Designing biosensor platforms to study glycosaminoglycan interactions

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Abstract

The importance of glycosaminoglycans (GAGs) has been highlighted in many areas of research during recent years. Not only are they important in providing structure and support to our tissues but they also function as interaction partners in the extracellular matrix and at the cell membrane. The chemical structure and most notably the high negative charge of GAGs give them unique and interesting features, but also makes studying GAG-related interactions challenging. Biofunctional immobilization of GAGs to surfaces opens up for the use of many surface sensitive techniques that could provide detailed information about how these molecules regulate tissue growth and maintenance as well as the development of diseases.

In this thesis, different methods for immobilizing GAGs to surfaces have been investigated with the aim of studying GAG-related interactions. Two surface modifications have been used: supported lipid bilayers and self-assembled monolayers, both having suitable qualities for molecular immobilization and interaction studies. Immobilization of the GAGs hyaluronic acid and chondroitin sulfate was achieved using different methods. Immobilization was followed in real-time using quartz crystal microbalance with dissipation monitoring and surface plasmon resonance. Several GAG-interacting proteins were studied: e.g. bone morphogenetic protein-2, aggrecan, and virus glycoproteins.

Studying the interactions revealed interesting aspects of the interaction in itself, as well as highlighted important aspects regarding the surface immobilization of the GAGs. The interactions are highly dependent on the surface orientation of the GAGs and pros and cons with side-on versus end-on immobilization of GAGs are discussed in the thesis. The immobilizing technique, especially if functional groups are introduced on the GAG, also influences how the GAG is perceived by a secondary protein.

Potential applications of the GAG platforms are described; studying initial attachment of chondrocytes towards better cartilage implants and aggregation of platelets at a surface interface.

Keywords: glycosaminoglycans, extracellular matrix, molecular immobilization, interaction studies, QCM-D
Appended Papers

Paper I

Immobilization of Chondroitin Sulfate to Lipid Membranes and its Interactions with ECM Proteins


My contribution: I was responsible for designing and planning the experimental work. I performed all the experiments concerning coupling, characterization and protein interactions. I wrote the main part of the manuscript.

Paper II

Probing biofunctionality of surface immobilized GAGs

Noomi Altgärde,† Erik Nilebäck,† Laura De Battice, Jana Becher, Stephanie Möller, Matthias Schnabelrauch, Sofia Svedhem

†Authors contributed equally

Manuscript

My contribution: I was, together with E.N., responsible for designing and planning the experimental work. I performed the experiments concerning immobilization of GAGs for interaction studies using aggrecan, as well as additional physico-chemical characterization of the GAG layers. I took part in writing the manuscript.
Papers not included in this thesis

**Tuning cell adhesion by variation of pH during polyelectrolyte multilayer formation**

N. Aggarwal, G. Michanetzis, Y. Missirlis, N. Altgärde, S. Svedhem, T. Groth
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Abbreviations

GAG: glycosaminoglycan
HA: hyaluronic acid
CS: chondroitin sulfate
sHA: sulfated hyaluronic acid
HS: heparan sulfate
SA: streptavidin
SLB: supported lipid bilayer
SAM: self-assembled monolayer
ECM: extracellular matrix
QCM-D: quartz crystal microbalance with dissipation monitoring
SPR: surface plasmon resonance
BMP-2: bone morphogenetic protein-2
EDC: N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
NHS: N-hydroxysuccinimide
HSV: herpes simplex virus
gC: glycoprotein
# Introduction

The human body is a remarkable and complex creation where everything from our breathing to the shape of our nose is regulated by interactions of molecules. The movement of my fingers when writing this text is brought about by a flux of ions through the lipid membrane of nerve cells, causing energy conversion and conformational changes in motor proteins resulting in muscular contraction. Except from realizing the mere beauty in these molecular interplays, such knowledge is necessary for understanding diseases, recognizing them at an early stage (diagnostics) and finding ways to treat them (drug discovery/therapeutics).

The regulation of an organism’s function can be divided into three major areas: genomics, proteomics and glycomics. The first two concern the study of how our genes are regulated, translated into proteins, and the structure and function of these proteins. Glycomics, the study of sugars/carbohydrates, is the least studied discipline among the three, as a consequence of complicated synthesis and varying structure of GAGs. Despite this, they are by no means unimportant but have a multitude of functions in our bodies. Glycosaminoglycans (GAGs) are part of both the extracellular matrix and the cell membrane and have diverse functions like building up tissue structures, cell signaling and disease progression.

A variety of biosensor methods have been developed to study biomolecular interactions, where the biological event is transformed to a measurable signal. Depending on the method used, the molecules of interests might need to be labeled with e.g. a fluorescent or radioactive tag. Valuable information about if an interaction occurs or not can be gained, and where in e.g. a tissue section it takes place. However, to study the interaction more in detail and to get information about e.g. kinetics, a label might be disturbing. For this reason, label-free techniques have been developed. Many of these techniques are surface-based in order to increase control and sensitivity.

To confine the biomolecular interaction to a surface, immobilization of one of the reacting molecules is necessary. Often, and in this work, the immobilized molecule is called the ligand and the interacting molecule the analyte. The surface-based techniques can allow rapid and high throughput characterization, where the interaction between different analytes and a single ligand can be probed, either simultaneously or sequentially by regenerating the surface. This is very useful for investigating new and unknown interactions, but also for elucidating a single interaction when concentration, pH, and salt concentration need to be varied.

Appropriate immobilization of GAGs will allow studies of GAG-related interactions and how these molecules affect tissue growth and maintenance and the development of diseases. This knowledge, together with the immobilization protocol could lead to the development of new diagnostic tools, tissue-supporting implants, and cell substrates for tissue engineering.
1.1 Aim

The aim of my work is to investigate the function of GAGs, with a focus on GAGs found in bone and cartilage tissues. Thus, it is of interest to study how GAGs interact with other biomolecules crucial for tissue development and maintenance, like ECM constituents, growth factors, pathogens, cell membrane proteins or whole cells. To be able to study this with surface sensitive techniques, the GAGs need to be immobilized to a surface. The work presented here is hence aiming at creating platforms for suitable immobilization of GAGs. The usefulness of the platforms was evaluated by probing interactions with various proteins.

These results are part of FIND & BIND, an EU project aiming at investigating the role of carbohydrates in bone and cartilage tissues.¹

¹ The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007–2013) under Grant Agreement No. NMP4-SL2009-229292 (“Find & Bind”).
2 Glycosaminoglycans

2.1 Structure and in-vivo synthesis

Glycosaminoglycans (GAGs) are long, unbranched polysaccharide chains consisting of repeated units of disaccharides (Figure 1). The monomers consist of a 6-carbon ring with or without an attached amine group. A glycosidic bond links the monomers together. Two examples of GAGs are shown in Figure 2, hyaluronic acid (HA) and chondroitin sulfate (CS) consisting of the monosaccharides glucuronic acid and N-acetylgalactosamine. In CS, either of these can be sulfated.

![Figure 1. Part of the glycosaminoglycan family tree.](image)

![Figure 2. Molecular structure of the repeating disaccharide unit of a) hyaluronic acid (HA) and b) chondroitin sulfate (CS) type A.](image)

The GAG chain has a structural direction, having a reducing and a non-reducing end. The monomer at the reducing end is in equilibrium between an open form and a closed form (Figure 15). The open form presents an aldehyde that can react with a hydroxyl group on another monomer to elongate the chain.
Most GAGs are attached via their reducing end to a protein core, forming a proteoglycan. Proteoglycans can either be attached to the cell membrane or free in the extracellular matrix (ECM) as seen in Figure 3. They are synthesized and secreted by most vertebrate cells. The glycosylation, i.e. the attachment of GAGs to the core protein, begins in the endoplasmic reticulum and continues on its way through the Golgi apparatus. Here, the GAGs are also sulfated by sulfotransferases. The resulting pattern for this sulfation is not fixed or well understood. CS is divided into different types depending on where the sulfate groups are placed; A (chondroitin-4-sulfate), C (chondroitin-6-sulfate), D (chondroitin-2,6-sulfate) and E (chondroitin-4,6-sulfate). Type B used to be a CS type but is now called dermatan sulfate. Hyaluronic acid (HA) is an exception from other GAGs, as it is neither attached to a protein core, nor sulfated. HA is synthesized on the inner side of the plasma membrane by hyaluronan synthase and is then transported out to the ECM where it interacts with proteins and proteoglycans (Figure 3a).

Figure 3. Examples of GAGs in the ECM and at the cell membrane. a) The proteoglycan aggrecan, consisting of the GAGs chondroitin sulfate (CS) and keratin sulfate (KS) bind to the GAG hyaluronic acid (HA) together with a link protein (LP). b) The proteoglycan syndecan at the cell membrane consisting of CS and heparan sulfate (HS).

2.2 In-vivo function

One important function of GAGs in the ECM is their mechanical properties, giving support to the tissue as well as lubricating joints. They are also involved in many interactions mediating cell growth and proliferation, stabilizing growth factors, viral infection, and thrombosis regulation. The many possible sites for sulfation, discussed above, make GAGs very diverse in structure and a “sulfation code” has been suggested as a regulator of much of their function. For example, the ratio between CS-A and CS-C is fine-tuned during the different stages in embryonic development and also vary with age.

The involvement of GAGs in many, biologically vital mechanisms and the possibility of chemical alteration and functionalization make them an interesting focus for biosensor research. By immobilizing GAGs to surfaces, many of these interactions can be studied in detail. Investigating their interactions with growth factors can be useful in implant and scaffold design. Cellular attachment and signaling can be studied as GAGs function as signaling molecules. Important molecular aspects of viral infection and blood coagulation can also be studied.
3 Immobilizing biomolecules to surfaces

Finding a suitable way of immobilizing GAG derivatives to surfaces has been a major part of this project so far. General issues will be discussed along with a few examples of how immobilization could be made, both concerning modifications of the molecule and the surface. Finally, GAG immobilization in particular will be discussed.

Surface-based biosensor techniques can give deep insight into a specific interaction but for that to occur, care must be taken to preserve the molecule in its native form as much as possible. The introduction of specific functional groups onto the molecule under study might be necessary but should be performed in such a way that the interaction of interest is hindered as little as possible. When studying the function of an enzyme for example, the enzyme should not be immobilized at its binding pocket. Analyte binding to the background should be minimized in order to get as much information as possible about the analyte-ligand interaction. Consideration of the above comes down to choosing a good immobilizing technique and a good surface. The definition of “good” in this case depends on the answer to several questions:

- What surface sensitive technique will be use?  
  → A specific technique usually requires specific surfaces (e.g. gold or glass) and the immobilizing system needs to be compatible with this surface.

- How are the molecules expected to interact?  
  → Estimating how the interaction will take place gives clues on appropriate immobilization with respect to e.g. orientation and surface density.

- What variants of the ligand are available?  
  → Molecules functionalized in a specific way are not always commercially available and can be costly or cumbersome to synthesize.

Answers the above questions, and issues relating to this, will determine the immobilization method used.
3.1 Immobilization techniques

When studying the interaction between a ligand and an analyte, the stability of the surface-ligand binding needs to be trusted. By choosing chemically active groups on the surface and on the ligand, covalent bonds or strong non-covalent bonds between the two can be formed. The chosen technique should be as general as possible, to allow for the use of different ligands without changing the protocol. The techniques mentioned below are a few examples of commonly used immobilizing methods.

3.1.1 Amide bond formation using EDC/NHS

A common way of creating a covalent bond is by letting a carboxyl group react with a primary amine creating an amide bond, the same bond that links amino acids together in a protein.\(^{18}\) \textit{In vivo}, this reaction is brought about by the action of many enzymes and other molecules. \textit{In vitro}, the use of coupling reagents is necessary. For example, the carboxyl group is converted to an ester by the use of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). The ester can then react with the amine to form the amide bond. In order to increase the yield of this interaction, N-hydroxysuccinimide (NHS) is usually used together with EDC (Figure 4). It reacts with the EDC-ester, protects it from hydrolysis and makes a better leaving group for the subsequent reaction. Usually, carboxyl groups on the surface are activated and a ligand displaying primary amines is added. To avoid that the analyte also reacts with the activated surface, deactivation is normally performed using ethanolamine.\(^{19}\)

![Figure 4](image.png)

\textbf{Figure 4.} An amide bond created by reacting a carboxyl group on a surface and a primary amine on a ligand, with the use of EDC and NHS as coupling reagents.

3.1.2 Biotin-avidin coupling

The strong interaction (K\(_D\) \approx 10^{-15} \text{ M}) between biotin and avidin has been used for decades for immobilizing biomolecules to surfaces (Figure 8).\(^{20}\) Bacterially derived streptavidin (SA) can be used instead of avidin, also exhibiting a strong binding to biotin (K\(_D\) \approx 10^{-13} \text{ M}). It is non-glycosylated and has a pI closer to neutral pH (pI \approx 5-6) and therefore shows lower non-specific interactions. Neutravidin (pI = 6.3), a deglycosylated variant of avidin, is also sometimes used.\(^{21}\) All of the three variants have four binding sites for biotin. The biotin-avidin interaction has been used for a number of different systems and is, except for its strong binding, appreciated for its compatibility with different conditions in terms of pH, temperature, and denaturation.
3.1.3 Layer-by-layer assemblies

Building multilayered structures between two species of opposite charge is an easy way to build large molecular structures on surfaces.\(^{22}\) It requires no special equipment and has few limitations when it comes to substrates. By starting with a negatively charged surface, a polycation can be electrostatically adsorbed to the surface under the right buffer conditions. The surface becomes positively charged and a polyanion can be adsorbed in the same way (Figure 5). This straightforward fabrication makes it versatile in use for cell substrates and biomaterials. Even though the making of these layers is simple, the control and tuning of layer characteristics (final charge, roughness, stability) can be complicated.

![Figure 5. Formation of a polyelectrolyte multilayer via layer-by-layer assembly.](image)

3.2 Surface modifications for immobilization

3.2.1 Supported Lipid Bilayers

A lipid molecule has a hydrophilic (water-loving) head and a hydrophobic (water-hating) tail (Figure 6a). These characteristics make lipids self assemble when put in a solvent. In a water solvent, a spherical bilayer structure, a vesicle, will form where the heads are directed outwards and the tails into the bilayer (Figure 6b). This self-assembling is also the basis of the membrane that surrounds all the cells in our body. It consists of many types of lipids and also functional proteins and carbohydrates. Via the membrane, the cell communicates with surrounding cells and with the ECM.

![Figure 6. a) Schematic and chemical structure of a membrane lipid, here exemplified with POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. b) Lipids self-assemble into a vesicle in a water solution.](image)
Supported lipid bilayers (SLBs) are model mimics of the cell membrane and can be formed on solid supports, e.g. silica, by different methods, e.g. the vesicle-rupture technique shown in Figure 7. Lipids in a vesicle are fluid, and the fluidity is kept in the SLB by a thin layer of water between the support and the lipids. SLBs combined with surface-based sensor techniques are extensively used to study processes taking place at or near the cell membrane. Due to the mobility of the lipids, and the fact that SLBs are excellent in minimizing non-specific interactions, they are often modified to extend their usability for studying interactions involving e.g. cells, proteins and DNA.

3.2.2 Self-Assembled Monolayers

Another spontaneous process resulting in an ordered structure is the self-assembled monolayer (SAM), referring to a single layer of molecules on a solid support, the most used system being alkane thiols adsorbing on a gold surface (Figure 8). The semi-covalent bond between gold and sulfur in the thiol creates a close-packed, oriented system where a functional molecule can be added to the other end of the alkane chain. This functional group (e.g. carboxyl, amine or biotin) can be used to specifically bind other biomolecules or cells. It is preferred that binding only occurs to these functional groups, and the use of poly(ethylene) glycol (PEG) or oligo(ethylene) glycol (OEG) chains as part of the alkanes have been found to minimize unspecific binding of proteins.

Figure 8. Formation of a self-assembled monolayer. A gold surface is incubated in a thiol solution for >12h. The strong orientation of the thiols allows for functionalization of the other end, here exemplified with biotin (red dots) able to bind streptavidin in the next step.
3.3 Immobilization of glycosaminoglycans

Immobilization of GAGs and other carbohydrates to surfaces can be done either directly by pinning down the GAG itself, or indirectly by immobilizing the molecule which it is part of, e.g. a proteoglycan or a glycolipid. The indirect method allows for the use of the numerous immobilization strategies developed for proteins but with this method the interaction with the total GAG complex is studied. The direct method focuses the studied interaction to the GAG itself, requiring immobilization of a non-functionalized GAG (e.g. using the layer-by-layer method, section 3.1.3) or a functionalized variant.

Functionalization of the GAG allows for directed immobilization to a surface using suitable chemistry for that surface, e.g. amines (section 3.1.1), biotin (section 3.1.2) or thiols. It also gives the opportunity to tune the amount of binding sites on the molecules. Functionalization of GAGs is usually done to the carboxyl groups or the hydroxyl groups along the chain. Another possibility is to use the reducing end of the GAG (section 2.1) that in its open form presents an aldehyde group. Examples of functionalization can be seen in Figure 9.

![Figure 9](image-url)

Figure 9. Top: molecular structure of CS. The reducing end is in equilibrium between the closed and open form. Bottom: functionalization of CS an a) side-on and b) end-on configuration.
4 Glycosaminoglycan interactions

The interest for studying GAG interactions is increasing. This section is by no means a complete overview of the findings regarding this but is highlighting aspects about these complicated interactions. Examples of GAG-related interactions will be presented (4.2 & 0) and emphasized again in the result section.

4.1 General aspects

Much of GAG function is governed by interactions with other molecules, usually different kinds of proteins. The nature of proteoglycans encourages the formation of larger supramolecular assemblies where both the protein core and the GAG chains are available for interaction. As an example, the core protein of aggrecan forms aggregates by binding to HA together with a link protein (Figure 3a).\textsuperscript{39,40} The GAG chains in aggrecan are in turn thought to bridge between collagen molecules further extending this supramolecular assembly.\textsuperscript{41} Finding interacting sequences in the GAGs primary structure is not as straightforward as for proteins, since GAG synthesis is not coded in a template like DNA and is therefore not as defined. However, just as for proteins, GAGs have secondary structures, as they can adopt a helical structure stabilized by hydrogen bonds.\textsuperscript{9,42} The helix formation can create hydrophobic patches along the otherwise hydrophilic molecule, likely favoring binding both to other GAGs and to proteins. Hence there is likely also a tertiary structure, where multiple GAG chains can align in a β-sheet structure.\textsuperscript{43}

4.1.1 Multivalency

The affinity for a binding between a GAG and a protein at a single interaction site is usually very low and the overall strength of GAG-protein interactions, the avidity, is due to multivalency.\textsuperscript{44,45} Multivalency is important for strength and specificity in many biological interactions and can involve GAGs binding multiple proteins as well as proteins having multiple binding sites for GAGs (Figure 10). Multivalency complicates evaluation of an interaction, especially when the nature of the interaction is not fully known.\textsuperscript{46} If the analyte binds in a multivalent manner, the response will be highly dependent on the density of ligands on the surface. In these cases it is better to let the multivalent molecule be the immobilized ligand and usually when studying GAG-protein interactions, the GAG is immobilized.\textsuperscript{53} The multivalent nature of GAG interactions also suggests their involvement in cross-linked structures.\textsuperscript{47}

![Figure 10](image)

Figure 10. a) monovalent binding, b) a multivalent analyte binds to ligands on the surface, c) a multivalent ligand binds to analyte molecules from the solution, d) a multivalent analyte binds to a multivalent ligand with the possibility of crosslinking.
4.1.2 Specificity

The specificity of GAG-protein interactions is a complex question and debated in several review articles. First, the distinction between specific and non-specific interactions is not clear cut, especially when polyelectrolytes are involved. The specificity of GAG-protein interactions likely comes from hydrogen bonds, e.g., between the amino acid tyrosine and hydroxyl groups on the GAG, and from hydrophobic interactions. Just as for protein-protein interactions, geometrical arrangements of the interacting partners are thought to play an important role. Today only a few specific GAG-protein interactions are known and most of them concern the heparin sulfates. However, as more knowledge is gained on the sulfation patterns of GAGs and the way they govern protein interactions, specific interaction sequences are likely to be found in this.

4.1.3 Quantifying binding characteristics

For quantitative analysis of an interaction, binding constants are usually established. In simple terms, a biomolecular interaction can be explained by eq. 1:

\[
A + B \leftrightarrow AB \quad \frac{k_a}{k_d}
\]

where \(A\) and \(B\) are the two interacting partners and \(k_a\) and \(k_d\) are the association and dissociation rate constants, respectively. The equilibrium association and dissociation constant are defined as:

\[
K_A = \frac{[AB]}{[A][B]} \quad K_D = \frac{1}{K_A} = \frac{[A][B]}{[AB]}
\]

\(K_D\) is more often used in interaction analysis, where a low value indicates a strong interaction. If the interaction takes place at a surface, the binding of analyte \(A\) to ligand \(B\) can be related to the Langmuir adsorption isotherm and the rate of change in surface coverage can be explained by:

\[
\frac{d\theta}{dt} = k_a[A](\theta_{max} - \theta) - k_d\theta
\]

where the first part accounts for association to the surface and the second part for dissociation from the surface. \(\theta\) is the surface coverage at time \(t\) and \(\theta_{max}\) is the maximum surface coverage. At equilibrium, the rate of change is zero and solving for \(\theta\) gives:

\[
\theta = \frac{[A]\theta_{max}}{K_D + [A]}
\]
where \( k_d/k_a = K_D \). If \( K_D \) equals \([4]\) then according to eq. 4, \( \theta = \theta_{\text{max}}/2 \). In other words, \( K_D \) equals the bulk concentration at which half of the receptor on the surface are occupied.

Fitting measurement data to eq. 4 is only reasonable if the Langmuir model holds.\(^{50}\) The model comes with many prerequisites: pure samples, homogenous surface with all sites equal and non-cooperativity. Care must be taken as to not draw conclusions based on poorly fitting data. The Langmuir models can obviously be extended to include various other parameters. However, even if complicated models often provide a better fit, they do not necessarily give a more true explanation of the data.

### 4.2 GAG interactions with ECM proteins

Today, hundreds of proteins are known to interact with GAGs although the nature and significance of these interactions are not always known.\(^{45}\) Perhaps the most well-known, studied and used interaction is heparin acting as an anticoagulant by binding and activating anti-thrombin III.\(^{51}\) The GAG-interacting partner could be of structural importance, like collagen, or other polysaccharides and proteoglycans, enzymes, growth factors or chemokines.\(^{9,52}\)

**Collagen** in its many forms is the most abundant protein in the ECM and known to form aggregates with the other main ECM-constituents; proteoglycans and HA.\(^{41,53}\) Both the protein core and the extending GAG chain on the proteoglycans participate in this interaction. The sulfation patterns on the GAG chains matters for the interaction, where an over-sulfated variant of CS, CS-E, has a higher binding to most collagen proteins. Ionic interactions are likely dominating since there is a strong dependence on ionic strength and pH.\(^{41}\) The nature of collagen-GAG interaction is difficult to investigate due to the different types and forms of collagen and the fact that the whole proteoglycan takes part in the interaction. However, the supramolecular structures built up by collagen are likely to be important for tissue structure and are often used in tissue engineering research. It was recently shown that a complex of type I collagen and CS, with and without the growth factor BMP-4 was beneficial for increased bone growth.\(^{54}\)

**Aggrecan** is another important constituent in cartilage ECM. It forms supramolecular assemblies by binding through the N-terminal of the protein core to HA chains together with a link protein (Figure 3a).\(^{40}\) HA also has a cell surface receptor, CD44, that binds HA along with aggrecan aggregates, thus stabilizing the ECM.\(^{55}\) The stability of HA-aggrecan assemblies is pH dependent and they are therefore thought to be involved in the tissue response to inflammation.\(^{39}\) This is also supported by the fact that HA binds an inflammation-response protein, TSG-6.\(^{56}\) Elucidation of the interaction between HA and aggrecan could give valuable insight in how cartilage degenerative diseases like osteoarthritis initiate and progress.
Enzymes are not only involved in GAG synthesis within the cell but also regulate GAG levels in the ECM. More or less specific enzymes act on the different GAGs; heparinase I and II, chondroitinase ABC, hyaluronane lyases, and hyaluronidase, decreasing the amount of GAGs and hence regulating their functions. Some cancer cells secrete GAG degrading enzymes, indicating that proteoglycans are involved in cancer metastasis. As mentioned previously, the sulfation patterns of GAGs change as the tissue develops and ages, a reaction also governed by sulfatases.

GAGs are situated both in the ECM and at the cell membrane and therefore have an ideal placement to be involved in extracellular signaling events. Growth factors are substances that regulate cell behavior and participate in cell-cell communication. The crucial role of growth factors in tissue development has led to an increased use in research and in clinic. For example the growth factor bone morphogenetic protein-2, BMP-2, is used to enhance bone growth. The half-life of growth factors is short, causing problems when administering it to the tissue in solution. In vivo this is solved by interactions with ECM molecules that protect the growth factors from degradation by proteases. This led to the idea of combining growth factors with GAGs for administration, which proved to stabilize them and retain their functionality. The positively charged growth factors easily attach to the negatively charged GAGs but the exact mechanism behind this interaction is not fully understood. It has been suggested that heparin-like GAGs interacts specifically with the basic N-terminal of BMP-2 and that each BMP-2 molecule, being a dimer, could bind two GAG chains. Interaction between BMP-2 and the non-sulfated HA has also been seen, hypothesized as arising from ionic interactions. The 3D structure of BMP-2 has been shown and 3D rapid prototyping suggests that the homo dimer presents a helical binding pocket suitable for left handed helixes, a conformation that GAGs are energetically likely to adopt.
4.3 Interactions with viruses

Viruses are small pathogens that lack the ability of replicating. Their only way of multiplying is by hijacking a host cells replication machinery. Host cell infection starts with attachment to the surface of the cell and later penetration into it. The viral genome is then replicated within the cell. Virus proteins are synthesised and new virus particles can be created. After exiting the host cell, the new virus particles, virions, can infect other cells nearby. The virion is protected by a capsid, a protein core, and some virus, e.g. the herpes simplex virus (HSV), also have a lipid membrane as an outside cover, called the envelope. The envelope also contains many kinds of glycoproteins, which mediate both attachment and entry into the host cell. Since much of the host cell membrane displays different kinds of GAGs, it is perhaps not surprising that many viruses use these molecules to attach and direct their entry into the cell. Most HSV-attachment studies involve the binding of envelope glycoproteins to HS but binding has also been shown to CS. The GAG-binding region on the glycoproteins is thought to consist of clusters of positively charged amino acid and basic residues. These regions can bind electrostatically to negatively charged GAGs but there is likely also more specific hydrogen bonding involved. The spacing of sulfate groups along the GAG is also thought to be important. Understanding the mechanism behind attachment and entry of viruses could help in designing more general and efficient vaccines and anti-viral drugs.
5 Experimental techniques

In this section, techniques applied for studying formation and characterization of GAG platforms in the present work will be described.

5.1 Quartz Crystal Microbalance with Dissipation Monitoring

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D) is a surface-based mass sensitive technique often used in biosensing. The basis of QCM-D lies in the quartz, a piezoelectric material developing a charge potential when mechanical stress is applied. Conversely, movement of the crystal can be brought about if a potential is applied across a quartz crystal disc, which in QCM-D is achieved by putting gold electrodes on both sides of the crystal sensor. By applying an alternating potential, the QCM-D sensor will deform repeatedly in a so-called shear-thickness mode (Figure 11).

![Figure 11](image-url)

Figure 11. When an alternating potential is applied to an AT-cut quartz crystal (like a QCM-D sensor), the crystal deforms in a shear-thickness motion.

The applied potential will cause the crystal to oscillate at a certain frequency. The most stable oscillation occurs at the crystals resonance frequencies, where all the added energy is converted into motion. The resonance frequency for a QCM-D crystal occurs at 5 MHz (fundamental frequency) and odd multiples of this. During a measurement, all resonance frequencies are measured simultaneously. The sensitivity is highest in the centre of the crystal and the sensing depth is decreased for higher overtone numbers. When something adsorbs on the sensor surface, it is monitored as a decrease in the oscillating frequency (Figure 12).

![Figure 12](image-url)

Figure 12. Schematic QCM-D graph showing frequency and dissipation responses obtained during material adsorption. As material adsorbs, frequency (black) decreases and dissipation (light grey) increases. If the adsorbed layer is loose the dissipation is high (solid), if it is dense the dissipation is low (dashed).
In QCM-D, the applied potential is continuously switched on and off, which distinguishes this technique from other QCM techniques. When switched off, the oscillation decays and it is possible to measure the damping of the system, resulting in a parameter called dissipation, expressed in eq. 5:

\[
D = \frac{1}{2\pi} \times \frac{E_{dissipated}}{E_{stored}}
\]  

(5)

For thin, rigid layers (small \(D\)) the mass adsorbed on the sensor can be described by the Sauerbrey equation:

\[
\Delta m = -\frac{C \Delta f_n}{n}
\]  

(6)

were \(\Delta m\) is the change in mass, \(C\) is a crystal specific constant (17.7 ng/(cm²·Hz)), \(n\) is the overtone number and \(\Delta f_n\) the frequency change measured for that overtone.\(^{73,74}\) The Sauerbrey equation is the simplest and perhaps the most used way of estimating mass. Since this mass sensor is based on movement of the sensor, it will detect anything that is acoustically coupled to the sensor. That is not only the adsorbed film but also solvent that moves along with the film will be sensed. To estimate the mass of a film having high water content, the Sauerbrey equation cannot be used. Instead, a model taking the viscoelastic properties of the layer into account is suitable, e.g. by the use of Voigt elements.\(^{75}\) The fact that associated water is also measured makes it possible to measure highly hydrated systems that can be difficult to sense with e.g. optical techniques.\(^{76}\) Here, frequency data were always normalized with regard to the overtone number.

### 5.2 Surface Plasmon Resonance

Surface plasmon resonance is a phenomenon used for detecting adsorbed mass on a surface. A surface plasmon is a charge-density wave that can exist at the interface between two media where the dielectric constants are of opposite sign, e.g. between a free-electron metal and a dielectric.\(^{77}\) The plasmon can be excited by interaction with another energy wave in resonance, i.e. a light wave. The plasmon, and hence the resonance condition, is highly sensitive to changes at this interface, e.g. adsorption of a material with optical properties different from the bulk.

An SPR instrument is usually built according to the Kretschmann configuration (Figure 13).\(^{78}\)
A glass prism is coated with a thin metal film, usually gold. Light is applied through the prism such that it hits the interface at an angle above the critical angle, $\theta > \theta_c$ where total internal reflection occurs. Under these conditions no light is transmitted through the gold film but an electrical field wave is created, having an intensity that decays exponentially from the surface (the evanescent field).

The wave vectors for the plasmon, $k_{sp}$, and for the parallel component of the incident light, $k_x$ are given by:

$$k_{sp} = \frac{\omega}{c} \frac{\varepsilon_m(\omega)\varepsilon_a}{\sqrt{\varepsilon_m(\omega) + \varepsilon_a}} = [|\varepsilon_m| > |\varepsilon_a|] = \frac{\omega}{c} \sqrt{\varepsilon_a} = \frac{\omega}{c} n_a$$

(7)

$$k_x = \frac{\omega}{c} \sqrt{\varepsilon_g \sin \theta}$$

(8)

where $\omega$ is the angular frequency, $c$ is the speed of light, $\varepsilon_a$, $\varepsilon_m$ and $\varepsilon_g$ are the dielectric constants for the ambient medium, the metal, and the glass prism, $n_a$ is the refractive index of the ambient medium and $\theta$ is the incident angle of the light. For the plasmon and the light to be in resonance, $k_x$ and $k_{sp}$ need to be equal and energy is then transferred from the light to the plasmon, causing a dip in the intensity of the refracted light sensed by a detector. As molecules attach to the sensor surface, the plasmon will shift due to changes in the refractive index, $n_a$. According to $k_{sp} = k_x$, the angle of incidence, $\theta$, then needs to change. By scanning the incident angle until the resonance conditions are found, the new refractive index can be calculated.

When communicating SPR data, the displacement of the resonance angle is sometimes reported. For the commonly used BIAcore instrument, values are often given in resonance units (RU). The RU values depend on the change in refractive index, $\Delta n$, which in turn is related to the surface concentration of the adsorbed biomolecule. Mass, or surface coverage $\text{ng/cm}^2$, can be estimated using eq. 9.
\[ m = \frac{C_{SPR} \Delta RU}{\beta} \]  

where \( C_{SPR} \) is a constant accounting for the decay length of the evanescent field, the sensitivity of the instrument and the refractive index increment for the adsorbed substance, \( dn/dC \), and \( \beta \) a factor compensating for the distance from the sensing surface. To calculate the \( C_{SPR} \) values for immobilized GAG layers, it was assumed that \( C_{SPR} = 0.066 \text{ ng/cm}^2 \) for a protein with \( dn/dC = 0.18 \text{ ml/g} \) adsorbing on a flat gold surface and that \( C_{SPR} \) varies linearly with \( dn/dC \). For glycosaminoglycans like HA and CS, many different values for \( dn/dC \) can be found in the literature, ranging from 0.155-0.176 for HA and 0.129-0.17 for CS. This is likely due to measurements being made in different buffers and at different wavelengths. In this work, mean values of these literature values have been used to calculate usable \( C_{SPR} \) values. sHA is assumed to have the same \( dn/dC \) as CS as it is also highly sulfated.

Water associated with the sensed molecules is not measured with SPR, since only the difference from the surrounding media (containing water) is sensed. By subtracting modelled SPR masses from QCM-D masses (which include associated water), the water content of a layer of biomolecules can be estimated. Measurements with SPR are also often used to estimate specificity, affinity and kinetics for a given interaction.

5.3 Fluorescent Recovery After Photobleaching

Fluorescent recovery after photobleaching, (FRAP) is a convenient method to monitor the lateral diffusivity of fluorescently labelled molecules. This can be useful for different kinds of systems, but a typical application is to monitor the mobility of lipids in a lipid bilayer (section 3.2.1). One of the characteristics of a supported lipid bilayer is that it retains its fluidity when on a solid support. Changes in this fluidity can easily be tested using FRAP. A fraction of fluorescently labelled lipids is incorporated into the SLB, and a small spot in the SLB is bleached by a high intensity light pulse. The fluidity in the bilayer causes the florescence in the spot to recover by the diffusion of non-bleached fluorescent lipids into the bleached area and vice versa. By analysing the recovery, the diffusion constant can be calculated. Here, calculations based on the Hankel transform was used, a method less sensitive to noise than tradition methods.
5.4 Isoelectric point analysis

Analysis of surface charge is important in understanding the behaviour of materials used in diagnostics, implants and as cell culture substrates as it influences protein adsorption, complement activation and blood coagulation. As an indicator of surface charge, the ζ-potential is usually measured using streaming potential or streaming current. A common setup for these measurements is a channel consisting of two parallel plates made from the material to be analysed. When an electrolyte is present, a charged double layer occurs at the walls. As the electrolyte is pushed passed, a streaming current develops caused by displacements in the charged double layer. The net stream of ions can be measured directly by electrodes on each end of the channel and the ζ-potential can be calculated by eq. 10:

$$\zeta = \frac{dI}{dp} \cdot \frac{\eta}{\varepsilon \cdot \varepsilon_0} \cdot \frac{L}{A}$$  \hspace{1cm} (10)

where \(dI/dp\) is the slope of streaming current \((I)\) versus the differential pressure across the sample \((p)\), \(\eta\) is the viscosity of the solution, \(\varepsilon_0\) is the permittivity, \(\varepsilon\) is dielectric constants for the electrolyte, \(L\) and \(A\) the length and cross-section area of the channel respectively. The isoelectric point, i.e. the pH where the ζ-potential is zero, can be determined by measuring the ζ-potential during a pH titration.

5.5 Contact angle goniometry

The surface energy is another important property of biologically used materials. Simply speaking, surface energy can be considered as the energy needed to create a surface from the bulk material, i.e. the by breaking of chemical bonds. An easy estimation of the surface energy is by measuring the wettability by contact angle goniometry. A liquid droplet, typically water, is placed on the surface and the spreading of the drop will indicate the wettability (hydrophilicity/ hydrophobicity) of the surface (Figure 14). For homogenous surfaces, the surface energy can be estimated by repeating this measurement for liquids with different surface tension and plotting the contact angle \(\theta\) versus the liquid surface tension. The surface energy is then equal to the liquid surface tension when \(\cos(\theta) = 1\).

![Figure 14. Contact angles for surfaces with different wettability; a) a super-hydrophilic surface with \(\theta<10^\circ\), b) a hydrophilic surface with \(\theta<90^\circ\), c) a hydrophobic surface with \(\theta>90^\circ\).](image-url)
6 Summary of results

This section shortly explains the main experimental results, most of which are included in the appended papers. Since a variety of GAG derivatives, surface modifications and immobilization techniques have been used during this work, an overview of this will be presented in section 6.1. A more detailed description on the GAG platforms used, how they were developed and characterized is presented; looking both at the immobilization itself (6.2) as well as the effect of immobilization on subsequent interactions (6.3). Finally, an example of how the developed GAG platforms can be used to study more complex biological systems will be presented (6).

6.1 Toolbox for immobilization of glycosaminoglycans

Figure 15. An overview of the GAGs used in this study; including modifications and immobilization. Thumbnails from this figure will be used throughout the results section to refer to the GAG and immobilization strategy used.
6.1.1 Surface modifications

As surface modifications for GAG immobilization, both SAMs and SLBs were used.

Two kinds of SLBs (section 3.2.1) were used, either presenting carboxyl groups (SLB-COOH) or amines groups (SLB-NH₂). The bilayers were formed by vesicle rupture on silica surfaces, characterized in QCM-D by frequency and dissipation shifts of \( \Delta f = -26 \text{ Hz} \) and \( \Delta D < 0.5 \cdot 10^{-6} \) respectively. Formations of these SLBs are summarized in Figure 16 and discussed in detail in paper I.

![Figure 16. QCM-D frequency and dissipation shift for formation of SLBs used in paper I.](image)

The development of a suitable biotinylated SAM (section 3.2.2) for QCM-D measurements has been described previously, and is also explained in paper II. This surface modification was used for QCM-D and SPR measurements. In all cases, the SAMs were prepared \textit{ex-situ} and binding of SA was performed \textit{in-situ} (Figure 8).
6.1.2 Glycosaminoglycans

The GAGs used in this study were supplied from INNOVENT (Jena, Germany), a collaborating partner within the project FIND & BIND. To allow for directed immobilization, the GAGs were functionalized with hydrazid or biotin in a side-on or end-on configuration. HA is a non-sulfated GAG, whereas CS is sulfated. In order to study the effect of sulfation, HA was sulfated. HA with varying degrees of sulfation would be beneficial for many protein interaction studies. Also CS was extra sulfated. The degree of functionalization and sulfation is expressed in terms degree of substitution (DS) of possible sites on each repeating unit, i.e. each disaccharide. For example, introduced hydrazid groups are coupled to carboxyl groups on the chain (Figure 9). Each HA repeating unit has one carboxyl group, hence $DS_{hydr.}=1$ means that each repeating unit is tagged with one biotin, $DS_{hydr.}=0.5$ means every second repeating unit and so on. Introduced sulfate groups are coupled to hydroxyl groups on the GAG chains and the same reasoning applies regarding $DS_s$.

6.2 Design and characterization of GAG-platforms

The immobilizing strategies outlined above results in different structural properties of the immobilized GAGs. Some characteristics of the GAGs only become visible when studying GAG-related interactions and will be discussed in section 6.3.

6.2.1 Side-on immobilization of chondroitin sulfate

In paper 1, we wanted to take advantage of the benefits with using a SLB (section 3.2.1) for immobilization of CS. Two different approaches were used, that both resulted in side-on immobilized CS. In the first strategy, hydrazide functionalized CS (h-CS) was let to react with EDC/NHS-activated carboxyl groups on the (SLB-COOH), creating amide bonds (section 3.1.1). In the other strategy, the naturally occurring carboxyl groups on non-functionalized CS were instead activated and let to react with amine groups on a SLB (SLB-NH$_2$), also resulting in amide bonds. The immobilization was followed in real time by QCM-D (Figure 17).
The high charge of CS, together with possible chain stiffness caused difficulties in reaching high amounts of immobilized material. Buffer conditions, as well as functionalization degree and functional lipid ratio needed to be optimized. Immobilized amount of functionalized CS were approx. 25 ng/cm$^2$ using the Sauerbrey equation (eq. 6). This is reasonable for a thin CS layer immobilized predominantly in a side-on configuration. It was easier to increase the amount of immobilized CS on the amine bilayer (55 ng/cm$^2$), likely due to the increased amount of amine groups exerting an attractive force on the negative CS. The immobilizing method did not seem to affect the viscoelastic properties of the layers.

SLBs have a dynamic structure, which is often a benefit. However, this can pose problems when immobilizing long chains or large structures as lipid material could potentially be removed. Thus for comparison, h-CS was immobilized to SAM-COOH. Only small differences were seen when comparing the immobilized amount of CS to the SAM ($\Delta f = 2.3 \pm 0.5$ Hz, $\Delta D = 0.4 \pm 0.01 \cdot 10^{-6}$), and the SLB ($\Delta f = 1.5 \pm 0.4$ Hz, $\Delta D = 0.6 \pm 0.3 \cdot 10^{-6}$), suggesting that the fraction of DOPE-COOH lipids is a rather good measure of the number of carboxyl groups exposed at the surface and that the low response is not caused by removal of lipid material.

The lateral diffusion of lipids in the SLB was also assessed in fluorescence recovery after photobleaching (FRAP) experiments. The diffusion coefficient (D) of fluorescently labeled lipids was calculated, showing no significant difference between a functional bilayer and bilayer with immobilized CS, showing that the lipid molecules in the SLB retained their lateral mobility.
Figure 18. FRAP images of SLB-NH$_2$ 1% of fluorescently labelled lipid (NBD-POPC) without (left) and with (right) immobilized CS. After bleaching, 30 images were taken with a 5 s pause in between. The last image is shown to the right in each section.

6.2.2 End-on immobilization of glycosaminoglycans

An alternative immobilization configuration to side-on, is pinning the GAG end-on to the surface, similarly to how a GAG is attached to its protein core in a proteoglycan (Section 2.1 and 3.3). Here, the reducing end was used to attach a functional molecule at only one end of the GAG (Figure 9). As a continuation on the side-on immobilization strategy used above, CS was end-on functionalized with a hydrazide molecule. However, this did not lead to any detectable immobilized amounts on a SLB. Instead a biotin group was attached to the end and the GAG was immobilized to a SAM using the strong interaction between biotin and streptavidin (section 3.1.2). This strategy was evaluated in paper II.

Figure 19 shows immobilization of end-on biotinylated chondroitin sulfate (b-CS), hyaluronic acid (b-HA) and sulfated hyaluronic acid (b-sHA) to a monolayer of SA on a SAM, monitored with QCM-D.

Figure 19. QCM-D graph showing immobilization of b-HA (dashed line), b-sHA (solid line) and b-CS (dotted line) to SA on a SAM, and subsequent rinsing.
There is a difference in molecular weight between the used GAG derivatives (Table 1). However, the immobilized amounts seen with QCM-D do not scale with the difference in molecular weight. The hydration of GAGs is important in many tissues like cornea to give opacity, skin to give viscoelasticity, and cartilage to give damping (cartilage consists of about 75% water). To investigate whether the difference in immobilized amount in fact comes from a difference in layer water content, the same measurements were made using SPR (Figure 20). The amount of associated water can be estimated by subtracting the mass measured with SPR from that measured with QCM-D (section 5.2). \(^{28,85}\)

![Figure 20](image.png)

**Figure 20.** SPR sensorgram showing immobilization of b-HA (dashed line), b-sHA (solid line) and b-CS (dotted line) to SA on a SAM with subsequent rinsing.

In order to reach saturation of b-CS multiple injections were needed (not shown). The immobilized mass monitored by QCM-D (GAG + associated water), was estimated using the Voigt model. To estimate the mass of immobilized GAGs in SPR, eq. 9 (section 5.2) was used with \(\beta = 0.9\) to account for a distance of 5-10 nm from the sensing surface caused by the immobilization layer (SAM + SA). \(^{28}\) This, along with the estimated water content is summarized in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Mw</th>
<th>dn/dc</th>
<th>(C_{\text{SPR}})</th>
<th>(m_{\text{QCM-D}})</th>
<th>(m_{\text{SPR}})</th>
<th>water content [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-CS</td>
<td>20k</td>
<td>0.147</td>
<td>0.081</td>
<td>110</td>
<td>11.2 ± 1.7</td>
<td>90</td>
</tr>
<tr>
<td>b-HA</td>
<td>23k</td>
<td>0.160</td>
<td>0.074</td>
<td>1080</td>
<td>19.8 ± 1.1</td>
<td>98</td>
</tr>
<tr>
<td>b-sHA</td>
<td>30k</td>
<td>0.147</td>
<td>0.081</td>
<td>230</td>
<td>20.8 ± 1.6</td>
<td>91</td>
</tr>
</tbody>
</table>

The degree of hydration estimated for CS and sHA is roughly the same, although their sulfation differ by a factor of 3 (\(D_S(sHA) > D_S(CS)\)). The great difference between HA and sHA seen in QCM-D is likely due to a higher water content (98% versus 91%) based on these results. However, the molecular weight for sHA is higher which should result in a greater immobilized mass assuming the same grafting density. That
this is not the case and that the immobilized mass for CS is so low can be due to
strong electrostatic repulsion between the sulfate groups and conformationally more
constrained polymer chains. Increasing the ionic strength did not solve this issue.

To further characterize the GAG layers, \( \zeta \) potential measurements and contact
angle measurements were made. Results are summarized in Figure 21.

![Figure 21](image)

Figure 21. Measurements of a) \( \zeta \) potential dependence on pH and b) water contact angles for
surfaces displaying SA only, and SA with end-on immobilized GAGs. Solid line: SA, dashed
line: b-HA, dotted line: b-sHA.

For both measurements, there was a clear effect on surface characteristics upon
immobilization of GAGs. The difference between the GAG variants was however not
significant. The most striking difference between the derivatives lies in their sulfation.
For \( \zeta \) potential, the occurrence of sulfate groups is expected to lower the isoelectric
point due to a higher charge of the polymer. The influence of sulfate groups in the
wettability is less straightforward as both hydroxyl groups and sulfate groups are
expected to yield a very hydrophilic polymer. It is important to notice that both the
QCM-D and SPR results point to a difference in surface coverage, potentially also
influencing the results seen here. There could therefore be an effect from the
underlying SA layer, increasing the isoelectric point and the contact angle. Using
longer GAG chains could reduce this effect and potentially show a greater difference
between the derivatives.
6.3 Bio-functional evaluation of GAG-platforms

6.3.1 CS & type I collagen → Polyelectrolyte behaviour

To illustrate the polyelectrolyte nature of GAG-protein interactions, the interaction between CS and type I collagen was studied (Section 4.2, **Figure 22**).

![Figure 22](image)

**Figure 22.** QCM-D frequency and dissipation signals for addition of type I collagen in high (marked with squares) and low (without marks) ionic strength buffer to deactivated SLB-COOH 5% (dashed curves) and to SLB-COOH with immobilized h-CS (DS$_{hydr.}$ 0.2) (solid curves). Brackets indicate differences between sample and control for high and low ionic strength, respectively. (Gaps in curves are introduced to align the rinsing events; no information was added or removed.)

As expected for a polyelectrolyte interaction, it is highly dependant on ionic strength and pH. At pH 7 there was very little binding of type I collagen to CS (not shown) whereas at pH 5 there was a remaining binding between type I collagen and CS after rinsing in high ionic strength buffer. More binding was seen when collagen was added in low ionic strength buffer compared to a high ionic strength one. This experiment also shows the suitability of using the SLB as an immobilization background; very little collagen bound non-specifically when CS was not present. Collagen is often used as a positively charged linker when building layer-by-layer assemblies consisting of GAGs (section 3.1.3).

6.3.2 CS & BMP-2 → A growth factor reservoir

The interactions between GAGs and growth factors are interesting to study because of the importance in tissues and the potential of using a combination of GAGs and growth factors in therapeutics. CS has recently gained increased interest because of its role in bone and cartilage tissue and its interactions with different growth factors. The growth factor bone morphogenetic protein-2 (BMP-2) is frequently used in research,
primarily with the aim of stimulating bone growth and regeneration.\(^5\) The interaction between these two molecules was studied (Figure 23) and presented in paper I.

![Figure 23](image-url)

**Figure 23.** QCM-D frequency and dissipation signals when adding BMP-2 to h-CS immobilized to SLB-COOH 5% (left) and CS immobilized to SLB-NH\(_2\) 20% (right) (solid lines). Addition was made in both low (top) and high (bottom) ionic strength acetate buffer. Control measurement, in absence of CS, is marked with dashed lines. After addition, the system was rinsed with the acetate buffer just used (indicated in the graph), and later with PBS (not shown).

Although the dimer BMP-2 is a quite small molecule (26 kDa) and there are low amounts of CS immobilized on the surface, the binding induces large responses. Using both low and high ionic strength buffer distinguishes between non-specific and specific interaction; in low ionic strength buffer there is no difference between BMP-2 binding to CS or to the negatively charged background of SLB-COOH, increasing the ionic strength displays a lower but presumably specific interaction. When using the positively charged background SLB-NH\(_2\), there is no unspecific interaction as the positively charged BMP-2 is rather repelled. The large amounts of BMP-2 that bound to CS immobilized on SLB-NH\(_2\) compared to on SLB-COOH at high ionic strength can in part be due to the added mass of CS when using this strategy. Also, CS is here expected to be more in its native form, as it has not been functionalized with hydrazide. Structural rearrangements are likely to occur as CS and BMP-2 interact, as
the reaction did not reach equilibrium during the used conditions. The appearance could also be due to an aggregation of BMP-2 induced at the sensor surface. A cross-linking behavior between GAGs and proteins has been shown for other systems, like HA and the dimer TSG-6.\textsuperscript{47} Two binding sites for the GAG heparin have been suggested\textsuperscript{63} and a similar interaction with CS could therefore be likely. Whether this interaction is of a cross-linking nature or if the CS chain can accommodate itself in a binding pocket of BMP-2\textsuperscript{66} is too early to say. However, this study illustrates the crucial role of GAG immobilization in elucidating more complex interaction mechanisms. Also, as BMP-2 is not dissociating from CS upon rinsing, the system could potentially be used as a growth factor-presenting cell culture substrate.

6.3.3 HA & aggrecan  Building the ECM structure

To study the interaction between HA and aggrecan, HA was immobilized in several ways and the interaction was studied with QCM-D (Figure 24, Table 2), presented in paper II.

![Figure 24](image_url)

Figure 24. Aggrecan binding to surface immobilized HA in three different ways; end-on biotinylation (full and half coverage), side-on biotinylation, and side on without introduced functional groups.

<table>
<thead>
<tr>
<th>MW [kDa]</th>
<th>HA binding</th>
<th>Aggrecan binding</th>
<th>Aggrecan binding %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δf [Hz]</td>
<td>ΔD (10\textsuperscript{-6})</td>
<td>Δf [Hz]</td>
</tr>
<tr>
<td>20</td>
<td>28.3 ± 0.5</td>
<td>6.4 ± 0.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20</td>
<td>6.5 ± 1.0</td>
<td>1.6 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>90</td>
<td>28.1</td>
<td>3.7</td>
<td>1.0</td>
</tr>
<tr>
<td>15</td>
<td>13.0 ± 6.9</td>
<td>1.4 ± 0.9</td>
<td>3.1 ± 2.4</td>
</tr>
</tbody>
</table>
Because the amount of surface-bound HA depended on the immobilization method, aggregan binding was normalized to the mass of the HA layer underneath. Bound masses were estimated using the Sauerbrey equation. In view of the viscoelastic nature of the adlayer, these values do most likely not reflect accurate absolute values. They do however allow for a valid comparison between the different samples. When studying GAG-protein interactions, the end-on configuration is often considered to be the most natural since it resembles the structure of a proteoglycan. However, HA is not found in this configuration, but is free in the ECM. In line with this and the likely orientation of the HA-aggrecan interaction (Figure 3a), side-on immobilization of non-functionalized HA gives the highest interaction with aggrecan. For side-on immobilization of biotinylated HA, there is most likely too many biotin groups present on the molecule to enable an interaction. For end-on immobilization, there is likely sterical hindrance for the highly hydrated aggrecan molecules to reach the side of the HA chains, supported by that decreasing the surface coverage increases the interaction. To better estimate the actual masses bound, comparison with SPR measurements is in progress.

6.3.4 HA & hyaluronidase → Probing biofunctionality

From the results presented above, it is clear that the immobilizing strategy strongly affects GAG-protein interactions. Many ECM components reacting with GAGs are either not commercially available or often expensive. There is hence a need for cheaper evaluation of GAG immobilization. In paper II, the enzyme hyaluronidase (HAase, section 4.2) was investigated for its potential as a cheap evaluation protein. HAase randomly cleaves the β-1,4-glycosidic bond in HA under acidic conditions, yielding tetra- or hexasaccharide products.4-6 HA with different degrees of biotinylation were immobilized on a SAM and degradation caused by HAase was studied in QCM-D, seen as loss of mass. Degradation using HAase also highlight the effect of high biotinylation as side-on biotinylated HA was not degraded, hence not recognized as HA. End-on biotinylated HA was almost fully degraded, hence not experiencing the sterical hindrance to the same extent as aggrecan. Full degradation could potentially be reached by reducing the surface coverage. Bulk degradation by HAase using HPLC showed that the differences are in fact due to functionalization rather than surface immobilization.
6.4 Applications of the GAG platform

6.4.1 Virus infection via GAG attachment

Many enveloped viruses use glycoproteins on their membrane for initial attachment to a host cell. Attachment is vital for entry into the cell and replication of the viral genome, enabling spreading of the virus. Anti-viral drugs can hinder the viral infection either by targeting the attachment mechanism of the viral particle or its replication inside the host cell. Studying virus strains resistant to these drugs is important for developing new and better drugs, as well as for learning more about the infectious mechanism of a specific virus.

Here, glycoproteins type C (gC) was isolated from the envelope of herpes simplex virus-1 (HSV-1). A wild-type strain, KOSc, was used as well as an anti-viral drug resistant strain, AC1. gC from KOSc has a mucin-like region in its N-terminal that is thought to bind to sulfated GAGs at the host cell membrane. gC from AC1 has a deletion in this mucin-like region and is because of this therefore expected to have an altered interaction with GAGs on the host cell.

gC from KOSc and AC1 was added at different concentrations to GAG-presenting surfaces and the binding was studied using SPR. Equilibrium responses as a function of protein concentration are plotted in Figure 25, I) as dependent of immobilized GAG and in II as depending on gC. For further experimental details, see appendix A.
Figure 25. I) Binding of gCs from HSV-1 strain KOSc (empty circles) and from AC1 (filled circles) to surface immobilized sHA, CS and HA. II) Binding of gCs from HSV-1 strain KOSc and AC1 to surface immobilized sHA (solid line), CS (dotted line), and HA (dashed line). (Bally, M., Altgärde, N., Trybala, E., Svedhem, S., Höök, F., Bergstöm, T., in progress)

To compare the binding, the dissociation constant $K_D$ was estimated by $K_{0.5}$ (grey, dotted line in Figure 25 II) (see section 4.1.3). It was found to be approx. 5 nM for binding of gC from KOSc and 70 nM for the binding of gC from AC1 to sHA and CS. The low binding of gC to non-sulfated HA, which was expected, made estimations of $K_{0.5}$ difficult. There was no apparent difference in the binding affinity between sHA and CS for the gCs, but the saturation values differ greatly. There is lower amounts of CS on the surface, about 2/3 of the response value for sHA, which is roughly the difference in saturation seen for the gC from AC1. The difference in saturation values seen for gC from KOSc are greater than that, about 2/6. The sulfation on sHA is synthetically derived, and about 3 times higher than the natural sulfation occurring on
CS. This difference between the GAG derivatives seems to be sensed more by gC from the wild-typ strain KOSc than by gC from the mutant strain AC1.

A higher affinity between sulfated GAGs and gC from KOSc is apparent. Furthermore, the amount of material binding of the mutated gC from AC1 is much greater. The difference in R\textsubscript{max}, also associated with a slower dissociation rate (Figure 26), could suggest an aggregation of this gC. Both gCs include a hydrophobic transmembrane region that could induce micelle formation. Also, since gC from AC1 lacks the mucin-like region likely repulsing other nearby gCs, micelle formation could be more pronounced for gC from AC1.

![Figure 26](image)

*Figure 26.* Dissociation from surface immobilized sHA of gC from a) KOSc and b) AC1 at a gC concentration of 44.6 nM. (Bally, M., Altgärde, N., Trybala, E., Svedhem, S., Höök, F., Bergstöm, T., in progress)

The above results can be linked to cell-infecting experiments for the two HSV-strains. Although lacking the mucin-like region, AC1 still binds to and infects cells. However, they do not release as well from the cell surface (Bergström et. al., unpublished results). Release will affect both entry and exit fro the virus particle; as to strong binding hinders the viron from going into the cell and could stop newly formed viruses on their way out from the cell. This is coherent with what is seen here, the gC from AC1 binds in high amounts but is not released. The mucin-like region does not seem to be a requisite for GAG-binding, but it rather has a crucial modulating ability, allowing the virus particles to also be released from the cell surface.

K\textsubscript{0.5} is only a true estimation of K\textsubscript{D} if the Langmuir equation holds, e.g. when there is no cooperativity in the binding (see section 4.1.3). In view of the shape of the curves in Figure 25, cooperativity is likely at least for the gC From AC1. Cooperativity can be estimated by fitting to extensions of the Langmuir equation, e.g. the Hill equation or investigating the derivative of the binding curve. Analysis of this is on-going.
7 Looking back and looking ahead

The overall aim of my work is to investigate the function of glycosaminoglycans (GAGs) in bone and cartilage tissue, in term of how they interact with proteins, glycoproteins and cells. In order to do this in a controlled way, we wanted to immobilize the glycosaminoglycans to a surface. This proved to be a project on its own.

What technique should be used? And what surface? How does immobilization affect subsequent interactions? How does orientation of the GAGs affect subsequent orientation? How do one quantify interactions between GAGs and proteins?

In paper I, these issues were discussed for immobilization of chondroitin sulfate to supported lipid bilayers via amine coupling. There are clear benefits of using the lipid bilayers as an immobilizing layer because of the low unspecific interaction. In the paper, the influence of functional groups was investigated. In order to accomplish end-on immobilization instead of side-on, another immobilization strategy was evaluated in paper II, namely biotin-streptavidin immobilization on a self-assembled monolayer. This was evaluated in paper II. Throughout the project, interactions with various proteins have been investigated. These studies were very helpful in determining important aspects of the underlying GAG layer. For example, binding of aggregan and hyaluronidase to immobilized GAGs (paper II) shows the effect of both functionalization and orientation where different aspects are important for different proteins. The end-on immobilized GAGs were successfully used to study binding mechanisms of virus glycoproteins. A setup like this provides valuable complementary results to conventional virus research.

An interesting field in GAG research is the combination with growth factors, aiming towards cell experiments. In paper I, the high binding of the growth factor bone morphogenetic protein-2 to chondroitin sulfate was shown. These studies will be continued using end-on immobilized GAGs and investigating the effect of sulfation. This will also be beneficial in a study together with Prof. Anders Lindahl at the department of clinical chemistry and transfusion medicine, Sahlgrenska University Hospital, for studying the initial attachment of chondrocytes. The use of a cross-linked hyaluronic acid scaffold has shown promising results for clinical applications, and the initial chondrocyte attachment to hyaluronic acid and other GAGs is interesting for improved scaffold design.

Another possible application area for the GAG-platforms developed during this work is to study platelet (thrombocyte) activation studies, which can provide fundamental information on blood coagulation and related diseases. Currently used techniques for the quantification of platelet aggregation are based on light aggregometry, electrical impedance aggregometry or thromboelastometry, all performed under static or quasi-dynamic conditions. In order to mimic the conditions found in human cardiovascular system it is important to perform studies under flow conditions and on surfaces.
mimicking biological surfaces, e.g. lipid membranes or ECM components. Combining QCM-D with light microscopy could give valuable information for such a system, studying the effect of different surface coating on platelet activation and coagulation. This setup was used to study platelet adhesion on end-on immobilized hyaluronic acid. Hyaluronic acid has been suggested as a coating for stents to prevent stent-induced blood cloths.\textsuperscript{17} Essentially no binding of platelets to HA was seen using the suggested setup (Figure 27).

![Figure 27](image-url)

**Figure 27.** Addition of platelet rich plasma to hyaluronic acid immobilized end-on on streptavidin modified self assembled monolayer. (Kunze, A., Altgärde, N., Hesse, C., Svedhem, S., in progress)
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10 Appendix A

Experimental section for GAG-Virus protein interaction

**Herpes simplex virus-1 glycoproteins.** Plaque purified wild-type strain of herpes simplex virus-1 (HSV-1), KOSc, was subjected to ten passages in african green monkey kidney (GMK AH1) cells in the presence of PI-88, a sulfated polysaccharide known to inhibit HSV attachment to cells. Glycoproteins for wild-type HSV-1 and escape variants were isolated. AC1, a HSV-1 strain that survived PI-88 was found to have deletion of 83 aa in the N-terminal region on glycoproteins gC. The deleted region lies in a mucin-like region of the gC hypothesized to be involved in the attachment of HSV-1 to cells. gC from KOSc and AC1 were aliquoted and frozen, and dissolved in PBS before measurements.

**Studying HSV glycoprotein-GAG interactions.** KOSc and AC1 were dissolved in PBS and added at different concentrations to surfaces presenting end-on biotinylated-CS, b-HA, and b-sHA. A flow of 5 ul/min was used for equilibrium measurements, 60 ul/min for kinetic measurements.