BIOMIMETIC INTERFACES FOR SURFACE SENSITIVE DRUG DISCOVERY TECHNIQUES

Niko Granqvist

ACADEMIC DISSERTATION

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Supervisors:  Professor Marjo Yliperttula  
Division of Biopharmaceutics and Pharmacokinetics  
Faculty of Pharmacy  
University of Helsinki  
Finland

Dr. Tapani Viitala  
Division of Biopharmaceutics and Pharmacokinetics  
Faculty of Pharmacy  
University of Helsinki  
Finland

Reviewers:  Dr. Eng. Krzysztof Noworyta  
Department of Physical Chemistry of Supramolecular Complexes  
Institute of Physical Chemistry Polish Academy of Sciences  
Poland

Professor Yvonne Perrie  
Aston Pharmacy School  
Aston University  
United Kingdoms

Opponent:  Dr. Sci. (Tech.) Lasse Murtomäki  
Department of Chemistry  
School of Chemical technology  
Aalto University  
Finland

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ABSTRACT

For the last few decades, the expenses of pharmaceutical development and drug discovery have been constantly increasing whereas the amount of new pharmaceutical products reaching the market has been diminishing. The drug discovery methods today rely heavily on different screening technologies in the early discovery phase. High-throughput screening is usually the dominant approach along with different computational methods, but these methods lack the ability to monitor the interactions between drugs and cells in real-time. The ability to measure drug-cell interactions and cell responses during drug stimulation in real-time could provide complementary kinetic information to traditional methods already used in drug discovery. This time-resolved information should help to build a better mechanistic understanding of the effect of drug formulation design on the drug release actions, the drug delivery process and the efficacy of the drug, especially when it comes to new biological drugs and nanoparticle formulations.

This dissertation addresses challenges in developing functional surfaces and analysis methods based on the surface plasmon resonance technique for pharmaceutical research purposes. The research in this thesis spans from traditional drug-protein interaction studies and preparation of cell model surfaces to interaction studies with living cells. An approach where proteins were immobilized in a hydrogel was used for studying the interaction kinetics between protein kinase Cε and both an activating and an inhibiting single-chain antibody. The affinities determined for the interactions were able to predict the level of activation or inhibition in subsequent cell culture assays.

This thesis also presents two types of new analysis methods, i.e. label-enhanced and multi-wavelength surface plasmon resonance (SPR) methods were developed in order to improve the sensitivity of bioassays and accuracy for characterizing ultra-thin films, respectively. The label-enhanced SPR method was shown to improve assay sensitivity up to 100-fold, whereas the multi-wavelength SPR analysis provided the means to characterize organic layers in the range from a few nanometers to hundreds of nanometers, i.e. layer thicknesses of relevance to biological membranes and hydrogels.

New surface coating chemistries based on dextran and thiol-PEG were also developed in this thesis in order to enable the preparation of robust biomimetic membranes by vesicle spreading or adsorption. The dextran-based and PEG-based coatings promoted supported lipid bilayer and adsorbed vesicle layer formation, respectively. The new analysis approaches developed in this thesis were further utilized in order to characterize the optical properties of the formed lipid layers on the dextran- and PEG-based coatings. Finally, a new analytical approach for signal processing of the real-time and label-free SPR measurements performed together with living cells is introduced which provides the mean to differentiate between para- and transcellular cell absorption routes of drug molecules.

This dissertation contributes to the pharmaceutical research field by introducing new measuring tools, improved in vitro biomimetic models and new approaches for
processing of the signal from label-free measurements in order to provide relevant real-time and complementary information to traditional drug development and discovery tools. This will hopefully benefit the pharmaceutical research field and possibly enable a more efficient development of new pharmaceuticals and therapies in the future.
ACKNOWLEDGEMENTS

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Liljalle
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>Au</td>
<td>gold</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Caco2</td>
<td>heterogeneous human epithelial colorectal adenocarcinoma cells</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CMD</td>
<td>carboxymethyl dextran</td>
</tr>
<tr>
<td>d</td>
<td>thickness</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl cellulose</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DPI</td>
<td>dual polarization interferometry</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EggPC</td>
<td>Egg phosphatylcholine</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>IAM</td>
<td>immobilized artificial membrane</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffered saline</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical cancer cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
</tr>
<tr>
<td>HepG2</td>
<td>human hepatoblastoma cells</td>
</tr>
<tr>
<td>HSPC</td>
<td>hydrogenated soy phosphatylcholine</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>k</td>
<td>complex part of refractive index</td>
</tr>
<tr>
<td>ka</td>
<td>on rate of interaction</td>
</tr>
<tr>
<td>kd</td>
<td>off rate of interaction</td>
</tr>
<tr>
<td>KD</td>
<td>affinity of interaction</td>
</tr>
<tr>
<td>LB</td>
<td>Langmuir–Blodgett</td>
</tr>
<tr>
<td>MDCK II</td>
<td>Madin-Darby canine kidney type II cells</td>
</tr>
<tr>
<td>ň</td>
<td>complex refractive index ((\tilde{n} = n + ik))</td>
</tr>
<tr>
<td>n</td>
<td>real part of refractive index</td>
</tr>
</tbody>
</table>
NHS  N-hydroxysuccinimide
NIR  near infrared
OWLS  optical waveguide lightmode spectroscopy
PAMPA  parallel artificial membrane permeation assay
PAH  poly(allylamine hydrochloride)
PBS  phosphate buffered saline
PEG  polyethylene glycol
PEG-SAM  polyethylene glycol polymer modified with thiol
PEI  poly(ethyleneimine)
PEM  polyelectrolyte multilayer
PKCε  Protein kinase C epsilon
PLL  poly(L-lysine)
PM-IRRAS  polarization-modulated infrared reflection adsorption spectroscopy
POPS  palmitoyl-oleyl-phophatylserine
POPC  palmitoyl-oleyl-phosphatylcholine
PS-PMMA  poly(styrene)-poly(methyl methacrylate) copolymer
PSS  poly(styrene sulfonate)
QCM  quartz crystal microbalance
RAC  receptors for activated C kinase
RI  refractive index
RWG  resonant waveguide grating
SA  stearic acid
SDS  sodium dodecyl sulphate
SE  spectroscopic ellipsometry
SiO2  silicon dioxide
SLB  supported lipid bilayer
SPR  surface plasmon resonance
SSC  saline sodium citrate buffer
SVL  supported vesicle layer
TIR  total internal reflection
UAc  Uranyl Acetate
UV-VIS  absorbance spectroscopy using ultra-violet to visible light range
VHH  light chain antibody
1 INTRODUCTION

The ultimate goal of drug discovery is to produce a safe and effective drug for a given condition. Though modern high-throughput synthesis methods can produce massive amounts of new compounds for evaluation as drug leads, and computational methods for predicting chemical and biochemical interactions have been improving tremendously during the last few decades, there is still a lot to learn how a chemical affects a real biological system. As an unknown chemical compound cannot be tested in a human, because it is ethically extremely unacceptable, there is always a need to go through a series of \textit{in vitro} and \textit{in vivo} models before finding a clinically acceptable drug lead.

Biomimetic is a word used to describe that a human-made system is copying, imitating or adapting a natural biological system. Though most commonly used in describing for example new functional materials that mimic natural structures, like super hydrophobic surfaces created by mimicking lotus leaves, the term is often used for describing its other common use i.e. modelling biological systems through their components. The closest biomimetic model for the complete human physiology is an animal physiology, but using animals is both ethically questionable and expensive, so the amount of animal tests is kept as low as possible during drug development. Basically most tests performed during drug development could be called biomimetic tests, as for example the simple solubility tests utilizing model gastrointestinal fluids. The reason why these tests are biomimetic is that the prediction ability form \textit{in vitro} tests to the \textit{in vivo} situation of the given assay is usually the better the closer it mimics the real biological environment. An extremely good example of this is the parallel artificial membrane permeation assay (PAMPA) which predicts the gastrointestinal absorption of drugs in human much better than the octanol-water partitioning coefficient, especially for charged molecules \cite{1}. However, the knowledge, understanding and the ability to manipulate biological compounds is still rather limited, making it either completely impossible or too difficult and expensive to fully model biological systems. Therefore, there is an increasing need for a better understanding of how to prepare and manipulate simplified biomimetic interfaces for the purpose of drug discovery and development.

For a given drug development assay, it is always the interplay of simplicity, prediction ability towards the desired \textit{in vivo} situation and cost which determines how useful it is in drug development. Taking into account the above, there is a clear demand for improved assays, especially as the complexity of new drug molecules and formulations that enters the market is increasing, formulations are more specific in their treatment profile compared to earlier ones, and the increased use of nanotechnology in drug formulations are pushing the limits of the capabilities of current \textit{in vitro} assays \cite{2}.

There are two interesting types of assays which are commonly utilized in drug development. The first ones include the cell monolayer assays that model biological interfaces and membranes, e.g. Caco-2 and MDCK II that are mainly used as small intestine models \cite{3-7}, but also as many other tissue models, such as blood-brain-
Introduction

barrier [8,9], retinal epithelia [10] and epidermal [11] models. The second type of assays are different kind of supported lipid bilayers (SLBs) which can be used to model different biological membranes such as the cell wall [12] or the mitochondria membrane [13], or as a stabilizing environment for membrane proteins and receptors like G-protein coupled receptors (GPCRs) [14], as well as for passive drug permeation prediction like PAMPA [15]. So far, these measurements have mostly been used as static measurements which produce information in timescale of hours, such as drug or nanoparticle permeation of cell monolayers, and provide only limited information. Currently, the SLBs do not show enough biomimetic ability which limits their use as an in vitro platform in the drug development cycle for predicting the the final in vivo situation.

This work focuses on developing biomimetic interfaces and combining these with real-time and label-free detection methods to create new types of platforms and complementary approaches to traditional in vitro assays for drug discovery and drug development. The protocols and synthesis for preparing biomimetic interfaces based on supported lipid bilayers (SLB) and living cell monolayers on sensor surfaces for surface sensitive detection techniques (i.e. SPR and QCM) were developed and optimized. The ultimate goal is to provide complementary platforms for the future that could improve the in vivo predictability of purely interaction-based assays like PAMPA and ELISA, and to provide complementary real-time information for current traditional static cell-based assays, such as the Caco-2 and MDCK-II monolayers. Hence, the immediate goals of this work were to create a new and simplified protocol for preparing biomimetic air-stable SLBs for an easier large-scale industrial adaptation, and to optimize the immobilization protocols of living cells on the sensor surfaces. These biomimetic interfaces in combination with surface-sensitive techniques are anticipated to provide powerful platforms, which can measure biochemical and biophysical interactions in real-time and in conditions that take into account the dynamic processes present in biological systems, thus increasing the amount of information available through traditional cell-based assays. Additionally, fundamental non-labelled signal analysis was addressed in detail in order to correlate the effect of biochemical and biophysical phenomena on the optical signal. This was achieved by studying a traditional biochemical affinity interaction, developing an analysis approach for characterizing thin and thick organic layers, and by utilizing a new hybrid method combining the benefits of both non-labelled and labelled optical detection.
Figure 1 An illustration of the different barriers that are present and need to be taken into account during the drug development process. Some general methodology and especially label-free tools have been highlighted for the different steps. The process is not always linear but commonly involves repetition of previous stages, as indicated by the bidirectional arrows. The focus of this dissertation is in the two middle sections dealing with in vitro biochemical and biophysical interaction measurement tools.
2 REVIEW OF THE LITERATURE

2.1 SURFACE SENSITIVE DETECTION METHODS

There are several surface sensitive detection techniques, which are based on either a mechanical, optical or electrical detection of compounds. A common factor for these methods is that they detect compounds in contact with a surface or within a few hundreds of nanometers from the surface in a label-free manner. The different methods have of course their own strengths and weaknesses that are related to the physical principles of the method in question, and the practical limitations of the instrumentation arising from the physics, engineering and economical aspects. A few examples of the most commonly used methods have been summarized in Table 1, and a short description of each method follow.

The surface-sensitive label-free methods are in practice able to measure in real-time. While for example a mass spectrometer, UV-VIS and other spectrophotometric technologies are in a broader sense label-free, they are what is called sampling techniques. This means that a sample of buffer, supernatant or something else needs to be removed from the system under study, before it can be detected. The practical maximum sampling frequency for these instruments is in the order of minutes, and more commonly hours or days. The real-time label-free methods have a sampling rate of seconds or less. In practice this means that the label-free technologies can achieve measurements of kinetic phenomena in a totally different magnitude than sampling methods.

A significant difference between the predominant drug research methods and the label-free methods is that most of the label-free detection methods are flow-through systems instead of cuvette-based, as a well plate is also in principle a form of cuvette. This means that all traditional methods such as PAMPA, Caco-2 cell permeation and microscopy methods are performed under static or semi-static conditions. Flow-through systems have two advantages over the well plates: A dynamic flow shear on the surface that mimics physiological conditions [16], and constantly maintained sink conditions even with extremely low sample concentrations. The sink condition is an important factor to consider when designing an assay, because if the concentration gradient in the experiments is not constant, then the kinetics of the experiment are affected and become non-linear as a function of time. In a flow system, the concentration is constantly “replenished”, and therefore sink conditions is ensured during the whole experiment.
Table 1. Some examples of commonly used biosensors and characterization technologies in pharmaceutical research.

<table>
<thead>
<tr>
<th>Method</th>
<th>Type</th>
<th>Thickness characterization</th>
<th>Visco-elasticity</th>
<th>Optical properties</th>
<th>Surface coverage</th>
<th>Biosensors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomic force microscopy</td>
<td>Mechanical</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>[17,18]</td>
</tr>
<tr>
<td>Quartz crystal microbalance</td>
<td>Mechanical</td>
<td>Limited</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Limited</td>
<td>[19,20]</td>
</tr>
<tr>
<td>Dual polarisation interferometry</td>
<td>Optical</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Indirect</td>
<td>Yes</td>
<td>[21,22]</td>
</tr>
<tr>
<td>Ellipsometry</td>
<td>Optical</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Indirect</td>
<td>Limited</td>
<td>[23,24]</td>
</tr>
<tr>
<td>Optical waveguide lightmode spectroscopy</td>
<td>Optical</td>
<td>Indirect</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>[22]</td>
</tr>
<tr>
<td>Surface Plasmon Resonance</td>
<td>Optical</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Indirect</td>
<td>Yes</td>
<td>[25-27]</td>
</tr>
<tr>
<td>Current measurement</td>
<td>Electrical</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Point-of-care</td>
<td>[28,29]</td>
</tr>
<tr>
<td>Impedance spectroscopy</td>
<td>Electrical</td>
<td>Limited</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>[30,31]</td>
</tr>
</tbody>
</table>
2.1.1 MECHANICAL METHODS
Quartz crystal microbalance (QCM) and atomic force microscopy (AFM) are mechanical methods that can be used for characterizing layers on the nanoscale. A unique and common feature for these higher-end mechanical methods is the ability to measure viscoelastic properties of thin films.

AFM is in practice purely an imaging tool, and in most cases it produces information about surface structure and thickness. While AFM is an effective imaging method for dry samples, it is still an extremely challenging method for measuring samples in a liquid environment [32,33,18]. In interfacial research, AFM is often used to probe the membrane morphology on a substrate [17], or certain binding events with large constituents such as nanoparticles or large macromolecules [34].

QCM utilizes AT-cut single-crystalline quartz sensors for measuring bound mass on surfaces through changes in the resonance frequency of the crystal [35]. When several overtones are simultaneously being measured, the thickness and viscoelastic properties can be modelled for nanoscale layers (by assuming the density of the sample) [36,37]. QCMs are also sometimes used as biosensor tools, but the need to use the whole quartz sensor without damping it makes the flow systems relatively large, and therefore limits the usefulness of the method for kinetic biosensor measurements. The literature is quite rare and shows a clear difference in information quality when comparing the use of QCM as a biosensor tool to the most commonly used methods, i.e. the optical biosensors [20,38,39]. The QCM has established itself as a fairly standard tool in SLB work where the capability of monitoring viscoelasticity enables an easy determination of the morphology of the deposited lipid layer (i.e. bilayer or vesicle) [40,41]. Consequently, the QCM is commonly used for SLB related biochemical or biophysical interactions research. A notable feature to highlight for the QCM again is its capability of probing viscoelasticity of adsorbed layers, which also enables to detect the effect of drugs on the membrane viscoelasticity during drug partitioning into the membrane [42].

2.1.2 ELECTROCHEMICAL METHODS
Electrochemical biosensors usually utilize the measurement of a change in an electrical property, such as potential (potentiometric), current (amperometric), conductivity of a medium (conductometry) or impedance (impedimetric) for determining a binding property [29]. Electrochemical biosensors are the most common type of biosensors for a simple reason – there are several clinically relevant quick tests based on electrochemical detection, the most common probably being the blood glucose level test [28]. However, electrochemical sensors are based on catalytic electron creating reactions and on electron counting (current measuring). Therefore, they are generally not suitable for monitoring biochemical or biophysical interactions, because most of these interactions do not create any electrons. A more general electrochemical biosensing technique is impedance spectroscopy, which measures the ability of ions to move close to the electrode [29]. Basically, the more
resistive barrier the ions find, the lower the ion mobility. This can be used to detect biochemical interactions quite easily. However, due to the timescale and other practical issues such as electrode structure and subsequent flow cell geometry, the impedance spectroscopy is more useful in quantitative analysis than in kinetic characterization. Impedance sensors and electrodes are quite simple, and can even be prepared by printing them on paper [43]. This makes them more versatile than the mechanical or optical biosensor methods, as well as more viable candidates for consumer point-of-care applications even though not so lucrative as research tools.

2.1.3 OPTICAL METHODS
Most of the surface-sensitive label-free methods are based on an optical interfacial property called the evanescent field [22]. The evanescent field is a weak electromagnetic field that is created into a dielectric interface under total internal reflection conditions (i.e. the light incident angle is larger than the critical angle). For practical measurement purposes, this phenomenon needs to be enhanced with some other means, like multiple reflections (e.g. waveguides sensors used in optical waveguide lightmode spectroscopy (OWLS) and dual polarization interferometry (DPI)), or free electron excitation on a dielectric-metal interface (e.g. surface plasmon resonance, SPR). In principle, the evanescent field methods detect the optical density (i.e. refractive index, RI) or a change in it, inside the evanescent field, which extends approximately ½ a wavelength of the incident light to the measurement medium. The most typical applications of these are different molecule-molecule interactions, where the kinetics and binding affinity are determined [38,39]. Application areas which have hundreds of publications utilizing these evanescent field based methods are different biomolecule screens, such as antibody [44], nucleotide [45] and drug screens [46]. More advanced and interesting emerging applications involve the use of different SLB assays for screening drugs or other biochemical or biophysical interactions with both liposomes [47] and lipid bilayers [21]. Another new emerging application, that is highly relevant for drug screening, is the use of living cells in combination with the evanescent field based methods for developing different label-free cell assays, for example for studying cell response to drug stimulus [48].

The ellipsometer is another common optical nanoscale detection method. An ellipsometer does not utilize the evanescent field but measures the change in the polarization of light when it is reflected from a surface. Due to its physical limitations, ellipsometry is still mostly used for characterizing thin films in air or vacuum. This, because the light beam is passing through the media above the sample, which means that if ellipsometer is to be used for measuring sample layers in liquid media, then the sampling fluidics need to be approximately 1 000 times larger than with the evanescent field methods (i.e. mL vs. μL) [23]. Ellipsometry is most commonly utilized in hard material science for the characterization of inorganic coating properties, but there are also some applications in the biochemical materials and biochemical and physical interactions field particularly when combined with other methods such as QCM [49,50].
As all the optical detection principles is in practice based on a change in the refractive index, the molecules that resemble the medium (e.g. sugars that have a RI close to that of water-based buffers) will be difficult to detect with these methods. Fortunately, most biological compounds like proteins, lipids and nucleic acids have a relatively high RI (i.e. within a range of 1.4 – 1.5) and can effectively be detected with all the optical detection methods. This thesis provides a more in-depth discussion of the SPR method in the next chapter, because the SPR method has been the dominant method in most biochemical and biophysical interaction research in the past. It was also selected as the main detection method in this thesis because of the versatility offered by the physical principles and optical configuration utilized in the method.

2.2 ANALYSIS OF THE SPR SIGNAL

One of the most interesting label-free technologies is the SPR technology, because of its versatility which is enabled by the simple fundamental physics of the method [22,51,52]. Surface plasmons are particle waves of the free electron plasma on a metal surface, which can be excited by p-polarized light under the resonance condition. The resonance condition depends on the dielectric properties of the surrounding medium, and the electrical properties of the metal. This resonance condition can be described mathematically by Maxwell equations for a multilayer optical system [27]. A general answer for a multilayered system linked to measurable or controllable variables can be solved easily by modern computer-based calculative methods. The Maxwell equations and the matrix formalism needed for analysing multilayer systems has been described several times in literature in detail [27,54,51,55], and there are also dedicated software packages available for this [56], and is not discussed in further detail in this thesis.

The physics behind the SPR phenomenon enable a wide range of applications from a biosensor of molecular interactions [38,39] to measurements of organic and inorganic thin films [27,53]. However, the SPR method as such has been mostly used as a pure biochemical interaction analysis method [38,39], and a highly unexploited area of SPR is its use in other pharmaceutical research areas, such as monitoring drug, protein or nanoparticle interactions with cell surfaces and cells. Unfortunately there are some gaps in the knowledge how the optical signal in SPR is affected by biophysical and biological phenomena. The actual reason for this is mostly instrumentation-related – the market leader in SPR instruments focuses completely on screening biomolecular interactions and on biosensor applications, and most of the instrumentation on the market is specialized only for that purpose, which has limited the development of the method for other application areas [52].

Because of this, there is a lack of both theoretical and empirical knowledge of SPR in nanoscale layer characterization, which needs to be addressed before new applications of the method can be adopted more widely. Therefore, the basic theoretical principles of the optical physics of SPR and biochemical interaction kinetics will shortly be introduced below. This also contains some discussions on how the SPR can be more effectively used as a thin film characterization tool, and how to
correlate the optical signal detected by SPR with the behaviour and responses of living cells immobilized on an SPR sensor during drug stimulus.

2.2.1 MULTIVARIABLE SPR ANALYSIS
When solving the SPR results for thickness \( d \) and real refractive index \( n \), it becomes clear that there is no unique answer for the resonance condition wave vector \( k_{sp} \) when a sample do not absorb light (i.e. complex part of the refractive index \( k = 0 \)). In such a case, only a continuum answer can be found where the \( n \) and \( d \) are non-separable and proportional to each other:

\[
k_{sp} \propto n \cdot d.
\]

This of course makes the determination of layer thickness from SPR data dependent on the quality of the literature value of the refractive index, and vice versa. This is problematic especially in the case of ultrathin films, as they do not always follow the properties of bulk materials [57]. Two different solutions to this issue have been proposed so far: A measurement of the layers in two different media with a large difference in their refractive indexes (such as air and water) [27], or a measurement with two different optical conditions (such as the use of two different light wavelengths with angle-scanning SPR) [58-60].

The two-media method is quite effective, giving a large difference between the two continuums and has the ability to determine the layer thickness from the SPR spectrum with high precision [27]. A significant limiting factor for this method is, however, that the sample needs to be stable in both media, and it should also not swell or collapse in either media.

The two-wavelength approach does not suffer from the same drawback as the two-media method. However, the difference between the continuum solutions is much smaller than with the two-media method, and the results of the analysis are less precise [58-60]. The two-wavelength method also requires an additional parameter to be used, i.e. the wavelength dependency of the refractive index. While this parameter is relatively constant between similar materials [61], there is still some variation in it which can cause a systematic error in the analysis.

It is apparent that neither method is still sufficiently robust to fulfill all characterization needs that arise with biological interfaces, because of instability (in dual media analysis) or lack of reference values (dual wavelength analysis). Thus, there is a need for more advanced analysis methods that could bypass these limitations when determining \( d \) and \( n \) of sample layers.

2.2.2 SPR SIGNAL IN CELL-INTERACTIONS
Label-free techniques have been adapted recently into studying cell-interactions with various compounds and surfaces. Several techniques have been proposed for this, among them different evanescent field detection methods including SPR [48]. However, a common feature of the evanescent field detection methods is that the
penetration depth of the evanescent field is approximately ½ of a wavelength of the incident light, meaning that it in most cases have a maximum penetration depth of 250-500 nm. This is much less than the cell thickness, and the sensitivity in the evanescent field also decays exponentially when moving away from the sensor surface [52,55]. This means that the evanescent field only probes the bottom part of the cell. There have been some attempts to improve this with near infrared SPR [62,63], but the scanning depth with near infrared SPR is still much less than the common cell diameter.

Based on the penetration depth of the evanescent field, it is clear that direct binding interactions that happen on the cell surface cannot be detected. The logical and commonly accepted paradigm is that the cell responds to the binding stimulus indirectly by shifting around its mass and organelles, which consequently affects the evanescent field. Thus, the detected signal from the binding event is an indirect signal from the cell’s response [48]. As SPR is a widely used method for detecting binding of a mass to a surface, it is commonly assumed that the behaviour of the SPR signal always displays similar trends as seen with general molecule-molecule interaction: a shift in SPR angle (or intensity at fixed angle) equals a binding. However, the cells do not necessarily move the mass towards the surface, which can also lead to a negative SPR signal change during cell stimulus [64]. Even more complicating is, that the basic SPR theory has been developed for systems that do not absorb light at the incident light wavelength and do not take into account that the cell organelles are in the size range that causes scattering of light. The light absorption will also affect the optical permeability of the sensor, and will therefore cause other changes in the optical signal. Upon closer inspection of the literature, it is clear that this phenomenon is poorly studied. Because of this, it has not been possible to properly explain the complex SPR signal behaviour with cells by traditional signal analysis of SPR or other evanescent field techniques. The unexplained non-linearity of the signal and a poor correlation between studies by different groups implicate that the effect of the behaviour of living cells on the signal detected by SPR is still poorly understood. Therefore, there is a need to improve the theoretical understanding of how cells influence the SPR signal. It is also apparent from the current literature that there are no well established protocols for cell culturing onto sensor surfaces and for retaining cell viability for the SPR based cell assays. Thus, there is a clear need for optimizing the measurements conditions and cell culturing protocols for successful SPR biosensing with living cells.

### 2.2.3 LABEL ENHANCED SPR

SPR and other label-free methods are commonly seen as very attractive biosensing techniques because they do not utilize labels for detection and can detect analytes by their inherent properties. However, this also means that there is no selectivity in the detection with label-free methods. For example, a change in salt concentration, additional compounds or temperature will interfere with the detected signal. Hence, the more sensitive the method, the more sensitive it is to these environmental effects. Another drawback of label-free methods is the sensitivity of detection: Commonly fluorescence- and radioactive-labelled detections are several decades more sensitive
than a label-free detection [65,66]. However, achieving such a sensitive labelled detection usually needs long signal gathering periods (minutes or more), meaning that the measurements are always static and not able to characterize dynamic processes [67].

Due to these reasons, hybrid technologies that combine label-free and labelled detection have been proposed. SPR fluorescence is an interesting example as it uses the SPR evanescent field for exciting the fluorescent molecule and measures the emitted fluorescence by an external photodetector. The drawback of it is of course the need of additional equipment, and possibly also a quenching effect of the plasmonic metal surface [52,68]. Several different nanoparticle-based labelling technologies have also been proposed [69-71]. While nanoparticle-based labelling technologies improve the molecular sensitivity they actually affect the kinetics of the detection significantly as the label is several times larger than the analyte. The use of a labelled analyte that absorbs light at the SPR wavelength has also been proposed briefly [72,73]. However, this approach has never been largely adapted for use in biosensing applications.

The use of labeled compounds in combination with SPR detection can complement the currently used SPR analysis approaches in some areas, such as detection of small molecular analytes. Therefore, this thesis also introduces a new visible-light dye-labelling method for SPR technology which improves the detection sensitivity of SPR and avoids the drawbacks of the current technology when detecting small molecular compounds.

2.2.4 PRINCIPLES OF KINETIC ANALYSIS

2.2.4.1 One-to-one interactions

The most simple biochemical interactions are single-site binding reactions, such as simple protein-protein or protein-drug interactions. They can mathematically be described by a simple one-to-one kinetic model (i.e. one analyte, one binding site).

\[
A + B \xrightarrow{k_a} AB \xleftarrow{k_d}
\]

where A and B are the two interacting components (i.e. immobilized ligand and analyte in solution), AB is the complex formed when they react and \(k_a\) and \(k_d\) are on- and off-kinetic constants, respectively.

When this kinetic model is derived for a regular chemical equilibrium the affinity of the binding at the equilibrium position can be calculated from any kind of signal from any instrument. If affinity is measured, the equation is treated as a chemical equilibrium, and the equilibrium constant is calculated at the state of equilibrium binding. Similarly, if the expression is treated as a chemical kinetic rate constant equation, we can solve for the kinetic parameters with differential equation against time. These methods are well known, and the reader should refer to previous
literature for details [74,52]. It is important to notice that the affinity is by definition related to the kinetic parameters \(k_d/k_a = K_D\), and several different kinetic constants can produce exactly the same affinity.

In practical experimental considerations, it is extremely important to take into account the minimum data requirements for calculating the affinity – wrong type of data might seemingly enable a calculation of kinetics and/or affinity, but actually have no unique solution and might instead give a large error related to the values produced by the analysis [75].

It should also be noticed that the kinetics of an interaction govern the timescale in which it can actually be accurately measured [75]. This can complicate the analysis, especially in the cases of slow off-rates as it can slow down the whole experimental process and lower the throughput of the assay. This is unfortunate, as a slow off-rate is normally desirable for a drug in order for it to give a good therapeutic response, whereas in the case of a non-target a slow off-rate is not desirable as it can cause unwanted side effects [76].

### 2.2.4.2 Complex interactions

It is always advisable to fit a dataset with the simplest possible model that describes the data adequately and which is physically meaningful to the dataset. However, there are many practical cases where the simple one-to-one kinetic model is not correct, and a more complex kinetic model needs to be applied. Aside from some practical instrument-related kinetic models (diffusion- and depletion-corrected kinetics), there are various kinetic models that are relevant in different cases [74,77].

The two most notable models are the so-called “bivalent” and “one-to-two” kinetic models [74]. The bivalent kinetic model has been developed to describe the situation where there is a second binding step or conformational change after the first binding event. A common example of such interactions is antibodies, which have two active binding sites. If the target sites in the antibodies are close enough, then both of these sites will bind sequentially. This kind of behaviour cannot be described by the one-to-one kinetic model, but needs a different expression:

\[
A + B \xrightarrow{k_{a1}} AB \xrightarrow{k_{d1}} AB' \xleftarrow{k_{a2}} AB' \xleftarrow{k_{d2}},
\]

where \(AB'\) is the product of the second binding step.

The one-to-two binding kinetic model describes a situation where there is one analyte but two binding sites on the target molecule or surface. For example, human serum albumin has multiple binding sites, and the drug warfarin binds to two of them. Also a heterogeneous immobilization of the target molecule on the surface, caused for example by an insufficient stability of the target, can lead to such situations. In this case, we have two independent binding reactions, which both create a signal in the assay.
Even though these models have been created for specific situations, they also work surprisingly well with more complex cases. Interactions of proteins with materials can mostly be modelled with the one-to-two model. In a physical sense there are much more than two different binding sites, but usually the two strongest are so dominant that it is not possible to detect other binding events [78].

2.3 BIOMIMETIC INTERFACES AND MEMBRANES

2.3.1 CELL MODEL MEMBRANES
Supported lipid bilayers (SLBs) and supported vesicular layers (SVLs) are surface-anchored and/or supported membrane structures composed of phospholipids, such as phosphatidyl cholines, sphingomyelins and other naturally occurring or synthetic lipids. While there are many different support strategies for the SLBs (and SVLs), only the methodologies that can be used with in situ label-free sensing techniques are considered here. The lipids are supported by a solid surface, and the support properties often influence the properties and morphology of the membrane structure (i.e. SLB or SVL). The SLBs are often classified based on the type of support structure used (i.e. polymer-supported, protein-tethered etc. SLBs [12]). However, in principle the support structure is only a mean to form the SLB, so for an end user studying biochemical or biophysical interactions of SLBs with e.g. proteins will not find this classification useful. SLBs can be either simple, single-component constructions (such as palmitoyl-oleyl phosphaditylcholine [40]), mixtures of several compounds (such as egg-extract phosphatidylcholines, charged phosphatidyserine and cholesterol [21]), or even complex membrane extracts with membrane receptors, proteins and polysaccharides [79].

![Figure 2](image)

**Figure 2** An artistic impression of the structure of a liposome (left) and a lipid bilayer (right).

2.3.2 ESTABLISHED TECHNOLOGIES UTILIZING BIOMIMETIC MEMBRANES
The most widely used applications of SLBs and SVLs are in the field of permeation screening in the early in vitro screening of drugs. The most important SLB-based standardized medium- or high-throughput screening methods that are widely used
are the PAMPA and immobilized artificial membranes (IAM) [15,1,80]. The golden standard methods still used for the intestinal permeation screening are cell culture methods [15,81]. Another interesting related method is the patch clamp, which is a kind of a hybrid between the cell and the SLB methods [82]. Label-free SPR biosensors with immobilized liposomes have also been introduced for drug permeation screening [83,84]. The methods have been summarized in Table2.

PAMPA utilizes a filter-supported SLB or SVL (i.e. a membrane deposited from an organic solvent, which forms a mixed barrier of the SLB and the solvent in the filter). The sampling in PAMPA is performed from acceptor and donor compartments in a well plate for determining the relative drug concentrations. The PAMPA membrane is in general not well defined and the organic solvents involved reduce the biological relevance of the method. Despite of this, PAMPA can usually predict passive permeation of drugs in a relatively good fashion, and definitely better than the traditional octanol-water partition assay, especially for charged lipids. A new approach similar to PAMPA in terms of filter supported SLB has also been proposed for predicting passive permeation of drugs [80]. This new approach does not use organic solvent and forms a tighter barrier than PAMPA. This solvent free PAMPA has been shown to give higher predictive ability of drug permeability compared to both human and Caco-2 cell line for passively permeating drugs [80].

The IAM method is basically a relative chromatography method, and functions exactly as any other chromatography methods. The permeation is assessed from a relative partition coefficient obtained from the retention time that the drug spends in the column. This means that the information is always relative to standard compounds used in the assay for calibration. The IAM performs well when used to predict the relative permeation of drugs of a homology series, but is not as useful if the sample set is structurally diverse [15,80].

The cell permeation assays also use a filter support, onto which the cells are cultivated as a tight monolayer. The cell methods for permeability studies are laborious and need much more equipment than the SLB methods, but they are also able to monitor the permeation of actively transported compounds and other transport-related interactions [15,1]. In the patch clamp method, a cell’s outer membrane is fixed to a glass capillary, creating a patch of the cell membrane which is then perturbated with the compounds of interest. Patch clamp has been shown to have an extremely high level of predictability for active transport and protein interactions, but it is a difficult method to perform in practice [82].

The traditional established methods are effective in providing information of the permeation and partitioning of drugs. However, they offer little information of the uptake mechanism of the drug, the effect of the drug on the cell membrane and of other biophysical interactions the drug has with the membrane. This is mostly due to the nature of the methods. They provide static information that is measured after the interaction event itself, and are unable to provide real-time information on the interactions, whereas the real-time biosensors can provide useful measurement methods which can provide complementary kinetic information to the traditional static methods for better understanding drug-cell surface interactions and their role on drug permeation and partitioning.
Table 2. *Examples of the measurement methods commonly used for studying and predicting drug partitioning and permeation during pharmaceutical development.*

<table>
<thead>
<tr>
<th>Method</th>
<th>Type</th>
<th>Main use</th>
<th>Morphology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanol-water</td>
<td>Well</td>
<td>Partition</td>
<td>Solvent</td>
<td>[1]</td>
</tr>
<tr>
<td>PAMPA</td>
<td>Well</td>
<td>Passive permeation</td>
<td>Mixed + solvent</td>
<td>[15]</td>
</tr>
<tr>
<td>Cell permeation</td>
<td>Well</td>
<td>Passive and active permeation</td>
<td>Cells</td>
<td>[3,1]</td>
</tr>
<tr>
<td>IAM</td>
<td>Chromatography</td>
<td>Partition</td>
<td>Affinity</td>
<td>[15]</td>
</tr>
<tr>
<td>“Solvent free” PAMPA</td>
<td>Well</td>
<td>Passive permeation</td>
<td>Bilayer or mixed</td>
<td>[80]</td>
</tr>
<tr>
<td>Patch clamp</td>
<td>Single cell</td>
<td>Receptor and transfer protein intr.</td>
<td>Cells</td>
<td>[82]</td>
</tr>
<tr>
<td>Label-free liposome assays</td>
<td>Flow-through</td>
<td>Receptor interaction</td>
<td>Liposomes</td>
<td>[83,84]</td>
</tr>
<tr>
<td>Label-free planar bilayer assays</td>
<td>Flow-through</td>
<td>Lipid bilayer interaction</td>
<td>Belayer</td>
<td>[21]</td>
</tr>
</tbody>
</table>
2.3.3 BIOCHEMICAL INTERACTIONS WITH LABEL-FREE DETECTION TECHNOLOGIES

The most common application for label-free biosensing especially for proteins and drug molecules is the molecule-molecule interaction screening. The yearly reviews of optical biosensors show that these methods have established themselves as standard methods in this area. Every year there are thousands of publications made by utilizing the label-free biosensors [38,39]. As the methods are quite established, there are a lot of standardized protocols and assay formats available both from instrument manufacturers and the scientific community. Unfortunately, the interactions are not always that simple, and sometimes a successful experiment will need either non-standard assays or a new surface chemistry design [26].

2.3.4 SUPPORTED LIPID BILAYERS FOR LABEL-FREE BIOSENSORS

2.3.4.1 Immobilized liposomes

SVLs are often convenient in applications which clearly do not dependent on membrane morphology or lipid diffusion inside the lipid bilayer. The passive drug permeation through the lipid layer and some membrane receptor interactions are examples of such applications. It is typically simpler to keep the liposomal morphology than form bilayers, because the liposomes are thermodynamically quite stable and there are assay protocols and commercial sensor coatings that support the liposomes without allowing them to fuse into a bilayer. The usual anchorings for liposomes are either hydrophobic linker molecules in thick hydrogels, such as the commercial “Biacore L1” sensor, which is based on a 100 nm thick carboxymethylxylonane (CMD) hydrogel with hydrophobic linkers [84], or specific linking systems such as avidin-biotin [12]. These are in general utilized with SPR detection technology, and during their use the lipid layer morphology is commonly disregarded.

The use of such tethered SVLs with SPR is quite an effective way to screen receptor interactions similar to other one-to-one interactions [14]. This is a relatively usual way of screening interactions in the cases where the proteins in question are not stable when removed from the membrane environment. There are some challenges with these assays, mostly concerning the protein concentration in the vesicles which is often too low. There are also some practical issues in creating effective reference chemistries for these assays. One of the interesting applications of SVLs is detecting a molecule passing through pores through a lipid membrane, as demonstrated by Brändén et al. who studied the transport of sucrose through melitin pores [85]. As mentioned earlier, SVLs in combination with SPR can also be used to screen selections of drugs for their partitioning into a lipid membrane similar to the
PAMPA assay. This was demonstrated by Baird et al. using a large set of commercially available drug compounds [84].

While the creation of SVLs for label-free biosensing purposes is quite well established, it is not easy to investigate or replicate the surface coating of commercial sensors and study its effect on the SVL formation due to the commercial nature of the sensors. The differences in the support chemistries used between various label-free technologies also indicate that a lot is still unknown about the SVL formation. Therefore, there is a need for clarifying the role of surface chemistry on the formation of SVLs and develop suitable chemistries for promoting the SVL formation in a reproducible manner.

2.3.4.2 Planar lipid bilayers

Planar solid supported lipid bilayers (SLBs) are in general more difficult to produce than SVLs. There are two main methods for producing SLBs: Self-assembly from liposome solution (i.e. vesicle spreading) or Langmuir-Blodgett deposition. The self-assembly method is more convenient in general, as the Langmuir-Blodgett technique is a quite time-consuming thin film fabrication technology. The current knowledge indicates that planar lipid bilayers are only formed when there are such interactions between the lipids and the surface that promote rupturing of liposomes, e.g. phosphocholine interaction with silicon dioxide surface in the presence of calcium [40], or incorporated biotin – avidin interactions [12]. Also, the self-assembly is quite demanding in terms of other conditions, such as pH, ionic strength, temperature and the liposome stability [41]. In general, even small amounts of stabilizing components in the liposome will prevent the self-assembly into lipid bilayers altogether, which can lead to a situation that only whole liposomes will be adsorbed on the surface.

The support structure under the SLBs also affects its usability for different interaction experiments. Extremely strong surface interactions between the support and the lipids will hinder the diffusion of the membrane components, and affect the interaction of the lipids with both the membrane incorporated functionalities and analytes in the surrounding liquid [12]. Similarly, insufficient space between the support and the SLB, such as with SiO$_2$-supported SLBs can lead to an incorrect folding or denaturation of membrane proteins [12].

Another challenge for a more general utilization of planar SLBs is the instability of the membranes when passing through an air-water interface. There are a few recent examples of stable lipid bilayer systems that can resist the transition through an air-water interface [86,87], but it seems that these methods are still extremely selective in terms of lipid composition and experimental conditions.

As a result, there is a clear demand for new surface supports in order to improve the properties of biomimetic membranes. The ultimate goal would be to find a general support and a method for the preparation of SLBs containing incorporated and non-denaturated membrane proteins or even extracted cell membranes, which would also have at least a decent air-stability.
2.3.4.3 Controlling supported lipid layer morphology

A morphology control of supported lipid layers between curved SVLs and planar SLBs might first seem unnecessary. There have, however, been some clear indications that some biochemical reactions between the SLB and analytes do not take place in the same way if the morphology of the lipid layer is not right [21,88]. Nanoparticle interactions with lipid layers probably also have some dependency on the lipid layer morphology, as the size of nanoparticles is often within the same size range as the size of liposomes. Nanoparticles are also affected by fluid dynamics in a greater extent than small molecules due to the much smaller diffusion coefficient of e.g. small drug molecules compared with nanoparticles.

As discussed previously, the control of the morphology of supported lipid layers between SVLs and planar SLBs is actually a quite challenging task. In general the self-assembly of a planar SLB is a rather sensitive process in terms of lipid composition, sensor coating, temperature and flow. Many protocols for preparing SLBs actually seem to be limited to a specific coating–lipid combination, such as the most common protocols of the use of phosphocholines to form planar SLBs on SiO$_2$ surfaces [41] and use of polyethylene glycol to trigger vesicle fusion for liposomes containing non-charged phosphatidylethanolamine lipids [79]. As one can imagine, the specificity of these reactions greatly limits the amount of possible lipid compositions to be used to form planar bilayers.

One important factor to consider when discussing lipid layer morphology is the possibility to detect the formation of the lipid bilayer, and the capability to distinguish between a planar SLB and a SVL. The QCM technique is extremely sensitive for monitoring the SLB formation, as there is a large change in surface bound mass when a vesicle ruptures, consequently releasing the water inside the vesicle. A general conception has been that optical methods are not at all able to detect the rupturing of vesicles in a similar manner as the QCM technique. There is even a report by Keller et al. that claim that detecting vesicle rupture with optical techniques is impossible altogether [40]. While this article opened up nearly a whole new research field with SLBs utilizing the QCM-liposome self-assembly, the SPR work used as reference in the report is questionable as the measurement conditions and the surface are not identical. The QCM work was done with SiO$_2$-coated sensors, while the SPR work most probably was performed with gold-coated sensors. It seems more likely that the inability not to see the self-assembly of a bilayer with SPR in the report by Keller et al. is more due to the instrument used and not to the SPR method itself.

While fluorescence recovery after photobleaching (FRAP) and AFM are often used for verifying SLB formation, they are ex situ methods and require that the sensor surface is removed from e.g. the QCM and SPR instrument and flow channels. This means that the lipid layer passes through an air-water interface during the transfer of the sensor surface between different characterization instruments. This is often problematic for the stability of the SVL or SLB on the sensor surface. Similarly, when performing the lipid layer deposition ex situ, then the conditions of the liposome self-assembly are not comparable to conditions inside the QCM and SPR instrument and flow channels, especially in terms of flow which is known to be an important factor influencing the self assembly [19,16]. But, if we actually make an optical model of a
liposomal layer, and compare it with a similar model of a planar SLB, we can see that there is actually a clear difference which should be detectable in the SPR if the instrument output is more flexible than only a time-signal sensorgram [16]. Especially when utilizing multiple-wavelength SPR it should be possible to provide a clear differentiation between SLB and SVL formation [53]. Furthermore, the utilization of the anomalous behaviour of the SPR signal when trapping compounds that absorb light inside the liposomes should provide an alternative mean for differentiating between SLB and SVL formation.

![Figure 3](image)

**Figure 3** An artistic impression of supported lipid bilayers on (A) a hard supporting surface, (B) a thin hydrogel support and (C) a supported vehicular layer on a hydrogel support.

### 2.3.5 CELL MONOLAYERS FOR LABEL-FREE BIOSENSORS

It is of interest to use cell monolayers in surface-specific interaction experiments because the whole biological machinery of the cells is present and intact, which is not the case with SLBs. This makes it possible to monitor such cell processes with these cell assays which are dependent on the cell machinery. This is of course also possible with traditional well plate assays. However, by combining cells with label-free technologies gives access to a different timescale (i.e. from seconds to hours) and enable an integration of dynamic conditions. Some interesting studies have recently been reported where label-free biosensing is combined with cells, for example for monitoring cell toxicity of compounds [90], receptor mediated signaling [91] and endocytic vesicle formation [92].

Extremely interesting areas where the label-free cell assays can be applied are nanoparticle-cell interactions. The size of the nanoparticle makes it much more sensitive to the dynamic environment than small molecules or proteins, since the shear and diffusion affects the mass transfer of nanoparticles to a larger extent than small molecules or proteins. Also, the ability to monitor cell processes in the timescale not achievable in sampling-based experimental setups is a clear advantage, because this can provide biophysical information of such interactions and cell phenomena that is not easily achieved with other methods.

Label-free biosensing with cells is a new and promising field, which is suffering from the lack of understanding of both signal transduction and measurements methodologies. The methods, however, show great promise in providing new information of interactions and biophysics of cells in a timescale that has not been
previously accessible. There is a clear need to improve the understanding of both signal transduction and the cell culture protocols for label-free biosensing in order to make these label-free assays more appealing for a wider user base.
2.4 REFERENCES


Review of the literature


3 AIMS OF THE STUDY

The general aim of the research described in this thesis was to improve current- and develop new biomimetic platforms that could be utilized in future studies of biochemical and biophysical interactions between drugs, biomacromolecules and cells.

The specific aims were:
- to develop label-free biochemical detection assay surfaces for challenging analytes
- to improve the label-free detection sensitivity and specificity through a hybrid labelled technology
- to establish an analysis methodology based on multiple-wavelength SPR signal analysis for determining optical properties of both nanoscale and thick layers
- to develop a platform for preparing biomimetic interfaces for real-time measurements of cell surface interactions
- to establish a correlation between the SPR signal and the living cell response upon stimulus with drugs
Abstract

Dysregulation of PKCε is involved in several serious diseases such as cancer, type II diabetes and Alzheimer’s disease. Therefore, specific activators and inhibitors of PKCε hold promise as future therapeutics, in addition to being useful in research into PKCε regulated pathways. We have previously described llama single chain antibodies (VHHs) that specifically activate (A10, C1 and D1) or inhibit (E6 and G8) human recombinant PKCε. Here we report a thorough kinetic analysis of these VHHs. The inhibiting VHHs act as non-competitive inhibitors of PKCε activity, whereas the activating VHHs have several different modes of action, either increasing Vmax and/or decreasing Km values. We also show that the binding of the VHHs to PKCε is conformation-dependent, rendering the determination of affinities difficult. Apparent affinities are in the micromolar range based on surface plasmon resonance studies. Furthermore, the VHHs have no effect on the activity of rat PKCε nor can they bind the rat form of the protein in immunoprecipitation studies despite the 98% identity between the human and rat PKCε proteins. Finally, we show for the first time that the VHHs can influence PKCε function also in cells, since an activating VHH increases the rate of PKCε translocation in response to PMA in HeLa cells, whereas an inhibiting VHH slows down the translocation. These results give insight into the mechanisms of PKCε activity modulation and highlight the importance of protein conformation on VHH binding.

4.1 INTRODUCTION

Protein kinase C (PKC) is a family of serine/threonine kinases that regulate several signaling pathways in cells. The ten PKC isozymes have distinct biological functions and are divided into three groups based on cofactor requirements [1]. All of the PKC isozymes are regulated by phosphatidylserine (PS). In addition, conventional PKCs (α, βI, βII and γ) are activated by Ca\textsuperscript{2+} and diacylglycerol (DAG), novel PKCs (δ, ε, η and θ) require only DAG for activation, and atypical PKCs (ζ and 1/λ) are insensitive to both DAG and Ca\textsuperscript{2+} [2]. Conventional and novel PKC isozymes translocate to the plasma membrane when DAG or its surrogate, phorbol 12-myristate 13-acetate (PMA), which is often used as a PKC activator in cellular assays, become available [3]. In addition to cofactor binding, PKC activity is also regulated by priming phosphorylations of three conserved phosphorylation motifs [1] and protein-protein interactions such as binding to receptors for activated C kinase (RACKs) [4].

PKCε plays essential roles in a variety of signaling systems including those regulating proliferation, differentiation, gene expression, metabolism, transport, and muscle contraction [5]. Therefore, it is not surprising that its dysregulation is implicated as a player in several serious diseases including cancer [6], [7], diabetes mellitus [8], [9] and Alzheimer’s disease [10].

In cancer, PKCε is considered a transforming oncogene that can contribute to malignancy either by enhancing cell proliferation or by inhibiting cell death [6]. PKCε has been found to be overexpressed in tumor-derived cell lines and in tumor specimens from various organ sites, and is considered to be the PKC isozyme with the greatest oncogenic potential [11]. Furthermore, in vitro studies have shown that overexpression of PKCε increases proliferation, motility and invasion of fibroblasts or immortalized epithelial cell lines [7]. One of the mechanisms by which PKCε controls cell division is through its role in cytokinesis. PKCε associates with 14-3-3 scaffold proteins to regulate abscission, a process which requires PKCε kinase activity [12].

In type II diabetes, PKCε has been identified as one of the proteins involved in insulin resistance [13]. Activated PKCε reduces the insulin receptor (IR) gene promoter activation, decreasing the number of IR’s on the cell surface, thereby leading to a decrease in insulin sensitivity [8]. The decrease in IR numbers on the cell surface is mediated by the transcription factor HMGA1, which is inhibited from binding to the IR promoter by a phosphorylation catalyzed by PKCε [8], [14].

In Alzheimer’s disease (AD), PKCε activators, cyclopropanated fatty acid derivatives DCP-LA and DHA-CP6, have been found to reduce amyloid β levels by enhancing the degradation of amyloid precursor protein (APP) [15], whereas overexpression of APP in turn decreases the levels of both membrane-bound active PKCε and cytosolic inactive PKCε in three different cell lines [16]. Moreover, overexpression of constitutively active PKCε leads to increased secretion of the neuroprotective peptide sAPP, which is cleaved from APP by α-secretase [17]. Preliminary animal studies support the role of PKCε in Alzheimer’s disease, since PKCε activation in a transgenic mouse strain containing familial AD mutations was found to prevent amyloid plaques, synaptic loss and cognitive deficits [18].

PKCε is considered a desirable drug target for the treatment of cancer, AD and diabetes among other diseases. However, since different PKC isozymes can have
different or even opposing roles in the same process [19], any therapeutic agents would have to be PKCε isozyme specific in order to have the desired therapeutic effect. The group of Dr. Mochly-Rosen has described the identification and characterization of a PKCε translocation inhibitor (εV1-2) [20] and a PKCε agonist peptide (ψεRACK) derived from the PKCε RACK [21]. Furthermore, they have shown that other peptides derived from the C2 domain of PKCε have the potential to act as PKCε agonists or antagonists [22].

We have previously reported the selection and screening of another class of PKCε specific activators and inhibitors, namely VHHs [23]. VHHs are the antigen binding regions of llama single chain antibodies that contain three complementary determining regions (CDRs) involved in antigen binding [24]. VHHs are highly soluble and stable, antigen-specific, and easy to produce [25]. They tend to have nanomolar affinities to their target antigens, and VHHs with affinities even in the picomolar range have been described [24]. Due to their unique structure, VHHs can also recognize conformational epitopes such as enzyme active sites that cannot be recognized by conventional antibodies. Furthermore, especially the long CRD3 loops of VHHs could serve as perfect leads for the design of new peptide drugs against various enzymes [25]. These advantages of VHHs compared to conventional antibodies, together with the positive data from the first clinical trials carried out with VHHs, indicate that VHHs are promising therapeutics, which will undoubtedly contribute to medicine in the future [26].

Here we report further details of the PKCε specific VHH activators (A10, C1 and D1) and inhibitors (E6 and G8) described previously [23]. Based on surface plasmon resonance (SPR) studies, the three activators and two inhibitors have affinities in the micromolar range. Furthermore, we show that the VHHs display species specificity since they do not bind the rat PKCε despite the 98% identity between the human and rat proteins. These VHHs were also tested in kinase activity assays to determine the Michaelis-Menten kinetics of activation or inhibition. Finally, we show that the VHHs have an effect on PKCε activity in a cellular context, since the activator A10 increases both the rate and degree of PKCε translocation in response to PMA stimulation in HeLa cells, whereas the inhibitor G8 slows down PKCε translocation. The results presented here give insight into the mechanisms of PKCε activation or inhibition by VHHs and highlight the conformation specific nature of the binding between these VHHs and their target protein. Moreover, the results demonstrate that these VHHs expressed inside HeLa cells as intrabodies have the ability to influence PKCε translocation, a step that is required for PKCε activation.

4.2 RESULTS

4.2.1 AFFINITY MEASUREMENTS USING SURFACE PLASMON RESONANCE

We have already shown by immunoprecipitation (IP) and kinase activity assays that the VHH activators and inhibitors of PKCε bind the human PKCε protein [23]. In the present study we further characterized the VHHs by determining their affinities to PKCε. Therefore, affinity measurements with surface plasmon resonance (SPR)
technology were performed. First, we tried to determine the affinities using Biacore SPR technology (GE Healthcare, UK), which is commonly used to study the interactions of VHHs and their antigens [27]–[29]. A CM5 chip was coated with human recombinant PKCε using standard amino-coupling, and binding of VHHs to PKCε was studied in a Biacore T100 instrument. None of the tested VHHs bound to PKCε in this setup (data not shown). Next, each VHH was amino-coupled to the surface of a CM5 chip and the binding of PKCε to the flow cell surface was studied. This setup also failed, since PKCε bound to the surface of the reference flow cell as well as the VHH-coated flow cells (data not shown). These results were in strong contrast to the enzyme-linked immunosorbent assay (ELISA) and IP results described previously [23].

The Bionavis SPR Navi 200-equipment was then used to study binding affinities. When PKCε was amino-coupled to the surface of the flow cell, none of the VHHs showed binding to PKCε, as was the case with Biacore. However, when the dextran hydrogel was amine-functionalized using ethylene diamine and PKCε was carboxyl-coupled to the surface of the flow cell, VHH binding to PKCε was detected (figures 1 and 2). The VHHs were injected in serial dilutions with five different concentrations for every VHH. The middle concentration was injected twice and served as an internal control. The resulting data was analyzed with TraceDrawer 1.3 from Bionavis.

The binding of VHHs A10, C1, D1 and E6 to PKCε was best fitted with second order Langmuir binding models based on the forms of the binding curves. Therefore, two association constants (kₐ values), two dissociation constants (kₐ values) and two affinities (Kₐ values) were calculated for each of these VHHs. The most likely explanation for the second order models is that PKCε was present in two or more different conformations on the surface of the flow cell, and binding of the VHHs to two of these conformations, with the strongest interaction affinities to VHHs, could be detected. An alternative explanation is that the VHH samples contained two different proteins that bound PKCε, but this is highly unlikely since the purity of the VHH samples was always checked on a Coomassie stained protein gel and found to be over 95% (data not shown). The G8 data was fitted with a first order Langmuir model, resulting in single kₐ, kₐ and Kₐ values for this VHH.

Out of the activators (A10, C1 and D1), C1 had the highest affinities for PKCε coupled to the surface of the flow cell (figure 1B and table 1), namely 3.38 μM and 7.3 μM. D1 had affinities of 44.2 μM and 7.91 μM (figure 1C and table 1) and ranked second in affinity among the PKCε activating VHHs. A10 had the lowest affinities of the three activators (25.4 μM and 104 μM; figure 1A and table 1). In kinase activity assays, C1 caused the greatest increase in PKCε activity, followed by D1 and A10 [23]. Since C1 had both the highest affinity of the three activators and led to the greatest increase in PKCε activity, followed by D1 and A10, the affinities measured here for the three activators support the results from kinase activity assays.
Kinetics of PKCε Activating and Inhibiting Llama Single Chain Antibodies and Their Effect on PKCε Translocation in HeLa Cells

Figure 1  SPR sensograms and fits for PKCε activating VHHs. SPR sensograms and fits for second-order Langmuir binding models are shown for VHHs A10 (A), C1 (B) and D1 (C). The VHH injection time was 3 min, followed by a dissociation time of 5 min. The surface was regenerated with an injection of 10 mM NaOH for 3 min, followed by a stabilization time of 5 min between each VHH injection. Five concentrations of each VHH were used, with the middle concentration injected twice as an internal control. The VHH concentrations (in µg/ml) are marked adjacent to each fit on the right hand side of the figure.
Figure 2  SPR sensograms and fits for PKCε inhibiting VHHs.

SPR sensograms and fits for a second-order Langmuir binding model of VHH E6 (A) and a first-order Langmuir binding model of VHH G8 (B). The VHH injection time was 3 min, followed by a dissociation time of 5 min. The surface was regenerated with an injection of 10 mM NaOH for 3 min, followed by a stabilization time of 5 min between each VHH injection. Five concentrations of each VHH were used, with the middle concentration injected twice as an internal control. The VHH concentrations (in µg/ml) are marked adjacent to each fit on the right hand side of the figure.

Of the two inhibitors (E6 and G8), E6 (figure 2A) was a better binder of PKCε immobilized to the flow cell surface than G8 (figure 2B). The affinities of E6 to PKCε in this setup were 587 nM and 9.71 µM, whereas the KD value for G8 was calculated to be 102 µM (table 1). As was the case with the activators, the obtained affinity constants support the results from kinase activity assays, where E6 is a more potent inhibitor of PKCε than G8 [23].

Table 1.  Association and dissociation constants for the interaction of VHHs with PKCε obtained from SPR measurements.

<table>
<thead>
<tr>
<th>VHH</th>
<th>$k_4$ 1 (1/(M*s))</th>
<th>$k_2$ 1 (1/s)</th>
<th>$K_D$ 1 (M)</th>
<th>$k_2$ 2 (1/(M*s))</th>
<th>$k_4$ 2 (1/s)</th>
<th>$K_D$ 2 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10</td>
<td>2.95x10^-6 (+4.31x10^-6)</td>
<td>7.50x10^-4 (+7.62x10^-4)</td>
<td>2.54x10^-3 (+1.76x10^-4)</td>
<td>1.01x10^-6 (+1.98x10^-6)</td>
<td>1.05x10^-5 (+3.22x10^-6)</td>
<td>1.04x10^-5 (+7.21x10^-6)</td>
</tr>
<tr>
<td>C1</td>
<td>3.09x10^-6 (+1.60x10^-6)</td>
<td>1.04x10^-3 (+3.56x10^-4)</td>
<td>3.38x10^-4 (+2.25x10^-4)</td>
<td>1.12x10^-6 (+4.11x10^-7)</td>
<td>8.21x10^-7 (+2.41x10^-6)</td>
<td>7.30x10^-7 (+3.08x10^-6)</td>
</tr>
<tr>
<td>D1</td>
<td>2.41x10^-6 (+2.89x10^-7)</td>
<td>1.07x10^-3 (+2.00x10^-4)</td>
<td>4.42x10^-4 (+8.80x10^-5)</td>
<td>5.51x10^-6 (+1.27)</td>
<td>4.35x10^-7 (+9.02x10^-7)</td>
<td>7.91x10^-7 (+1.82x10^-6)</td>
</tr>
<tr>
<td>E6</td>
<td>5.22x10^-6 (+2.30x10^-6)</td>
<td>3.06x10^-3 (+6.60x10^-4)</td>
<td>5.87x10^-4 (+4.78x10^-4)</td>
<td>1.53x10^-6 (+4.59x10^-7)</td>
<td>1.49x10^-7 (+5.10x10^-7)</td>
<td>9.71x10^-7 (+3.64x10^-6)</td>
</tr>
<tr>
<td>G8</td>
<td>1.11x10^-6 (+7.11x10^-7)</td>
<td>1.13x10^-3 (+4.01x10^-4)</td>
<td>1.02x10^-4 (+1.58x10^-5)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
4.2.2 SPECIES SPECIFICITY OF PKCε ACTIVATING AND INHIBITING VHHS

We have previously shown that VHHs A10, C1 and D1 increase human recombinant PKCε kinase activity, whereas VHHs E6 and G8 decrease kinase activity [23]. Rat brain extract is often used as an alternative source of PKC for experiments such as kinase activity assays, since it is known to contain many of the PKC isozymes, including PKCε [30], [31]. However, when kinase activity assays with the VHH activators and inhibitors of PKCε were performed using rat brain extract, no effect on kinase activity was seen (data not shown), even though based on Western blotting PKCε was present in the rat brain extract (figure 3A). The most likely explanation for this is that the VHHs do not bind the rat PKCε protein.

Figure 3 PKCε in rat brain extract.

(A) 15 µg of rat brain extract was separated on a SDS-PAGE gel. PKCε was detected with anti-PKCε and HRP-conjugated goat anti-mouse antibodies. (B) Immunoprecipitations were performed with rat brain extract using a commercial anti-PKCε antibody (IgG Ab) and VHHs. PKCε (marked with an arrowhead) is visible at 90 kDa on lane 1. The bands at 55 kDa and 25 kDa on lane 1 represent the heavy and light chains of the anti-PKCε antibody. The bands at 16 kDa for A10, C1, D1, E6 and G8 represent the VHHs. A sample of uncoated protein A sepharose beads was included as a negative control (lane 2 = ctrl).

All of the VHHs that have been shown to have an effect on human PKCε kinase activity are able to immunoprecipitate human recombinant PKCε from Sf9 cell lysate [23]. To test whether the VHHs can also bind the rat PKCε protein despite the fact that they cannot influence its kinase activity, IPs were performed with rat brain extract. In addition, an IP with a commercial anti-PKCε antibody known to bind the rat form of the protein was included as a control. The five VHHs and the commercial anti-PKCε antibody were successfully captured by protein A beads (figure 3B). However, rat PKCε was only immunoprecipitated by the commercial anti-PKCε antibody and not by any of the VHHs. Therefore, the VHH activators and inhibitors of PKCε do not bind the rat PKCε protein, and hence cannot have an effect on its kinase activity. These results suggest that the VHHs are species-specific towards human PKCε, and confirm the very high specificity of the VHHs to human PKCε versus other PKC isozymes, an issue which could be a concern with peptide and other small molecule activators or inhibitors.
4.2.3 KINETIC MEASUREMENTS OF PKCε ACTIVATION AND INHIBITION

To characterize the kinetics of PKCε activation or inhibition by VHHS, kinase activity assays were performed with varying concentrations of the substrate peptide. The VHH concentration was kept constant (1 μg/well) for each experiment. When substrate concentrations are varied, the resulting data can be used to calculate the Michaelis-Menten kinetics of the activation or inhibition.

Results from the PKCε kinase activity assay with PKCε activators PS and 1,2-dioctanoyl-sn-glycerol (DOG; a DAG analogue) show that the three VHHs that act as PKCε activators have different mechanisms of activation (figure 4A and table 2). VHH A10 leads to increased PKCε activation by almost doubling the V_max value, or the maximum rate achieved by the system (141 nmol/min/mg for control and 253 nmol/min/mg for A10), whereas it has almost no effect on the K_m value of the reaction (figure 4A). In contrast, VHHs C1 and D1 have a much smaller effect on the V_max, but they decrease the K_m value of the system from 424 μM for the control, to 81 μM for C1 and 126 μM for D1. A lower K_m value indicates that the reaction is faster relative to the V_max, so C1 and D1 seem to increase the speed of the reaction instead of the maximum rate of the reaction. Since the K_m value is influenced both by the affinity of the enzyme to the substrate and the rate at which the substrate bound to the enzyme is converted to the product, the lower K_m value measured with VHHs C1 and D1 could indicate either an increase in the affinity or the rate at which the substrate is converted to the product.

Figure 4 Kinetics of PKCε activation by VHHs A10, C1 and D1.

The kinase activity of full-length PKCε in the presence (A) and absence (B) of PKC activators DOG and PS was measured with varying MARCKS substrate concentrations. The VHH concentration was constant (1 μg/well) for each experiment. The data is presented as percentage maximal control activity (control activity with 1000 μM substrate) ± SEM and represents at least 3 independent experiments, each with duplicates. Note that the V_max values for the VHHs have not been reached yet, see table 2 for analysis.

The results for the activators were similar for the kinase activity assay that was performed with full-length PKCε without activators PS and DOG (figure 4B). Since VHHs A10, C1 and D1 have no effect or a very small effect on the activity of the catalytic domain alone [23], the Michaelis-Menten kinetics of the activators on the catalytic domain were not determined.

Based on the Michaelis-Menten constants obtained for the two PKCε inhibiting VHHs E6 and G8, VHH E6 is a more efficient inhibitor of PKCε than G8 is. In the assay using the full-length PKCε protein with the activators DOG and PS present
I Kinetics of PKCε Activating and Inhibiting Llama Single Chain Antibodies and Their Effect on PKCε Translocation in HeLa Cells

(figure 5A and table 2), E6 decreases the $V_{\text{max}}$ from 141 nmol/min/mg (control) to 29 nmol/min/mg, whereas G8 leads to a more moderate decrease ($V_{\text{max}}$ of 113 nmol/min/mg).

When the PKCε activators DOG and PS are not included in the assay (figure 5B), the difference between E6 and G8 is less and even G8 decreases the $V_{\text{max}}$ by almost half. When the catalytic domain of PKCε is used instead of the full-length protein (figure 5C), the inhibition of kinase activity by E6 is so great that the $K_m$ value cannot be reliably measured. In this case, E6 decreases the $V_{\text{max}}$ from 120 nmol/min/mg to 6.7 nmol/min/mg. G8 is also a more potent inhibitor of the catalytic domain than the full-length protein, since it decreases the $V_{\text{max}}$ of the reaction almost 3-fold.

Figure 5  Kinetics of PKCε inhibition by VHHs E6 and G8.
(A–B) The kinase activity of full-length PKCε in the presence (A) and absence (B) of PKC activators DOG and PS was measured with varying MARCKS substrate concentrations. (C) The kinase activity of the catalytic domain of PKCε was measured with varying MARCKS substrate concentrations. The VHH concentration was constant (1 µg/well) for each experiment. The data is presented as percentage maximal control activity (control activity with 1000 µM substrate) ± SEM and represents at least 3 independent experiments, each with duplicates. The catalytic domain activity (C) with G8 is an exception with only 2 independent experiments with duplicates.

Figure 6  $K_m$ and $V_{\text{max}}$ values for PKCε activating and inhibiting VHHs.

<table>
<thead>
<tr>
<th>PKCε with activators PS and DOG (n≥3)</th>
<th>PKCε without activators PS and DOG (n≥3)</th>
<th>Catalytic domain (n≥2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td>Control</td>
<td>424</td>
<td>141</td>
</tr>
<tr>
<td>A10</td>
<td>348</td>
<td>253</td>
</tr>
<tr>
<td>C1</td>
<td>81</td>
<td>191</td>
</tr>
<tr>
<td>D1</td>
<td>126</td>
<td>158</td>
</tr>
<tr>
<td>E6</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>G8</td>
<td>260</td>
<td>113</td>
</tr>
</tbody>
</table>
4.2.4 ANALYSIS OF PKCε INHIBITION BY VHHS E6 AND G8

The mechanism of PKCε inhibition by VHHS E6 and G8 was studied with kinase activity assays with varying substrate and VHH concentrations. We have previously shown that the binding site of both E6 and G8 is in the catalytic domain of PKCε [23]. Therefore, Sf9 lysate expressing the catalytic domain of PKCε was used for these assays.

The data from these assays was analyzed using non-linear regression models but is represented as a Lineweaver-Burk plot to allow for easy visualization of the $K_m$ and $V_{max}$ values. As can be seen from figure 6, with both VHHs the $K_m$ of the reaction remains about the same when the VHH concentration increases. However, the $V_{max}$ decreases as the VHH E6 or G8 concentration increases. In the controls without VHH, the $V_{max}$ is 46.7 nmol/min/mg, whereas at the highest VHH concentrations used in this experiment, the $V_{max}$ is only 17.0 nmol/min/mg for E6 (figure 6A) and 15.9 nmol/min/mg for G8 (figure 6B).

According to the Michaelis-Menten kinetics, when the apparent $K_m$ remains about the same but the $V_{max}$ decreases with increasing inhibitor concentrations, the inhibition is non-competitive [32]. Therefore, E6 and G8 appear to be non-competitive inhibitors of PKCε that do not compete with the substrate peptide MARCKS for binding to PKCε.

Figure 7  E6 and G8 are non-competitive inhibitors of PKCε.
The activity of the catalytic domain of PKCε was measured with varying MARCKS substrate concentrations and varying concentrations of VHHs E6 (A) and G8 (B). The data was analyzed using non-linear regression and the Michaelis-Menten kinetics model and represents 3 independent experiments, each with duplicates. The data is presented as a Lineweaver-Burk plot to allow for the easy visualization of $K_m$ and $V_{max}$ values.

4.2.5 EFFECT OF ACTIVATOR A10 AND INHIBITOR G8 ON PKCε TRANSLOCATION

PKCε is known to translocate to the cell membrane in response to PMA stimulation [3], [33]. In order to study whether the VHHs have an effect on the translocation of PKCε, one of the activating VHHs (A10) and one of the inhibiting VHHs (G8) were cloned to a mammalian expression vector and a C-terminal mCherry-tag was introduced to the sequence. HeLa cells were then double-
transfected with PKCε-EGFP and the A10-mCherry or G8-mCherry plasmids, or an mCherry control plasmid. Translocation studies were performed with a confocal microscope 24 hours after transfections by stimulating cells with 100 nM PMA and monitoring the cellular localization of PKCε-EGFP and mCherry constructs for 30 minutes.

In cells transfected with the mCherry control plasmid, about 70% of PKCε-EGFP remained in the cytoplasm 10 minutes after PMA stimulation (figure 7A and D). Strikingly, in cells transfected with the PKCε activator A10-mCherry, only 50% of PKCε-EGFP was still present in the cytoplasm at this time point (figure 7B and D). In contrast, in cells transfected with the PKCε inhibitor G8-mCherry, 90% of PKCε-EGFP was still present in the cytoplasm of the cells 10 minutes after PMA stimulation (figure 7C–D).

After 20 minutes, about 55% of PKCε-EGFP was present in the cytoplasm in mCherry transfected control cells. The amount of green fluorescence remained constant in the cytoplasm after this time point (figure 7A and D). In cells transfected with A10-mCherry, only about 40% of PKCε-EGFP was present in the cytoplasm 20 minutes after PMA stimulation. As was the case with the mCherry control transfected cells, the amount of fluorescence in the cytoplasm remained at the same level from 20 to 30 minutes in A10-mCherry transfected cells (figure 7B and D). In cells transfected with G8-mCherry, 70% of PKCε-EGFP remained in the cytoplasm of the cells at 20 minutes after PMA stimulation. In these cells, more PKCε-EGFP translocated to the membranes during the last 10 minutes of the experiments, since at the end of 30 minutes around 55% of PKCε-EGFP remained in the cytoplasm of G8-mCherry transfected cells (figure 7C–D). Even though a clear difference in translocation speed could be seen between mCherry transfected control cells and cells transfected with the PKCε inhibitor G8-mCherry, this difference did not reach statistical significance at any of the time points. The difference in PKCε-EGFP translocation between control cells transfected with mCherry and A10-mCherry transfected cells reached statistical significance at 20 and 30 minutes after PMA stimulation. Therefore, the PKCε activator A10 increases both the rate and the extent of PMA-induced PKCε translocation in HeLa cells, whereas the inhibitor G8 slows down the rate of PKCε translocation from the cytoplasm to the membranes.
Figure 8 Activator A10 increases and inhibitor G8 decreases the rate of PMA-induced PKC\(\varepsilon\)-EGFP translocation in HeLa cells.

(A–C) Representative images of HeLa cells transfected with PKC\(\varepsilon\)-EGFP and mCherry (A), A10-mCherry (B) or G8-mCherry (C) taken with a confocal microscope at 1, 10, 20 and 30 minutes after adding 100 nM PMA. (D) Quantification of PKC\(\varepsilon\)-EGFP translocation from the cytoplasm over time. Data is presented as percentage relative fluorescence in the cytoplasm ± SEM from at least 2 independent experiments with 4–6 cells per experiment (mCherry n = 4, A10-mCherry n = 3, G8-mCherry n = 2). The difference between cells transfected with the mCherry control plasmid and cells transfected with A10-mCherry was statistically significant (\(p<0.05\)) at 20 and 30 minutes (denoted with *).
VHH antibodies generally have affinities comparable to those of conventional antibody fragments, with $K_D$ values in the nanomolar range [25], and VHHs with affinity constants as low as 100 pM have been described [24]. The high affinities displayed by most VHHs are one of the main advantages of VHH antibodies in research and drug development. However, here we report affinity constants for PKCe activating and inhibiting VHHs ranging from 587 nM to 104 μM.

One reason for the relatively low affinities described here could be the fact that only one round of VHH selections was carried out to obtain PKCe binders [23]. However, selections were done from an immune VHH library and usually antigen affinities of VHHs from immunized libraries are 10–100 times better than the affinities of VHHs isolated from naïve or synthetic libraries [25].

Another factor that probably contributes to the relatively low $K_D$ values is the fact that these VHHs can only bind the native form of PKCe. The VHHs can bind human PKCe in immunoprecipitations and kinase activity assays, but not in Western blots where the PKCe protein has been denatured [23]. Furthermore, the VHHs tested here show relatively weak binding in VHH ELISAs (Summanen et al., unpublished results), where PKCe has been coated on the wells of 96-well plates.

Assays such as ELISA and SPR, where the antigen has to be immobilized on a surface in order to measure an interaction, can be problematic when conformation dependent interactions are studied [34]. When the protein is immobilized using functional groups such as $\text{–NH}_2$ or $\text{–COOH}$ groups, the protein molecules are randomly oriented on the surface [35]. Therefore, only some of the protein molecules will be present in an orientation that can be recognized by the interaction partner, in this case the VHHs. Furthermore, when the interaction between randomly immobilized protein and the surface is too strong, there is a possibility of protein denaturation [35].

Since the affinities of the five VHHs studied here could not be measured at all with Biacore or Bionavis SPR when PKCe was amino-coupled to the chip, it is clear that the orientation of PKCe in the chip is crucial for measuring binding. Binding was observed when PKCe was carboxyl-coupled instead, but also in this case only some of the coated PKCe molecules would have been in the correct orientation and conformation. The critical role of protein orientation in SPR measurements is emphasized by the finding that oriented immobilization of an antibody increased its immunobinding efficacy approximately two-fold compared to standard amino-coupling [36]. Therefore, the best option would have been to immobilize PKCe to the flow cell in a controlled orientation via for example a His-tag [35], [37], but due to technical restrictions we were not able to perform such measurements.

As the interaction between the VHHs and PKCe seems to be conformation dependent, the affinity constants measured here are not likely to be the absolute affinities of these VHHs to PKCe in the solution phase. The reported $K_D$ values should therefore not be compared to the affinities reported for VHH antibodies elsewhere. However, the obtained affinity constants can be used for internal comparison to determine which PKCe binders display the strongest interaction to PKCe. The scientific value of the $K_D$'s reported here is evident from the fact that the affinities do
support the data obtained from other experimental setups. Particularly, VHH C1, the strongest activator of PKCε, also has the strongest affinity for PKCε among the three activating VHHs. Furthermore, E6, which is a more potent inhibitor of PKCε kinase activity than G8, also has a higher affinity for PKCε than G8.

We also showed that in addition to the VHHs being conformation dependent, they seem to be species-specific as well. While all five VHHs bind human PKCε and either increase or decrease its kinase activity, they have no effect on the kinase activity of rat PKCε, nor did they bind it in an IP. Both human and rat (Rattus norwegicus) PKCε proteins are 737 amino acids in length, and identical for 726 of these amino acids (98%). Within the catalytic domain, where all of the VHHs described here bind to, the human and rat proteins differ in only eight amino acids. One would expect PKCε specific antibodies to bind to both proteins since the differences between them are so small. However, as reported here, the VHHs do not bind rat PKCε, which again demonstrates how subtle differences in amino acid composition or protein conformation play a critical role in the binding of these PKCε activating and inhibiting VHHs. Further mapping of the VHH binding site will show if the observed species specificity is due to a specific amino acid substitution or a small conformational difference between the human and rat proteins.

The kinetics of the PKCε activation and inhibition were also studied in more detail. We show here that the three activators A10, C1 and D1 increase PKCε activity in different ways. A10 nearly doubles the maximum rate of the reaction, whereas C1 and D1 have almost no effect on the V_max but increase the speed of the reaction relative to the V_max, as is evident from the smaller K_m values reported for these VHHs. These results were similar with and without the PKC activators DOG and PS present in the assay. We know from previous studies [23] that all of the activating VHHs bind the catalytic domain of PKCε. Since the VHHs can increase PKCε kinase activity in an in vitro assay without any additional proteins present, it seems likely that the VHH binding somehow stabilizes the active conformation of PKCε. In order to determine the method of PKCε activation for each VHH, the exact binding sites for each VHH must be studied.

There are also differences between the two PKCε inhibiting VHHs E6 and G8. Based on the kinase activity assay results reported here, E6 is a more potent inhibitor of PKCε, since it leads to a larger decrease in the maximum rate of the reaction. With full-length PKCε, the V_max is only around 20% of the control with E6, whereas G8 has a much smaller effect on full-length PKCε activity. When the catalytic domain is used instead of the full-length protein, G8 also displays a larger degree of inhibition. This supports previous results [23], where G8 was found to be a better inhibitor of the catalytic domain alone than the full-length protein, possibly because in the full-length protein, the G8 binding site could be partially concealed.

According to Michaelis-Menten kinetics, both E6 and G8 are non-competitive inhibitors of PKCε, since increasing VHH concentrations had no effect on the apparent K_m of the system but demonstrated clear decreases in V_max. Therefore, we can rule out the substrate-binding site from the possible binding sites of E6 and G8 within the catalytic domain of PKCε. However, as is the case with the activating VHHs, there are several possible mechanisms by which E6 and G8 can have an effect
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on PKCε kinase activity. A more detailed explanation of PKCε inhibition warrants a study into the exact binding sites of E6 and G8 in the catalytic domain of PKCε.

Remarkably, we also demonstrated for the first time that the PKCε activating and inhibiting VHHs can influence PKCε activity when expressed inside HeLa cells. Upon PMA stimulation, PKCε translocates from the cytoplasm to the plasma membrane, as was shown with EGFP-tagged PKCε in HeLa cells. The activating VHH A10 expressed inside HeLa cells with a C-terminal mCherry-tag increased both the rate and the degree of PKCε translocation compared to the control. On the other hand, the inhibiting VHH G8 decreased the rate of PKCε translocation in response to PMA. Since PKCε translocation is required for activation, we can conclude that the VHHs can influence PKCε activity also in a cellular context. These results highlight the potential of activity modulating VHHs in PKCε research and drug development. Furthermore, the observed cellular effects suggest that the affinities of the VHHs to PKCε are in fact better than the micromolar affinities obtained from SPR experiments. Inside cells, both PKCε and the VHHs will be properly folded, allowing the VHHs to bind to their conformational epitopes on PKCε surface.

The results described here provide important additional information about the VHH activators and inhibitors of PKCε. In addition to the peptide-based PKCε agonists and translocation antagonists [20], [21], these VHHs are the only strictly PKCε isozyme specific activators and inhibitors described so far. Since the different PKC isozymes can have overlapping and sometimes even opposing roles in many biological processes, such isozyme specific compounds that influence kinase activity are crucial in studying the role of PKCε in various contexts. Furthermore, PKCε specific VHHs could in the future be developed into therapeutics against diseases such as cancer or type II diabetes, or the CDR regions of VHHs could be used to design novel peptide-based therapies against these life-threatening diseases.

4.4 MATERIALS AND METHODS

4.4.1 MATERIALS

Mercaptoundecanol, epichlorohydrin, dextran (500 kDa from *Leuconostoc* spp.), bromoacetic acid, N-ethyl-N′-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), phosphate buffered saline (PBS) tablets, and ethanolamine were all obtained from Sigma Aldrich (St. Louis, MO). Gold-coated SPR sensor slides were obtained from BioNavis Ltd (Tampere, Finland).

4.4.2 PRODUCTION AND PURIFICATION OF VHHS

Monoclonal VHH antibodies were produced and purified as previously described [23]. Briefly, VHH production in *E. coli* JM109-strain was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 30°C. Periplasmic fractions were prepared by freezing the bacterial cell pellets for 1 h at −80°C to break the outer membrane of *E. coli* and resuspending cells in 10 ml of phosphate buffered saline (PBS), followed by mixing for 2 h at 4°C. VHHs were
purified from the periplasmic fraction using the his-tag and Talon Metal Affinity Resin (Clontech, CA) and eluted with 300 mM imidazole. Eluted VHHs were dialysed against PBS overnight at 4°C and stored at −20°C until used.

4.4.3 EXPRESSION OF PKCε IN SF9 CELLS

Human full-length PKCε and its catalytic domain (amino acids 298–737) were produced in Sf9 cells using the baculovirus expression system (Bac-to-Bac, Invitrogen, Carlsbad, CA). The cloning of PKCε constructs and baculovirus stock production has been described before [23]. For expression of recombinant PKCε, Sf9 cells were infected with an optimized amount of baculovirus stock and grown for 48 h at 27°C in suspension. The collected cells were washed with PBS and frozen until used. Crude cell lysates were prepared by resuspending cells in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 0.5 mM EGTA and 0.1% Triton X-100, supplemented with a protease inhibitor cocktail (Complete, Roche, Basel, Switzerland) and centrifuging for 15 min at 4°C at 16,200 g. Protein concentrations of the supernatants were determined by Bradford assay (Sigma-Aldrich, St. Louis, MO) and used for kinase activity assays as described below.

4.4.4 SURFACE PLASMON RESONANCE

The affinity measurements were performed with BioNavis SPR Navi 200 (BioNavis Ltd, Tampere, Finland). Carboxymethylated dextran hydrogel for ligand immobilization was self-synthesized according to the BioNavis protocol. First a self-assembled monolayer of mercaptoundecanol was formed on clean gold-coated SPR sensor slides in an overnight reaction in ethanol and rinsed thoroughly. The sensor was then left to react for 3 h with epichlorohydrin (2% v/v) in 0.1 M NaOH, whereafter it was rinsed with Milli-Q H2O, transferred to 30 g/l solution of dextran in 0.1 M NaOH and left to react for 24 h. After washing thoroughly with Milli-Q H2O the sensor was immersed in 0.5 M bromoacetic acid in 2 M NaOH for 24 h. After this reaction the sensor was thoroughly washed with Milli-Q H2O and stored at +8°C until used in protein immobilization reaction.

Protein immobilization to the hydrogel was performed with reverse activated ester synthesis according to the BioNavis protocol. In brief, the immobilization was performed in situ in the instrument using Sigma’s PBS (0.01 M phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4) as background and injection buffer. A flow rate of 20 μl/min and an injection time of 8 min was used for all injections. Reference surface was created in flow channel 2 in parallel with the protein immobilization. The channel was treated in exactly the same manner as the sample channel, except that instead of PKCε protein blank PBS was injected.

The flow cell surface was cleaned with an injection of a solution containing 2 M NaCl and 10 mM NaOH. Activation of the surface was performed by an injection of a solution consisting of 200 mM EDC and 50 mM NHS. Ethylene diamine (10 mg/ml) was injected in order to amine-functionalize the dextran hydrogel. PKCε was diluted to 10.5 μg/ml with the EDC/NHS activation solution, mixed well and immediately
injected to the instrument. Protein immobilization of approximately 80 pg/mm² was observed.

The experiments were performed in HBS (Hepes buffered saline; 20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.001% Tween-20) measurement buffer, with a temperature of 21°C and a flow rate of 20 μl/min with 8 min injection times. Serial dilutions of the VHHs (A10 and C1: 5 μg/ml to 80 μg/ml; D1 and E6: 6.25 μg/ml to 100 μg/ml; G8: 12.5 μg/ml to 200 μg/ml) were injected, as is required for kinetic analysis of molecular interactions [38]. NaOH (10 mM) was found to be an effective regeneration agent for the system, and was used as regeneration solution between each consecutive injection.

4.4.5 RAT BRAIN EXTRACT PREPARATION

Two rats were asphyxiated with CO₂ gas and then decapitated. The skulls were cut open and the brain tissue was scraped into ice cold PBS. The brain tissue was then homogenized with Dounce tissue homogenizer in buffer containing 10 mM HEPES pH 7.5 and 2 mM EDTA. The homogenized tissue was centrifuged at 1000 g for 10 min at 4°C. The resulting supernatant was centrifuged further for 1 h at 40 000 g at 4°C. The extract was then poured into an ion exchange column containing diethylaminoethyl cellulose (DEAE) in column equalization buffer (10 mM HEPES pH 7.5, 2 mM EGTA and 2 mM EDTA). The column containing the extract was extensively washed with column equalization buffer and the remaining bound proteins were subsequently eluted with buffer containing 10 mM HEPES pH 7.5, 2 mM EGTA, 2 mM EDTA, 200 mM NaCl, and 10 mM β-mercaptoethanol. The eluted protein fractions were combined and the protein content was determined. The brain extract was stored at −20°C after addition of 50% glycerol (final concentration).

4.4.6 WESTERN BLOTS

To confirm the presence of PKCe in rat brain extract, 15 μg of protein from rat brain extract was separated by SDS-PAGE gels and blotted onto a PVDF-membrane. The blot was probed with 1:1000 dilution of mouse anti-PKCε antibody (BD Biosciences, NJ), followed by a 1:4000 dilution of HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, CA).

4.4.7 IMMUNOPRECIPITATIONS OF PKCe FROM RAT BRAIN EXTRACT

Immunoprecipitations (IPs) were done to check whether the VHHs can bind the rat PKCe protein. IPs were started by incubating 30 μl of Protein A sepharose CL-4B beads (GE Healthcare, United Kingdom) washed once with immunoprecipitation buffer (IPB; 25 mM Tris-HCl pH 7.5, 0.1% Triton X-100) with 3 μg of VHH in 1 ml of IPB for 1 h at 4°C with continuous shaking. Simultaneously, a sample of protein A sepharose was also coated with 1 μg of commercial PKCe antibody (BD Biosciences, NJ) in 1 ml IPB and was used as a positive control. The antibody-coated protein A beads were blocked with 1% BSA in IPB for 15 min at 4°C and washed once with IPB.
Rat brain extract (200 μg/sample) was added to the beads and incubated overnight with continuous shaking at 4°C. The beads were washed 4 times with IPB and resuspended in 15 μl of 2× Laemmli sample buffer. Samples were loaded onto 15% SDS-PAGE gels for separation of proteins, electrotransferred to PDVF membranes and the membranes were then incubated with anti-PKCε (BD Biosciences, NJ) or anti-tetra His (Qiagen, Venlo, the Netherlands) antibodies followed by HRP-conjugated anti-mouse antibodies (Santa Cruz Biotechnology, CA).

**4.4.8 KINASE ACTIVITY ASSAYS**

Kinase activity assays were carried out as described before [23]. Briefly, kinase activity was determined by measuring the incorporation of [γ-32P] into a PKC substrate peptide MARCKS (FKKSFKL). For determining K\textsubscript{m} and V\textsubscript{max} values, 5 μg of protein from Sf9 cell lysate expressing full-length PKCε or PKCε catalytic domain was pre-incubated with 1 μg of VHH and the substrate (0 μM–1000 μM) in a total volume of 25 μl for 10 min at 30°C in a 96-well plate. Reaction mix (75 μl/well) was added, yielding final concentrations of 10 mM HEPES pH 7.5, 7 mM MgCl\textsubscript{2}, 0.25 mM EGTA, 100 μM cold ATP and 0.3 μM [γ-32P]ATP. Activity of the full-length PKCε was measured with and without PKC activators phosphatidylserine (40 μg/ml) and 1,2-dioctanoyl-sn-glycerol (DOG; 8 μg/ml). Kinase reactions were performed for 5 min at 30°C, after which 25 μl/well was pipetted to a P81 cation exchange paper (Whatman, Kent, United Kingdom). The papers were washed with 75 mM phosphoric acid, dried and placed in scintillation tubes with scintillation fluid. Radioactivity was measured by liquid scintillation counting (1414 Winspectral, Wallac, Finland). For the analysis of PKCε inhibition by E6 and G8, the assay was performed in the same way, except that only the catalytic domain of PKCε was used and VHH concentrations/well ranged from 20–161 nM (E6) and 21–668 nM (G8). Rat brain extract (1 μg/well) was used as an alternative source of PKCε.

**4.4.9 CLONING OF VHH-MCHERRY CONSTRUCTS AND PURIFICATION OF PLASMID DNA**

The activator A10 and the inhibitor G8 were cloned into the pcDNA3.1+ mammalian expression vector (Invitrogen, Carlsbad, CA) with a C-terminal mCherry tag. The mCherry plasmid was a generous gift from Prof. Roger Tsien (University of California, San Diego, CA). First, the mCherry-sequence was cloned into the pcDNA3.1+ vector using the BamHI and EcoRI restriction sites. The resulting mCherry-pcDNA3.1+ plasmid was verified by sequencing.

The cDNA for the VHHs was PCR amplified from Pax50 bacterial expression vectors using the forward primer 5′-GGCGCTAGCATGGCAGAGGTGCAG-3′ and the reverse primer 5′-GGCAGATCTCCCGTGATGGTGATG-3′ to introduce the NheI and BglII restriction sites. The His\textsubscript{6}-tag that was on the C-terminus of the VHHs on the Pax50 expression vector was included in the cloning, so that the His\textsubscript{6}-tag is situated between the VHH and mCherry on the pcDNA3.1+ expression vector. The PCR amplified VHH fragments were digested with NheI and BglII and cloned into the mCherry-pcDNA3.1+ expression vector using the NheI and BamHI sites (BglII
and BamHI have complementary sticky ends). The resulting VHH-His6-mCherry constructs were verified by sequencing and the plasmids were produced in the E. coli strain JM109.

Plasmid DNA for mammalian cell transfections was purified from E. coli cells using the PureYield™ Plasmid Midiprep System (Promega, Fitchburg, WI). To improve the purity of the eluted DNA, a subsequent ethanol precipitation step was performed and the dried DNA was diluted in TE buffer. The plasmid DNA was diluted to a concentration of 1 μg/μl and stored at −20°C. The PKCe-EGFP plasmid, which was a kind gift from Prof. Peter Parker (Cancer Research UK, London Research Institute), was produced and purified as described above.

4.4.10 CELL CULTURE

Human cervical cancer HeLa cells (CCL-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). For transfections and treatments, DMEM without FBS was used. Cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide (CO₂).

4.4.11 HELA CELL TRANSFECTIONS AND TRANSLOCATION STUDIES

For transfections, HeLa cells were seeded to 6-well plates (350 000 cells/well in 2 ml of FBS-supplemented DMEM) and incubated overnight to allow attachment. Double transfections of PKCe-EGFP and mCherry, A10-mCherry or G8-mCherry were carried out in serum-free medium with the FuGENE HD transfection reagent (Roche, Penzberg, Germany) according to the manufacturer's instructions. Translocation studies were performed at 37°C with a Leica SP2 AOBS confocal laser scanning microscope 24 hours after transfections. Double transfected cells expressing both fluorescent proteins were chosen for the experiments, and the 488 nm argon ion laser and the 561 nm He-Ne laser were used for the detection of EGFP-tagged PKCe and VHH-mCherry constructs, respectively. Typically the translocation of PKCe-EGFP was monitored in approximately 4–6 cells per experiment. Once double-transfected cells were located under the microscope, 100 nM PMA was carefully added to the cells. Images from the same cells were taken for 30 min every 30 sec. Translocation of PKCe-EGFP after PMA addition was quantified by measuring the relative fluorescence intensity in a region of interest with a diameter of 5 μm placed in the cytoplasm of each cell.

4.4.12 STATISTICAL ANALYSIS

SPR kinetic analysis of the results was performed with TraceDrawer 1.3 for BioNavis Ltd (Tampere, Finland). The measurements were double referenced, meaning that each sample was referenced using a blank reference channel on line and also 0-samples were measured and referenced from all sensograms during data analysis. Double referencing is a common procedure in SPR biosensor
experiments [38]. The sensograms were fitted with either first order or, when appropriate, second order Langmuir binding models in the TraceDrawer software.

The data from kinase activity assays was analyzed and Michaelis-Menten kinetics were calculated with GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA) software using non-linear regression. The translocation of PKCε-EGFP in HeLa cells was quantified using Leica confocal LAS AF Lite software (Leica Microsystems, Wetzlar, Germany). The statistical significances in translocation speed in cells transfected with the mCherry and VHH-mCherry constructs were calculated with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) using a one-way Anova with Dunnett's post-test. Statistical significance was denoted with * when $p<0.05$.

4.5 ACKNOWLEDGMENTS

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4.6 AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MS NG CB EE. Performed the experiments: MS NG. Analyzed the data: MS NG. Contributed reagents/materials/analysis tools: MY CTV. Wrote the paper: MS NG RKT JB CB EE.

4.7 REFERENCES

I Kinetics of PKCε Activating and Inhibiting Llama Single Chain Antibodies and Their Effect on PKCε Translocation in HeLa Cells


Abstract

Surface plasmon resonance (SPR) is a well-established optical biosensor technology with many proven applications in the study of molecular interactions as well as in surface and material science. SPR is usually applied in the label-free mode which may be advantageous in cases where the presence of a label may potentially interfere with the studied interactions per se. However, the fundamental challenges of label-free SPR in terms of limited sensitivity and specificity are well known. Here we present a new concept called label-enhanced SPR, which is based on utilizing strongly absorbing dye molecules in combination with the evaluation of the full shape of the SPR curve, whereby the sensitivity as well as the specificity of SPR is significantly improved. The performance of the new label-enhanced SPR method was demonstrated by two simple model assays: a small molecule assay and a DNA hybridization assay. The small molecule assay was used to demonstrate the sensitivity enhancement of the method, and how competitive assays can be used for relative affinity determination. The DNA assay was used to demonstrate the selectivity of the assay, and the capabilities in eliminating noise from bulk liquid composition variations.

5.1 INTRODUCTION

Label-free biosensors based on a variety of physical transduction principles, e.g.,
optical, electrochemical and gravimetric transduction, are well-established research
tools. Among optical biosensors, the sensors based on surface plasmon resonance
(SPR) have gained the most widespread acceptance within application areas such as
protein-protein interaction studies and drug screening, including fragment screening
[1–3]. Ever since the pioneering SPR biosensor work of Liedberg et al. [4] and of
Flanagan and Pantell [5], considerable scientific effort has been invested into
improving the analytical performance—in particular the analytical sensitivity—of SPR
instruments. Today, SPR is a highly mature technology, and there are thousands of
research papers published on applications of SPR-based biomolecular interaction
analysis [6,7].

The most distinct advantage of label-free sensor methods is the actual absence of a
label that may potentially alter the chemical properties of the analyte and interfere
with the biochemical binding event. However, it is well known that this absence of a
label inherently causes SPR, like other label-free methods in general, to display
certain performance limitations, most notably concerning sensitivity and specificity.
Even though the sensitivity of SPR instruments has been steadily refined over the last
20 years, it still does not compare favourably with label-based methods like e.g.,
fluorescence and radiochemical methods. The sensitivity of SPR instruments may
often be inadequate when dealing with small molecules, low concentrations, weak or
slow binding events, or in cases where there are limited amounts of biochemically
active binding partners immobilized on the sensor surface [8–10]. It has been
discussed [11] if the sensitivity of SPR instruments can be further improved or
whether the theoretically achievable sensitivity limit has already been reached.
Additionally, in many practical situations, noise sources such as temperature and
pressure variations and variations in the composition of the bulk liquid, may
dominate over pure instrument noise and thus limit the practically achievable
detection limit [12]. When it comes to specificity, SPR, like most other
label-free methods, is a universal detection method that detects any substance that
binds to the surface, irrespective of the identity of the substance. Non-specific
binding of unidentified substances, notably proteins, from the sample solution that
interferes with the detection of the actual analyte is a very common problem [13].

Label-based methods, e.g., fluorescence, generally show superior sensitivity and
specificity as compared to label-free methods. However, fluorescence may show other
disadvantages apart from the potentially interfering presence of the label per se.
Fluorescence-based sensor methods generally show inferior quantitative robustness,
and are hampered by phenomena like quenching, photobleaching, and
environmentally induced variations of the quantum yield [14]. In particular, in the
case of SPR-excited fluorescence, the presence of the gold surface of the SPR sensor
slide, which is a prerequisite for the strong, exciting evanescent field of the sensor,
may cause severe quenching of the fluorophore [3,15]. Also, the highly specific nature
of fluorescence detection may in many cases prove to be a limitation, since the
binding of non-fluorescent substances, e.g., in the preceding immobilization step,
cannot be monitored or quantified.
With a view on the limitations of label-free as well as label-based methods presented above, we have developed the concept of label-enhanced SPR sensing. The concept is based on utilizing strongly absorbing dye molecules in combination with the evaluation of the full shape of the SPR curve. The sensitivity, on a mass basis, is significantly enhanced as compared to conventional label-free SPR. The influence of noise factors like temperature, pressure, and bulk liquid composition variations is also significantly reduced by using label-enhanced SPR sensing. The specificity, with respect to the label, is very high, which reduces the problem of non-specific binding. Additionally, label-enhanced SPR can be run simultaneously and in parallel with label-free SPR on standard SPR hardware. This means that all capabilities of conventional SPR, like universal monitoring of all binding steps, including immobilization, and the full real-time and kinetics capabilities, are completely retained. Consequently, all the advantages of label-free and label-based sensing are combined on one single instrument platform. In this paper, we outline the theory and the principles of label-enhanced SPR, and demonstrate the improved sensitivity and specificity using two simple biochemical model systems: a small molecule assay and a DNA hybridization assay (Figure 1).

![Figure 1](image)

**Figure 1** Artistic illustrations of the assays used in the study. (a) Small molecule assay: Sensor consisting of BSA (blue) and avidin (black), used in the competitive assay of biotin (green) and labelled (red) biotin; (b) DNA hybridization assay: The sensor had the same basic structure as in the small molecule assay, but single-stranded DNA was bound through biotin to the surface-bound avidin, and either non-labelled or labelled complementary DNA was introduced as the sample.

### 5.2 EXPERIMENTAL SECTION

#### 5.2.1 THEORY

The full quantitative theory of SPR, which takes its origins in Maxwell’s equations, has been dealt with in considerable detail elsewhere [16–19], therefore, only a simplified approach will be used here to outline the foundation of label-enhanced SPR. The approach is based on the Kretschmann optical configuration (prism coupling), which is the predominant optical configuration of SPR instruments today. The optical system consists of a glass sensor slide or prism, which is covered by a thin layer of gold in immediate contact with a dielectric medium, *i.e.*, the sample, into which the evanescent field of the surface plasmon wave extends. Light from a monochromatic light source is guided through the prism on the back of the gold layer.
and the intensity of the reflected light from the gold layer is monitored in the angular domain. This results in a graph of intensity as a function of angle, which shows a pronounced minimum due to light absorption and excitation of surface plasmons at a specific combination of light wavelength and reflection angle. This function is called the SPR curve or the SPR dip. In this case, the following equation is valid:

\[ n_p \sin \theta = \text{Re} \left[ \frac{\varepsilon_m \varepsilon_s}{\varepsilon_m + \varepsilon_s} \right] \] (1)

where \( n \) denotes refractive index, \( \theta \) denotes the angle of minimum intensity (due to surface plasmon excitation), \( \varepsilon \) denotes the complex permittivity, and subscripts \( p \), \( m \), and \( s \) denote the prism, metal, and sample, respectively. Equation (1) is the quantitative equation used in conventional, label-free SPR. \( n_p \) and \( \varepsilon_m \) are constant at a constant wavelength. The imaginary part of \( \varepsilon_s \) can be neglected for non-absorbing samples, which reduces \( \varepsilon_s \) to \( n_s^2 \). Consequently, the angle \( \theta \) becomes a simple function of \( n_s \), i.e., the weighted average of the refractive index in the evanescent field zone, which is a measure of the amount of substance bound to the sensor surface. For absorbing samples, \( \theta \) depends also on the imaginary part of \( \varepsilon_s \), i.e., on the absorption coefficient \( a_s \) of the sample. However, it is found both from theory and from experiments that this dependency is rather small [20–22]. Thus:

\[ \theta = f(n_s, a_s) \approx \theta_0 + k_1 (n_s - n_{s,0}) + k_2 (a_s - a_{s,0}) \] (2)

where the second expression is a linear approximation in the shape of a two-dimensional Taylor expansion around an initial value \( \theta_0 \) and \( k_1 \) and \( k_2 \) are simple constants. The linear approximation is usually assumed to be valid within the normal working range of SPR [23]. However, even though the influence of the absorbance term in Equation (2) is rather small, an absorbing sample will influence not only the angle \( \theta \), but the entire shape of the SPR curve. The main effect of light absorption in the sample is a broadening of the curve due to attenuation of the surface plasmons; the higher the absorption, the higher the attenuation and the curve broadening. The full derivation of the shape of the curve is quite lengthy [19], and it is sufficient to state here that the dip width depends primarily on the absorbance \( a_s \) and to a smaller extent on the refractive index \( n_s \) according to the approximate equation:

\[ W = 4\gamma / (n_p \cos \theta) \] (3)

where \( W \) is the angular half-width of the SPR curve (the full angular width of the SPR curve at 50% reflectivity) and \( \gamma \) is a factor that depends primarily on the absorbance of the sample. In analogy with Equation (2), this can be written as:

\[ W = g(n_s, a_s) \approx W_0 + k_3 (n_s - n_{s,0}) + k_4 (a_s - a_{s,0}) \] (4)

Note that the \( n_s \) term dominates in Equation (2), while the \( a_s \) term dominates in Equation (4). Equations (2) and (4) may now be differentiated:
\[ \Delta \theta = k_1 \Delta n_s + k_2 \Delta a_s \]
\[ \Delta W = k_3 \Delta n_s + k_4 \Delta a_s \] (5)

Equation (5) is a simple linear equation system with two unknowns: \( n_s \) and \( a_s \). Hence, both unknowns can be solved by measuring \( \Delta \theta \) and \( \Delta W \). The constants \( k_1 - k_4 \) are empirical constants that appear through the Taylor expansions. They depend on the measurement wavelength, the optical properties of the prism, and the initial value of the sample refractive index, but are true constants at a defined experimental setup.

The \( n_s \) term is the ordinary refractive index signal measured in conventional SPR. However, a significant improvement of the sensitivity can be obtained by using label-enhanced SPR and by selecting the label to be a dye molecule with an anomalously high refractive index at the measurement wavelength \([24–26]\). According to the Kramers-Kronig relations of fundamental optics \([25]\), the maximum value of the refractive index of an absorbing compound appears at a slightly longer wavelength than the absorption maximum.

The \( a_s \) term is a measure of the absorbance, \( i.e. \), a highly specific measure of the amount of dye-label adsorbed onto the sensor surface. This is the basis of the high specificity of label-enhanced SPR. Except in the very unlikely case of non-specific binding of coloured substances from the sample solution, the \( a_s \) term is unaffected by non-specific binding. Also, since most sources of noise, e.g., temperature, pressure, and bulk composition variations, do not contribute to the \( a_s \) term, this term will show very low system noise, which also contributes to the improved analytical sensitivity.

Thus, in order to maximize the sensitivity of label-enhanced SPR, it is advantageous to select the dye label to have as high a refractive index and as high an absorption coefficient as possible at the measurement wavelength. In order to fulfil both these criteria, the dye should be selected to have an absorption maximum at a slightly shorter wavelength than the SPR measurement wavelength at hand. However, from a more practical point of view, other properties of the label also come into play, like e.g., solubility, molecular weight, molecular charge, chemical reactivity, hydrophobicity/hydrophilicity, chemical stability, photostability, purity, and cost of synthesis.

### 5.2.2 MATERIALS

Sodium hydroxide, 20× saline sodium citrate buffer (SSC), sodium dodecyl sulphate (SDS), dimethylsulfoxide (DMSO), sucrose, biotin (native biotin), avidin, and biotin-labelled bovine serum albumin (biotin-BSA) were obtained from Sigma-Aldrich (Helsinki, Finland). Biotin-labelled 25-mer DNA oligonucleotide probe (probe DNA) and complementary 25-mer DNA oligonucleotide (native DNA) were obtained from IDT (Helsinki, Finland). Biotin labelled with dye B12 (labelled biotin) and complementary 25-mer DNA oligonucleotide labelled with dye B10 (labelled DNA) were obtained from Episentec (Solna, Sweden). 2×SSC used in the experiments was diluted from 20× to a 2× working solution (SSC, 20 mM sodium citrate, 300 mM NaCl, pH 7.4) using Milli-Q grade water with a resistivity of 18.2 MΩ·cm.

SPR gold sensor slides were obtained from BioNavis (Tampere, Finland). The SPR sensors were used immediately after cleaning with a hydrogen peroxide-ammonia-
water solution according to the protocol suggested by the manufacturer. Shortly, the sensors were cleaned in a boiling 1:1:5 solution of hydrogen peroxide-ammonia-water for 10 min, washed carefully with plenty of water, and wiped with a cotton-tipped applicator wetted with a 4% SDS solution followed by drying with nitrogen. The SPR instrument used in the measurements was a BioNavis SPR Navi 200-L, equipped with 785 nm and 670 nm light sources. The liquid handling in the experiments was performed using the built-in peristaltic pump and 12-port chromatography injector, and the flow cell used had 1 μL inner volume. All measurements were performed at 25 °C.

The SPR data was analysed using the SPR Navi Data Viewer and the EpiGrammer™ (Episentec) programs. Conventional or “standard” SPR sensorgrams, monitoring purely the refractive index $n_s$, are reported as $\Delta \theta$ values in units of degrees. Enhanced sensorgrams, displaying the absorption coefficient after solving the linear equation system of Equation (5), are reported as $a_s$ values. The $a_s$ values may mathematically also be reported in units of degrees. However, after solving Equation (5), in which the constants may vary somewhat due to varying experimental conditions, it is not physically meaningful to compare these $a_s$ units to “standard” degrees. Therefore, $a_s$ values are reported in arbitrary units (a.u.) instead.

5.2.3 SMALL MOLECULE ASSAY

The sensor slides for the small molecule assay were prepared in situ in the instrument by using a freshly cleaned sensor slide. 2×SSC with 5% DMSO added for solubility enhancement was used as the running and sample buffer for all measurements. The buffer flow was 50 μL/min, and the duration of all sample injections were 2 min. The sensor surfaces were first functionalized with biotin-BSA by injecting 100 μg/mL of biotin-BSA and allowing the protein to spontaneously self-assemble on the gold. This was followed by two subsequent injections of 100 μg/mL of avidin. After the baseline had stabilized, either pure samples of native biotin or labelled biotin, or samples of mixed native biotin and labelled biotin (total biotin concentration 50 μM; mixing ratios 0.0, 0.11, 0.25, 0.4, 0.6 and 1.0 native/labelled), were injected. The concentrations used were high compared to the biotin affinity, and were meant to fully saturate the binding during the relatively short injection time. Since the sensor slides were not regenerable after the biotin injections, a new sensor slide was used for each mixing ratio, and since the amount of avidin varied between sensor slides, the signals of the analyte injections were normalized relative to the avidin binding signal for each sensor slide (Table 1). The use of non-regenerable sensor slides is of course not very practical in a high-throughput assay, but served well to demonstrate the performance in the present experiments. The biotin binding signal was averaged over a two minute interval after the passing of the sample injection pulse through the system.
Table 2. Results of labelled and native biotin competitive interaction assay. The amount of immobilized avidin was used to normalize the label-enhanced SPR signal. The ratio (b/a) is the ratio of native biotin concentration (b) to labelled biotin concentration (a). The results are plotted in Figure 2.

<table>
<thead>
<tr>
<th>b/a</th>
<th>Immobilized Avidin (mdeg)</th>
<th>Enhanced Signal (a.u.)</th>
<th>Normalized Signal (a.u.)</th>
<th>1/Normalized Signal (a.u. $10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>39.0</td>
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<tr>
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<td>117</td>
<td>4.4</td>
<td>0.0376</td>
<td>26.59</td>
</tr>
</tbody>
</table>

5.2.4 DNA HYBRIDIZATION ASSAY

The sensor surface for the DNA detection assay was prepared in situ in the instrument by using a freshly cleaned sensor slide. 2×SSC was used as the running and sample buffer for all measurements. The buffer flow used was 100 μL/min, and the duration of all sample injections was 2 min. The sensor surface was first functionalized with biotin-BSA by injecting 100 μg/mL of biotin-BSA and allowing the protein to spontaneously self-assemble on the gold. This was followed by two subsequent injections of 100 μg/mL of avidin and 25 μg/mL of biotinylated probe DNA. After the baseline had stabilized, several subsequent injections of native DNA and labelled DNA at a concentration of 25 μM were performed, with 10 mM sodium hydroxide regeneration injections between the sample injections.

In the noise reduction experiments (Section 4.4), hybridization of labelled DNA oligonucleotides was performed as above, but with sucrose in the concentration range 0%–0.3% added to the DNA sample solutions.

5.3 RESULTS AND DISCUSSION

5.3.1 SMALL MOLECULE ASSAY: SENSITIVITY

A first set of experiments was designed to demonstrate the enhanced sensitivity of label-enhanced SPR. Firstly, a 400 μM sample of native biotin was injected onto an avidin sensor slide, and the sensorogram was registered in conventional SPR mode. The result is shown in the upper panel of Figure 1. After the initial baseline, there is a large, negative injection peak, but after the sample plug has passed, there is no detectable signal from biotin binding. Secondly, a 5 μM sample of labelled biotin was injected onto another avidin sensor slide, and the sensorogram was registered in the enhanced, absorbance-measuring mode. The result is shown in the lower panel of Figure 2. There is a small overshoot from the injection, but after the sample plug has passed, there is a strong, stable signal from the binding of labelled biotin. The signal-to-noise ratio of the binding signal, as compared to the noise (standard deviation) of
the initial baseline, is about 100. Thus, in effect, the analytical sensitivity with respect to biotin binding is enhanced $100\times$.

It is to be noted that the avidin sensor slide provides a low-capacity surface, consisting essentially of a monolayer of avidin molecules, and consequently a low signal is to be expected upon biotin binding. Molecules of the size of biotin (244 Da) can, under optimized conditions, be detected using conventional SPR when binding to a high-capacity surface based on a three-dimensional, hydrophilic network (e.g., a dextran matrix) [27]. However, the present experiments were not designed to show the absolute signal level achievable for biotin binding, but rather the sensitivity enhancement achievable using dye labelling.

![Image](image.png)

**Figure 2** Comparison between signal generation of native biotin (upper panel) and labelled biotin (lower panel), demonstrating the sensitivity enhancement of the label-enhanced SPR method. The standard sensorgram of native biotin (400 µM in PBS) does not produce any detectable signal after the injection (the negative peak is due to the bulk effect), while the enhanced sensorgram of labelled biotin (25 µM in running buffer) produces a strong signal with a signal-to-noise ratio of about 100.

Even though the binding of even small molecules in many cases can be detected using standard SPR, such monitoring often requires specialized and unfavourable conditions. The detection often requires immobilization of a large amount of binding partner, e.g., protein, on the sensor slide surface and a high density of surface binding sites, which may cause problems with steric effects and crowding (and even avidity effects for multivalent binders) [28]. In the important application field of drug screening on cell membrane receptors, for example, the densely packed environment in a dextran matrix is far from the biologically relevant environment of physically isolated receptor proteins in an essentially flat membrane surface. Also, as is well known, a high density of surface sites regularly causes problems with mass transport limitation effects in kinetic analysis [3], and in particular so since the diffusion is further restricted in a dense matrix as compared to free solution. Standard SPR detection in drug or fragment screening generally requires high concentrations of the
screened compounds in solution to enhance the detectability [29,30], which leads to solubility problems and high non-specific binding. Consequently, it would be desirable to enhance the sensitivity of SPR detection of small molecules for a number of reasons.

The high concentration of native biotin as compared to labelled biotin in the present experiments was used to ensure saturation of native biotin binding, and to eliminate the risk that labelled biotin would yield a stronger signal simply due to a higher binding constant to the surface. However, this risk was later ruled out during the experiments presented in Section 4.2.

5.3.2 SMALL MOLECULE ASSAY: LINEARITY AND COMPETITION

A second set of experiments was designed to show the linearity of the label-enhanced SPR method and its applicability to competitive analysis. Mixtures of varying ratios of native and labelled biotin, but at a constant total biotin concentration of 50 μM, were injected onto avidin sensor slides. The sensorgrams were registered in the enhanced mode, which only measures the binding of the dye-labelled biotin. The results are presented in Table 1 and in Figure 3, in which the inverted signal is plotted vs. the native/labelled biotin ratio. The standard deviation (of the inverted signal) between replicates was 0.38 units in these experiments. The residual experimental variability around the linear regression model in Figure 2 was 0.8%. Since the data in Figure 3, in accordance with the theory of competitive analysis (cf. Equation (6)), yields a straight line, the experiments serve to show that the label-enhanced SPR concept is applicable to competitive analysis. Thus, even though the biotin binding cannot be directly detected in the present system according to Section 4.1, the concentration of biotin in solution can be indirectly determined using competitive analysis. The competitive format has the potential to improve the detection limit of small molecules using SPR by about 100×.

Based on the very plausible assumption that the binding of the small biotin molecules to well-defined, discrete binding sites on the avidin molecules follows a competitive Langmuir adsorption isotherm, the data may be fitted to the following equation [31]:

\[
\frac{1}{\text{Signal}} = \frac{1}{(KC)} + \left(\frac{b}{a}\right) \frac{k_b}{(k_a \ K \ C)}
\] (6)

where C is the total surface binding capacity of biotin, K is an arbitrary sensitivity constant of labelled biotin, k_a and k_b are the equilibrium adsorption constants of labelled and native biotin, respectively, and a and b are the solution concentrations of labelled and native biotin, respectively. The ratio of the binding constants, k_b/k_a, calculated from Equation (6), was 5.5, indicating that the affinity of native biotin is 5.5 times higher than that of the labelled biotin. This difference is not unexpected, since the biotin binding sites of avidin are expected to show the highest binding affinity for native biotin, but, most importantly, this affinity difference does not reduce the practical usefulness of the competitive assay format.
Equilibrium interaction analysis of the competitive binding of native biotin and labelled biotin. The data points are described well by a linear regression line \( (R^2 = 0.992) \) as predicted by Equation (6). The intersection and slope of the linear regression line were used to calculate the relative equilibrium binding constant of labelled biotin.

The applicability to different assay formats is of critical importance for the usability of label-enhanced SPR. In many cases, when analysing complex samples of biochemical origin, it may simply not be possible to selectively label only the analyte, and in other cases, when there is a risk that the label will interfere with the binding event, it may not be desirable to label the analyte. In such cases, labelling of an analyte analogue combined with indirect monitoring of the binding event in a competitive assay may be used. As is well established, competitive assays can be used to determine both concentration affinity constants, and kinetic reaction constants [32–34]. To measure concentration, also inhibitive assays (a.k.a. competition in solution) and sandwich assays may be used. Both of these assay formats use a binder of the analyte which is labelled [6].

The good linear fit of the data in Figure 3 to Equation (6) also serves to justify the linear approximations made in Equations (2) and (4) in Section 2. The higher affinity for the native biotin as compared to the labelled biotin obtained from the competition assay verifies that the risk discussed in Section 4.1, i.e., that the high signal obtained for labelled biotin would be due to a higher affinity for labelled biotin compared to the native biotin, can be safely ruled out.

### 5.3.3 DNA HYBRIDIZATION ASSAY: SPECIFICITY

A third set of experiments was designed to validate the enhanced specificity of label-enhanced SPR. Native and labelled 25-mer DNA oligonucleotides were alternately hybridized to a complementary strand immobilized on the sensor slide surface. The hybridization sensorgrams are shown in Figure 4, where the upper panel shows the standard SPR sensorgram and the lower panel shows the enhanced sensorgram. Note
that both sensorgrams represent the same run. The different injections are detailed in the figure caption.

The most obvious feature of Figure 4 is the high specificity of labelled DNA as compared to native DNA in the enhanced sensorgram. There is a strong, stable signal from the labelled DNA (injections 2 and 3 at times 60 and 79 min), but no discernible signal from the native DNA (injections 1 and 4 at times 45 and 93 min). There are remaining features from the sodium hydroxide regeneration injections even in the enhanced sensorgram. This is due to the fact that these injections cause very large disturbances of the refractive index, which obviously fall outside the linear range of the method.

Figure 4  Standard (upper panel) and enhanced (lower panel) sensorgrams of the hybridization of native and labelled DNA oligonucleotides. The enhanced sensorgram has been calculated from the standard SPR sensorgram according to Equation (5). Native DNA is injected at 44 and 93 min. Labelled DNA is injected at 60 and 79 min. Sodium hydroxide regeneration solution was injected at 47, 52, 70, and 85 min.

The second feature is the absence of baseline drift in the enhanced sensorgram. This drift, which most probably is due to desorption of loosely bound protein from the surface during sodium hydroxide injections, is clearly seen in the standard sensorgram. The improved stability of the baseline in the enhanced sensorgram is another corollary of the high specificity, since the desorbed protein in itself does not absorb light at the SPR wavelength used.

The signal enhancement effect is less apparent in this case than in the case of biotin in Sections 4.1–4.2. The reason for this is that the oligonucleotides as such are much heavier (about 10 kDa) than the biotin (244 Da), thereby causing a relatively stronger signal in the standard sensorgram. Still, the signal-to-noise ratio of the labelled DNA in the enhanced sensorgram is 2.5 times higher than that of the native DNA in the standard sensorgram. There are many applications where the sensitivity of standard SPR may be adequate, but where the improved specificity offered by dye-labelled SPR may be a pronounced advantage, e.g., when analysing large proteins,
like antibodies at low concentration, in samples showing a high non-specific binding of proteins.

5.3.4 DNA HYBRIDIZATION ASSAY: NOISE REDUCTION

A fourth set of experiments was designed to demonstrate the immunity of label-enhanced SPR to noise emanating from variations of the bulk solution environment, or more specifically from variations in the bulk liquid composition. Repeated hybridizations of dye-labelled DNA to a complementary DNA strand immobilized on the sensor slide surface were performed with different concentrations of sucrose added to the buffer. The sucrose additions were selected to represent large variations in the composition, and consequently the refractive index, of the buffer. Figure 5 shows standard sensorgrams of DNA only, sucrose only, and DNA with added sucrose, as well as enhanced sensorgrams of a number of DNA hybridizations in the presence of varying amounts of sucrose.

Figure 5 clearly shows that the strong disturbances from the sucrose additions in the standard sensorgrams are absent in the enhanced sensorgrams. The large bulk refractive index variations induced by sucrose are efficiently eliminated, which is yet another embodiment of the high specificity of label-enhanced SPR. It is rather apparent that the hybridization kinetics, obscured in the standard sensorgrams, can easily and robustly be quantified in the enhanced sensorgrams. The DNA hybrids are so stable that the hybridization level can still be measured after the passing of the injection pulse, but for fast associating or dissociating species, it is essential to be able to discern the surface interaction process from the refractive index disturbance caused by the sample injection pulse.
Most often, it is impossible to exactly match the bulk refractive index of the injected sample with that of the running buffer. This problem is especially pertinent in connection with small molecule drug screening or fragment screening using SPR [29,35], where fast and weak interactions combined with the inherent low signal levels of small molecules may cause detection problems. The compounds to be screened are most often stored in DMSO, and it is practically impossible to exactly match the DMSO concentration of all samples with that of the running buffer. Therefore, bulk disturbances are commonly encountered in standard SPR. There are existing procedures for minimizing the influence of bulk disturbances in standard SPR, but these require double or triple referencing with a reference flow channel, blank samples and the creation of calibration curves composed of several injections of DMSO at different concentrations [36,37]. It is quite clear that there is a great interest in simplifying these procedures in order to decrease the complexity, experimental time, and uncertainty of drug screening.

The present experiments demonstrate how refractive index variations due to composition variations of the bulk solution can be efficiently eliminated using label-enhanced SPR. Other dominating noise factors in SPR originate from temperature and pressure variations of the bulk solution. Since such variations cause the same kind of bulk refractive index variations as do composition variations, it seems safe to assume that also temperature and pressure noise can be efficiently reduced, even though this is not explicitly demonstrated by the experiments.

5.3.5 A COMPARISON OF SIGNAL ENHANCEMENT TECHNIQUES FOR SPR

There are a number of suggestions in the literature on the use of heavy labels, e.g., metal and plastic nanoparticles or high molecular weight compounds, to enhance assay sensitivity by a mere mass increase [38–40]. However, these suggestions show a number of limitations as compared to dye-label enhancement. Firstly, and most importantly, non-absorbing labels do not offer the improved specificity that absorbing labels do. Secondly, slow diffusive mass transport and steric hindrance exclude the use of heavy labels in kinetic analysis. Thirdly, the steric and entropic interference with biochemical binding events is much more pronounced for large labels, like e.g., latex particles, than for dye labels that are small molecules, excluding the use of heavy labels in equilibrium analysis. And fourthly, simple and reliable methods to achieve a controlled 1:1 coupling of biomolecules to larger particles are scarce, while there exists a rich and well-established range of methods for the controlled labelling and purification of molecular species [41]. Consequently, the use of heavy labels to increase the analytical sensitivity in SPR is generally limited to specialized cases of mere concentration analysis.

Another way to improve the sensitivity of SPR is to utilize surface plasmon fluorescence. However, fluorescence methods are plagued by the quenching problems discussed in Section 1 [3,15], while quantitative label-enhanced SPR, based on absorbance of light rather than on fluorescence, offers a much more robust method that is immune to quenching problems. Also, the highly specific nature of fluorescence detection does not allow the monitoring and quantification of non-labelled binding steps, like e.g., the important receptor immobilization step. It would,
in principle, be possible to combine standard SPR and surface plasmon fluorescence in one single instrument [42], but such an instrument would be optically complex and expensive. To the best of the authors’ knowledge, no such combined commercial instrument exists today.

The sensitivity enhancement effect demonstrated using label-enhanced SPR is, quite naturally, largest for small analyte molecules. In the present work, we demonstrate a $100\times$ increase of the achievable signal-to-noise ratio for the 244 Da biotin model molecule. For larger analyte molecules, with a molecular weight of a few kDa, e.g., peptides or oligonucleotides, the sensitivity increase is on the order of $10\times$, which is still a sizeable improvement for critical applications. For full size proteins, larger than about 25 kDa, no signal enhancement effect is obtained using a single dye label, but in this case multiple labelling may be used to further enhance the signal. However, such large molecules usually generate enough signal to be conveniently analysed by conventional SPR. Nevertheless, the improved specificity is a benefit of label-enhanced SPR irrespective of the size of the analyte molecule. This improved specificity may be advantageous also in the analysis of antibodies and other heavy proteins in crude samples, e.g., serum samples or culture media samples, with a high level of non-specific binding.

5.4 CONCLUSIONS

The label-enhanced SPR method can improve the analytical sensitivity of small-molecule detection in SPR about 100-fold. By working in the competitive mode, the detection limit in small molecule analysis can be improved analogously even without direct labelling of the analyte. The method exhibits a high specificity with respect to the labelled compound, which may be of importance also in assays where the sensitivity of standard SPR is adequate, but where there is a high level of non-specific binding. A consequence of the high specificity is also that noise emanating from refractive index variations of the bulk liquid is eliminated. Since label-enhanced SPR can be run simultaneously and in parallel with conventional SPR, all the capabilities of label-free SPR and label-enhanced SPR can be combined on one standard SPR instrument platform. Consequently, the combination of label-free and label-enhanced SPR is foreseen to significantly widen the application field of optical biosensors within biotechnology, biochemistry, and drug discovery.

5.5 ACKNOWLEDGMENTS

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5.6 REFERENCES


II Label-Enhanced Surface Plasmon Resonance: A New Concept for Improved Performance in Optical Biosensor Analysis


Abstract

A three-wavelength angular-scanning surface plasmon resonance based analysis has been utilized for characterizing optical properties of organic nanometer-thick layers with a wide range of thicknesses. The thickness and refractive index were determined for sample layers with thicknesses ranging from subnanometer to hundreds of nanometers. The analysis approach allows for simultaneous determination of both the refractive index and thickness without prior knowledge of either the refractive index or the thickness of the sample layers and without the help of other instruments, as opposed to current methods and approaches for characterizing optical properties of organic nanometer-thick layers. The applicability of the three-wavelength angular-scanning surface plasmon resonance approach for characterizing thin and thick organic layers was demonstrated by ex situ deposited mono- and multilayers of stearic acid and hydrogenated soy phosphatidylcholine and in situ layer-by-layer deposition of two different polyelectrolyte multilayer systems. In addition to the three-wavelength angular-scanning surface plasmon resonance approach, another surface plasmon resonance optical phenomenon, i.e., the surface plasmon resonance waveguide mode, was utilized to characterize organic sample layers whose thicknesses border the micrometer scale. This was demonstrated by characterizing both in situ layer-by-layer deposited polyelectrolyte multilayer systems and an ex situ deposited spin-coated polymer layer.

6.1 INTRODUCTION

The determination of organic ultrathin film properties, i.e., thickness \(d\) and refractive index \(n\), in the range of 0.1–100 nm is still a challenging task, especially when reaching the lower end range of 0.1–10 nm. \([1, 2]\) The lower end range is highly relevant for chemical sensing, organic optics, and electronics, as well as characterizing and measuring biological processes and biological barrier layers, such as cell walls, cell membranes, and lipid bilayers. \([3, 4]\) Furthermore, relatively thick layers from 400 nm to a few micrometers are also relatively difficult to characterize, as they fall into a gap between nano- and macrocharacterization techniques. Examples of layer structures with a few micrometer thickness are, for instance, bacteria, cell types, such as red blood cell monolayers, many natural structural components, such as cellulose cell walls in plants, and biologically relevant hydrogels and structural or fibrillar proteins such as actin, collagen, and keratin fibers. \([5]\) There are several techniques that provide accurate information on organic thin film properties and are commonly used for thin film characterization, e.g. ellipsometry, \([7]\) spectroscopic ellipsometry (SE), \([1]\) surface plasmon resonance spectroscopy (SPR), \([3]\) quartz crystal microbalance (QCM), \([1]\) and atomic force microscopy (AFM). \([8]\) However, in many cases several different techniques have to be simultaneously employed to obtain a reliable estimate of \(d\) and \(n\) of ultrathin and thick organic films. \([1]\)

Ellipsometry and SE utilize polarized light for determining \(d\) and \(n\) of thin organic layers. In the case of SE the dispersion coefficient for materials is also obtained. \([1, 7]\) However, when using monochromatic light for determining \(d\) and \(n\) for thin organic layers by ellipsometry, these two parameters are inseparable. Therefore, it is necessary to know, assume, or measure with some other techniques either \(d\) or \(n\). Even with a known \(n\), it is still difficult to obtain accurate values for organic layer thicknesses below 10 nm with ellipsometry. \([9]\) With SE it is possible to independently determine both \(d\) and \(n\) for organic layers, but for organic ultrathin films where \(2\pi dn/\lambda < 1\), the relative error of \(d\) becomes linked to the initial assumption of \(n\) for the layer, and a unique determination of both \(d\) and \(n\) becomes impossible. \([1]\) When the thickness of the sample layers approaches the micrometer range, the output vector \((\Psi, \Delta)\) obtained from ellipsometry measurements will have a periodicity, which complicates the analysis of such sample layers with single-wavelength ellipsometry. This is, however, not a major issue in SE due to multiple output vectors as a function of the wavelength. \([7]\) Characterizing sample layers with ellipsometry or SE with samples immersed in liquid is also often challenging, because of the optical setup required. The incident light and reflected light need to travel through optical windows, as well as the surrounding medium and the sample layer. This creates challenges in engineering flow systems with low volume and good flow control and prevents the use of opaque liquids. \([7]\)

QCM on the other hand is a mechanical technique which makes use of an AT-cut quartz resonator oscillating at its resonance frequency. \([10]\) It can be used to measure the amount (mass) and mechanical properties (viscoelasticity) of sample layers deposited on top of the quartz crystal resonator by measuring the change in frequency and dampening of the oscillation. If the change in frequency and the
dampening of oscillation are measured at several overtones, the thickness, density, and viscoelasticity of the sample layer can be obtained by theoretical modeling. [11] However, for accurate modeling in QCM also the density of the material should be known, which is practically never accurately known for ultrathin films but is often estimated from bulk properties. [1] Furthermore, the sensing depth of QCM in liquid media is approximately 300 nm, and highly viscoelastic layers with thicknesses in the range of 200–500 nm easily dampen the crystal oscillation completely.

AFM is a mechanical technique where an oscillating or stationary cantilever probe is brought in close contact with the sample surface until van der Waals force repulsion starts to affect the oscillation amplitude or deflection of the cantilever. The oscillation amplitude and/or deflection of the cantilever can then be used to directly measure the topography, as well as the viscoelasticity and viscoelasticity distribution in the sample layers. With AFM it is also possible to measure interaction forces, nanoscale friction, conductivity, and other properties depending on what type of probes are used for the measurements. To accurately measure sample layer thicknesses with AFM, it is necessary to use a mask or some other method to create an internal reference within the sample layer. Additionally, the film thickness obtained by AFM will depend on the tip force or tapping strength used during the measurement, especially for soft and fluidlike films where the tip can penetrate through the sample layer. Another challenge with AFM is that performing in situ measurements, especially in liquid media, is not at all a trivial procedure. [8, 12, 13]

SPR is an optical method where the free electron plasma on a metal surface is excited by using p-polarized visible light. The excitation of surface plasmons can be monitored as a function of the incoming light angle or a change in the wavelength of light. In the most common optical configuration for SPR, i.e., the Kretschmann configuration, the light is coupled to the metal layer through a glass prism from one side, while the sample and the outside medium in contact with the sample are located on the opposite side. This configuration enables signal detection which does not have any interference from the surrounding media as the incoming light is not passing through the media or sample layer as is the case with ellipsometry. [14] To obtain $d$ and $n$ information on a sample layer, a single-wavelength SPR angle versus light reflection intensity spectrum can be fitted using multilayer models in the same way as in ellipsometry. In the cases where the sample layer does not absorb light at the wavelengths used for detection, the same limitations also occur for SPR as for ellipsometry; i.e., $d$ and $n$ are not separable variables. [15, 16] However, it has previously been shown that if the SPR spectra are measured in two different media, [15, 17] or the SPR measurement is performed with two different wavelengths of light and the dispersion relation is known, then a unique $d$ and $n$ can be calculated for the sample layer. [17-20] Usually the limit of thickness determination of sample layers with SPR is taken to be around 300 nm on the basis of the fact that the plasmon evanescent field that decays exponentially penetrates approximately $\frac{1}{2}$ wavelength of the incident light into the media. [21] However, when the sample layer thickness approaches and exceeds $\frac{1}{2}$ times the wavelength of light used for detection, then a harmonic type of waveguide behavior of the SPR begins, which can be used to uniquely determine $d$ and $n$ of the sample layers. [22-24]
III Characterizing Ultrathin and Thick Organic Layers by Surface Plasmon Resonance Three-Wavelength and Waveguide Mode Analysis

In this study we demonstrate how to utilize the SPR phenomenon for determining optical properties of ultrathin and relatively thick organic layers by using a three-wavelength SPR approach for (a) determining the thickness and refractive index of ultrathin films and (b) determining the thickness and apparent refractive index of thick layers in the SPR waveguide mode. This has been achieved by investigating several model systems consisting of well-known ultrathin Langmuir–Blodgett (LB) films, thick polyelectrolyte multilayer films, and a thick spin-coated polymer film.

6.2 MATERIALS AND METHODS

6.2.1 MATERIALS
Hydrogenated l-α-phosphatidylcholine (HSPC; >99%) was obtained from Avanti Polar Lipids, and stearic acid (SA; ≥98.5%), uranyl acetate dihydrate (UAc; ≥98%), poly(styrenesulfonate) sodium salt (PSS; \(M_w = 70\) kDa), poly(allylamine hydrochloride) (PAH; \(M_w = 15\) kDa), poly(ethyleneimine) (PEI; \(M_w = 750\) kDa), poly(l-lysine) (PLL; \(M_w \approx 300\) kDa), and hyaluronic acid (HA; \(M_w = 1500\) kDa) were obtained from Sigma-Aldrich. NaCl, Tris, NH₃OH (30%), and H₂O₂ (30%) were of analytical grade and obtained from Sigma-Aldrich. All chemicals were used as received. All water used was Milli-Q grade with a conductivity of 18 MΩ cm⁻¹ and a total organic content of <4 ppm.

6.2.2 THREE-WAVELENGTH SPR MEASUREMENTS
Three-wavelength surface plasmon resonance measurements were performed with an SPR Navi 200-L instrument equipped with two light source pairs providing 655 and 782 nm, as well as 670 and 783 nm, wavelengths and an autosampler accessory (Oy BioNavis Ltd., Tampere, Finland). SPR sensors were gold-coated sensors (∼50 nm) with a chromium adhesion layer (∼2 nm) and were obtained from BioNavis Ltd. SPR sensors were cleaned before use by boiling them for 15 min in an NH₃ (30%)/H₂O₂ (30%)/H₂O (1:1:5, v/v) oxidizing solution.

All three-wavelength SPR experiments were processed using the BioNavis Dataviewer software. Multilayer models for sample layer analysis were done by using the Winspall 3.02 software. [25] The three-wavelength and two-medium cross point analyses were performed by using Microsoft Office Excel 2007.

6.2.3 COMPRESSION ISOTHERMS AND LANGMUIR–BLODGETT FILM DEPOSITION
A KSV Minitrough instrument (KSV-NIMA, Biolin Scientific Oy, Espoo, Finland) was used for all compression isotherms and Langmuir–Blodgett deposition experiments. The experiments were carried out at 23 °C using a thermostated Teflon trough (330 mm × 75 mm) and 50 μM UAc in the subphase. The isothermal compressions were started 10 min after the monolayer substance was spread onto the subphase. A constant barrier compression speed of 7.5 cm²/min was used during the whole
monolayer compression. For LB depositions the monolayer was first left to stabilize for 10 min at the predetermined surface pressure before the deposition procedure was started. The speed for the deposition of mono- and multilayers of SA and HSPC was 5 mm/min. The deposited layers were allowed to dry in air for at least 15 min before the SPR spectra at different wavelengths of the sample layer were measured. After the SPR spectra were measured, the multilayer buildup was continued using the same sensor. SA monolayers were deposited at surface pressures of 15 and 45 mN/m and multilayers at a surface pressure of 45 mN/m. HSPC monolayers were deposited at surface pressures of 10 and 30 mN/m and multilayers at a surface pressure of 30 mN/m.

$d$ and $n$ of the deposited LB films were determined by three-wavelength SPR analysis from SPR spectra measured of 1, 3, 5, 7, 9, and 11 deposited layers of SA, and from SPR spectra of 1 and 3 deposited layers of HSPC. Additionally, $d$ and $n$ of 11 SA and 3 HSPC layers were determined by two-medium SPR analysis from SPR spectra measured in both air and water.

6.2.4 PM-Irras Measurements
Polarization-modulated infrared reflection absorption spectroscopy (PM-Irras) is a reflection-based infrared tool where $p$- and $s$-polarized spectra are simultaneously measured for a sample deposited on an IR reflecting substrate, such as an air–metal interface. [26] If the substrate is a good electrical conductor (e.g., gold), there is an enhancement effect of molecular dipoles perpendicular to the surface and an elimination of dipoles parallel to the surface. This effect can be used to qualitatively determine the orientation and packing of molecules on the metal surface.

PM-Irras measurements were performed with a KSV-NIMA PMI 550 instrument (KSV-NIMA, Biolin Scientific Oy, Espoo, Finland). PM-Irras spectra of LB monolayers of SA and HSPC deposited on the SPR gold sensor at different surface pressures were recorded at an incident light angle of 80°, which gives the highest sensitivity for gold substrates. The maximum retardation wavelength was set to 3000 cm$^{-1}$ for all measurements.

6.2.5 Polyelectrolyte Multilayer Deposition
The polyelectrolyte multilayer (PEM) depositions were performed and monitored in situ in the SPR Navi 200-L instrument. All PEM buildup experiments were performed in a 0.15 M NaCl, 20 mM Tris buffer with a pH of 7.4 and at a temperature of 20 °C. The flow rate used in the experiments was 50 μL/min, the sample contact time was 4 min, and the washing time between oppositely charged polyelectrolyte injections was 10 min. One layer of PEI (0.1 mg/mL) was first deposited on gold as an adhesion layer for all PEM experiments. After this, the PEMs were formed with sequential deposition of PSS and PAH (0.1 mg/mL) for PSS–PAH PEM formation or HA and PLL (0.1 mg/mL) for HA–PLL PEM formation.

The PSS–PAH PEM formation was measured up to 20 bilayers using both SPR light source pairs and up to 120 bilayers using the 782 and 655 nm SPR light source pair. The measurement was paused, and all the fluidics were thoroughly cleaned with
III Characterizing Ultrathin and Thick Organic Layers by Surface Plasmon Resonance Three-Wavelength and Waveguide Mode Analysis

pure water every 40 bilayers to avoid clogging of the samples in the microfluidics of the instrument.

d and n of the forming PEM layers were determined by three-wavelength SPR analysis from SPR spectra measured for 3, 5, and 10 bilayers for PSS–PAH, for 2 and 5 bilayers for HA–PLL, and for a relatively thick PSS–PAH multilayer consisting of 120 bilayers.

6.2.6 EX SITU PREPARED SPR WAVEGUIDES

A waveguide was prepared on the SPR sensor by spin coating 5 wt % PS–PMMA in toluene solution at 1000 rpm. The spin-coated polymer layer was allowed to dry in ambient room temperature and humidity for 30 min before the SPR spectrum of the sample layer was measured. The PS–PMMA used was a random copolymer with a 24% fraction of MMA determined by 1H NMR and an Mₖ of 500 kDa determined by size exclusion chromatography. The copolymer was a kind gift from the Laboratory of Polymer Chemistry, Department of Chemistry, Faculty of Science, University of Helsinki. The SPR spectrum of the spin-coated polymer layer was measured in air at ambient humidity and room temperature by using a wavelength of 670 nm. The analysis of the optical properties of the waveguide was performed with the Winspall 3.02 software.

6.3 THEORY

6.3.1 SPR VECTOR FUNCTION

Surface plasmons are particle waves of the free electron plasma on a metal surface, which can be excited by p-polarized light under the resonance condition (Figure S1, Supporting Information). A theoretical mathematical description for the resonance condition can be obtained by solving the Maxwell equations for a multilayer optical system. [15] A detailed mathematical description of the SPR phenomenon is available in the Supporting Information(section SI). A general solution for a multilayered system linked to measurable or controllable variables can be obtained by using a transfer matrix formalism of 2 × 2 matrices. The overall formalism has already been published several times, and it is not in the scope of this paper to discuss it in detail again. [15, 16]

In practice, this matrix formalism is solved by mathematical fitting tools or by dedicated software tools developed for it, such as Winspall. [25]

6.3.2 MULTIVARIABLE SPR EXPERIMENTS

Multivariable SPR analysis has previously been described for two-variable systems. [18-20] These approaches have not been widely accepted, and the full analysis described in previous literature is mathematically quite complicated. However, with the ability today to accurately model and calculate multilayer optical
system responses from the SPR spectrum by using matrix formalism, we can simplify the overall process and mathematical analysis for solving the sample layer properties.

As stated previously, the SPR spectrum measured in one set of conditions is in practice not sensitive to the unique differences in \(d\) and \(n\), and only a continuum solution for the surface plasmon wave vector (\(k_{sp}\)) proportional to \(d\) and \(n\) can be deducted:

\[
k_s \propto n \times d. \tag{1}
\]

In practical experiments we can assume that \(k_{sp}\) contains all the information and constants that cause the differences in the experimental SPR spectra measured at several wavelengths or in two different media. Hence, we can simplify the relationship for mathematical purposes in the following way. If we measure the SPR spectrum in two different media with a large enough difference in \(n\), then a unique solution for the final sample layer can be relatively easily calculated from the intersection of the two continuum solutions when \(d_1 = d_2 = d\) and \(n_1 = n_2 = n\), i.e.

\[
\begin{align*}
\{k_{sp1} &= n_1 \times d \\
k_{sp2} &= n_2 \times d \}
\end{align*}
\tag{2}
\]

A similar but slightly more complex approach is to use a multiwavelength approach, as \(n\) also has a wavelength dependency, i.e., \(dn/d\lambda\). However, for relatively small changes, this relationship can to a good approximation be assumed to be linear (Supporting Information, section SII, Figure S2). \cite{27} Hence, this approximation allows us to find a unique solution for \(d\) and \(n\) by solving the following equation system:

\[
\begin{align*}
k_{sp1} &= n_{\lambda_1} \times d \\
k_{sp2} &= n_{\lambda_2} \times d, \\
k_{sp3} &= n_{\lambda_3} \times d
\end{align*}
\tag{3}
\]

where

\[
n_{\lambda_2} = n_{\lambda_1} + \frac{dn}{d\lambda} \times (\lambda_2 - \lambda_1); \quad n_{\lambda_3} = n_{\lambda_1} + \frac{dn}{d\lambda} \times (\lambda_3 - \lambda_1), \tag{4}
\]

and

\[
\begin{align*}
k_{sp1} &= n_{\lambda_1} \times d \\
k_{sp2} &= \left(n_{\lambda_1} + \frac{dn}{d\lambda} \times (\lambda_2 - \lambda_1)\right) \times d. \\
k_{sp3} &= \left(n_{\lambda_1} + \frac{dn}{d\lambda} \times (\lambda_3 - \lambda_1)\right) \times d
\end{align*}
\tag{5}
\]

If even more wavelengths are available, then in theory it should be possible to use a true Cauchy relation instead of the linear approximation of \(dn/d\lambda\). It is worth mentioning that the discussion above is only valid for sample layers that do...
not absorb light at the wavelengths used for SPR spectrum measurement, i.e., for $k = 0$, which is often the case for organic sample layers. If $k \neq 0$, then there is actually a unique solution for the sample layer in the $k_{sp} = d(n + ik)$ space, and the above approach would be unnecessary.

6.3.3 SPR WAVEGUIDE

A so-called SPR waveguide mode can be utilized in SPR sensing when relatively thick dielectric layers are deposited on the SPR sensor surface, i.e., when the thickness of the sample layer is $>1/2\lambda$ of the incident light. When using the Kretschmann configuration, it is even possible to couple surface plasmons and optical waveguide modes so that the excitation light and the guided wave modes are phase matched. [23] The effect of matching these conditions has been discussed previously for thin and loose hydrogels [23] and for dense spin-coated polymers. [22] The SPR waveguide coupling can theoretically be investigated and analyzed by using the same mathematical models as for standard SPR as described above.

6.3.4 RESULTS AND DISCUSSION

6.3.5 COMPRESSION ISOHERMS AND LANGMUIR–BLODGETT DEPOSITION

The purpose of utilizing Langmuir and Langmuir–Blodgett methods in this study is shortly discussed in the Supporting Information (section SIII). Parts A and B of Figure S3 (Supporting Information, section SIII) show the compression isotherms of SA and HSPC measured on pure water and UAc-containing subphases. UAc was chosen as the subphase counterion as it has been shown to enable the deposition of more than one monolayer of phospholipids on a solid substrate. [28, 29] The compression isotherms reveal that the UAc expands the liquid state of both the SA and HSPC monolayers compared with the monolayers measured on a pure water subphase. This is an effect opposite what is usually encountered with multivalent counterions, such as Mn$^{2+}$, Cd$^{2+}$, and Tb$^{3+}$. [30] Also, the area per molecule is slightly larger for both monolayers on a UAc-containing subphase compared with the pure water reference. These effects can be attributed to the complex state of uranium(IV), which forms (UO$_2$(OH)$_2$)$_{2^{2-}}$ or UO$_2$(CO$_3$)$_{2^{2-}}$ complexes at pH 5.6 used in the experiments shown in Figure S3. [31] The formation of these uranyl complexes consequently makes the counterion larger than simple multivalent ions. Therefore, the uranyl counterion complexes become the limiting factor in the packing of the monolayers instead of the hydrophobic lipid tail of the amphiphilic substances in the monolayer. [32, 33] However, the UAc counterion clearly stabilizes and rigidifies the monolayer similar to other multivalent counterions, which can be seen from the increase in the maximum pressure that can be achieved in the compression isotherm. The UAc counterions in the subphase thus enabled us to deposit mono- and multilayers of both SA and HSPC monolayers on SPR sensor slides for further optical characterization of ultrathin organic layers. The transfer ratios of all the LB depositions were close to 1 (data not shown), which indicates that the transfer of the
mono- and multilayers was complete and that the transferred layers were intact and of good quality.

The PM-IRRAS (Supporting Information, section SIII, Figure S3C,D) and isotherm (Supporting Information, section SIII, Figure S3A,B) data show that the SA monolayer has a more ordered packing than the HSPC monolayer at higher surface pressures and that the order in the SA monolayer increases with increasing surface pressure. The difference in the SA and HSPC monolayer properties should clearly be reflected in the optical properties of the monolayers. Furthermore, the SA monolayer deposited from a UAc subphase is not as highly ordered as an SA monolayer deposited from a Cd$^{2+}$-containing subphase, which exhibits a crystalline hexagonal packing. [33] For optical measurements such as SPR and ellipsometry, a slightly more random order makes comparison between the two different molecules more reliable. For example, crystalline hexagonal packing of SA with cadmium[27] has a relatively large effect on the optical properties of the layer, [17] which is reflected as an anomaly large dispersion coefficient for the Cd–stearate monolayer.

### 6.3.6 THREE-WAVELENGTH AND TWO-MEDIUM SPR ANALYSIS OF ULTRATHIN FILMS

The SPR full angle scans measured for SA and HSPC mono- and multilayers were separately fitted with a multilayer model in Winspall for each thickness, wavelength, and appropriate surrounding medium for the measurement to gather the $d$–$n$ continuum solutions for the layers. The procedure started with finding the optimized optical properties for the pure SPR sensor slide by accurately fitting the background SPR full angle scan measured for the same SPR sensor slide used for mono- and multilayer depositions. The optical properties of the pure SPR sensor slide were then kept constant and used as the starting point for finding the optical properties of the deposited sample layers. Parts A–C of Figure 1 show examples of the results of the Winspall fits to the SPR full angle scans measured in air with 655, 670, and 782 nm laser wavelengths for the pure SPR sensor slide and one SA monolayer deposited at 45 mN/m. Figure 1D on the other hand shows the SPR full angle scans measured in both air and water with a 783 nm laser wavelength for the pure SPR sensor slide and for 11 SA monolayers deposited at 45 mN/m.

For the three-wavelength analysis, the $d$ and $n$ continuum solutions were plotted against each other, and the $dn/d\lambda$ value was varied manually until a matching cross point between the wavelength pairs was found (Supporting Information, section SIV, Figure S4). In the case of the two-medium analysis the continuum solution was directly obtained by determining the cross point in the $d$–$n$ plot obtained by analyzing the SPR full angle spectra of SA and HSPC multilayers measured in both air and water. The cross point determined through the two-medium analysis is also the unique solution for $d$ and $n$ of the corresponding multilayer. Tables 1 and 2 summarize the results from the two-medium and three-wavelength analyses for these ultrathin films, respectively.
III Characterizing Ultrathin and Thick Organic Layers by Surface Plasmon Resonance Three-Wavelength and Waveguide Mode Analysis

![Figure 1](image1)

Figure 1  SPR full angle scans of the pure SPR sensor background (blue solid lines) and a single deposited SA monolayer (red solid lines) with corresponding Winspall fits (symbols and black dashed lines) measured with (A) 655 nm, (B) 670 nm, and (C) 783 nm laser wavelengths. (D) Measured SPR full angle scans of 11 SA layers measured with a 783 nm laser wavelength, both in air (solid lines) and in water (dashed lines). Inset tables show the optical parameters used for the Winspall fits (1 = prism, 2 = chromium, 3 = gold, 4 = sample layer, 5 = air)

![Figure 2](image2)

Figure 2  Example of finding the intersection points from thickness \((d)\) versus refractive index \((n)\) plots of (A) 1 monolayer of HSPC in air using the three-wavelength analysis and (B) 11 layers of SA with the two-medium analysis. The arrows emphasize the intersection point found in the graphs.
Table 1. Thickness (d), Refractive Index (n), Dispersion \( (dn/d\lambda) \), Thickness per Layer (d/layer), and Error Estimates (±) Obtained from the Two-Medium Analysis for HSPC and SA Multilayer Films Deposited at 30 and 45 mN/m, Respectively

<table>
<thead>
<tr>
<th>HSPC</th>
<th>d (nm)</th>
<th>&quot;±&quot; (nm)</th>
<th>n(780nm)</th>
<th>&quot;±&quot;</th>
<th>dn/d(\lambda) calculated</th>
<th>d/layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 LB 655 nm</td>
<td>8.86</td>
<td>0.01</td>
<td>1.506</td>
<td>0.001</td>
<td></td>
<td>2.95</td>
</tr>
<tr>
<td>3 LB 670 nm</td>
<td>8.03</td>
<td>0.01</td>
<td>1.552</td>
<td>0.001</td>
<td></td>
<td>2.68</td>
</tr>
<tr>
<td>3 LB 782 nm</td>
<td>8.65</td>
<td>0.01</td>
<td>1.525</td>
<td>0.001</td>
<td>0.00015</td>
<td>2.88</td>
</tr>
<tr>
<td>3 LB 783 nm</td>
<td>7.65</td>
<td>0.01</td>
<td>1.599</td>
<td>0.001</td>
<td>0.00037</td>
<td>2.55</td>
</tr>
<tr>
<td>AVG</td>
<td>8.30</td>
<td></td>
<td></td>
<td></td>
<td>0.00026</td>
<td>2.77</td>
</tr>
<tr>
<td>STD</td>
<td>0.55</td>
<td></td>
<td></td>
<td></td>
<td>0.18</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SA</th>
<th>d (nm)</th>
<th>&quot;±&quot; (nm)</th>
<th>n(780nm)</th>
<th>&quot;±&quot;</th>
<th>dn/d(\lambda) calculated</th>
<th>d/layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 LB 655 nm</td>
<td>28.76</td>
<td>0.01</td>
<td>1.512</td>
<td>0.001</td>
<td></td>
<td>2.61</td>
</tr>
<tr>
<td>11 LB 670 nm</td>
<td>27.84</td>
<td>0.01</td>
<td>1.531</td>
<td>0.001</td>
<td></td>
<td>2.53</td>
</tr>
<tr>
<td>11 LB 782 nm</td>
<td>29.26</td>
<td>0.01</td>
<td>1.509</td>
<td>0.001</td>
<td>2.6E-05</td>
<td>2.66</td>
</tr>
<tr>
<td>11 LB 783 nm</td>
<td>28.60</td>
<td>0.01</td>
<td>1.520</td>
<td>0.001</td>
<td>9.0E-05</td>
<td>2.60</td>
</tr>
<tr>
<td>AVG</td>
<td>28.62</td>
<td></td>
<td></td>
<td></td>
<td>0.00006</td>
<td>2.60</td>
</tr>
<tr>
<td>STD</td>
<td>0.59</td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Thickness (d), Refractive Index (n), Dispersion \( (dn/d\lambda) \), Thickness per Layer (d/layer), and Error Estimates of the Cross Point Determination (±) Obtained from the Three-Wavelength Analysis in Air for HSPC and SA Monolayers Deposited at Two Different Surface Pressures and for SA Multilayers Deposited at 45 mN/m

<table>
<thead>
<tr>
<th>HSPC</th>
<th>d (nm)</th>
<th>&quot;±&quot; (nm)</th>
<th>n(780nm)</th>
<th>&quot;±&quot;</th>
<th>(dn/d\lambda) (1/nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 LB 10 mN/m</td>
<td>3.4</td>
<td>0.10</td>
<td>1.404</td>
<td>0.001</td>
<td>0.0002</td>
</tr>
<tr>
<td>1 LB 30 mN/m</td>
<td>3.7</td>
<td>0.10</td>
<td>1.334</td>
<td>0.001</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SA</th>
<th>d (nm)</th>
<th>&quot;±&quot; (nm)</th>
<th>n(780nm)</th>
<th>&quot;±&quot;</th>
<th>(dn/d\lambda) (1/nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 LB 15 mN/m</td>
<td>2.4</td>
<td>0.10</td>
<td>1.584</td>
<td>0.5</td>
<td>0.0002</td>
</tr>
<tr>
<td>1 LB 45 mN/m</td>
<td>3.88</td>
<td>0.70</td>
<td>1.351</td>
<td>0.07</td>
<td>0.00015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SA 45 mN/m</th>
<th>d (nm)</th>
<th>&quot;±&quot; (nm)</th>
<th>n(780nm)</th>
<th>&quot;±&quot;</th>
<th>(dn/d\lambda) (1/nm)</th>
<th>d/layer (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 LB</td>
<td>3.88</td>
<td>0.70</td>
<td>1.351</td>
<td>0.07</td>
<td>0.00015</td>
<td>3.88</td>
</tr>
<tr>
<td>3 LB</td>
<td>8.8</td>
<td>0.20</td>
<td>1.471</td>
<td>0.0099</td>
<td>0.00015</td>
<td>2.94</td>
</tr>
<tr>
<td>5 LB</td>
<td>12.7</td>
<td>0.08</td>
<td>1.551</td>
<td>0.01</td>
<td>0.00015</td>
<td>2.54</td>
</tr>
<tr>
<td>7 LB</td>
<td>17.9</td>
<td>0.29</td>
<td>1.536</td>
<td>0.0049</td>
<td>0.00015</td>
<td>2.56</td>
</tr>
<tr>
<td>9 LB</td>
<td>21.9</td>
<td>0.09</td>
<td>1.571</td>
<td>0.00075</td>
<td>0.00015</td>
<td>2.43</td>
</tr>
<tr>
<td>11 LB</td>
<td>26.4</td>
<td>0.34</td>
<td>1.573</td>
<td>0.0078</td>
<td>0.00015</td>
<td>2.40</td>
</tr>
<tr>
<td>AVG of LB 5-11</td>
<td>0.20</td>
<td>1.558</td>
<td>0.0057</td>
<td></td>
<td></td>
<td>2.48</td>
</tr>
<tr>
<td>STD of LB 5-11</td>
<td>0.136</td>
<td>0.018</td>
<td>0.004</td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
</tbody>
</table>
It is interesting to notice concerning the three-wavelength analysis (Table 2) that the thicknesses of the SA monolayers deposited at low and high surface pressures show large differences in their values, while the differences in the thicknesses obtained for the HSPC monolayers at low and high surface pressures are much smaller. This is in good agreement with the compression isotherms (Figure S3A,B, Supporting Information) and PM-IRRAS data (Figure S3C,D) of SA and HSPC monoalyers, which clearly indicated that the SA monolayer undergoes a structural ordering in the form of increased trans conformers along the hydrocarbon chain when the surface pressure is increased from 15 to 45 mN/m. On the other hand, the structural changes in the HSPC monolayer were merely due to a slight change in the tilt angle of the hydrocarbon chains with respect to the SPR sensor slide surface.

As can be seen in Figure 2A, all the $d-n$ continuum solutions obtained by the three-wavelength analysis in air did not necessarily cross perfectly as would be expected from a purely theoretical point of view. The reason for this was most probably the fact that the wavelength pairs in the SPR instrument used in this study actually measure the sample layers from two physically different spots. To take this uncertainty into account in the analysis, the parameter values were estimated graphically as the average of the two cross points in the $d-n$ plots for different laser pairs (Supporting Information, section SIV), and the difference between the average of the cross points and the actual cross points has been given as an error estimate (“±”) in the values provided for each analysis in Tables 1 and 2.

The three-wavelength analysis of single deposited monolayers of both HSPC and SA shows relatively high uncertainty in the obtained values, and it seems to overemphasize the thickness of the first layer compared with the two-medium analysis (Tables 1 and 2). However, the individual thicknesses obtained for SA mono- and multilayers plotted against the layer number in Figure 3A show an excellent linear dependency, as well as a transition of the intersection with the $y$ axis at 1.7 nm. The transition is on the order of magnitude of the peak-to-peak roughness of 2 nm of the SPR sensor slide (Supporting Information, section SV, Figure S5). The linear trend of the individual analyses of the SA mono- and multilayers confirms that the three-wavelength analysis behaves as expected aside from the deviation in the first layer. The slope in the $d$-layer number plot of the SA mono- and multilayers gives a thickness of 2.24 nm/layer, while the three-wavelength analysis for SA multilayers with five or more deposited layers gives an average thickness of 2.48 ± 0.08 nm (Table 2). This is in good agreement with the value of 2.6 ± 0.05 nm obtained from the two-medium analysis (Table 1). The average layer thicknesses obtained from both the three-wavelength and two-medium analyses correspond well to the theoretical length of 2.5 nm of SA, [33] the thickness of 2.66 ± 0.05 nm reported for Cd–stearate monolayers, [17, 33] and the average thickness between 2.66 and 2.79 nm/layer obtained with ellipsometry for arachidic acid/uranyl acetate multilayers. [34]
The individually extracted refractive indices from the three-wavelength analysis for the SA mono- and multilayer films plotted against the layer number in Figure 3B show a nonlinear increasing trend in the refractive index values, which levels out after five deposited layers. This can be explained by a space-filling model where the first couple of deposited layers partially follow the roughness of the SPR sensor surface, and then the layers gradually bridge the gaps and finally start to deposit more smoothly with better organization and orientation when more layers are deposited. This probably also causes the slightly lower thickness of 2.24 nm/layer for the SA monolayers obtained from the slope in Figure 3A compared with the individually determined average thicknesses obtained from the three-wavelength and two-medium analyses.

The results obtained from the three-wavelength analysis indicate that this approach detects the real thickness and the apparent (layer density dependent) refractive index of the ultrathin layers. The two-medium analysis, on the other hand, probes the real refractive index and the apparent thickness of the sample layers. This is surprising, as usually similar optical methods are thought to give an apparent thickness of a virtual full layer, which is of course always lower than the real layer thickness if there is any roughness present in the system. The three-wavelength SPR analysis on the other hand uses multiple and simultaneous probing wavelengths which interact with the system slightly differently. This means that, although the dispersion coefficient has to be empirically determined, or assumed if only two wavelengths are used, the three-wavelength analysis actually determines the real layer thickness more accurately than the single-wavelength analysis where the refractive index has to be completely assumed or is taken from literature sources for bulk materials. Regarding the dispersion coefficient, it is obvious that the uranyl acetate counterion clearly has a smaller effect on the refractive index of the condensed monolayers than the cadmium ions, which earlier showed a very large $dn/d\lambda$ value of 0.00463 for Cd–stearate. [17] The dispersion coefficient values obtained for the ultrathin films in this study are close to those of other organic
compounds, such as palmitic acid \((dn/d\lambda \approx 0.00005)\) \cite{35} and different polymers \((0.00008–0.00003)\), \cite{36} and those obtained for the polyelectrolyte multilayers (see the SPR analysis of polyelectrolyte multilayers section below). This difference is probably due to the fact that the Cd–stearate monolayer is arranged in a hexagonal crystalline lattice, while uranyl acetate and other complex counterions result in a less ordered packing in the monolayers, and therefore, the optical properties of the layer are not affected by the crystallization.

### 6.3.7 THREE-WAVELENGTH SPR ANALYSIS OF POLYELECTROLYTE MULTILAYERS

Two types of polyelectrolyte multilayers, i.e., PSS–PAH and HA–PLL, with different growth characteristics and properties were chosen for three-wavelength analysis. The thickness of the PSS–PAH polyelectrolyte multilayer is known to grow relatively slowly for each additional layer, but with quite dense layers. \cite{37} The thickness of the HA–PLL polyelectrolyte multilayer grows very fast, forming relatively low density layers compared with the PSS–PAH multilayer. \cite{38} As the PEM deposition can include tens or hundreds of layers, only a few layers close to the beginning of the depositions were characterized in detail. The actual three-wavelength characterization of the polyelectrolyte multilayers followed the same procedure as described earlier for the LB deposited SA and HSPC mono- and multilayers, with the exception that the SPR full angle spectra for the polyelectrolyte multilayers were measured in liquid media and not in air as was the case for the LB films. Table 3 summarizes the results obtained for the thicknesses and refractive indices for 3, 5, and 10 bilayers of PSS–PAH and 2 and 5 bilayers of HA–PLL polyelectrolyte multilayers.

### Table 3. Thickness \((d)\), Refractive Index \((n)\), Dispersion \((dn/d\lambda)\), Thickness per Layer \((d/\text{Layer})\), and Error Estimates \(\pm\) Obtained from the Three-Wavelength Analysis for PSS–PAH and HA–PLL Polyelectrolyte Multilayer Films

<table>
<thead>
<tr>
<th>No. of bilayers</th>
<th>(d) (nm)</th>
<th>(\pm) (nm)</th>
<th>(n(780\text{nm}))</th>
<th>(\pm)</th>
<th>(dn/d\lambda) ((1/\text{nm}))</th>
<th>(d/\text{layer}) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS:PAH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9.2</td>
<td>0.5</td>
<td>1.495</td>
<td>0.010</td>
<td>0.00003</td>
<td>3.07</td>
</tr>
<tr>
<td>5</td>
<td>16.8</td>
<td>0.5</td>
<td>1.481</td>
<td>0.005</td>
<td>0.00003</td>
<td>3.36</td>
</tr>
<tr>
<td>10</td>
<td>35.0</td>
<td>1</td>
<td>1.470</td>
<td>0.005</td>
<td>0.00002</td>
<td>3.50</td>
</tr>
<tr>
<td>120</td>
<td>400.0</td>
<td></td>
<td>1.501</td>
<td></td>
<td></td>
<td>3.33</td>
</tr>
<tr>
<td>((782\text{ nm}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>404.0</td>
<td></td>
<td>1.503</td>
<td></td>
<td></td>
<td>3.37</td>
</tr>
<tr>
<td>HA:PLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25.2</td>
<td>3</td>
<td>1.356</td>
<td>0.001</td>
<td>0.00003</td>
<td>12.61</td>
</tr>
<tr>
<td>5</td>
<td>67.1</td>
<td>3</td>
<td>1.351</td>
<td>0.001</td>
<td>0.00005</td>
<td>13.42</td>
</tr>
</tbody>
</table>
The characteristic behavior of the two different polyelectrolyte multilayers is very clear; i.e., the thickness per layer for the HA–PLL polyelectrolyte multilayer is always at least 4 times larger than the thickness per layer for the PSS–PAH polyelectrolyte multilayer and already reaches a thickness of ~67 nm after five deposited bilayers. The thicknesses per layer for both polyelectrolyte multilayers also increase slightly with the number of layers, which is often the case because the first few layers during polyelectrolyte multilayer buildup usually grow in a nonlinear fashion. [37, 38] Furthermore, the refractive indices obtained for the HA–PLL polyelectrolyte multilayers are clearly smaller than for the PSS–PAH polyelectrolyte multilayers. The results from the three-wavelength analysis of the polyelectrolyte multilayers are in good agreement with the facts that HA–PLL polyelectrolyte multilayers exhibit exponential growth characteristics, forming low-density layers. [38] PSS–PAH has been reported to exhibit a linear growth pattern with high-density layers. [37] The thickness values obtained from the three-wavelength analysis for the polyelectrolyte multilayers correlate very well with previously reported thickness values of 4 nm/layer for PSS–PAH polyelectrolyte multilayers, especially when taking into account that the concentration used for the polyelectrolyte multilayer buildup in the present study was 1/10 of the concentration normally used. [37]

The results obtained from the three-wavelength analysis for both the LB films and the polyelectrolyte multilayers indicate that the SPR technique can effectively be utilized for characterizing optical properties of ultrathin films without previous knowledge of the system. While the examples in this study still show some uncertainty in the results, the three-wavelength analysis approach is rather easy to perform, while the level of the uncertainties can be easily estimated and evaluated for acceptance. Critical assessment of the limits of detection and accuracy of the three-wavelength SPR analysis presented in this study in comparison to established methods such as ellipsometry or SE have proven difficult because of insufficient literature available. However, a careful evaluation of published results on ultrathin film measurements indicates that the SPR three-wavelength analysis approach is able to characterize thinner layers with less uncertainty for the extracted thickness and refractive index values. Additionally, the ability of the three-wavelength SPR analysis to determine the real thickness instead of apparent thickness obtained from single-wavelength and two-medium analysis could be extremely beneficial in the future when characterizing ultrathin films in the range of a few to tens of nanometers.

Schoch and Lim recently published a study [39] where SPR was used for analyzing the layer thickness by utilizing a noninteracting sample which probes the excluded volume created by the layers on the sensor surface. Their study showed a capability of determining the real thickness and apparent (layer density dependent) refractive index of thin organic layers similar to that of the three-wavelength analysis approach presented in the current study. As stated by Schoch and Lim, self-assembly of polymers into loose solvated polymer brushes tends to occupy a space related to their hydrodynamic size, and use of the bulk material refractive index is clearly incorrect for such systems. Therefore, any approach that can take into account the material density related refractive index is always more correct than using bulk refractive indices. The benefit of their approach is that it does not need any knowledge about the underlying optical system (sensor structure), but it is a relative measurement,
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unlike the analysis approach presented in the current study. However, the approach of Schoch and Lim requires a lot of knowledge about the sample layer properties, reference cells, and a noninteracting probe sample, which actually makes it quite a tedious measurement to perform in practice. The analysis approach presented in the current study does not need any of these. Furthermore, the molecular probes approach utilized by Schoch and Lim is suitable mostly for liquid-phase measurements, and there will always be an issue of finding completely noninteracting samples with sufficiently high refractive index. After all, most noninteracting samples, such as polysaccharides and polyethylene glycol, are materials with extremely low refractive index, which produce low signal responses in SPR. In addition, Schoch and Lim claim that their approach bypasses the refractive index constraint in SPR. However, one of the most important properties of organic layers is the real refractive index, which should not be neglected, especially for layer-related optical applications. From another point of view, the refractive index itself reflects the integrity of the deposited layers, because the refractive index is sensitive to the molecular packing density of the sample layers.

6.3.8 SPR ANALYSIS OF RELATIVELY THICK FILMS

It is possible to build very thick and dense layers with polyelectrolytes under proper experimental conditions and by the choice of the polyelectrolytes used for the layer buildup. It has been shown that the PSS–PAH polyelectrolyte pair can be used to prepare polyelectrolyte multilayers consisting of hundreds of layers, partly because of its ability to form densely packed layers with linear growth characteristics. [37] Figure 4A shows a time sensogram of the change in SPR minimum angle measured with a 655 nm laser wavelength for 110–120 PSS–PAH bilayers. Every single peak in the time sensogram represents an injection of an oppositely charged polyelectrolyte followed by a rinsing period. The time sensogram clearly shows that the signal from the growing PSS–PAH polyelectrolyte multilayer was still behaving in a linear fashion when the SPR waveguide mode minimum was monitored. It is also clear from the sensogram that the SPR waveguide mode is still sensitive enough to measure the addition of nanometer-scale layers on top of the waveguide layer, and not only for detecting changes in the layer density in the form of sample absorbing into the waveguide as shown earlier. [23, 24]

Interestingly, the PSS–PAH polyelectrolyte multilayer induced an SPR waveguide when approximately 100 bilayers had been deposited, which could be seen as a dramatic decrease in the SPR peak intensity, as well as the appearance of an additional peak at lower angles compared with the main SPR peak (Figure 4B). The waveguide formed by the PSS–PAH polyelectrolyte multilayer also showed a significant wavelength dependency (Figure 4B,C). The SPR resonance peak in the SPR full angle scan measured with the 655 nm laser wavelength has progressed enough to higher angles to be clearly distinguishable from the additional waveguide peak appearing in the critical angle region of the SPR spectrum (Figure 4B). The SPR full angle spectrum measured with a 782 nm laser wavelength for the same layer thickness shows that the main SPR resonance peak and the waveguide peak in the critical angle region can still be detected as an overlapping optical phenomenon
(Figure 4C). However, an overlapping critical angle and the main SPR resonance peak can still be used to calculate the thickness of the layer.

**Figure 4**  
(A) Time sensogram of the change in SPR minimum angle measured with a 655 nm laser wavelength for 110–120 bilayers. SPR full angular scans (solid red lines) and corresponding Winspall fits (dashed black line) for 120 bilayers of PSS–PAH polyelectrolyte multilayers measured with (B) 655 nm and (C) 782 nm laser wavelengths. (D) SPR full angle scan of a spin-coated PS–PMMA copolymer layer measured in air with a 670 nm laser wavelength (red solid line) and the corresponding Winspall fit (black times signs, dashed black line) plotted together with a pure gold background (blue solid line). The inset table in (D) shows the optical parameters used for the Winspall fit (1 = prism, 2 = chromium, 3 = gold, 4 = PS–PMMA layer, 5 = air).

Actually, whenever a layer is thick enough to induce an SPR waveguide mode in the SPR full angle spectrum, then there is only one solution for the real thickness and the apparent (layer density dependent) refractive index, which means that there is no need to assume one or the other. The thicknesses and refractive indices obtained by fitting the SPR full angle spectrum measured for the 120 bilayer thick PSS–PAH polyelectrolyte multilayer with two different laser wavelengths of 655 and 782 nm were 404 and 400 nm and 1.503 and 1.501, respectively (Table 3). The thickness per layer is also in good agreement with the values obtained for the PSS–PAH polyelectrolyte multilayers consisting of 5 and 10 bilayers. The refractive index obtained for the thick PSS–PAH polyelectrolyte multilayers was on the other hand slightly larger than the refractive indices obtained for 5 and 10 bilayers. This is a consequence of the fact that polyelectrolyte multilayers that form dense layers tend to compact their structure with increasing layer number by excluding excess water from
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The resonant waveguide peaks actually retain the thickness development after its formation, which is evident from the waveguide node minimum shift (Figure 4A). This makes it possible to use them to characterize both the thickness and refractive index of thick organic layers or as a detection element in biosensor assays, which has also previously been shown in the literature. [22, 23]

Finally, it was also possible to measure waveguides deposited ex situ in a fashion similar to that for the in situ prepared waveguides of PSS–PAH polyelectrolyte multilayers. This allows for the determination of the real thickness and apparent (layer density dependent) refractive index for other types of relatively thick layers as well. Figure 4D demonstrates this for a spin-coated PS–PMMA copolymer layer. The SPR full angle scan measured in air with a 670 nm laser wavelength now shows all together three distinct peaks caused by the thick spin-coated PS–PMMA layer. The thickness and the refractive index determined for the PS–PMMA layer were 765.7 nm and 1.554, respectively (inset table in Figure 4D). The refractive index of the PS–PMMA layer is in very good agreement with the weighted average of the refractive indexes of pure PS (1.58425 at 670 nm) and PMMA (1.48741 at 670 nm) when considering that PS–PMMA contains 24% PMMA, i.e., 1.561. The slight nonzero imaginary part in the refractive index for the PS–PMMA layer indicates that there is some loss of light in the material, which may occur, for example, when light is reflected from an inhomogeneous surface or cracks in the spin-coated film.

6.4 CONCLUSIONS

A three-wavelength angular-scanning surface plasmon resonance based analysis has been demonstrated to be an efficient approach for determining ultrathin films thicknesses within the range that is difficult to access with other methods. The applicability of the multiparametric SPR (MP-SPR) for measuring the film thickness and optical property determination with relatively thick films using SPR waveguide modes was also demonstrated. These results can be utilized in characterizing and building different detection platforms for sensor development, life sciences, and drug development, as well as for obtaining fundamental information about the optical properties of different biological and organic systems. A one-instrument approach for accurately characterizing such layers without the help of any other instrument was successfully presented in this work. A critical assessment of the limits of detection and accuracy of the three-wavelength SPR analysis compared to the established ellipsometry or SE methods could not be done due to insufficient literature in the area. A careful evaluation of the few publications with results on ultrathin film measurements indicates, however, that the SPR three-wavelength analysis approach is able to characterize thin layers with good accuracy for the resulting thickness and refractive index values.

The ability to perform SPR measurements at multiple wavelengths allows both for an accurate characterization of ultrathin films and also for characterizing relatively thick organic layers in the micrometer range. By proper wavelength selection in the future, it should also be possible to almost completely cover the SPR “blind spot”, in
which the SPR angle is too high to be modeled, but the waveguide resonance mode has not yet been reached.

6.5 REFERENCES


III Characterizing Ultrathin and Thick Organic Layers by Surface Plasmon Resonance Three-Wavelength and Waveguide Mode Analysis

6.6 APPENDIX: SUPPORTING INFORMATION

6.6.1 SI THEORY OF SPR

Surface plasmons are particle waves of the free electron plasma on a metal surface, which can be excited by p-polarized light under the resonance condition (Figure S1). A theoretical mathematical description for the resonance condition can be obtained by solving the Maxwell equations for a multilayer optical system [1], which provides the following mathematical solution for the resonance condition:

\[
\frac{\omega}{c} \sqrt{\varepsilon_0 \sin \theta} = \varepsilon_0 \sin \theta \left( \frac{\varepsilon_1 \varepsilon_2}{\sqrt{\varepsilon_1 + \varepsilon_2}} \right),
\]

(S1)

where \( \omega \) is the angular frequency of light, \( c \) is speed of light in vacuum, and \( \varepsilon_0, \varepsilon_1 \) and \( \varepsilon_2 \) are the permittivities of the prism, SPR metal layer and the adjacent medium, respectively.

\[ k_x \neq k_{s0} \Rightarrow \text{No plasmon excitation} \]
\[ k_x = k_{s0} \Rightarrow \text{Plasmon excitation} \]
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**Figure S1.** Schematic representation of the Kretschmann configuration of the SPR used in the study. A) When the projected wave vector of the incident light do not match the wave vector of surface plasmons no excitation occurs. B) At a certain incident light angle the projected wave vector of the incident light matches the wave vector of surface plasmons, which is dependent on the wavelength of light used and the dielectric properties of the prism ($\epsilon_0$), metal layer ($\epsilon_2$) and the surrounding medium ($\epsilon_1$).

The permittivity and refractive index of materials can be used in their complex forms:

$\varepsilon = \varepsilon' + i\varepsilon'' \quad \text{(S2)}$

$\tilde{n} = n - ik \; ; (k > 0). \quad \text{(S3)}$

Where $\varepsilon$, $\varepsilon'$ and $\varepsilon''$ are the complex permittivity, real- and imaginary part of the complex permittivity, respectively. $\tilde{n}$, $n$ and $k$ are the complex refractive index, real- and imaginary part of the complex refractive index, respectively. Permittivity and refractive index have the following relationship:

$\tilde{n} = \sqrt{\varepsilon} \quad \text{(S4)}$

$\varepsilon = n^2 + k^2. \quad \text{(S5)}$

A general answer for a multilayered system linked to measurable or controllable variables can be solved using a transfer matrix formalism of $2 \times 2$ matrices. The overall formalism has already been published several times, and it is not in the scope of this article to discuss it in detail again. [1,2]

In practice this matrix formalism is solved by mathematical fitting tools, or by dedicated software tools developed for this, such as Winspell [3].

### 6.6.2 SII REFRACTIVE INDEX LINEARITY APPROXIMATION

So called Cauchy materials, such as most polymers and metal oxides, have a nonlinear dependency between wavelength and the refractive index. However, in a relatively small wavelength region this dependency can be approximated as linear, which is apparent from Figures S2. The refractive index ($n$) obtained from Kasarova et al. [4] has been fitted with a linear regression in Figure S2. The separately marked triangles emphasize the measurement wavelengths used in the SPR analysis in the current study. It can be seen from Figure S2 that the red-visible region refractive index dependency on wavelength can be approximated as the residual of the linear fit which is approximately 2% (R-value), and visually one can see that the linear fit also describe the relevant region with good correlation.
Figure S2. Wavelength dependency of the refractive index for three different polymers (plastics). The Red lines are linear fits to the data in the wavelength region between 0.6 – 0.8 μm. The colored triangles emphasize the wavelengths used in the SPR analysis in the current study.

6.6.3 SIII LANGMUIR AND LANGMUIR-BLODGETT TECHNIQUE AND PM-IRRAS CHARACTERIZATION OF MONOLAYERS

In this study we utilized the Langmuir-Blodgett technique for preparing well defined thin organic films with controlled number of layers in order to clarify how well a multi-wavelength SPR detection could be used for simultaneous determination of the
thickness and refractive index of ultrathin films in the thickness range 1-10 nm. The Langmuir-Blodgett (LB) technique is a well established method that enables controlled depositions of mono- and multilayers of a water insoluble organic amphiphilic substance floating on an air-water interface [5]. Deposition takes place by single or repeated immersions of a solid substrate through the floating monolayer at a controlled molecular packing density. Thus, the LB technique allows to precisely controlling the number of deposited layers and consequently the thickness of thin organic amphiphilic sample layers on solid substrates. Often multivalent counter ions are used in the subphase beneath the floating monolayer in order to facilitate the deposition of good quality mono- and multilayers [6-9].

**Figure S3.** A) Compression isotherms for SA and HSPC monolayers on pure water (blue solid lines) and \(10^{-5}\) UAc subphases (red solid lines). PM-IRRAS spectra of the CH-region of B) SA and C) HSPC monolayers deposited on the SPR sensor slide at different surface pressures; low surface pressure (blue solid lines) and high surface pressure (red solid lines).
PM-IRRAS measurements

PM-IRRAS spectra measured for organic ultrathin films deposited on IR reflective substrates have proven to provide valuable information about the orientation, conformation and structural order of the molecules in the sample layer, even for single monolayers [10-11]. These properties on the other hand have an influence on the thickness and optical properties of ultrathin organic layers determined by optical techniques, such as SPR, especially in the case of the first deposited layers. In order to detect any clear differences in orientation or structural order in the deposited monolayers of SA or HSPC we collected PM-IRRAS spectra for monolayers deposited onto SPR sensor slides from UAc containing subphases at two different surface pressures (Figure S3B and S3C).

The PM-IRRAS spectra for the C-H stretching mode region measured for both SA and HSPC monolayers show bands at 2965 cm⁻¹, 2920 cm⁻¹, 2880 cm⁻¹ and 2850 cm⁻¹, which are assigned to the CH₃ asymmetric stretch, CH₂ asymmetric stretch, CH₃ symmetric stretch and CH₂ symmetric stretch vibrations, respectively [12-13]. For the SA monolayer an increase in the surface pressure, i.e. an increase in packing density, shifts the IR band at 2920 cm⁻¹ slightly towards lower wavenumbers, whereas the band at 2880 cm⁻¹ grew more discrete and shifted to slightly higher wavenumbers. These changes in the PM-IRRAS spectra indicates that the SA monolayer possess an increasing number of trans conformers along the hydrocarbon chain for the monolayer deposited at higher surface pressure [14]. This correlates with an increasing order in the SA monolayer at higher surface pressure, which should be reflected in the optical properties of the monolayer. In the case of the HSPC monolayer the PM-IRRAS spectra did not show any shift for the band at 2920 cm⁻¹, whereas the bands at 2965 cm⁻¹, 2880 cm⁻¹ and 2850 cm⁻¹ shifted to slightly higher wavenumbers. This indicates that the molecules in the HSPC monolayer obtain a slightly more perpendicular orientation against the SPR sensor slide surface without an increase in order when deposited at higher surface pressure. The isotherm behavior of the HSPC and SA monolayers also supports this, as no clear liquid-solid transformation can be seen for the HSPC monolayer, while the SA monolayer exhibits this transition at 40 mN/m.

SIV THREE WAVELENGTH ANALYSIS METHOD

The thickness (d) versus refractive index (n) continuum solutions for the sample layer deposited on the SPR sensor slide were obtained by using Winspall [3] to fit the SPR full angle scan measured at a specific laser wavelength. The optical properties of the sample layer were then fitted by fixing the previously optimized optical properties for the pure SPR sensor slide, and then keeping n constant while fitting d for the sample layer. This was then repeated for n-values between 1.2-1.6 with an increment of 0.2. The d-n continuum solutions obtained by this procedure for a HSPC monolayer are shown in Figure S4A for the three different wavelengths used in this study. Hereafter, the d-n curve for 655 nm was kept in place and the d-n curves for all the other wavelengths were shifted by varying the dn/dλ value manually so that the two cross-points for the different laser pairs were as close as possible to each other, both in the d and n direction (Figure S4B). The positions of cross-point #1 and cross-
point #2 were then used to calculate the average cross-point, which then provided \(d\) and \(n\) for the sample layer in question. The difference between the average cross point and the actual cross points (i.e. cross-point #1 and cross-point #2) was then taken as the error estimate (‘±’) in the values provided for each analysis.

**Figure S4.** A) The \(d\)-\(n\) continuum solutions obtained at different SPR laser wavelengths for a HSPC monolayer deposited at 30 mN/m. B) Illustration of the optimal cross-points found after shifting the \(d\)-\(n\) continuum solutions for 670 nm, 783 nm and 782 nm by \(\frac{dn}{d\lambda} = 0.0002\) with respect to \(d\)-\(n\) continuum solution for 655 nm. The circle in B) represents the error estimate form the cross-point analysis.

### 6.6.5 SV SPR SENSOR ROUGHNESS

The information about the roughness of the SPR sensors slide has been kindly provided by the sensor manufacturer. The roughness has been measured using AFM analysis. \(R_q\) average of 5 independent measurements was 0.890 nm. It is apparent from the Gaussian-shaped thickness population in Figure S5 that approximately 70-90% of the thickness is within ±1 nm from the average thickness.
**Figure S5.** AFM image and height histogram of a pure SPR sensor slide surface.

### 6.6.6 REFERENCES


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6.7 APPENDIX: ADDITIONAL INFORMATION

The multiple wavelength SPR analysis presented in section 5.3.2 can also be expanded from the three-wavelength into a general solution with N-possible wavelengths. In such case, it is also possible to use a true Cauchy dispersion relation instead of the linear approximation of dn/dλ. The previous considerations would be transferred into a general solution with N wavelengths:

\[
\begin{align*}
  k_{sp1} &= n_{\lambda1} * d \\
  k_{sp2} &= n_{\lambda2} * d \\
  k_{sp3} &= n_{\lambda3} * d \\
  & \vdots \notag \\
  k_{spN} &= n_{\lambdaN} * d 
\end{align*}
\]

where

\[
n_{\lambdaN} = B + \frac{c}{\lambda^2} + \frac{d}{\lambda^4} + \cdots,
\]

that can be in most cases simplified into the first two terms:

\[
n_{\lambdaN} = B + \frac{c}{\lambda^2}
\]

The three wavelength solution has been empirically tested in this dissertation, and was found to function well in both liquid and gaseous media down to single molecular monolayer thicknesses. The general Cauchy dispersion is currently only a theoretical consideration at the moment due to limitations in current instrumentation. But as it seems to be clearly beneficial to add additional probing
wavelengths, it is quite probable that a commercial instrument manufacturer will soon come up with systems capable of carrying such measurements. The evaluation of the above general theory will need to wait for such situation.
7 IV CONTROL OF THE MORPHOLOGY OF LIPID LAYERS BY SUBSTRATE SURFACE CHEMISTRY

Abstract

In this study, surface coatings were used to control the morphology of the deposited lipid layers during vesicle spreading, i.e. to control if liposomes self-assemble on a surface into a supported lipid bilayer or a supported vesicular layer. The influence of the properties of the surface coating on the formation of the deposited lipid layer was studied with quartz crystal microbalance and two-wavelength multi-parametric surface plasmon resonance techniques. The control of the lipid self-assembly on the surface was achieved by two different types of soft substrate materials, i.e. dextran and thiolated polyethylene glycol, functionalized with hydrophobic linkers for capturing the lipid layer. The low-molecular-weight dextran-based surface promoted a formation of supported lipid bilayers, while the thiolated polyethylene glycol-based surface promoted a supported vesicular layer formation. A silicon dioxide surface was used as a reference surface in both measurement techniques. In addition to promoting the supported lipid bilayer formation of known lipid mixtures, the dextran surface also promoted a supported lipid bilayer formation of vesicles containing the cell membrane extract of human hepatoblastoma cells. The new dextran-based surface was also capable of protecting the supported lipid bilayer against dehydration when exposed to a constant flow of air. The well established quartz crystal microbalance technique was effective in determining the morphology of the formed lipid layer, while the two-wavelength surface plasmon resonance analysis enabled a characterization of the adsorbed supported lipid bilayers and supported vesicular layers.

7.1 INTRODUCTION

The understanding of different biochemical interactions present in human physiology is one of the primary goals of modern biochemistry and pharmaceutical research. Many of the methods and approaches for studying biochemical interactions use full systemic experiments on either human or animal physiology. Human or animal experiments are difficult to perform both from an experimental and an ethical point of view. Especially analyzing in vivo results as well as evaluating different causalities is often very complicated. Therefore, both the study of biochemical interactions and the current pharmaceutical development relies heavily on different in vitro experimentations[1,2]. Most of these in vitro methods are either relatively simple and do not mimic the in vivo situation very well, such as the parallel artificial membrane permeation assay (PAMPA)[2,3], or are quite complicated, making the control of the system difficult, as is the case with most cell screening assays[4,5]. Furthermore, these in vitro methods often use fluorescently labeled compounds, do not enable measurements in real-time and rely on secondary detection techniques. The overall efficiency (cost per new drug entering the market) is decreasing constantly, partly because an effective transfer of new formulations from laboratory scale to the clinic is very slow[6]. Hence, there is a clear need for new, more efficient and/or cost-effective methods for screening biochemical interactions that could provide complementary information to already existing in vitro methodologies, as well as reduce the need of ethically questionable in vivo studies.

Supported lipid membrane structures, i.e. supported lipid bilayers (SLBs) and supported vesicle layers (SVLs), are excellent biomimetic systems because they closely resemble cell membranes and other biological barriers consisting mostly of phospholipids. It is possible to incorporate membrane proteins, receptors and other biologically relevant molecules into these lipid membrane structures to mimic biological membranes with specific functionalities[7]. For example, it is possible to prepare asymmetrical SLBs so that the mobility of the lipids and other components is very near to that of the membranes found in the nature. The biological similarity of such cell membrane models can be excellent in the context of interaction research[8]. Traditionally SLBs and SVLs are prepared directly on a solid support by vesicle fusion or vesicle adsorption, respectively[9]. This method is often used in combination with several label-free detection techniques, such as quartz crystal microbalance (QCM), dual polarization interferometry (DPI) and surface plasmon resonance (SPR).

The common applications of cell membrane model systems are in membrane biophysics for SLBs[9] and in studying biochemical interactions between soluble compounds and lipids or membrane proteins for SVLs[10]. The SLBs and SVLs have also been introduced for pharmaceutical research[8,11,12]. But there are still several challenges such as control of the morphology between SLB and SVL, incorporation of membrane proteins and other membrane components of interest, as well as the method robustness and the lack of assays showing all these three properties together. The control of morphology has been extensively studied on inorganic supports, for example SLBs are readily formed on SiO2 substrate and SVLs on Au and TiO2[9,13]. Also specific linking chemistry, such as biotin-avidin, HisTag, nucleotides and ionic interactions between polyelectrolytes and charged lipids have been used to bind lipid
bilayers on solid support[14,15]. However, often the challenge with both SLBs and SVLs is that the formed lipid layers are unstable and sensitive for irreversible denaturation of the membrane structure upon transition through the air-water interface[16]. Improvements in the stability of SLBs against this denaturation have recently been demonstrated by using cholesteryl functionalized hydrated polymer supports[17], strongly-interacting metal chelated SLBs[18] and by “sandwiching” SLBs between the support and proteins[16] or PEG[19]. In the case of using linking chemistry in formation of SLBs or SVLs one of the counterpart is also left on top of the final lipid layer[14], which can influence the surface properties and interactions of the lipid layer. The advantage of using hydrophobic linkers such as alkane chains or cholesterol immobilized on the support is that they do not introduce unwanted counterparts on top of the lipid layer[14,17].

In this study we have focused on improving the control of the morphology of the supported layer (SLB vs. SVL) as well as the air stability of supported lipid membranes using polymeric supports. Furthermore, our aim was to enable an SLB formation of vesicles composed of known lipid mixtures, or a mixture of cell membrane extract of human hepatoblastoma cells (HepG2) and known lipid mixture in order to prepare SLBs with high biological relevance for biochemical and pharmaceutical applications. The support materials for anchoring either SLBs or SVLs were loose networks of hydrated polymers functionalized with hydrophobic linkers acting as a cushion between the supported lipid membrane structure and the substrate. The surfaces were prepared from commercially available dextran and a custom-synthesized thiol containing polyethylene glycol–polymer (PEG-SAM). Silicon dioxide (SiO2) surface was used as a reference support due to its well-known ability to promote SLB formation by vesicle spreading[9]. Three different known lipid mixtures and one cell membrane extract composition were used to study the type of lipid structure that was promoted by the different supports.

### 7.2 MATERIALS AND METHODS

#### 7.2.1 MATERIALS

Sodium chloride, sodium hydroxide, calcium chloride, 50% hydrogen peroxide, concentrated ammonia, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 11-mercaptopoundecanol, epichlorohydrin, bromoacetic acid, decylamine, EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbo diimide hydrochloride), NHS (N-Hydroxysuccinimide), ethanolamine, PBS (phosphate buffered saline, tablet P4417) and Dextran (Mr = 6kDa from Leuconostoc spp.) were obtained from Sigma-Aldrich (Helsinki, Finland). B-03 labeled biotin was obtained from Episentec Ab (Sollentuna, Sweden). EggPC (Egg phosphatidylcholine), POPS (palmitoyl-oleyl-phophatidylserine), cholesterol (from lamb wool) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) were obtained from Avanti Polar Lipids (Alabama, USA). Ethanol (95%) was obtained from Altia Corporation (Rajamäki, Finland). Hellmanex II was obtained from VWR Finland (Helsinki, Finland). All water used in the experiments had a resistivity of 18.2 MΩ/cm.
The PEG-SAM was synthesized from a commercial PEG45–polymer with OH-end groups and a molecular weight of 2 kD, obtained from Polysciences Inc (Pennsylvania, USA). The synthesized PEG-SAM had the general structure of H2C=CH-(CH2)9-PEG-(CH2)10-SH, or an analogous disulfide dimer. The reagents and methods used in the synthesis of the PEG-SAM are described in detail in the Supporting information section S1.

Gold and SiO2-coated SPR sensors were obtained from BioNavis (Ylöjärvi, Finland), and similarly coated 5 MHz QCM crystals were obtained from Q-Sense Inc./BiolinScientific (Västra Frölunda, Sweden).

7.2.2 SURFACE SYNTHESIS

Two different lipid-binding surface coatings were synthesized on gold coated sensors for SPR and QCM measurements. The surface coatings on both the SPR and QCM sensors were simultaneously prepared in the same reaction vessels in order to have as identical coatings as possible for both sensors. The dextran based surface was selected because the polysaccharide structure is close to the natural polysaccharides found in the cell membranes. The small 6 kDa size should make relatively thin and dense layers upon grafting to a surface and there are suitable chemical pathways to post modify it into a lipid anchoring surface[20,21]. The PEG-SAM was selected because PEG is well known for its biocompatibility and low interaction with SLBs and there are suitable chemical pathways which allow end-group modification of the PEG prior to grafting it to the support surface (see Supporting information S1). Both of these polymers have been shown to work well with SLB and SVL layers in previous literature[8].

The dextran coated (Dex6kDa) surface in this study was synthesized with slight modifications as described by Summanen et al.[20], which is a modification of the method by Löfås and Johnsson[21], i.e. 6kDa Dextran at a concentration of 300 g/L was used instead of 500 kDa Dextran at a concentration of 30 g/L, and 2 M bromoacetic acid was used instead of 0.5 M bromoacetic acid. The sensors were first cleaned by keeping them in a boiling H2O2:NH3:H2O (1:1:5)-solution for 10 minutes and then washed thoroughly with ultrapure H2O. Hereafter, the sensors were immersed in a solution of 5 mM mercaptoundecanol in an 8:2 ethanol:water-solution for 24 hours. The sensors were then allowed to react for 3 h with epichlorohydrin (2% v/v) in 0.1 M NaOH rinsed with water, transferred to a 300 g/L solution of dextran in 0.1 M NaOH and left to react for 24 h. After this the sensors were washed thoroughly with ultrapure H2O and immersed in 2.0 M bromoacetic acid in 2 M NaOH for 24 h, after which the sensors were thoroughly washed with ultrapure H2O and stored at +4°C. The surface immobilized carboxylated dextran was then functionalized with decylamine by utilizing EDC/NHS activation chemistry. The carboxylated dextran surface was first activated by treating it for 10 minutes with a 80 mg/mL:20 mg/mL EDC/NHS in PBS buffer. Immediately after this, the activated dextran surface was treated with a 30 mg/mL decylamine suspension in PBS for 10 minutes. The surface was finally deactivated with a 1 M ethanolamine solution, washed thoroughly with ultrapure H2O and ethanol, dried and stored dry in +4 °C for later use.
The PEG-SAM surface coating was prepared on gold coated sensors by using a simple self-assembly protocol. The sensors were first cleaned by keeping them in a boiling H2O2:NH3:H2O (1:1:5)-solution for 10 minutes, then washed thoroughly with ultrapure H2O and dried with nitrogen gas. The sensors were then immersed in a 2 mg/mL ethanol solution of the PEG-SAM for 24 h. After the formation of the self-assembled PEG-SAM layer the sensors were finally washed thoroughly with ethanol, dried carefully with nitrogen and stored dry in +4 °C for later use.

### 7.2.3 CELL MEMBRANE EXTRACT PREPARATION

Cell membrane extract was produced from human hepatoblastoma cells (HepG2) derived from the liver tissue of a fifteen year old male (HB-8065, ATCC-LGC Promochem, USA). The cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 100 mM sodium pyruvate in a humidified atmosphere containing 5% CO2 at 37° C. About 0.86 g HepG2 cells were allowed to melt on an ice well. Cell pellets were suspended into a harvest buffer (50 mM Tris-HCl /300mM mannitol, pH7). The cell suspension was centrifuged at 800 x g for 5 min at 4 °C and the supernatant was thrown away. This step was repeated twice. The residue was suspended in a membrane buffer (50 mM Tris-HCl/50mM mannitol and 2mM EGTA, pH7). Cells were then homogenized by using a cell homogenizer by 40 strokes and incubated on ice for 1 h. The solution was centrifuged at 800 x g for 10 min at 4 °C, and the supernatant was taken and further centrifuged at 15 000 x g for 1 h at 4 °C. Again, the supernatant was taken and centrifuged at 100 000 x g for 75 min at 4 °C. Finally, the supernatant was removed and the pellets were weighed and stored at -75 °C until used. Qualitative test for the presence of proteins in the extracted cell membranes was performed by using a Bio-Rad Protein Assay Reagent kit (Cat.#500-0006), which verified that the cell membrane extract contained a substantial amount of protein.

### 7.2.4 LIPOSOme PREPARATION

Three different lipid compositions were used for the liposomes for the SPR and QCM studies. The EggPC was chosen as the majority of the lipids in prokaryotic cell membranes are phosphatidylcholines[22], and it offers a good combination of saturated and non-saturated lipids that are physiologically relevant. The POPS was added to the lipid mixture to simulate the natural negative charge that is exhibited in natural prokaryotic cell membranes. The cholesterol was added to the lipid mixture in order to mimic the natural cell membranes even further as it is one of the major components controlling the rigidity of cell membranes. Liposomes were prepared with the sonication method[23]. The lipid compositions were 100 % EggPC, 75:25 % EggPC:POPS and 70:25:5 % EggPC:POPS:Cholesterol by molar ratio. A labeled liposome containing 100% EggPC (EggPC+Label) liposome and 1 μg/mL of B-03 labeled biotin in the hydration buffer was also prepared for SPR experiments.

Shorty, the liposomes were prepared by first drying the lipid mixture with a nitrogen flow, and rehydrated to a total lipid concentration of 1 mg/mL with a
HBS+Ca\textsuperscript{2+} buffer (20 mM HEPES, 150 mM NaCl, 3 mM CaCl\textsubscript{2}, pH 7.4). Hydration was allowed to take place for one hour with mixing every 15 minutes. After the hydration, the liposomes were sonicated with a Vibra-Cell VCX 750 sonicator (Sonics & Materials Inc., Newtown, CT, USA) in an ice-water bath until the solution became clear, which typically took 5-15 minutes depending on the lipid mixture. The quality of the prepared liposome solution was always checked with a Malvern Zetasizer 3000HSA dynamic light scattering (DLS) instrument (Malvern Ltd., Malvern, UK). A hydrodynamic radius below 50 nm and a monomodal size distribution for the liposomes were required in order to accept the prepared batch for later use. The number averaged size of the liposomes obtained from DLS measurements were 27 nm for EggPC, 27 nm for EggPC+PS, 39 nm for EggPC+PS+Chol and 27 nm for the EggPC+Label, respectively. The CONTIN analysis of the size distributions are presented in the supporting information (Figure S3). The liposome stock solutions were stored at +4 °C and used within 5 days.

Labeled EggPC liposomes were prepared exactly as the other liposomes, except that 1 μL of 1 mg/mL B-03 labeled biotin in pure water solution was added to the HBS+Ca\textsuperscript{2+} hydration buffer during the hydration step.

The liposomes containing the HepG2 membrane extract were prepared with a method adapted from the work of Dodd et al.[24]. The HepG2 membrane extract from the centrifugation pellet was dispersed in a HBS+Ca\textsuperscript{2+} buffer to form a 10 mg/mL stock solution, and then sonicated for 20 minutes in the same conditions as the other liposomes in this study. This extract was then mixed with a 10 mg/mL pre-sonicated stock solution of EggPC liposomes with a mass ratio of 4:10 extract:EggPC forming a total lipid concentration of 0.14 mg/mL. This solution was then sonicated for 5 minutes at the same conditions as described before for other liposomes in this study in order to mix the different liposome populations. The sonicated membrane extract mixture was always used for the QCM and SPR measurements within the same day of preparation. DLS measurements of the resulting HepG2-EggPC liposomes gave a number averaged size of 63 nm. The HepG2 cell membrane extract was mixed with EggPC because in general natural total extracts do not fuse into SLBs due to the stability of natural extracts, but addition of more unstable lipid such as EggPC can results in a bilayer formation, as described by Dodd et al.

### 7.2.5 QCM MEASUREMENTS

The QCM measurements were performed with a KSV QCM-Z500 instrument (KSV Instruments, Helsinki Finland) at 20 °C. The flow rate used for the measurements was 250 μL/min and the running buffer was HBS (20 mM HEPES, 150 mM NaCl, pH 7.4). The frequency and dissipation changes for the 3\textsuperscript{rd}, 5\textsuperscript{th}, 7\textsuperscript{th} and 9\textsuperscript{th} overtones (marked from here on as F3, F5, F7 and F9) were recorded during all experiments. The results were analyzed by using the KSV QCM-Z500 software (version 3.4).

The silicon dioxide QCM sensors were washed in situ in the flow channel with sequential 5 min injections of 20 mM CHAPS, 2% Hellmanex II, 95 % ethanol and ultrapure H\textsubscript{2}O[25]. The experiments were performed by first measuring a baseline with the running buffer for approximately 10 minutes, then injecting a 0.1 mg/mL liposome solution for 8 minutes followed by a 10-minute rinse period with the
running buffer. Samples were measured consecutively with a wash sequence between the samples.

The Dex6kDa and PEG-SAM surfaces were washed in situ in the flow channel before the measurements by injecting 20 mM CHAPS for 5 minutes followed by rinsing with ultrapure H₂O. The measurement sequence was the same as for the SiO₂-coated QCM sensors, with the exception that a 5-minute ultrapure H₂O injection was added after the flush period with the running buffer, followed by a second flush period with the running buffer.

The QCM thickness analysis of the lipid layers was performed by using either the Sauerbrey- or a viscoelastic model (equivalent circuit analysis) for the deposited lipid layers[26]. It was necessary to use two different models for QCM analysis, because the Sauerbrey model describes the thickness of a rigid layer (such as an SLB), and the equivalent circuit analysis provides the thickness and mechanical properties of a viscoelastic layer. Constant parameters used in the modeling were: density of lipid/liposome layer 1.0 g/mL, density of buffer 0.9986 g/mL and viscosity of buffer 0.890 mPa s.

### 7.2.6 SPR MEASUREMENTS

The SPR measurements were simultaneously performed at wavelengths of 670 and 785 nm with an MP-SPR instrument SPR Navi 200-L (BioNavis, Ylöjärvi, Finland). The measurements were performed at 20 °C and with a flow rate of 30 μL/min. The flow rate for the SPR measurements was selected so that the hydrodynamic flow conditions in the SPR flow channel matched the flow conditions in the QCM flow channel[27]. Theoretically, the SPR flow channel used in this study would have a 9 times higher surface shear stress than the QCM flow channel if the same flow rate were used in both flow channels.

SiO₂ SPR sensors were washed in situ in the flow channel with sequential 3-min injections of 20 mM CHAPS, 2% Hellmanex II, 95 % ethanol and ultrapure H₂O. The experiments were performed by first measuring the baseline with the running buffer for approximately 10 minutes, then injecting a 0.1 mg/mL liposome solution for 8 minutes, followed by a 10-minute rinse/flush period with the running buffer. Samples were measured consecutively with a wash sequence in between the samples. The Dex6kDa and PEG-SAM surfaces were washed in situ in the flow channel before the measurements by injecting 20 mM CHAPS for 5 minutes. The SPR measurements did not need the same ultrapure H₂O rinsing treatments as the QCM measurements.

The air stability of the EggPC+PS+Chol lipid bilayers on Dex6kDa was measured by running air for 10 min at a flow rate of 200 μL/min over the SPR surface. After exposing the lipid bilayer to air, the surface was rewetted with the same HBS buffer, and the return of the baseline was recorded. The full SPR angular range was continuously monitored during this period, and the change to air and back to the buffer was confirmed in real-time from the critical angle (total internal reflection angle, TIR from now on) shift induced in the SPR angular spectrum when going from water-based buffer to air and back to the buffer.

The thickness (d) and the real refractive index (RI) of the lipid layer structures were calculated by using the SPR Navi LayerSolver software v. 0.16 (BioNavis Ltd,
Ylöjärvi, Finland). The software uses the well-known Fresnel equation formalism for calculations[28], but allows to simultaneously process multiple SPR spectra in a single calculation which has earlier been performed in several calculation steps[29,30]. In this work the optical modeling was performed by using SPR angular spectra measured at 670 and 785 nm at the same time point, and by linking all the thicknesses as common variables, while the complex refractive index was put either as an independent variable (for background) or as a linearly dependent variable between the two wavelengths used[30].

7.3 RESULTS AND DISCUSSION

7.3.1 QCM MEASUREMENTS

The QCM measurements in Figure 1 shows that the liposomes of the three known lipid compositions spread as a bilayer on the SiO$_2$ sensor surface. The three lipid compositions exhibited the typical adsorption-bursting behavior often seen in QCM experiments during vesicle spreading when SLBs are formed[9,31]. The overlap of the normalized overtones (or more precisely, the lack of any difference between the overtones), the normalized frequency level of 25 Hz and the fact that the changes in dissipation values are less than $2 \times 10^{-6}$ (supporting information, Figure S4) indicates that good quality lipid bilayers were formed[9]. The QCM measurements also revealed that the simple \textit{in situ} wash cycle of CHAPS, Hellmanex II, ethanol and ultrapure H$_2$O used between consecutive measurements in this study was sufficient to clean the sensor completely, and it was not necessary to wash the sensors with piranha (conc. H$_2$SO$_4$;H$_2$O$_2$ 3:1) as is commonly suggested[9]. This enables a faster repetition of measurements and improves the safety of the preparative steps for vesicle spreading studies on SiO$_2$ surfaces.

A similar initial adsorption-bursting behavior seen on the SiO$_2$ surface was also clearly seen for the vesicles composed of known lipids, when they interact with the Dex6kDa surface (Figure 2; red, black and blue lines). On the other hand, the normalized frequency level and the overlap of the overtone frequencies clearly show that complete lipid bilayers were not spontaneously formed on the Dex6kDa surface, and the deposited lipid layers remain as mixtures of SLBs and SLVs (Figure 2). However, treating the lipid layers deposited on the Dex6kDa surface with H$_2$O triggers a process which results in the formation of almost perfect lipid bilayers, which is indicated by the normalized frequency levels of approximately 25 Hz and the overlap of the normalized overtone frequencies. The dissipation results also support these findings (Supporting information, Section S3, Figure S5). The mechanism of the triggered SLB formation caused by the H$_2$O injection is not clear, and it seems to be specific for the lipid composition. It is probable that the mechanism is due to osmotic stress induced by the ionic strength gradient over the liposome membrane. [32,33].
The H$_2$O triggered SLB formation could also be used to prepare an SLB from the vesicles composed of a mixture of the HepG2 cell membrane extract and EggPC (green lines in Figure 2). The higher level and the poorer overlapped of the normalized overtone frequencies for the SLB of the HepG2-extract compared to the known lipids could be expected. This, because the HepG2 cell membrane extract contains intact cell membrane proteins, saccharides and other components, which increase the size and viscoelasticity of the SLB of the HepG2-extract compared to the SLBs with known lipids. A similar approach of including cell membrane extract in an SLB (i.e. natural extract from *Escheria Coli* bacteria mixed with fluid EggPC) has been previously demonstrated by Dodd *et al.*[24]. However, even though Dodd *et al.* managed to immobilize some kind of a supported lipid bilayer on SiO$_2$, surface it was not a good quality SLB according to their QCM-D results. However, it was possible to use similar approach with small modifications in this study in order to spread the HepG2-extract mixed with EggPC into a bilayer.
Figure 2  QCM Δf/N vs. time sensograms at F3, F5, F7 and F9 for all the four vesicle formulations used in this study during interaction with the Dex6kDa surface. (Left to Right: Red – EgPC ; Black – EgPC+PS ; Blue EgPC+PS+Chol ; Green – HepG2-extract). The horizontal line marks the position where Δf/N = 25 Hz. The arrows indicate events in the sensogram: a) injection of liposomes b) end of injection of liposomes c) injection of water d) end of injection of water. The bilayer formation was triggered by treating the deposited lipid layers with H2O. The final frequency change (25 Hz) and the overlap of the overtones indicate that the liposomes rupture and form lipid bilayers on the Dex6kDa surface.

When interacting with the PEG-SAM surface, all the vesicles in this study behaved completely differently than with the two previous surfaces. The PEG-SAM surface clearly promoted the formation of SVLs, which can be seen from the significantly larger normalized overtone frequency changes compared to the typical 25 Hz for an SLB as well as from the fact that the normalized overtone frequencies do not overlap at all (Figure 3). This indicates that the deposited lipid layer was a visco-elastic layer composed of adsorbed vesicles. Furthermore, the H2O treatment that was capable of transforming the (partial) SVLs adsorbed on the Dex6kDa surface did not result in the formation of SLBs with the PEG-SAM surface. The dissipation results for the PEG-SAM surface also shows a completely different behavior compared with the Dex6kDa or SiO2 surfaces (Supporting information, Section S3, Figure S6) further indicating that the PEG-SAM surface promotes SVL formation and not SLB formation.
It should be noted that in order to properly prove SLB formation with the QCM, more than one measured overtone should be analyzed and compared, and the difference between the normalized overtones should be as small as possible. Apart from reaching a final frequency level of 25 Hz and negligible dissipation changes [31], the overlap of the normalized overtones is also an important indicator for showing the absence of liposomes. The sensitivity of a single overtone frequency for differentiating between an SLB and an SVL becomes smaller as the overtone frequency gets higher. At the 9th or the 11th overtones the frequency changes for a 5 MHz crystal is nearly identical for an SLB and an SVL due to the shear wave penetration depth of the higher overtone frequencies compared to the lower ones [26]. For example, the shear wave penetration depth for the 3rd overtone for a 5 MHz crystal in pure water at 25 °C is 138 nm compared to 79 nm for the 9th overtone.

The QCM thickness analysis of the SLBs formed on the SiO$_2$ and and on Dex6kDa surfaces was performed by using the Sauerbrey equation. Additionally, the thickness of the SLB/SVL formed both before and after water treatment on both the Dex6kDa and PEG-SAM surfaces was analyzed by using the visco-elastic model. The results of the analysis, i.e. thickness from Sauerbrey analysis, and the thickness, the elastic modulus ($\varepsilon$) and viscous modulus ($\eta$) from the viscoelastic analysis, are presented in Table 1.

These Sauerbrey QCM thickness analysis show that approximately equal SLBs from all the known lipid compositions were readily formed on the SiO$_2$ surfaces, whereas SLB formation on the Dex6kDa surface only takes place after ultrapure
water treatment. Applying the visco-elastic modeling to the lipid layers on the Dex6kDa surface after water treatment produced systematically slightly larger thicknesses compared to the Sauerbrey analysis. The thickness and elastic moduli of the SLBs on the Dex6kDa surface were generally significantly higher than those of the SVL layers on Dex6kDa before water treatment, which supports the conclusion that SLBs are formed on the Dex6kDa surface after the water treatment. The thickness of the HepG2 extract mixture on the Dex6kDa surface after ultrapure water treatment is slightly higher while the elasticity is lower compared to the thickness of the known lipid mixtures. However, the thickness for the HepG2 extract mixture after ultrapure water treatment is still significantly smaller than any of the lipid mixtures before water treatment. The HepG2 results are reasonable when considering that there is membrane protruding proteins present in the SLB formed from the HepG2 mixture which easily could contribute to a thickness increase of 1-2 nm and a higher viscoelasticity due to protruding proteins incorporated in the bilayer which traps water. The PEG-SAM surface clearly retains the SVL morphology both before and after water treatment according to the QCM modeling results. However, decrease in thickness and an increase in elasticity indicates that some fraction of the liposomes on the PEG-SAM surface actually are fused into an SLB, but a significant portion of the adsorbed liposomes still retains the SVL morphology.

It is also worth noting that the thicknesses of the SVLs on the Dex6kDa surface before ultrapure water treatment are clearly smaller compared to the SVLs on the PEG-SAM surface. This is a strong indication that the liposomes deform to a larger extent on the Dex6kDa surface than on the PEG-SAM surface, thus inducing larger stresses to the liposomes on the Dex6kDa surface and therefore facilitating SLB formation when changing the osmotic conditions during ultrapure water treatment. One of the explanations for a larger deformation of liposomes on the Dex6kDa surface compared to the PEG-SAM surface could be that there is a difference in the density and availability of the hydrophobic chains which causes differences in the interaction strength between the surfaces and the adsorbed liposomes.
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Table 1. Result from the QCM thickness modeling. Sau = Sauerbray equation and V-E= visco-elastic modeling (equivalence circuit modeling). *) The Sauerbray equation produces false values because it assumes a rigid film, but as shown as shown from the viscoelastic modeling, the HepG2 extract seems to behave as an SLB with protruding proteins.

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<th>(d) (nm /Sau)</th>
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<td>EggPC+Chol</td>
<td>9.8</td>
<td>0.80</td>
<td>0.00245</td>
<td></td>
</tr>
</tbody>
</table>

7.3.1.1 Promotion of SLB or SVL formation by the substrate

The QCM results showed that there was a large difference between the Dex6kDa and PEG-SAM surfaces in promoting either SLB or SVL formation, even though both surfaces are relatively thin hydrogel supports with a thickness in the range of a few nanometers. The Dex6kDa clearly promoted the formation of SLBs and the PEG-SAM promoted SVL formation. The most probable explanation for this is the type of surface morphology the polymers form on the sensor surface, and the effect of different synthesis routes causing the lipid anchoring groups to be distributed differently in the Dex6kDa and PEG-SAM layers. The measurement conditions used in this work were selected so that they would promote SLB formation on the SiO2 surface[9]. The measurement protocols were also kept constant during all measurements and it is unlikely that these would have a significant effect on the morphology control in this work. Furthermore, the Dex6kDa and PEG-SAM surfaces
should be non-charged due to the way they are produced, whereas the wettability of
the surfaces show a clear difference, i.e. contact angle of ultrapure water for the
surfaces were 56° for PEG-SAM and 71° Dex6kDa. Therefore, the surface charge
cannot have a large role in the promotion of either SLBs or SVLs on the different
polymer surfaces synthesized in this study, while the wettability originating from the
different distribution of hydrophobic linker chains in the polymer surfaces probably
is more significant.

The Dex6kDa was formed from a highly concentrated solution, where the
polymers are in a semi-entangled conformation and should also form a similar
network upon surface linking[34]. Furthermore, the hydrocarbon chains (i.e. decyl
chains) were also post-synthesized into the formed dextran hydrogel, which most
probably modifies mainly the outer surface portion of the hydrogel. Thus, the overall
synthesis process should lead to a relatively dense and flat dextran surface with easily
accessible hydrocarbon chains. The higher contact angle of Dex6kDa compared to the
PEG-SAM and the change in the contact angle as a result of the decyl binding to the
Dex6kDa also indicate that the decyl-chains are more exposed in the Dex6kDa than
in the PEG-SAM surface.

The PEG-SAM was formed by a self-assembly reaction with a custom synthesized
PEG polymer having pre-modified end groups. While the dominant species in the
PEG polymer mixture were thiol- and analogous disulfides of the PEG-polymer with
one hydrocarbon chain (i.e. un-10-decene), the PEG polymer mixture also contained
di-thiols and unthiolated polymers. The concentration of the PEG polymer mixture
was relatively high during the PEG-SAM formation, but was still within a range where
polymers behave mostly as individual coils rather than as an entangled network[34].
Due to steric interactions the self-assembly of polymers under such conditions should
form a more “mushroom” like surface with individual polymer coils rather than a
dense polymer brush structure[35]. This kind of behavior is also supported by an
other study where similar PEG self-assembly were studied with SPR for PEG
thickness and density as a function of molecular weight, which is also proportional to
polymer coil dimensions[36].

Taken together the promotion of SLB formation on the Dex6kDa and SVL
formation on the PEG-SAM surface is quite reasonable. The brushlike Dex6kDa
hydrogel most probably forms a rather homogeneous and smooth surface with a
sufficient amount of easily accessible hydrocarbon chains, which promotes SLB
formation, whereas the mushroomlike PEG-SAM surface is probably not so
homogeneous and smooth with less accessible hydrocarbon chains, thus promoting
the vesicles to retain their shape on the surface.

7.3.2 SPR MEASUREMENTS

The SPR measurements did not show as pronounced bursting behavior for any of
the surface/lipid compositions as was seen in the QCM measurements (Figure 4).
This is reasonable, as the QCM technique is sensitive to the water content in the
adsorbed layer while SPR is not, and SVLs carry a significant amount of water
trapped in the liposomes. Despite of this, a small bursting behavior which follows the
general shape of the SLB-formation steps in QCM measurements was detectable in
Control of the morphology of lipid layers by substrate surface chemistry

The level of the SPR signal acquired during the deposition of the vesicles corresponds well with the expected values for a lipid bilayer when evaluated with an optical model of the systems (Supporting information, Section S4, Table S2)[22]. The difference between the signal level of SiO$_2$ and Dex6kDa SLBs can be explained by the effect caused by the support structure on the optical signal of the SLB. In an optical model an SLB adsorbed on a spacer hydrogel support structure gives approximately 15% less signal compared with an SLB adsorbed on a dense SiO$_2$ surface (Supporting information, Section S4, Table S2). This was approximately also the difference observed in the actual SPR measurements (Figure 4).

The SPR measurements with the vesicles containing the dye-label did not largely differ from the pure EggPC lipid composition on the surfaces that were expected to promote the SLB formation (i.e. SiO$_2$ and Dex6kDa – EggPC red trace and label+EggPC orange trace in Figure 4). However, the difference in the SPR signal between the EggPC and the label+EggPC lipid compositions during adsorption to the PEG-SAM surface was large. This is expected in the case of SVLs, because when a compound that absorbs light is trapped in the vesicles this will appear as a much higher RI index compared to vesicles without the light-absorbing compound. This is due to the anomalous behavior of the refractive index when compounds that absorb light in the same wavelength region used for the SPR measurements are present.[37-39]. This further confirms the earlier findings from QCM measurements, i.e. that the SiO$_2$ and Dex6kDa coatings promote SLB formation and the PEG-SAM coating promotes SVL formation. The small difference between the SPR signal levels of the Dex6kDa and SiO$_2$ surface for the EggPC and label+EggPC lipid formulations is probably due to the extra space provided by the hydrogel under the SLB, which traps a small amount of the labeled material in the hydrogel. On the other hand, in the case of the SiO$_2$ surface the SLB is formed on a rigid surface with a minimal space between the surface and the SLB, which does not allow any labeled material to be trapped between the surface and the SLB.

The label+EggPC SPR measurements also show that it was not necessary to use the water triggering step used in the QCM measurements for SPR measurements in order to form SLBs of the known lipid compositions on the Dex6kDa surface. This is probably due to the fact that the shear stress in the SPR flow channel is actually higher compared to the QCM flow channel, because the heights of the two flow channels used in the calculations for synchronizing the flow conditions are in reality not as well defined and precise as assumed. On the other hand, the HepG2-extract lipid composition still required a water triggering step in the SPR measurements in order to form an SLB on the Dex6kDa surface. Furthermore, there was a clear difference in the absolute SPR signal level measured for the SLB of the HepG2-extract lipid composition (green line in Figure 4) compared to the other lipid compositions. The reason for this is that the HepG2-extract lipid composition contains more cholesterol, as well as membrane proteins and other cell membrane components which are not present in the known lipid compositions. This also makes the vesicles prepared from the HepG2-extract lipid composition more stable and visco-elastic[40], which consequently requires some triggering step such as the water treatment to induce SLB formation.
Figure 4  SPR angle vs. time sensograms measured with a wavelength of 670 nm during interaction of all the five lipid vesicle formulations with the three different surfaces used in this study. From left to right – SiO2 surface, PEG-SAM, Dex6kDa and HepG2 membrane extract on Dex6kDa. The different lipid compositions are marked with colors as follows: EggPC – Red ; EggPC+PS – Black ; EggPC+PS+Chol – Blue ; EggPC + Label – Orange and HepG2-extact – Green. The arrows indicate events in the sensogram: a) injection of liposomes b) end of injection of liposomes c) injection of water d) end of injection of water. The SPR is sensitive to the refractive index of the lipids (density and packing), as well as to the effect of the supporting surface structure on the evanescent field (Supporting information, Section S4, Table S2). This makes it relatively difficult to determine if a bilayer is formed or not. However, the measurements with the label clearly show a large difference between the PEG-SAM and the other two surfaces, which correlates with the QCM measurements (Fig1-3). The HepG2-extract has a higher SPR signal than the other lipid compositions on the Dex6kDa surface because it also contains a higher amount of cholesterol, as well as membrane proteins and other cell membrane components that are not present in the known lipid compositions.

7.3.2.1 Two-wavelength SPR analysis

A two-wavelength analysis for determining the thickness (d) and refractive index (RI) of the adsorbed lipid structures was performed with the LayerSolver software for all the known lipid compositions. Background parameters were calculated from the spectra taken 1 minute before the vesicle injection, and the sample spectra were taken 10 minutes after the vesicle injection was ended. The optical parameters obtained from the background spectra and examples of the optical fits to the background spectra can be found in the supporting information Table S1 and Figure S7. It was not possible to accurately determine or separate the optical properties of the Dex6kDa and PEG-SAM layers in the background spectra, because their contribution to the overall background spectra was very small. Hence, the optical parameters for the background spectra in the case of Dex6kD and PEG-SAM surfaces should be considered as “apparent” parameters, which means that they are not physically correct but they are still accurate enough for the analysis purpose in this study. This
in combination with the effect caused by the support structure on the optical signal discussed above (Supporting information, Section S4, Table S2) means that the results from the two-wavelength SPR analysis for the lipid layer structure formed on the Dex6kDa and PEG-SAM surfaces should only be considered as qualitative and reflect only the differences inside the same series. The results from the two-wavelength SPR analysis for the lipid layer structures formed on the SiO2 surface do not suffer from this drawback, and can be viewed as accurate. The results of the two-wavelength SPR analysis for the different lipid compositions and support surfaces are presented in Table 2.

The d and RI values obtained for the SLBs on SiO2 in this study correlate well with results obtained with a similar surface in dual polarization interferometry by Lee et al. [22], especially when taking into account the difference in the lipids used. The lipid mixtures used in this study were natural extracts (EggPC) which are mixtures of many components with a high amount of unsaturated fatty acid chains, while the work of Lee et al. was performed with fully-saturated di-myristoyl phosphatidylcholine (DMPC) as the main component. It should also be noted that while the reference in the work by Lee et al. also relies on results obtained from a secondary technique (i.e. neutron scattering for thickness determination) for analyzing both the refractive index and thickness for their lipid mixtures, we have obtained similar results in this study without a need for similar references. Both the current study and literature show that the cholesterol-containing lipid mixture has a higher thickness, which can be explained by the condensing effect of cholesterol in the SLB. The cholesterol situates between the fatty acid tails of the phospholipids in the SLB, and forces them to stand up straighter than they would without the presence of cholesterol[41]. This effect was more pronounced with the longer unsaturated chains in this work compared with saturated alkane chains used by Lee et al.

As discussed above, the d and RI values obtained for the actual Dex6kDa and PEG-SAM surfaces have to be taken as qualitative rather than quantitative. Even so, the d and RI values obtained for the lipid layers on the Dex6kDa surface support the fact that an SLB was formed with all the different lipid compositions used in this study, which is further supported by the QCM results. The d and RI values for the SLBs on the Dex6kDa surface were slightly higher but within a reasonable range when compared to the values obtained for the SiO2 surface. This might depend on the influence of the background during optical modeling as discussed above, or alternatively the SLBs might ripple on the Dex6kDa surface consequently displaying a slightly larger thickness compared to the SiO2 surface. It might also be possible that the higher RI obtained for the SLBs on the Dex6kDa surface compared to the SiO2 surface indicates that the lipids can pack more tightly on the Dex6kDa surface than on the SiO2. However, this can only be speculated, as the results obtained from the optical modeling were too uncertain for drawing stronger conclusions, because of the issues related to the background uncertainty and the spacing layer sensitivity (Supporting information, section S4).

The lipid layers on the PEG-SAM surface clearly shows higher d and lower RI compared to the two other surfaces, which was expected on the basis of the QCM results. This supports the earlier findings that the PEG-SAM surface rather promotes SVL than SLB formation. However, the d:s were quite small compared to the vesicle
sizes. This can be explained either by the uncertainty in the background used during optical modeling, or most probably because the SVLs deform into disc-like vesicles upon absorption on the PEG-SAM surface which consequently is reflected as a much smaller thickness than would be expected for full non-deformed vesicles.

The results from the two-wavelength SPR analysis correlate very well with the results obtained from the QCM thickness analysis. Hence, these SPR results support the conclusions drawn from the QCM results on the SLB and SVL formation on the different surfaces used in this study. The results are also supported well by earlier studies with similar experiments reported in the literature [22]. While the QCM was an easier method for detecting the SLB formation as such, it is quite apparent that the two-wavelength SPR analysis approach was more sensitive to slight changes in the lipid layer thickness and density than the QCM experiments even though the SPR analysis was performed in a qualitative intra-series manner. However, together these two methods seem to offer an excellent combination for studying the biophysical properties of different membranes and other adsorbed or layered materials.

Table 2. Lipid layer parameters obtained from optical modeling of SPR measurements.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Lipid</th>
<th>d</th>
<th>n (670nm)</th>
<th>n (785nm)</th>
<th>dn/dL (-1/nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiO2</td>
<td>EggPC</td>
<td>4,70</td>
<td>1,4421</td>
<td>1,4381</td>
<td>0,000035</td>
</tr>
<tr>
<td>SiO2</td>
<td>EggPC+PS</td>
<td>4,63</td>
<td>1,4431</td>
<td>1,4387</td>
<td>0,000038</td>
</tr>
<tr>
<td>SiO2</td>
<td>EggPC+Chol</td>
<td>5,61</td>
<td>1,4236</td>
<td>1,4191</td>
<td>0,000039</td>
</tr>
<tr>
<td>Dex6kDa</td>
<td>EggPC</td>
<td>5,11</td>
<td>1,4735</td>
<td>1,4680</td>
<td>0,000048</td>
</tr>
<tr>
<td>Dex6kDa</td>
<td>EggPC+PS</td>
<td>5,16</td>
<td>1,4729</td>
<td>1,4673</td>
<td>0,000049</td>
</tr>
<tr>
<td>Dex6kDa</td>
<td>EggPC+Chol</td>
<td>6,38</td>
<td>1,4401</td>
<td>1,4371</td>
<td>0,000026</td>
</tr>
<tr>
<td>PEG-SAM</td>
<td>EggPC</td>
<td>10,79</td>
<td>1,3977</td>
<td>1,3975</td>
<td>0,000002</td>
</tr>
<tr>
<td>PEG-SAM</td>
<td>EggPC+PS</td>
<td>13,84</td>
<td>1,3813</td>
<td>1,3804</td>
<td>0,000008</td>
</tr>
<tr>
<td>PEG-SAM</td>
<td>EggPC+Chol</td>
<td>8,69</td>
<td>1,3928</td>
<td>1,3882</td>
<td>0,000040</td>
</tr>
<tr>
<td>PEG-SAM</td>
<td>EggPC+Label</td>
<td>10,55</td>
<td>1,4031</td>
<td>1,4013</td>
<td>0,000016</td>
</tr>
</tbody>
</table>

7.3.2.2 Air stability of the SLB

The air stability for the EggPC-PS-Chol lipid mixture on the Dex6kDa surface was tested in triplicate by flowing air through the SPR microfluidic system at a nominal speed of 200 μL/min. The SPR flow channels have an internal volume of 1 μL, meaning that the gas content in the flow channel was replenished 3.33 times per second. Other lipid mixtures or surfaces used in this study were not tested, because SLBs formed on the SiO2 surface are known to be unstable during transition through the air-water interface[16]. The air-stability of the SVLs adsorbed on the PEG-SAM surface was not studied because SLBs are often more desirable biomimetic surfaces than SVLs for use with surface-sensitive detection techniques because of their well defined structures, which consequently makes the analysis of the signal responses
with SLBs much easier compared to SVLs. The less complex lipid mixtures were also left out from the air-stability studies because the main challenge in preparing biomimetic surfaces by vesicle adsorption is to form air-stable SLBs with cholesterol containing vesicles, as well as for vesicles with lipid compositions resembling natural cell membranes as close as possible.

When the SPR flow channel was filled with air this caused a large transition in the SPR angular spectra, where the SPR peak typical for water was transformed into a multimodal mixed angular spectrum. During this time it was not possible to track the SPR peak minimum. However, the crossing of the air-water interface was clearly indicated by the shift of the critical angle of the SPR peak to the range typical for water. Due to the difficulty in tracking the SPR peak during air exposure, the time period for the air flow in the measurements was cut from the SPR sensograms (Figure 6). Figure 6 clearly shows that the SPR signal levels returned close to the original signal level after the air treatment of the EggPC+PS+Chol SLB. An air-stability of 86% was obtained for the EggPC+PS+Chol SLB by comparing the averaged SPR signal level before the air injection to the averaged SPR signal level after the air treatment. The measurements were performed with a constant flow of air through the flow channel for 10 minutes, which means that the EggPC+PS+Chol SLB was also continuously drying during the experiment. This indicates that the EggPC+PS+Chol SLB on the Dex6kDa surface had an excellent resistance against drying upon exposure to air and upon transition through the air-water interface.

![Air Stability 670 nm](image)

**Figure 5** Normalized SPR minimum angle vs. time sensograms of air stability measurements of the EggPC+PS+Chol lipid mixture adsorbed on the Dex6kDa surface. The three repetition sensograms have been normalized to the deposition plateau (interval starting at 20-25 minutes), and the air stability in percentage has been characterized from the magnitude of the returning signal (interval at 45-50 minutes). The air treatment was also recorded on line, but was cut out of the sensogram for clarity. The presence of air in the flow channel was confirmed by the shift in the TIR angle during the measurement (not shown). The three repetitions gave an average of 86% stability of the deposited EggPC+PS+Chol SLB.
7.3.3 COMPARISON OF SURFACES

The QCM and SPR results indicate, that the Dex6kDa surface offers a platform for SLB deposition similar to the SiO$_2$ surface, which was utilized as a reference surface in this study. The PEG-SAM surface however promotes SVL deposition. While both the Dex6kDa and the PEG-SAM surfaces are hydrophilic and highly hydrated, the difference in promoting different lipid morphology was most probably due to the differences in the inherent structure, density and location of the hydrophobic groups of the hydrogels.

Both of the hydrogel-based sensor surface coatings could be regenerated by a simple CHAPS detergent washing cycle, while the SiO$_2$ surface needed a much harsher and complex washing procedure before reuse. This is also a beneficial property for the screening of interactions, as it allows a faster experimental cycle and increases the throughput of the assay. The throughput, along with a simpler automation caused by the less-complicated wash cycle, makes the new Dex6kDa and PEG-SAM surfaces viable candidates for wide applications in the screening of biochemical interactions and in pharmaceutical research. This kind of a reusable sensor structure offers even greater benefits in several application areas when compared to non-regenerable sensor structures, e.g. biotin-avidin based sensors.

The air stability exhibited by the SLBs on the Dex6kDa surface was found to be good. This property is important in a wider application of SLBs within interaction research and pharmaceutical development, as it allows resistance to sampling mistakes (i.e. dissolved gases out-gassing in microfluidics), more advanced samples (air-bubble trapped samples) or even a pre-deposition of SLBs and a storage of them. A similar air stability has also been shown for supports containing Langmuir-Blodgett deposited monolayer with a Zr$^{2+}$-ion top-layer, which actually required that palmitoyl-oleyl phosphatic acid is included into the SLB mixture[18]. The benefits of the SLB supported with Dex6kDa over the Zr$^{2+}$-ion approach are a wider lipid functionality, a regenerability and a simpler synthesis. Another similar approach has been introduced by using cholesterol in a PEG matrix as linking groups[17]. This approach was similar to the Dex6kDa approach in this study, but the air stability was performed in much milder conditions (i.e. a careful transfer of the substrate and SLB through air-water interface), while in this study the stability was demonstrated under a constant air flow. An approach where SLBs have been “sandwiched” between proteins or PEG has shown good results for the SLB air stability. However, the approach often requires certain groups or macromolecules to be added to the SLB formulation[16,19]. This can cause interference in measuring the interactions or other properties of the SLBs, which further makes the Dex6kDa surface developed in this study a more viable approach for preparing SLBs for any applications studying these properties.

7.4 CONCLUSIONS

This work describes the synthesis of two new surface coatings that promotes different lipid layer morphologies during vesicle spreading on the supporting surface. The low-molecular-weight dextran-based Dex6kDa surface showed good performance in
promoting SLB formation with both known lipids, as well as with a mixture containing the natural membrane extract of HepG2 cells. The low-molecular-weight self-assembled PEG-SAM surface promoted SVL formation on the surface with all lipid compositions tested in this study. It was also possible to demonstrate that the Dex6kDa surface effectively protects the SLB layer from dehydration after deposition. The air-stability allows to develop more robust SLB based experimental assays, thereby increasing the probability to further develop SLBs as useful platforms for a wide range of applications. The simple synthesis procedures of a surface combined with air-stability for the deposited lipid layers makes it possible to achieve more flexible and robust SLBs for applications in areas such as drug discovery and development, biosensing and biophysics.

In addition, this study further demonstrates the utilization of the relatively rare multiple-wavelength SPR method. The two-wavelength SPR analysis used provided results which correlated with the well established QCM technique, as well as with other optical techniques used for SLB characterization. This study also shows that the combination of both SPR and QCM methodologies provides high quality data with a large amount of information for characterizing nanometer-scale systems. It is apparent that the use of both techniques is especially beneficial in biophysics and similar applications, due to the quality and magnitude of the information required for these applications.

7.5 ACKNOWLEDGEMENTS

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Supporting Information Available:
S1 – Polymer end group modification of PEG
S2 – DLS size distribution graph
S3 – Impedance-QCM dissipation results
S4 – SPR Signal dependency on coating material
S5 – Optical constants of two-wavelength SPR analysis
7.6 REFERENCES


7.7 SUPPORTING INFORMATION

7.7.1 S1 – END GROUP MODIFICATION OF PEG POLYMER

Materials
All chemicals used in the synthesis were obtained from Sigma-Aldrich (Helsinki, Finland), if not stated otherwise. PEG₄₅, Mw = 2 kDa and –OH end groups at both end, was obtained from Polysciences Inc (Pennsylvania, USA) and ethanol was obtained from Altia (Rajamäki, Finland). The water used was of 18.2 MΩ/cm resistivity. Solvents were distilled over molecular sieves and chemicals were dried in vacuum dessicator before use.
Synthesis of 1-tosyloxyundec-10-ene
1-tosyloxyundec-10-ene was synthesized using a known method from literature [1]. Briefly, 10-undecen-1-ol (40 mmol), 4-toluenesulfonyl chloride (125 mmol) and triethylamine (250 mmol) were dissolved in 300 mL of dichloromethane (DCM). The dichloromethane was purged with nitrogen before addition of the reactants, and the reaction mixture was purged with nitrogen all through the reaction. The mixture was allowed to react 4 hours at room temperature (RT).

The solvent was removed using a rotary evaporator. DCM was added, and the mixture was extracted twice with 1 M HCl. The organic phase was separated and dried with sodium sulphate (anhydrous). The solids were filtered out and the organic phase was evaporated in a rotary evaporator and the residue was dried overnight in a vacuum dessicator. Further purification was performed using column chromatography (silica, DCM:cyclohexane 75:25, Rf 60%). The colorless final product (1-tosyloxyundek-10-ene) was dried overnight in a vacuum.

The purity of the product was checked with 1H NMR, and was determined to be close to 100%. The yield was determined to be 58%.

Synthesis of PEG-SAM
The synthesis protocols were adapted from literature [1,2].

First the end-groups of PEG45 were modified to form an intermediate product carrying double bonds in both ends. PEG45 (0.5 mmol) and 1-tosyloxyundec-10-ene (3 mmol) were dissolved into 25 mL of distilled and nitrogen purged DMF. Five mmol of NaH was then carefully added to the mixture (Note! Reacts violently with water, handle with care). The reaction mixture was stirred for one hour at RT, then the temperature was raised to 90 °C and the reaction was carried out for 3 days. The reaction was stopped by lowering the temperature back to RT, and carefully neutralizing the remaining NaH with a small amount of methanol.

The raw product was dissolved in a small amount of THF and precipitated twice into ice cold diethyl ether. The product was then dried in a vacuum. The purity of the intermediate product was checked with 1H NMR, and it was calculated that approximately 88% of all end groups were converted with the double bond in the reaction. Yield was determined to be 50%.

The intermediate product (0.28 mmol) and thioacetic acid (35 mmol) were dissolved into 15 mL of distilled and nitrogen purged THF. The mixture was stirred under nitrogen flow for 30 minutes, after which a catalytic amount of azobisisobutyronitrile (AIBN) was added to the mixture. The reaction mixture was heated to 70°C and allowed to react overnight.

The residue was precipitated in diethyl ether and collected by centrifugation at +8 °C. A clear solid intermediate product was collected and dried. This intermediate product was dissolved into 15 mL of ethanol, and 15 mL of saturated sodium methoxide in methanol was added to the mixture. The reaction mixture was allowed to react overnight at RT with vigorous stirring.

The solvent was evaporated and the crude product was dissolved in THF and the solids were removed by filtering. The liquid was evaporated and the product was finally purified by passing it through a plug of silica (Chloroform:methanol 80:20).
Finally, the product was dried in a vacuum. The yield of the product was 70% by mass.

The final product was characterized with $^1$H NMR, size exclusion chromatography (SEC, in THF and against polystyrene standards), and matrix assisted laser desorption-ionization time of flight mass spectrometry (MALDI TOF MS) using sodium fluoroacetate and dihydroxybenzoic acid as the matrix. The NMR results confirm that both thiol- and double-bonds existed in the product, but also that some of the product was dimerized with disulfide bridges (Figure S1). The dimerization was also confirmed by SEC, which showed two separate peaks. Treatment with sodiumborohydrate could remove the disulfide shift in the $^1$H NMR, confirming the first impression on partial dimerization to disulfide. The MALDI TOF MS did not show the dimerization, but as the MALDI TOF method involves a relatively large energy transfer, it probably broke the disulfide during the measurement. The MALDI TOF MS was used to characterize the product, and yielded a $M_n = 2174$ Da and a $M_w = 2275$ Da and a PDI = 1.05 (Figure S2). The MALDI TOF MS also showed that the most common molecular weight (highest intensity of the distribution) was 2341 Da, which was the theoretical molecular weight for the desired PEG product with unsymmetric end-groups (i.e. thiol and double bond).

![Figure S1](image)

**Figure S1.** $^1$H NMR spectrum of the synthesized PEG-SAM polymer.
**Figure S2.** MALDI TOF MS spectrum of the synthesized PEG-SAM polymer.
7.7.2 S2 - DLS SIZE DISTRIBUTION.

Figure S3. The number-average size distribution results for CONTIN analysis. Red – EggPC, Blue EggPC+PS, Green EggPC+PS+Chol and Purple HepG2:EggPC.

7.7.3 S3 IMPEDANCE BASED QCM: DISSIPATION RESULTS

The KSV QCM-Z500 utilizes impedance based analysis for measuring dissipation of samples [3], unlike the patent-protected dissipation technology of QCM-D from Q-Sense. While both can measure viscoelastic properties of the samples, the measurements are physically quite different and they cannot be assumed to be 100% comparable. Still, relative information of the degree of viscoelasticity should be comparable and valid between the different technologies, and this was how they have been compared in this work. Comparing the similarities and differences of the
The dissipation results support the findings of the QCM-frequency in terms of SLB or SVL formation on the surface. The 9th and 11th overtones for the measured dissipation were discarded from the figures, because they were so noisy that they interfered with the visualization of the other overtones. However, the dissipation measured at the 9th and 11th overtones also showed the same trends and dissipation levels as the overtones shown. The dissipation changes measured for the SiO₂ (Figure S3) and the Dex6kDa (Figure S4) surfaces that the morphology of the lipid layer was an SLB at the end of each deposition experiment. The dissipation changes measured for the PEG-SAM surface (Figure S5), on the other hand, indicate that the morphology of the lipid layer was an SVL layer. These results correlate with the dissipation responses for SLB and SVL layer formation in the literature.

**Figure S4.** Dissipation changes during vesicle spreading on the SiO₂ surface for EggPC (red) EggPC+PS (black) and EggPC+PS+Chol (blue) for the 3rd, 5th and 7th overtones. Figure 1 in the original article corresponds to this dissipation figure. The vertical lines have been drawn to response level 0 and 5 to help in visualizing the response level between the dissipation graphs (Figures S3-S5).
Figure S5. Dissipation changes during vesicle spreading on the Dex6kDa surface for EggPC (red) EggPC+PS (black), EggPC+PS+Chol (blue) and HepG2-extract (green) for the 3\textsuperscript{rd}, 5\textsuperscript{th} and 7\textsuperscript{th} overtones. Figure 2 in the original article corresponds to this dissipation figure. The vertical lines have been drawn to response level 0 and 5 to help in visualizing the response level between the dissipation graphs (Figures S3-S5).

Figure S6. Dissipation changes during vesicle spreading on the PEG-SAM surface for EggPC (red) EggPC+PS (black) and EggPC+PS+Chol (blue) for the 3\textsuperscript{rd}, 5\textsuperscript{th} and 7\textsuperscript{th} overtones. Figure 3 in the original article corresponds to this dissipation figure. The vertical lines have been drawn to response level 0 and 5 to help in visualizing the response level between the dissipation graphs (Figures S3-S5).
7.7.4 S4 – THE INFLUENCE OF THE COATING MATERIAL ON THE SPR SIGNAL

As the penetration depth of the SPR field and the SPR sensitivity both depend on the distance between the surface and the dielectric constant of the coating [4], a rough estimate of the effect on SPR signal magnitude was performed by optical simulations of the layer. The optical model used for simulations consisted of an SPR sensor with optical parameters obtained from optical modeling of an actual SPR sensor (Glass/Adhesion/Gold layers in Table S1). The surface coating was assumed to be either 10 nm of SiO$_2$ ($n = 1.56$, $d = 10$ nm), or a loose dextran hydrogel ($n = 1.34$, $d = 10$ nm). A hypothetical lipid bilayer was included in the model on top of this, with an assumed $n = 1.50$ and thickness of either 4 nm or 5 nm [5]. The SPR measurement wavelength was set to 670 nm, which is the wavelength of the standard laser in the SPR instrument used in this study. The modeling was performed by using the Winspall 3.02 software [6]. The theoretical SPR signal changes obtained in this way for the different surfaces and lipid bilayer thicknesses are shown in Table S2.

The results of the simulations shown in Table S2 reveal that a “spacing” hydrogel produces approximately 75% less signal in an SPR measurement under the assumed conditions when compared with a rigid SiO$_2$ surface. As the model is relatively rough, it does not perfectly reflect the real experimental situation. For example, the Dextran hydrogel thickness and RI are only rough assumptions. Despite of this, it is possible to conclude from the results that the hydrogel support surface most probably will produce a visibly smaller SPR signal change than the SiO$_2$ surface during SLB formation.

### Table S1. Optical parameters used in the SPR models. A is the surface support layer (10 nm of SiO$_2$ with $n = 1.56$ or 10 nm of hydrogel with $n = 1.34$). The SLB layer thickness ($B$ in the table) used was either 4 nm or 5 nm.

<table>
<thead>
<tr>
<th>Layer</th>
<th>d</th>
<th>n</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>Inf</td>
<td>1.5202</td>
<td>0</td>
</tr>
<tr>
<td>Adhesion</td>
<td>1.62</td>
<td>3.098</td>
<td>1.734</td>
</tr>
<tr>
<td>Gold</td>
<td>48.76</td>
<td>0.227</td>
<td>3.775</td>
</tr>
<tr>
<td>Support</td>
<td>A</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>SLB</td>
<td>B</td>
<td>1.50</td>
<td>0</td>
</tr>
<tr>
<td>Buffer</td>
<td>Inf</td>
<td>1.334</td>
<td>0</td>
</tr>
</tbody>
</table>
Table S2. Theoretical changes in the SPR angle during SLB formation on the SiO₂ surface or on the dextran hydrogel surface, and the signal ratio as a function of thickness. The simulations were performed with two different thicknesses of the SLB in order to see if it had any effect on the signal ratio.

<table>
<thead>
<tr>
<th>SLB thickness</th>
<th>SiO₂</th>
<th>Dextran</th>
<th>Ratio (Dex6kDa/SiO₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5nm</td>
<td>1,021</td>
<td>0,759</td>
<td>0,744</td>
</tr>
<tr>
<td>4nm</td>
<td>0,816</td>
<td>0,602</td>
<td>0,738</td>
</tr>
<tr>
<td>AVG</td>
<td></td>
<td></td>
<td>74 %</td>
</tr>
</tbody>
</table>

7.7.5 S5 – OPTICAL CONSTANTS OF THE TWO-WAVELENGTH SPR ANALYSIS

The optical parameters obtained for the backgrounds in the two-wavelength calculations are shown in Table S3. An example of the fitting results for the two-wavelength SPR analysis is presented in Figure S6.

Table S3. The optical constants obtained for the background fits and used for the following lipid layer characterization with two-wavelength SPR analysis. “A” denotes the variable parameter during lipid layer characterization.

<table>
<thead>
<tr>
<th></th>
<th>SiO₂</th>
<th>Dex6kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>d</td>
<td>n(670) n(785)</td>
<td>k(670) k(785)</td>
</tr>
<tr>
<td>Glass</td>
<td>Inf</td>
<td>Inf</td>
</tr>
<tr>
<td>Cr</td>
<td>2.59</td>
<td>3,8648 4,9861</td>
</tr>
<tr>
<td>Au</td>
<td>49,65</td>
<td>0,2695 0,3005</td>
</tr>
<tr>
<td>SiO₂</td>
<td>6,96</td>
<td>1,5773 1,5900</td>
</tr>
<tr>
<td>Lipid A</td>
<td>A A A A</td>
<td>0,0000 0,0000</td>
</tr>
<tr>
<td>Buffer</td>
<td>Inf</td>
<td>Inf</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PEG-SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>d</td>
</tr>
<tr>
<td>Glass</td>
</tr>
<tr>
<td>Cr</td>
</tr>
<tr>
<td>Au</td>
</tr>
<tr>
<td>SiO₂</td>
</tr>
<tr>
<td>Lipid A</td>
</tr>
<tr>
<td>Buffer</td>
</tr>
</tbody>
</table>
IV Control of the morphology of lipid layers by substrate surface chemistry

Figure S7. Measured data and typical fitting results obtained for background (red and blue) and lipid layer (orange and green) when fitted with simultaneous two-wavelength SPR analysis. The example is for a SiO₂ surface with EggPC lipid bilayer.

7.7.6 REFERENCES

Abstract

In vitro cell-based assays are widely used during the drug discovery and development process to test the biological activity of new drugs. Most of the commonly used cell-based assays, however, lack the ability to measure in real-time or under dynamic conditions (e.g. constant flow). In this study a multi-parameter surface plasmon resonance approach in combination with living cell sensing has been utilized for monitoring drug-cell interactions in real-time, under constant flow and without labels. The multi-parameter surface plasmon resonance approach, i.e. surface plasmon resonance angle versus intensity plots, provided fully specific signal patterns for various cell behaviors when stimulating cells with drugs that use para- and transcellular absorption routes. Simulated full surface plasmon resonance angular spectra of cell monolayers were compared with actual surface plasmon resonance measurements performed with MDCKII cell monolayers in order to better understand the origin of the surface plasmon resonance signal responses during drug stimulation of cells. The comparison of the simulated and measured surface plasmon resonance responses allowed to better understand and provide plausible explanations for the type of cellular changes, e.g. morphological or mass redistribution in cells, that were induced in the MDCKII cell monolayers during drug stimulation, and consequently to differentiate between the type and modes of drug actions. The multi-parameter surface plasmon resonance approach presented in this study lays the foundation for developing new types of cell-based tools for life science research, which should contribute to an improved mechanistic understanding of the type and contribution of different drug transport.

8.1 INTRODUCTION

Current drug discovery paradigms are slowly shifting from the reductionism thinking approach towards a more holistic approach [1,2]. The ability to examine living cells in physiologically relevant environments, to monitor drug induced cell stimuli, and differentiating between different drug delivery routes are of utmost importance for improving our mechanistic understanding during the drug discovery and development processes [2–5]. Therefore, cell-based assays have gained increased popularity compared to biochemical assays in drug discovery and development. Although cell-based assays are more complex and less specific than biochemical assays, they facilitate the measurements of mode of action, pathway activation, toxicity, and phenotypic responses of cells mediated by exogenous stimuli. However, established in vitro cell-based assays are static and laborious and cannot measure real-time interactions on the cellular level. They often rely on labelled materials for imaging or detection purposes, and they require a secondary detection technique where the final quantification is based on UV- or fluorescence spectroscopy, mass spectrometry, radiometry or chromatographic techniques. Thus, a development of new in vitro cell-based assay methodologies and approaches which enable direct detection, and real-time, non-invasive, label-free and continuous high sensitivity monitoring of cell responses to exogenous stimuli, would be desirable.

Several label-free techniques have recently been developed for studying cell-substrate adhesion, cell-cell interactions, cell migration and volume changes in cells [6–14], as well as for monitoring living cell activity (e.g. cellular metabolism, toxicity, receptor mediated signaling and endocytic vesicle formation) [15–26]. Among the label-free techniques developed for probing the activities and interactions of living cells, optical techniques that utilizes evanescent waves, i.e. surface plasmon resonance (SPR) and resonant waveguide grating (RWG), have attracted a great deal of interest. This is probably because they are widely spread and have established themselves as powerful techniques for biosensing applications. However, the evanescent wave measuring techniques generally penetrate approximately ½ of the incident light wavelength into the surrounding medium. Thus, for a visible light source, a 300 nm penetration depth with an exponential decay of sensitivity as a function of distance from the sensor surface is commonly achieved [27]. This means that in living cell sensing, the evanescent wave technique only probes the bottom part of the cell layer. Attempts to improve the penetration depth have been made by utilizing near infrared (NIR) SPR [12, 24], but despite of this the active scanning range is still well below the common cell diameter.

An advantage of SPR compared to RWG is that SPR systems are capable of measuring in constant and controlled flow conditions, and depending on the optical setup of the SPR instrument, it is even possible to extract thickness and refractive index information on the (cell) layers through optical modeling of the full SPR spectrum [27, 28]. SPR has established itself as a powerful technique for providing affinity and kinetic information of target-based biomolecular interactions [29, 30]. However, several studies have demonstrated that SPR is also a powerful tool for real-time monitoring of living cell interactions, and for studying different cellular processes without the use of labeling agents [15, 16, 18, 19–22, 24, 26]., So far all SPR
interaction studies with living cells are performed by measuring and analyzing only changes either in the main SPR peak angular position or in the reflection intensity at a fixed angle near the main SPR peak minimum. This probably origins from one or both of the following reasons: 1) Traditionally SPR has almost solely been used for routine biomolecular interaction analysis based on reflectance or angular changes, and the living cell sensing is therefore forced into the same thinking patterns, and/or 2) the SPR instruments used for living cell studies do not provide any other information than reflection intensity at fixed angle or angular change information, which does not allow for any other type of analysis.

The full SPR angular spectra have successfully been used in modeling optical properties and thicknesses of both thin organic and inorganic layers [27, 28, 31]. However, a highly unexploited approach of SPR is to measure the full SPR angular spectra in real-time in order to fully utilize its shape or key parameters (i.e. SPR peak angular position, SPR peak minimum intensity and the changes in the total internal reflection region) for studying drug interaction processes with cellular targets. This might not be critical when considering traditional biomolecular interactions, but it should play a significant role in living cell sensing. Therefore, analyzing multiple parameters from the full SPR angular spectra would be of interest in order to try to obtain a better quantitative or even qualitative understanding of how SPR could be utilized for living cell sensing. No studies have so far made use of real-time monitoring of the full SPR angular spectra and utilized it for analyzing real-time drug-cell interactions.

Herein, changes in simulated full SPR angular spectra induced by varying different optical parameters are compared with actual SPR measurements of drug-MDCK II cell interactions in order to elucidate the signal responses in living cell sensing with SPR. An understanding of cell-analyte responses is established through optical modeling of different sections of the cell monolayer, and by examining the changes taking place in the full SPR angular spectra caused by the introduction of an analyte. The simulated SPR angular spectra responses from a cell monolayer are then compared with the measured full SPR angular spectra of an actual cell monolayer composed of MDCKII cells. Finally, a new qualitative analysis method demonstrating how the multi-parameter SPR approach enables to distinguish between passive (trans- and paracellular) drug absorption processes during drug-cell interactions is presented.

### 8.2 EXPERIMENTAL SECTION

#### 8.2.1 SPR THEORY

The working principle of the SPR technique is based on utilizing visible light to excite free electrons on a surface of a metal, which in turn causes surface plasmons to travel along the metal surface also creating an evanescent field to the adjacent medium in contact with the metal. The surface plasmon excitation takes place when certain conditions regarding the optical properties of the system and incident light angular frequency are matched, resulting in a high absorption of the incident light. The most
common way of fulfilling these conditions is to use the so-called Kretschmann configuration (Figure 1A), which enables the detection of plasmon excitation from a sharp dip in the refracted light intensity (Figure 1B). The total internal reflection (TIR) region is sensitive to the optical properties of the media outside the evanescent field ($\varepsilon_{\text{bulk}}$), whereas the main SPR peak angular position and intensity are highly sensitive to the optical properties of the media within the evanescent field ($\varepsilon_1$) [27, 28].

Figure 1  Kretschmann configuration and key parameters obtained from the full SPR angular spectra.
A) A simplified chart of the Kretschmann configuration enabling plasmon excitations and SPR measurements. The intensity of the reflected light from a monochromatic light source is measured as a function of incident light angle ($\theta$). The light passes from a high refractive index medium (glass, $\varepsilon_0$) to a low refractive index medium (air or liquid, $\varepsilon_1+\varepsilon_{\text{bulk}}$). In between, the light is reflected from an interface containing a metal with a high density of free electrons and an optimal thickness for plasmon excitation (gold 50 nm, $\varepsilon_2$) to a photodetector. The surface plasmons on the metal surface are excited at a certain incident light angle ($\theta$) and the evanescent field created by the plasmon extends to the adjacent low refractive index medium ($\varepsilon_1$) where samples are introduced to the system. B) A schematic full SPR angular spectrum showing the positions of the TIR region, the main SPR peak angular position and the main SPR peak minimum intensity.
A theoretical mathematical description for the surface plasmon resonance condition for a multilayer optical system can be obtained by solving the Maxwell equations. This results in the following solution for the resonance condition:

\[
\frac{\omega}{c} = \frac{\varepsilon_0 \sin \theta}{\sqrt{\varepsilon_0 \frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2}}}
\]  

where \( \omega \) is the angular frequency of light, \( c \) is the speed of light, \( \theta \) is the angle of the incident light and \( \varepsilon_0, \varepsilon_1 \) and \( \varepsilon_2 \) are the permittivity of the prism, of the SPR metal layer and of the adjacent medium, respectively (Figure 1). The permittivity (\( \varepsilon \)) and refractive index (\( n \)) of the materials can be written in their complex forms as following:

\[
\varepsilon = \varepsilon' + i\varepsilon''
\]

\[
\tilde{n} = n - ik; \quad (k > 0)
\]

and the permittivity and the complex refractive index also have the following relationship:

\[
\tilde{n} = \sqrt{\varepsilon}
\]

\[
\varepsilon = n^2 + k^2
\]

A general answer for the Maxwell equations for multilayered systems linked to measurable or controllable variables can be solved by using the transfer matrix formalism of 2×2 matrices. The overall mathematical formalism has been published several times, and it is not in the scope of this article to discuss it in detail [28, 31]. In practice, this matrix formalism is solved by mathematical calculations and fitting tools, or by taking advantage of dedicated software tools developed for this (e.g. Winspall) [32]. Fitting the full SPR angular spectra then provides information that can be used to characterize sample properties, such as the real refractive index, the thickness and the light absorbance properties of different analyst materials at the surface [28, 33, 34].

### 8.2.2 SIMULATION OF FULL SPR ANGULAR SPECTRA

The Winspall software (version 3.02) [32] was used throughout this study to simulate the full SPR angular spectra. The optical parameters for the fixed components in the simulations were as following: Glass prism, \( n = 1.5294, k = 0 \) and thickness = \( \infty \); Chromium adhesion layer, \( n = 3.1085, k = 3.4873 \) and thickness = 1.53 nm, Gold layer, \( n = 0.2262, k = 3.7639 \) and thickness = 50.59 nm. The optical parameters for the chromium adhesion and the gold layer were extracted from a Winspall fit to a measured full SPR angular spectrum of a real and thoroughly cleaned gold-coated SPR sensor slide immersed in water. All simulations were done with the sample layer immersed in a bulk medium resembling water with \( n = 1.3299 \) and \( k = 0 \). The
incident light wavelength used in the simulations was 670 nm, which is the same wavelength as in the SPR device used in this study.

8.2.3 CELL CULTURE
Madin-Darby canine kidney (MDCKII) cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (Gibco) supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C in a 5% CO₂ incubator.

Immobilization of cells on surface plasmon resonance sensor
Gold-coated SPR sensor slides were obtained from Bionavis Ltd. (Tampere, Finland). Before the experiments the sensors were first cleaned by boiling them for 5 min in a solution containing 1 part of 30% ammonia hydroxide solution (Sigma), 1 part of 30% hydrogen peroxide (Sigma) in 5 parts of Milli-Q-water. Hereafter, the sensors were rinsed thoroughly with Milli-Q-water and dried with nitrogen. Finally, the SPR sensor slides were autoclaved before cell immobilization.

The immobilization of MDCKII cells on the SPR sensor slides were performed by first treating confluent cell layers in cell culture flasks with 0.25% trypsin/EDTA in DPBS, followed by a re-suspension of the cells in the cell culture medium. The SPR sensor slide was then placed in a cell culturing polystyrene petri dish with a cell growth area of 8.8 cm² and 3 ml of the cell suspension was pipetted on top of the SPR sensor slide. Cells were then allowed to attach and grow on the SPR sensor slide in an incubator in a controlled environment until they were confluent.

8.2.4 VIABILITY OF CELLS ON SPR SENSOR SLIDES
The trypan blue test was performed on cells cultured both in tissue culture treated polystyrene wells as a reference, and on cells cultured directly on SPR sensor slides. After 24 h of culturing the medium was carefully removed, and the cells were washed with DPBS before detaching them with a 0.25% trypsin/EDTA solution. Cells were then resuspended in cell culture medium and a Trypan blue solution (Gibco) was added to the cell suspension in a ratio of 1:1 (v:v) in order to stain the dead cells blue. The non-colored cells (viable cells) were counted with a Cedex XS cell counter (Roche Diagnostics Oy).

8.2.5 TEST COMPOUNDS IN LIVING CELL SURFACE PLASMON RESONANCE ANALYSIS
Propranolol hydrochloride (Sigma-Aldrich) and D-mannitol (Fluka) were used as test compounds in the SPR interaction studies with living cells. Each test compound was diluted in a buffer composed of Hank’s Balanced Salt Solution (HBSS, Gibco) supplemented with 10 mM Hepes (Sigma) and adjusted to pH 7.4 with 1 M NaOH (running buffer).
8.2.6 SURFACE PLASMON RESONANCE ANALYSIS
Interaction experiments between test compounds and immobilized MDCKII cells were performed using a multiparameter SPR device (MP-SPR Navi 200, BioNavis Ltd, Tampere, Finland). The cells were cultured on the SPR sensor slide for 3–4 days before analysis. Just before the measurements, the whole flow path of the SPR device was filled with the running buffer. Once the cells had reached confluency on the SPR sensor slide, they were once washed with the running buffer. After this, the sensor slide was quickly inserted into the instrument before the cell layer could dry. The experiments were performed under a constant flow rate of 10 μl/min, which was controlled by a syringe pump accessory. The interaction between cells and test compounds were measured by injecting the compound of interest for 6 to 10 min followed by a rinsing period of 10–20 minutes with pure running buffer. All the experiments were performed at 20 °C by using the angular scan mode. The angular scan range during the experiments was between 60–78°. With this scan range the angular scan mode provided a full SPR angular spectrum every four seconds. At the end of each experiment, the SPR sensor slide was examined under an optical microscope in order to evaluate the cell monolayer integrity after the interaction experiments.

8.3 RESULTS

8.3.1 IMMOBILIZATION OF MDCKII CELL MONOLAYERS ON SPR SENSOR SLIDES
Real-time drug-cell interactions were monitored with SPR by immobilizing a monolayer of MDCKII cells on the SPR sensor slide while continuously measuring the full SPR angular spectrum during cell stimulation with propranolol and D-mannitol. For this purpose, it was of utmost importance to optimize the cell immobilization protocol, because the surface coverage of cells on the SPR sensor slide has a dramatic influence on the shape of the full SPR angular spectra [12, 16, 24]. The morphology of MDCKII cells seeded directly on SPR sensor slides and polystyrene surfaces (used as a reference) was found to be the same (Fig. 2A–D). Different cell seeding densities from 5×10⁴ cells/cm² to 1×10⁵ cells/cm² on the SPR sensor slide revealed the following: 1) The lowest cell seeding density of 5×10⁴ cells/cm² was not sufficient to form a fully confluent cell monolayer and large cell free areas could be seen in the microscopy image (Fig. 2B), 2) the highest cell seeding density of 1×10⁵ cells/cm² showed some cell-condensed clusters (Fig. 2D), and 3) an intermediate cell seeding density of 7×10⁴ cells/cm² was optimal for immobilizing a uniform, almost cluster-free and fully confluent cell monolayer on the SPR sensor slide (Fig. 2C). The optimum cell seeding time for achieving confluent MDCKII cell monolayers on SPR sensor slides was also determined to be 3–4 days.
Stimulation experiments with drugs showed that the MDCKII cell monolayers on the SPR sensor slides remained confluent with hardly any changes in morphology after being exposed to the drug at a flow rate of 10 μl/min in the SPR flow channel (Fig. 2E). The flow experiments clearly demonstrated that there is no need to use any adhesion promoter in order to successfully immobilize and retain a confluent
MDCKII cell monolayer on the SPR sensor slide for SPR interaction measurements. The trypan blue cell viability test showed that the MDCKII cells remained viable on the SPR sensor slides after 24 h of cell culturing (Fig. 2F).

Thus, the optimized cell seeding conditions allowed to consistently preparing uniform, fully confluent and viable MDCKII cell monolayers on the SPR sensor slides. This ensures that no significant contributions to the SPR signal will be caused by cell spreading, cell division, cluster formation or overgrown cell monolayers.

8.3.2 SIMULATED FULL SPR ANGULAR SPECTRA OF CELL MONOLAYERS

In the majority of biomolecular or biosensing interaction studies with SPR, the sample layer thickness is well below the penetration depth of the evanescent field. In such a case, changes in the real part of the refractive index \((n)\) will to a good approximation reflect the mass of the analyte within the sample layer. This is exemplified with the simulated full SPR angular spectra in Figure 3A. This figure shows that the main SPR peak angular position will shift to higher angles when the refractive index \((n)\) of a sample layer increases from 1.45 to 1.5. On the other hand, if \(n\) would have been kept constant in the simulation in Figure 3A, then an increase in the sample layer thickness would have caused a corresponding increase in the main SPR peak angular position angle. This is the case, as long as the layer thickness is smaller than the penetration depth of the evanescent field. A much less frequently characterized property when using SPR is the imaginary part of the refractive index \((k)\), which is linked to the absorbance or scattering of light by the sample layer. Figure 3B shows the changes in simulated full SPR angular spectra caused by an increase in \(k\) from 0 to 0.05 in the sample layer. It is clear that the change in \(k\) induces an increase in the main SPR peak minimum intensity, which means that less of the light used to excite surface plasmons actually can do so. The reason for this is that the sample absorbs or scatters light, which consequently changes the conditions for exciting surface plasmons. In such a case the optical properties of the system start to deviate from the optimum conditions for surface plasmon excitation described by equation (1). This in turn leads to a situation where an increasing amount of light is reflected back instead of exciting the surface plasmons.

Figure 3C shows another exotic optical behavior of the simulated full SPR angular spectra. When the sample layer thickness reaches the corresponding thickness of the penetration depth of the SPR evanescent field, then the main SPR peak angular position is shifted to very high angles and a second peak starts to appear in the vicinity of the total internal reflection (TIR) angle. After this a further increase in the sample layer thickness does not induce any major changes in the main SPR peak angular position, but the second peak in the vicinity of the TIR angle will become more pronounced. This behavior of the full SPR angular spectra is due to the formation of a waveguide on the SPR sensor slide [34, 35]. Figure 3C also reveals that the waveguide peak grows stronger when the layer thickness approaches the wavelength of the light used in the simulation (i.e. 670 nm), or when the sample layer thickness is close to twice the penetration depth of the evanescent field.
Figure 3  Simulated full SPR spectra for optical changes within the evanescent field and thick sample layers. Behavior of simulated full SPR angular spectra when A) changing the real and B) changing the imaginary parts of the refractive index components, and C) for very thick (waveguide) sample layers. The following parameters were used for simulations: A) sample layer thickness: 10 nm, k = 0 and n varied from 1.45–1.5, B) sample layer thickness: 10 nm, n = 1.45 and k varied from 0.00–0.05, and C) n = 1.38, k = 0 and sample layer thickness varied from 400–700 nm.
When a cell monolayer is taken as the sample layer in SPR it is expected that the behavior of the full SPR angular spectra should differ dramatically compared to a sample layer with a thickness well below the penetration depth of the evanescent field. The cell monolayer is a very thick water rich layer with a thickness of a few to tens of micrometers, and with a refractive index very close to water. Thus, the thickness of the cell monolayer is much larger than the penetration depth of the evanescent field. Figure 4A shows a schematic representation for the sample layer used in simulating full SPR angular spectra of cell monolayers. In order to clarify the effect of changing different optical properties in a cell monolayer, the cell monolayer is theoretically split into three sections, i.e. a thin section in the magnitude of evanescent field close to the sensor surface ($n_{ef}$, 500 nm), a thick section consisting of the rest of the cell ($n_{cell}$, 3000 nm), and an infinite bulk medium layer ($n_{bulk}$, buffer).

When the full SPR angular spectra of a cell monolayer based on the layer structure in Figure 3A was simulated, it was found that a cell monolayer should form a weak waveguide on the SPR sensor slide (Fig. 4B–E). This is indicated by the small peaks and wavy curves in the TIR region (see Figure 3). An increase in the real part of the refractive index of a cell monolayer ($n_{ef}$) within the evanescent field (region III in Fig. 4A) resulted in an increase in the main SPR peak angular position (Fig. 4B), with negligible changes in the main SPR peak minimum intensity and TIR region (inset in Fig. 4B). Similarly, an increase in the complex part of the refractive index ($k_{ef}$) induces an increase in the main SPR peak minimum intensity and a decrease in the intensity around the TIR angle, but no changes in the main SPR peak angular position (Fig. 4C and 4D). It is worth noting that 10 times smaller refractive index changes are needed for the cell monolayer compared to a thin sample layer (see Figure 3) in order to induce the corresponding changes in the main SPR peak angular position or main SPR peak minimum intensity.

If the $n_{cell}$ for the cell monolayer outside the evanescent field (region II in Fig. 4A) was increased in the simulations, then no changes were seen in the main SPR peak angular position, but the TIR angle increased slightly (Fig. 4D). Correspondingly, when $k_{cell}$ was increased in the same region, then no changes were seen in the main SPR peak minimum intensity (Fig. 4E). The intensity changes in the TIR region also showed a rather complex behavior with several inflection points at which the intensity changes reversed direction. However, the intensity changes around the TIR angle (~62°) became negligible when $k_{cell}$ reached 0.002–0.003 (inset in Fig. 4E) when compared to the intensity changes induced by changes in $k_{ef}$ within the evanescent field (inset of Figure 4C). Additionally, no changes in the main SPR peak angular position or the SPR peak minimum intensity were found in cases where the complete cell monolayer thickness ($d_{ef} + d_{cell}$) was varied between 2000–6000 nm or when only $n_{bulk}$ of the bulk medium layer was varied between 1.330–1.355 (Figure S1 and S2). In these cases there were also only negligible changes in the TIR region when the cell layer thickness was larger than 3000 nm, which is still much smaller than the actual thickness of a cell.
IV: Flowing buffer region (bulk) 
\( n_{\text{bulk}} = n_{\text{cell}} - n_{\text{bulk}}, d_{\text{bulk}} = \infty \)

III: Upper region of cell monolayer 
\( n_{\text{cell}} = n_{\text{cell}} - n_{\text{cell}}, d_{\text{cell}} = 3000 \text{ nm} \)

II: Evanescent field / Lower region of cell monolayer 
\( n_{\text{evf}} = n_{\text{evf}} - n_{\text{cell}}, d_{\text{evf}} = 500 \text{ nm} \)

I: SPR sensor slide / Gold layer 
\( n_{\text{cell}} = n_{\text{evf}} - n_{\text{bulk}}, d_{\text{cell}} = 50 \text{ nm} \)

Figure 4  Simulated full SPR spectra for optical changes within different regions of a cell monolayer.

A) Schematic representation of the sample layer used in simulating full SPR angular spectra of cell monolayers. The cell monolayer was theoretically split into three sections in order to clarify the effect of changing different optical properties in it: 1) a thin section in the magnitude of evanescent field close to the sensor surface (\( n_{\text{evf}}, d_{\text{evf}} = 500 \text{ nm} \)), 2) a thick section consisting of the rest of the cell (\( n_{\text{cell}}, d_{\text{cell}} = 3000 \text{ nm} \)), and 3) an infinite bulk medium layer (\( n_{\text{bulk}}, d_{\text{bulk}} = \infty \)). Simulated full SPR angular spectra when changing; B) the real (\( n_{\text{cell}} \)) and C) the imaginary (\( k_{\text{cell}} \)) parts of the refractive index for a cell monolayer within the evanescent field, D) the real part of the refractive index (\( n_{\text{cell}} \)) for a cell monolayer not within the evanescent field and E) the imaginary part of the refractive index (\( k_{\text{cell}} \)) for a cell monolayer not within the evanescent field. Insets in B-E are more detailed views of the TIR regions. The following parameters were used for simulations: B) \( d_{\text{cell}} = 3000 \text{ nm}, k_{\text{cell}} = 0.002, n_{\text{cell}} \) varied from 1.340–1.345, \( d_{\text{evf}} = 500 \text{ nm}, n_{\text{evf}} = 1.34, k_{\text{evf}} = 0.002 \), C) \( d_{\text{cell}} = 3000 \text{ nm} \) with \( n_{\text{cell}} = 1.340, k_{\text{cell}} \) varied from 0–0.005, \( d_{\text{evf}} = 500 \text{ nm}, n_{\text{evf}} = 1.34 \) and \( k_{\text{evf}} = 0.002 \), D) \( d_{\text{cell}} = 3000 \text{ nm}, k_{\text{cell}} = 0.002, n_{\text{cell}} = 1.34, d_{\text{evf}} = 500 \text{ nm}, k_{\text{evf}} = 0.002 \) and \( n_{\text{evf}} \) varied from 1.340–1.345, and E) \( d_{\text{cell}} = 3000 \text{ nm}, k_{\text{cell}} = 0.002, n_{\text{cell}} = 1.34, d_{\text{evf}} = 500 \text{ nm}, n_{\text{evf}} = 1.34 \) and \( k_{\text{evf}} \) varied from 0–0.005.
8.3.3 DRUG-CELL INTERACTION ANALYSIS WITH SURFACE PLASMON RESONANCE

The successful immobilization of MDCKII cell monolayers on the SPR sensor slides was verified before each interaction measurement with drug compounds by first measuring the full SPR angular spectrum of the cell monolayer. Figure 5A shows a typical full SPR angular spectrum measured for a MDCKII cell monolayer. The full SPR angular spectrum for an MDCKII cell monolayer shows large shifts in the main SPR peak angular position, in the main SPR peak minimum intensity and in the shape of the TIR region compared to a pure SPR sensor slide. This confirmed the presence of a MDCKII cell monolayer on the SPR sensor slide. The main SPR peak angular position for the MDCKII cell monolayer was at 71.35° and the TIR region had a smooth shape located at ~64°. These were very close to the main SPR peak angular position of 71.85° and the TIR region location of ~62° for a simulated full SPR angular spectrum for a cell monolayer (see Fig. 4). This actually implicates that the MDCKII cell monolayer forms a low density waveguide on the SPR sensor slide. The differences in the angular position of the TIR region between measured and simulated full SPR angular spectra is a direct consequence of the fact that the simulated spectrum for a cell monolayer assumes a completely homogenous layer with a uniform thickness, whereas these assumptions are not necessarily valid for an actual living cell monolayer.

After verifying the quality of the MDCKII cell monolayer the MDCKII cells were stimulated with the test compounds and the actual full SPR angular spectra were measured as a function of time. Figure 5B shows a typical sensogram of the main SPR peak angular position for an experiment with confluent MDCKII cell monolayer when cells were exposed to increasing concentrations of propranolol and D-mannitol. The main SPR peak angular position for both compounds was displaced to lower angles during stimulation. However, when the cells were rinsed with pure running buffer the SPR angle started to increase and it took approximately 5 minutes for the baseline to stabilize. The main SPR peak angular position remained at higher values after stimulating the MDCKII cells with propranolol, whereas it returned to the baseline level after stimulation with D-mannitol. This suggests that a certain fraction of the propranolol remains in the cell monolayer after each stimulation, and that D-mannitol is almost completely removed from the cell monolayer after stimulation, regardless of the concentration used. The changes in the main SPR peak angular position were clearly concentration dependent for propranolol, but not for D-mannitol, for which the changes in the main SPR peak angular position were almost constant for all tested concentrations. These results indicate that propranolol and D-mannitol have different modes of interaction with the MDCKII cells which is reflected in the changes in the main SPR peak angular position. No significant changes in the main SPR peak angular position were measured when propranolol or D-mannitol was allowed to interact with a pure gold SPR sensor slide. This verifies that the main SPR peak angular position changes measured during interaction of the test compounds with the MDCKII cells actually reflect real drug-cell interactions and not drug-gold interactions.
Figure 5  SPR signal responses during drug stimulation of MDCKII cells. A) Measured full SPR angular spectra of a pure gold coated SPR sensor slide (grey line) and MDCKII cell monolayer immobilized on the SPR sensor slide (black line). B) Measured changes in the angular position of the SPR peak minimum as a function of time when a MDCKII cell monolayer was stimulated with propranolol (blue line) and D-mannitol (red line). C) Focused part of full SPR angular spectra showing the main SPR peak curves measured before (black line), during (red line) and after (blue line) stimulating a MDCKII cell monolayer with 25 µM propranolol. D) Measured changes in the SPR peak minimum intensity as a function of time when a MDCKII cell monolayer was stimulated with propranolol (blue line) and D-mannitol (red line). In figure C) and D) the downward arrows represent the time of sample injections, and upwards arrows represent the injection of buffer without sample.

The changes in the main SPR peak angular position when stimulating the MDCKII monolayer with propranolol and D-mannitol are surprisingly large, especially when considering the molecular weight of these compounds (i.e. 259.34 g/mol for propranolol and 182.17 g/mol for D-mannitol). This is in accordance with the simulated full SPR angular spectra for cell monolayers in Figure 4, where it was shown that very small changes in n or k in the cell monolayer induced rather large variations in the main SPR peak angular position and the main SPR peak minimum intensity.

Interestingly, when MDCKII cells were stimulated with propranolol it was found that not only the main SPR peak angular position changed during stimulation, but also the main SPR peak minimum intensity changed significantly (Fig. 5C–D). In the case of D-mannitol only slight or no changes were observed in the main SPR peak
minimum intensity during the MDCKII cell stimulation (Fig. 5D), even though the main SPR peak angular position during cell stimulation with D-mannitol still showed a negative shift (Fig. 5B). These results further indicate that propranolol and D-mannitol have different modes of interaction with the MDCKII cells, which is reflected not only in the main SPR peak angular position but also in the main SPR peak minimum intensity. Thus, instead of analyzing only the main SPR peak angular position changes during drug-cell interactions it would be more useful to analyze both the main SPR peak angular position and the main SPR peak minimum intensity and plot these against each other in order to better understand and distinguish between the mode of interaction between different drugs during cell stimulation.

Figure 6 shows a series of plots of changes in the main SPR peak angular position versus main SPR peak minimum intensity from at least three repetitions and for several concentrations when MDCKII cells were stimulated with propranolol and D-mannitol. The difference between the behavior of the two compounds in terms of angle and intensity is very clear. Propranolol shows large changes in both angle and intensity which result in curves with significant slopes. D-mannitol on the other hand shows in most cases very small changes in intensity, leading to curves with a more horizontal appearance in these plots. The same trend in the intensity versus angle plots is repeated in the same way for each compound at all the concentrations tested.

The simulated full SPR angular spectra for a cell monolayer suggest that the TIR region should also reflect changes in the cell monolayer during drug stimulation (see Fig. 4C–E). When examining the TIR region in more detail during drug stimulation of MDCKII cells we found that the TIR angular position followed the same trend as the main SPR peak angular position when stimulating the MDCKII cells with propranolol or D-mannitol, i.e. larger changes for propranolol compared to D-mannitol (Figures S3 and Figure S4A). On the contrary, the reflection intensity of the TIR angular position (~64°) showed a reverse trend compared to the main SPR peak minimum intensity, i.e. significant positive changes for propranolol and very small positive changes for D-mannitol (Figures S3 and Figure S4). These results are in sound accordance with the behavior of a cell monolayer described by the simulated full SPR angular spectra in Figure 4, where it was shown that the TIR angle and the main SPR peak angular position changes in the same direction with changes in $n$, and the reflection intensity in the TIR region changes in the opposite direction than the main SPR peak minimum with changes in $k$. Finally, when the TIR angular position was plotted against the reflection intensity of the TIR angular position, we found that the curves displayed similar slopes for both propranolol and D-mannitol, but the magnitude of the curve was clearly larger for propranolol than for D-mannitol (Figure S4C).

Figure 6  Change in SPR peak angular position versus minimum intensity during drug stimulation of MDCKII cells.
Individual repetitions of the SPR measurements in a concentration series from three repetitions when MDCKII cell monolayers were stimulated with propranolol and D-mannitol, respectively: 2.5 nM (A: propranolol, F: D-mannitol), 250 nM (B: propranolol, G: D-mannitol), 2.5 μM (C: propranolol, H: D-mannitol), 25 μM (D: propranolol, I: D-mannitol) and 250 μM (E: propranolol, J: D-mannitol).
8.4 DISCUSSION

In this study, there were several reasons for using MDCKII cells and the two passively absorbing drugs utilizing different absorption routes. We anticipated at the beginning of our studies that the SPR signal responses with cells would be very complex if all kinds of drug transport processes were present. Therefore, the aim was to simplify the experimental design as much as possible by choosing a combination of cells and drugs which are already widely used specifically for passive (para- and transcellular) drug permeation studies. The choice of MDCKII cells was mainly based on the fact that they have low expression of drug transporters and little metabolic activity, which makes it a valuable cell line, especially for studying passive drug transport processes [36, 37]. D-mannitol and propranolol were chosen as model drugs because they are known to utilize different passive drug absorption routes, i.e. propranolol uses the transcellular and D-mannitol uses the paracellular absorption route [38, 39]. With this we could ensure that no other competing drug absorption processes, i.e. active transport and efflux of drugs, were present during our studies. In this way we could concentrate on studying and distinguishing the differences in the SPR signal responses which were solely due to passive drug absorption processes, without the interference from multiple competing processes.

Several studies have shown that the surface coverage of cells on the gold SPR sensor slide has a dramatic influence of the shape of the full SPR angle curve [12, 16, 24]. Therefore, in order to obtain repeatable SPR analyses and avoid unspecific interactions of the test compounds, not originating from cell interactions; it is of utmost importance to find a cell immobilization protocol that enables a repeatable preparation of confluent monolayers on the SPR sensor slide. In this study we found that MDCKII cells adhered and proliferated on gold and formed a confluent cell monolayer on the bare gold SPR sensor slide (Figure 2). We also found that no cell adhesion promoter was needed to form confluent monolayers of MDCKII cells on gold. Furthermore, Trypan blue tests showed that the gold SPR sensor slide surface is not cytotoxic for MDCKII cells (Figure 2F).

After reaching confluency, the MDCKII cells started to form clusters on the smooth gold SPR sensor slide surface. By examining the microscopy images it seemed that liquid had gathered under the cells at the clustering points. Cells are generally attached to surfaces in focal, close, and extracellular matrix contacts, each with its own characteristic separation distance from the surface [40]. As a result, cell plasma membranes are normally 10–100 nm away from the substrate surface depending on cell types and culturing conditions. However, due to the cluster formation in the case of MDCKII cells in this study the plasma membranes could have been even further away in those zones, which probably have a small effect on the quality of the measured full SPR angle spectrum of the MDCKII cell layer. This could explain why the small waveguide node appearing in the simulated full SPR angle spectra for a cell monolayer (Figure 4) is not properly visible in the measured full SPR angle spectrum of the MDCKII cells (Figure 5A).

Other cells than MDCKII have previously also been cultured on bare gold for SPR cell studies. These studies include cell lines such as human melanoma [22], human basophilic KU812 [41], RBL-2H3 rat mast and PAM212 mouse keratinocyte cells [19].
Moreover, Robelek and Weger[13] have grown MDCKII cells directly on SPR sensor surfaces as a confluent monolayer to study volume changes of cells. Based on the literature and the results in this study it seems that there is no general coating procedure or protocol for cell adhesion and preparation of confluent cell monolayers on gold coated SPR sensor slides, and therefore it is necessary to optimize these conditions for each cell line separately before performing SPR interaction measurements.

According to the full SPR angular spectra simulations a cell monolayer should form a low density waveguide with the main fundamental SPR peak minimum visible at relatively small angles compared to the thickness of the system (Figure 4). Additionally, the next node(s) remain close to the total internal reflection region, never progressing far from there. The actual full SPR angular spectrum measured for a confluent MDCKII cell layer (Figure 5A) resembled the simulated spectrum for a cell monolayer surprisingly well (Figure 4). This suggests that a monitoring of the shape of the full SPR angular spectrum thus provides a means to verify cell monolayer integrity during SPR interaction measurements. The full SPR angular spectra simulations also revealed that the sensitivity towards optical changes in a cell monolayer is enhanced by the fact that the cell covers the complete SPR evanescent field region, which enables the detection of extremely small changes in the living cell monolayer during cell stimulation. This has already been experimentally shown and indicated by other studies [15, 19, 20], but no studies have so far attempted to give an explanation of the implications of this on the full SPR angular spectra and how this could possibly be further utilized for living cell sensing with SPR.

The literature of SPR interaction measurements involving immobilized cells on the sensor surface are quite contradictory – in some cases the injection of the analyte results in positive SPR responses [15, 19, 20, 24, 26] and in other cases in negative SPR responses[18–21]. A widely accepted simplification for the measured SPR signal is that the main SPR peak angular position (or the intensity change at a fixed angle) is linearly proportional to the mass change in the evanescent field. This is also evident from the basic physics of the SPR phenomena [27] and the simulations in Figures 3A and 4B. Based on this it has been suggested that the SPR responses with cells based on measuring the changes in the main SPR peak angular position (or the intensity change at a fixed angle) originates from mass redistribution within the cells [19]. While possible and even probable, such a mass redistribution in cells should lead into changes both in the refractive index, and in the apparent light absorption of cell layers as different cell organelles and structures shift within the cell. This mass distribution could indeed induce either negative or positive changes in the SPR responses measured by monitoring only the main SPR peak angular position (or the intensity change at a fixed angle), depending on if the cytoskeletal mass migration in the cells is in the direction away from or towards the SPR evanescent field region during cell stimulation. This is also actually implicated by the simulations in Figure 3 and 4, and supported by the studies by Cuerrier et al., Chabot et al. [18, 21] and Yashunsky et al. [12]. Cuerrier et al. and Chabot et al. showed that morphological changes in cells, i.e. contraction of cells, induce a negative SPR shift in the reflection intensity measured at a fixed angle, while Yashunsky et al. showed that
cell spreading induces an increase in the reflection intensity measured at a fixed angle with mid infra-red SPR.

However, none of the studies mentioned above have considered the changes in the main SPR peak angular position together with changes in the main SPR peak minimum intensity. It is clear from the simulated spectra in this study that changes both in the main SPR peak angular position and in the main SPR peak minimum intensity, and preferably also changes in the TIR region should be simultaneously monitored in order to fully understand the origin of the SPR responses caused by changes in the whole cell monolayer during drug stimulation of cells. This is because an increase/decrease in $n$ will increase/decrease the main SPR peak angular position (Figure 4B) about twice as much as the TIR angle position (Figure 4D). Furthermore, an increase/decrease in $k$ will be reflected in an increase/decrease in the main SPR peak minimum intensity and mainly a decrease/increase in the intensity around the TIR (Figure 4C and Figure 4E).

Often small molecular drugs or other compounds used for stimulating cells do not have significant absorptive properties in the wavelengths used in SPR devices and should generally cause a positive SPR response when measuring only the main SPR peak angular position (or the intensity change at a fixed angle). Thus, the simulations in Figure 4 suggest that a utilization of multiple parameters extracted from the full SPR angular spectra measured in real-time during cell stimulation would provide a means to determine the origin of the SPR response. In other words, this could provide a way to distinguish whether the SPR response 1) originates only from the accumulation of the stimulant in the cell layer, 2) is due to morphological changes of the cells or cytoskeleton mass redistribution within the cells, or 3) is a combined effect of 1) and 2). The plots of the main SPR peak minimum intensity versus the main SPR peak angular position in Figure 6 and the changes in the TIR region (Figures S3 and Figure S4) actually highlight that there is a clear difference in the interaction modes of propranolol and D-mannitol with the MDCKII cells. It is known that propranolol utilizes the transcellular pathway and D-mannitol utilizes the paracellular pathway when absorbed through a cell monolayer [38, 39]. The graphical representation in Figure 6 in combination with the changes in the TIR region (Figure S4C) thus provides the means to differentiate between the modes of action of drugs with cells.

When examining Figure 5B, Figure 5D and Figure 6A–E in more detail it is obvious that there is a clear negative shift in both the SPR peak angular position and the SPR peak minimum intensity during cell stimulation with propranolol when examining each concentration separately. After stimulation, both the main SPR peak angular position and the main SPR peak minimum intensity return to a higher level than before stimulation. This indicates that some propranolol accumulates in the cells as expected because of its ability to diffuse transcellularly into cells. It is worthwhile to note from Figure 5B, Figure 5D and Figure 6E that the change in both the main SPR peak angular position and the main SPR peak minimum intensity quickly becomes positive during stimulation of the MDCKII cell with the highest concentration for propranolol (i.e. 250 μM). These results for propranolol indicate that a stimulation of the cells with a low concentration of propranolol first induces a cell contraction accompanied by a mass redistribution away from the evanescent field.
region leading to a negative shift in both of the SPR responses. Hence, the SPR responses for low concentrations of propranolol probably originate from morphological and mass distribution changes of the cells, rather than from propranolol absorbed into the cell, since the amount of absorbed propranolol is not sufficient to render the SPR signals positive. A stimulation of the MDCKII cells with the highest concentration of propranolol causes an initial negative shift in both SPR responses. This suggests that a stimulation with propranolol first induces cell contraction accompanied by a mass redistribution away from the evanescent field region. Hereafter, both SPR responses become positive, which is probably a consequence of a cell spreading accompanied by a mass redistribution towards the evanescent field region and an accumulation of propranolol in the cells. Similar behavior has been shown for HEK-293 cells stimulated with angiotensin II by correlating the change in the SPR intensity at a fixed angle with phase-contrast microscopy imaging [18].

In the case of D-mannitol (Figure 5B, Figure 5D and Figure 6F–J), a clear negative shift is only seen in the angular position of the SPR peak minimum during cell stimulation, while the SPR peak minimum intensity only changes very little or not at all. After cell stimulation, the main SPR peak angular position returns to the same level as before stimulation. This could be expected because D-mannitol diffuses through cell layers by the paracellular pathway, and no accumulation of D-mannitol is expected in the cell monolayer or within the cells. However, it was surprising to see that the SPR peak angular position during cell stimulation with D-mannitol showed a negative shift even though there was no change in the main SPR peak minimum intensity. This could indicate that D-mannitol indeed induces a cell contraction in order to utilize the paracellular pathway, but the contraction is not sufficiently large to induce any cell mass redistribution within the cell. This perception is also supported by the fact that the changes in the main SPR peak angular position are basically constant for all concentrations and much smaller in the case of D-mannitol compared to propranolol (Figure 5B and Figure 6). Furthermore, the smaller changes in the TIR region (Figure S3 and Figure S4) indicate a smaller mass redistribution within the cells in the case of D-mannitol compared to propranolol. This is also supported by the simulations in Figure 4C–E, which suggest that the contribution to the TIR region origins mainly from the cell monolayer outside the evanescent field and does not have any significant contributions from morphological changes of the cells. This could mean that the TIR region merely reflects the mass redistribution within the cell monolayer.

The results from our study presented here convinced us that the change in the main SPR peak angular position reflects both drug accumulation and morphological changes in the cell monolayer, and that the change in the main SPR peak minimum intensity is mainly due to mass redistribution within the cells. Thus, based on the results in this work, we suggest the following two possible scenarios for the SPR responses in living cell sensing: 1) An accumulation of a drug, e.g. D-mannitol, in the cell layer which induces only a slight contraction of the cells but no mass redistribution in the cells, would result in a negative change in the SPR peak angular position, but it would not result in a change in the SPR peak minimum intensity. 2) An accumulation of a drug, e.g. propranolol, in the cell layer which induces a
contraction of the cell layer and a mass redistribution in the cells, would result in a negative change in both the SPR peak angular position and the SPR peak minimum intensity. If taking into account all other possible options, there could be two additional scenarios for the SPR responses in living cell sensing which could not be addressed by the experimental design in this work. Namely, 1) a simple accumulation of a drug in the cell layer which does not induce any morphological changes in the cell layer, would result in a positive change in the SPR peak angular position, but no change in the SPR peak minimum intensity, and 2) an accumulation of a drug in the cell layer which induces a spreading of the cell layer, and a mass redistribution in the cells would result in a positive change in both the SPR peak angular position and the SPR peak minimum intensity.

Conclusively, it is rather obvious that the origins of the SPR responses are very complex when combining SPR with living cells. In this work we have presented an attempt to better understand the SPR responses in living cell sensing by comparing optical modeling of cell monolayers with multiple parameters extracted from full angular SPR spectra recorded in real-time during cell stimulation with two model drugs. Simulated full SPR angular spectra in combination with changes in the main SPR peak angular position, the main SPR peak minimum intensity, and changes in the TIR region indicated that the change in the main SPR peak angular position, traditionally used in SPR studies, does probably not only reflect simple mass accumulation or mass redistribution within the cells. Instead, it probably also reflects morphological changes in the cell layer, which actually could dominate the main SPR peak angular position response. The change in the main SPR peak minimum intensity, on the other hand, is suggested to reflect which type of change in the cell layer (morphological and/or mass redistribution) causes the changes in the main SPR peak angular position.

Finally, plotting the main SPR peak angular position versus the main SPR peak minimum intensity showed specific signal patterns. These signal patterns are suggested to reflect the type of drug absorption route utilized by the drug. We believe that the results in this study provide a step forward to an improved understanding of the signal responses in living cell sensing with SPR. This can open up new opportunities for utilizing the SPR technology in a broader context in the field of life sciences, for example as a tool for providing real-time complementary information for traditional in vitro cell assays in order to obtain a better mechanistic understanding of drug-cell interactions on a cellular level.
8.5 SUPPORTING INFORMATION

Figure S1. Simulated full SPR angular spectra demonstrating that very large changes in the complete cell monolayer thickness \((d_{ef} + d_{cell})\) do not affect the main SPR peak position. It is worth noting that the complete cell monolayer thickness has to change dramatically before it induces any significant changes in the shape of the TIR region. The following parameters were used for simulations: \(n_{ef} = n_{cell} = 1.34\), \(k_{ef} = k_{cell} = 0.002\) and \(n_{bulk} = 1.3299\), \(k_{bulk} = 0\).

Figure S2. Simulated full SPR angular spectra demonstrating that changes in \(n_{bulk}\) of the bulk medium layer above the cell monolayer do not affect the main SPR peak and induce only very small changes in the shape of the TIR region. The following parameters were used for simulations: \(n_{ef} = n_{cell} = 1.34\), \(k_{ef} = k_{cell} = 0.002\) and \(k_{bulk} = 0\).
Figure S3. Measured full SPR angular spectra at selected time points after starting the stimulation of a MDCKII cell monolayer with A) 25 μM D-mannitol and B) 25 μM propranolol. Th time points for recording the full SPR angular spectra in A) were t = 0 min (black solid line), t = 2 min (red solid line), t = 4 min (blue solid line), t = 13 min (black dashed line), and in B) t = 0 min (black solid line), t = 2 min (red solid line), t = 5 min (blue solid line), t = 17 min (black dashed line).

**Figure S4.** A) Change in the TIR angle position measured as a function of time during stimulation of a MDCKII cell monolayer with 25 μM Propranolol (blue line) or D-mannitol (red line). These results suggest that there is a much higher mass redistribution away from the cell monolayer region within the evanescent field (Fig. 4A, region III) for propranolol than for D-mannitol. B) Change in the intensity at TIR angle position measured as a function of time during a stimulation of a MDCKII cell monolayer with 25 μM Propranolol (blue line) or D-mannitol (red line). These results indicate that there is a much higher analyte accumulation and mass redistribution towards the cell monolayer region outside the evanescent field (Fig. 4A, region II) for propranolol than for D-mannitol. C) Change in the intensity at TIR angle position versus change in TIR angle position for 25 μM Propranolol (blue line) or D-mannitol (red line) during stimulation of a MDCKII cell monolayer. Note that the slopes of these curves are the same, while the magnitude is clearly different indicating that an overall larger mass redistribution within the cell monolayer takes place during stimulation with propranolol than with D-mannitol. The same slope of these curves strongly suggests that the TIR region of the full SPR angular spectrum actually merely reflects accumulation of analytes and mass redistribution within the cell monolayer, but does probably not have any contribution from the adhesion and contact area of the cells.
8.6 AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: TV NG MR MY. Performed the experiments: SH NG TV. Analyzed the data: TV NG. Contributed reagents/materials/analysis tools: TV NG MY. Wrote the paper: TV NG.

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8.8 REFERENCES


The label-free biosensor technologies have during the last decades established themselves as relatively common tools for biochemical interaction studies. While the label-free methods become more standardized, there still remain partially unexplored areas that could allow the methods to be utilized even better. The purpose of this thesis was to address several challenges of label-free methods that have not been widely studied, and to add new tools into the toolbox of biological and pharmaceutical scientists.

While detection of simple biochemical interactions has been well established, especially for SPR-based biochemical assays, there are still several challenges in the experimental design and most notably in the immobilization chemistry for functionalizing the sensor surfaces. Publication I in this thesis presents a solution to the issue where the general protein immobilization protocols to CMD hydrogels did not allow to determining the affinity between protein kinase C ε and single-chain antibodies due to a steric hindrance or a denaturation of the immobilized protein. Thus, an uncommon alternative protein binding solution, where the dextran hydrogel was functionalized with an amine instead of carboxylic acid, was utilized in order to avoid the steric not to modify the immobilized protein structure which consequently allowed to determine the affinity.

Another challenge in various interaction measurements where label-free detection is used is the assay sensitivity, as well as the fact that label-free detection methods are sensitive to changes in the surrounding environment. Publication II in this thesis presents a new hybrid-labelled method which can be used in tandem with label-free SPR detection, and which allows for significant improvements in the sensitivity and more importantly in the specificity of the detection. This enables simpler sensor structures for biosensing applications with SPR, and the use of difficult samples with non-optimal binding or optical properties.

Biomaterials, like most other material science and engineering fields, are constantly moving towards the utilization of nanoscale layers. The use of the SPR phenomenon as an analysis tool for ultrathin film has largely been overlooked. Publication III in this thesis establishes the basis for using the SPR technique as an analysis tool for nanoscale layers through theoretical considerations and empirically proving the effectiveness with several different nanotechnological layered systems.

SLBs have been established as synthetic biomimetic models for biological membranes, but the current assay methodologies lack the control and robustness of the lipid membrane. In publication IV a new surface chemistry is presented that is capable of promoting formation of SLBs of complex lipid compositions which offers a platform for preparing more robust SLB assays with partial air-stability.

The biomimetic detection assays are approached from another direction in publication V, i.e. established pharmaceutical cell-based assays are adapted to a flow-through label-free detection technology for drug-cell interactions studies. While combining cell assays and a label-free technology is not a completely new idea, there have been a lot of uncertainties and unexplained differences in interpreting the signal.
responses measured during cell stimulation. Publication V adds knowledge for better understanding the optical SPR signal behaviour when cells are stimulated with drugs, thus explaining a lot of the interpretation differences in the literature. The new analysis approach of the SPR signal also provided the mean for differentiating between the passive permeation routes of model drug compounds.

The use of surface-sensitive label-free detection technologies in combination with various biomimetic sensing surfaces clearly indicated that these platforms are promising approaches as new type of in vitro drug development tools that could provide additional information to traditional assays for improving our mechanistic understanding when developing new drugs. These platforms may also enable a more ethical, efficient and cheaper drug development process in the future, consequently resulting in more effective and safer drugs. The new knowledge obtained in this thesis for a better understanding of the behaviour of the optical evanescent field detection system with different biomimetic surfaces, the analysis approaches developed for thin film characterization and the new surface chemistry protocols for creating biomimetic assays was not aimed for any specific application. However, the new knowledge will hopefully promote further development and utilization of non-labelled detection techniques as new and more efficient research tools for biotechnological and pharmaceutical scientists.

The technologies and analysis approaches presented in the literature survey and the individual research publications in this dissertation are not yet ready for commercialization. While the basic physics and chemistry are founded for a given methodology, there is still a lot of work to be done for optimizing, integrating and validating these technologies into widely accepted methodologies. Despite of this, this dissertation acts as a valuable addition to the existing knowledge, and provides the basic knowledge and hints for further development of surface-sensitive label-free and real-time research methods for life sciences. This could in general benefit the human society through a better understanding of our own physiology, and through improved medical care.