THESIS FOR THE DEGREE OF LICENTIATE OF PHILOSOPHY

Virus-cell membrane interactions

Binding studies of Herpes Simplex Virus using surface-sensitive techniques

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CHALMERS UNIVERSITY OF TECHNOLOGY

Göteborg, Sweden 2016

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Printed at Chalmers Reproservice
Göteborg, 2016

Cover picture: *Illustration of the model system used in this thesis to study herpes-glycosaminoglycan interactions. Biotinylated glycosaminoglycan chains are end-grafted to a sensor surface consisting of a supported lipid bilayer and a monolayer of streptavidin. The arrangement of the chains is designed to mimic the brush-like architecture of glycosaminoglycans in the extracellular matrix and close to the cell surface. Binding and release of individual virus particles is probed.*

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Abstract

Viruses are parasites capable of infecting all forms of life. They lack the ability to replicate by themselves and therefore hijack the replication machinery of cells to produce new viral copies, called virions. Two steps of this replication cycle are of particular interest in the frame of this thesis: the initial attachment of the virus to the cell membrane and the release of the virus from the cell membrane after infection. One virus for which these mechanisms are still under investigation is the herpes simplex virus (HSV). HSV is commonly known for causing blisters on the skin or mucosa of the lips, mouth or genitals. In rare cases, it can migrate to the central nervous system, causing meningitis or encephalitis. HSV is an enveloped DNA virus that binds to the cell membrane via interactions between viral glycoproteins and cell-surface sulfated polysaccharide chains, called glycosaminoglycans (GAGs).

The main focus of the work presented here was the interaction between HSV and surface immobilized GAGs. The individual GAG chains were end-grafted to a sensor surface to mimic the brush-like architecture of GAGs found close to the cell surface. Total internal reflection fluorescence microscopy (TIRFM) was used to extract information on binding kinetics and mobility of single fluorescently labeled HSV particles. Two aspects of the HSV-GAG interaction were studied in detail: First, the influence of the sulfation of the GAG chains on the binding characteristics, and second, the role of glycosylation of the viral glycoproteins. Binding studies of HSV to different GAGs showed that the degree of sulfation of the GAG influences the binding affinity of HSV. Furthermore, single particle tracking (SPT) analysis revealed that HSV diffuses on the GAG surface and that this mobility is influenced by the affinity of the HSV-GAG bonds. Finally, experiments involving virus mutants demonstrated that the glycosylation of the viral glycoproteins plays a critical role in the release of HSV virions from the surface of infected cells.

Taken together, two aspects modulating the interaction between the herpes simplex virus and glycosaminolgycans were identified in this thesis, providing new insights into the mechanisms that regulate the initial attachment of the virus to the cell membrane and its release from the cell membrane after infection. In addition, the mobility of the HSV particles observed with the model system suggests that the virus is able to diffuse on the cell membrane *in vivo*.

Keywords: herpes simplex virus, glycosaminoglycans, TIRF microscopy, binding kinetics, single particle tracking

Appended Papers

PAPER I

Binding kinetics and lateral mobility of herpes simplex virus type 1 on end-grafted sulfated glycosaminoglycans

Nadia Peerboom, Stephan Block, Noomi Altgärde, Olov Wahlsten, Matthias Schnabelrauch, Tomas Bergström, Marta Bally

In manuscript.

My contribution: I designed and planned the experiments, which I performed myself, analyzed the data and wrote the main part of the manuscript.

PAPER II

Mucin-like region of herpes simplex virus type 1 attachment protein glycoprotein C (gC) modulates the virus-glycosaminoglycan interaction

Noomi Altgärde, Charlotta Eriksson, Nadia Peerboom, Tuan Phan-Xuan, Stephanie Moeller, Matthias Schnabelrauch, Sofia Svedhem, Edward Trybala, Tomas Bergström, Marta Bally

Journal of Biological Chemistry, 290(35), 21473-21485, 2015.

My contribution: I designed and planned the TIRF experiments and performed the data analysis. I wrote the corresponding methods section in the manuscript.

PAPER III

Herpes Simplex Virus Type 2 Mucin-like Glycoprotein mgG Promotes Virus Release From the Surface of Infected Cells

Edward Trybala, Nadia Peerboom, Beata Adamiak, Malgorzata Krzyzowska, Jan-Åke Liljeqvist, Marta Bally, Tomas Bergström

Submitted.

My contribution: I designed and planned the TIRF experiments, which I performed myself, analyzed the data and wrote the corresponding part of the manuscript.

List of abbreviations

AFM	Atomic Force Microscopy
CS	Chondroitin Sulfate
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
GAG	Glycosaminoglycan
GMK	Green Monkey Kidney
GSL	Glycosphingolipid
HA	Hyaluronic Acid
HS	Heparan Sulfate
HSV	Herpes Simplex Virus
KS	Keratan Sulfate
MSD	Mean-squared Displacement
NMV	Native Membrane Vesicle
RNA	Ribonucleic Acid
SA	Streptavidin
sHA	sulfated Hyaluronic Acid
SLB	Supported Lipid Bilayer
SPR	Surface Plasmon Resonance
SPT	Single Particle Tracking
TIRF(M)	Total Internal Reflection Florescence Microscopy

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1
Introduction

*"Nothing in life is to be feared, it is only to be understood." – **Marie Curie***

Viruses are ubiquitous on earth; they are found in their hosts, but also in water, soil and air. We have only discovered a very small fraction of all existing viruses. One reason for this is that the majority of viruses do not cause any effect on their host, even if permanently residing within their genomes. More than 8% of the human genome is in fact estimated to be of viral origin[1]. Viruses are even believed to have played a crucial role in the origin of life billions of years ago and in the evolution to more complex forms of life, thanks to their ability to transfer genes between cells.

Despite their possible role at all levels of life and their potential use as anti-bacterial or anti-cancer agents*, viruses are mainly known for the diseases and deadly outbreaks they cause around the world. The Ebola virus outbreak in West Africa between 2013 and 2015 has caused almost 30 000 cases, of which about a third were fatal†. The ongoing epidemic of Zika virus has been reported in 58 countries in the Americas and the Pacific‡. Also in Sweden we observe the effects of virus outbreaks: About 10 000 cases of influenza (Influenza A, Influenza B and A/H1N1) are registered yearly during the winter season in Sweden§.

A virus infection starts with the transmission of a number of virus particles between hosts. This transmission generally occurs via vectors (insects and ticks for example), via food and water, or directly from person to person (coughing and sneezing, needles with contaminated blood or via sexual contact for example). Once inside the host, the virus needs to replicate in order to survive. For this purpose, it hijacks the replication machinery of the cell and produces new virus particles (called virions) that exit the cell to infect new hosts. This replication cycle starts with attachment of the virus to the cell membrane, continues with genome replication and virus assembly inside the cell and finishes with the release of the new virions. For many viral species, the molecular interactions between the virus and the cell membrane during virus attachment and release are not fully understood yet. Identifying which mechanisms and which molecules regulate these interactions is however a key step in developing ways to inhibit this infection with anti-viral drugs.

Certain viruses can mutate fast and therefore make existing therapies and vaccines inefficient. In addition, new virus strains can emerge when a virus changes host and undergoes evolutionary changes[2]. Luckily, different virus strains can sometimes use very similar mechanisms for cell infection in general and membrane attachment and release in

* Due to their ability to infect and kill cells current research investigates the potential of genetically modified viruses to infect and destroy tumor or bacteria cells.
† Source: World Health Organization (www.who.int): Ebola Situation Report, 12 May 2016.
‡ Source: World Health Organization (www.who.int): Zika Situation Report, 12 May 2016.
§ Source: Folkhälsomyndigheten (www.folkhalsomyndigheten.se): Influensarapport (Säsongssammanfattning).

particular. A well-established therapy for one virus strain can therefore potentially be modified in case of an outbreak of a new virus strain that shows structural similarities to the first one. This calls for the investigation of the processes involved in viral attachment and release to and from the cell surface and for finding the missing puzzle pieces to complete the knowledge of virus-cell membrane interactions.

In this work we have studied the case of the herpes simplex virus (HSV). HSV is a very widespread human virus. Taken together, its two serotypes are estimated to affect around 90% of the population worldwide[3]. It is known to bind to sulfated glycosaminoglycans, long polysaccharide chains present at the cell surface and in the extracellular matrix, in its initial step of attachment. Given the high amount of infected people, the similarities to other common viruses (HIV for example) and the missing knowledge about the mechanisms regulating attachment and release, HSV is an interesting virus to study.

The aim of this work is to characterize the interactions between HSV and glycosaminoglycans (GAGs) with the goal of gaining further knowledge of the mechanisms that are involved in the initial step of virus attachment to the cell membrane and in the release of new virions from the cell membrane after replication. To achieve this goal, we use a simple model of the cell surface that makes it possible to study HSV-GAG interactions in a controlled way and with the option to change both architecture and physiochemical properties of the carbohydrate surface. To gain a comprehensive view of the parameters modulating the interaction two experimental approaches will be discussed in this thesis: first of all, the physiochemical properties of the GAG surface are modified, using for example GAGs with different sulfation patterns. This makes it possible to investigate how the GAG sulfation influences the interaction with the HSV particles. Secondly, we take advantage of viruses presenting mutations in their glycoproteins to study the contribution of certain glycoproteins to the HSV-GAG interactions. The main techniques used in this work are Surface Plasmon Resonance (SPR), to monitor in real-time the step-by-step assembly of the GAG surfaces, and Total Internal Reflection Fluorescence (TIRF) microscopy to detect and analyze single virus binding events to the surface.

The next two chapters provide a background to the field of study: Chapter 2 presents the biological aspects while chapter 3 looks at the studied biological processes from a physicist's point of view. Chapter 4 gives an overview of the two techniques (SPR and TIRF) used in this work. Chapter 5 summarizes and discusses the results of the different papers. Finally, chapter 6 concludes this thesis with an outlook of my work.

2
Background in Biology

"Biology is the study of complicated things that have the appearance of having been designed with a purpose."
– Richard Dawkins

The world of biology relies on a fascinating interplay between complex mechanisms regulating life. There appears to be an infinite number of things yet to be discovered, which has stimulated the appearance of more and more interdisciplinary research approaches applied to biology. Starting from very small and simple organisms, life has evolved throughout time to very complex forms of life and our understanding of their complexity is still limited.

The human body in particular is the subject of much attention since the processes happening inside us directly affect our health. We are one of the most complex living organisms created by nature, built up by more than 10^{13} cells that are constituted of lipids, proteins, carbohydrates and nucleic acids, forming the four fundamental building blocks of life[4], [5]. These classes of macromolecules are responsible for different functions and orchestrate together, in a perfectly designed network, the processes that make our body function. When some of these processes malfunction, fundamental knowledge of the human biology, together with the development of new techniques, can then help improving the life quality.

All living beings on earth are directly related to each other, which means that very simple organisms, like a bacteria for example, can share common processes to eat, breathe and reproduce as the human body[6]. Both are built up from the fundamental building blocks of life. For this reason we have a lot of knowledge to gain from small organisms and research in biology is conducted at every length scale. The length scale we focus on in this thesis is the nanometer range. It is the scale of viruses and the cell membranes they interact with during cell infection.

In this chapter the cell membrane and the structure and functioning of viruses are presented. Then, the Herpes Simplex Virus and the characteristics of its interactions with the cell membrane will be introduced and discussed.

2.1 The cell membrane

The cell membrane, also known as plasma membrane, is an essential component of the cell. It separates the content of the cell from the outer environment and acts as a barrier that controls the passage of molecules in and out of the cell. The cell membrane is a dynamic and flexible structure, built up mainly from *lipids* and *proteins* (figure 2.1).

Lipids are amphiphilic molecules. They consist of a hydrophilic (polar) head group and a hydrophobic (nonpolar) hydrocarbon tail. In order to minimize the contact between the

hydrophobic tails and the surrounding water molecules, lipids arrange into distinct supramolecular assemblies when in an aqueous environment at a high enough concentration. Lipids account for approximately half of the mass of most animal cell membranes[7]. The most abundant lipids are the phospholipids. The main characteristic of this type of molecules is that they generally contain two hydrocarbon chains, giving a cylindrical shape to the lipid. This geometry is the reason why most phospholipids spontaneously assemble into 5 nm thick, double-layered sheets, called bilayers. Individual lipids can diffuse freely within the bilayer with lateral diffusion coefficients on the order of 10^{-8} cm^2/sec[8]. The diffusion of lipids within the cell membrane is generally about one order of magnitude slower[8].

The remaining half of the membrane mass mainly comes from the membrane proteins that are responsible for most of the membrane's functions. Proteins are very diverse in structure and in the way they are attached to the membrane. Some are covalently linked to the lipid bilayer and reside in the cytosol or in the extracellular matrix. So-called transmembrane proteins on the other hand are amphiphilic molecules and cross the whole membrane (with the hydrophobic parts being oriented towards the hydrocarbon tails of the lipids). The structure of a membrane protein is directly linked to its function. Transmembrane proteins are for example responsible for the transport of molecules across the membrane. Other proteins (both transmembrane proteins or proteins on the outer cell surface) serve as receptors for signal molecules outside of the cell. The binding of a molecule to a receptor protein then usually results in a physiological response of the cell.

The concept of a fluid membrane was first proposed in the early 70s by Singer and Nicolson. They suggested a so-called *fluid mosaic model* that applies for most biological membranes. This model describes the cell membrane as a two-dimensional fluid, made of proteins embedded in a lipid matrix[9]. These new insights were of great importance for understanding the structure and functionality of biological membranes. Singer's and Nicolson's model turned out to be too simplistic and was therefore refined in 1982, when Karnovski et al. demonstrated that lipid molecules are, despite their lateral mobility, not homogeneously distributed in the membrane and that the lateral mobility of most proteins was constrained, as opposed to freely diffusing. They suggested that lipids form tightly packed microdomains within the membrane, thus creating heterogeneous structures of high significance for the functionality of the membrane[10]. These microdomains are generally known as lipid rafts.

2.2 Carbohydrates

Carbohydrates, also referred to as sugars, are the third building block of cells. They are one of the major components of the extracellular matrix (ECM), a layer of extracellular macromolecules extending from the membrane and surrounding all eukaryotic cells. They are also found attached to the cell membrane, all-together forming the *glycocalyx*, a layer of high complexity, which plays a key role in determining how a cell interacts with its surroundings. Unlike proteins, carbohydrates are not coded for in the cellular DNA, but are synthesized by the cell via enzymatic reactions. They reflect the status of the cell: If a cell undergoes changes due to an infectious disease or cancer for example, its carbohydrate composition is usually altered. For this reason, carbohydrates can be used as biomarkers for certain physiological conditions of a cell[11].

Carbohydrates exist in different forms (figure 2.1): They can be attached to proteins (more than half of all proteins are glycosylated[12], i.e. they carry sugar moieties), but also to certain lipids (~1% of all lipids found in a generic mammalian cell[13]). They are then called glycoproteins and glycolipids, respectively. Some carbohydrates occur as long, unbranched polysaccharide chains, composed of repeating disaccharide units. These chains are either attached to a protein core (called a proteoglycan) or secreted freely into the ECM.

Glycosylation is the addition of one or more monosaccharide units to a protein or lipid[13]. This process accords additional information, structure and function to these molecules. Glycoproteins are either *N*-Linked (this type of glycosylation occurs on all membrane-associated glycoproteins) or *O*-linked referring to the component the sugar unit is linked to (nitrogen or oxygen). For glycolipids, the monosaccharide units constitute the head group of the lipid, meaning that the glycans are oriented towards the ECM. The most abundant glycolipid in mammalian cells are glycosphingolipids (GSLs). They play a role in cell-cell recognition, cell surface reception and messaging[13].

The components of the ECM that we will focus the most on in this thesis are the *glycosaminoglycans* (GAGs). They are the main form of polysaccharides in mammalian cells. These long linear sugar chains exist both close to the cell surface and in the extracellular matrix, therefore often serving as receptor sites for diverse biomolecules and pathogens. They also modulate cell adhesion, differentiation, migration and proliferation[13]. Despite their structure made from repeating units of disaccharides, GAGs are highly heterogeneous molecules. This is due to postsynthetic modifications of the chain – mainly through addition of sulfate groups. The sulfation patterns on the GAG chains have been shown to be specific for certain tissues, development stages of the cell and disease conditions[14], [15]. As we will discuss later, the sulfation motifs on the chain ("sulfation code" of the GAG) also influence the interactions with viruses[16]. In this work, we focus on three different types of GAGs: hyaluronic acid/hyaluronan (HA), heparan sulfate (HS) and chondroitin sulfate (CS). HA is not sulfated and therefore has the simplest chemical composition. With up to 10 000 disaccharide units, it is by far the longest GAG that exists. Unlike HS and CS, HA is not bound to a protein core. HS and CS are made of 10 to 100 dissaccharide units[17]. They differ in their disaccharide units, their sulfation patterns and their location in the ECM or on the cell surface. HS is mainly found attached to membrane proteoglycans (for example perlecan, agrin and syndecan). Syndecan also carries CS chains. In contrast, around 100 CS chains are covalently bound, in a brush-like configuration, to the proteoglycan aggrecan, present in the ECM and thus located further away from the cell membrane[18] (figure 2.1).

As already mentioned, glycoconjugates often serve as receptors for viruses[19]. The influenza virus for example binds to sialic acid residues on the cell surface[20]. Many viruses use GAGs for their initial attachment to the cell. Well-known examples are the human immunodeficiency virus (HIV)[21], the Ebola virus[22], the human papillomavirus (HPV)[23] and the herpes simplex virus (HSV)[24]. The sulfation code of the GAGs and their location in the ECM play an important role in this binding process, as we will discuss further in this thesis.

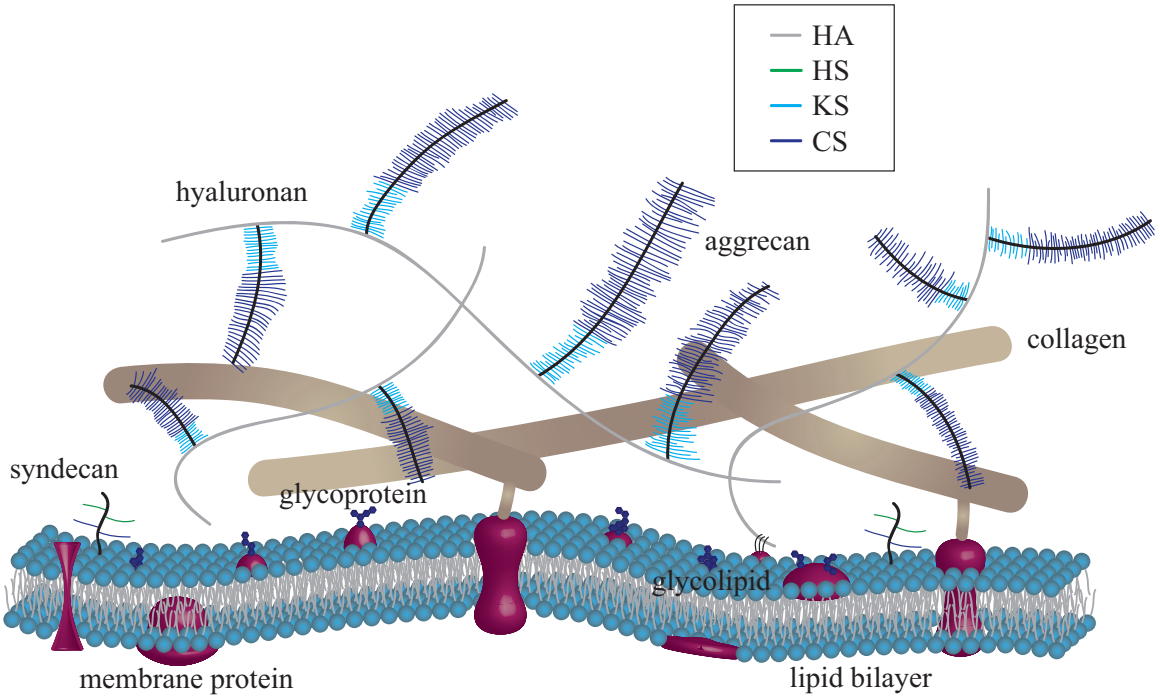


Figure 2.1: Cell membrane containing lipids and proteins together with the extracellular matrix, displaying collagen, proteoglycans and glycosaminoglycans.

2.3 What is a virus?

A virus is by its simplest definition a small infectious agent that uses the cell's replication machinery to replicate. It can infect all species found on earth, from animal cells, to plant cells and bacteria. The diameter of a single virus particle, called virion, is in the nanometer range, with the smallest virus being around 20 nm (parvovirus). So-called superviruses can have characteristic diameters up to half a micrometer: the largest known virus, the mimivirus, has a diameter of about 400 nm with 100 nm long filaments extending from the capsid[1], [2].

A virus is usually formed by a protein capsid protecting the genome. The genome can be either DNA or RNA and both can be either single or double stranded. Some viruses are enveloped, which means that a lipid membrane surrounds the protein capsid. This membrane is derived either from the plasma membrane of its host cell or from one of the inner membranes of the cell. This lipid envelope embeds the proteins involved in virus attachment, entry and release.

For a virus to survive it must replicate. To do so, it infects host cells, which turn into factories producing new virions. The replication cycle of a virus is divided into different steps[2]: The first step is the *attachment*, during which viral proteins bind to specific attachment factors on the cell membrane. More viral proteins and cell membrane receptors will then come into play for successful virus *entry* into the cell. The virus enters through the cell either by endocytosis (a process during which the plasma membrane bends around the virion and

pinches off into the cytoplasm) or by fusion of the viral envelope with the cell membrane. Once inside the cell, the virus has to synthesize new viral components and replicate its genome. Synthesis of new components is done by *transcription* of the genetic information into mRNA, *translation* of this information into sequences of amino acids to form new proteins and *transport* of viral components to different locations within the cell. Genome *replication* usually takes place in the cell nucleus but can also be carried out in the cytoplasm for certain viruses (most RNA viruses). Finally, all the components *assemble* to form a new virion that exits the cell in the final step called *release*. For enveloped viruses, the release process can occur via budding at the plasma membrane, which is enriched with specific viral proteins. In this case, the virus acquires its viral envelope by deforming the membrane into a bud enveloping the rest of the viral components. Some viruses acquire their envelope by budding through the nuclear membrane. They are then transported out via vesicles that fuse with the plasma membrane. In certain types of infection, the cell bursts to release the virions.

How specific a viral infection is to the cell that is infected generally depends on the type of virus. A cell that can be infected by a certain type of virus and permit its replication is called a permissive cell. A permissive cells needs to meet a series of requirements for successful virus infection[2]: First of all the cell membrane must have attachment factors and entry receptors specific for that type of virus. Second, the cell must contain all the components necessary for virus replication (proteins and enzymes for example). This last requirement generally restricts the number of permissive cells for viruses with small genomes that almost entirely depend on the cell's replication machinery to copy their genome. Viruses with larger genomes are usually able to synthesize their own proteins and enzymes needed for the replication process inside the cell[1].

The fate of an infected cell depends on the type of virus infection, which can be divided into four groups[1]: *Acute or lytic infections* produce new virions at a high rate and result in rapid cell death. *Persistent or chronic infections* are long-term infections with a slow virus production. In *latent or proviral infections*, the viral genome resides in an inactive state within the cell. Finally, in *transforming infections*, the cell's growth properties are altered, which can lead to the development of cancer.

2.4 The herpes simplex virus

There are eight different human herpesviruses. As an adult person, the probability to have been infected with at least one of them is very high. A common characteristic of herpesviruses is that their infections often become latent, remaining for a lifetime with new outbreaks every now and then. The best-known examples are the *herpes simplex virus* (HSV), causing blisters on the skin or mucosa of the lips, mouth or genitals, and the *varicella-zoster virus*, responsible for chickenpox. HSV, which we focus on in the frame of this thesis, has two serotypes: HSV-1 and HSV-2. HSV-1 and HSV-2 preferentially reactivate from oral and genital sites respectively, both serotypes are however able to infect either site[25].

The herpes simplex virus is an enveloped virus with a double-stranded DNA genome. With around 150 kbp[26], [27], the genome of HSV is relatively large, which means that it encodes for many different proteins, all assigned to specific functions. Twelve different species of

glycoproteins are found in the viral envelope. Each one of them is designed to play a role in one or several virus replication steps[28].

Virus attachment is mediated by glycoproteins gC and gB binding to cell surface heparan sulfate (HS) and chondroitin sulfate (CS). gC-1 (glycoprotein gC of serotype HSV-1) is the main attachment protein of HSV-1 and it binds to HS[29] and CS[30], [31]. It is however not essential for successful cell infection, since it has been shown that gB-1 mediates binding for gC-1 deficient HSV-1 virions[32]. The situation is different for HSV-2, where gB-2 (glycoprotein gB of HSV-2) is considered the main attachment protein[33], [34]. Virions that are deficient in both gC and gB show drastically reduced infectivity[32]. This is however believed to be partially due to the need of gB for viral entry[35]. Fusion between the viral envelope and the plasma membrane is believed to be the main pathway for HSV entry into the cell[35]. Fusion is triggered by glycoprotein gD (and additionally gB and gH/gL heterodimers) binding to entry receptors on the cell membrane. Three different classes of entry receptors have been identified[36]: HVEM (herpesvirus entry mediator), nectin-1 (both serotypes) and nectin-2 (HSV-2 only) and 3-*O*-sulfated HS (HSV-1 only). An alternative pathway for viral entry via endocytosis and fusion at low pH with the endosomal membrane has been shown for HeLa and CHO cells[37], thus suggesting that HSV has two distinct pathways for viral entry, depending on the host cell.

In this work, we focus on two key steps of the HSV replication cycle: the initial attachment of the virus to the cell membrane and the release of the progeny virions from the cell membrane. These two processes are closely linked to each other: while there is a need for strong interactions that bind the virus particle to the cell membrane to achieve viral entry, these same interactions must be overcome in order to detach the newly assembled virions from the cell membrane. If not, all progeny virions would get trapped on the cell membrane in a dead-end infection. The sialic acid binding influenza virus produces an enzyme (neuraminidase) that degrades the sialic acid moieties on the cell surface to balance this interaction[38]. A similar mechanism, based on the HS degrading enzyme heparanase (HPSE), has recently been suggested for HSV-1[39]. Hadigal et al. indeed demonstrated that HS expression on the cell surface is drastically decreased after infection and that this effect is a result of an upregulation of active HPSE upon infection. It is interesting to note that the genome of the influenza virus codes for its receptor degrading enzyme neuraminidase, while HSV is not known to encode any enzymes, but would take advantage of a host-enzyme to facilitate viral egress.

In this thesis we will discuss an alternative mechanism for regulation of attachment and release of HSV, related to the existence of a highly glycosylated region, named mucin-like region due to its structural similarity with mucins. Interestingly, a mucin-like region was found close to the GAG-binding site on gC-1 but it was not present on gC-2[40]. This region was later shown to affect binding kinetics of gC-1 to immobilized HS[41]. This aspect is further discussed in paper II. A similar structure has also been found on glycoprotein gG of HSV-2. Paper III discusses its potential role in viral egress.

3
Background in Physics

"Nothing happens until something moves." – Albert Einstein

Nature is governed by universal laws of physics. Four different fundamental forces have been identified to rule over all objects surrounding us. These forces have been used to explain Newton's famous apple falling from the tree or a lightning in the sky for example. They have also been applied to very big scales to describe the movement of planets and galaxies, as well as to very small scales, to understand how the nucleus of an atom holds together.

In the same way that atoms and planets obey the laws of physics, living matter does too. In fact, all processes regulating life are ruled by physics (entropy in particular, which we will come back to soon). This highlights the importance and need of studying biological processes from a physicist's point of view.

In this chapter we start with discussing the laws of thermodynamics and their importance in biology. We then discuss specificity and multivalency of receptor-ligand interactions and describe the formalism of binding kinetics. Finally, there will be a section about single particle diffusivity and tracking, a further topic of direct relevance to this thesis.

3.1 Thermodynamics

A cell is the fundamental unit of structure in all organisms. It is a very dynamic entity that is constantly changing. An equilibrium state, from a classical physics perspective, is therefore difficult to apply to any biological system. The processes happening inside the cell occur at very different time scales though, which makes it possible to consider isolated "quasiequilibrium" states ruled by the laws of thermodynamics[5].

Cells need to store and transform energy to be able to use it for all the reactions that require energy. The source of energy is food (fats, proteins, carbohydrates), or sunlight for plant or bacteria cells for example. In this last example the energy provided by the absorbed photons is transformed into chemical energy used for the cell's metabolism. During this process, the law of conservation of energy (first law of thermodynamics) has to be fulfilled.

Also the second law of thermodynamics plays an important role in biology. It teaches us the concept of free energy. The Gibb's free energy is defined as follows:

$$G = H - TS \tag{3.1}$$

The enthalpy H is the internal energy of the system and T is the temperature. S is the entropy of the system. It is a measure of the disorder of a system, or, more precisely, a measure of the number of microscopic configurations a system can exist in for a given macroscopic state[5]:

$$S = k_B \ln W \tag{3.2}$$

where k_B is the Boltzmann constant and W is the number of microstates. Every system strives for minimizing its free energy and an interaction takes place spontaneously if $\Delta G < 0$. This explains for example the hydrophobic effect and therefore the arrangements of lipids and proteins into well-defined structures.

3.2 Specificity and multivalency

The forces that tie molecules together can be of different nature. The strongest intermolecular interactions are the covalent bond and the electrostatic force. The main difference between these two forces, other than that the first one is of chemical and the second one of physical nature, is their directionality. The covalent bond is a directional force, meaning that the molecules orient themselves in well-defined angles. The electrostatic force on the other hand is non-directional and therefore less *specific* than the covalent bond[42]. Other examples of non-directional forces involved in intermolecular interactions are van der Waals forces, hydrophobic interactions, hydrogen bonds and electric dipole interactions[42].

The specificity of an interaction is defined as the ability of that interaction to occur only between a biomolecule A and another biomolecule B. If A is also able to bind to a third biomolecule C, but prefers to bind to B, we talk about *selectivity* instead. High specificity can also be achieved for non-directional forces if the three-dimensional arrangement of the two binding partners is favorable to that interaction (figure 3.1a). This is for example the case for antibody/antigen interactions[43], [44].

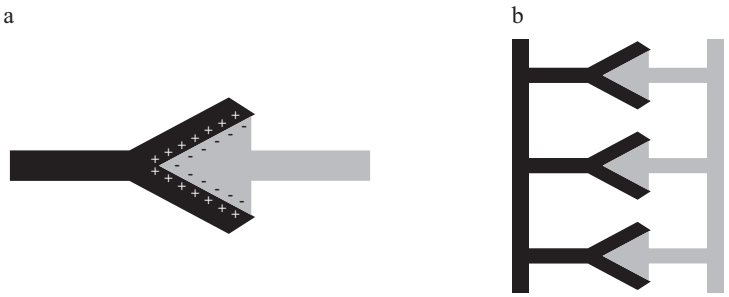


Figure 3.1: Illustration of the concept of specificity and multivalency. a) Non-directional forces can achieve high specificity if the three-dimensional arrangement is in favor of the interaction. b) Multivalent interactions can be collectively stronger than the separate monovalent interactions they are composed of.

Interactions occurring between the cell membrane and a variety of entities binding to it (extracellular vesicles, viruses, polyvalent molecules, …) are often composed of multiple weak interactions between several recognition sites on both the membrane and the binding

entity. In this case we talk of *multivalency* (figure 3.1b). The strength of a multivalent interaction depends on the number of ligand-membrane receptor bonds and its specificity is highly affected by the density and spatial arrangement of receptors in the cell membrane. This mechanism therefore allows for control of the interactions the cell membrane engages into.

Considering that viruses present several copies of binding proteins on their capsid or envelope, it is generally believed that the interaction between a virus and the cell membrane is of multivalent nature[45]. This would mean that stable attachment to the cell membrane can only be achieved if multiple viral proteins (taking the role of keys) connect to receptors on the cell surface (the locks). How well the viral keys fit into the cellular locks then determines the specificity of the interaction. The dynamics of multivalent virus-cell membrane interactions has been studied for Simian virus 40 for example[46]–[48]. Multivalency has also been suggested for a number of other viruses, like influenza and HSV[45], [49].

3.3 Quantifying binding kinetics

To quantify and compare the strength of an interaction between a receptor and a ligand we use binding rate constants. Since the assays used in this work are all based on surface-sensitive methods, we will focus on the case of ligands in solution, binding to receptors immobilized on a surface (figure 3.3a).

We will first consider a simple interaction between a ligand L and a receptor R, forming the complex LR.

$$L + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} LR \tag{3.3}$$

k_{on} and k_{off} are the reaction coefficients for binding and release respectively, also called association and dissociation rate constants. They can be related to the activation energies $\Delta E_{on/off}$ of association and dissociation respectively by writing the following expression:

$$k_{on/off} = A\, exp\left(-\frac{\Delta E_{on/off}}{k_B T}\right) \tag{3.4}$$

Here T is the temperature of the system and A represents the number of collisions per unit time and concentration of ligands in the case of k_{on}, and the number of dissociation attempts per second for k_{off}. We define $K_D = k_{off}/k_{on}$. K_D is expressed in molar and has a low value for a high affinity interaction. It can be related to the Gibbs free energy (see figure 3.2):

$$K_D = k_{off}/k_{on} = A^*\, exp\left(-\frac{\Delta E_{off} - \Delta E_{on}}{k_B T}\right) = A^*\, exp\left(-\frac{\Delta G}{k_B T}\right) \tag{3.5}$$

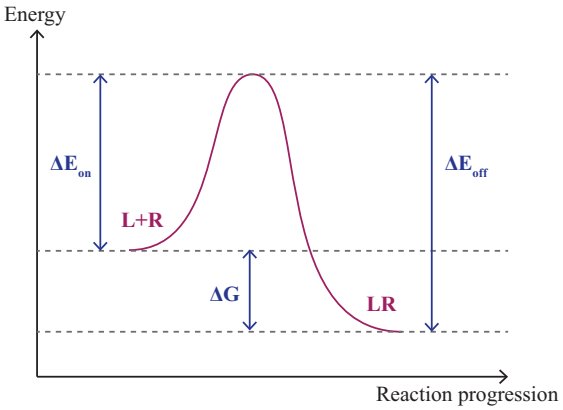


Figure 3.2: Arrhenius plot: The reaction constants for association and dissociation can be related to the activation energies for both processes and thus to the Gibbs free energy according to equations 3.4 and 3.5.

The rate of binding depends on two mechanisms: the rate of reaction, *i.e.* how fast the reaction between L and R occurs, and the diffusion of the ligand molecules in solution, *i.e.* the mass transport. One of these processes usually occurs much faster than the other, making one the limiting factor of the binding event. For this reason a system is either classified to be *reaction-limited* or *diffusion-limited* (mass transport-limited)[50].

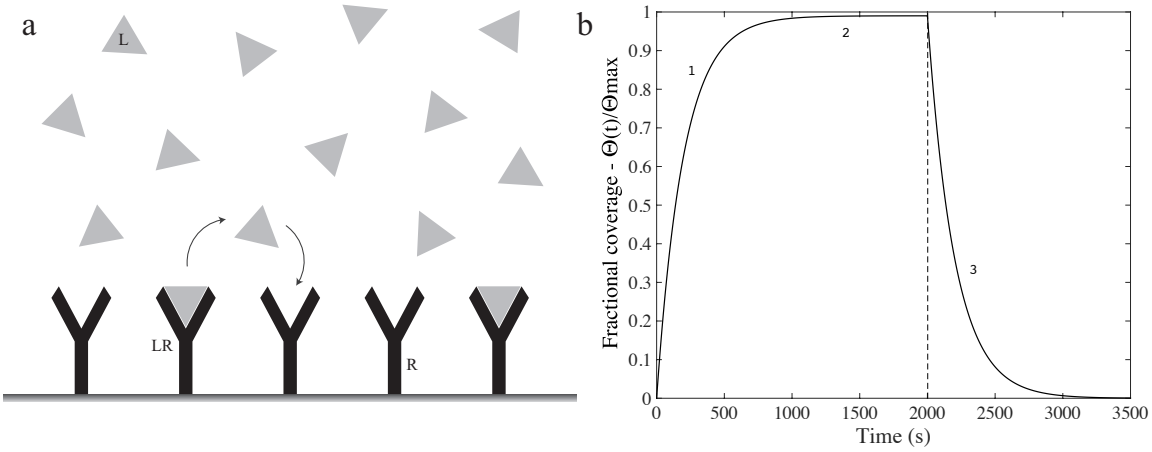


Figure 3.3: Illustration of the Langmuir model. a) Ligands in solution bind to and release from receptors immobilized on a surface. b) The fractional coverage expressed as a function of time can be divided into three parts: 1. Ligands binding to an empty surface 2. Equilibrium conditions (total rate of change is zero) 3. Exponential decay of bound ligands during rinsing step (no ligands in bulk solution).

A reaction-limited system is characterized by fast diffusion and reversible binding events, meaning that the ligand molecules constantly bind to and release from the surface. The number of ligands bound to a receptor is given by $\Theta(t)$ and the rate of change is written as:

$$\frac{d\Theta(t)}{dt} = k_{on}C\,(\Theta_{max} - \Theta(t)) - k_{off}\Theta(t) \tag{3.6}$$

C is the concentration of ligands in solution and Θ_{max} is the total number of receptors on the surface.

To solve this differential equation we consider two cases of different boundary conditions. The first case is *association* to an empty surface $\Theta(0) = 0$. The solution is then given by:

$$\Theta(t) = \frac{k_{on}C\Theta_{max}}{k_{on}C+k_{off}}\left[1 - exp\left(-\left[k_{on}C + k_{off}\right]t\right)\right] \quad (3.7)$$

This expression is known as the Langmuir isotherm. At $t \to \infty$ equilibrium is reached, meaning that the rates of binding and release are equal. Equation 3.7 then becomes after rearrangement:

$$\frac{\Theta_{eq}}{\Theta_{max}} = \frac{C}{C+K_D} \quad (3.8)$$

For *dissociation*, the boundary condition becomes $\Theta(0) = \Theta_{eq}$ and we have $C = 0$. The solution of equation 3.6 is then expressed as an exponential decay:

$$\Theta(t) = \Theta_{eq}exp\left(-k_{off}t\right) \quad (3.9)$$

The Langmuir model (figure 3.3b) does not take into account the depletion of ligand molecules close to the surface. If ligand molecules diffuse slowly, this depletion zone is larger and the system is diffusion limited. In that case, the number of bound ligand is given by the Ilkovic equation[50]:

$$\Theta(t) = 2C_0\sqrt{\frac{Dt}{\pi}} \quad (3.10)$$

C_0 is the concentration of ligands in the bulk solution and D is the diffusion coefficient, as defined in the next section.

It is worth recalling that the rate of change in equation 3.6 includes both on and off rates. Techniques based on ensemble averaging (like Surface Plasmon Resonance for example, described in chapter 4), cannot eliminate release events to study pure association to a sensor surface. With techniques based on single particle detection however, association and dissociation can be analyzed independently of each other. This is the case for Equilibrium Fluctuation Analysis, a method based on TIRF microscopy that we use in this work[51]. If we eliminate the negative term (release) in equation 3.6 and assume that $\Theta_{max} \gg \Theta(t)$, we get the association rate:

$$\frac{d\Theta(t)}{dt} = k_{on}C\Theta_{max} \quad (3.11)$$

This shows that the association rate is directly proportional to the association rate constant k_{on}, the concentration of ligands in solution C and the number of receptors on the surface Θ_{max}.

3.4 Diffusivity and Single Particle Tracking

When microscopic particles are suspended in a fluid, they move randomly in the solution. This movement is called *Brownian motion*, named after the botanist Robert Brown, who in 1827 observed pollen grains in water under a microscope and saw small particles randomly moving in the cavities of the pollen grain, filled with water. The underlying mechanism behind this motion was described 78 years later by Albert Einstein. He suggested that the observed random movement of suspended particles in solution is due to collisions with the surrounding molecules of the solution[52]. The latter ones are in constant movement due to their thermal energy of the order of $k_B T$. The diffusion coefficient for the particles is given by the Stokes-Einstein equation*:

$$D = \frac{k_B T}{6\pi\eta r} \tag{3.12}$$

where is η the dynamic viscosity of the medium and r the hydrodynamic radius of the particle. As can be seen from this equation, the diffusion coefficient of the particle is directly related to its size. Measuring the diffusion coefficients of particles undergoing Brownian motion thus makes it possible to calculate their size distribution. Several techniques take advantage of this principle to determine the size distribution of nanoparticles in solution. One example is Nanoparticle Tracking Analysis (NTA)[53].

In Single Particle Tracking (SPT) the trajectories of individual particles are analyzed to extract information about diffusivity and type of motional behavior. This technique is often used in combination with fluorescence microscopy and based on the fact that the position of single fluorescent objects can be determined with a localization precision in the nanometer range, by applying a Gaussian fit to their intensity profiles[54]. The same principle is used for tracking studies based on light scattering by individual particles. SPT has been very popular to study mobility of lipids and proteins in cell membranes, being an alternative to widely used ensemble-averaging techniques, like Fluorescence Recovery After Photobleaching (FRAP) for example†. A common approach in SPT analyses is to calculate the mean-squared displacement (MSD) of the tracked particles. The MSD is a measure of the deviation of the particle position in relation to a reference position over time. It is defined as:

$$MSD(\Delta t) = \langle (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \rangle \tag{3.13}$$

for $n = 1,2,3,\dots,N$. The particle located in (x_i, y_i) will be at position (x_{i+n}, y_{i+n}) after n frames. $\Delta t = n * movie\ time\ interval$ is called lag-time. N is an arbitrary number, but should in general be chosen smaller than ¼ of the total frame number, to avoid falsification of MSD values at high lag-times due to too few data points[55]. An illustration of how the MSD is determined for a particle trajectory is showed in figure 3.4a.

* The Stokes-Einstein equation is valid for fluids of low Reynolds number.

† In a FRAP experiment, a small region of a fluorescent sample is photobleached to measure the diffusion of molecules in and out of the bleached spot [83]–[85].

For a particle performing a random walk in a two-dimensional plane the MSD curve is linear:

$$MSD(\Delta t) = 4D\Delta t \tag{3.14}$$

We then talk about normal diffusion and the diffusion coefficient is estimated from a linear fit of the MSD curve. Equation 3.14 can be generalized to account for anomalous diffusion, the case when the normal diffusion of the particle is hindered:

$$MSD(\Delta t) = 4D\Delta t^{\alpha} \tag{3.15}$$

where $\alpha = 1$ for normal diffusion and $\alpha < 1$ for anomalous diffusion.

For confined diffusion we observe an asymptotic MSD curve:

$$MSD(\Delta t) = A_c[1 - C_1 \exp(-4C_2 D\Delta t / A_c)] \tag{3.16}$$

A_c is the area of the confinement and the constants C_1 and C_2 are given by the geometry of the confinement. Finally, the MSD for diffusion under directed motion is given by:

$$MSD(\Delta t) = v^2\Delta t^2 + 4D\Delta t \tag{3.17}$$

where v is the velocity of the directed motion. Figure 3.4b illustrates how the MSD curve is interpreted to classify the trajectories into the different types of diffusion.

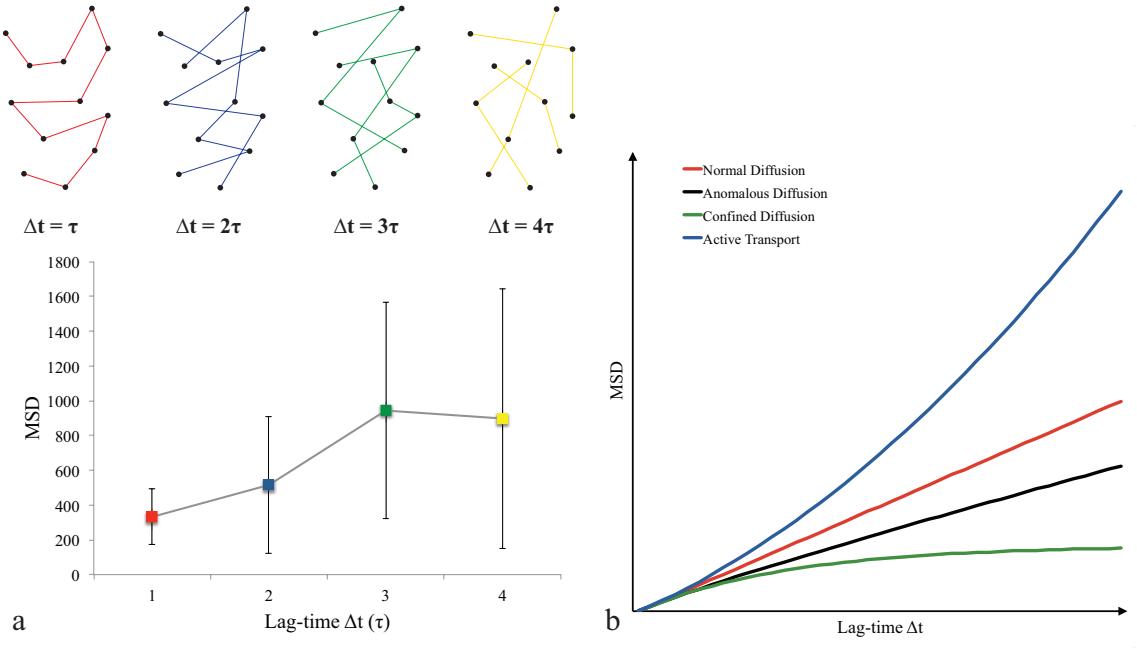


Figure 3.4: Mean squared displacement (MSD), inspired from[56]. a) Illustration of how an MSD curve is obtained for an arbitrary trajectory and $\Delta t = \tau, 2\tau, 3\tau, 4\tau$. The error-bars illustrate how the method suffers from big statistical errors at long lag times. b) Different modes of diffusion (normal diffusion, anomalous diffusion, confined diffusion and active transport) can be determined by the behavior of the MSD curve (see main text for corresponding equations).

4
Experimental Techniques

"I suppose it is tempting, if the only tool you have is a hammer, to treat everything as if it were a nail." – *Abraham Maslow*

Experimental assays for studying biomolecular interactions often rely on surface-sensitive techniques. The basic working principle of such a device is that a receptor molecule is immobilized on a sensor surface and a signal is obtained upon binding with a ligand from the bulk solution. The signal can be of optical, acoustical or chemical nature for example, depending on the type of technique that is used. Surface-sensitive techniques have been widely used to study cell membranes for example. Supported lipid bilayers, created by spontaneous rupture of lipid vesicles on a glass surface, make it possible to create a simplified version of the cell membrane and to study isolated biomolecular recognition events.

Two surface sensitive techniques, used in this work, are described here: Surface Plasmon Resonance (SPR) and Total Internal Reflection Fluorescence Microscopy (TIRFM). The first one is based on ensemble averaging, while the second one detects single binding events.

4.1 Surface Plasmon Resonance (SPR)

Surface Plasmon Resonance (SPR) is a widely used technique in biosensing applications. It was introduced in the early 80s when Liedberg et al. demonstrated its potential for gas detection and antibody adsorption[57]. Today SPR is a very popular technique to study protein-ligand interactions in particular in the context of drug development[58]. It allows the monitoring in real-time of the refractive index change caused by adsorbing molecules to a sensor surface, making it possible to extract information about binding kinetics and affinity of the studied biomolecular interaction.

SPR takes advantage of surface plasmons, which are collective oscillations of free electrons of a metal, arising at the interface between the metal and a dielectric medium when excited by light under certain conditions. The electromagnetic waves coupled to this oscillation are called surface plasmon polaritons. They propagate along the interface and generate an evanescent field decay on both sides of the interface[59]. The dispersion relation of this two-dimensional waves is given by[60]:

$$k_{sp} = \frac{\omega}{c}\left(\frac{1}{\epsilon} + \frac{1}{\epsilon_m}\right)^{-1/2} \tag{4.1}$$

where ω is the angular frequency, c the speed of light, ϵ_m the real part of the dielectric constant of the metal at the given frequency and ϵ the dielectric constant of the second medium. Given the nature of the two media, we have $\epsilon_m < 0$, $\epsilon > 0$ and $|\epsilon_m| \gg |\epsilon|$. Equation 4.1 can be simplified to:

$$k_{sp} = \frac{\omega}{c}\sqrt{\epsilon} = \frac{\omega}{c}n \tag{4.2}$$

with n being the refractive index of the dielectric medium. If we consider an incident light beam impinging under an angle θ on the interface, the parallel component of the wave vector is given by:

$$k_x = \frac{\omega}{c}\sqrt{\epsilon}\, sin\theta \tag{4.3}$$

Excitation of surface plasmons requires phase matching of the wave vectors k_{sp} and k_x[59]. As can be seen from equations 4.2 and 4.3, this is impossible since k_x is always smaller than k_{sp}. To obtain phase matching a different geometry has to be used. If we now consider a three-layer system instead, consisting of a thin metal film sandwiched between two insulation media of different dielectric constants ϵ_a and ϵ_g, equations 4.2 and 4.3 become:

$$k_{sp} = \frac{\omega}{c}\sqrt{\epsilon_a} = \frac{\omega}{c}n_a \tag{4.4}$$

$$k_x = \frac{\omega}{c}\sqrt{\epsilon_g}\, sin\theta = \frac{\omega}{c}n_g sin\theta \tag{4.5}$$

Thus making the solution $k_{sp} = k_x$ possible if $n_g > n_a$. In SPR, a so-called Kretschmann configuration[61] is used, where a glass prism is coated with a thin (~50 nm) metallic film, usually made of gold (figure 4.1). The light beam hits the interface of the metallic film with an angle higher than the critical angle of total internal reflection (see next section about TIRF Microscopy for the theory about total internal reflection). When resonance is achieved, the surface plasmons will be excited at the interface between the metal and the ambient medium (usually water). The evanescent field generated by the surface plasmon resonance along the z-axis penetrates the ambient medium by a couple of hundred nanometers, thus making SPR a surface sensitive technique.

The main working principle of SPR is that when molecules adsorb to the metal/water interface, the refractive index of the ambient medium n_a will change and resonance will occur at a different angle θ, according to equations 4.4 and 4.5:

$$k_{sp} = k_x \iff \theta = \arcsin\left(\frac{n_a}{n_g}\right) \tag{4.6}$$

SPR therefore senses small changes in refractive index due to molecular adsorption at the interface causing a shift of the angle at which resonance is obtained. The coverage of adsorbed mass can be related to the refractive index change and the difference in resonance angle using[62], [63]:

$$\Delta\Gamma = \frac{d\,\Delta n}{(dn/dC)} = \frac{d}{S(dn/dC)\,[1-\exp(-d/\delta)]}\Delta deg \qquad (4.7)$$

In this expression, d is the film thickness, S the sensitivity of the instrument expressed in degrees per refractive index unit, (dn/dC) the refractive index increment per biomolecule concentration in solution and δ the decay length of the intensity of the evanescent field.

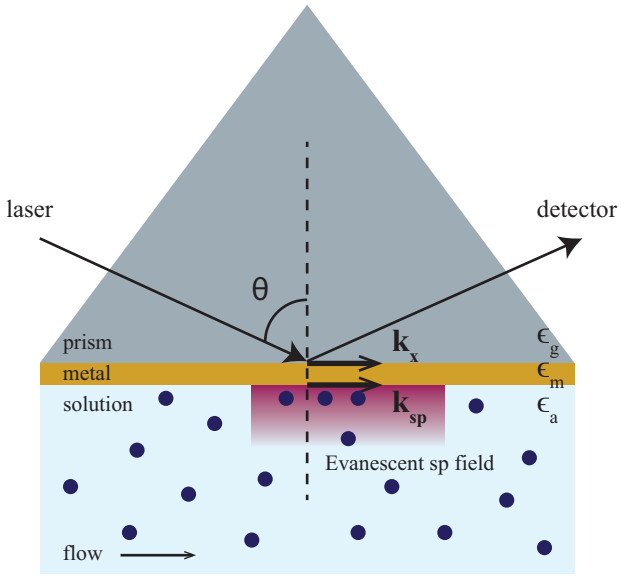


Figure 4.1: Working principle of SPR using the Kretschmann configuration. A thin metal film coated onto the backside of a glass prism is illuminated with a laser beam in total internal reflection. At a certain angle, surface plasmon resonance is achieved and an evanescent field is created at the metal/solution interface. A change in refractive index upon binding of molecules to the surface will result in a change of resonance angle.

4.2 Total Internal Reflection Fluorescence Microscopy

Total Internal Reflection Fluorescence Microscopy is a technique combining three key elements: Microscopy, Fluorescence and Total Internal Reflection. All of these methods had been widely used before Daniel Axelrod combined them to image cell structures in contact with a solid substrate[64] in the early 80s. TIRF microscopy has been used in a wide range of applications since then, many of them being of biological nature. The full theory behind TIRFM was first described in 1984 by Axelrod et al.[65]. The main working principle is that only a thin layer above the surface is illuminated thanks to the total internal reflection (TIR) setup. In that way, the fluorescent signal from out-of-plane particles is successfully suppressed. TIRFM can be seen as an extension to regular fluorescence microscopy, which means that the molecules to be imaged have to be fluorescently labeled beforehand.

Fluorescence is the emission of light by a molecule, called fluorophore, excited by light of a certain wavelength. The fluorophore, initially in the ground energy state S_0, will absorb the energy of the incoming photon to reach the next higher energy state S_1. The energy of the photon must correspond to the energy gap between the two states and typically lies in the visible light spectrum. S_1 being an unstable energy state, the molecule will return to the

ground state via a relaxation process. This relaxation is divided into a vibrational relaxation process and a radiative relaxation process, emitting a photon of lower energy (longer wavelength) than the excitation photon. Figure 4.2 illustrates this process with a Jablonski diagram. The typical lifetime of fluorescence, defined as the average time between excitation and return to the ground state, is around 10 ns[66].

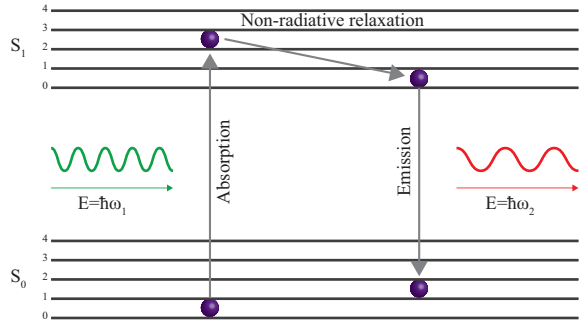


Figure 4.2: Jablonski Diagram illustrating the principle of fluorescence. A fluorophore in the ground state S_0 is excited to the higher energy state S_1 upon absorption of the energy of an incoming photon. During relaxation a photon of longer wavelength (lower energy) is emitted.

Fluorescence microscopes take advantage of the difference in excitation and emission wavelengths. The white light from the light source passes an excitation filter on the way to the sample that selects a range of wavelengths matching the excitation wavelength of a specific fluorophore. Before reaching the camera, the light passes a second filter, called emission filter, so that only the light emitted from the fluorescent molecules, and not the excitation light, is collected for the image (see figure 4.4).

The element that distinguishes TIRF from regular fluorescence microscopy is the total internal reflection (TIR) setup. To explain the principle of TIR we use geometrical optics. Snell's law describes how a light beam behaves when impinging on an interface of two media with different refractive indexes n_1 and n_2:

$$n_1 \sin\theta_1 = n_2 \sin\theta_2 \tag{4.8}$$

θ_1 and θ_2 are the angle of incidence and angle of refraction, respectively. This formula shows how the angle of refraction depends on the angle of incidence and the refractive indexes of the two media. If the second medium is of lower refractive index ($n_2 < n_1$), the refracted beam will travel along the interface of the two media at $\theta_1 = \theta_c$. This angle, called critical angle, is given by:

$$\theta_c = arcsin\left(\frac{n_2}{n_1}\right) \tag{4.9}$$

For angles above the critical angle ($\theta_1 > \theta_c$) the incident beam is totally reflected at the interface. This situation is called total internal reflection and represented schematically in figure 4.3.

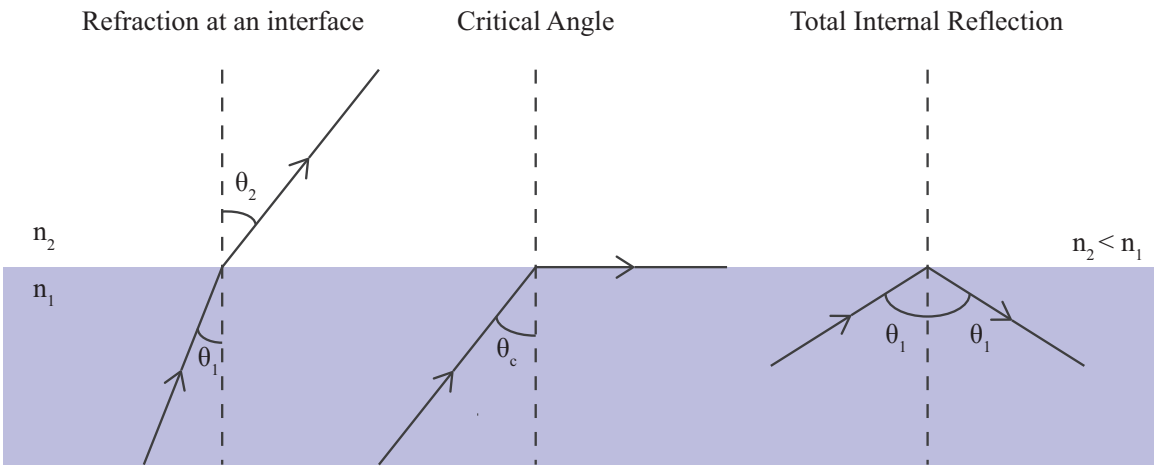


Figure 4.3: Illustration of Snell's law and the principle of total internal reflection. For incident angles higher than the so-called critical angle, the incident beam is totally reflected at the interface with a lower refractive index medium.

An evanescent field is then created at the interface, extending a small distance into the optically thinner medium. The intensity I of this evanescent field at a distance z from the interface is given by[67]:

$$I(z) = I_0 e^{-z/d} \tag{4.10}$$

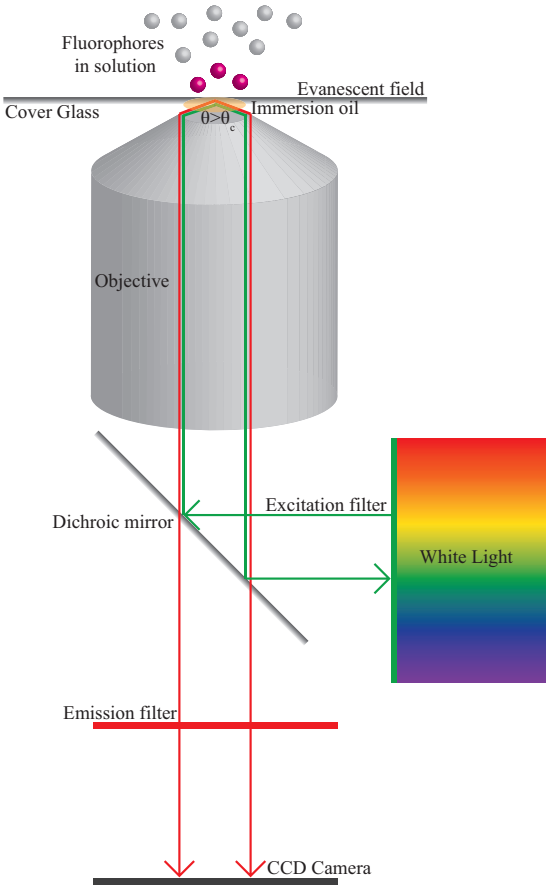
I_0 is the intensity at the interface and the characteristic exponential decay depth d is defined as:

$$d = \frac{\lambda}{4\pi n_2}\left(\frac{sin^2\theta_1}{sin^2\theta_c} - 1\right)^{-1/2} \tag{4.11}$$

with λ being the wavelength of the incident light.

The exponential decay depth d is usually on the order of the wavelength λ or smaller, meaning that only fluorescent molecules within a couple of hundred nanometers away from the surface will be excited by the incident light. Fluorophores in the background solution will not be taken into account. This principle is the reason that TIRFM is a surface sensitive technique very well suited for a wide range of surface-based assays. A schematic of the working principle of TIRF microscopy is shown in figure 4.4, together with a picture of a TIRF microscope.

a



b

Figure 4.4: TIRF Microscopy. a) Working principle including the three main key elements: Fluorescence, Optical Microscopy and Total Internal Reflection. b) Picture of a TIRF Microscope (source: www.nikon.com).

5
Results

"Experimentation is the least arrogant method of gaining knowledge. The experimenter humbly asks a question of nature." – ***Isaac Asimov***

The main question addressed in this thesis is how the herpes simplex virus interacts with glycosaminoglycans in its initial step of cell membrane attachment and during viral egress after infection. Attachment and release are two key steps in the replication cycle of the virus that are closely connected to each other. Strong attachment is needed for the virus to engage into cell entry, while this interaction needs to be overcome during release of newly produced virions. Understanding which mechanisms regulate this counterplay is an important step towards the development of anti-viral agents that inhibit HSV infections.

To address this question, an assay based on surface-immobilized glycosaminoglycans and total internal reflection fluorescence microscopy was developed. This assay is presented in the first section of this chapter. It is then applied to investigate two main aspects of the HSV-GAG interaction as further discussed below. The first one is the influence of the sulfation of the different GAGs on binding kinetics and virus mobility on the surface. The second aspect presented in this work is the role of the viral glycoproteins, and in particular their glycosylation, in regulating attachment and release of HSV.

5.1 Description of the assay

The first step in the development of the assay is the immobilization of glycosaminoglycans onto a sensor surface. The strategy used here takes advantage of the high affinity between biotin and streptavidin to immobilize the GAG chains in an end-on configuration[68], [69]. In that way a brush-like configuration that mimics the architecture of GAGs found close to the cell surface is created. The different components of the sensing platform are shown in figure 5.1. To immobilize the sugar chains, a supported lipid bilayer (SLB) is first formed on the sensor chip. A fraction of the lipids in the bilayer have a biotin group linked to their head groups, making it possible for streptavidin molecules to attach to the SLB. Finally end-biotinylated GAG chains bind to the streptavidin layer.

The GAGs used in this work were hyaluronic acid (HA), sulfated hyaluronic acid (sHA), chondroitin sulfate (CS) and heparan sulfate (HS). HA is the only GAG that is not sulfated and was therefore used as a negative control for the HSV binding to sulfated GAGs. CS and HS are native GAGs, presenting a high degree of heterogeneity in their sulfation patterns. As discussed in chapter 2, these patterns are believed to have an importance in regulating the binding of viruses to GAGs. To study this importance, we used sHA as comparison. This GAG originates from HA chains that were artificially sulfated. It therefore presents a more homogeneous distribution of sulfate groups that is very different from the enzymatically driven sulfation of CS and HS.

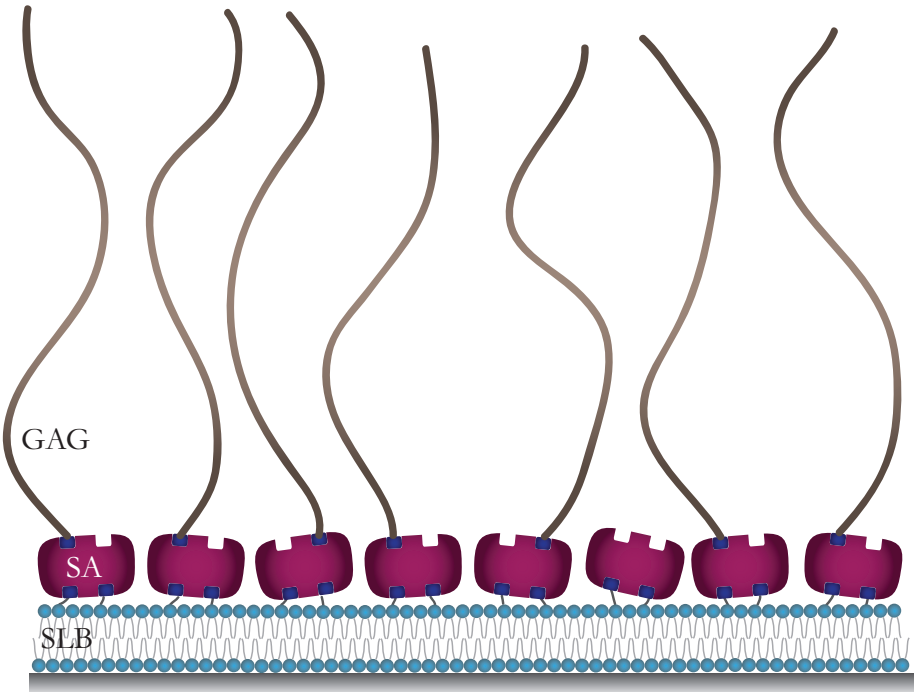


Figure 5.1: End-on immobilization of glycosaminoglycan chains onto a sensor surface via biotin/streptavidin to create a brush-like architecture. The streptavidin molecules are bound to a supported lipid bilayer containing a fraction of biotinylated lipids.

This surface functionalization could be monitored in real-time using surface plasmon resonance (SPR). This was done in **paper I** and served as method to determine surface densities of the GAG chains. The highest chain density was obtained for HS; CS and sHA yielded ~14% and ~24% of HS coverage, respectively. These findings were consistent with previously reported results[68], [69].

To be able to observe single HSV particles binding to the GAG adlayer in TIRF microscopy, the virus was fluorescently labeled. For this we used a dye that inserts into the lipid envelope of the virus. In that way, and thanks to the surface sensitivity of TIRF microscopy, single HSV particles binding to the GAG surface could be detected and effectively discriminated from the ones in solution. To study the kinetics of the interaction, we analyzed the recorded movies in terms of association of HSV particles to the surface according to equation 3.11 and dissociation from the surface according to equation 3.9. In addition, we studied the mobility of the viruses with means of single particle tracking in **paper I**.

5.2 Influence of the sulfation of glycosaminoglycans

The first aspect of the HSV-GAG interaction studied with the GAG platform was the influence of sulfation of the GAG chains on HSV binding (**paper I**). HSV is known to bind to HS and CS, which are both sulfated GAGs. The sulfation of the GAG chain therefore appears to be a requirement for HSV binding and the degree of sulfation, i.e. the number of sulfation groups present on the GAG chain, is likely to influence the binding strength. A

second hypothesis is that, in addition to the degree of sulfation, the distribution of the sulfate groups on the GAG chain could play a role. To verify these hypotheses, HSV binding to CS, HS and sHA was studied and compared. The main difference between these molecules is their degree of sulfation, which is approximately three times higher for sHA in comparison to CS and HS, and the distribution of the sulfate groups in patterns (CS and HS) or in a homogeneous fashion (sHA).

The recorded movies were first analyzed in terms of the number of particles associating to the surface over time and the number of particles dissociating from the surface over time. The first observation of this study is that HSV indeed binds to sulfated CS, HS and sHA, but not to non-sulfated HA, since more than 10 times less virus particles associated to the HS surface in comparison to sHA. Analysis of the kinetics revealed that association rates of HSV are in a similar range for all three GAGs with somewhat more particles binding to CS. According to equation 3.11, the binding rate is directly proportional to k_{on} and the number of receptors on the surface. Taking into account the higher chain density for sHA in comparison to CS (~2 fold), this result indicates that k_{on} is higher for CS, despite the higher degree of sulfation on sHA (~3 fold). This result supports the above stated hypothesis that not only the degree of sulfation of the GAG chain, but also its type of sulfation influences the affinity of the HSV interaction.

Analysis of the dissociation of particles revealed that only very few particles leave the surface. Indeed, less than 0.5% of the bound HSV particles were observed to dissociate from the surface, for all GAG surfaces. This is in agreement with the idea that quasi-simultaneous breakage of all bonds, is of small likelihood for such a multivalent interaction. Furthermore, this observation indicates that HSV particles get trapped on the GAG chains, making these molecules very efficient attachment factors for viruses.

It appeared from the recorded TIRF movies that a fraction of the viruses exhibits lateral mobility on the GAG adlayer. Single particle tracking (SPT) was performed to determine the diffusion coefficients of the moving particles, as described earlier in chapter 3. This SPT analysis revealed that the tracked particles generally show higher diffusion coefficients on native CS and HS in comparison to sHA. Furthermore, mobility studies on low-density sHA surfaces (chain density reduced to approximately 50%), showed somewhat faster diffusion in comparison to high-density sHA. Altogether, these observations indicate that the HSV mobility is influenced both by the surface density of sulfate groups and the type of sulfation of the GAG chain.

In conclusion, **paper I** showed that the binding affinity of HSV to surface immobilized GAGs is influenced by the type and degree of sulfation of the GAG chains. More precisely, the results suggest that the sulfation patterns on native GAG chains play a role in promoting and modulating the binding behavior of HSV. In addition, lateral mobility of single HSV particles was observed on all GAG surfaces. This stochastic "wobbling" movement was thought to be due to the gradual exchange of bonds between the virus and the GAGs, and appeared to be generally faster on the less sulfated GAGs. We believe that this type of movement is likely to occur *in vivo* during cell infection, when a virus is first recruited to the cell membrane to then move along the membrane until firmly attaching to it.

5.3 Role of the glycosylation of the viral glycoproteins

In **paper II** and **III** the role of the glycosylation of the viral glycoproteins on HSV binding was investigated. As discussed in chapter 2, so-called mucin-like regions have been found on certain glycoproteins. These highly glycosylated regions have been shown to play an important role in evasion of the virus from the host's immune system[70]–[72]. The idea that mucin-like regions could also play an essential role in HSV-GAG interactions emerged from experiments based on serial HSV infection cycles of cultured cells in the presence of the GAG mimetic muparfostat (PI-88), serving as inhibitor of the infection. Both HSV-1 and HSV-2 selected virus mutants that were resistant to inhibition after several passages. Interestingly, in both cases, the mutation affected the mucin-carrying glycoprotein of the virus. For the HSV-1 mutants the mucin-like domain of the gC glycoproteins was deleted[73], while the HSV-2 mutants were completely lacking the mucin-carrying gG glycoproteins[74]. These findings suggest that the mutants interact differently with GAGs as compared to the wild types and that the mucin-like domains play a role in the HSV-GAG interaction.

Paper II discusses the role of the mucin-like region on the gC glycoprotein of HSV-1. The binding kinetics of purified gC glycoproteins from HSV-1 mutants (gCΔmuc) to surface immobilized sHA and CS in comparison to native gC were studied using SPR (**paper II**). The main conclusions of these studies were that gCΔmuc had less of a propensity to bind to the GAG layer in comparison to native gC but that once bound, the gCΔmuc–GAG complex was more stable compared to gC. To further investigate the role of the mucin-like region on gC we studied the association of HSV-1 mutants (KOS-gCΔmuc) to immobilized CS and compared the results to the association of native HSV-1 (KOS). As expected, the mutant HSV-1 particles associated less to the GAG surface in comparison to the wild-type virus (~15% of the KOS association). These findings demonstrate that the mucin-like region on gC-1 modulates the attachment and release of HSV-1 to GAGs.

In **paper III** the case of HSV-2 is discussed. While for HSV-1 the selective deletion occurred on the main attachment glycoprotein (gC-1) of the virus, the mutation of HSV-2 concerned a glycoprotein formerly not known to be involved in virus attachment (gG). In addition, the HSV-2 mutants were lacking the entire gG glycoprotein, contrarily to HSV-1 that selected mutants with truncated gC's. Cell culture experiments showed that gG deficient HSV-2 mutants were able to infect cells but yielded a ~200 fold decreased number of viruses in the cell culture medium in comparison to the HSV-2 wild type. This observation was due to HSV-2 mutants getting trapped on the surface of infected cells, as confirmed by liberation of particles to wild type level by GAG mimetic muparfostat. To complement these findings we used TIRF microscopy to study HSV-2 mutant association and dissociation to surface immobilized CS chains. The gG deficient HSV-2 particles demonstrated better association to the GAG layer but poorer dissociation in comparison to the wild type strain. Taken together, these observations suggest that gG balances the virus interaction with GAGs by acting like a shield that reduces the accessibility to the binding proteins gC-2 and gB-2 and thereby preventing trapping of the virus on the surface of infected cells.

Both **paper II** and **III** demonstrate the role of mucin-like regions of viral glycoproteins in mediating HSV interactions with GAGs. These highly glycosylated regions form extended

structures, frequently containing negatively charged sialic acid residues. We therefore believe that steric hindrance and electrostatic repulsion with the negatively charged sulfate groups on the GAG chains contribute to reversibility of the HSV-GAG interaction, preventing virus trapping on the cell surface. The mechanisms used by the two serotypes however appeared to differ from each other. The location of the mucin-like region was very different, since it was found on the main attachment glycoprotein for HSV-1, but on a separate glycoprotein for HSV-2. This is probably the reason why the deletion of the mucin-like compound (or the entire glycoprotein carrying this compound in the case of HSV-2) resulted in lower association rates for HSV-1 mutants, but higher association rates for HSV-2. For both virus types however, the mucin-like region appeared to play a crucial role in virus release from the surface of infected cells, since its absence reduced the ability of the virus (**paper III**) or the purified glycoproteins (**paper II**) to dissociate from the GAG surface.

6
Outlook

"Science never solves a problem without creating ten more." – ***George Bernard Shaw***

How does the herpes simplex virus interact with the cell membrane during the initial step of virus attachment? And how does it release again from the membrane after cell infection? These were the two main questions addressed in this thesis. In an attempt to answer these questions we described an assay based on the surface immobilization of glycosaminoglycans, the main attachment factors of HSV, which allows for probing the interactions of single HSV particles to the GAG chains. The results, described in the previous chapter, showed that the two aspects we studied, namely the sulfation of the GAG chains and the glycosylation of the viral glycoproteins, both strongly influenced the HSV-GAG interaction.

This chapter discusses different methods that can be used to help further understanding the mechanisms used by the herpes simplex virus to bind to the cell membrane. The first section describes a method based on native cell membranes that has already led to promising preliminary results and the second section gathers different ideas for future directions, all aiming at answering the two fundamental questions stated above.

6.1 Supported native membranes

The assay described in this work was based on a model system that aimed at mimicking the brush-like architecture of glycosaminoglycans close to the cell surface and in the extracellular matrix. This system has the advantage of isolating the type of interaction to be studied (HSV-GAG interaction in this case), while allowing for modification of a series of different parameters (receptor density and degree of sulfation for example). When occurring in nature, during the infection of a cell, the interaction of HSV with the cell surface is however more complex than in our model system and involves a series of attachment factors, cell membrane receptors and viral glycoproteins. To include this complexity into our study, it is very valuable, in addition to our model system, to probe HSV binding in a more native-like environment, taking all ligands involved into account.

Recent work has demonstrated the possibility of extracting native membrane material from cells and incorporating this material into supported-lipid bilayers (SLBs): Pace et al. for example described a method for SLB formation from native membrane vesicles (NMVs), obtained via mechanical cell-lysis[75]. This method takes advantage of synthetic vesicles to facilitate the formation of the SLB on a glass substrate. A polymer cushion is used with the aim of "lifting-up" the membrane from the substrate and preserving membrane protein mobility.

Forming a supported lipid bilayer that contains native cell membrane material is advantageous for several reasons. First of all, it allows for more native-like interaction studies

that are compatible with surface-based techniques like total internal reflection fluorescence (TIRF) microscopy, used in this thesis. Second, it includes all the cell membrane components without the complications of working with whole cells.

Motivated by the promises of such a system, we developed a platform (figure 6.1a) based on the formation of supported native membranes extracted from *green monkey kidney* (GMK) cells, a cell line that is commonly used for HSV infection studies[76]. The final goal is to use this platform as a complement to our model system (the GAG platform) to probe the binding of single HSV particles to the cell membrane. The GMK cells were mechanically lysed to form native membrane vesicles, which were then sonicated together with synthetic vesicles to form hybrid vesicles, as described by Pace et al.[75]. This sonication step is performed to assure mixing of the two vesicle species, crucial for SLB formation. Successful SLB formation of the hybrid vesicles was confirmed by observing, with TIRF microscopy, the rupture of a fluorescently labeled fraction of the vesicles.

As a first application, we used this platform to probe the inhibition of the HSV-1 virus with heparin, a GAG that binds to the viral glycolipids, thereby hindering the interaction of the virus with the membrane. Figure 6.1b shows a dose-response curve of this inhibition test. This curve was obtained by plotting the association rates of HSV-1 particles to the native bilayer against the concentration of heparin. The characteristic sigmoidal shape of this curve[77] demonstrates the successful inhibition of HSV-1 binding with heparin. As a continuation of this project, we plan to use this platform to test different viral inhibitors as drug candidates, and to study the contribution to binding kinetics of the different biomolecules involved in the binding of the virus to the cell membrane. In addition, different cell lines, that are for example negative in HSV attachment factors, will be considered for these studies. Furthermore, this platform could potentially be used to study fusion of the viral envelope to the cell membrane under acidic conditions for example, similar to a reported study of pH dependent fusion of influenza to synthetic SLBs[78].

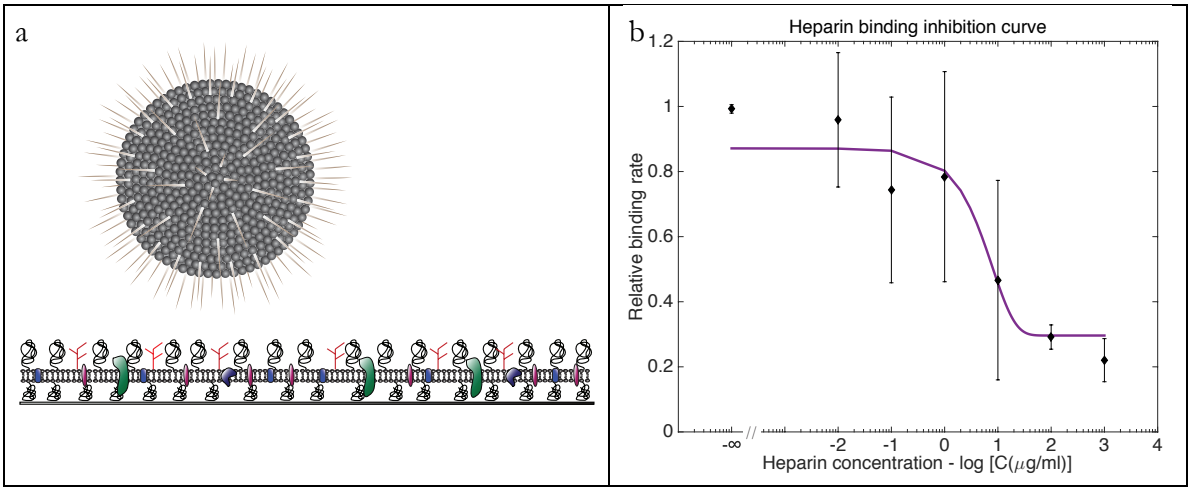


Figure 6.1: a) Schematic of HSV binding to a supported native membrane. b) Heparin inhibition curve showing the association rate of HSV-1 to the native SLBs for increasing heparin concentrations. Association rates were obtained from the slopes of the cumulative association plots (equation 3.11).

6.2 Other possible future directions

Many aspects discussed in this thesis and in the appended papers would be interesting to study further. One of them is the role of glycoprotein gG-2 in the attachment and release of HSV-2 to and from the cell membrane. As discussed in **paper III** and in the previous chapter, gG-2 was found to play a major role in mediating the release of HSV-2 virions from the surface of infected cells. This finding was unexpected, since gG-2 is not one of the known attachment proteins of HSV-2 (which are gB-2 and gC-2). To clarify the roles of the individual glycoproteins of HSV-2, it would be interesting to purify the proteins and perform binding studies to immobilized GAGs in SPR for example. In a similar way to the SPR studies on purified HSV-1 glycoproteins in **paper II**, these studies would make it possible to measure the affinity of the glycoproteins to the GAG chains and to determine if gG-2 is a GAG-binding glycoprotein.

Another possible direction of my research work could be to further study the binding kinetics of the HSV-GAG interaction. As discussed in this thesis, viruses are known to interact with cell membrane receptors and attachment factors in a multivalent manner. HSV is therefore expected to bind to multiple GAGs simultaneously. To be able to fully describe and understand this binding mechanism, it would be of great interest to be able to measure the number of GAG-bonds the virus forms, as well as their individual strengths. A technique that could be of use in this quest is atomic force microscopy (AFM). AFM was introduced in the 80s to image surfaces with atomic resolution[79] and has today become an attractive tool to study protein-ligand interactions[80]. This is partially due to the possibility of functionalizing the AFM tip with a ligand to measure interaction forces between the ligand and a receptor[81]. In such a force-induced pulling experiment the AFM tip is first approached to the receptor until ligand and receptor bind. The tip is then retracted from the surface to measure the force required to break the bond and generating a so-called force-distance (F-D) curve[80]. From this curve, and from theoretical modeling, one can then extract the energy barrier for rupture (ΔE_{off}) and the dissociation rate constant k_{off}[82], according to equation 3.5. Furthermore, in case of multivalent interactions, this method allows for determination of the number of bonds and the dissociation rate constants k_{off} of each individual bond. By attaching a single HSV particle to the AFM tip, one could therefore probe the interaction strength of the virus with the GAG surface and determine the number of formed bonds.

7
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Acknowledgements

It is time to say *thank you* to all the people that have helped me reaching this important step on the road to the PhD.

First of all I would like to express my gratitude to *Marta Bally*, my supervisor. Thank you for accepting me as your first PhD Student. Your presence (both in person and on Skype) is of enormous help in my work. Thank you for your encouraging words after a failed experiment and for always motivating me to give my best. I would also like to thank *Fredrik Höök*, my examiner, for wise words and interesting discussions.

Many thanks go to my collaborators from Sahlgrenska: *Tomas Bergström* and *Edward Trybala* for interesting scientific discussions, and *Maria Johansson* for preparing all the material I use in my experiments and for teaching me how to handle viruses without endangering myself and others.

I would also like to acknowledge all co-authors and collaborators that contributed to my publications and ongoing work. Thank you to *Noomi*, for the good times inside and outside the lab and for answering all my questions about glycosaminoglycans, *Stephan*, the single particle tracking genius, *Hudson*, the native membrane expert and *Eneas*, my master student.

Many thanks go to the current and past members of the *Biological Physics Group* for making this a very enjoyable workplace. I am very happy to be part of such a great group of friendly, smart and helpful people. Thank you for interesting and unexpected conversation topics during lunch and fika breaks that truly refresh your mind after extensive thesis writing. For the group trips and the afterworks, and for not just being colleagues but friends.

A special thank you goes to *Olov*, for helping me with basically everything that concerns this PhD journey. For trying to teach me the Swedish language and culture and of course for proofreading this thesis! Thank you also to *Nagma* for the tip with the filter columns and *Mokhtar* for the help with Matlab that made all of this possible!

Thanks to my current and past office-mates for promoting both fun times and productivity.

I would like to express my gratitude to my parents for their support and for teaching me the importance of education. I appreciate that you started liking my new home as much as I do and that you realized that it is not actually that far away! Thank you also to my sisters and my friends from back home, I wish I could spend more time with you. Danke für alles! Merci pour tout!

Finally, "ett stort tack" to my friends in Sweden for all the good times, and to *Stefan* for great support and for always believing in me.