THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROBING BIOMOLECULAR RECOGNITION AT THE SINGLE MOLECULE LEVEL

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Cover picture

Schematic illustration of DNA-modified- and cell membrane-derived liposomes interacting with a DNA- and protein-modified surface in the presence of evanescent-field illumination.

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Abstract

Specific recognition between biomolecular partners contributes to a multitude of biochemical signaling within and between cells. The aim of this work has been to investigate the possibility to probe biomolecular recognition reactions at the smallest possible scale, the single molecule level. The ability to do so provides unique possibilities to understand and acquire information of biological systems utilizing lower analyte concentrations and/or obtain information hidden in ensemble measurements.

By exploring several of the unique properties of liposomes, we have in this work developed assay formats capable of analyzing individual molecular interactions. Using dye-labeled liposomes as optical signal enhancers in a total internal reflection fluorescence microscopy (TIRFM) setup, we managed to detect sequence-specific hybridization of individual DNA molecules with fM sensitivity. In doing so, we demonstrated that with single molecule resolution and surface chemistries that minimize unspecific binding, the sensitivity is no longer limited by signal-to-noise but the actual surface coverage on the sensor. In addition, by monitoring the residence time of thousands of individual recognition events in parallel, kinetic information could be acquired from coverage fluctuations at stagnant liquid conditions. In this way the need for controlled liquid handling upon addition and removal of analyte-containing solutions is eliminated, which not only offers simplicity (i.e. no liquid-flow system is required), but also enable compatibility with high-throughput formats, such as microtiter plates. Taking further advantage of the fact that liposomes are compatible with the important class of cell membrane-residing proteins, we extended the assay to investigate binding kinetics of ligand interactions with membrane receptors. Monitoring individual binding events also provides possibilities for parallel detection of multiple interaction partners. In exploring this aspect, we have taken the first steps in the development of a new means to perform multiplexed biorecognition analysis using imaging mass spectrometry. In this case, liposomes were, in addition to signal enhancers, also used as target-specific chemical fingerprints for multiplexed readout.

Finally, future and ongoing extensions of the liposome-based assay are presented, illustrating two clinically relevant applications, screening for virus detection and mass spectrometry-based immunohistochemistry.

Keywords. *single molecule, molecular recognition, liposome, cell membrane, DNA, membrane receptor, multiplexing, barcode, total internal reflection, TIRFM, time-of-flight secondary ion mass spectrometry, TOF-SIMS*

List of appended papers

The thesis is based on the work contained in the following articles.

I. Single-molecule Detection and Mismatch Discrimination of Unlabeled DNA Targets
Gunnarsson, A., Jönsson, P., Marie, R., Tegenfeldt, J. and Höök, F.
Nano Letters, 8 (1), 183-188, ***(2008)***

II. Kinetic and Thermodynamic Characterization of Single Mismatch Discrimination using Single-molecule Imaging
Gunnarsson, A., Jönsson P., Zhdanov, V. and Höök, F.
Nucleic Acids Research 37 (14), e99 ***(2009)***

III. Kinetics of Ligand binding to Membrane Receptors from Equilibrium Fluctuation Analysis of Single Binding Events
Gunnarsson, A., Dexlin, L., Wallin, P., Svedhem, S., Jönsson, P., Wingren, C. and Höök, F.
Submitted to Angewandte Chemie Int. Ed.

IV. Spatial-Resolution Limits in Mass Spectrometry Imaging of Supported Lipid Bilayers and Individual Lipid Vesicles
Gunnarsson, A., Kollmer, F., Sohn, S., Höök, F. and Sjövall, P.
Analytical Chemistry, 82 (6), 2426–2433 ***(2010)***

V. Liposome-Based Chemical Barcodes for Single Molecule DNA Detection Using Imaging Mass Spectrometry
Gunnarsson, A., Sjövall, P. and Höök, F.
Nano Letters, 10 (2), 732–737, ***(2010)***

Publications not included in this thesis

i. **Dynamic Microcompartmentalization of Giant Unilamellar Vesicles by Sol gel transition and Temperature induced shrinking/swelling of poly(N-isopropyl acrylamide)**
Markström, M., Gunnarsson, A., Owe Orwar and Aldo Jesorka
Soft Matter 3 (5), 587-595, ***(2007)***

ii. **Supported Lipid Bilayers, Tethered Lipid Vesicles and Vesicle Fusion investigated using Gravimetric, Plasmonic and Microscopy Techniques**
Höök, F., Stengel, G., Dahlin, A., Gunnarsson, A., Jonsson, M., Jönsson, P., Reimhult, E., Simonsson, L., and Svedhem, S.
Biointerphases, 3 (2), FA108-FA116, ***(2008)***

iii. **Simulation of Dissociation of DNA Duplexes Attached to the Surface**
Zhdanov, V., Gunnarsson, A., Höök, F.,
Central Eur J. of Physics, 8 (6), 883-892 ***(2010)***

iv. **Molecular Motors on Lipid Bilayers and Silicon Dioxide: different Driving Forces for Adsorption**
Albet-Torres, N., Gunnarsson, A., Persson, M., Balaz, M., Höök, F. and Månsson, A.
Soft Matter, 6 (14), 3211-3219 ***(2010)***

v. **Accumulation and Separation of Membrane-Bound Proteins using Hydrodynamic Forces**
Jönsson, P., Gunnarsson, A. and Höök, F.
Anal. Chem., 83 (2), 604-611 ***(2011)***

vi. **Formation and Applications of Planar Supported Lipid Bilayers derived from Cell Membranes**
Simonsson, L.†, Gunnarsson, A. †, Wallin, P., Jönsson, P. and Höök, F.
† authors contributed equally
Submitted

Patent applications

i. **Method of fabricating lipid bilayer membranes on solid supports**
Gunnarsson, A., Simonsson, L., Jönsson, P., Reimhult, E., Roca, M. and Höök, F.
filed to Patent och registreringsverket

Contribution to the appended papers

I. I designed, optimized and performed the experimental work. I performed the data analysis in collaboration with P. Jönsson who made the image analysis software. I wrote the main part of the paper.

II. I designed, optimized and performed the experimental work. I performed the data analysis in collaboration with P. Jönsson. V. Zhdanov made the Monte Carlo simulations. I wrote the main part of the paper.

III. I designed optimized and performed the experimental work. L. Dexlin assisted me with the cell culture and the FACS analysis. P. Jönsson aided me with the data analysis. I wrote the main part of the paper.

IV. I designed, optimized and performed the experimental work together with P. Sjövall. I wrote the main part of the paper.

V. I designed, optimized and performed the experimental work together with P. Sjövall. I wrote the main part of the paper.

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"Nature, to be commanded, must be obeyed"

Francis Bacon

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Introduction

Interactions between biomolecules are essential for all reactions occurring in the living cell, such as DNA replication, protein synthesis and signal transduction. Every day, every second, in every cell of the human body, millions of biomolecular interactions occur simultaneously. One example is the hybridization of two single-stranded sequences of DNA that form the famous double helix structure discovered by Watson and Crick in the 1950's[1]. Other examples are protein-protein and ligand-receptor interactions that occur on the surface of the cell membrane, resulting in signaling cascades that can alter the function or even the fate of the cell. Molecular interactions also play a key role in diseases, such as aggregation of proteins and peptides in the brain, causing illnesses such as Alzheimer's and Parkinson's disease. It is thus clear that the ability to study bimolecular interactions is an essential part of understanding life as well as future development to improve it, primarily through drug discovery and biomedical diagnostics. In response to this insight, the last decades has witnessed a rapid development of novel techniques that enable sensitive analysis of biomolecular recognition. These techniques have, in turn, provided an essential platform for the entire biotechnology revolution. However, although rapid progress has been made, several challenges still remain.

One such challenge is related to the urgent need for simultaneous analysis of multiple biomolecules in so-called multiplexed assays. The need for multiplexing in dealing with complex systems, such as the living cell, becomes obvious from a glance at the human genome which contains approximately three billion DNA base pairs that code for roughly 25 000 different proteins, not including post-translational modifications. Whether the purpose of a multiplexed analysis is related to large scale genomic or proteomic investigations or drug screening, the principle need is similar. For example, functionality in the cell typically involves hundreds of genes that need to be analysed in parallel through measurements of RNA expression levels. Similarly, monitoring changes in protein levels as a means of early disease diagnostics often requires analysis of a number of proteins in order to acquire reliable results.

To further grasp the enormous challenge of analysing proteomes, one must consider the large dynamic range in protein expression levels. With relative abundances that differ more than ten orders of magnitude (mg/ml-fg/ml or μM-aM)[2], detection of disease-specific biomarkers, which are typically low abundant (<nM), rapidly becomes a search for a needle in a haystack. In many cases the absolute abundance of these biomarkers in blood and tissue are even below the detection limit of current methods. Hence, improvements in early disease prevention will require new detection principles with sensitivities in the fM-aM concentration range. The challenge to obtain improved sensitivity is however far from trivial. In particular, the reversible nature of most biomolecular interactions imply that in equilibrium, only a certain fraction of the total number of molecules in a solution will be bound to its counterpart at every instant in time. For a typical antibody-antigen interaction, which is considered to be a relatively strong biomolecular interaction, this means that only one molecule in a million will be bound at low fM concentrations. Additional factors such as specificity, i.e. the ability to *only* detect the molecule of interest, must also be taken into consideration since bioanalytical tools often rely on the inherent specificity of biomolecules created by nature, the prime examples being single stranded DNA or monoclonal antibodies. Specificity also depends on a multitude of external parameters such as ionic strength, temperature etc, which influence, for example the electrostatic interaction potential between biomolecules. Hence, small differences in biomolecular interactions that cause similar, but not identical, binding patterns can often be masked by other effects such as relative abundance.

An additional challenge related to studies of biomolecular recognition is the limited number of techniques available for investigating the sub-class of proteins associated with the cell membranes. This class represents approximately one third of all proteins identified in the human genome[3] but represent over two-thirds of all drug targets[4]. As a consequence, large scale multiplexing capacity is a prerequisite also in this case since pharmaceutical compound libraries used in early phases of drug screening often contain millions of small molecules[5]. The prime reason for the slow progress with respect to analysis of drug/ligand dependent membrane protein function in comparison with similar studies of water-soluble proteins stems from the hydrophobic nature of membrane-associated biomolecules. This property makes them incompatible with techniques that are appropriate for water soluble molecules, since an aqueous environment causes the hydrophobic nature of the membrane proteins to misfold and, as a consequence, potentially lose function. Furthermore, lack of solubility often results in problems with protein aggregation. Methods to circumvent this problem typically rely on extraction of membrane proteins from the cell membrane using detergents which allow them to be separated and purified by, for example conventional chromatography. Another strategy is to genetically modify the protein to express only the water-soluble part of the protein. However these strategies are cumbersome, inefficient and may still damage or alter the structure and function of the molecules. In addition, recent observations suggest that preserving the natural cell membrane environment with its large flavour of constituents is essential for proper protein function[6].

In the work described in this thesis, we have made an attempt to address the three challenges described above by developing a new surface-based detection assay. In doing so, we have focused on means to probe biomolecular recognition at the level of

individual binding events. This was accomplished by using liposomes as target-specific, nanometer-sized optical and chemical enhancer entities. Utilizing the liposomes as carriers for various recognition elements (DNA or membrane proteins), we have explored several potential advantages with such a system, including enhanced signal-to-noise ratios, resistance against photobleaching, compatibility with membrane proteins and their ability to encode chemical information for multiplexed analysis.

In brief the five appended papers in this thesis describe the development of a surface-based sensor format aiming at probing biomolecular recognition at the level of single molecules. The work can be divided into three main objectives.

I. The first objective (paper I and II) was the development of a surface-based sensing platform for biomolecular recognition with single molecule sensitivity. Using this platform we investigated the fundamental challenges and potential benefits of such an assay with focus on the kinetics of biorecognition.

II. The second objective (paper III) was to utilize an inherent advantage of the sensing platform in order to probe biomolecular interactions between water-soluble proteins and proteins embedded in the cell membrane. Also for this objective, efforts were emphasized on exploring the kinetics of biorecognition.

III. The third and final objective (paper IV and V) was to transfer the sensing format into a multiplexed assay with the ability of simultaneous and parallel biorecognition analysis. As one of the first examples of multiplexed analysis at the single molecule level, the work may potentially also contribute to the development of miniaturized sensor platforms with ultra high density ($>10^6$ probes/mm^2).

"He who only understands chemistry does not understand that either"

Georg C. Lichtenberg

2

Biomolecular interactions

The living cell is often called the building block of life. In order to sustain life; thousands of chemical reactions occur simultaneously in the cellular machinery. Cells can respond and adapt to their surrounding, grow and proliferate or even initiate self-destruction through apoptosis. All of these functions are regulated by various biomolecular interactions. In the nucleus, DNA replication and transcription is tightly controlled by transcription factors that either activate or suppress gene expression. In the cytosol, protein-protein interactions between enzymes such as kinases enable signal transduction and formation of protein complexes with specific functions. At the cell surface, hormones interact with cell membrane receptors, thus triggering signaling pathways that can alter the function of cells. This chapter provides an overview of the basic concepts and theory that govern intermolecular interactions between biomolecules.

2.1 Recognition, affinity and specificity

Within the living cell, interactions can occur between a variety of different biomolecules such as DNA-DNA, DNA-protein, protein-protein, protein-peptide and protein-ligand interactions. In this thesis work, I have focused on two types of interactions; DNA-DNA hybridization and protein-protein interactions.

Interactions between proteins occur at the protein-protein interface provided by energetically beneficial interactions between molecular groups on the interacting partners. These interactions are typically categorized as ionic (electrostatic), polar or dispersion (van der Waals) forces. The strength of the interaction define the affinity of the ligand for its target while the structural precision of the interaction (i.e. how well the ligand fits into the binding pocket of the target) is primarily related to the specificity of

the interaction. For proteins that act as receptors (e.g. cell membrane receptors), the binding of a ligand often induces a conformational change that can trigger a chemical reaction (e.g. allosterically regulated enzymes), induce flux of ions (e.g. ligand-gated ion channels) or change the affinity of a second ligand to the same receptor (cooperative binding). Ligands that activate receptors are generally referred to as agonists, while competitive inhibitors of these ligands are called antagonists.

In contrast to protein-protein interactions in which electrostatic forces in many cases favors the interaction, DNA hybridization always faces electrostatic repulsion as the DNA backbone is highly negatively charged. However, a combination of favorable hydrogen bond formation between individual base pairs and base stacking in the double helical structure contribute to the overall stability of hybridization. The interaction is further stabilized by the entropic gain of excluded water molecules in the core of the helix. The affinity of DNA hybridization increases with the length of the complimentary sequence. G-C interactions contribute to a higher degree to the overall stability of the duplex in comparison to A-T interactions. However, in contrast to popular belief, the additional hydrogen bond (three versus two for G-C and A-T interactions respectively) has only a minor contribution. Instead, base-stacking dominates the contribution to the overall stability of the double helix[7].

2.2 Kinetics of molecular interactions

Biomolecular interactions can typically be described as reversible interactions. The affinity of a ligand for its binding partner relates to the strength of the interaction, characterized by the Gibbs free energy, and can be quantified by the equilibrium dissociation constant, K_d. The affinity of a biomolecular interaction typically represents the total contribution from a series of intermolecular interactions at the interface between the two molecules.

The simplest model of a binding reaction between a ligand (A), and its binding partner (B), can be described as.

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} AB$$

where k_{on} and k_{off} are the rate constants for association and dissociation respectively. If one of the two molecular entities (B) is immobilized on a surface, binding of ligand (A) will in this case follow the Langmuir kinetic model. This model describes the relation between the time dependence of the coverage of molecules on the surface and the concentration and affinity of the binding partners according to

$$\frac{d\Theta}{dt} = k_{on} C(\Theta_{\max} - \Theta) - k_{off}\Theta \qquad (2.1)$$

where Θ is the surface coverage of the bound ligand, Θ_{max} is the maximum surface coverage (or the maximum number of available binding sites per surface area), and C is the concentration of the analyte in solution. Eq. 2.1 can be solved analytically, giving

$$\Theta = \frac{k_{on} C \Theta_{\max}}{k_{on} C + k_{off}} \left(1 - e^{-\left(k_{on} C + k_{off}\right) t}\right) \tag{2.2}$$

At equilibrium binding conditions ($t \rightarrow \infty$), this expression becomes

$$\Theta = \frac{k_{on} C \Theta_{\max}}{k_{on} C + k_{off}} = \Theta_{\max} \frac{1}{1 + \frac{K_d}{C}} \tag{2.3}$$

where $K_d = k_{off}/k_{on}$ is the equilibrium dissociation constant or affinity constant. As seen from Eq. 2.3 the equilibrium dissociation constant is defined as the ligand concentration when half of the binding sites of the particular binding partner are occupied (dotted line in Fig 2.1).

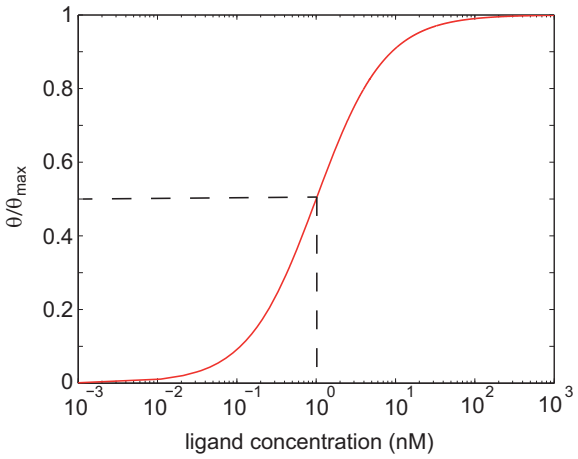

Figure 2.1. Relative surface coverage of bound ligand as a function of ligand concentration in the bulk. K_d for this particular example is 1 nM.

Although not discussed in this thesis, it should be noted that other, more complex models for molecular binding exist. These models account for e.g. cooperative binding, which means that the affinity of the interaction changes with the amount of bound ligand[8].

2.3 Thermodynamics of molecular interactions

A reduction in free energy is the driving force for all molecular interactions. Investigations of the thermodynamic parameters that underlie recognition reactions is not

only of fundamental interest in order to understand complex biological interactions, it has also a central role in drug discovery[9]. In the latter case, such investigations provide quantitative information of the drug-target interaction which facilitates interpretation of differences in binding affinity between potential drug candidates.

The underlying driving forces for biomolecular recognition events are governed by the interplay between enthalpy, ΔH, and entropy, ΔS, which can be described by Gibbs free energy, ΔG.

$$\Delta G = \Delta H - T\Delta S \tag{2.4}$$

where T is the temperature of the system. Molecular interactions that result in a favorable decrease in enthalpy due to formation of new chemical bonds are often counteracted by a decrease in entropy of the formed complex. However, complex formation also leads to displacement of solvent molecules (typically H_2O) from the interaction interface into the bulk which results in an increase in entropy of the system. The relative gain or loss in free energy of these processes determines if the interaction is enthalpy- or entropy driven. Together with input of energy, the interplay between enthalpy and entropy generally explains how living organism can form and maintain ordered structures. The increase in entropy due to reorganization of solvent molecules is particularly well described for amphiphilic molecule such as lipids and surfactant and known as the hydrophobic effect. This phenomenon will be further discussed in the next chapter on lipid membranes.

Gibbs free energy is also related to the equilibrium constant according to

$$K_d = e^{-\frac{\Delta G^0}{RT}} \tag{2.5}$$

where ΔG^0 is the standard Gibbs free energy and R is the gas constant. While Gibbs free energy describes the thermodynamic equilibrium of the binding reaction as a function of temperature, the Arrhenius equation describes the temperature dependence of the rate constants, k_{on} and k_{off} for the same reaction:

$$k_{on} = A_{on} e^{-\frac{E_{a,on}}{RT}} \tag{2.6}$$

$$k_{off} = A_{off} e^{-\frac{E_{a,off}}{RT}} \tag{2.7}$$

where $E_{a,on}$ and $E_{a,off}$ are the activation energy barriers for the association and dissociation reaction respectively and A_{on} and A_{off} are the pre-exponential factors. Hence, by determining the rate constant as a function of temperature, the activation energy of the binding (or unbinding) reaction can be extracted. The method developed in this thesis work has been focused on primarily investigating dissociation rate constants and associated activation energies for DNA hybridization (papers I and II) and protein-protein interactions (paper III).

"It takes one thousand nanobiologists to make one microbiologist"

Sydney Brenner

3

Lipid membranes

A central part of this thesis work involves cell membranes and mimics thereof. Paper I and II describes the use of liposomes (also called lipid vesicles) as signal enhancers in fluorescence microscopy. Paper III extends this concept further by using the liposome not only as a supramolecular enhancer element, but also as a carrier for membrane-associated proteins acting as one of the recognition partners. Furthermore, paper III also utilizes the inert property of planer supported lipid bilayers (SLB) as a surface modification that prevents unspecific binding of biomolecules. Paper IV and V explores yet another attribute of liposomes namely the possibility to chemically modify and subsequently analyze their chemical composition using imaging mass spectrometry.

This chapter introduces the basic features of the cell membrane as well as two commonly used mimics thereof; the liposome and the planar SLB. In addition, methods for producing liposomes, SLBs and proteoliposomes directly from the cell membrane of living cells are briefly discussed.

3.1 The cell membrane

In Nature, the cell membrane plays a fundamental role for all living cells, providing a barrier for passive diffusion of solutes, thereby separating the interior of the cell from its exterior environment. The cell membrane is also responsible for selected transport of material in and out of the cell. This function is primarily provided by proteins embedded within the membrane. In fact, more than 30% of all proteins in the cell are in one way or the other associated with the cell membrane[10]. Many of the membrane residing proteins are receptors that govern the cell response from outer stimuli or cell-cell signaling. A detailed understanding of the sophisticated functions and chemical pathways orchestrated by this class of proteins is not only of fundamental interest, but also of great importance

for the pharmaceutical industry. This is emphasized by the fact that more than two-thirds of existing drugs are directed against plasma-membrane proteins[4] and almost half of the 100 best-selling drugs on the market target membrane receptors[11].

The main constituent of the cell membrane is the amphiphilic lipid molecule consisting of a polar headgroup and one or several (typically two) hydrophobic fatty acid tails. Depending on the lipid, the headgroup is either positively or negatively charged or zwitterionic (the net change depends on the pH). When exposed to a water solution, the lipids spontaneously form various structures primarily driven by a hydrophobic association between the tails, which protect them from the surrounding water. Among the most common structures is the lipid bilayer membrane consisting of two layers of lipids, both pointing their hydrophobic tails inwards, while exposing the polar headgroups to the surrounding polar solvent (typically H_2O). Support for the existence of a lipid bilayer as the building block of the cell membrane was introduced already in the 1920s by Gorter and Grendel[12]. They demonstrated that the area occupied by the molecular constituents in the cell membrane was twice as large at an air-water interface (at which the lipids form a monolayer), as compared to the area of the original cell membrane.

Traditionally, biological membranes have been described with the fluid mosaic model in which membrane proteins are dissolved and allowed to diffuse laterally in the plane of the membrane[13]. However, during the last decades, evidence have accumulated suggesting that the lipid portion of cell membranes have a more complex functional role than to merely provide a laterally fluid scaffold for the membrane proteins. This notion is strengthened by the fact that a cell membrane contain on the order of a hundred different lipids, suggested to play an important role in, for example, endo- and exocytosis[14] and membrane protein activity[15].

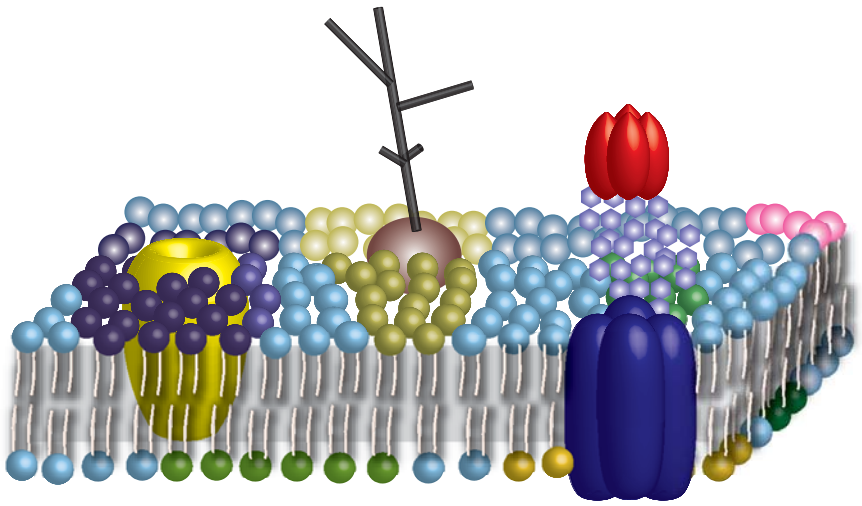

Figure 3.1. Schematic illustration of the complexity of the cell membrane exemplifying a few constituents such as ion channels (yellow), carbohydrate-modified proteins, integral (blue and peripheral (red) membrane proteins as well as multitude of different lipid species. (©2011 L. Simonsson)

In this work, we have utilized simplified mimics of the natural cell membrane, and made use of the possibility to vary the lipid composition by will. The majority of the work is based on phospholipids, which are the main constituent of biological cell membranes, and in particular phophatidylcholine (PC). This lipid has a zwitterionic headgroup, which is neutral at physiological pH, and two fatty acid tails. Other important lipids used in this work are rhodamine-labeled phosphatidyl ethanolamine (Rh-PE) which was used in all fluorescence measurements and a deuterated form of PC (D^{13}-DPPC), in which all hydrogen atoms in the headgroup are exchanged for deuterium. The latter was used in the mass spectrometry measurements presented paper IV and V to enable separate chemical identification of the traditional PC-lipid and the deuterated version. Below follows a brief survey of the typical mimics of the cell membrane, their use in science in general and for studies of membrane-associated recognition in particular.

3.2 Membrane model systems

Detailed biophysical studies of the cell membrane are often hindered by the complexity of cell membranes, consisting of thousands of different constituents. In order to reduce this complexity, numerous methods to produce mimics of the cell membrane have been developed. Two of the most commonly used model membrane structures schematically illustrated in Fig 3.2, are planar supported lipid bilayers and liposomes which are spherical lipid bilayers enclosing an inner volume. In its simplest form, these model systems consist of a single type of lipid which, driven by the hydrophobic effect, spontaneously arrange into a bilayer structure in an aqueous environment.

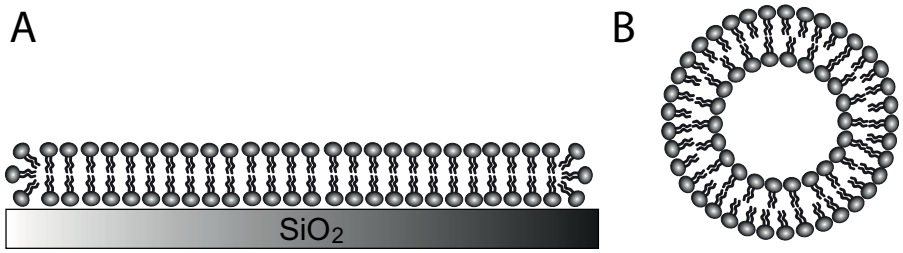

Figure 3.2. Schematic image of two membrane models systems, A) a planar supported lipid bilayer on SiO_2 and B) a liposome

3.2.1 Supported lipid bilayers

Planar lipid membranes on solid supports are typically formed by liposome adsorption and subsequent fusion into a continuous sheet[16]. The mechanism, which only occurs on a handful of substrates including SiO_2, is believed to be dominated by three stages. Initially individual liposomes adsorb to the surface, where they subsequently, at a critical coverage, undergo rupture to form small bilayer patches which eventually merge with adsorbed and later arriving non-ruptured liposomes to form a continuous bilayer[17,18]. In comparison with suspended bilayer assemblies, SLBs offer several

advantages such as ease of preparation, stability and compatibility with a wide range of surface-based analytical tools. During the last decades, SLBs have been used as cell membrane mimics in a variety of applications including investigations of the mechanical properties of membranes[19,20], phase separation and domain formation[21,22] and questions related to cell adhesion and communication[23,24].

More complex supported membranes containing e.g. membrane proteins can also be prepared by spreading proteoliposomes on solid supports[25] or by preparing SLBs and subsequently incorporate genetically engineered proteins with anchor molecules such as glycosylphosphatidylinositol (GPI)[26]. However, a major limitation of planar lipid bilayers on solid supports arises when transmembrane proteins are incorporated. This stems primarily from the close proximity between the lipid membrane and the surface. The lack of liquid space between the solid support and the lipid membrane often result in loss of lateral mobility and function of the incorporated proteins[25,27]. Different strategies have been employed to address this limitation, such as formation of SLBs on soft water rich polymer cushions[28], or tethering of lipid bilayers with long spacer molecules[29].

Another important feature of SLBs that also deserves to be mentioned is the inherent anti-sticking property, which makes the SLB a suitable surface modification that prevents unspecific binding of many proteins as well as lipid assemblies such as liposomes[30]. This attribute was explored in paper III to prevent unspecific adsorption of liposomes and cytosolic proteins.

3.2.2 Liposomes

The liposome, consisting of a self-enclosed lipid bilayer ranging in diameter from tens of nanometers to several micrometers, is another frequently used membrane model system. Liposomes are classified according to their size and lamellarity, typically categorized as small unilamellar vesicles (SUV, typically 20-200 nm in diameter) or giant unilamellar vesicles (GUV, Ø >1 μm). In both cases, the interior region is enclosed by a single lipid bilayer. SUVs can be produced using a variety of techniques such as extrusion which is one of the most common. Using this method, the lipids are first dissolved in an organic solvent such as chloroform or methanol and dried in a flow of nitrogen before rehydrated in buffer, mixed and extruded through a pore-containing polycarbonate membrane[31]. In this way, the size of the liposomes can easily be tuned by the pore size of the membrane. The liposomes used throughout this thesis work were SUVs made by the extrusion technique.

A major benefit of using liposomes as scaffolds for membrane protein studies is the fact that liposomes provide liquid space on both sides of the membrane. Therefore, in contrast to SLBs, large transmembrane proteins, containing both a cytosolic and a extracellular domain, will have a better chance to preserve their native conformation. This potential advantage is explored in paper III, in which the membrane receptors were located in suspended liposomes while the ligand (which is typically the suspended interaction partner) was covalently attached on the surface.

A limitation of using liposomes as membrane mimics is related to their often minute size, which hinders access to their interior volume with, for example, electrodes for measurements of charge translocations events. In this respect, planar supported

membranes (or GUVs) offer a significant advantage by providing physical access to both side of the membrane, which also allow the surface to be used as one of the electrodes[32].

3.2.3 Liposome conjugates

To create complex functionality, cell membrane mimics can be conjugated to a variety of recognition molecules, including proteins, peptides, nucleic acids and carbohydrates. These chemically modified liposomes have found application areas within many different disciplines of science. One of the more interesting examples are liposomes coated with recognition molecules such as antibodies for targeted delivery of encapsulated drugs[33-35]. DNA and antibody conjugated liposomes have also been explored in the context of biomolecular sensing, which is discussed in more detail in chapter 4.

Even in situations when molecular transfer across a cell membrane is not in focus, a solid support may still offer certain advantages, such as in surface-based biomolecular sensing applications. In this case, tethered liposomes are an interesting alterative to planar supported membranes. In particular, DNA-modified liposomes have been used to tether liposomes to functionalized surfaces in order to generate protein microarrays for soluble[36] and membrane-associated proteins[37]. One promising feature of such a sensing format is the potential to use the DNA sequence recognition as a mechanism for self-sorting onto DNA functionalized chips[38-40]. Modification approaches involve lipids with reactive headgroups to covalently attach the DNA[39] or cholesterol-anchored DNA that spontaneously incorporate into the lipid bilayer[41]. The advantage of the latter is the possibility to post-modify the liposomes with a rather accurate control of the DNA density[42], which was utilized in paper I, II and V.

In addition, by incorporating a fraction of fluorescently labeled lipids, the liposomes can be easily monitored using fluorescence microscopy and used as enhancer elements for biomolecular sensing applications as discussed in detail in chapter 5.

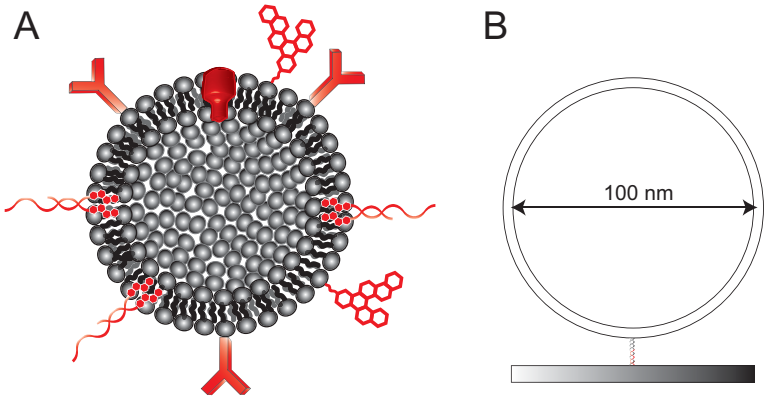

Figure 3.3. A) Schematic representation liposome conjugated to various recognition molecules such as DNA, antibodies, carbohydrates and membrane proteins. B) Illustration of a surface-immobilized liposome (Ø = 100 nm) tethered via a single 45mer DNA duplex, drawn in relative scale.

3.3 Proteoliposomes

The liposomes discussed so far have been based on purified lipids that are assembled into bilayer structures prior to retroactive modifications such as introduction of membrane-associated proteins. However, before insertion of the membrane protein into the liposome (so-called membrane-protein reconstitution) can be achieved, the membrane protein of interest must be isolated from the plasma membrane. This typically requires multiple time-consuming purification and enrichment steps, including initial isolation of the plasma membrane from other cellular constituents and organelles[43]. In this context, removal of contaminating cytoplasmic proteins that are present in much higher abundance in the crude cell extract is a challenge. Furthermore, purification and enrichment typically rely on methods such as liquid chromatography or ultracentrifugation that use water (or other polar solvents) as the main dissolving agent. Due to the hydrophobic nature of membrane proteins, solubilization using surfactants[44] is therefore a necessity. However, surfactants often introduce additional complications in downstream analyses related to the stability of the protein structure as well as interference caused by the surfactant itself[44]. Nevertheless, for detailed studies of protein function such as transport through ion channels, the reduced complexity of a system with reconstituted membrane proteins, as compared to a native membrane, is often preferred. In addition, pre-formed liposomes enable precise control of the lipid composition surrounding the protein.

An alternative route to acquire proteoliposomes that contain the cell membrane constituents of interest is to extract liposomes (or cell membrane fragments) directly from the membrane of living cells. Such proteoliposomes, referred to in this thesis as cell membrane derived liposomes, may contain essentially all components that were once present in the original cell membrane. A description of the multitude of methods and protocols that are used to produce this type of proteoliposomes is beyond the scope of this thesis. Protocols typically involve vesiculation[45], in which liposomes are released from the cell membrane upon exposure of aldehydes or sonication[46], in which large membrane fragments form suspended liposomes upon exposure of ultrasound that disrupt the membrane. In this work, we instead produced the cell membrane derived proteoliposomes utilizing the extrusion technique described above[31]. Here, the liposomes were formed by pushing intact cells through the porous membrane which mechanically disrupts the membrane. Although this technique is commonly used to produce liposomes from purified lipids, it has previously also been used to produce proteoliposomes from crude cell membrane suspension[47].

In contrast to suspensions containing liposomes with reconstituted proteins, liposomes derived directly from extruded cells contain an enormous complexity. This offers both advantages and disadvantages. For instance, previously unknown membrane constituents that are crucial for the interaction to be biologically relevant are naturally included. However, the complexity may also introduce molecules that interfere with the interaction of interest. Hence, although the simplified preparation protocol adds a significant practical advantage, the complexity of the liposome suspension puts additional demands on the assay development. This holds particular true for surface-based formats, in which case unspecific binding of lipid structures or high abundant soluble proteins is a major concern. This challenge is specifically addressed in paper III, in which a surface

modification that display resistance towards unspecific binding of crude cell extracts but provides specificity for the interaction under investigation was developed.

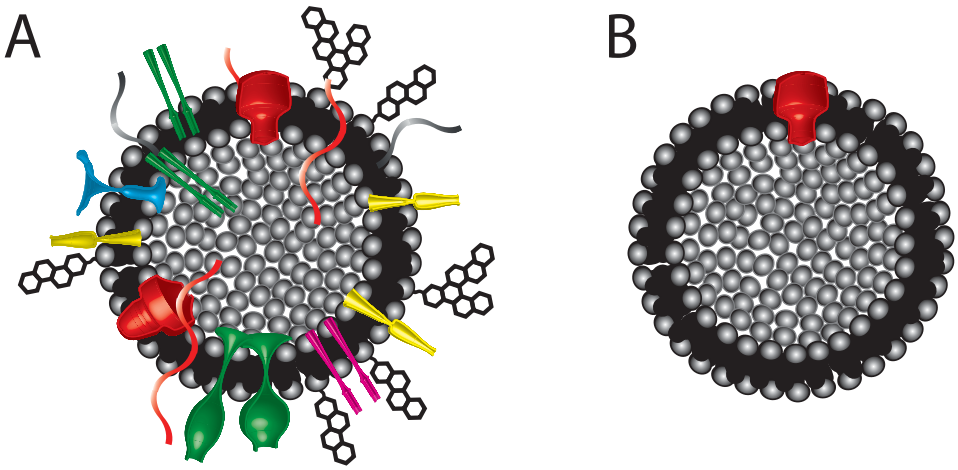

Figure 3.4. Schematic illustration of the complexity of a liposome derived directly from the membrane of a cell (A) in comparison to a liposome with single reconstituted proteins (B).

"Not everything that can be counted counts, and not everything that counts can be counted"

Albert Einstein

4

Biomolecular sensing

The wide range of analytical tools that are used to investigate biomolecular interactions cannot all be covered in this thesis. Instead, I will focus on a group of techniques that are specifically developed to probe intermolecular interactions between biomolecules. These techniques are often referred to as bioanalytical sensors. A bioanalytical sensor is a device incorporating a biological recognition element and a transducer mechanism which translates the biomolecular interaction into an detectable output signal[48]. The transducer is often based on optical, acoustical, electrical/electrochemical or magnetic readout principles. Bioanalytical sensors can be divided into different subcategories depending on the nature of the biological recognition element. Catalytic-based sensors utilize the intrinsic action of biological enzymes in which the target (substrate) concentration can be quantified by measuring the product formation of the enzymatically driven reaction, the most prominent example being the well-established glucose sensors. Affinity-based biosensors utilize an immobilized recognition element, a probe, such as a single stranded DNA, an antibody or a membrane receptor that specifically bind its interaction partner, the target. Combined with a sufficiently sensitive transducer mechanism, which translates the binding reaction into a detectable signal, the presence of specific target molecules in complex solutions can be analyzed. This chapter will focus on affinity-based sensors and primarily those that are surface-based, since this has been the main theme of this thesis work.

4.1 Sensing formats – solution versus surface

Methods for detection and identification of biomolecules can either be surface-based or operate in solution. In the former case, one of the interaction partners is typically immobilized on a surface. In contrast, solution-based concepts operate at conditions

under which both interaction partners are freely diffusing in solution. Both alternatives offer advantages and disadvantages. An obvious benefit with solution-based methods is the fact that these techniques typically mimic the situation *in vivo*, in which both interaction partners are free to diffuse in three dimensions (not true for all interactions though). In contrast, immobilization of one of the interactions partners onto a surface might alter the characteristics of the interaction. This can, for example, be due to structural rearrangement of the molecule upon binding or electrostatic contributions from the surface. These phenomena become obvious when comparing rate and affinity constants for the same interactions in bulk and at surfaces[49] and was also observed for DNA hybridization by us (paper II) and others[50]. An additional limitation often connected with surface-based assays is denaturation of proteins upon immobilization, which is circumvented in solution-based assays[51].

However, despite obvious benefits of solution-based assays, surface-based techniques designed to probe biomolecular interaction have become very popular. This stems from a number of reasons. The most significant one is perhaps the numerous different transducer mechanisms that translate the biomolecular recognition event into a detectable signal. Depending on the choice of transducer, which are typically based on optical, mechanical, electrical and magnetic principles, different type of information can be retrieved. Furthermore, surface-based techniques offer the possibility to integrate the sensor in an array format for parallel readout as well as the possibility to inject and rinse with a number of consecutive solutions. In this way, binding and release of multiple ligands can be probed using the same immobilized interaction partner. The benefit of multiple injections also provides a convenient way of forming complex molecular architecture as shown in papers I-III and V.

Three important aspects need to be taken under consideration when designing surface-based affinity sensors. First, the sensor surface must contain functional probes that provide specificity and sufficiently strong affinity for the particular interaction partner or target molecule under investigation. Second, the underlying surface should provide an inert, repellant surface to avoid unspecific binding of other molecules. Third, the sensor must rely on a sensitive transducer mechanism since target molecules are often present at low concentrations (pg/ml or pM for many clinical relevant molecules[52]), resulting in low surface coverage even at saturated binding (see Fig 2.1).

The actual sensing format can either rely on end-point measurements, in which detection is accomplished after all the target molecules have bound or allow real-time monitoring of both the binding and release process (Fig. 4.1). The advantage of the latter is the possibility to extract kinetic information, which is discussed in more detail in the next section. Furthermore, the sensing format can rely on labeled molecules (e.g. utilizing fluorescent dyes) or operate in a label-free configuration. The disadvantage of the former is the necessity to label the target molecule of interest prior to detection, which introduces additional preparation steps as well as a risk to alter or disrupt the interaction. In contrast, label-free sensing relies on detection of the interaction without any chemical alteration of the target molecule. In this context it is important to note that the definition of label-free bioanalytical sensing is different in different communities. Researchers in favor of investigating interactions in solution often argue that surface-based techniques are generally not label-free, since the immobilized interaction partner is not freely diffusing but attached to a surface (acting as a label). Another often encountered view in the

surface-based community is that label-free sensing only constitutes techniques in which the transduction mechanism is able to detect the direct binding of unlabeled targets without subsequent identification using e.g. fluorescent antibodies. Although I have for simplicity adapted to the latter definition in the thesis, I would like to stress that the key point in label-free sensing is neither of the two above, but rather whether the actual target molecule is labeled or not. To clarify this view it is important to note that the choice of sensor format depends strongly on the purpose of the measurement, a factor that is often overlooked in bioanalytical sensor development. For instance, sole detection of low abundant biomarkers does not typically require the additional information provided by real-time measurements (e.g. binding kinetics). A yes/no outcome is often sufficient, preferably including quantitative information on target concentration. Instead, the key prerequisite is in this context sensitivity. The standard assay that fulfill this purpose is the enzyme-linked immunosorbent assay (ELISA)[53]. Fig. 4.1 illustrates a typical sandwich configuration of the ELISA, in which an immobilized capture antibody binds the target of interest. Detection is achieved via the addition of a primary- and enzyme-linked secondary antibody followed by readout upon addition of a substrate for the conjugated enzyme which leads to a change in color or fluorescence. Signal amplification via multiple cycles of each enzyme generate high sensitivity (pM-fM) but at the expense of excluding any kinetic information. High specificity is also ensured since recognition of two different antibodies is required for detection.

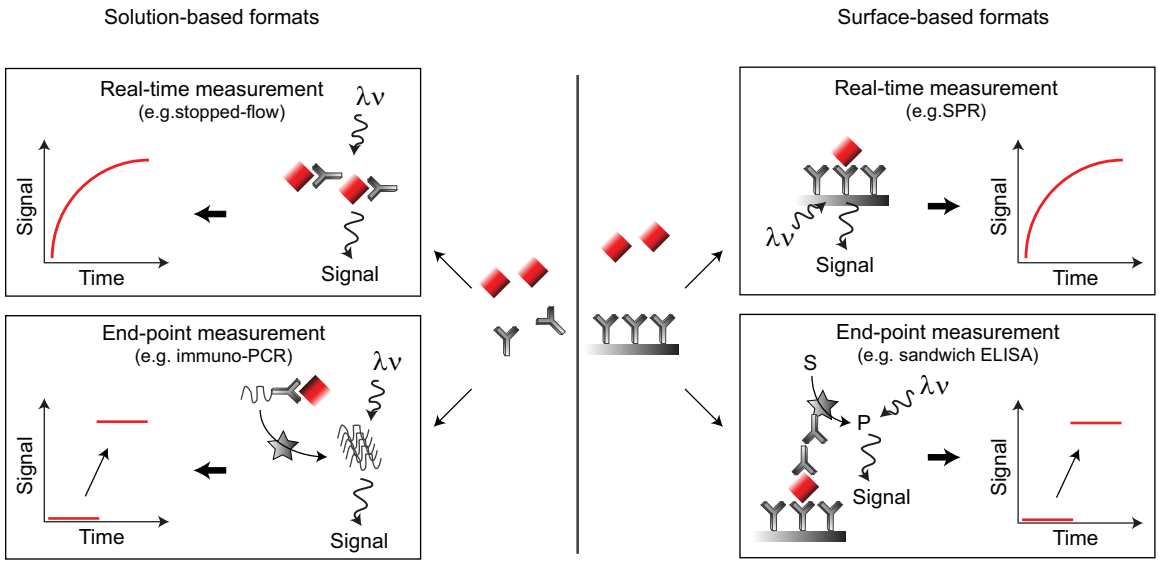

Figure 4.1. Schematic illustration of different sensing formats based on monitoring interactions in solution (left) or at surfaces (right). For both formats, detection can either rely on end-point measurements or allow real-time monitoring of the interaction. Typical examples of each format based on optical transducer mechanisms are shown for illustration purposes. End-point measurements often rely on signal amplification, such as enzymatic amplification (illustrated here as the star-shape figure), which increase the sensitivity.

In contrast, pharmaceutical drug screening typically rely on small molecules as the active compound. Introduction of a label, which has a similar size as the compound, will in this case most likely affect the interaction. Hence, label-free technologies that

measures physical contrast at a surface upon target binding, are preferred. Furthermore, biophysical parameters such as the kinetic behavior of the interaction are important entities when evaluating drug efficacy[54]. Hence, for this purpose, techniques that provide real-time measurements (Fig. 4.1) are preferred over assays based on end-point detection, despite the often lower sensitivity of the former.

4.2 Real-time measurements of binding kinetics

A major challenge within molecular biology research is to understand the mechanisms that control biomolecular interactions, which regulate a variety of processes in the cell. Identification and characterization of such interactions are important in order to understand these processes at the molecular level. In particular, the binding kinetics of molecular interactions determines the effect or duration of a specific interaction. For instance, the immune response is critically dependent on the association and dissociation rate of antibody-antigen binding[55]. Kinetic information is also important for successful drug design[56], in which recent studies suggest that the drug-target residence time have major implications for the activity and performance of the drug[54].

A multitude of techniques have been developed that enable biomolecular recognition events to be monitored in real-time, which thus enable extraction of kinetic rate constants. Due to the high sensitivity of fluorescence-based techniques (a single fluorophore can be detected with sensitive optical microscopy or spectroscopy), several methods such as fluorescence after photo bleaching (FRAP)[57], Förster resonance energy transfer (FRET)[58] and Fluorescence correlation spectroscopy (FCS)[59] have been extensively used to investigate binding kinetics of various interaction partners. These techniques rely on introduction of labels (typically conjugated to the molecule of interest), but as discussed above, there are also techniques that offers label-free detection of biomolecular interactions in real-time. Common examples include instruments based on surface plasmon resonance (SPR)[60], optical waveguide lightmode spectroscopy (OWLS)[61] and quartz crystal microbalance (QCM)[62]. However, in comparison with fluorescence-based assays, these techniques suffer from lower sensitivity. Accordingly, analyte concentrations in the μM-nM regime are typically required. Fig. 4.2 illustrates a typical experiment in which the immobilized interaction partner is exposed to, during flow, a solution containing the recognition partner. The surface coverage is monitored in real-time during injection and rinsing. Assuming that the Langmuir binding model described above applies, the rinsing curve can be fitted with an exponential curve to extract k_{off}.. Similarly, the binding curve upon injection can be fitted to Eq. 2.2 for extraction of k_{on} (and k_{off}). These numbers can subsequently be used to estimate the affinity ($K_d = k_{off}/k_{on}$). In addition, K_d is often estimated using Eq. 2.3 by monitoring the equilibrium coverage at different concentrations of the interaction partner.

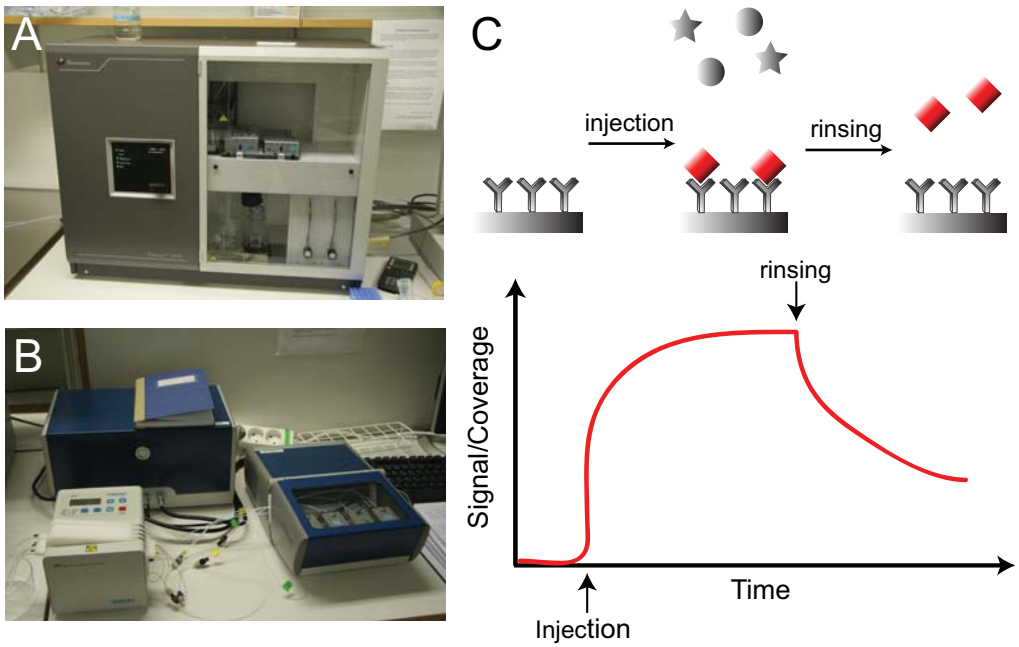

Figure 4.2. Example of surface-based bioanalytical instrument. (A) SPR instrument (Biacore 2000, Sweden) (B) QCM-D instrument (Q-sense, E4, Sweden) (C) Schematic illustration of a typical measurement in which the immobilized biomolecule (illustrated as an antibody) is exposed to a flow of liquid containing the recognition partner (blue). The surface coverage is monitored in real-time during injection (primarily for extraction of k_{on}), at equilibrium (for extraction of K_d) and during rinsing (for extraction of k_{off})

4.3 Probing membrane-associated interactions

With two thirds of the existing drug targets being membrane-associated proteins[4], the ability to measure drug interactions with this group of biomolecules is of major importance for the pharmaceutical industry. In particular, the ability to measure the residence time ($\tau=1/k_{off}$) of the drug-target complex is being recognized as a key parameter for evaluating drug efficacy[54,63,64]. The reason for this is that drug binding *in vivo* occurs under transient, non-equilibrium, conditions and can therefore not be represented by an assay that rely on equilibrium coverage (K_d) determinations. Yet, as a consequence of the lack of techniques compatible with kinetic studies of membrane receptors, drug-target interactions are often quantified by screening assays that rely on affinity (K_d) measurements (i.e. no kinetics data). Hence, sufficient information to assess the potency of drug candidates is not necessarily provided.

Significant efforts have therefore been undertaken to transfer the success of surface sensitive techniques, such as SPR spectroscopy, to probe binding kinetics of water-soluble proteins[65-67] into analogous screening assays of membrane-associated receptors. However, these studies are often hindered by the low concentration of membrane receptors in natural cell membranes which result in low surface densities of immobilized receptors accompanied with low signal-to-noise levels. Furthermore, even if relatively

high protein concentrations are produced, the lack of suitable methods to achieved high surface densities while preserving the hydrophobic environment is apparent.

In response to this, various strategies to increase the surface concentration of immobilized membrane receptors, and thereby increase the signal-to-noise upon ligand binding have been developed. These strategies include membrane protein presenting virus particles[68], reconstituted membrane proteins in amphipathic polymers[69] or planar lipid bilayers[70]. Although promising, these approaches are practically cumbersome, still provide low signal-to-noise and/or suffer from not preserving the natural cell membrane environment. An alternative strategy to improve the sensitivity and thereby the signal-to-noise ratio is to use single molecule approaches. Such approaches open up new possibilities for kinetic analysis of membrane-receptor-ligand interactions as shown in paper III and discussed in detail in chapter 5.

4.4 Parallel analysis of multiple interactions

The ability to detect multiple targets simultaneously and thereby significantly reduce the measurement time is a central issue when analyzing complex biological samples. For example, analyzing the entire human genome consisting of approximately three billion DNA base pairs[71] requires simultaneous analysis of millions of DNA fragments. Similarly, analysis of the human proteome would require analysis of >25 000 species[72], excluding various post-modifications, alternative splicing etc that would significantly increase this number. Up to now, two main approaches have been adopted, microarray technology and, more recently, detection schemes based on target-specific barcodes. The two approaches are schematically illustrated in Fig 4.3.

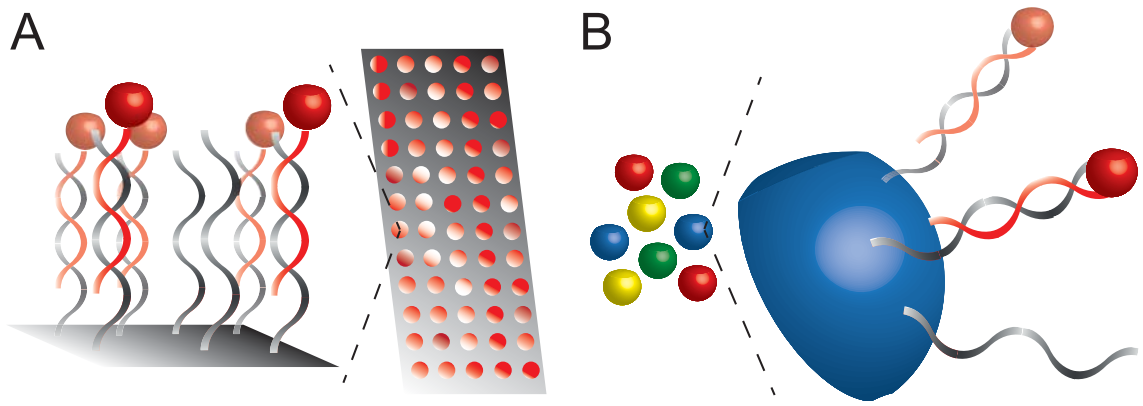

Figure 4.3. Schematic illustration of (A) DNA microarray and (B) microsphere-based suspension array. The labeled target sequence (red) enable fluorescence readout in both situations. However, decoding is accomplished either by spatial information in A or via fluorescently color-coded microspheres in B.

The dominating methodology is based on ordered arrays of surface-immobilized capture molecules, in which the position of the capture molecule, or probe, enable decoding of the specific target molecule that was detected. This technology has matured

primarily within analysis of nucleic acids with the recent development of DNA microarrays. These arrays contain thousands of different capture molecules (i.e. single stranded DNA with varying nucleotide sequence) immobilized onto a solid support[73]. Fluorescence readout of state-of-the-art DNA microarrays with a spot size of ~10 μm^2[74] enable simultaneous analysis of up to 10^5-10^6 different probes[75].

While DNA microarray technology potentially enables simultaneous analysis of entire genomes, the protein equivalent, typically an antibody microarray[76] still suffers from several shortcoming, such as protein denaturation and poor long-term stability[77]. These negative effects originate primarily from unwanted protein-surface interactions. Development of new detection principles in combination with new surface chemistries will be critical in order for protein microarray technology to be reliably integrated into high-throughput (>1000 probes/mm^2)[78] and routine applications.

The alterative methodology relies on target-specific fingerprints (barcodes) associated with each type of capture molecule. These assays can either be based on encoded microrcarriers consisting typically of polymer beads carrying different probe molecules or involve molecular hybrid systems in which the probe molecule is directly conjugated to a separate molecule, acting as the fingerprint. The most common version of the latter assay is based on antibody-DNA conjugates. Here, antibody binding to the target of interest is followed by rinsing of unbound probes which facilitates selection of the specific reporter DNA sequence that is conjugated to the bound antibody. The reporter DNA, acting as a barcode, is subsequently amplified by the polymerase chain reaction (PCR) and detected. Thanks to the possibility of amplifying the DNA barcode sequence, through PCR, the sensitivity of this type of assay, termed immuno-PCR, is thousand fold higher than detection of the antibody directly[79]. Although sensitive, immuno-PCR suffers from time-consuming washing and amplification steps as well as the necessity of complex conjugation chemistry. To increase the multiplexing capacity and to improve the speed of analysis, encoded microrcarriers containing not only the probe molecule, but also a barcode signal, has been developed. In contrast to microarray technology, this assay format enables multiplexed measurements in suspension[80]. The potential of multiplexed encoding using fluorescence have been extensively investigated using for example microspheres containing precisely controlled ratios and concentrations of different organic dyes[81,82] or quantum dots[83,84]. Readout of both barcode and binding event is typically accomplished via multi-colour flow cytometry and these so-called suspension arrays have matured into the commercial market.

Readout of suspension arrays can also be accomplished by immobilizing the microcarriers on a surface. One example of such a so-called random array[85] use functionalized microspheres that are randomly assembled into an array of microwells, in which each well can be addressed separately by e.g. an optical fiber. The advantage of such an approach as compared to standard microarrays is that the probes do not need to be predefined on the surface. Using molecular self-assembly, the sensor surface can be functionalized with a large number of different probes in a single mixing step, thus simplifying fabrication as well as facilitating high density (spot size ~10 μm^2) arrays. A unique 25mer DNA sequence connected to each bead enable decoding of a large number of different barcodes using a sequential hybridization scheme with a set of different fluorescent labels[86].

In contrast, direct decoding of beads based on their inherent fluorescent fingerprint is generally limited to tens of different targets[80] due to spectral overlap between fluorescent dyes and variations in bead quality. To circumvent this drawback, encoding strategies using other means than fluorescence has been explored. For instance, Raman-active dyes in close proximity to gold nanocarriers can provide more narrow-band fingerprints for spectroscopic decoding than fluorescence, thus potentially improving the multiplexing capacity[87]. An even more promising approach utilizes gold nanocarriers with different DNA sequences as unique barcodes, in analogy with immuno-PCR, providing an essentially limitless multiplexing capacity[88,89]. This type of assay has demonstrated very high sensitivity (fM-aM detection limit) but suffers from multiple preparation steps, including amplification and separation procedures using magnetic beads.

We have in this thesis work explored a potentially attractive alternative to overcome the limited multiplexing capacity of fluorescence-based formats. Using mass spectrometry, we (paper V) and others[90] have investigated the use of nanocarriers with different chemical composition that act as chemical fingerprints (in analogy to optical fingerprints in fluorescence-based assays) for multiplexed readout. These approaches rely on a random array format. However, in contrast to the fiber bundle microwell arrays described above, simple planar substrates were used. Although MS in principle allows for direct identification of biomolecules in a complex sample[91], the sensitivity is often a limiting factor, reducing the applicability to high-abundant species[2]. In contrast, by analyzing the chemical composition of the nanocarrier which contains thousands of barcode molecules associated with each target molecule, large signal enhancement and thus increased sensitivity can be achieved. In fact, using high resolution imaging MS, individual nanocarriers can be analyzed simultaneously which, as discussed in detail in chapter 7, opens up for exciting possibilities for multiplexed assays down to the level of single molecules.

"The only way of discovering the limits of the possible is to venture a little way past them into the impossible"

Arthur C Clarke

5

Biomolecular sensing at the single molecule level

Major efforts have been devoted to the quest of investigating the behavior of biomolecules with the ultimate resolution namely on the level of individual molecules. The motivation for these efforts strongly depends on the research field and the specific questions. However, in general, the ability to analyze a biological system at the level of single molecules have provided researchers with an entirely new set of investigations that was previously not possible using techniques that measures properties of molecular ensembles. For example, single molecule investigations (i) reveal population heterogeneity, (ii) enable precise localization with nanometer accuracy in spatially distributed samples such as a cell, (iii) enable investigations at low copy numbers, observed for the majority of molecules in the living cell, thus eliminating the need for enrichment as well as (iv) facilitate direct measurements of mechanical properties including forces, generated by, for example, molecular motors.

5.1 Probing biomolecular recognition at the single molecule level

The pursuit for single molecule resolution has also been a driving force within the field of biomolecular sensing. The most obvious reason being the potentially increased sensitivity. The advent of micro- and nanofabrication have boosted the development of a variety of surface-based techniques that provide label-free studies of biomolecular interactions at extremely low concentrations (~fM)[92] in some cases reaching even single molecule sensitivity[93,94].

To understand the challenge of engineering sensors with high sensitivity, one must consider several factors that influence the signal such as the relation between affinity (characterized by K_d) and the surface coverage (Eq. 2.3). With state of the art label-free instruments, such as SPR, the sensitivity is around 0.1 ng/cm^2. For a typical protein with a molecular weight of 50 kDa, this corresponds to 10^5 molecules per 100×100 um^2 at equilibrium. Taking into account the footprint of a typical immobilized IgG antibody (10×10 nm^2), the sensor surface (100×100 μm^2) can, under optimized conditions harbor a maximum of 10^8 capture molecules. Even for a surface with an exceptionally high fraction of active capture molecules this would correspond to a relative surface coverage of 0.1%. Following the reasoning above, this yields a detection limit (assuming a K_d=1 nM) of around 1 pM (see Eq. 2.3), given that equilibrium is reached within a reasonable time scale.

The relation between target concentration and the corresponding surface coverage becomes particularly interesting when taking into account the area of the sensor surface. Following the same reasoning as above, only one out of a million immobilized capture molecules will be occupied at a target concentration of 1 fM. For a micron sized sensor (100×100 μm^2), this means that 100 target molecules will be bound at equilibrium. Detection in this case will therefore essentially require single molecule sensitivity. By shrinking the sensor dimensions to the nanoscale (<100×100 nm^2) in order to potentially enhance the sensitivity of the transduction mechanism, will result in a surface coverage of target molecules far below a single molecule (10^{-4}). If single molecule sensitivity can in this way be reached, detection will have to rely on fluctuation analysis over extended time periods, the length of which will be determined by the strength of the interaction. Despite this, groups have reported fM detection limits using single nanowire sensors[92,95] with similar sensor areas as in the example above. This suggests that additional effects, such as e.g. field-induced electrostatic interactions that locally increase the target concentration may in this case have played a role[96].

In light of the discussion above I would like to advocate in this thesis is that a bioanalytical sensor optimally designed for high sensitivity should have a sufficiently large surface area to accommodate numerous capture probes, while at the same time retain single molecule resolution. Such a sensor format was in fact recently reported using optical microcavities[94] demonstrating real-time label-free detection at aM target concentrations. The impressive sensitivity is accomplished by monitoring changes in resonance of whispering gallery resonators upon biomolecular binding.

Following the same argument of molecular resolution while preserving a large sensor area, I have in this thesis work developed a sensing assay with single molecule sensitivity that is compatible with field of views of >100×100 um^2. High sensitivity (fM) was achieved using a sandwich assay, in which a secondary recognition element was conjugated to a liposome as schematically illustrated in Fig. 5.1. With the liposome being fluorescently labeled, time-resolved observation of individual binding events was accomplished using a TIRFM setup. The combination of evanescent-based illumination and imaging enables statistics from thousands of simultaneous binding events to be collected from equilibrium coverage fluctuations in the presence of suspended liposomes. In this way, information about the kinetics of the interaction could also be extracted. Various aspects of this assay format are discussed in more detail in the following sections

in an attempt to illustrate potential benefits of a liposome-based single molecule sensing platform over alternative single molecule concepts.

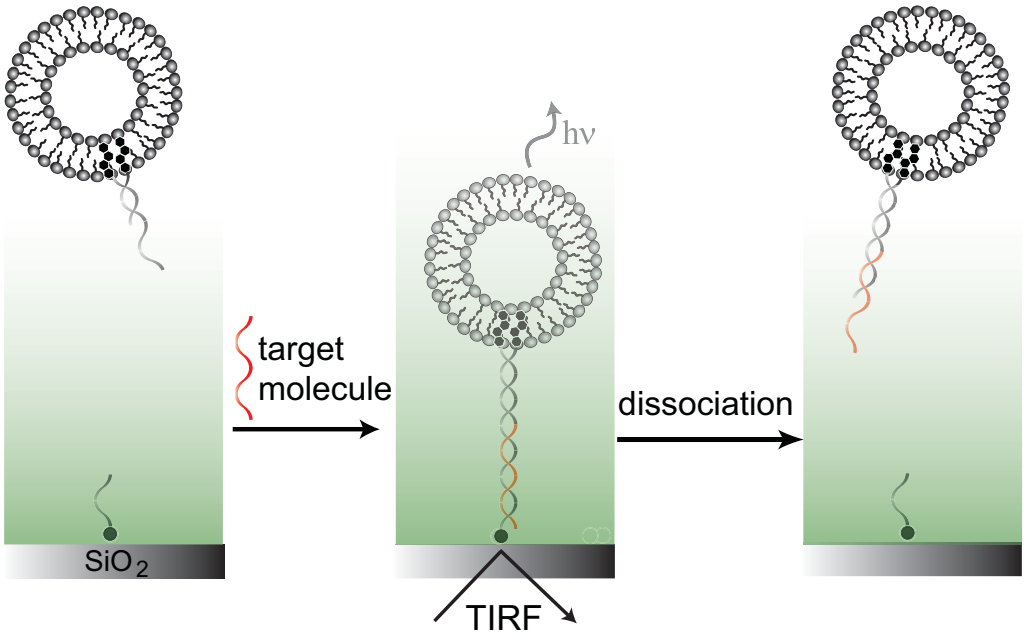

Figure 5.1. Schematic illustration of the liposome-based single molecule assay. DNA-modified, fluorescently labeled liposomes are immobilized on the probe-modified surface in the presence of the unlabeled single stranded target sequence. Due to the evanescent field provide by the TIR illumination, immobilized liposomes are detected in the presence of suspended liposomes. Each individual recognition event is monitored over time to enable extraction of the residence time.

5.2 Interaction kinetics at the single molecule level

Although recent development of surface-based techniques has provided label-free studies of biomolecular interactions, with sensitivities down to the single molecule level[93,94], dynamic studies of single molecules are typically performed using fluorescent-based methods (such as TIRFM and FCS). In addition to provide single molecule resolution, these methods also offer spatial resolution down to the nanometer scale. However, one limitation with fluorescence-based imaging approaches, besides the need for conjugation of external labels, is the fact that they suffer from relatively rapid photobleaching of the fluorophores. This typically limits the observation time to a few seconds[97], and hence, they are only suitable for low-affinity interactions with rapid dissociation kinetics ($k_{off} > 0.1\ s^{-1}$). Various enhancer elements can be used to improve the signal-to-noise ratio and prolong the visualization time, thus circumventing the problem of rapid photobleaching. One of the more prominent examples of such enhancers are quantum dots[98]. Using advanced surface-functionalization, these nanocrystals can be made water-soluble and biocompatible. Compared with conventional fluorophores,

quantum dots display narrow, symmetric emission spectrum and are photochemically stable. Various examples of biomolecular detection using bioconjugated nanocrystals have been reported in the literature[99,100]. Most of these assays, however, are solution-based and rely on ensemble measurements. This observation is probably attributed to the fact that bioconjugated quantum dots often suffers from unspecific binding to surfaces when used in surface-based formats as well as emission blinking, the latter complicating studies of single binding events. In this work we have explored fluorescently-labeled liposomes as an alternative enhancer element. Although composed of organic dyes, the possibility to easily modify each liposome with thousands of fluorophores makes it resistant to photobleaching which extend the observation time of individual liposomes to hours (paper I and II). Furthermore, in contrast to quantum dots, liposomes show excellent anti-sticking properties to suitably functionalized surfaces. This inert property contributes to the low unspecific binding of liposomes in surface-based biorecognition assays which is a prerequisite for sensitive detection.

The use of liposomes for signal enhancement have been previously explored in different sensing formats (see [101] for review), including fluorescence enhancement in ELISA-type assays[102] or frequency shift enhancement in QCM[103]. However, regardless of whether detection is based on intact or lysed liposomes (releasing its detectable content), signal enhancement is only achieved via end-point measurements. In fact, with a few exceptions[104] the majority of assays that is compatible with detection of low (<pM) target concentrations rely entirely on end-point measurements, thus excluding kinetic information.

In contrast, the liposome-based assay described in this thesis demonstrates, besides high sensitivity, capabilities of following the dynamics of single molecule interactions for high affinity binders, such as DNA hybridization and antibody recognition. Furthermore, the single molecule resolution enable measurements at stagnant liquid conditions which eliminate the need of controlled liquid exchange since neither the rate of binding upon injection nor release upon rinsing must be recorded in order for kinetic data to be extracted. However, the sensor format also suffers from certain limitations. Although the sandwich format provide high sensitivity, it may obscure the extraction of absolute rate constants since more than one reversible bond is typically required to immobilize the liposomes (paper I-II). Furthermore, the assay format only allows *direct* extraction of k_{off}. This is due to the low surface coverage of liposomes required for successful image analysis which prevents an accurate determination of the maximum coverage. Hence, in order to extract k_{on} (and K_d), one must independently estimate the surface coverage of capture molecules which may vary somewhat from one experiment to the next.

Despite these limitations, the extraction of kinetic data presents additional opportunities such as determination of the thermodynamic properties of the interaction by varying the temperature. An example of the type of information obtained from such work is illustrated here by considering the thermodynamics of DNA hybridization, which was examined in paper II.

The thermodynamic properties of DNA hybridization in solution have been investigated since the 1950s[105] using methods such as differential scanning calorimetry (DSC) and isothermal mixing calorimetry (ITC) [106]. This has enabled the development of theoretical models capable of predicting the thermodynamic properties of arbitrary sequences[107]. Furthermore, studies of the temperature dependence on the kinetic rate

constants of DNA hybridization in bulk[108] and with surface-based techniques[50,109] have provided insights related to the activation energies of the association and dissociation process. In this thesis work, we showed that similar information can be retrieved from single molecule imaging studies (paper II). The main advantage with such an approach is the elimination of multiple injections and rinsing processes. Measurements at varying temperatures can be performed sequentially, without any regeneration of the surface, which is otherwise a necessity for thermodynamic investigations based on kinetic measurements. Besides improvements in reproducibility, such an approach also limits sample consumption, which is often a major concern for calorimetric measurements.

5.3 Membrane receptor-ligand interactions at the single molecule level

As discussed above, the difficulty to probe binding kinetics of membrane receptor-ligand interactions using surface-based assays is primarily due to insufficient sensitivity while still maintaining the receptor in a lipid environment. Single molecule approaches thus offers an attractive alternative, providing high sensitivity and compatibility with native cell membranes either as whole cells or proteoliposomes. In addition, techniques based on observation of individual recognition events are capable of operating under stagnant liquid conditions; while still enable extraction of binding kinetics.

Perhaps the most straightforward approach is to fluorescently label the ligand under investigation and monitor its interaction with the receptor using high resolution fluorescence microscopy. However, besides the risk that the label might alter the interaction, single molecule imaging techniques are typically limited to low affinity interactions (k_{off}>10^{-1} s^{-1})[97] due to rapid photobleaching.

An alternative approach is to use fluorescence correlation spectroscopy (FCS)[110] which measures the correlation of fluorescence intensity fluctuations. FCS is often applied in a confocal microscope setup in which the intensity fluctuations are probed in the small focal volume determined by the focused light. With such as setup, intensity fluctuations that originate from individual molecules diffusing into the focal volume can be detected and the diffusion coefficient can be determined. Interactions between biomolecules can be investigated using FCS by distinguishing the diffusion coefficient of bound and unbound biomolecular complexes. The technique has been used to study protein binding in living cells[111] and ligand binding to proteoliposomes[112]. By temporally resolving the relative amounts of free and bound fluorescently labeled ligand, association rate constants, k_{on}, upon injection and affinity constants, K_d at equilibrium can be determined[113]. However, to obtain dissociation rate constants, k_{off}, competitive binding using a large access (typically > μM) of unlabeled ligands is required[114]. Furthermore, since the data acquisition requires a certain time interval to collect enough statistics, low affinity interactions (k_{off}>10^{-1} s^{-1}) are typically not accessible.

In this work, we have explored an alternative, surface-based approach to investigate the kinetics of membrane receptor-ligand interactions. Besides the advantages offered by a surface-based formats, the approach circumvents limitations related to the residence time. The assay is an extension of the liposome-based assay format described earlier.

Using fluorescently labeled cell membrane-derived liposomes, carrying the receptor of interest; we monitored their interaction with a ligand modified surface, as schematically illustrated in Fig. 5.2.

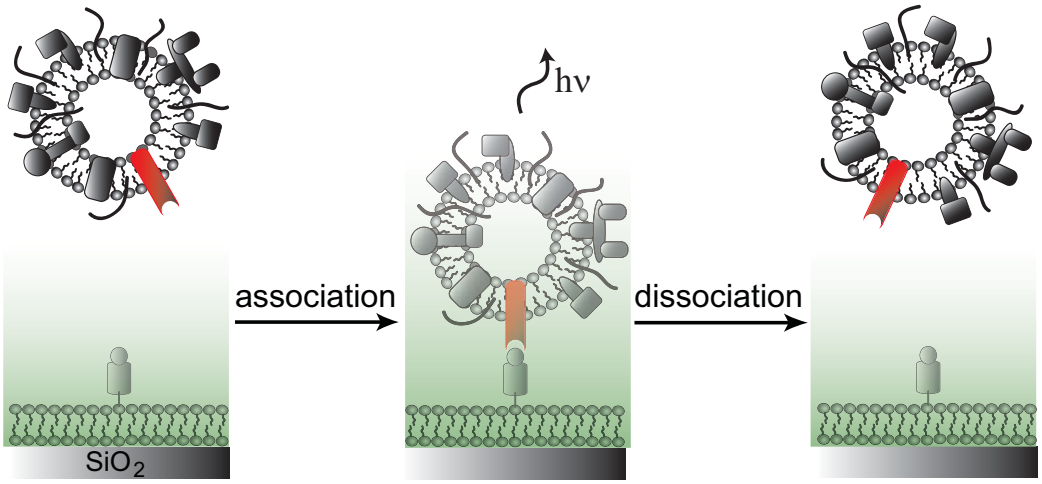

Figure 5.2. Schematic illustration of the membrane receptor-ligand interaction assay. The fluorescently stained liposomes carrying the membrane receptor of interest (red) associate and dissociate from the surface-immobilized ligand at stagnant liquid conditions. Due to the evanescent field provided by the TIR illumination, immobilized liposomes are detected in the presence of suspended liposomes. Each individual recognition event is monitored over time to enable extraction of the residence time.

5.4 Multiplexed detection at the single molecule level

Standard two-dimensional microarrays as well as commercially available suspension arrays lack the sensitivity required to detect single recognition events. Up to date, only a few attempts have been made to carry out multiplexed analysis at the level of single molecules. One approach involved DNA origami, in which single-stranded DNA segments are folded into three dimensional structures[115], to fabricate molecular analogues of polymer bead microcarriers used in suspension arrays. Detection and decoding was achieved by analyzing the shape of the DNA structures in the presence of hybridized DNA targets using atomic force microscopy (AFM). Although the fabrication process, which is based on self-assembly, is rapid and produces a large number of probes, the analysis is cumbersome, sequential and hence very slow. Another interesting approach is to make use of rolling circle amplification. Target detection initiates the amplification of long DNA sequences (complementary to the DNA target) which can be labeled selectively by different fluorescent probes and analyzed with fluorescence microscopy[116]. However, as already mentioned, fluorescence-based methods are severely limited in terms of multiplexing capacity due to spectral overlap.

To overcome this limitation we have explored yet another potential benefit of the liposome-based assay introduced in this thesis. The idea is to use the lipid composition of

each liposome as a chemical barcode for multiplexed detection, as illustrated in Fig. 5.3. Using high resolution mass spectrometry imaging, which enables single liposome resolution (paper IV), each recognition event can be detected and decoded based on its chemical fingerprint.

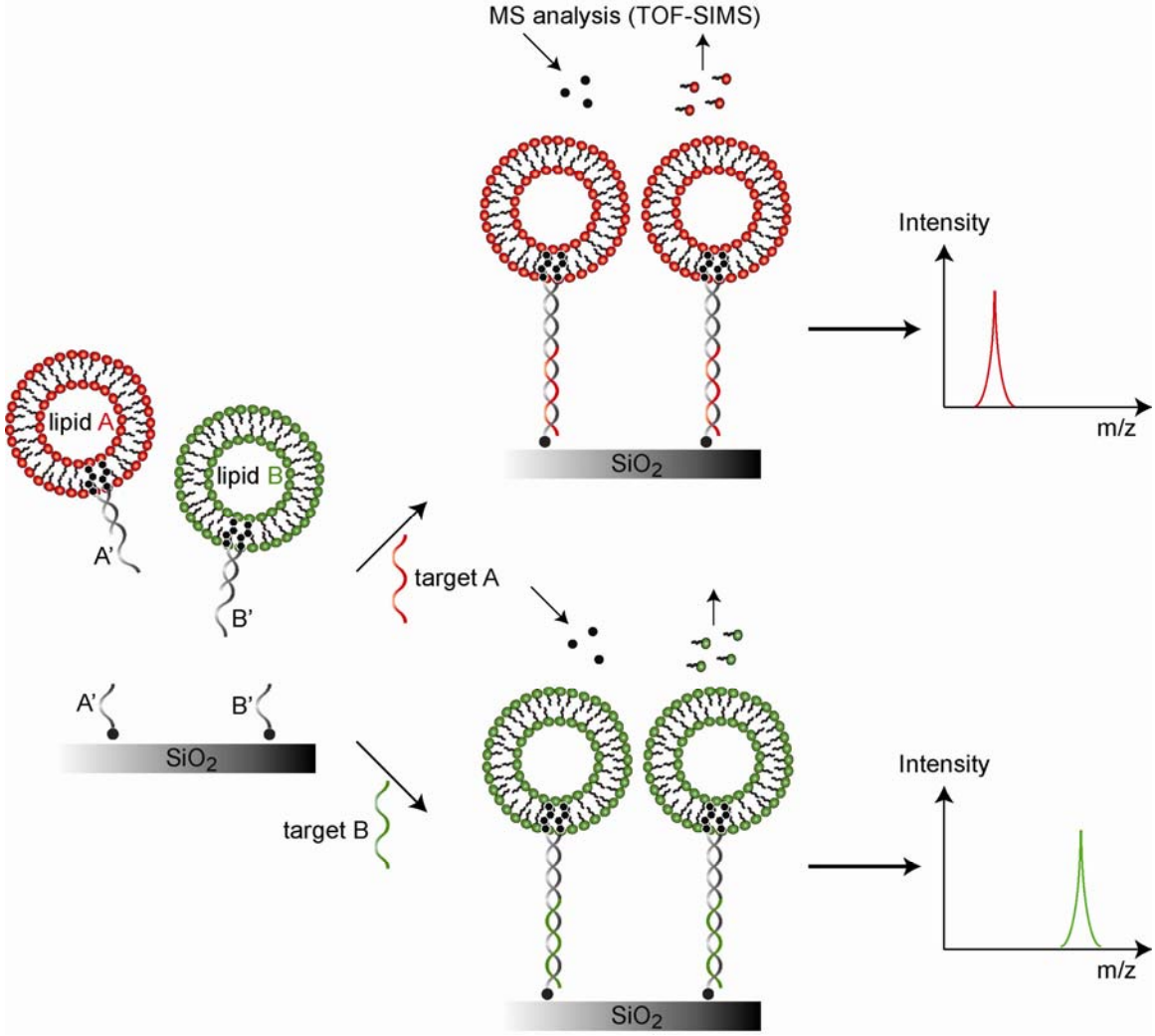

Figure 5.3. Schematic illustration of the multiplexed single molecule assay. DNA-modified liposomes with different lipid composition are immobilized on the probe-modified surface upon hybridization with the unlabeled single stranded target. The chemical composition of the liposomes, acting as chemical fingerprints can be analyzed by imaging mass spectrometry. Using high resolution mode, chemical identification of individual liposomes can be accomplished which, under certain conditions, correspond to detection of single recognition events.

"The trouble with measurements is its seeming simplicity"

Unknown

6 Methodology

This chapter gives a brief background to the instruments and experimental setups used in this thesis work. Although a wide range of techniques have been applied such as ultracentrifugation, SPR; QCM-D, nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), optical spectroscopy, flow-cytometry (FACS) etc, only the two main techniques, namely total internal reflection fluorescence microscopy (TRIFM) and time-of-flight secondary ion mass spectrometry (TOF-SIMS) will be discussed in detail. The use of fluorescence microscopy to image and spatially resolve, for example, liposomes on surfaces is well established[18], while the prime technical contribution of this thesis work is related to means to analyze and spatially resolve individual liposomes using TOF-SIMS. Using the assay format illustrated in Fig. 5.1, TIRFM enable dynamics of individual molecular interactions to be monitored in real-time by following the binding and unbinding of surface-immobilized liposomes. In contrast, TOF-SIMS, being an ultra high vacuum technique, is only compatible with end-point measurements. However, chemical information can be extracted without the need of external labels, which enable chemical analysis of individual surface-immobilized liposomes. An additional section in this chapter briefly describes the image analysis of the TIRF micrographs, which was used for the data extraction in paper I-III.

6.1 Total internal reflection fluorescence microscopy (TIRFM)

Among the techniques that enable investigations of molecular interactions at the single molecule level, fluorescence microscopy is probably the most common. This technique typically relies on imaging of fluorescently tagged molecules, in which the main obstacle to achieve high sensitivity is background noise from molecules that is not in focus. Hence, by confining the excitation volume, observation of individual labeled

molecules can be achieved. Two main approaches have been adopted, confocal microscopy and total internal reflection fluorescence microscopy (TIRFM). In TIRFM the excitation volume is confined to the interface between a high and low refractive index medium which reduces the emission of light from molecules in solution resulting in a significant decrease of the background intensity (noise)[117]. In the TIRFM setup used in this work the excitation light is confined to an evanescent field at a glass-liquid interface, which extends only a few hundred nanometers into the bulk solution.

6.1.1 Introduction to total internal reflection fluorescence

Total internal reflection occurs when a beam of light passing through an optically dense medium encounters an interface to a lower refractive index medium on the distal side at an angle of incidence that is larger than a certain critical angle, as illustrated in Fig. 6.1. The critical angle, θ_c, is determined from Snell's law as the angle of incidence corresponding to an angle of refraction equal to 90 degrees.

$$\theta_c = \arcsin\left(\frac{n_1}{n_2}\right) \tag{6.1}$$

where n_1 is the refractive index of the low refractive index medium (e.g. the liquid) and n_2 is the refractive index of the optically denser medium (e.g. the transparent glass substrate). When the angle of incidence is greater than the critical angle, the beam is completely reflected at the interface. The light however still interacts with the glass-liquid interface. The result is an evanescent field at the interface which extends only a few hundred nanometers into the liquid medium. The extension of the evanescent field along the z-axis is dependent on the incident angle and the wavelength of the excitation light and given by:

$$I(z) = I(0)\exp(-z/\delta) \tag{6.2}$$

where $I(0)$ is the intensity at the interface. The decay constant, δ is a function of the two refractive indices and the wavelength of the incident light.

$$\delta = \frac{\lambda_0}{4\pi}\left(n_2^2 \sin\theta - n_1^2\right)^{-1/2} \tag{6.3}$$

As a result of the local evanescent field (typically ~100 nm), only fluorophores in very close proximity of the surface will be excited, thereby reducing background fluorescence from molecules in solution, as illustrated in Fig. 6.1.

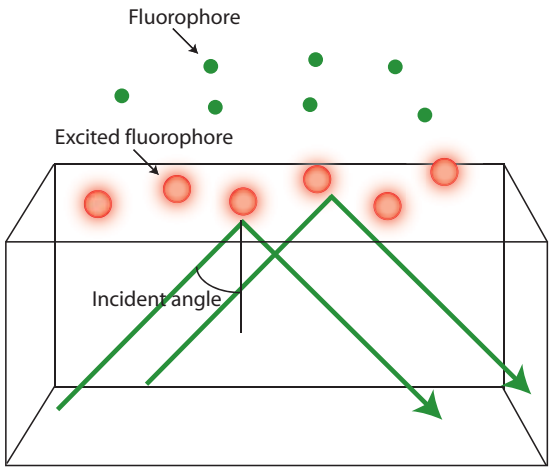

Figure 6.1. Principle of total internal reflection fluorescence. The incident angle is larger than the critical angle and total internal reflection occurs at the glass-liquid interface producing an evanescent field that stretches only a few hundred nanometers into the bulk liquid. As a result, fluorophores in solution will only be excited in close proximity of the surface, thereby reducing background fluorescence from molecules in solution.

6.1.2 Total internal reflection fluorescence microscopy - experimental setup

Total internal reflection fluorescence microscopy (TIRFM) was developed in the 1980s by Daniel Axelrod[118]. TIRFM uses the evanescent wave to selectively excite fluorophores in close proximity of the glass-liquid interface. Due to the advantage of low background, TIRFM has been used extensively in cell biology studies for visualization of the basal plasma membrane of cells[119] since selective excitation of molecules at the cell surface significantly reduces the interference from molecules inside the cell. However, the low background has also made TIRFM a useful technique to observe fluorescence from individual molecules, making it an important tool for biophysics and quantitative biology[120]. Fig. 6.2 shows the two main configurations used to achieve TIRF in an ordinary inverted microscope; (i) prism-based TIRF and (ii) objective-based TIRF. In prism-based TIRF the evanescent field is excited using a laser directed at a high angle of incidence. Since the refractive index of air is lower than that of glass, a prism in optical contact with the glass slide is required to achieve an angle of incidence that is large enough for TIR to occur at the glass-liquid interface of the sample. The emitted fluorescent light is collected through the objective as in ordinary fluorescence microscopy. In contrast, using objective-based TIRF, both the emission and excitation light is directed through the objective. Using an objective with a high numerical aperture (typically NA >1.4) the sample can be illuminated at an angle required for TIR excitation. The emitted fluorescence is collected by the objective and projected onto the CCD camera. As in the case of prism-based TIRF, no excitation light will be transmitted at the glass-liquid interface leaving only the evanescent field associated with the TIR to excite fluorophores in close proximity of the surface. Both configurations were used in this thesis work.

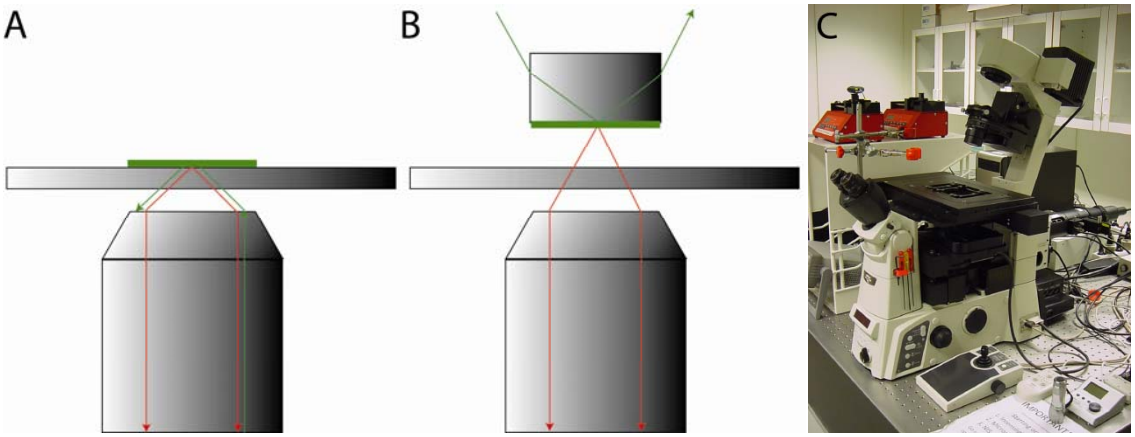

Figure 6.2. A) Schematic illustration of objective-based TIRFM with green excitation light and red emission light. The thick green line represents the excitation volume. B) Schematic picture of prism-based TIRFM with green excitation and red emission light. C) Inverted TIRFM microscope (Nikon Ti Eclipse).

6.2 Image analysis

Due to the limited scope of the thesis, this methodology section describes the image analysis performed in paper I-III, but will not give any broader perspective of image analysis strategies in general.

As described in chapter 5, TIRFM was used to monitor individual association and dissociation events by observing the binding and unbinding of fluorescently labeled liposomes at the surface. The liposomes were selectively modified with a recognition element such as single stranded DNA (paper I and II) or contained the membrane receptor of interest (paper III). In order to analyze the TIRF micrographs collected over time, automated image analysis was implemented using Matlab®. Since each liposome contains approximately a thousand fluorophores, the signal-to-noise level is much higher compared to other fluorescently-based single-molecule studies, in which the molecule of interest is directly labeled with one or a few fluorescent dyes[97]. Each liposome that exceeds a certain intensity threshold (set by the user) is counted as bound. However, liposomes that are not immobilized via molecular recognition but merely enter the evanescent field for an instant due to diffusion in close proximity of the surface can still be recorded in a single micrograph. These events should however be excluded from the binding events, indicating recognition. The latter can be distinguished from the non-bound liposomes since they, in contrast to the non-bound liposomes, will remain on the same position at the surface over several frames. To avoid false positives, only liposomes that remain immobilized on the surface for at least three consecutive frames are included in the analysis.

Each bound liposome is followed over time in order to detect their release from the surface, from which their corresponding residence time can be determined. If the intensity of a bound liposome drops below the dissociation threshold (typically 25% of the detection intensity threshold) it is considered dissociated. Since all immobilized

liposomes will be slightly affected by bleaching, the drop in intensity between two consecutive frames, must be larger than a specified value (typically half of the detection intensity threshold) to be counted as a detached liposome. A small fraction (typically <1%) of the total population of liposomes will reach intensities below the dissociation threshold due to bleaching, rather than release. Such liposomes are considered bleached and are not included in the analysis of the dissociation events and the estimation of k_{off}. Furthermore, to accurately determine the residence time, liposomes that were already bound to the surface at the start of the measurements are not used in the analysis.

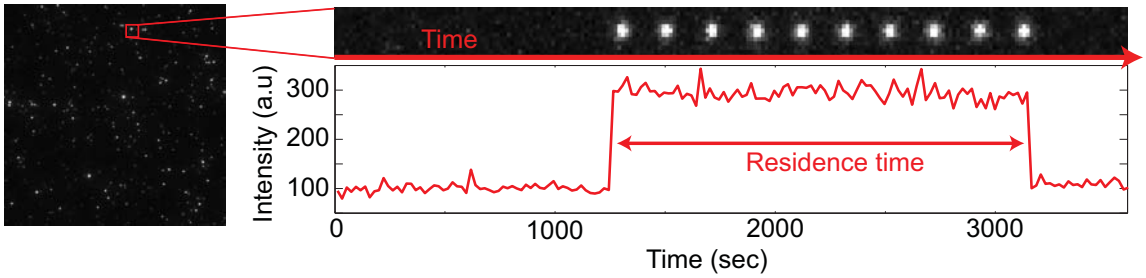

Figure 6.3. Fluorescence micrograph snapshot and kymograph (displaying every 10th frame) with corresponding intensity profile of a small subsection (6×6 μm), highlighting a single liposome interacting with the surface. Detection of individual binding events was obtained by distinguishing bound liposomes from non-interacting liposomes that disappear from the surface during the time between two consecutive frames.

Due to the finite time-span of the measurement, liposomes that bind in the late part of a measurement will only be accounted for if their residence time is short (i.e. they detach before the end of the measurement). Hence, liposomes with long residence times will be underrepresented in the statistical analysis. In order to compensate for this, two different approaches were applied. In paper I, a modified expression was used to represent the dissociation curves, compensating for the underrepresentation of long binding times. In paper II and III an alternative approach was used, in which the total measurement time is divided into two parts of equal duration. All liposomes that bind to the surface at any frame in the first half of the total measurement time are accounted for. These liposomes are subsequently monitored for the same period of time, which is half of the total measurement time. In this way, liposomes with longer residence times will be equally accounted for and no additional compensation is necessary as long as bleaching can be controlled. Both approaches yield the same result in terms of dissociation constants, but the simplicity of the latter approach, in which no additional expressions must be included in the curve fitting, makes this method more attractive in practical situations. Note, that the kinetic extraction is independent of at which point in time the actual measurement started since only new liposomes that bind during the measurement are included in the analysis.

6.3 Time-of-flight secondary ion mass spectrometry (TOF-SIMS)

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is a surface sensitive mass spectrometry technique developed in the early 1980s[121]. In a simplified way, it can be viewed as a microscope providing chemical information of the molecules on the surface with a high lateral resolution. The technique is highly surface sensitive since the total signal originates from the first molecular layers of the sample. This chapter will introduce the basic concepts of imaging mass spectrometry using TOF-SIMS and address the advantages and challenges of the technique. Furthermore, the use of TOF-SIMS for analysis of biological samples is discussed in the context of lipids and lipid membranes, which have, so far, been the main applications of the technique for biological samples.

6.3.1 Principles of time-of-flight secondary ion mass spectrometry

The principle components in a TOF-SIMS instrument is schematically illustrated in Fig. 6.4. A focused and pulsed ion beam is directed towards the sample surface and upon impact, the high energy ions, so-called primary ions, cause the ejection of molecules and fragments from the sample surface. A minor fraction of the ejected particles will be ionized, called secondary ions, which allows for their subsequent electrostatic collection and acceleration into the time-of-flight analyzer. Inside the analyzer, the ions travel in a field-free region at a constant kinetic energy, which is the same for all the ions with the same charge (in TOF-SIMS almost all secondary ions have single charge, + or -). With the kinetic energy given by $mv^2/2$, the velocity of the secondary ions will depend on their mass (or strictly, the mass-to-charge ratio, m/z). Since all ions travel the same distance in the TOF analyzer, and they were all formed simultaneously upon impact of the primary ion pulse on the sample surface, lighter ions will move faster and thus reach the detector at an earlier time as compared to the heavier ones. By adding the signal from many pulses and keeping track of the flight time of each detected secondary ion, a mass spectrum containing separate peaks for each type of secondary ion is obtained.

The produced secondary ions display a varying degree of fragmentation ranging from entire molecular ions down to atomic species. As a general rule, smaller fragments exhibit higher yields than larger fragments but typically contain less chemical information. In contrast, large fragments or molecular ions enable precise chemical identification of the molecules in the sample. Since monatomic ion sources, such as In and Ga, can be focused with high precision, they have been commonly used in imaging TOF-SIMS, providing a spatial resolution of down to 100-200 nm. However, for biological samples, these high energy primary ions result in poor yield of larger fragments, obscuring the identification of different biomolecules[122]. The introduction of cluster primary ion sources, such as Au_n and Bi_n, greatly improved the possibility to analyze biomolecules by producing increased yields of high-mass secondary ions. Using these ion sources, large biomolecules, such as phospholipids, can be imaged at submicrometer lateral resolutions.

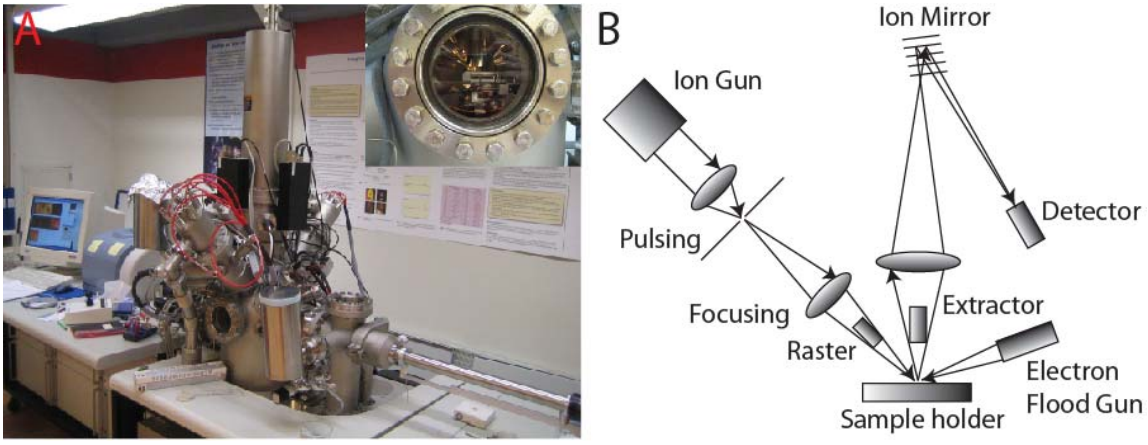

Figure 6.4. The TOF-SIMS instrument. A) TOF-SIMS IV (ION-TOF GmbH, Germany). B) Schematic principle. Primary ions from the ion gun are focused onto the sample in a raster pattern in short pulses. After each pulse the extractor collects the ionized fragments that are dislocated from the surface. The fragments are separated according to their mass-to-charge (m/z) ratio in the time-of-flight analyzer before reaching the detector.

Depending on the complexity of the sample and which type of information that is desired, TOF-SIMS analysis can be performed in two different modes, with the instrument optimized for either high mass resolution or high spatial resolution. The pulse length of the primary ions determines the mass resolution, which means that the shorter the pulse, the higher the mass resolution. By squeezing the primary ion pulse (bunched mode) into a shorter pulse, higher mass resolution ($m/\Delta m$ ~5,000) can be achieved, discriminating for instance between Si (m/z 27.977) and C_2H_4 (m/z 28.032). The bunching, however, is made at the expense of a larger beam focus, i.e., reduced spatial resolution, which in this case is around 2-5 μm[123]. In contrast, to achieve maximum spatial resolution, bunching is turned off and the primary ion beam is focused via additional apertures (burst alignment mode), reaching below submicron resolution. This, however, is at the expense of a reduced mass resolution ($m/\Delta m$ ~300). The relatively low mass resolution in the burst alignment mode is typically not a problem for materials with few chemical constituents, which yield a few well separated peaks, but could pose a serious problem for complex biological samples containing a multitude of molecular entities.

One of the shortcomings of the TOF-SIMS technique is the limited quantitative information, which means that the absolute signal intensity cannot be directly correlated to the abundance of a specific molecular entity on the analyzed surface. Although the signal in principle is proportional to the surface concentration of the monitored compound, the absolute signal also depends on other factors, most notably the so-called matrix effect. Matrix effects can be explained as variations in yield of various secondary ions due to the chemical environment of the analyzed molecules. Biological samples are often complex, multi-component systems, and are therefore likely to cause complications in the quantitative analysis due to matrix effects. Even for lipid model systems, suppression and enhancement of specific fragments have been observed, which stress the importance of careful analysis of the obtained data[124].

6.3.2 Sample preparation

TOF-SIMS is an ultra high vacuum technique, which makes sample preparation of biological samples a crucial issue. Many different procedures have been developed and evaluated, the most common ones being based on various freeze-drying procedures. In this approach, the water-immersed biological sample is rapidly frozen by dipping it into a cryo-chamber containing liquid propane (~ -180 °C) or liquid nitrogen (-196 °C). The rapid freezing prevents the water from forming large ice crystals, which could otherwise damage the native structure of the sample. The sample is then moved, in the frozen state, to a vacuum chamber where the frozen water is slowly removed by sublimation.

6.3.3 Analysis and imaging of lipids in TOF-SIMS

With the development of new cluster ion sources, resulting in higher yields of large secondary ions (typically a few hundred atomic mass units, amu)[125], TOF-SIMS has emerged as an important tool for analysis of biomolecules. Recent studies using TOF-SIMS have demonstrated the possibility of mapping the spatial distribution of a variety of lipids and cholesterol in biological tissues[126-128]. Phosphatidylcholine (PC) is one of the most frequently studied lipids, partly because of its biological relevance and high abundance in cell membranes, but also due to several characteristic secondary ion peaks with high secondary ion yields. Fig. 6.5 shows a positive ion TOF-SIMS spectrum of a PC bilayer on SiO_2, highlighting three characteristic peaks at m/z 86, m/z 166 and m/z 184, which all originate from the phosphocholine head group. Thanks to the improved yields of large secondary fragments when using cluster primary ions, it is also possible to obtain a clear signal of the molecular peak of PC[129]. The inset shows the molecular ion at m/z 760 which illustrate the high specificity but also the significantly reduced yield as compared to fragments of lower mass.

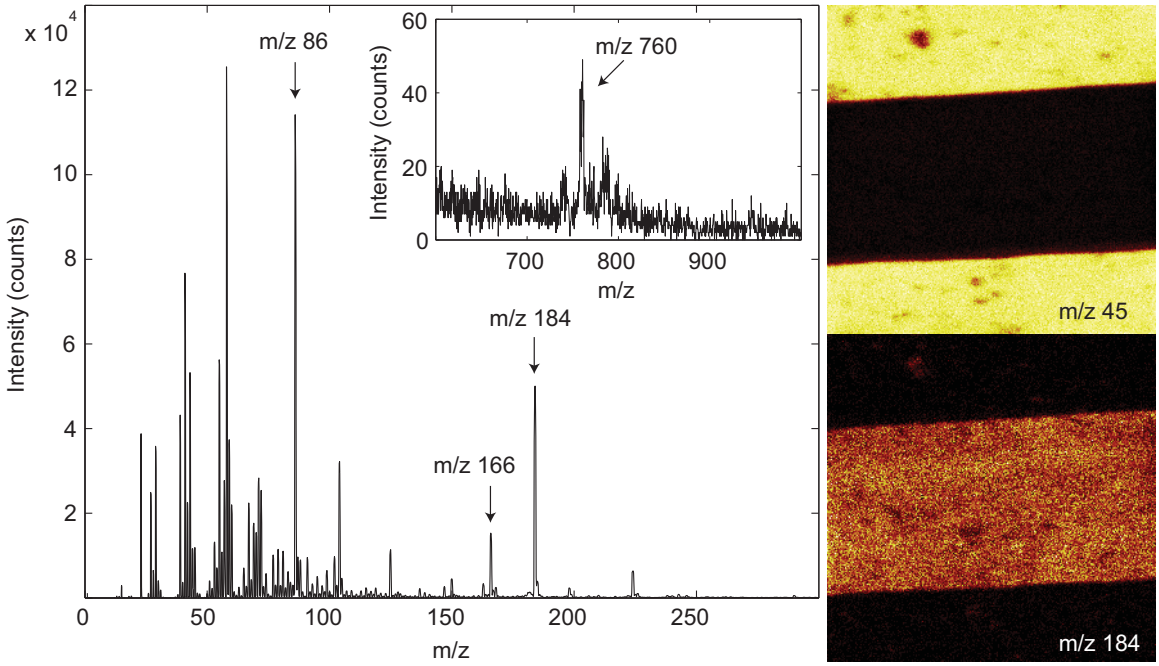

Figure 6.5. Positive TOF-SIMS spectrum of a PC bilayer showing three characteristic peaks at m/z 86, m/z 166 and m/z 184. The inset shows the peak of the molecular ion. The two ion images show the signal intensities of a peak characteristic for thiol-PEG, at m/z 45, and of a peak characteristic for PC at m/z 184, respectively. Field of view is 200×200 μm^2.

Fig 6.5 also illustrates the imaging capability of the TOF-SIMS instrument, by which the spatial localization of specific fragments can be visualized. In this example, fragments representing the PC lipid (m/z 184) and the polymer (thiol-PEG) surface modification (m/z 45) are shown. The detailed chemical information is obtained by analyzing the sample in the so-called static mode[123] which means that the sample is only exposed to low primary ion doses that do not cause significant molecular damage on the sample surface during the analysis. In contrast, dynamic SIMS is carried out at high primary ion dose densities, typically using a continuous primary ion beam (as opposed to the pulsed beam used in TOF-SIMS), which cause severe molecular damage to the sample surface. The chemical information in dynamic SIMS is therefore normally limited to atomic and very small secondary ion fragments. The advantage of using a continuous beam, however, is that it can be well focused and recent work has demonstrated the distinction of co-existing gel and liquid phases in model membranes at a resolution of roughly 100 nm[130]. Although an exceptionally high lateral resolution can be achieved, the molecular damage necessitates isotope labeling of the lipids in order to assign a particular mass to its parental molecule. In doing so, one of the main advantages with mass spectrometry techniques, namely the possibility to analyze unlabeled samples, is lost. To circumvent this drawback, significant efforts have been focused on improving the lateral resolution in TOF-SIMS without sacrificing the possibility to analyze sufficiently large fragments. Primarily, these improvements rely on the new cluster ion sources mentioned above, which allow for a high lateral resolution while providing a sufficiently high yield of large fragments and molecular ions to enable label-free identification of biomolecular species.

"If your experiment needs statistics, then you ought to have done a better experiment"

Ernest Rutherford

7

Summary of appended papers

This chapter summarizes the main results in the appended papers. It is divided into three sections; each addressing one of the three challenges in bioanalytical sensing described in the introduction, namely (i) high sensitivity, (ii) ability to probe binding kinetics of membrane receptor-ligand interactions and (iii) multiplexed detection.

7.1 Detection and dissociation kinetics of single DNA molecules with high sensitivity

The challenge of probing biomolecular recognition with high sensitivity was addressed by the development of an assay format with single molecule sensitivity. Previous work in the group had shown that liposomes could easily be modified using cholesterol-conjugated DNA that spontaneously insert into the lipid membrane[41]. Establishing DNA as an excellent model system for biorecognition, we designed a sandwich assay in which unlabeled single-stranded DNA targets mediated the immobilization of DNA-modified, fluorescently-labeled liposomes onto a probe-modified surface. The fluorescent enhancement of the liposomes, containing approximately a thousand fluorophores, resulted in high signal-to-noise ratios for individual biorecognition events also under conditions in which each interaction was mediated by a single DNA hybridization.

Furthermore, we demonstrated that with single molecule resolution, the sensitivity of the assay is no longer limited by single-to-noise but rather the actual surface coverage, as long as unspecific binding is negligible. In reality, however, proper surface chemistry, or specifically, the capability to minimize unspecific binding while ensuring specific binding, is in fact the limiting factor as the surface coverage is decreased at lower concentrations (Eq. 2.3). Using a polymer-coated surface consisting of biotinylated

PLL-*g*-PEG, we managed to demonstrate an inert surface to DNA and liposome adsorption and hence we were able to show sequence specific detection of individual DNA targets at 10 fM concentrations (paper I).

Due to the reduction of fluorescence background provided by the evanescent-field illumination of the TIRFM, immobilized liposomes could be detected in the presence of suspended liposomes. Besides sensitive detection, this feature enabled us to probe binding kinetics of the biorecognition and in particular the dissociation kinetics by monitoring the residence time of individual immobilized liposomes. Exploring several aspects of this possibility (such as the thermodynamic behavior of DNA hybridization at surfaces, paper II), perhaps the most striking one was the ability to distinguish different DNA sequences based on their kinetic behavior. Typically, sequence identification using solid state hybridization technology such as DNA microarrays; rely on the ability that different sequences recognize its perfect match on the chip. However, tiny sequence variations such as single nucleotide mismatches or single nucleotide polymorphism (SNP) can often cause difficulties, especially if the relative concentrations of the different sequences differ substantially. This is because surface coverage (which is proportional to the signal) is related to target concentration (Eq. 2.3). Small signals due to mishybridization can therefore be enhanced by larger relative amounts of mismatched sequences. In contrast, we demonstrate that mismatch discrimination based on differences in dissociation kinetics is unaffected by concentration variations and hence potentially more reliable (paper II). In fact, we could discriminate a single nucleotide mismatch by a factor of two difference in dissociation rate constant as shown in Fig 7.1.

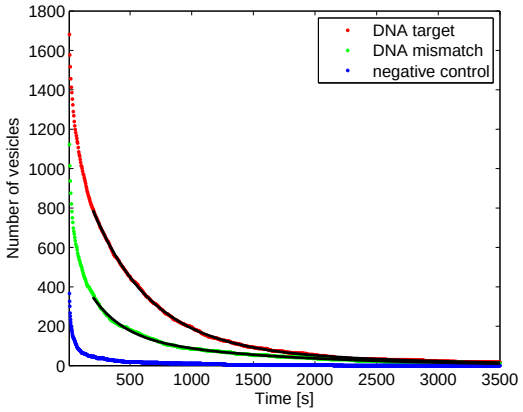

Figure 7.1. Typical dissociation trends plotted as the number of liposomes that still remain bound after a certain time for a fully complementary sequence (red) and the same sequence containing a single mismatch. The dissociation trends were obtained by simply converting the residence time histogram generated from each measurement i.e. the difference in the number of liposomes between each time interval corresponds to the magnitude of subsequent bars in a residence time histogram. Detection of specific recognition events was obtained by distinguishing bound liposomes from non-interacting liposomes that disappear from the surface during the time between two consecutive frames (see section on image analysis). The dissociation rates were fitted to double exponential functions $f(t) = A_1\exp(-k_{off,1}t) + A_2\exp(-k_{off,2}t)$ (black lines).

7.2 Dissociation kinetics of membrane receptor-ligand interactions

To further explore the unique opportunities provided by a liposomes-based assay format we attempted to address the challenge of probing membrane receptor-ligand kinetics in a natural cell membrane environment. We selected two high-performing human recombinant single chain Fv (scFv) antibodies (clone c10 and cb26)[131] from the n-CoDeR library[132] as ligands directed against two key immunological plasma membrane receptors. Since similar ligands from the same library are currently being explored in clinical settings (by Bioinvent AB), the two recombinant antibody fragments used in this work are excellent biopharmaceutical model systems.

We demonstrated, by simple procedures; the production of liposomes derived directly from fluorescently labeled cell membranes and verified by QCM-D that these proteoliposomes contained the two membrane receptors of interest (paper III). In order to improve the sensitivity and be able to extract kinetic information, individual binding events of fluorescently labeled cell membrane derived liposomes were monitored by TIRFM, in analogy with the DNA assay. Thanks to the evanescent-field illumination, the residence time of bound liposomes could be obtained from an equilibrium fluctuation analysis at stagnant liquid conditions, despite the presence of suspended liposomes in the solution. Particularly important in these experiments was the successful development of high-performing surface chemistry that eliminated unspecific binding reactions since a non-purified suspension of liposomes derived directly from extruded cells was used. We demonstrated that a SLB containing a small fraction of chemically active lipids provide an inert background that prevents nonspecific liposome binding while enables a gentle means to immobilize the ligand.

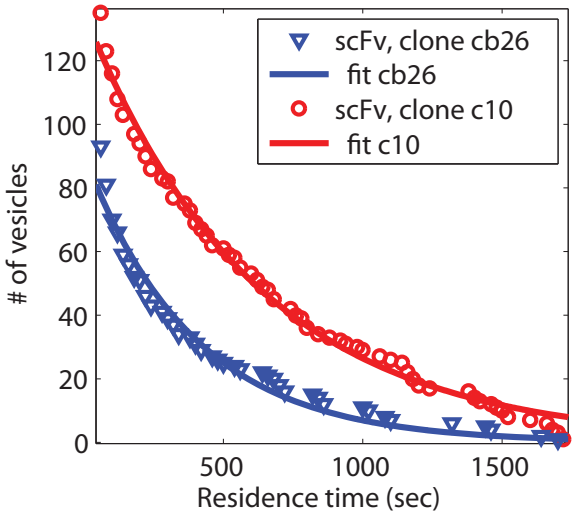

Figure 7.2. Typical dissociation trends for two different membrane receptor-ligand interactions extracted from individual time traces of cell membrane derived liposomes interacting with the surface-immobilized ligand (scFv). The dissociation rates were fitted to single exponential functions $f(t) = A\exp(-k_{off}t)$ (solid lines).

From single exponential fits, the dissociation rate constants were determined, which were in good agreement with the dissociation rate constants previously reported for scFv interactions with water soluble compounds[133]. The fact that the equilibrium coverage fluctuation analysis is performed at stagnant liquid conditions makes the assay compatible with high-throughput formats such as microtiter plates.

Although high sensitivity and hence low sample consumption are obvious benefits, the nature of the inverted assay (immobilized ligand and receptor in solution) also poses certain limitations such as the uncertainty of the number of membrane receptors per liposome. More than one receptor per liposome facilitates multivalent interactions, i.e. the liposome is immobilized on the surface by more than one recognition pair, which may obstructs an accurate kinetic analysis.

7.3 Multiplexed detection of individual DNA molecules using imaging mass spectrometry

To address the final challenge mentioned in the introduction, namely multiplexed detection at the level of single molecules, we adopted a random array approach relying on molecular self-assembly. Also in this study, sequence-specific DNA hybridization was used as a model system for biorecognition. The basic idea was to analyze the chemical composition of individual DNA-immobilized liposomes and use the information as molecular fingerprint for parallel readout. The first step included fabrication of a substrate and development of a sample preparation procedure that enabled TOF-SIMS analysis of individual liposomes on the sensor surface. Using this procedure, we demonstrated that individual lipid objects with a diameter of a few hundred nanometers could be imaged and chemically indentified (paper IV).

To investigate if the concept could be used for multiplexed biomolecular sensing, two liposome populations, consisting of either POPC or D^{13}-DPPC lipids (PC with deuterated head group), each modified with a specific recognition sequence were introduced. As an initial proof-of-concept, the TOF-SIMS instrument was only used for surface sensitive chemical analysis (i.e. no imaging). From such a measurement we could demonstrate simultaneous detection and identification of two different DNA sequences via identification of the lipid content on the surface, as shown in Fig 7.3 (paper V).

For a sample incubated only with target DNA sequence A, the (top) spectrum showed a clear signal of POPC liposomes only, i.e., liposomes modified with the complementary cholesterol conjugate of target DNA A. The inverse pattern was observed in the next spectrum, originating from a similar sample but incubated only with DNA sequence B. Finally, we observed signals from both types of lipids for the third sample which was incubated with both A and B, which demonstrated the functionality of the concept.

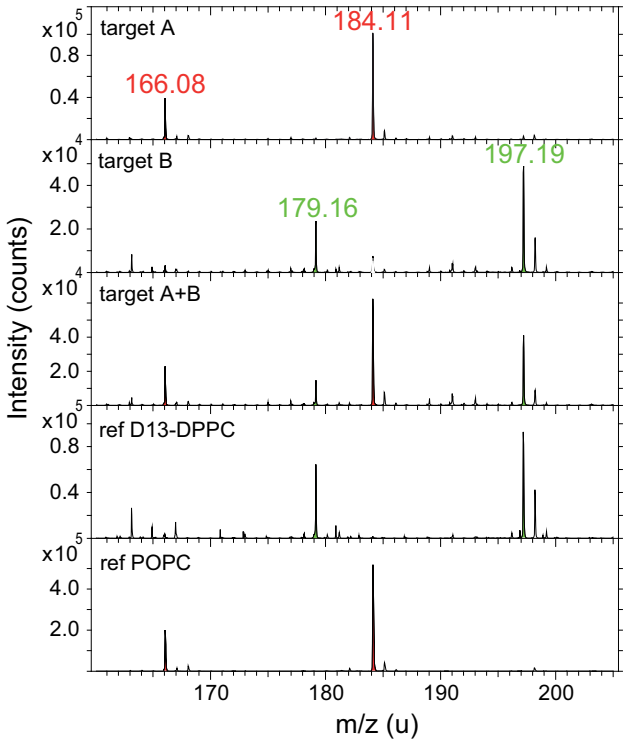

Figure 7.3. Positive ion spectra for three different samples exposed to different target DNA sequences (A, B or both) and two reference spectra for the lipid in each liposomes population. Important mass peaks are color coded to simplify interpretation. Red and green peaks are characteristic for POPC and D^{13}-DPPC respectively.

Although the high mass resolution of the TOF-analyzer enable distinction of many different compounds, the sensitivity and the multiplexing capacity without imaging are likely to be limited by mass interferences between different barcode signals and fragment ions from other molecules on the surface. However, utilizing the high resolution imaging capability of the TOF-SIMS instrument, individual liposomes can be located and chemically identified, as shown in Fig 7.4. In addition to offer single molecule detection capability, the identification of individual liposomes provides a considerable advantage when it comes to the ultimate multiplexing capacity. This advantage stems from the elimination of overlapping signals from liposomes with different chemical composition and thus enables lipid species to be mixed within one liposome. This unique possibility dramatically increases the library of possible barcodes reaching, under reasonable assumptions, beyond a million.

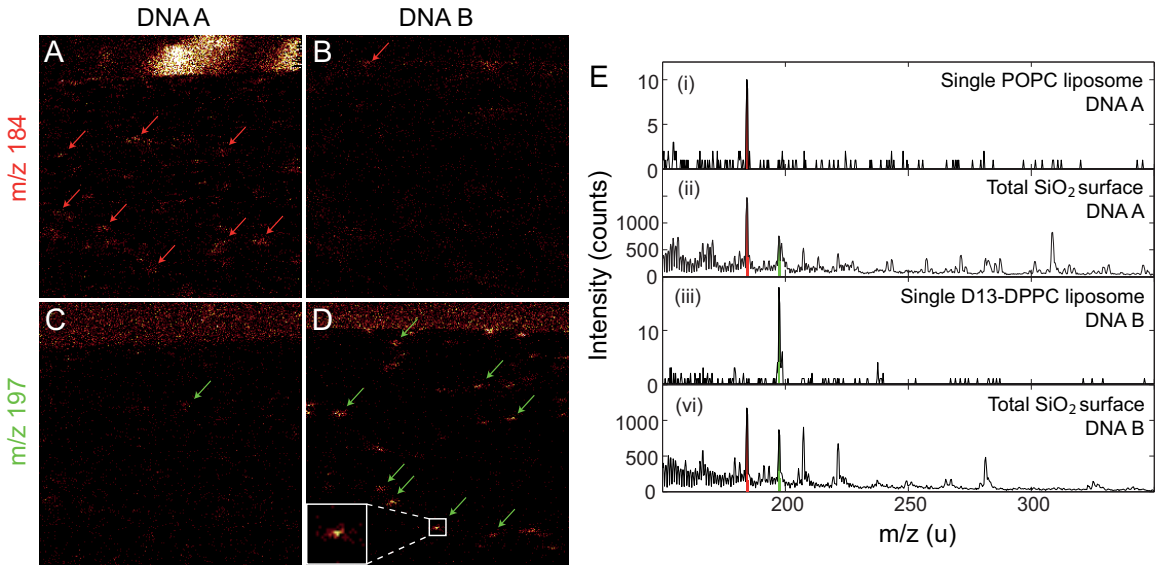

Figure 7.4. Ion images of individual liposomes (Ø~300 nm) on two different substrates incubated with 100 pM DNA target A (A,C) or DNA target B (B,D). The top and bottom rows show ion images for m/z 184 (specific for POPC) and m/z 197 (specific for D^{13}-DPPC), respectively. Small, high intensity spots [inset] represent individual liposomes (some of which are marked with red [POPC] and green [D^{13}-DPPC] arrows to simplify interpretation). Field of view is 50 x 50 µm. E) Spectra from a single (i) POPC and (iii) D^{13}-DPPC liposome and spectra from the total area in ion image (ii) A and (iv) D. The POPC (m/z 184) and D^{13}-DPPC (m/z 197) specific peaks are colored in red and green, respectively.

Another striking observation is that the single liposome resolution actually enhances the sensitivity, which in the non-imaging measurements is limited by background signal from other lipids. A comparison of the two lowest spectra in Fig 7.4 originating from a single liposome or the entire field of view clearly demonstrate a reduction in background signal (in this case POPC, red) for the single liposome spectrum. In addition, the ability to count individual recognition events potentially enables quantitative measurements, which is otherwise a major obstacle for MS analysis in general (i.e. not only in TOF-SIMS).

"Predictions can be very difficult
-especially about the future"

Niels Bohr

8

Perspectives and outlook

At this point, I hope I have convinced the reader of some of the potential benefits of bioanalytical sensing at the single molecule level. In this chapter I will discuss in some detail two potential extensions of the liposome-based assay format presented in this thesis.

8.1 Future applications

The assay format described in this thesis provides several potential benefits in comparison with alternative bioanalytical tools such as high sensitivity (~fM) while preserving the possibility to extract kinetics information. The single molecule resolution also enable acquisition of kinetic information at equilibrium binding conditions, thus eliminating the need for controlled liquid handling making it compatible with high-throughput formats such as microtiter plates. Furthermore, the elimination of liquid flow and multiple sample injections for sequential measurements make the assay highly attractive in terms of minute sample consumption. The assay format is also appealing due its relative simplicity which relies on self-assembly principles which, in combination with a high multiplexing capacity provided by the imaging MS analysis, could even compete with state-of-the-art microarray technology in terms of both density and number of probe molecules.

In this context, it is also important to note general limitations of the assay format. While direct determination of k_{off} is relatively straightforward, extraction of k_{on} and K_d is more difficult to access. This is primarily related to the readout principle which relies on image analysis that requires spatially separated objects. Due to the resolution limits of any optical microscope, all liposomes cannot be analyzed at maximum coverage, which in turn prevent direct determination of K_d (and indirectly k_{on}).

With that said, two other aspects are perhaps the most attractive features of the liposome-based assay, namely the compatibility with membrane-associated interactions and the possibility to perform multiplexed detection on intrinsically disordered samples such as random arrays, cells or even tissue sections. One way to utilize the latter benefit is schematically illustrated in Fig. 8.1, which could potentially be an interesting alternative to traditional immunohistochemistry. Here, antibody-modified liposomes with different chemical composition are used as fingerprints for parallel detection of individual proteins on a tissue section. Although widely applied in the medical community, traditional immunohistochemistry, which typically based on dye-conjugated antibodies or structure specific dyes (such as DNA-intercalating dyes) still faces several limitations. These limitations include (i) the maximum number of different constituents that can be analyzed simultaneously, (ii) the difficulty of co-analyzing protein and lipid distributions and perhaps more importantly, (iii) the need for prior knowledge of what to detect (i.e. what antibodies to use) and (iv) the risk to exclude important information that was present in the sample but not screened for.

An alternative approach to optical methods is to use imaging MS. One of the main difficulties with an MS approach, however, is the trade-off between spatial resolution and detectable mass range. To clarify this, imaging matrix-assisted laser desorption ionization (MALDI) enable soft ionization and hence identification of entire protein molecules (typically 20-100 kDa) but only at a spatial resolution of approximately 100×100 μm^2. In contrast, TOF-SIMS imaging provides a much higher lateral (and depth) resolution on the order of ~100 nm but at the expense of molecular fragmentation which obstructs protein identification in complex samples. Our approach is therefore to combine two concepts, enabling indirect protein detection and identification (down to level of single proteins) to be accomplished via liposome-based chemical barcodes (Fig 8.1) while maintaining the high lateral resolution provided by the TOF-SIMS. This assay format provides several benefits as compared to traditional immunohistochemistry such as (i) unlimited multiplexing capacity and perhaps more importantly, (ii) the ability to simultaneously acquire the intrinsic signal from unknown species in the sample such as lipids and carbohydrates. This enables unique possibilities to investigate co-localization of protein- and lipid distributions with a high spatial resolution as illustrated in Fig. 8.1, (note that Fig. 8.1B and 8.1C are separate TOF-SIMS ion images of the lipid distribution in tissue and surface-immobilized liposomes but the overlay in D is false and is only present to illustrate the idea).

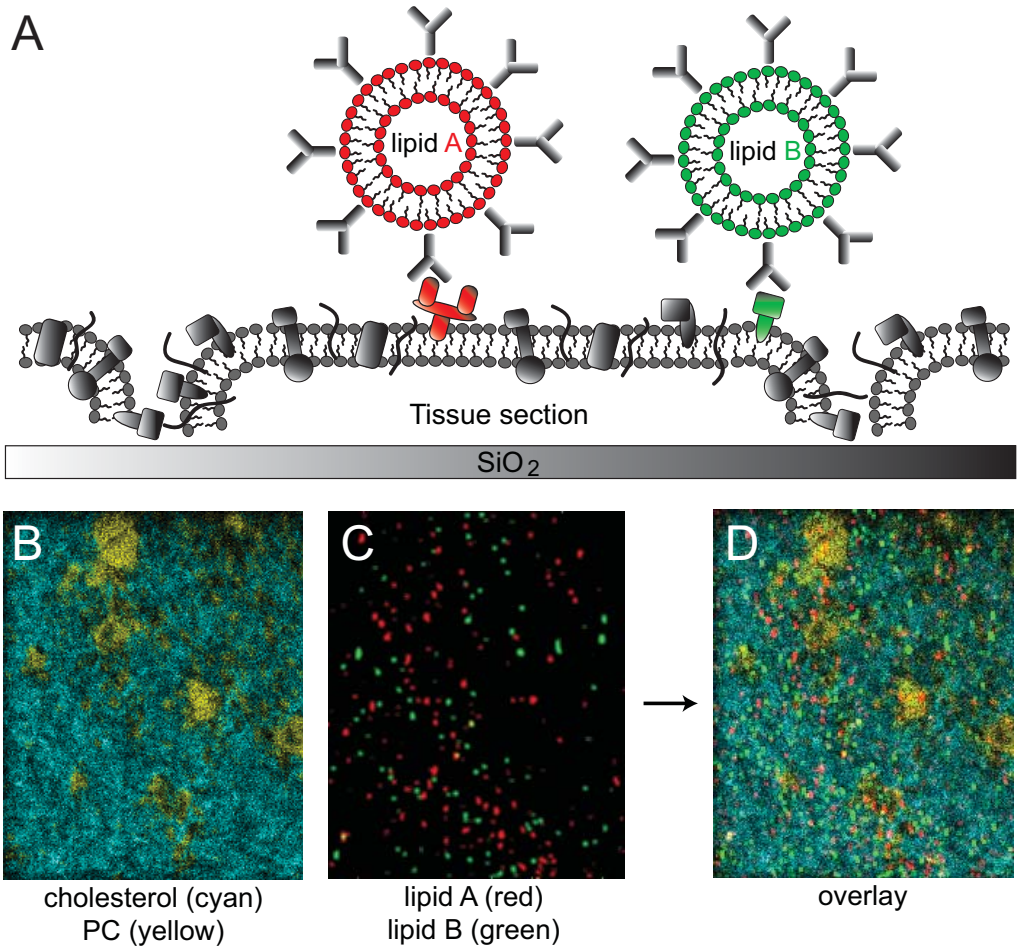

Figure 8.1. (A) Schematic illustration of mass spectrometry based immunohistochemistry. Antibody-conjugated liposomes with different chemical composition act as chemical fingerprints for multiplexed protein detection on a tissue section. B) Ion image of lipid and cholesterol distribution in rat brain tissue. C) Ion image showing detection and chemical identification of individual liposomes with two different chemical compositions. D) False overlay of data from B and C for illustration purposes showing simultaneous detection of lipid and multiple protein species.

A second outlook relates to the compatibility with membrane-associated interactions. Although the first steps were already taken in paper III, various other types of membrane-associated interactions can be probed in a similar format. I will highlight one such extension which is already work in progress and relates to the investigation of virus interactions with, so far, glycolipid-modified model membranes. The modified liposome assay is schematically illustrated in Fig. 8.2, in which a glycolipid-modified SLB facilitate surface-immobilization of virus particles. Detection is accomplished by addition of fluorescently-labeled, glycolipid-modified liposomes that interact with the virus particles in a transient manner which enable extraction of kinetic information. The microtiter plate-compatible assay format allows potential screening of unkown ligands as well as inhibitors for drug/vaccine application. Simultaneously, it provides detection of single virus particles.

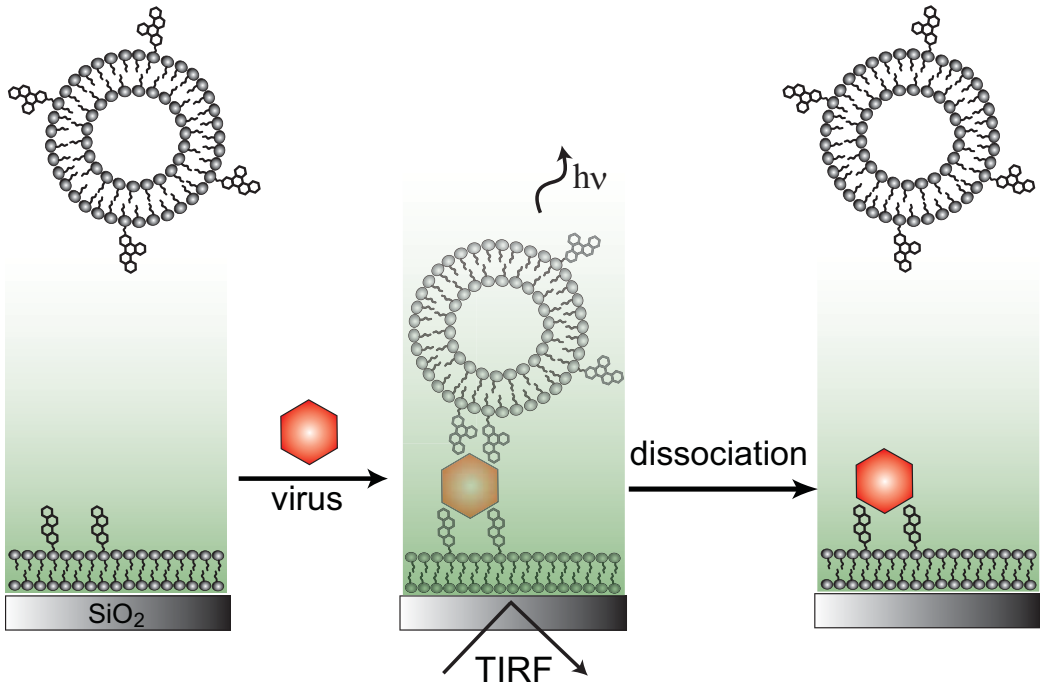

Figure 8.2. Schematic illustration of the liposome-based single virus interaction assay. Glycolipid-modified fluorescently labeled liposomes are immobilized on the carbohydrate-modified SLB in the presence of bound virus particles. The transient interaction can be monitored over time which enables extraction of binding kinetics under stagnant liquid conditions.

Furthermore, this assay format also provides unique opportunities to investigate multivalent interactions[134] (such as virus-cell membrane interactions), in which the single molecule resolution may play an important role. Besides sensitivity, one of the strengths of the assay is the ability to simultaneously probe both rapid and slowly dissociating populations. For simplicity, assume two populations of binders (liposomes) with radically different residence times ($k_{off,1} = 10^{-1}$ s^{-1} and $k_{off,2} = 10^{-3}$ s^{-1}) due to variations in e.g. multivalency. Using the Langmuir binding model it can be shown that the relative equilibrium coverage of the two populations is directly related to the ratio of the dissociation rate constants (Eq. 8.1), given the reasonable assumption that the association rate constants, k_{on}, are the same for both populations.

$$\frac{\Theta_1}{\Theta_2} = \frac{k_{off,2}k_{on,1}}{k_{off,1}k_{on,2}} = \frac{k_{off,2}}{k_{off,1}} \qquad if\ k_{on,1} = k_{on,2} \tag{8.1}$$

This implies that for the two populations above, approximately 99% of the total signal (coverage) in an ensemble measurement (using for instance QCM-D or SPR) originates from the slowly dissociating population. To probe kinetic processes of the rapidly dissociating population therefore require excellent signal-to-noise which in reality is often not the case. In contrast, monitoring the dynamics of individual liposomes will include statistics from both (or potentially many) populations. In fact, in this case the

rapidly dissociation population will contribute with the majority of the dissociation events.

In summary, I hope I have illustrated with the included papers and the two future applications mentioned above that the liposome-based assay described in this thesis may contribute to future development and understanding in the field of biomolecular recognition studies.

"People who write obscurely are either unskilled in writing or up to mischief"

Herbert M. McLuhan

9

Acknowledgements

It has come to my understanding that this chapter is the selective section in any thesis read by most people. I have therefore decided to put additional effort in writing this particular chapter.

As always, I would like to start with a big thank you to my main supervisor, Fredrik Höök. Although you made me move back and forth between Gothenburg and Lund four (!) times in less than two years, I'm happy that I finally decided to stay in your group. You have been a great supervisor in many aspects, not only as a mentor in science but also in other aspects of my professional life.

I would also like to thank my co-supervisor, Peter Sjövall. You have been a great asset as a bridge between academia and the outside world. I can't remember all the journeys to SP in Borås, hoping for new results at the end of the day. But we finally managed to succeed and now we even got a VR grant to continue our project! Thanks for not giving up on me.

I would also like to mention Jonas Tegenfeldt, who introduced me to TIRFM and in doing so, partly initiated the microscopy activities in Fredrik's group. Looking at the microscopy activities today and all the equipment in the group, it was quite an important moment.

Moving on to the other members of our group I would especially like to thank the "Lund crew". It's been quite a journey! As the other groups at FTF in Lund always pointed out; "there is something special about the Biogroup". Maybe it was the Biosport, maybe it was the Friday beer or maybe simply the relaxed atmosphere at the afternoon coffee break? Peter Jönsson, you have been an invaluable source of knowledge for me. Thanks for all the help with the image analysis, the many discussions on "jumping vesicles", the "pushing bilayer" collaboration and last but not least, for being a great friend. Magnus Jonsson, the oldest (and wisest?) guy in the Lund crew. Although we never actually had any projects together you are "like a brother to me", or at least that's what they claim. Although I thought I knew quite a few "snapsvisor", I apparently missed

the best one (“Järnvägsbommar”), which you taught me on several occasions. And finally Lisa, the last member of the “Lund crew”, what would my PhD studies have been without you? How many vacations did we spend together so far? Is it approaching twenty? And how many parties did we enjoy together? Is it approaching a hundred? And now we even have a common research project, what more is there say?

Of course, my collaborators in Sweden and abroad as well as the rest of the group members in both Lund and Gothenburg deserve an acknowledgment. Thanks for all the great coffee breaks. In particular you, Marta should have an extra credit for your brave attempt to continue on the winding road of single liposome analysis. Best of luck and watch out for those multiexponentials!

And finally a big hug to my wife and family. Thanks mom and dad! I know how badly you wanted to be acknowledged in this thesis ☺ so here it is! I hope you finally understand a little bit of what I have been trying to achieve during the last couple of years. And last but not least, a big kiss to you, Jenny, my wife. I could of course write something (sissy) like “you have always been a great support” but now when I think of it, have you really? Instead I want to thank you for turning your life upside down for me and moving back to Gothenburg. I wrote in my licentiate thesis that I might marry you one day. And so I did. And to keep up with this tradition I will give another vow this time. Who knows, one day we might have children together.

"Science knows only one commandment
-contribute to science"

Bertolt Brecht

10

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