproteins without adequate removal of fats and cholesterol from the macrophages by high density lipoprotein (HDL). Though lowering circulating low density lipoprotein (LDL) and cholesterol levels can slow atherosclerosis progression and even cause regression (Skogsberg et al., 2008). However the precise etiology of atherosclerosis is not established.

Vink et al studied the effect of oxidized low-density lipoproteins (Ox-LDL) on the endothelial glycocalyx layer of hamster cremaster muscle capillaries (Vink & Duling, 1996). They demonstrated that clinically relevant dose of Ox-LDL partially degrade endothelial glycocalyx layer and decrease its dimension as shown in Figure 1.11. Additionally Ox-LDL increase platelet-endothelial cell adhesion (Vink et al., 2000). The effects of Ox-LDL on endothelial glycocalyx layer are inhibited by the administration of superoxide dismutase (SOD) and catalase, this suggests that increased amounts of oxygen-derived free radicals mediate Ox-LDL-induced degradation of the endothelial surface layer and lead to the loss of endothelial cell anti-adhesive properties.
Figure 1-11 Light microscopy images of circulating red blood cells in a hamster cremaster muscle capillary. The gap between red blood cells and luminal endothelial cells was considered to represent endothelial glycocalyx and associated macromolecules. A) RBC-Endothelial cell gap dimension before the administration of Ox-LDL. B) A decrease in RBC-Endothelial cell gap dimension at 24 minutes, after bolus injection of Ox-LDL. (Adapted from (Vink et al., 2000).

Van den Berg et al investigated the effects of two different atherogenic risk factors on the endothelial glycocalyx dimension: the vascular site and atherogenic diet (van...
den Berg *et al.*, 2006). It has been proposed that atherosclerotic lesions within the arterial tree develop at predictable vessel geometries, e.g., arterial branching and curvatures as this causes local flow instabilities and separations (Zarins *et al.*, 1983). Such lesions can be detected and visualized as changes in vascular wall properties and quantified as intima-to-media ratios (IMR). Increases in IMR have been found to be associated with increased cardiovascular risk factors and atherosclerosis. Van den Berg *et al.* assessed this risk factor using the mice common carotid artery as a model for low risk region and the mice internal carotid artery sinus region as a high-risk region for atherosclerosis. They demonstrated an inverse relation between glycocalyx thickness and intima–media ratio. This relation can be linked to a reduction of vascular protective properties of the endothelial glycocalyx at sites with higher atherogenic risk. A thinner glycocalyx was observed in the internal carotid sinus region compared to the common carotid artery, the thinner glycocalyx was shown to be accompanied by greater IMR and a thicker subendothelial layer therefore more susceptible to atherosclerosis. Furthermore it was shown that a high-fat, high-cholesterol diet reduced the endothelial glycocalyx layer dimensions, as shown in Figure 1.12.
Figure 1-12 Electron micrographs of Alican blue stained luminal surface of mice carotid arteries. Image C and D show the common carotid and internal carotid sinus regions in apoE*3 mice on atherogenic diet. Image A and B show the common carotid and internal carotid sinus regions in mice on normal diet. Note that in image C compared to A, glycocalyx dimension at the common carotid area is significantly reduced. Adapted from Figure 2 in (van den Berg et al., 2006).

1.3.2 Diabetes

Diabetes is characterised with hyperglycaemia and insulin resistance or absence, cardiovascular disease is a common complication of diabetes, the high levels of glucose in the circulating blood impair the endothelial vessel wall and the vessels are more susceptible to atherosclerosis. It was reported by Nathan et al, patients with
diabetes were randomly allocated to two treatment groups, in which one group received conventional treatment and the other group received an intensive treatment (Nathan et al., 2003). Then ultrasonography was used to detect the vascular intima-media thickness which was a measurement of the atherosclerosis risk. The study reported that patients receiving the conventional treatment had a mean intima-media thickness larger than the group receiving the intensive therapy, which demonstrated the damaging effect of hyperglycaemia on blood vessels. Hyperglycaemia is also shown to inhibit 67% of nitric oxide synthase enzyme activity in bovine aortic endothelial cells (Du et al., 2001). Increased degradation of glycosaminoglycans had also been demonstrated in hyperglycemic conditions this may suggested a link between some macromolecules in endothelial glycocalyx and the pathologies associated with diabetes (Ceriello et al., 1983).

Nieuwdorp et al investigated the impact of hyperglycemia on the endothelial glycocalyx in healthy volunteers (Nieuwdorp et al., 2006). They provided an in vivo measurement of systemic endothelial glycocalyx and measured the effect of acute hyperglycaemia and type I diabetes on the endothelial glycocalyx. They demonstrated that the systemic glycocalyx volume was almost 50% less in patients with type I diabetes when compared to healthy volunteers; this value for the systemic glycocalyx was further reduced in diabetics with microalbuminuria. These results suggest that the endothelial glycocalyx is reduced systemically due to hyperglycaemia, however in a recent study by Michel& Curry, questions were raised
about the robustness of the method used by Nieuwdorp et al to measure the systemic volume of the endothelial glycocalyx (Michel & Curry, 2009). One of the questions involved the plasma-volume measurements by Nieuwdorp et al which were based on the distribution volume of circulating red cells and the hematocrit of blood taken by venepuncture. These problems were made more complex by the evidence that plasma and red cells have different distribution patterns within the microvasculature (Eppihimer & Lipowsky, 1994). Further studies are needed to determine the effect of hyperglycemia and diabetes on endothelial glycocalyx layer dimensions and the role of this layer in the mechanism of the vascular disease which is associated with the diabetes.

1.3.3 Inflammation

The partial degradation of the endothelial glycocalyx layer by Ox-LDL may provide a clue to the inflammatory process associated with atherosclerosis. Leher et al demonstrated that the systemic bolus injection of Ox-LDL was associated with increased adhesion of leukocytes to the endothelium of small arterioles and venules and this can be prevented by vitamin C or SOD (Lehr et al., 1993). This was further supported by the observations that SOD prevents Ox-LDL–induced leukocyte adhesion and albumin leakage to the vascular endothelium (Liao & Granger, 1995).

Henry and Duling designed an experiment to test the inflammatory response of the
endothelial glycocalyx to inflammation using tumor necrosis factor- alpha (TNF-α) (Henry & Duling, 2000). In the intact hamster cremaster microvessels, fluorescein isothiocyanate (FITC) - labeled Dextran 70, 580, and 2,000 kDa were excluded from a region extending from the endothelial surface to 0.5 µm into the lumen. This exclusion zone was assumed to represent the dimensions of the endothelial glycocalyx layer. When the TNF-α was applied to the microvessels, it significantly increased the penetration rate of FITC- Dextran 40, FITC- albumin, and FITC- IgG into the glycocalyx and caused a significant increase in the intraluminal volume occupied by flowing red blood cells which were usually excluded from the this layer. The adhesion of white blood cells to the endothelial cells increased during the TNF-α application. This result demonstrated that inflammatory cytokines such as TNF-α could cause disruption of the endothelial apical glycocalyx. Additionally Mulivor and Lipowsky reported that intestinal ischemia/reperfusion led to significant reduction of endothelial glycocalyx thickness in rat mesenteric venules, most likely due to shedding of GAG chains (Mulivor & Lipowsky, 2004).

1.4 Aim and Objectives

The working hypotheses of this PhD project are stated as followed:

The glycocalyx layer in vitro requires sufficient time to develop before the cultured endothelial monolayer can be used to study cell responses to flow and blood cells with physiological relevance.
A secondary hypothesis is that the endothelial glycocalyx layer has a very different mechanical property to the cell membrane. There is a need to assess its Young’s Modulus directly.

This PhD project aims to investigate the temporal development and spatial distribution of the endothelial glycocalyx \textit{in vitro} using confocal imaging and AFM indentation tools, and to gain insights of effects of shear stimulation on the distribution of the glycocalyx.

In order to build up the properties of the endothelial glycocalyx, the objects of the project are:

- Develop a convincing method to observe and degrade endothelial glycocalyx \textit{ex vivo} and \textit{in vitro}, and compare the difference before and after the enzyme degradation. Immunofluorescent confocal images are taken with the glycocalyx components staining (heparan sulphate and sialic acid residue); both two and three dimension images are presented to for studying the thickness of the glycocalyx layer. Heparanase III and neuraminidase are used to degrade the corresponding glycocalyx component and compares are made between the cells with and without the glycocalyx.

- Observe and assess the process of endothelial glycocalyx growth \textit{in vitro} from the very beginning of the culture until three weeks. Compare the glycocalyx distribution difference during the whole culture period. The three dimensional
confocal images are taken throughout the three weeks of culture, on both normal HUVECs and the ones with neuraminidase treatment. In addition, the distribution of the glycocalyx is compared among all these data and the development pattern of the endothelial glycocalyx are suggested.

• Measure the Young’s modulus of the HUVECs membrane using AFM indentation, both endothelial cells with and without glycocalyx layer are examined in this experiment. The indentations take place on endothelial cells at different days of culture and on different locations of endothelial cell membrane.

• Observe the endothelial glycocalyx under shear stress conditions. The HUVECs are exposed to shear stress of 12 dyn/ cm² for 24 hours, and the glycocalyx distribution is monitored throughout the following 24 hours. The glycocalyx redistribution is evaluated by the percentage area of the cell membrane covered by the glycocalyx and by the intensity ratio of the glycocalyx at different areas of the cell membrane.

• Investigate the endothelial glycocalyx recovery process after enzyme degradation. The HUVECs are treated with neuraminidase to degrade the sialic acid residual, and the reallocation of the glycocalyx layer are monitored during the 24 hours after the degradation under either static or shear flow conditions.
Chapter 2. Materials and method

2.1 Cell culture

2.1.1 Preparation of the culture medium

HUVEC culture medium is prepared under aseptic conditions. The following reagents are added to a 500ml M199 medium (Invitrogen, Paisley UK):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>working concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal bovine serum</td>
<td>10%</td>
<td>Invitrogen, Paisley UK</td>
</tr>
<tr>
<td>β-endothelial cell growth factor</td>
<td>1ng/ml</td>
<td>Sigma, Poole UK</td>
</tr>
<tr>
<td>Endothelial cell growth supplement from bovine neural extract</td>
<td>3µg/ml</td>
<td>Sigma, Poole UK</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1.25µg/ml</td>
<td>Sigma, Poole UK</td>
</tr>
<tr>
<td>Heparin</td>
<td>10U/ml</td>
<td>Sigma, Poole UK</td>
</tr>
<tr>
<td>penicillin</td>
<td>100 U/ml</td>
<td>Sigma, Poole UK</td>
</tr>
<tr>
<td>streptomycin</td>
<td>100µg/ml</td>
<td>Sigma, Poole UK</td>
</tr>
</tbody>
</table>

Table 2.1 Reagents for HUVEC culture including M199 culture medium and various growth factors
After adding all the reagents to M199 medium, the culture medium is sterilized by filtering through a 0.22µm pore sterile filter (Millipore) and separated into 50ml aliquot for future use.

2.1.2 Preparation of the cell flasks or cover slips

Cell culture flasks are coated with Collagen Type I, 5-10µg/ml (Sigma) at 37°C for 1 hour. After coating, collagen solution is discarded and flasks are gently washed with Phosphate buffered Saline (Sigma) once.

2.1.3 HUVECs

HUVECs, purchased from Lonza, are cultured at 37°C in a 5% CO₂ incubator. Culture medium is changed every 2-3 days.

2.1.4 Trypsinization of adherent cells

After reaching 80% confluence, HUVECs are sub-cultured using trypsin (0.25 % trypsin-EDTA, Sigma). Culture medium is removed and cells are gently washed with 3-5 ml PBS for 10 seconds. After discarding PBS, 2-3 ml trypsin is added and rotated through the whole flask. The trypsin is then removed and cells are checked under the microscopy. After cells are detached from the flask, 5ml culture medium is added to stop the trypsinization. Cells are centrifuged at 1000rpm for 5 minutes. The
pellet is re-suspended by culture medium and placed in new flasks at a density of 5000 cells/ml. Culture medium is changed every 2-3 days. HUVECs are discarded after 10 passages.

2.1.5 Cryopreservation and reawakening of HUVECs

For future usage, HUVECs are preserved in liquid nitrogen. After confluence, cells are trypsinized and centrifuged as described earlier. The pellet is resuspended by the following medium: 20%FBS + 70% HUVECs culture medium + 10% Dimethyl sulphoxide (DMSO, sigma).

Cells are put into cryo-vials and placed into a cryo-box (Nalgene freezing container, Nalgene, Hereford UK) and stored in a –80°C freezer overnight. The following day, the vials are transferred into a liquid nitrogen cryobank for long term storage.

To reawake HUVECs, a vial is taken from the liquid nitrogen and put in a 37°C water bath. During the process, the vial is gently shaken until the medium is totally thawed. It is then centrifuged at 1000rpm for 5 minutes to discard the DMSO. The pellet is resuspended with 5ml HUVECs culture medium. After cell counting, the HUVECs are placed in a collagen pre-coated culture flask at a 5000 cells/cm² concentration. The culture flask is placed in a 37°C, 5% CO₂ incubator. Cell culture medium is changed every 2-3 days.
2.2 Confocal microscopy

2.2.1 Preparation of the ex vivo & in vitro samples

Thoracic aortas are harvested from C57BL/6 mice (2 to 4 months old, 20 to 30 grams body weight) and the aortas are either sectioned for cross section observation, or cut open and pinned upward for enface observation of the endothelial glycocalyx.

HUVECs are seeded on sterilized collagen type I coated cover slips. After different periods of culture, HUVECs are ready for immunofluorescent observations.

2.2.2 Immunofluorescent staining

The aorta segments are fixed by 4% paraformaldehyde for 10 minutes at room temperature, followed by 20% rabbit serum blocking for 30 minutes at 37°C. HepSS-1 (HepSS-1, US biological) is used to target heparan sulfate or sialic acid component of glycocalyx. CD144 (Santa Cruz) is applied to target the endothelial tight junction. After an hour of staining at 37°C, DAPI (Sigma & Aldrich) is applied for 10 minutes at 37°C.

Cultured HUVECs are stained using wheat germ agglutinin (WGA-FITC, Sigma & Aldrich), which is used to bind to N-acety-D-glucosamine and sialic acid component of the glycocalyx. The endothelial cytoplasm is stained by Cell Tracker Red CMTPX
(Invitrogen), followed by Hoechst 33342 (Sigma & Aldrich) for cell nucleus staining.

Both the *ex vivo* aortas and *in vitro* HUVECs are kept in 10% serum culture medium after the staining, ready for confocal microscopy (Leica Microsystems, Wetzlar, Germany).

### 2.3 Atomic Force Microscopy (AFM)

#### 2.3.1 The AFM and cantilever

Atomic force microscopy is used to map out the morphology of the HUVECs. Figure 2.1 is a schematic drawing of the AFM cantilever scanning through the HUVEC monolayer. The cantilever tip reflects a laser beam and the oscillations of the tip are recorded by a position sensitive detector.
Figure 2.1 Schematics of AFM probing of HUVECs *in vitro*. The cantilever tip is scanning through the endothelium membrane, the reflection of a laser beam on the cantilever tip is detected and the oscillations of the tip are recorded by a position sensitive detector.

HUVECs are cultured in collagen type I coated round cover slips (d = 13 mm). The cover slip is placed on a microscopic liquid sample stage. Measurements are carried out using an atomic force microscope (NT-MDT Ntegra System), as shown in Figure 2.2.
B) Scanning measuring head
- Photodiode adjustment screws
- Laser adjusting screw
- Manual approach knob
- Positioning adjusting screw

C) Liquid cell
- Sample stage with magnetic sample holder

D) Probe holder seat for operating in liquid
Figure 2.2 The atomic force microscopy system. A) The broad view of the overall system, including the anti-vibration table (Halcyonics), the microscopy and the AFM; B) The close up look of AFM system; C) The close up look of the AFM system below the scanning measuring head; D) The probe holder that located on the bottom of the scanning head.

The cantilever (Olympus, OMCL-RC800PSA-1) has a pyramidal shaped tip and the end of the tip is hemispherical shaped with a radius of 20nm (shown in Figure 2.3).

Figure 2.3 The SEM imaging of the AFM cantilever over view. The short cantilever is being used in the experiment with length of 100nm and width of 20nm.
2.3.2 Two AFM scanning modes used for imaging and mechanical property examination

The most commonly used AFM scanning modes are contact mode, non contact mode and tapping mode. In our study, tapping mode is used for HUVECs topography imaging and contact mode is used for mechanical properties examination (Figure 2.4).

Under the tapping mode, the AFM cantilever oscillated with high amplitude under the assembled cantilever’s resonant frequency. When the cantilever approaches the sample surface, the oscillation reduces when the tip begin to tap the surface, this is caused by the energy loss during the contact. During the scanning process, the cantilever oscillation amplitude increases when passing a down fold. On the contrary, the amplitude decreases when passing a bump area. This motion allows the cantilever gently tapping the sample surface without damaging the surface, yet providing delicate details of the surface morphology.

During the contact mode operating, the AFM cantilever and sample surface remain in close contact. The amplitude of the cantilever remains at a set value, which maintains the same force between the tip of the cantilever and the sample. This mode has been widely used for mechanical properties measurements.
2.3.2 Cell morphology imaging using AFM

The HUVEC morphology are measured using the tapping mode of the AFM (Muller & Dufrene, 2008), as shown in Figure 2.5. Figure 2.5A is the height image; the brightness represented the height of HUVECs with the brightest areas represent the top of the cells above the nucleus. Figure 2.5B shows the phase image. It provides a clear boundary at the edge of the cell and reflects the mechanical property of the HUVEC surface.

Figure 2.5 AFM scanning images using the tapping mode. A) Height image; B) Phase image. The brightness of height image provide topographic information of the cells and phase image provide information of the edge of the cells.
Indentation tests are carried out under contact mode on samples within 1 hour of removal from the incubator. During AFM probing, samples are kept in the culture medium.

2.3.3 The force-distance curve

The Hertz model for indentation on a homogeneous soft sample by a stiff cone is used to analyze the force-distance curve (Hertz, 1882; Sato *et al.*, 2004):

\[ F = \delta^2 \frac{2}{\pi} \frac{E}{(1-\nu^2)} \tan \alpha \]

where \( F \) is the applied loading force, \( \delta \) is the indentation distance, \( \nu \) is the Poisson ratio of the tissue sample and is assumed to be 0.5 as cells are considered to be incompressible, \( \alpha \) is the half opening angle of the tip of the AFM cantilever and \( E \) is the Young’s modulus.

2.4 Shear stress stimulation

2.4.1 Design of the static flow chamber

A custom made parallel flow chamber is used for HUVEC shear stress stimulation. The sketch is shown in Figure 2.6. The hydrostatic pressure is provided by the height difference between the upper and lower reservoirs. A peristaltic pump returns the culture medium from the lower reservoir to the upper one, completing the flow circuit.
Figure 2.6 Schematic drawing of the static flow bioreactor. It contains a pulsatile pump, an air filter, a parallel flow chamber and two reservoirs.

### 2.4.2 Calculation of the shear stress

For steady laminar flow between two parallel plates, the wall shear stress on the plate is determined using the following equation (Depaola et al., 1992):

$$\tau = \frac{6\mu Q}{Wh^2}$$

where $\tau$ is the wall shear stress (dyn/cm²), $\mu$ is viscosity of the culture medium, $Q$ is the flow rate (ml/s), $h$ is channel height (0.25mm of silicon gasket) and $W$ is channel width (20mm of custom made chamber).
The height difference between two reservoirs and the according flow rates are measured. The shear stress is calculated and shown in Table 2.2.

<table>
<thead>
<tr>
<th>Height difference, h (cm)</th>
<th>Flow rate, Q (cm³/s)</th>
<th>Shear stress, τ (dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.038</td>
<td>3.7</td>
</tr>
<tr>
<td>10</td>
<td>0.088</td>
<td>8.6</td>
</tr>
<tr>
<td>15</td>
<td>0.132</td>
<td>12.8</td>
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<td>20</td>
<td>0.185</td>
<td>18.0</td>
</tr>
<tr>
<td>25</td>
<td>0.227</td>
<td>22.0</td>
</tr>
</tbody>
</table>

Table 2.2 Calculation of the shear stress. The shear stress is deducted from the height difference from the two reservoirs and the flow rate.

The plot of shear stress vs. reservoirs height difference is drawn based on the measured data, Figure 2.7, and the shear stress of 12 dyn/ cm² is set for the following HUVECs stimulation.
Figure 2.7 The relationship between the height difference of reservoirs and the wall shear stress.

2.4.3 Shear stimulation of HUVECs

HUVECs are seeded on cover slips and after day 4, 7, 10, 14 and 21 in culture, they are exposed to a steady shear stress of 12 dyn/cm². The pH of the culture medium is kept by gassing the medium with a mixture of 95% air and 5% CO₂ and the temperature is maintained at 37°C.
Chapter 3. Confocal imaging of the endothelial glycocalyx ex vivo

3.1 Introduction

Endothelial cells line the entire luminal surface of blood vessels and are in direct contact with the blood. They play vital roles in vascular regulation and blood cell activation and migration during physiological and pathological processes (Middleton et al., 1997; Cai & Harrison, 2000). Dysfunction of endothelial cells is a key factor in the initiation and progression of vascular diseases, such as atherosclerosis (Davignon & Ganz, 2004). At the luminal surface of the endothelium, the glycocalyx was first observed by Luft using electronic microscopy with ruthenium red staining, which revealed a layer of up to 20nm thick (Luft, 1966). More recent studies showed this layer to have a brush-like structure and was between several hundreds nanometers and a few microns in thickness (Squire et al., 2001).

The endothelial glycocalyx contains negatively charged molecules such as proteoglycans, glycosaminoglycans (GAGs), glycoprotein and plasma proteins (Oohira et al., 1983; Jackson et al., 1991). The molecules that make up the glycocalyx are continuously synthesized by and shed from endothelial cells in a dynamic process (Lipowsky, 2005; Gouverneur et al., 2006b). The endothelial glycocalyx serves a number of functions in the vascular system: 1) permeability control of fluid and solute filtration and absorption across the vessel wall (Michel &
Kendall, 1997; Weinbaum, 1998; Levick & Michel, 2010); 2) modification and amplification of the shear stress on endothelial cells by the circulating blood (Wang & Parker, 1995; Mochizuki et al., 2003); 3) regulation of interactions between blood cells and vascular endothelial cells (Damiano, 1998; Zhao et al., 2001); in addition, 4) the glycocalyx functions as a mechanotransducer for the endothelial cytoskeleton (Secomb et al., 2001; Florian et al., 2003; Tarbell et al., 2005; Gouverneur et al., 2006a). It has been reported by several laboratories that manipulation of the glycocalyx results in the dysfunction of the endothelial mechanotransduction. For example, van den Berg et al reported that atherogenic diet mice had much thinner glycocalyx layer than the normal diet ones, making them at higher risk of developing atherosclerosis (van den Berg et al., 2006).

The methodology of glycocalyx staining of both fixed and fresh mice thoracic aortas is established in experiments reported in this chapter.

3.2 Methodology

3.2.1 Preparation of the mice thoracic aortas

All animal experiments were performed according to protocols approved by the institutional committee for use and care of laboratory animals. C57BL/6 mice (2 to 4 months old, 20 to 30 grams body weight) were anesthetized and thoracic aortas were harvested. Before the staining, the aortas were prepared in two different ways for
either cross sectional or enface observation of the vessel (Figure 3.1). For cross sectional studies of the vessel (Figure 3.1 A), the aorta was immersed in Tissue-Tech OCT embedding compound (Sakura Finetek 4583) and then snap frozen in liquid nitrogen. Cross sections of the aorta between 5 and 10µm in thickness were prepared (Cryo-Star, HM560 MV, Microm International GmbH) and stored in a -80°C freezer. For the enface observation (Figure 3.1 B), the fresh aorta was cut-open along the axis of the vessel and unfolded onto a Petri dish with the luminal surfaces on the top, this observation was taken place on unfixed aorta samples.

![Figure 3.1 Preparation of the ex vivo aorta samples. A) Cross section vessel sample; B) Cut open vessel sample for enface observation of the luminal surface of the aorta.](image)

### 3.2.2 Enzyme treatment of the mice aortas

Two different types of enzyme (both from Sigma & Aldrich) were used to degrade glyocalyx components of the mice thoracic aorta, as shown in Table 3.1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Component of cleavage</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparinase III from 1-4 linkages between hexosamine</td>
<td>0.5 U/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>and glucuronic acid residues in heparan sulfate</td>
<td></td>
</tr>
<tr>
<td>heparinum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>N-acetyl neuraminic acid and sialic acid</td>
<td>1U/ml</td>
</tr>
<tr>
<td>from Clostridium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>perfringens</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Enzymes used in the experiment, including heparinase to cleave heparan sulfate residues and neuraminidase to cleave the sialic acid.

Heparinase type III (Sigma & Aldrich) cleaves heparan sulfate GAGs (Thi et al., 2004). The sectioned aorta segments were gently washed twice using serum-free M199 medium and incubated with 0.5U/ml heparinase III for 30 minutes at 37°C. The control group was kept in the HUVEC culture medium.

Neuraminidase from clostridium perfringens (Sigma Aldrich) cleaves N-acetyl neuraminic acid residues of glycoproteins and targets specifically the sialic acid component of the endothelial glycocalyx. The enface samples were treated with neuraminidase according to the protocol by Baker et al (Barker et al., 2004). Serum-free M199 medium was used to wash the sample gently twice before it was incubated with 1U/ml neuraminidase for 10 minutes at 37°C. The control group remained in the HUVECs culture medium.
3.2.3 Immuno-fluorescent staining of the mice aortas

Wheat germ agglutinin (WGA-FITC) was used to bind to N-acetyl-D-glucosamine and sialic acid component of the glycocalyx (Barker *et al.*, 2004; Megens *et al.*, 2007). Heparan sulfates were stained by Heparan Sulfate antibody FITC (HepSS-1, US biological) (Thi *et al.*, 2004). Endothelial cell tight junctions were stained using CD144 (Santa Cruz) and cell nucleus were stained using DAPI (Sigma Aldrich).

The aorta segments were fixed by 4% paraformaldehyde for 10 minutes at room temperature, followed by 20% rabbit serum blocking for 30 minutes at 37°C. After that, HepSS-1 or WGA-FITC and CD144 were applied on the segment for one hour at 37°C. DAPI was then applied for 10 minutes at 37°C. Segments were washed three times by serum free M199 after staining and kept in 10% serum culture medium, ready for confocal microscopy (Leica Microsystems, Wetzlar, Germany).

3.3 Results

3.3.1 The glycocalyx on fixed mice aortas sections

Following the sectioning of the aorta, the glycocalyx is stained using HepSS-1, as showed in Figure 3.2. From the luminal side, aorta vascular wall consists of several layers of cells: endothelial cells, internal elastic lamina, smooth muscle cells (Figure 3.2 A). Several parallel green fluorescence layers can be seen in Figure 3.2 B: the
first layer from the luminal side is endothelial cells and the following layers are smooth muscle cells. The dark green thick layer between endothelial cells and smooth muscle cells is internal elastic lamina. From this figure, both endothelial cells and smooth muscle cells appear uniformed glycocalyx layers.
Figure 3.2 HepSS-1 immunofluorescence staining of endothelial glycocalyx *ex vivo*.

A) An overview of the cross section aorta, Bar = 40μm; B) A closer look of the highlighted area in A), Bar = 8μm. The green staining indicates the heparan sulfate residue of the glycocalyx.

Sectioned samples of the fixed mouse thoracic aorta are stained in the following procedure.

As shown in Figure 3.3, HepSS-1 (green) targets the Heparan sulfate of the extracellular matrix that includes the glycocalyx of the endothelial and the smooth muscle cells. CD144 (red), which targets endothelial cell tight junctions, indicates the endothelium of the aorta and appears at the outmost layer of the vessel wall (as shown in the merged image of Figure 3.3 A). HepIII treatment of the exercised aorta is performed before the sectioning of the aorta, therefore cleavage of heparan sulfate GAGs happens predominantly to the endothelial glycocalyx that is directly accessible by HepIII. This is seen in the merged image of Figure 3.3 B, where the endothelium of the vessel (as indicated by the CD144 red staining) has hardly any green staining, indicating that most of the endothelial glycocalyx layer has been cleaved by HepIII.
Figure 3.3 *Ex vivo* HepSS-I (green) and CD144 (red) staining of the cross sectioned mouse thoracic aortic. Bar = 5μm. A) control, B) after HepIII treatment. The merged images of the green and red indicate the heparan sulfate staining of endothelial cells.

### 3.3.2 Enface observation of endothelial glycocalyx

Mouse thoracic aortas are cut open along the axis of the vessel and pinned onto a Petri dish with the endothelium facing upwards. They are then treated as the following procedure:

As shown in Figures 3.4, the green strained heparan sulfate containing layer, seeing in A), becomes much less expressed after HepIII treatment, as shown in panel B). When comparing the intensity of the green dye in heparinase III treated groups to that in control groups, as shown in Figure 3.4 C), a statistically very significant decrease (*p < 0.01*) can be observed following enzyme perfusion of the aortic endothelium. The *ex vivo* results show that the staining and enzyme treatment protocols for the glycocalyx as well as the confocal image system work properly in our study.
3.3.3 Reconstructed 3D enface images of the endothelial glycocalyx in unfixed aortas

Freshly harvest and unfixed mouse thoracic aortas have been used in Figure 3.5. Different to experiments in Figure 3.4, wheat germ agglutinin (WGA-FITC) is used to bind to the heparan sulfate and hyaluronic acid of the unfixed tissue samples. Three dimensional confocal images are taken at a series of depths (at $\Delta z = 200\text{nm}$ interval along the $z$ axis). The main enface panel shows the fluorescent image in the x-y cross section at a given $z$ location (rather than stack images). The two smaller panels reveal the structure of the glycocalyx layer along the x-z (bottom panel) and y-z cross
sections (side panel) as indicated by dashed lines in the enface image. The intact glycocalyx layer, seen in Figure 3.5 A), has a similar expression to that in Figure 3.4 A). The green stained layer is approximately 1–2μm in depth and is confined to the surface of the endothelium, as indicated by the red stained endothelial specific tight junctions. For neuraminidase treated samples, shown in Figure 3.5 B), there is little green stained layer in the enface image. Here, nuclei of endothelial cells and smooth muscle cells are stained blue. The bottom and side panels in Figure 3.5 B) reveal largely green free cross sections in the x-z and y-z planes. These ex vivo results agree to previous findings where the thickness of endothelial glycocalyx layer is estimated to be between several hundred nanometers to several microns (van den Berg et al., 2003; van den Berg et al., 2006; Chappell et al., 2009).
Figure 3.5 3D confocal images of the glycocalyx layer on freshly harvested and unfixed mouse thoracic aorta. The main panel shows the enface image at a given z depth. The bottom and side panels show the x-z and y-z cross-sectional images respectively. Bar = 10μm, in the main panel. A) control, B) after neuraminidase perfusion of the aorta.
3.4 Discussion

Previous studies have reported a wide range of values on the thickness of the glycocalyx layer. This is partly due to different species and different types of blood vessels used in experiments, and partly due to different preparation techniques employed. Most notably the dehydration and fixation process for conventional electron microscopy are likely to collapse all but the protein cores of proteoglycans and water crystals may also form (Adamson & Clough, 1992). For example, Vink’s group reported a 0.2 - 0.5μm hairy structure of the glycocalyx layer in ventricular myocardial capillaries of the rat heart using electron microscopy (van den Berg et al., 2003). Megens and co-workers, based on their 3D reconstruction images using two-photon laser-scanning microscopy, reported that in mice mesenteric arteries, the glycocalyx layer was ~4.5μm and covered two thirds of the entire endothelial surface area (Megens et al., 2007). The debate on differences between the endothelial glycocalyx in vivo and in vitro has also been there for some time, not only on the thickness of the glycocalyx, but also on the functionality of the in vitro glycocalyx layer. Chappell et al reported that HUVECs possessed the glycocalyx structure only in their ex vivo samples and not on cultured cells (Chappell et al., 2009). In a more recent paper by Ebong et al, the glycocalyx layer on cultured bovine aortic endothelial cells and rat fat pad endothelial cells have been observed using rapid freezing/freeze substitution transmission electron microscopy (Ebong et al., 2011).
Based on this unconventional approach, the endothelial glycocalyx layer was estimated to be approximately 5–10μm, i.e. ~100 folds thicker than what was observed on conventionally preserved endothelial cells. Their observation of stained heparan sulfates using confocal microscopy showed similar thickness (i.e. ~1.4μm) of the glycocalyx layer as reported in the current study.

In the *ex vivo* confocal images, CD144 staining shows a notable difference between the control and enzyme treated groups. In Figure 3.3, the CD144 staining in panel A can only been seen partly and is relatively weaker in comparison to panel B. Similar difference is seen in Figure 3.4. CD144 reveals a continuous and cobblestone pattern for the endothelial cell junctions for enzyme treated groups. In contrast, CD144 staining of the control group appears to be intermittent. This may be caused by the glycocalyx layer in the control group, which restricts the access of CD144 dye to cell tight junctions. In enzyme treated groups, the glycocalyx layer has been diminished. Cell tight junctions are exposed and directly accessible to CD144.
Chapter 4. Spatiotemporal distribution of the endothelial glycocalyx in vitro

4.1 Introduction

Ever since the dense carbohydrate layer that coats cell surface was revealed in 60s, the glycocalyx has been studied with growing interest (Luft, 1966; Rambourg & Leblond, 1967). Although there are continuing debates on the appearance of the glycocalyx layer between in vivo and the cultured models, in vitro endothelial monolayers have been used widely to study both the structure and functions of the glycocalyx (Yao et al., 2007; Ebong et al., 2011).

Because of the wide variety of the membrane constitutions of the glycocalyx, different components have been target. Sialic acid is one of the most commonly observed components; Linnartz et al stained terminal sugar residue sialic acid residues of the primary neuronal glycocalyx, in order determine the microglial-mediated clearance function (Linnartz et al., 2012). Baker et al used WGA-FITC for N-acetylneuraminic (sialic) acid staining to map out the glycocalyx on HUVECs (Barker et al., 2004). Other components have also been reported: for example, Stevens et al used HepSS-1 to immunostained heparan sulfate (HS) layer of the bovine lung microvascular endothelial cells, and their 3D reconstruction results revealed a dense HS layer across on the cell surface with a thickness ~ 2.8μm (Stevens et al., 2007).
Different constitutional components of the glycocalyx layer can be specifically digested using different enzymes, for example, neuraminidase for the sialic acid residue, heparanase for HS and hyaluronidase for hyaluronic acid (HA). Thi et al reported that following GAGs digestion from HUVECs in vitro using heparinase III, effects of the shear stress on the endothelial cytoskeleton was abolished (Thi et al., 2004; Yao et al., 2007). The role of glycocalyx as a mechanotransducer under shear stress can be inhibited after degrading the HS, HA and sialic acid. Shear induced nitric oxide (NO) production was also blocked following the glycocalyx degradation (Pahakis et al., 2007).

In this chapter, the spatial distribution of the endothelial glycocalyx layer is observed using laser scanning confocal microscopy. HUVECs of different days in culture are studied to reveal the temporal development of the glycocalyx layer. Comparisons are made between the controls and enzyme treated groups.

4.2 Methodology

4.2.1 Sample preparation

HUVECs (primary pooled) were purchased from Lonza (Lonza Cologne AG, Germany) and cultured at 37°C in 5% CO₂ incubator. After reaching 80% confluence, HUVECs were trypsinized with 0.25% trypsin and seeded on cover collagen type I
coated glass slide. HUVECs between passage 1 (P^1) and 10 (P^{10}) were used for experiments. Figure 4.1 shows the morphology of the P^3 HUVECs. Cells reached 90% confluent after 7 days in the culture medium and showed typical cobble-stone shape.

![Figure 4.1 Confluent HUVEC (passage 3) after 7 days in the culture medium.](image)

HUVECs, cultured for 1, 3, 5, 7, 14 and 21 days were investigated in immunofluorescent experiments. Results were compared to those of enzyme (5U/ml of neuraminidase for 30 minutes) treatment groups respectively. HUVECs were stained using the following protocol:

- **Live Cells**
- **treat A**: HUVEC culture medium, 30min
- **treat B**: Neuraminidase, 5U/ml, 30min
- **stain**: Cell Tracker Red CMPTX, Wheat Germ Agglutinin, Hoechst 33342
4.3 Results

4.3.1 Immunofluorescent staining of the endothelial glycocalyx

Multicolor immunofluorescent labeling of live HUVEC monolayer (14 days) on a cover slip is shown in Figure 4.2. The sialic acid component of the glycocalyx is stained green using wheat germ agglutinin (WGA-FITC). The endothelial cytoplasm is stained red with Cell Tracker Red CMTPX. The cell nucleus is stained blue by Hoechst 33342. Figure 4.2 is a 2D cross section image of the endothelial cells at a given z distance, where the sialic acid staining appears on the membrane of the HUVECs.

Figure 4.2 2D immunofluorescent image of HUVECs (14 days of culture). Sialic acid residue is stained using WGA-FITC staining in green, endothelium cytoplasm is stained using CellTracker CMPTX in red and nucleus is stained using Hoechst 33342 in blue.
In order to examine the distribution of the glycocalyx on the entire endothelial membrane, three dimensional reconstructed confocal images are taken from the top of the cell to the glass slide. A serial of single confocal sections are selected from the top to the bottom of the endothelial cells. Each section acquired is 200nm apart in the z-axis. As an example, Figure 4.3 A shows a series of x-y cross sectional images of a group of 14 day HUVECs. On the first few sections, the sialic acid staining (WGA-FITC, green) shows a small area near the nucleus. As the confocal scanning moves towards the bottom of the endothelial cells, WGA-FITC staining presents a ring pattern surrounding the nucleus. These serial images indicate the sialic acid staining located on the membrane of the endothelial cells. The stack images are shown in Figure 4.3 B), where the sialic acid, cytoplasm and nucleus are shown separately in the top left, top right and bottom left panels respectively. The merged image is shown in the bottom right panel. It can be seen that the sialic acid staining covers the entire cell membrane.
Figure 4.3 Sequential confocal images of 14 days live HUVECs. Green = sialic acid stained by WGA-FITC; Blue = nucleus stained by Hoechst 33342; Red = cytoplasm staining by cell tracker. A) x-y cross sectional images from the top to the bottom of HUVECs. \( \Delta z = 200 \text{nm} \). Bar = 20 \( \mu \text{m} \). B) Stack images of the sequential images. Bar = 5 \( \mu \text{m} \).

4.3.2 Spatial distribution and temporal development of the endothelial glycocalyx

The distribution of endothelial glycocalyx varies as the culture period progresses. In this study, HUVECs cultured for different period of time are observed, up to 21 days, see Figure 4.4. At the very beginning of the cell culture, e.g. by the end of day 1 shown in A), the sialic acid appears only on part of the edge areas of the cell membrane. By the end of day 10, as shown in B), the sialic acid can be seen both on the edge area and towards the apical area; however, there seems to be a denser staining near the edge area than that on the apical area of the cell membrane. By the end of three weeks, as seen in C), the sialic acid can be seen to cover the entire cell membrane.
Figure 4.4 Development of the endothelial glycocalyx layer between day 1 (A), day 10 (B) and day 21 (C). Bar = 5 μm.

Images of HUVECs of day 1, 3, 5, 7, 14 and 21 are studied and shown in Figure 4.5. The main panel shows the x-y cross section at a given z location, the two side panels show the reconstructed x-z and y-z cross sections. At the early days of culture, i.e. from day 1 to day 5, the majority of the green dye can only be seen on the edge area of the cells. As time progresses, e.g. by the end of day 7, the green dye appears not only near the edge of cells but also towards the apical area, although it is still patchy on the membrane above the endothelial nucleus. By the end of day 14, the green dye can be seen over the entire cell membrane. This uniformed coverage of glycocalyx remains the same until 21 days of culture. From the z section images (shown as the bottom and side panels), the thickness of the glycocalyx layer on HUVECs in vitro is between 300nm and 1μm. This layer is thinner compared to the one on the ex vivo
samples, but after 14 days in culture, it covers the entire surface of the cell membrane, forming a continuous layer.
Figure 4.5 Spatial distribution of the glycocalyx layer on live HUVECs in vitro and its temporal development from day 1 to day 21. The main panel shows the x-y cross sectional image at a given z depth. The bottom and side panels show the x-z and y-z cross sectional images respectively. Bar = 10μm, in the main panel.

4.3.3 The endothelial glycocalyx images following enzyme treatment

Neuraminidase is used to cleave the neuraminic acid component of the glycocalyx, including the sialic acid residues and N-acetyl-D-glucosaminyl residues. HUVECs are treated with neuraminidase of 5U/ml for 30min. A serial of confocal images are taken to observe the change in the appearance of the endothelial cells. Figure 4.6 shows the sequential and stack images of HUVECs (day 14), taken from the top of the cell to the glass slide with 200nm step change. The vast majority of the glycocalyx layer (seen in Figure 4.3B) has been abolished, leaving extremely patchy green dye near the edge area of the cells.
Figure 4.6 Confocal images of day 14 HUVECs after neuraminidase treatment. Green = sialic acid stained by WGA-FITC; Blue = nucleus stained by Hoechst 33342; Red = cytoplasm stained by cell tracker. A) Sequential confocal images were taken from the top of the cell to the glass slide with $\Delta z = 200$ nm. Bar = 50 $\mu$m. B) The stack image. Bar = 5 $\mu$m.

Figure 4.7 presents 3 cross sectional images of the bottom right panel in Figure 4.6 B). The main panel shows the x-y cross sectional image at a given z depth, and the bottom and side panels are the x-z and y-z cross sectional images respectively. In comparison to 14 day images in Figure 4.5, most of the glycocalyx layer has been digested following neuraminidase treatment. The HUVECs of different culture days are also treated with neuraminidase, shown in Figure 4.7, at day 7, 14 and 21 do not present a layer of the green dye after they are stained by WGA, indicating that the enzyme has cleaved away most of the glycocalyx from the cell membrane.
Figure 4.7 The x-y (main panel), x-z (bottom panel) and y-z (side panel) cross sectional images of the merged stack image in Figure 4.6B, the day 14 HUVECs. Bar = 10μm, in the main panel.

4.4 Discussion

Chappell et al. reported that the thickness of HUVEC glycocalyx was approximately 900nm in their ex vivo samples, whereas it was less than 30 nm in their in vitro ones (Chappell et al., 2009). The major components of glycocalyx, i.e. heparan sulfate and syndeacan-1 were observed on both the ex vivo and in vitro samples. The observed extremely thin glycocalyx layer in their in vitro samples may be due to their fixation preparation for electron microscopy. Insufficient culture time (i.e. 9-10 days in their study) may have also contributed to the small value. In a more recent study by Ebong et al., the glycocalyx layer on cultured bovine aortic endothelial cells and rat fat pad endothelial cells were observed using rapid freezing/freeze substitution transmission electron microscopy (Ebong et al., 2011). Based on this unconventional approach, the endothelial glycocalyx layer was estimated to be approximately 5-10μm, i.e. ~100 folds thicker than what was observed on conventionally preserved endothelial cells. Their confocal microscopy observation of stained heparan sulfates in the glycocalyx, however, showed the glycocalyx layer was approximately ~1.4μm, similar to the result reported in this chapter.
The study using confocal microscopy shows the development of the endothelial glycocalyx over time. The growth appears to initiate from the edge of cells and develops to the apical region. It takes up to two weeks to cover the entire cell membrane (i.e. region above the nucleus). The mechanism underlying this spatial distribution and temporal change needs further investigation, but it seems functionally beneficial as the glycocalyx layer covers the intercellular gaps for the endothelium to fulfill its role as a selective semi-permeable membrane for the circulating blood. The 3D reconstructed images, although limited by the confocal resolution, indicate the thickness of the glycocalyx layer is approximately 1μm. This finding agrees reasonably well to previous studies.
Chapter 5. Mechanical properties of the endothelial glycocalyx \textit{in vitro}

5.1 Introduction

Cell mechanical properties are linked to its cell functions, such as cell morphology, adhesion, migration and signaling (Guck \textit{et al.}, 2005; Yamazaki \textit{et al.}, 2005; Sheetz, 2006). Various approaches were applied to detect the mechanical properties of single cell, including micropipette aspiration, cytoindenter, magnetic or optical tweezers, and last but not least the atomic force microscopy (AFM) (Svoboda & Block, 1994; Guilak \textit{et al.}, 1999; Shin & Athanasiou, 1999). Since 1980s, AFM has been used to investigate properties of biological cells (Drake \textit{et al.}, 1989; Henderson, 1994). Using the AFM for biological material studies has the advantage of topographic characterization of molecular scale features, piconewton force sensitivity (Fisher \textit{et al.}, 2000; Chaudhuri \textit{et al.}, 2009). Elastic properties of endothelial cells have been studied using AFM, e.g. Sato \textit{et al} estimated the elasticity of HUVECs by applying the Hertz model, and reported the Young’s modulus changes with time during the early stage of cell culture (Sato \textit{et al.}, 2001; Sato \textit{et al.}, 2004). In addition, a softer edge region of the cell was observed with lower elasticity, where the Young’s modulus was $5 \pm 0.2$ kPa with the thickness of $0.418 \mu m$ and $49 \pm 1.4$ kPa with the thickness of $1.51 \mu m$.

Kataoka \textit{et al} interacted monocytes with endothelial cells and evaluated the mechanical property affected by the interaction. They reported the elastic modulus of HUVECs decreased after 2hrs monocytes application, the elastic modulus of the
center of HUVECs dropped from $2.7 \pm 1.8$ kPa to $1.6 \pm 0.6$ kPa, which indicated the softer HUVECs after monocytes and endothelial cells interaction (Kataoka et al., 2002).

The elasticity of other cell types has also been widely reported: the enzymatically-isolated chondrocytes were reported having Young’s modulus of approximately 0.6 kPa, which was significantly lower than the pericellular matrix of 1.54 kPa (Guilak et al., 1999); Rotche et al reported the BALB 3T3 fibroblasts elastic modulus of $1.01 \pm 0.4$ kPa (Park et al., 2005); and the elastic modulus of human chondrosarcoma cells were approximately 2kPa which varied within cell lines of different aggressiveness. (Darling et al., 2007) to list a few. Mechanical properties of the glycocalyx have not been studied until very recently. O’Callaghan et al measured the Young’s modulus of the glycocalyx on bovine lung microvascular endothelial cells (BLMVECs) and reported a value of $0.26 \pm 0.03$ kPa (O’Callaghan et al., 2011). However, to the best of our knowledge, there has been no experimental study to evaluate how the Young’s modulus of the spatial changes of the glycocalyx layer on the cell membrane and how it varies with time. As the interface between the circulating blood and the endothelium, the glycocalyx mediates flow induced shear stress on endothelial cells. The mechanical property of the glycocalyx layer is essential for us to understand its many biological functions.

In this chapter, I investigate the Young’s modulus of the glycocalyx layer using AFM
nano indentation. The Young’s modulus of the glycocalyx is calculated from the results on the HUVEC cell membrane with or without the glycocalyx layer.

5.2 Methodology

5.3.1 Calibration of the cantilever

A rectangular cantilever with a pyramid shaped tip is used for both topographic imaging and indentation of cells. The cantilever, shown in Figure 5.1 A, is 100 μm in length and 20 μm in width, with a known spring constant (0.38 N/m). The tip is pyramid shaped with a base length of 5.2 μm and a height of 2.9 μm (Figure 5.1 B). The half opening angle is approximately 41 degrees. The end of the tip has a semi-spherical shape with a radius of approximately 20nm, as shown in Figure 5.2 C.
Figure 5.1 SEM images of the AFM cantilever. A) The rectangular cantilever; B) The pyramidal shaped tip; C) The end of the tip is of a semi spherical shape.

The calibration of the cantilever is carried out before indentations on cells. The deflection-piezo displacement curve on the glass surface is recorded. Figure 5.2 presents an indentation test on the glass surface close to endothelial cells (the yellow dot on the height image in the left panel) and the deflection-piezo displacement curves during the on-set (red) and off-set (blue) phases of the indentation. The high value of stiffness of the glass induces a steep slope in the indentation curves as seen in the right panel. This slope is used to convert the recorded electric current I (nA, y axis) to the cantilever deflection (μm, x axis)
Figure 5.2 AFM indentation on a glass surface for cantilever calibration. Left panel shows the yellow dot as the location of indentation on the glass surface, and right panel shows the corresponding deflection-piezo displacement curves.

The force between the AFM tip and cell sample ($F$, Newton) is determined by the cantilever deflection in volt ($V$, volt), cantilever deflection sensitivity ($\alpha$, meter / volt) and the cantilever’s spring constant ($k$, Newton / meter) (Cappella & Dietler, 1999):

$$F = k \times \alpha \times V$$

It needs to be noted that the piezo displacement in Figure 5.2 is not the displacement of the tip against the testing sample, but the distance between the sample surface and the rest position of the cantilever, as shown in Figure 5.3.
Figure 5.3 Sketch of AFM cantilever deflection during indentation and the geometric relationship. The indentation depth $h$, cantilever deflection $\delta_c$, and tip-sample distance $D$:

$$h = |D| - \delta_c$$

- $h$: indentation depth
- $\delta_c$: deflection of the cantilever
- $D$: distance between the tip and sample

The indentation depth ($h$) is governed by the deflection of the cantilever ($\delta_c = V \times \alpha$) and tip-sample distance ($|D|$):

$$h = |D| - \delta_c = |D| - \frac{F}{k}$$

- $h$: indentation depth
- $\delta_c$: deflection of the cantilever
- $D_0$: the distance between the initial surface position and the position of force-free beam tip
- $D$: the reading of current surface position of sample, i.e. distance between current surface position and the position of force-free beam tip
The force-distance curve can be plotted from the raw deflection-piezo displacement data. As shown in the left panel of Figure 5.4, indentation points on HUVECs are chosen from the center of the cell (i.e. above the nucleus) to the edge of the cell. The right panel in Figure 5.4 shows the deflection-piezo displacement curves for indentation at the centre of the cell. The red curve represents indenting process, and the blue curve represents retracing process. Unlike the deflection-piezo displacement curves for indentation on the glass slide, where the loading and unloading curves overlap each other, the unloading curve of cellular samples decreases much more rapidly as the tip of the cantilever moves away. This is due to the viscoelastic property of HUVECs. The cell membrane cannot fully recover immediately after AFM indentation and the ‘dip’ at ~0.5 µm of displacement in the unloading curve may reflects the pulling force on the cantilever when the connection between the cell membrane and the AFM tip breaks up.

Figure 5.4 Deflection-piezo displacement curves of AFM indentation on HUVECs. Left panel: locations of indentation tests on HUVECs; Right panel: typical
deflection-piezo displacement curves during indentation.

Figure 5.5 shows an example of force - distance curve during indentation on a 14 days HUVEC. The indentation location is at the centre of the cell (i.e. above the nucleus). At the beginning of the indentation, the piezo is at the lowest position and the AFM cantilever is away from the sample surface and the force stays at 0 nN. When the tip of the cantilever makes contact with the sample surface, the force increases gradually as the indentation proceeds.

![Figure 5.5 An example force - distance curve during AFM indentation on HUVECs.](image)

5.3.2 Calculation of the Young’s modulus

The Young’s modulus is calculated from the following equation (Sato et al., 2004):

\[
F = \delta^2 \frac{2}{\pi} \frac{E}{(1-\nu^2)} \tan \alpha
\]
where $F$ is the applied loading force, $\delta$ is the indentation distance, $\nu$ is the Poisson ratio of the tissue sample (assumed to be 0.5 in this study), $\alpha$ is the half opening angle of the tip of the AFM cantilever and $E$ is the Young’s modulus.

The glycocalyx is considered as a simple isotropic, homogeneous and incompressible layer. The tip of the cantilever has a hemisphere shape with a radius of 20 nm. The cell membrane is considered as a deformable flat surface. In order to minimize possible influence of the cytoplasm and cytoskeleton to the results, only raw data from the first 200 nm of indentation depth are used in all the calculations.

5.3 Result

5.3.1 The HUVECs imaging under height and phase mode

Both the height and phase images are taken simultaneously in the study to gain information on the cell surface. In Figure 5.6 A), the brightness of the image indicates the height of the cell surface. Details of this topography can be seen on the 3D reconstructed height image in Figure 5.6 B). The phase image in Figure 5.6 C) provides contrast of the change in the height and gives much better indication of the location of the edge of cells. In later studies, the cell surface was divided into three regions: the ‘apical’ region is the area above the endothelium nucleus (shown between 2 black lines), and the rest area is divided equally into the ‘middle’ (as indicated by red lines) and the ‘edge’ (as indicated by blue lines) regions. This definition makes a spatial comparison of the cell membrane surface achievable.
Figure 5.6 AFM imaging of the HUVECs. A) Height image showing the topographic information of the cell surface, B) reconstructed 3D topography of HUVECS based on A), C) Phase image presenting the edge of the cell, the cell surface was divided equally into three divisions, apical, middle and edge.

5.3.2 The Young’s modulus of the endothelial glycocalyx

In Figure 5.7, Young’s moduli of the cell membrane at three different locations on the cell membrane are presented. HUVECs at the end of day 3, 7, 14 & 21 in culture are used. Comparisons are made between cells with and without the glycocalyx layer, i.e. between control groups and corresponding groups after the neuraminidase treatment.

Comparing the control groups only in day 3, 7, 14 & 21, it is seen that the Young’s modulus of the cell membrane decreases with the time, from approximately 2.93kPa (±1.16 kPa, n = 27) at day 3 to 1.20 kPa (±0.51 kPa, n = 26) at day 7, further decreases to 0.35 kPa (±0.15 kPa, n = 28) at day 14, then remains unchanged at 0.33 kPa (±0.19 kPa, n = 27) to day 21. This reflects the development of the glycocalyx layer, which is a more flexible structure, on the cell membrane and results in progressively reduced values in the Young’s modulus as the layer develops with time. After day 14, the glycocalyx layer is well developed and the value remains unchanged. However, in the neuraminidase treated groups, the Young’s modulus are bigger in all cases in comparison to their respective control groups. These increases are statistically
significant (p < 0.05, denoted by *) except for day 3, and are statistically more significant (p < 0.01, denoted by **) for day 14 and day 21. A close look at the control group at day 7 reveals that the difference in Young’s modulus between the apical (1.54±0.58 kPa, n = 9) and the edge (0.69±0.55 kPa, n = 8) of the cell is significantly different (p = 0.012). This is consistent with earlier results from the confocal microscopy, where the glycocalyx layer at day 7 is mainly around the edge of cells and less well developed in the apical region above the cell nucleus. Compare the neuraminidase treated groups at day 14 and day 21 with their respective control groups, the stiffness of HUVEC cell membrane increases by more than 6 folds, i.e. from approximately 0.34 kPa to 2.13 kPa, when the glycocalyx layer is cleaved away.

Figure 5.7 The Young’s modulus of the HUVEC membrane in vitro. HUVECs
cultured for 3, 7, 14 & 21 days are tested using AFM indentation. Comparisons are made between different locations (i.e. apical, middle & edge) on the cell membrane, as well as between the control groups (Con) and neuraminidase treated groups (Nase) at different days (from Bai & Wang, 2012).

The Young’s modulus of the glycocalyx layer can be evaluated by comparing the change in the value of the cell membrane Young’s modulus with and without the glycocalyx. The flexible glycocalyx layer on a deformable cell membrane can be simplified to a two springs in series system. In such a system, the overall spring constant of the system \( k \), can be expressed as

\[
k = \frac{k_1 k_2}{k_1 + k_2}
\]

where \( k_1 \) and \( k_2 \) are spring constants of the two individual ones respectively. Our results suggest that the cell membrane Young’s modulus without the glycocalyx layer is approximately 2.13kPa (average of day 14 & 21), and with the glycocalyx layer, the overall Young’s modulus is ~0.34kPa (average of day 14 & 21 control groups). This leads to the Young’s modulus of the glycocalyx layer alone to be approximately 0.39kPa.

5.4 Discussion

There has been no previous report on mechanical properties of the endothelial glycocalyx layer until a very recent independent study by O’Callaghan et al on
bovine lung microvascular endothelial cells (O'Callaghan et al., 2011). The Young’s modulus of the glycocalyx reported by O’ Callaghan was 0.26±0.03 kPa, which agrees to our measurement on HUVECs. However, our study further demonstrates the spatial variation or the temporal development of the glycocalyx layer on the cell membrane.

AFM indentation of HUVECs at day 7 (the control group) shows a statistically significant difference in the Young’s modulus between the edge and apical regions of the cell surface. This difference indicates an uneven spatial development of the glycocalyx on the cell membrane. As time progresses, e.g. at day 14 and day 21, the difference in the Young’s modulus at different locations on the cell surface becomes very small. This is consistent to our confocal microscopy results, where development of the glycocalyx layer is initially around the edge of the cell, then spreads out to cover the apical area above the nucleus.

The young’s modulus equation used in this study is based on Hertz theory (Hertz, 1882), which is an original work in contact mechanics that investigates the contract stress between two curved surfaces. It has been widely used for analyzing force-distance curves in AFM indentation.

Several simplified assumptions are made using the Hertz theory for the Young’s modulus calculation, including considering the endothelial glycocalyx as an isotropic,
homogeneous and linear elastic material. Several studies suggest that cell elastic properties using Hertz model are largely depend on the cell thickness, indentation thickness and AFM cantilever tip geometry (Dimitriadis, et al., 2002; Rico et al., 2005; Bilodeau, 1992). When applying a small indentation depth, a large strain is anticipated near the cantilever tip, causing the invalidation of the small strain analysis, especially with a large indentation depth that comparable to the cell height.

One of the possible improvements for the Young’s modulus calculation is to apply computational modelling. The force-displacement curve can be reproduced by simulating the AFM indentation test using finite deformation models. The finite element method modelling provides corrections and empirical formulae, which gives much more precise evaluations of the cell mechanical properties (Dimitriadis, et al., 2002; Kang et al., 2007).

This study gave quantitative insights into the spatial distribution and temporal development of the endothelial glyocalyx layer in vitro, and improved our understanding of the glyocalyx recovery mechanism which was of fundamental importance in vascular pathophysiology.
Chapter 6. Effects of the shear stress on the endothelial glycocalyx in vitro

6.1 Introduction

The endothelial glycocalyx is in direct contact with blood flow and is exposed to flow induced shear stress in vivo. As a mechanotransducer, the glycocalyx modifies the stress on endothelial cells and transforms it to the endothelial cytoskeleton (Damiano & Stace, 2002; Mochizuki et al., 2003). On the other hand, shear stress induces the deformation of the endothelial cytoskeleton and the glycocalyx, triggering a number of signaling events (Davies, 1997). It is known that the shear stress activates endothelial cell morphology changes within minutes (Stamatas & McIntire, 2001; Helmke & Davies, 2002). As a mechanotransducer, the glycocalyx transduces the strain to the endothelium in response to the shear stress. The glycocalyx can influence cell proliferation and cytoskeleton deformation under shear flow. Yao et al reported that the degradation of endothelial glycocalyx abolished the alignment of endothelial cells after 24 hour of steady flow, and the effect of shear stress on cell proliferation was abolished in the absence of the glycocalyx (Yao et al., 2007). A number of studies have demonstrated that the depletion of different GAGs components of the glycocalyx, i.e. heparan sulfate proteoglycan (HSPG), hyaluronic acid (HA), chondroitin sulfate (CS), are responsible for the significant drop of the flow-dependent NO production (Mochizuki et al., 2003; Lopez-Quintero et al., 2009). One of the earliest in vivo studies by Hecher et al (1993), reported a 64% reduction of
shear-dependent NO release when neuraminidase was used to remove sialic acids from the endothelial glycocalyx of rabbit femoral arteries. Similar phenomenon has also been observed in the in vitro studies. Tarbell et al exposed endothelial cells to different enzymes to selectively degrade various GAGs component: Hep III for HSPG, neuraminidase for sialic acid, chondroitinase for CS and hyaluronidase for HA. Their study showed that the depleting of HS, HA and SA caused the blockage of NO production, but not CS, and this might be related to glypican-caviolae-eNOS mechanism of the shear induced NO transduction (Pahakis et al., 2007).

There have been a limited number of studies on the glycocalyx recovery following enzyme degradation in vivo, for example, Potter et al focused on the glycocalyx shedding potentially caused by inflammation and studied the time recovery course after the shedding (Potter et al., 2009). In this study, a flow chamber was employed to examine the effect of the shear stress on the glycocalyx (Bai et al., 2010). The same immunofluorescent staining method was applied to observe the endothelial glycocalyx after the shear exposure (Bai & Wang, 2012). The percentage area of the cell membrane covered by the glycocalyx was used as an indication of the distribution of the glycocalyx on the cell membrane, and compared the value before and after 24 hours shear stimulation. The uneven distribution of the glycocalyx between the apical and edge regions was examined by the ratio of the intensity of glycocalyx staining at these two areas. The recoveries of the glycocalyx following enzyme degradation with and without shear stimulation were also investigated in this chapter.
6.2 Methodology

6.2.1 The shear flow system and recovery process of the HUVECs

A custom made steady flow bioreactor, as shown in Figure 6.1 A), was used to provide the shear flow to HUVECs. This is similar to the one used in the previous study (Bai et al., 2010). The system was composed by two reservoirs, a peristaltic pump, an interchange air nozzle and a rectangular parallel flow chamber (Figure 6.1 B). The hydrostatic pressure was provided by the height different of the two reservoirs; hence the corresponding shear stress was calculated.
Figure 6.1 Images of the steady flow bioreactor. A) the compositions of the bioreactor, including two reservoirs, air filter nozzle, peristaltic pump and a parallel plate flow chamber; B) zoom in photo of the flow chamber.

The rectangular parallel flow chamber was created by sandwiching a silicon gasket (0.25mm in thickness) between the HUVECs layer on the glass slide and a
polymethylmethacrylate (PMMA) transparent slide; the chamber was then secured by aluminum plates, as shown in Figure 6.2. The area inside of the parallel chamber was 70 mm in length and 20 mm in width.

![Figure 6.2 The sketch of a rectangular parallel flow chamber. The cell layer was sandwiching by glass slide and PPMA slide with a silicon gasket in middle, then secured by upper and lower plate.](image)

In this study, HUVECs were subjected to a shear stress of 12 dyn/cm². The pH was kept by gassing the flow system with a mixture of 95% air and 5% CO₂ and the temperature was maintained at 37°C (Zeng et al., 2006). Cells were monitored during the shear stimulation and for 24 hours afterwards. Control groups with static medium were concurrently performed. After the shear stress stimulation, HUVECs were kept in static culture medium, allowing 0, 2, 4, 8 or 24 hrs to recover respectively.
6.2.2 Recovery of the glycocalyx following enzyme treatment

Neuraminidase from clostridium perfringens (Sigma Aldrich) was used in the study. It cleaves N-acetyl neuraminic acid residues of glycoproteins and targets specifically the sialic acid component of the endothelial glycocalyx. Cultured HUVECs were treated with neuraminidase according to the protocol by Baker et al. (Barker et al., 2004). Serum-free M199 medium was used to wash the sample gently twice before it was incubated with 5U/ml neuraminidase for 30min at 37°C. Whereas the control group remained in the HUVECs culture medium. The recovery process was took place in the culture medium with 10% serum under two difference conditions for 24 hours: static medium or steady flow with a shear stress of 12 dyn/cm². At the end of the recovery, HUVECs were stained and observed under the confocal microscope.

6.2.3 Calculations of the percentage area glycocalyx and the intensity ratio

Wheat germ agglutinin (WGA) was used to bind to N-acetyl-D-glucosamine and sialic acid (SA) component of the glycocalyx (Barker et al., 2004; Megens et al., 2007). The endothelial cytoplasm was stained by Cell Tracker Red (Invitrogen). Endothelial cell nucleus was stained using Hoechst 33342 (Sigma Aldrich).

Cultured HUVECs were briefly washed using serum free M199. WGA-FITC and cell tracker red were applied to live cells for 15min at 37°C before Hoechst 33342 was applied for 5min. Live cells were washed three times by serum free M199 after
staining and kept in 10% serum culture medium, ready for confocal microscopy (Leica Microsystems, Wetzlar, Germany).

The detailed washing process after WGA-FITC staining was performed in the following sequence:

First, WGA-FITC staining solution was discarded. Then fresh M199 medium was replaced and the cells were immersed in medium for 5 minutes. The washing procedure was repeated again before the confocal observation.

Both the control cells and enzyme treatment cells were use the same procedure. The enzyme treatment group, using neuraminidase to cleave the sialic acid component of the sialic, which WGA-FITC targeted, appeared no WGA-FITC staining. This proved that the washing procedure washed off all the non-specific binding of WGA-FITC.

The intensity of the SA was analyzed using the software Image J (National Institutes of Health, USA).

In order to present the distribution of the endothelial glycocalyx, two different methods of fluorescence quantifications were performed:

1) **The percentage area**, A, of the cell membrane covered by the glycocalyx. Firstly, stack images of each color were created, as shown in Figure 6.3 A). They were transformed to black and white images using the threshold function of the ImageJ
software. The background pixels were set as black and the colored pixels were set as white, regardless of the brightness of different pixels (Figure 6.3 B).

Figure 6.3 Confocal images of day 14 HUVECs after 24 hours of shear stimulation. A) Stack images of the glycocalyx (top), cytoplasm (middle) and nucleus (bottom) of the
endothelial cells; B) Corresponding images in black and white.

The percentage area of the cell membrane covered by the glycocalyx was calculated
as the ratio of the green dye (WGA) area over the total area of the cell, i.e. the sum of
areas of WGA (SA), CTR (cytoplasm) and Hoechst (nucleus):

\[
A\% = \frac{A_{WGA}}{A_{WGA} \cup A_{CTR} \cup A_{Hoechst}} \times 100\%
\]

Figure 6.4 A diagram to show areas of different color staining, the merged staining
area is considered as the total area of the cell.

2) **The ratio of WGA intensity**, \( R \), between the apical (marked as A in Figure 6.5)
and the edge (marked as B & C) regions of HUVECs. The ratio indicates the
evenness of the glycocalyx distribution.

\[
R = \frac{I_{apical}}{I_{edge}}
\]
Figure 6.5 Areas of HUVECs used for intensity compare, the cell surface was divided equally into three divisions, apical (area above the nucleus), middle and edge.

The values A and R are calculated at different time points (0, 2, 4, 8 or 24 hrs) following the end of shear stimulation to study the recovery of the glycocalyx on HUVEC membrane.

6.3 Result

6.3.1 Distribution of the endothelial glycocalyx effects of the shear stress

HUVECs are seeded on glass slides for 13 days before 24 hours of steady shear stress stimulation. Their recovery are studied at different time point, i.e. 0, 2, 4, 8, 24hrs of
recovery. HUVECs are stained using WGA for the SA, cell tracker red CMTPS for the endothelial cytoplasm and Hoechst 33342 for the cell nucleus. They are observed using an invert microscope. In Figure 6.6 A, HUVECs are elongated in the flow direction and are parallel to each other. After 24 hours of recovery, in Figure 6.6 B, HUVECs appear to have recovered from their elongated shape. Figure 6.6 C is the control, in which HUVECs have been cultured for 14 days in static medium. WGA staining is evenly distributed on the entire cell membrane and the thickness of the glycocalyx layer is approximately 1μm. Confocal image of HUVECs cultured for 13 days in static medium then for 24 hours in steady shear flow (12 dyn/cm²) are shown in Figures 6.6 D-H. They are for recovery time t = 0, 2, 4, 8 and 24 hrs respectively. At t = 0hr, the majority of the WGA staining is distributed near the edge of the cell membrane, away from the nucleus. This appearance remains for t = 2hrs (see Figure 6.6 E). However, at t = 4hrs, WGA staining can be seen on top of the nucleus. As time progresses, more WGA dye is observed in the apical region of the cell membrane. After 24 hours recovery, WGA staining appears to cover the entire cell membrane, indifferent to that of the control group.
Figure 6.6 Morphological changes of HUVECs (A) right after 24 hours shear stimulation with no recovery; (B) after 24 hours recovery in static medium. In
confocal images, the main panels show enface images at a given z depth. The side panels show the x-z and y-z cross sectional images respectively. Bar = 10μm, in the main panel. C) The control group, D)- H) At t = 0, 2, 4, 8 and 24 hrs during recovery in a static medium. HUVECs are cultured for 13 days in static medium then subjected to 24 hrs shear flow at 12 dyn/cm².

6.3.2 Recovery of the endothelial glycocalyx after shear flow

The percentage area of the cell membrane that is covered by the WGA dye is calculated at t = 0 hr and 24 hrs following shear stimulation, and compared to the control. In Figure 6.7, HUVECs of different days in culture are studied. The 4D, 7D, 10D, 14D and 21D groups represent HUVECs that have been cultured in static medium for 3, 6, 9, 13 and 20 days respectively and are then subjected to 24 hours shear flow at 12 dyne/cm². The control groups show increases in the area from approximately 46.78 % (±7.01%, n=6) at 4 days to 80.39% (±6.99%, n=6) at 21 days. This is consistent to the earlier observation on the spatiotemporal development of the glycocalyx layer in Figure 4.5. Immediate after shear stimulation, i.e. 0 hour recovery, the area covered by the glycocalyx shows significant decreases (p < 0.05, denoted by *) from the control in all groups except for the 4D group. Following 24 hours recovery in the static medium, the percentage area increases in all groups and the values exceeds that of their controls.
Figure 6.7 The percentage area of the cell membrane covered by the glycocalyx. HUVECs cultured for 4, 7, 10, 14 and 21 days are investigated. In all the groups, cells are cultured in static medium initially and are subjected to 12 dyn/cm$^2$ shear stress for the last 24 hours (except the control groups, which are not subjected to shear flow. The Red and Blue bars indicate values at $t = 0$ hour and $t = 24$ hours of recovery following shear stimulation respectively. * denotes statistical significance, $p < 0.05$.

In Figure 6.8, we plot detailed time-recovery course of the glycocalyx layer on the cell membrane. Only 7D, 14D and 21D HUVECs are shown. The percentage areas increase with the recovery time for all groups. A rapid recovery during the early period was observed, i.e. 0 - 4 hours, which is followed by a much slower recovery between 8 - 24 hours. Linear curve fitting of data in these two periods show slopes of 5.04%/hr, 4.24%/hr and 2.85%/hr between 0 - 4 hrs, and 0.17%/hr, 0.64%/hr and 0.38%/hr between 8 - 24 hours for 7D, 14D and 21D groups respectively. They
indicate rapid WGA recovery within 4 hours after the shear stimulation. On the other hand, comparison of the recovery speed between the 7D, 14D and 21D groups shows that the 7D group has the fastest recovery between 0 - 4 hours, whereas the 21D group has the slowest.

Figure 6.8 Recovery of the glycocalyx with time after shear stress stimulation. HUVECs cultured for 7, 14 and 21 days are tested.

6.3.3 Intensity ratio of WGA between the apical and edge regions of the cell membrane

The intensity ratio (R) of WGA between the apical and the edge regions of the endothelial cell membrane indicates the relative distribution of the glycocalyx between the two areas. In Figure 6.9, we evaluate the changes in R during the 24 hours recovery period after shear flow. As in Figure 6.7, five groups of HUVECs are
used. They have been cultured for different lengths: 4D, 7D, 10D, 14D and 21D. The control groups show the R value increases from 0.466 (±0.087, n=6) for 4D to 0.871 (±0.077, n=6) for 21D. This increase represents a more evenly distributed glycocalyx layer on the HUVEC membrane with time, as demonstrated in our earlier study in Figure 4.5. Following 24 hours exposure to shear flow, R decreases in all groups in comparison to their controls, and the decrease is significant for 10D, 14D and 21D groups. This indicates a redistribution of the glycocalyx from the apical region to the edge region of the cell membrane. After 24 hours recovery in static medium, R increases to their control values. For 4D and 7D groups, R increases to above their control. The change in R value within the 24 hours recovery period is significant except for the 4D group. In early days of HUVEC, there is not much glycocalyx on the apical region of the cell membrane; hence the redistribution is less significant.

Figure 6.9 Intensity ratio of WGA between the apical and edge areas of HUVECs. Comparison to control is made at 0 hr and 24 hrs after shear stimulation. HUVECs
cultured for 4, 7, 10, 14 and 21 days are studied.

6.3.4 Recovery of the glycocalyx following its depletion by enzyme

The project further studied the recovery of the endothelial glycocalyx after enzyme treatment under two different conditions: either in static medium or in a flow chamber under shear stress of 12 dyn/cm². In Figure 6.10, 14 days old HUVECs are used in the study. After neuraminidase treatment, sialic acid staining of HUVECs shows little expression of the glycocalyx layer (see Figure 6.10 A). After 24 hours recovery under the static condition, Figure 6.10 B) shows a glycocalyx layer covering the entire surface of the HUVEC membrane. In comparison, after 24 hours recovery under the shear flow condition, Figure 6.10 C) shows HUVECs elongate along the flow direction, and sialic acid staining appears predominately near the peripheral area of the cell membrane, with patchy staining on top of the nucleus.
Figure 6.10 Recovery of the glycocalyx on 14 days old HUVECs after neuraminidase treatment. Bar = 10μm, in the main panels. A) Control image of HUVECs treated with neuraminidase; B) HUVECs recovered in static medium for 24 hours; C) HUVECs recovered in shear flow (12 dyn/cm²) for 24 hours.

Changes in the percentage area of the cell membrane covered by the glycocalyx are shown in Figure 6.11. Following neuraminidase treatment, nearly 80% of the HUVEC membrane is free of WGA staining. 24 hours recovery in a static medium shows significant increase in the area covered by the glycocalyx in all cell groups (i.e. 4D - 21 D). In comparison, there is also significant increase of the area covered by the glycocalyx after 24 hours recovery under 12 dyn/cm² shear flow, but the increase is less than that in a static medium.
Figure 6.11 Percentage area of HUVEC membrane covered by the glycocalyx after neuraminidase treatment and 24 hours recovery. HUVECs cultured for 4, 7, 10, 14 and 21 days are tested. 24 hour recoveries in either a static medium or under 12 dyn/cm² shear stress are compared.

The ratio of WGA intensity between the apical and edge areas of the HUVECs is shown in Figure 6.12. For all groups of HUVECs, neuraminidase treatment (i.e. controls) results in sparsely distributed glycocalyx on the cell membrane. The close to unity value of the ratio (0.949 ±0.087, n=30) indicates that the neuraminidase degrade SA compound evenly all over the cell membrane. 24 hours recovery in a static medium shows the intensity ratio decreases in all HUVEC groups. The significant decrease of 4D and 7D groups may indicate a more rapid recovery of the glycocalyx near the cell-cell junction areas than that in the apical area of the membrane. 24 hours recovery under shear stress results in even smaller values of the intensity ratio for all
HUVEC groups, but the difference is not statistically significant. Under such condition, the glycocalyx reappear predominately near the cell-cell junction areas. It has been also noted that the intensity ratio is bigger for HUVECs that have been cultured longer (e.g. 21D vs. 7D), indicating a more even reappearance of the glycocalyx for longer cultured HUVECs.

Figure 6.12 Ratio of WGA intensity between the apical and edge regions of HUVEC membrane. Controls are after neuraminidase treatment. Comparisons are made following 24 hours recovery in either a static medium or under 12 dyn/cm² shear stress. HUVECs cultured for 4, 7, 10, 14 and 21 days are used in the study.

6.4 Discussion

In this chapter, by comparing the reconstructed x-z and y-z cross sections images, the effect of a shear flow on the distribution of the glycocalyx was examined. The
resolution of the reconstructed cross section images were determined by the optical slice thickness and axial resolution. The Optical slice thickness is determined using the following equation:

$$\text{Optical slice thickness} = \sqrt{\left(\frac{0.88 \lambda_{em}}{n - \sqrt{n^2 - NA^2}}\right)^2 + \left(\frac{\sqrt{2} PH}{NA}\right)^2}$$

Where $\lambda_{em}$ is the emission wavelength of the laser, $NA$ is the numerical aperture of the objective lens (NA=1.4), $n$ is the refractive index of the immersion medium (n=1.515 of the glass and immersion oil), $PH$ is the pinhole size (PH=1).

The optical slice thickness can be improved by using immersion oil between the subject lens and the cover slip to increase the refractive index $n$, the smaller the pinhole size also contribute to a thinner optical slice thickness.

The resolution of confocal microscopy can be calculated on the basis of the following formulae:

$$\text{Lateral resolution (x, y axis)} = \frac{0.51 \lambda_{exc}}{NA}$$

$$\text{Axial resolution (z axis)} = \frac{0.88 \lambda_{exc}}{(n - \sqrt{n^2 - NA^2})}$$

Where $\lambda_{exc}$ is the excitation wavelength of the laser, $NA$ is the numerical aperture of the objective lens (NA=1.4), $n$ is the refractive index of the immersion medium (n=1.515 of the glass and immersion oil).

The lateral and axial resolutions and optical slice thickness can be summarized in the
The resolution of confocal fluorescent images is limited mainly by diffraction of the laser. The microscope being used for this project has a resolution of approximately 200 nm in the x and y directions. In the z direction, the resolution is poorer at ~400 nm.

The redistribution of the glycocalyx reveals an adaptive mechanism under shear flow, in which the glycocalyx reorganizes itself towards the cell-cell junction areas (Yao et al., 2007). This pattern can be observed in Figure 6.6, where SA staining occurs predominately near the edge of the cell membrane following 24 hours shear flow stimulation at 12 dyn/cm².

In order to binarize confocal images, a threshold parameter was used in Matlab to transform multi-colored images to black and white. The pixels that had brightness less than 10% of the brightest pixel in each image were set as “0” and the rest was

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation wave length (nm)</th>
<th>Emission wave length (nm)</th>
<th>Optical slice thickness (nm)</th>
<th>lateral resolution (nm)</th>
<th>axial resolution (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst</td>
<td>340,</td>
<td>510</td>
<td>479.49</td>
<td>158.6</td>
<td>319.66</td>
</tr>
<tr>
<td>WGA-FITC</td>
<td>488</td>
<td>521</td>
<td>489.83</td>
<td>212.6</td>
<td>458.80</td>
</tr>
<tr>
<td>Cell Tracker</td>
<td>577</td>
<td>602</td>
<td>565.98</td>
<td>236.6</td>
<td>542.48</td>
</tr>
</tbody>
</table>
set as “1”. The number of pixels with the value “1” was counted as the area of the dye.

The threshold setting performed under the assumption that the brightness of dye is at least 10% higher than the brightness of the background noise. The “1” area of the binarized images may not represent the full fluorescent pixels with a high background noise or a extremely weak dye, making the brightness different between dye and background less than 10%. However, it is rarely the case of all the confocal images in this study.

It has been reported that the shear stress simulates the synthesis of glycocalyx. For example Arisaka et al (1994) studied GAGs synthesis on procine aortic ECs, and Elhadj et al (2002) studied the proteoglycan production by bovine aortic endothelial cells. Similar pattern has been reported on SA staining in this chapter, as shown in Figure 6.7. It needs to be noted that the total time of HUVECs in culture for the 24 hours recovery groups (i.e. SS + 24 hrs Re) is 1 day longer than their controls, so strictly speaking, they are not directly comparable. However, comparing the 4D group 24 hr recovery data (64.05 % ± 13.86%, n = 6) to the control of 7D group (62.17% ± 5.93%, n = 6), we note similar percentage area coverage of the cell membrane by the glycocalyx despite the fact that the shear stimulated HUVECs are 2 days younger in culture.
As reported in the earlier study (Bai & Wang, 2012), a fully developed glycocalyx layer requires approximately two weeks in culture. After that, the thickness and distribution of the glycocalyx layer have little further development. The finding is consistent to results shown in Figure 6.8:

a) the percentage area of the cell membrane covered by the glycocalyx at day 7 is notably smaller than those at day 14 and day 21;

b) in comparison, there is much less difference between day 14 and day 21; and furthermore,

c) the slope of recovery between 0 - 4 hrs for day 7 group is much bigger than that of day 14 or day 21 groups.

It has been reported by Kang et al that heparan sulfate proteoglycans recovers from Heparinase III degradation swiftly (Kang et al., 2010). Six hours after the Heparinase III treatment, there are 20% recovery of the glycocalyx as indicated by normalized HSPG intensity. Our results agree to Kang et al’s observation although different component of the glycocalyx layer (i.e. SA in our study rather than HSPG in Kang’s study) has been targeted by different enzyme (i.e. neuraminidase in our study rather than Heparinase III).

The ratio of intensity of WGA, R, between the apical and edge areas of the cell membrane shows a similar pattern of change. It decreases following 24 hrs recovery in static medium, indicating a faster recovery of the glycocalyx layer near the cell-cell
junction region than that in the apical region of the cell membrane. Shear stimulation results in further reduction in the R value.

One of the limitations is the steady shear flow used in this study, which doesn’t represent the physiological, pulsatile flow in the circulation. Another limitation is the dye used in the study, i.e. WGA, mainly stains the sialic acid component of the GAGs. The recovery process may not represent the entire glycocalyx layer. Despite these limitations, the study provides a number of useful insights on the recovery of the endothelial glycocalyx following shear flow and enzyme stimulations.
Chapter 7 Discussion and future work

The endothelial glycocalyx, as the interface between the blood flow and vascular endothelial cells, plays significant roles, e.g. permeability control, endothelial mechanobiology and cell - cell interactions. As yet, many of its properties remain unknown. There is continuing debate on the difference between the glycocalyx in vivo and in vitro. This PhD project uses ex vivo vessel segments first to establish experimental protocol before cultured cells are used. The finding on the spatial and temporal development of the glycocalyx on HUVECs has important implications for future in vitro studies, namely, cultured endothelial cells need to be given time to develop a healthy glycocalyx layer in order to study their responses to shear flow or interaction with blood cells.

The evaluation of the Young’s Modulus of the endothelial glycocalyx is key to establish function-property relationship of both endothelial cells and the glycocalyx. It provides direct information on the stresses on the cell membrane and that transmitted into the endothelial cytoskeleton. The evaluated Young’s modulus of the glycocalyx (~0.38 kPa) is 6 fold softer than the endothelial cell.

The endothelial glycocalyx is the interface between the blood and blood vessel. Physiologically, it is not only a permeability barrier but also a mechanotransducer to transform mechanical signals to the blood vessel. Because of these crucial functions
of the endothelial glycocalyx, its dysfunction is involved in pathological conditions, such as atherosclerosis & diabetes. A number of groups reported that under these pathological condition, the degradation of endothelial glycocalyx leads to the reduction of its protective and anti-adhesive properties. Based on these physiological and pathological backgrounds, this PhD project introduces steady shear flow to the endothelial cells and study the distribution of the glycocalyx under this more physiological condition compare to a static condition. In addition, the application of enzyme treatment to degrade glycocalyx mimics the pathological conditions where the glycocalyx undergoing reduction or patchy structure. The results show that the endothelial glycocalyx appears near the cell-cell junction area after the shear flow. The recovery of glycocalyx distribution happens swiftly (within hours) to both shear flow and enzyme treatments.

The key studies and findings are summarized below.

7.1 Observation of the endothelial glycocalyx ex vivo

- The study establishes the immunofluorescent methodology to profile the endothelial glycocalyx ex vivo;
- For fixed aorta samples, confocal microscopy results using HepSS-1 and CD144 staining show a uniform layer of the heparan sulfate on the endothelial cell membrane;
- WGA-FITC staining of unfixed aorta segments presents the sialic acid residues on endothelial cell membrane with a thickness of 1 - 2 μm;
- The glycocalyx layer can be abolished using enzymes: hepanaranese III for heparan sulfate and neuraminidase for sialic acid residues.

7.2 Spatial distribution and temporal evolution of the endothelial glycocalyx in vitro

- The study using HUVECs demonstrates temporal development of the glycocalyx in vitro. In early days after cell seeding, e.g. day 1 to day 5, the glycocalyx is observed only near the edge of the cells, i.e. cell - cell junction areas. As time progresses, the glycocalyx develops and is observed on the entire cell membrane by day 14;
- The distribution of the endothelial glycocalyx varies between the apical and edge areas of the endothelial cell membrane. Before day 7 after cell seeding, the sialic acid staining is seen only at the cell-cell junction areas; at day 7 to day 10, patchy sialic acid staining is observed on the apical region of the cell; after day 14, the entire cell membrane shows uniform sialic acid staining;
- The thickness of the glycocalyx layer on cultured HUVECs as indicated by sialic acid staining is between 300 nm to 1 μm.
The Young’s modulus of the endothelial glycocalyx

- The study employs AFM nano-indentation on the cell membrane to evaluate its mechanical properties. The morphology of HUVECs is established using the tapping mode of AFM. The center and the edge of the cells are determined using the height and phase images.

- The Young’s modulus of the HUVEC membrane treated with neuraminidase is approximately 2.13 kPa. This is around six folds greater than that of the untreated cells, at ~ 0.34kPa.

- The young’s modulus of the endothelial glycocalyx layer is evaluated from the above results as being approximately 0.38 kPa.

- The Young’s modulus of HUVEC membrane shows significant difference between the edge and center regions of the cell. This is consistent to the confocal imaging results, which show uneven spatial distribution of the glycocalyx layer on the cell membrane.

- There is no significant difference in the Young’s modulus between different locations on HUVEC membrane after day 14 of cell seeding. This agrees to confocal imaging results which show the endothelial glycocalyx layer on the entire cell membrane.
7.4 Recovery of the endothelial glycocalyx following shear stress or enzyme treatment

- The glycocalyx layer’s recovery is evaluated by using the following two methods: change in the percentage area of the cell membrane stained by WGA and the intensity ratio of WGA between the apical and edge areas on the cell membrane.

- Following 24 hours exposure to shear stress (12 dyn/cm²), the sialic acid staining is seen to appear predominantly near the cell-cell junction areas.

- Within 24 hours recovery in the static medium following shear stress stimulation, the glycocalyx layer appears on the entire surface of the cell membrane. The recovery speed at the early time, i.e. between 0 - 4 hrs is significantly higher than that in the later time, i.e. between 8 - 24 hrs.

- The endothelial glycocalyx recovers within 24 hours following neuraminidase degradation. Cells of different days in culture show different recovery patterns.

7.5 Future Work

The current study evaluates the Young’s modulus of the cell membrane with a fully developed endothelial glycocalyx layer and the membrane after the glycocalyx has been cleaved. It has been broadly reported that endothelial glycocalyx is involved under pathological conditions, e.g. atherosclerosis & inflammation. In these diseases, different from the healthy intact glycocalyx layer, the endothelial glycocalyx
undergoing degradation and reduce of the thickness, hence lead to loss of anti-adhesive properties, which can cause diseases such as atherosclerosis. AFM indentation can be performed on healthy blood vessel samples and atherosclerosis model vessel samples, the difference of Young’s modulus between healthy and dysfunctional glycocalyx can be set up as an indicator for atherosclerosis. In addition, blood vessels of different stage of the atherosclerosis can be evaluated using this method for further analysis.

In this thesis, the responses of endothelial glycocalyx to shear stress and neuraminidase stimulations are examined, as well as the recovery after these stimulations. A dysfunction of glycocalyx is mimicked in the study: applying neuraminidase to degrade glycocalyx layer, then letting the glycocalyx recovery under shear flow. A swift recovery, i.e. during the 24 hours shear flow, is observed by comparing the area percentage and intensity ratio of the glycocalyx before and after the shear stimulation. Further research can focus on the recovery of glycocalyx Ox-LDL-induced degradation *in vivo*, which linked to diseases such as inflammation and atherosclerosis.

A conventional confocal microscope was used in the current study for the endothelial glycocalyx. The resolution of the confocal fluorescence images is approximately 200 nm in the x and y directions and 400 nm in the z direction. This clearly limits our ability to assess the thickness of the glycocalyx layer. Stochastic
optical reconstruction microscopy (STORM) provides a possible solution to this problem, which gives three dimensional super high resolution confocal images with ~20nm lateral and ~50nm axial resolutions (Rust et al., 2006; Xu et al., 2012). The new Institute of Bioengineering is in the process of installing the super resolution confocal system, which will enable much more accurate observation of the glycocalyx layer.

For in vitro studies, a temperature sensitive dish can be used to avoid possible degradation of the glycocalyx layer by trypsin. The temperature sensitive dish is covered by the covalently immobilized polymer poly (PIPPAAm), which is hydrophobic at 37°C, allowing cells to attach and grow, and hydrophilic below 32°C, resulting in the release of adherent cells (Kushida et al., 1999; Yamato et al., 2007). Our laboratory has acquired this system. Future study will probe into the development of the glycocalyx layer by comparing cell passage using the conventional method with trypsin and this new temperature sensitive method.
Figure 7.1 Temperature sensitive plate to detach cultured cells (Kushida et al., 1999).

Mechanical properties of glycocalyx remain poorly understood until very recently. O'Callaghan et al (2011) and our study (Bai & Wang, 2012) reported the Young’s modulus of the endothelial glycocalyx layer to be between two and four hundreds Pascal. These studies assumed a homogeneous layer for the glycocalyx, hence evaluated the average property of the glycocalyx. Mechanical properties of the individual glycocalyx constitutions remain unknown. AFM with functionalized tips can be used to detect individual antibody-antigen recognition events (Dupres et al., 2005; Lower et al., 2009). The tip of the AFM cantilever can be functionalized with antibodies that specifically recognize a certain type of GAGs, such as heparan sulfate or hyaluronic acid. The retrace force between the endothelial cell surface and the cantilever can be measured to pinpoint mechanical property of different GAGs.

Another approach to detect the elasticity of the glycocalyx can be in-situ AFM-SEM combination for true tensile testing on individual fibres of the glycocalyx. This new technique is able to give direct visual observation during mechanical testing (Hang et al., 2011). The ex vivo vessel segments need to be rapid frozen to minimize the damage of the sample and avoid possible alternation of the structure. The stress-stain behavior of glycocalyx filament can be tested between an ex vivo vessel segment and a vacuum compatible glue fiber.
The immunofluorescent study *in vitro* reported in this thesis focused on the sialic acid, which is attached by glycoproteins of the cell membrane. Other components of the glycocalyx, e.g. GAGs and proteoglycans, are also heavily involved in the endothelial mechanotransduction, for example the syndecans and its heparan sulfate proteoglycans signal pathway. Future studies can also look into their distribution on the cell membrane and temporal development.
Appendix Matlab code for the percentage area calculation

clear;clc;
close all

XL.filename = 'area percentage';

filenames=dir(*.tif);

Arealist = zeros(numel(filenames)/3,1);
figuretype = zeros(numel(filenames)/3,1);
% namelist = zeros(numel(filenames)/3,1);
% namelist = uint8(numel(filenames)/3,1);
for r=1:numel(filenames)/3
    filename=sprintf('%03d.tif',r);
    %     filewritenameI5(r,1).name=filewriteI5;
    glycocalyx=sprintf('%03d-1.tif',r);
    %     filewritenameA1(r,1).name=filewriteA1;
    cytoplasma=sprintf('%03d-2.tif',r);
    %     filewritenameI12(r,1).name=filewriteI12;
    nucleus=sprintf('%03d-3.tif',r);
    %     filewritenameI13(r,1).name=filewriteI13;

    area_gly=0.0;
    area_all=0.0;
    iii=-1;
    bwx=0.0;
    bwy=0.0;
    bwz=0.0;

    imageInfo = imfinfo(glycocalyx);
    imageType = imageInfo.ColorType;
    if strcmp(imageType,'truecolor')
        iii=1;
        % read image of gly to X, cytoplasma to Y, and nucles to Z
        X=imread(glycocalyx);
        Y=imread(cytoplasma);
        Z=imread(nucleus);
% change RGB to INDEXED image.
% 256 is the level of color map.
[x,mapX]=rgb2ind(X,256);
[y,mapY]=rgb2ind(Y,256);
[z,mapZ]=rgb2ind(Z,256);

bwx=im2bw(x,0.1);
bwy=im2bw(y,0.1);
bwz=im2bw(z,0.1);

[a_x,b_x,c_x]=size(X);
[a_y,b_y,c_y]=size(Y);
[a_z,b_z,c_z]=size(Z);

elseif strcmp(imageType,'indexed')
    iii=2;
    [x,mapX]=imread(glycocalyx);
    [y,mapY]=imread(cytoplasma);
    [z,mapZ]=imread(nucleus);

    bwx=im2bw(x,0.1);
bwy=im2bw(y,0.1);
bwz=im2bw(z,0.1);

    [a_x,b_x,c_x]=size(x);
    [a_y,b_y,c_y]=size(y);
    [a_z,b_z,c_z]=size(z);
elseif strcmp(imageType,'grayscale')
    iii=3;
    [x,mapX]=imread(glycocalyx);
    [y,mapY]=imread(cytoplasma);
    [z,mapZ]=imread(nucleus);

    bwx=im2bw(x,0.1);
%    bwy=im2bw(y,0.1);
%    bwz=im2bw(z,0.1);

    [a_x,b_x,c_x]=size(x);
    [a_y,b_y,c_y]=size(y);
    [a_z,b_z,c_z]=size(z);

end
% figure(3)
% imshow(y,map)
% X(123,122,3)
% figure(1)
% imshow(X)
% change image to binary image. 0.5 is threshold
% bw=im2bw(X,0.5);
% figure(2)
% imshow(bw)

if strcmp(imageType,'grayscale')
    for i=1:a_x
        for j=1:b_x
            if (x(i,j)==0)
                area_gly=area_gly+1;
            end
        end
    end
    % area of all
    for i=1:a_x
        for j=1:b_x
            if (y(i,j)==0 || z(i,j)==0 || x(i,j)==0)
                area_all=area_all+1;
            end
        end
    end
else
    % area of glycocalyx
    for i=1:a_x
        for j=1:b_x
            if (bwx(i,j)==0)
                area_gly=area_gly+1;
            end
        end
    end
    % area of all
    for i=1:a_x
        for j=1:b_x
            if (bwy(i,j)==0 || bwz(i,j)==0 || bwx(i,j)==0)
                area_all=area_all+1;
            end
        end
    end
end
end
end
% %    test
%    for i=1:a_x
%        for j=1:b_x/2
%            x(i,j)=5;
%            y(i,j)=5;
%            z(i,j)=5;
%            if (y(i,j)==0 || z(i,j)==0 || x(i,j)==0)
%                area_all=area_all+1;
%            end
%        end
%    end
%
% silence the figure poping
%    figure(1)
%    bwx=im2bw(x,0.2);
%    imshow(bwx)
%    figure(2)
%    imshow(y,mapY)
%    figure(3)
%    imshow(z,mapZ)

    percentage=area_gly/area_all*100

    Arealist(r,1) = percentage;
    figuretype(r,1)=iii;
%    namelist(r,1) = filename;
end
% xlsxwrite(XLfilename,namelist,1);
xlsxwrite(XLfilename,Arealist,1,'A');
xlsxwrite(XLfilename,figuretype,1,'B');
Reference


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