Imaging surface plasmon resonance

Olof Andersson
During the course of the research underlying this thesis, Olof Andersson was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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Imaging surface plasmon resonance
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To my family
The central theme of this thesis is the use of imaging Surface Plasmon Resonance (iSPR) as a tool in the characterization of surfaces with laterally varying properties. Within the scope of this work, an instrument for iSPR analysis was designed and built. SPR is a very sensitive technique for monitoring changes in optical properties in the immediate vicinity of a sensor surface, which is very useful in biosensing and surface science research. We have employed SPR in the Kretschmann configuration, wherein surface plasmons are excited by means of an evanescent field arising from total internal reflection from the backside of the sensor surface. In iSPR, the signal is the reflectivity of TM-polarized light which is measured using an imaging detector, typically a CCD camera. Advantages of this technique include extreme surface sensitivity and, because detection is done from the backside, compatibility with complex samples. In addition, SPR is a non-labeling technique, and in imaging mode, a lateral resolution in the µm range can be attained.

The imaging SPR instrument could be operated in either wavelength interrogation mode or in intensity mode. In the former case, the objective is to find the SPR wavelength, λ_{SPR}, which is the wavelength at which the reflected intensity is at a minimum. In intensity mode, a snapshot of the intensity reflectance is taken at a fixed wavelength and incidence angle.

In biosensor science, the use of an imaging technique offers a major advantage by enabling parallelization and thereby increasing throughput. We have, for example, used iSPR in biochemical interaction analysis to monitor immobilization and specific binding to protein and synthetic polypeptide micro arrays. The primary interest has been the study of soft matter surfaces that possess properties interesting in the field of biomimetics or for applications in biosensing. Specifically, the surfaces studied in this thesis include patterned self-assembled monolayers of thioclates on gold, a graft polymerized poly(ethylene glycol) (PEG) based hydrogel, a dextran hydrogel, and a polyelectrolyte charge gradient. Our results show that the PEG-based hydrogel is very well suited for use as a platform in protein immobilization in an array format, owing to the very low unspecific binding. In addition, well defined microarray templates were designed by patterning of hydrophobic barriers on dextran and monolayer surfaces. A polypeptide affinity microarray was further designed and immobilized on such a patterned monolayer substrate, in order to demonstrate the potential of analyte quantification with high sensitivity over a large dynamic range.
Furthermore, iSPR was combined with electrochemistry to enable laterally resolved studies of electrochemical surface reactions. Using this combination, the electrochemical properties of surfaces patterned with self assembled monolayers can be studied in parallel, with a spatial resolution in the µm regime. We have also employed electrochemistry and iSPR for the investigation of potential and current density gradients on bipolar electrodes.
Popularvetskaplig sammanfattning

Det genomgående temat i denna avhandling är användandet av en optisk metod, avbildande ytplasmonresonans (iSPR), som ett verktyg för att karakterisera mönstrade molekylära skikt. SPR är en extremt känslig teknik som i huvudsak mäter förändringar i de optiska egenskaperna, såsom brytningsindex, inom ett avstånd mindre än 100-200 miljondels millimeter (nm) från en yta. Själva experimenten går ut på att mäta förändringen hos intensiteten av ljus som reflekteras mot baksidan av en tunn metallfilm, i allmänhet guld. Molekyler som binder till den tunna guldfilmen ger upphov till en förändring i ljusintensiteten vilket sedan kan relateras till koncentrationen av molekylerna. Denna teknik används flitigt inom forskningen för studier av interaktioner mellan biomolekyler eller för ytcharakterisering. Vi har utvecklat ett instrument för att mäta avbildande SPR, vilket i kombination med tekniker för mönstring ger möjlighet att samtidigt utföra ett stort antal analyser, något som är av intresse bland annat inom läkemedelsforskning eller diagnostik, där ökad analyskapacitet är starkt efterfrågat.

Med hjälp av en metod baserad på gummistämplar med mönster i storleksorden tusendels millimeter (µm) har vi skapat väldefinierade mikroskopiska rutmönster på tunna (∼50 nm) guldtytor, till vilka proteiner och korta polypeptider (synetiskt framställda kedjor av cirka 10-20 aminosyror) sedan har länkats fast. Med hjälp av vårt avbildande SPR-instrument har därefter interaktionerna mellan dessa immobiliserade molekyler och andra målmolekyler kunnat studeras.

Även inom andra områden kan avbildande ytplasmonresonans vara intressant, vi har bland annat studerat graderingar i elektrisk potential och ström i syfte att utveckla ett nytt verktyg för mönstring och karakterisering av molekylära skikt.

Ett antal olika typer av kemiska ytmodifikationer har används, bl.a. så kallade självorganiserande monoskikt som utgörs av ett enkelt molekylbäger med en tjocklek av ∼2-5 nm. Med hjälp av SPR har även de proteinavstötande/attraherande egenskaperna hos polymera skikt med en tjocklek i storleksordningen 5-100 nm studerats. Dessa typer av ytor är intressanta då de kan användas som modellsystem för att studera adsorption till olika ytskikt eller som plattformar för immobilisering av proteiner i matrisformat, ”protein micro-arrayer”, vilka kan bestå av tusentals unika proteinfläckar. Då proteiner har mycket viktiga uppgifter som funktionsreglerare i kroppen förväntras många arrayer få stor betydelse inom forskning som syftar till att kartlägga och förstå biomekylära mekanismer. Tillgängligheten till ytkemi som tillåter stabil immobilisering av proteiner med bibehållna biologisk funktion är då av yttersta vikt.
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_Olof Andersson, Linköping 2008_
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This thesis is based on the following papers, referred to in the text by their Roman numerals (I-VI). The papers VII-XI are related to this work but not included in the thesis.

**Paper I**

**Protein microarrays on carboxymethylated dextran hydrogels: immobilization, characterization and application**


**Author’s contribution**

Participated in planning and evaluation. Performed the imaging SPR and half of the fluorescence experiments.

**Paper II**

**Imaging SPR for detection of local electrochemical processes on patterned surfaces**


**Author’s contribution**

Responsible for planning, performing, and evaluating the experimental work. I wrote the manuscript.

**Paper III**

**Formation of molecular gradients on bipolar electrodes**


**Author’s contribution**

Shared equally with C.U. in planning, performing, and evaluating the experimental work. Responsible for the imaging SPR experiments and the thiol chemistry. Contributed to the writing.
Paper IV

A gradient hydrogel matrix for microarray and biosensor applications: an imaging SPR study

Author’s contribution
Responsible for planning, performing, and evaluating the experimental work. A.L. and T.E. participated in planning and evaluation and prepared some of the samples. I wrote the manuscript.

Paper V

Lateral control of protein adsorption on charged polymer gradients

Author’s contribution
Contributed significantly to the planning and evaluation of the experimental work. Responsible for the imaging SPR experiments. Contributed to the writing of the manuscript.

Paper VI

A multiple-ligand approach to extending the dynamic range of analyte quantification in protein microarrays

Author’s contribution
Responsible for planning, performing, and evaluating most of the experimental work. Co-supervised H.N. during his undergraduate diploma work, during which he synthesized the polypeptides and determined the affinities. I did a major part of the writing.
Additional publications, not included in thesis

Paper VII

Reversible Hydrophobic Barriers Introduced by Microcontact Printing: Application to Protein Microarrays

Paper VIII

Microarray production on polymeric hydrogels using microcontact printing

Paper IX

Photographed poly(ethylene glycol) matrix for affinity interaction studies

Paper X

Evaluation of the potential and current density distributions at electrodes for bipolar patterning

Paper XI

DNA chips with conjugated polyelectrolytes in resonance energy transfer mode
In January 2005, at the time of the American presidential inauguration campaign, researchers at the United States Naval Research Laboratory (NRL) in Washington DC where at hard work, collecting, preparing and analyzing samples from patients admitted at nearby military hospitals, exhibiting symptoms of flu. They were working on the “Silent Guardian” project, which purpose was the demonstration of the potential for around the clock large scale bio-surveillance, based on state of the art Deoxyribonucleic acid (DNA) microarray technology. The Silent Guardian demonstration succeeded in this sense, and dozens of natural pathogens were identified within 24 hours. Luckily, none of the biological agents commonly associated with bioterrorism was found in the clinical samples, but control experiments showed that they would have, were they present. What made the work of the scientists at NRL possible was the fast-paced progress within the recent decades in the field of biological microarray technology.

In the quest of understanding life, the role of the fundamental building blocks of DNA and proteins are of great significance. While DNA molecules provide the basic instructions, it is the proteins that are the laborers of the cells at the
molecular level. Studying the DNA may give great insights, but it is not until one
appreciates the role of the proteins that a more complete picture can be drawn.
Proteins, however, are intrinsically more difficult to work with than DNA. This is
because while DNA polymers are rather stable and fairly homogeneous in charge
and structure, proteins can possess a wide variety of properties relating to their
backbone composition. Additionally, a vital part of the function of proteins lies
in their secondary and tertiary structures, which are sensitive to harsh treatment.
This is one of the reasons why DNA microarrays have been around for a longer
period of time than protein arrays.

Recent large scale efforts within the research community, such as the Human
Genome Project (HGP), or the Swedish Human Protein Atlas (HPA) project, has been
grounded towards deciphering the code of life. The huge amount of information
made available through these and other projects alike can be used to gain a
deeper understanding on the fundamentals of life’s processes at the molecular
level. However, interpretation and implementation of this newly gained knowledge into actual applications or devices puts forward new requirements on the
techniques available. In pharmaceutical research, for example, in the screening
for disease biomarkers, or in the selection process of new drug candidates, there
is a great demand for high throughput instrumentation capable of accurate analysis and parallelization. One such technique, that could be used as a transducer element in screening studies of the kind performed at NRL, is imaging Surface Plasmon Resonance (iSPR).

The recurring theme of the papers included in this thesis is the use of iSPR as a
tool in the study of biomolecular interactions, and of surfaces possessing properties
that are of interest in biomolecular analysis. In Papers I, IV, V and VI, iSPR is used to study molecular binding and interactions with surfaces. Papers II and
III focus on the use of iSPR in combination with electrochemical techniques. The
iSPR instrument used throughout the papers (with the exception of Paper I) was
developed and built within the scope of this work.

The remainder of this chapter will be devoted to giving a general introduction
and motivation, while the goal of the subsequent chapters is to provide a theoretical
foundation for the included papers. It should be noted, although much of the
discussion is focused on biological interaction analysis, that iSPR is an equally
applicable technique in many other fields of chemical sensing.

1.1 Imaging methods in biosensing

Biosensors, which are a type of chemical sensors, have two main components. First,
there is a recognition part, wherein a chemical reaction or binding takes place which
ultimately gives rise to a signal. The second part is a transducer element at which
the sensor signal is transformed into an amount or equivalent measure.
1.1 Imaging methods in biosensing

array biosensor (Figure 1.1), the recognition part can be subdivided into smaller groups each of which provides a separate signal. In this case, the transducer must be capable of multiplexing, that is to provide means of separating the individual signals from one another. In the type of iSPR instrument employed in this thesis, this is accomplished by using an imaging detector. The advantage of an imaging approach, is that it allows for increased throughput by enabling more and many different analyses to be performed in parallel.

Many other types of imaging transducer technologies suitable for use in biosensors exist. These are for example based on optical, acoustical/mechanical or electronic read-out. For instance, Quartz Crystal Microbalance (QCM), which is based on measuring very small changes in mass, is a technique suitable for miniaturization and large numbers of quartz crystals could be arranged in an array format to facilitate parallelization. Ellipsometry, for example in Total Internal Reflection (TIR) mode, is an optical technique closely related to surface plasmon resonance. Ellipsometry under SPR conditions offers a very high sensitivity at the expense of a somewhat more intricate instrumental setup. The technique is also not limited to thin metal film substrates. Techniques based on labeling, such as fluorescence microscopy are traditionally strong within biosciences, much thanks to the intrinsic fluorescence of proteins, the large number of commercially available fluorescent probes and the ease of labeling of e.g. DNA. Much more information than mere quantification can be extracted from fluorescence measurements, for instance, fluorescence decay times can give information on the structure of biomacromolecules. The advantage of iSPR over the aforementioned techniques lies in the high surface sensitivity, and that there is no need for labeling. For non-labeling techniques, however, there is a high demand on functional surface chemistry, that provides specificity towards desired analytes while preserving sensitivity. Therefore, a lot of research within the biosensor field is devoted to the development of novel surface coatings.

Figure 1.1: Working principle of an array biosensor. The principal component is the recognition layer which captures analyte molecules from solution. The substrate can sometimes provide connectivity to or be a working part of the transducer.
1.2 Biomolecules and biomolecular interactions

The fundamental building blocks that form the various types of biomacromolecules are in themselves remarkably simple. For example, only four different nucleotides make up the DNA molecule, which is responsible for encoding all the genetic information in living organisms. Proteins, which are the laborers in cell biology, are comprised of 20 different amino acids which combine to form a polypeptide backbone, the primary structure of the protein. Much of the function of proteins, however, lies in their elaborate secondary and tertiary structures. While the covalent peptide bonds in proteins are very stable, the three-dimensional structure is very sensitive to the environmental conditions. A prerequisite for a successful protein-based biosensor surface is therefore the availability of a friendly, native-like environment. Other important biochemical building blocks are lipids, which constitute membranes, and carbohydrates, which are vital in energy storage and for many cellular processes. Because lipids and carbohydrates are abundant in the native environment of proteins, molecules with similar structure and properties are a natural template choice when designing protein friendly surfaces.

Affinity sensors

A large portion of biosensors are affinity-based, wherein the sensor signal originates from a specific interaction between a ligand ($L$) immobilized on a surface, and the analyte ($A$) molecule. For a monovalent interaction, the kinetics (association $k_a$, and dissociation $k_d$) of formation of the $LA$ complex is described by the following differential equation (EQN. 1.1):\(^7\)

$$\frac{d[LA]}{dt} = k_a[L][A] - k_d[LA]$$

(1.1)

At equilibrium, the rate of formation is zero and the equation reduces to:

$$\frac{k_d}{k_a} = \frac{[L][A]}{[LA]} = K_D$$

(1.2)

wherein $K_D$ is the dissociation constant. A low dissociation constant is a characteristic of high affinity sensors. If the binding process can be monitored over time, the kinetic parameters can be extracted by fitting to the response curves during the analyte injection and post-injection phases. Information on kinetics is important in many cases, for instance, it can provide thermodynamic information on the specific interaction. Knowing the kinetics of an interaction is also important in drug screening tests, where the affinity is a measure of the potentiality of a new drug candidate.

In terms of quantifying the analyte concentration, affinity sensors are most reliable when the concentration of the analyte is in the range $(0.1-10) \cdot K_D$.\(^6\) This means that when the analyte concentration is unknown, as is most often the case,
systematic dilution of the sample is required to obtain a concentration within the defined range. This calls for a series of experiments to be performed. A way of circumventing laborious sample preparation is to use an affinity array sensor wherein separate elements of the array have different affinities toward the analyte. If the affinity array spans a wide enough range, one can ensure that the analyte concentration is in the same order of magnitude as the $K_D$ of some of the array elements and, ideally, quantification can be made in a single experiment.\textsuperscript{8} Figure 1.2 shows an example of the SPR responses from three elements in an affinity array during the injection and post-injection phases. In this model system, the dynamic range of quantification was extended by using three ligands with affinities spanning two orders of magnitude towards the analyte. A large dynamic range can be particularly desired in biomarker screening studies, where concentrations are known to vary over three to four orders of magnitude in some cases.\textsuperscript{9} The concept of affinity arrays is explored further in Paper VI of this thesis.

**Protein microarray scaffolds**

Since a few years back, protein chips comprising several thousand proteins from the human proteome are commercially available.\textsuperscript{10} Commonly, these are based on physisorption to e.g. nitrocellulose membranes, or covalent linkage by functional groups on glass surfaces directed towards amine- or carboxyl groups on the proteins.\textsuperscript{11,12} While these methods of immobilization are convenient and fairly straightforward the ligands typically become randomly oriented in an uncontrollable fashion on the surface upon binding.\textsuperscript{13} It has been demonstrated that directed, i.e. tag-mediated or site-specific, immobilization methods which offer some control of the orientation of the active site of the ligands are superior in terms
of retained biomolecular activity. Such concerns are less important on three-dimensional array supports, which provide a flexible, natural-like environment for the protein ligands, and also offers immobilization capacity in excess to a monolayer. One such platform is the dextran based hydrogel developed for the Biacore SPR-based biosensors. In highly condensed microarrays, one concern which constrains the attainable immobilization density is the risk of cross-talk or contamination of neighboring spots. Ligands are commonly delivered to the surface by dispensation, a process described more fully in section 3.3. Various solutions to reduce cross-talk has been presented, for instance through the introduction of microwells or by surface energy modification of regions on the substrate. Some of the work in this thesis has been directed towards development of scaffolds for protein microarrays. In Paper I, we present a method of introducing hydrophobic barriers by micro stamping onto a dextran hydrogel chip (Figure 1.3B), which facilitates dense microarrays to be created on three-dimensional surfaces. A poly(ethylene glycol) based UV-patterned hydrogel matrix is demonstrated in Paper IV, wherein three-dimensional spots for immobilization are separated by a two-dimensional hydrophobic barrier region (Figure 1.3A). This polymer matrix particularly excels in terms of low levels of non-specific binding, and might also act as a kind of ‘molecular sieve’, separating biomacromolecules with regard to size. Further, a two-dimensional microarray platform based on biotin tag-mediated binding is employed in Paper VI.

1.3 On the significance of gradients

Diffusion, the spontaneous intermixing of molecular species, is a fundamental process ubiquitous in everyday life. Due to diffusion, concentration gradients or spatial molecular distributions will occur spontaneously at molecular system interfaces. Such gradient patterns are believed to play a large role in morphogenesis∗, first

∗From Greek: “Beginning of the shape”
1.3 On the significance of gradients

Figure 1.4: Imaging SPR study of protein (lysozyme and pepsin) adsorption to an amphoteric polymer surface. In A, an SPR image of the polymer gradient is shown. B shows the shift in SPR wavelength upon introduction of proteins in solution. (The data in this figure was adapted from Paper V).

...suggested by Turing in an interesting work from 1952. At a cellular level, gradients of chemoattractants are important for cell motility, and in guidance of axonal outgrowth. Synthetic gradients are very important tools for in vitro studies of such phenomena.

Surfaces with laterally varying properties can be used as means of increasing throughput in materials research, as an alternative to the manufacture of large numbers of discrete samples. Using gradients, a range of properties can be incorporated and studied in one single sample. An imaging method, such as iSPR, is an excellent tool in the study of interactions at such surfaces.

Among the first type of surface gradients to be manufactured were wettability gradients, based for instance on monolayers of alkanethiols on gold. There are many other approaches to the creation of chemical surface gradients, some of which are based on polymers. In this thesis, gradients were prepared using a top-down approach, by modification of a uniform surface film, in Paper III and using a bottom-up approach based on graft polymerization in Papers IV and V. An example of an SPR image, showing the adsorption of proteins to an amphoteric polymer surface with a laterally varying composition, is given in Figure 1.4. From this data, it is straightforward to determine the surface composition which, in this case, gives a minimum in protein adsorption.

Two comprehensive reviews on soft matter surface gradients were recently published.
General introduction
A little more than a century ago, Wood, at the Johns Hopkins University, observed what he referred to as anomalies in the reflection spectra from diffraction gratings when varying the angle of incidence.\footnote{In principle, these observations could be explained by the work of Fresnel in the first part of the 19th century and modeled using the important theory of Maxwell from 1873.} These anomalies were attributed to excitation of surface waves by Fano in 1941\footnote{In principle, these observations could be explained by the work of Fresnel in the first part of the 19th century and modeled using the important theory of Maxwell from 1873.} and theoretically explained by Ritchie in 1957.\footnote{In principle, these observations could be explained by the work of Fresnel in the first part of the 19th century and modeled using the important theory of Maxwell from 1873.} Otto, Kretschmann and Raether pioneered the modern work on Surface Plasmon Resonance (SPR) in the end of the 60’s, devising an experimental setup that allowed for excitation of plasmon waves on flat metal films.\footnote{In principle, these observations could be explained by the work of Fresnel in the first part of the 19th century and modeled using the important theory of Maxwell from 1873.} The Kretschmann type of setup is widely used in commercial SPR sensors of today. Because the conditions under which surface plasmon resonance occurs is extremely sensitive to the optical properties of the proximity, SPR can be used to probe for changes in the refractive index or thickness of surface films. This was exploited in the end of the 70’s by Pockrand and Swalen in thin film studies.\footnote{In principle, these observations could be explained by the work of Fresnel in the first part of the 19th century and modeled using the important theory of Maxwell from 1873.} The high sensitivity of SPR to changes in the optical parameters of thin films relates to the existence of an evanescent field associated with the surface plasmon. In a biosensor
context this was first explored in 1982 by Liedberg et al., who showed how SPR can be used in the study of biomolecular interactions at surfaces. Principally, in a light-mediated SPR experiment, the objective is to find the conditions under which coupling of the incident light to a surface plasmon mode occurs. A comprehensive review on applications of surface plasmon resonance was recently published.

In this chapter, the theoretical basis of surface plasmon resonance is presented and some different configurations and important characteristics are discussed.

2.1 Theory

A Surface Plasmon (SP) is a surface-bound electromagnetic wave that propagates along the interface of a metal and a dielectric. The origin of SP waves is longitudinal charge density fluctuations in the free electron gas (plasma). Therefore, a prerequisite for SP excitation is that the metal can be described by the free electron model. A schematic representation of an SP wave at a semi-infinite metal-dielectric interface is shown in Figure 2.1. The most frequently employed metals are silver and gold. Even though the resonance width of thin silver films is smaller than for gold, which theoretically leads to a higher sensitivity, the relative chemical inertness and the ease of functionalization of gold makes it the most applicable metal in biosensor contexts. Gold can be considered free-electron like only at optical wavelengths longer than about 500 nm because at higher energies, electron excitations can occur and the light will be absorbed. Aside from the book by Raether, several excellent reviews describing the theory behind surface plasmons can be found in literature. Most optics textbooks also treat surface plasmons in detail. In the following subsections some fundamental theory is presented and a theoretical model of an SPR sensor is given in section 2.2.

The interactions of electromagnetic waves with matter are described by Maxwell’s
2.1 Theory

In SI units these are:

\[
\nabla \times E + \frac{\partial B}{\partial t} = 0, \quad (2.1)
\]

\[
\nabla \times H - \frac{\partial D}{\partial t} = J, \quad (2.2)
\]

\[
\nabla \cdot D = \rho, \quad (2.3)
\]

\[
\nabla \cdot B = 0. \quad (2.4)
\]

wherein,

\[
D = \varepsilon \varepsilon_0 E, \quad (2.5)
\]

\[
B = \mu \mu_0 H, \quad (2.6)
\]

\[
J = \sigma E. \quad (2.7)
\]

in which \(\varepsilon\) denotes the permittivity*, \(\mu\) the permeability and \(\sigma\) the conductivity tensors. For isotropic materials these become scalar functions of frequency. When there are no external charges, \(\rho = 0\) and the current density \(J = 0\). At optical frequencies it is also generally assumed that the permeability \(\mu = 1\). Recall that \(c^2 = 1/\mu_0 \varepsilon_0\), Eqn. 2.2 then becomes:

\[
\nabla \times \mu_0 H - \frac{\partial E}{\partial t} \varepsilon/c^2 = 0.
\]

### Dispersion relation

In the most simple case, a single (TM-polarized) SP wave is bound at the interface between two (isotropic) semi-infinite media as shown in Figure 2.1. The electric and magnetic fields associated with the SP wave in this case are given by Eqn. 2.8 and Eqn. 2.9.

\[
E_j = (E_{xj}, 0, E_{zj})e^{i(k_x x - \omega t)\pm(i k_z z)} \quad (2.8)
\]

\[
H_j = (0, H_{yj}, 0)e^{i(k_x x - \omega t)\pm(i k_z z)} \quad (j = 1, 2) \quad (2.9)
\]

Wherein \(j\) denotes the material (1 for the metal and 2 for the dielectric), \(\pm\) indicates positive or negative \(z\)-axis and the positive lies in the dielectric. The first exponential term accounts for propagation along the interface (in positive \(x\)-direction), while the imaginary \(k_z\) describes the exponential decay in the \(z\)-direction. Since the SP wave is TM-polarized there is no electric field component in the \(y\)-direction (the vector set is \(E_{xj}, H_{yj},\) and \(E_{zj}\)). These fields are continuous at the boundary:

\[
E_{x1} = E_{x2}, \quad (2.10)
\]

\[
H_{y1} = H_{y2}, \quad (2.11)
\]

\[
\varepsilon_1 E_{z1} = \varepsilon_2 E_{z2}. \quad (2.12)
\]

*also denoted the dielectric function, \(\varepsilon(\omega)\).
This implies that $k_{x1} = k_{x2} = k_x$ (where medium 1 is the metal and 2 the dielectric). The Maxwell relations (Eqs. 2.1-2.4) applied on the waves in Eqn. 2.8 and Eqn. 2.9 lead to the following set of partial differential equations:

$$\frac{\partial E_{xj}}{\partial z} - i k_x E_{zj} - i \omega \mu_0 H_{yj} = 0,$$

(2.13)

$$\mu_0 \frac{\partial H_{yj}}{\partial z} - \frac{i \omega \varepsilon_j}{c^2} E_{xj} = 0,$$

(2.14)

$$\mu_0 \frac{\partial H_{yj}}{\partial x} + \frac{i \omega \varepsilon_j}{c^2} E_{zj} = 0,$$

(2.15)

$$\frac{\partial E_{zj}}{\partial z} + i k_x E_{xj} = 0. \quad (j = 1, 2) \quad (2.16)$$

Eqn. 2.14 together with the boundary conditions Eqs. 2.10-2.12 yield the following dispersion relation:

$$\frac{k_{z1}}{\varepsilon_1} + \frac{k_{z2}}{\varepsilon_2} = 0 \quad (2.17)$$

Combination of Eqs. 2.13-2.14, and Eqn. 2.16 gives the following relation for $k_z$:

$$k_{zj} = \sqrt{\frac{\omega^2 \varepsilon_j}{c^2} - k_x^2},$$

(2.18)

By substitution of Eqn. 2.18 in Eqn. 2.17 the dispersion relation for surface plasmons at a semi-infinite metal-dielectric interface is reached:

$$k_x = \frac{\omega}{c} \sqrt{\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2}},$$

(2.19)

**Propagation length and Probe depth**

Some notable features of SP waves can be extracted from Eqn. 2.19. Firstly, since the dielectric function of the metal is complex ($\varepsilon_1(\omega)$), while $\varepsilon_2(\omega)$ for the dielectric is real), the SP wavevector will have an imaginary part, $k_x''$. Therefore the surface plasmon is a damped wave and has a finite propagation length in the $x$-direction. The energy of the SP is absorbed and dissipated as heat in the metal film, the damping term is denoted $\Gamma_i = k_x''$ where the subscript $i$ indicates internal damping. Furthermore, the absence of propagating waves in the metal or dielectric requires that $k_z$ in Eqn. 2.8 and Eqn. 2.9 be purely imaginary. This implies that $\frac{\varepsilon_1 \varepsilon_2}{\varepsilon_1 + \varepsilon_2} > \varepsilon_2^*$. The wavevector of light incident through the dielectric medium is given by $k_{in} = \frac{\omega}{c} \sqrt{\varepsilon_2}$ and therefore $k_{in}$ will always be smaller than $k_x$. Consequently, surface plasmons can not be excited optically at a metal-dielectric interface by plane wave light incident through the dielectric. One way to circumvent this problem is to employ an Attenuated Total Reflection (ATR) configuration as will be described in more detail in section 2.3 v.i. The above findings are summarized in Figure 2.2

*Here we have assumed that the real part of the dielectric function of the metal is much larger than the imaginary part ($|\varepsilon'_1| \gg \varepsilon'_1$).
2.1 Theory

Figure 2.2: Schematic illustration of the electric field $E_z(z,x)$ at the interface of semi-infinite metal and dielectric (water) layers (at $\lambda = 633\text{nm}$). The field decays exponentially in the $z$-direction, the probe depth is in the sub-micron range for the dielectric and about an order of magnitude smaller for the metal film. The wave is attenuated in the direction of propagation $(x)$ over a distance of some 10-30 microns. Note that the $x$-axis has been cut off to illustrate the attenuation.

which schematically shows the exponentially decaying evanescent field and the attenuation of the SP wave propagating in the $x$-direction.

The intensity, which is directly proportional to the energy density, of the SP is given by the square of the electric field, for the fields in Eqn. 2.8 this yields (wherein $''$ denotes the imaginary part):

$$I_{zj} \propto e^{-2k''_{zj}z}, (j = 1, 2) \tag{2.20}$$

$$I_x \propto e^{-2k''_x x}. \tag{2.21}$$

Two important parameters of the SP wave are the propagation length $(L_x)$ and the probe depth$^*$ $(\delta_{zj})$. These are defined as the distances (along $x$ or $z$ respectively) at which the intensity of the electric field has dropped to a value of $1/e$ and are hence given by:

$$L_x = \frac{1}{2k'_x} \tag{2.22}$$

$$\delta_{zj} = \frac{1}{2k''_{zj}} (j = 1, 2) \tag{2.23}$$

The effect of a thin metal film

So far only the special case of a semi-infinite metal-dielectric system has been treated. Typically, in optical excitation of surface plasmons one deals with an asymmetric system, wherein the metal film is thin and supported on top of another, dielectric material (typically glass). A metal thickness of 50 nm, which is typical,

$^*$Distinguished from the penetration depth in that the latter applies to the electric field strength rather than the intensity (different value by a factor of two).
Surface plasmon resonance is of the same order of magnitude as the probe depth $\delta_z$ of the SP. Consequently, the evanescent field will penetrate the film and probe the substrate leading to an alteration in the resonant wavevector of the SP. The new wavevector can be expressed as:

$$k_x = k_x^\infty + \Delta k_x$$

wherein $k_x^\infty$ denotes the wavevector in the semi-infinite case. It is important to note that $\Delta k_x$ is a complex quantity which means that the propagation length will be influenced. There is now an additional complex term, $\Gamma_{rad} = \Delta k_x''$ (radiative damping term), caused by reradiation of the light associated with the SP.

In the next section a model that can be used to calculate the intensity reflectance of an asymmetric SPR system consisting of many thin film layers is presented.

2.2 Stratified medium matrix model

The general ATR SPR coupler is based on total internal reflection within a glass prism coated with a thin metal film. This type of SPR coupler can be optically modeled as a stratified medium wherein the outermost prism and ambient (generally water or air) layers skirts the intermediate thin metal layer. For biosensor applications, several additional thin film layers are incorporated in the model. These can be for instance; molecular linker layer, immobilized biomolecular layer, and analyte layer. In the following subsections, the Fresnel reflection formulas are used to calculate the intensity reflectance from a general $N$-layer stack. An alternative approach would be to analytically solve Maxwell’s equations as was done in the previous section, a tedious undertaking for a system of more than one layer. The matrix formulation, on the other hand, can easily be employed to solve for an arbitrary number of layers. Many descriptions of the matrix model exist in literature, see for example the work by Hansen, Azzam and Bashara or Abelés.

Assumptions

The basis of the stratified medium matrix model is a layer stack comprised of homogeneous layers connected through interfaces (Figure 2.3). In the general case, the stack will be comprised of $N + 1$ (smooth and perfectly parallel) layers, with varying thicknesses, $d_j$, and complex refractive indices $\tilde{n}_j = n_j + ik_j$. Note that, in this section, we will write the complex refractive index with a plus sign because of known problems in how for instance MATLAB will handle the complex algebra when a minus sign is used. The first (incident) and last layers are considered semi-infinite and non-absorbing. The incident light can be parallel- (denoted

*See the citations for a discussion about sign conventions.
Figure 2.3: Layer stack in the stratified medium model. The 0th and Nth layers are semi-infinite and have real refractive indices. The incident wave is p- (TM, ||) or s- (TE, ⊥) polarized. The planes show the interfaces, $I_{ij}$.

$p$, TM or ||) or perpendicular- (denoted $s^*$, TE or ⊥) polarized. The Cartesian coordinate system in Figure 2.3 defines the $z$-direction as parallel to the plane of incidence with the positive direction into the layer stack. Electric fields will be superscripted + for positive and - for negative $z$-direction corresponding to refracted and reflected waves respectively. For our purposes it is convenient (although not necessary) to consider all layers isotropic, hence all fields are independent of $x$ or $y$. Just as before, it is assumed that the permeability $\mu = \mu_0$ for all layers.

**Derivation**

Since there is no dependence on $x$ or $y$, we have for the total electric field amplitude at a certain distance along the $z$-axis:

$$E_{z_{\text{tot}}} = E_{z_{+}} + E_{z_{-}}$$  \hspace{1cm} (2.25)

Where the subscript indicates the $z$-dependence. Eqn. 2.25 holds for both TM-polarized and TE-polarized light respectively. For the relation between the electric field vectors at two points, $z_1$ and $z_2$ we have:

$$\begin{bmatrix} E_{z_{1+}} \\ E_{z_{1-}} \end{bmatrix} = \begin{bmatrix} M_{11} & M_{12} \\ M_{21} & M_{22} \end{bmatrix} \begin{bmatrix} E_{z_{2+}} \\ E_{z_{2-}} \end{bmatrix} = M \begin{bmatrix} E_{z_{2+}} \\ E_{z_{2-}} \end{bmatrix}$$  \hspace{1cm} (2.26)

*From the German word senkrecht, meaning orthogonal.*
in which $M$ denotes the scattering matrix. When the points $z_1$ and $z_2$ are located within the same layer the relation can be written as:

$$
\begin{bmatrix}
E^+_{z_1} \\
E^-_{z_1}
\end{bmatrix} = L_j
\begin{bmatrix}
E^+_{z_2} \\
E^-_{z_2}
\end{bmatrix}
$$

(2.27)

Wherein $L_j$ is the layer matrix of the $j$:th layer. Conversely, when $z_1$ and $z_2$ are located at the interface of separate adjacent layers $i$ and $j$ we introduce the interface matrix $I_{ij}^*$. We then have:

$$
\begin{bmatrix}
E^+_{z_1} \\
E^-_{z_1}
\end{bmatrix} = I_{ij}
\begin{bmatrix}
E^+_{z_2} \\
E^-_{z_2}
\end{bmatrix}
$$

(2.28)

In the general case where $z_1$ and $z_2$ are within separate non-adjacent layers we can write:

$$
\begin{bmatrix}
E^+_{z_1} \\
E^-_{z_1}
\end{bmatrix} = I_{i(i+1)} L_{(i+1)(i+2)} \cdots L_{(j-1)j} I_{(j-1)j}
\begin{bmatrix}
E^+_{z_2} \\
E^-_{z_2}
\end{bmatrix}
$$

(2.29)

The scattering matrix in the case of $N$ consecutive layers is hence given by:

$$
M = \left( \prod_{j=1}^{N-1} I_{(j-1)j} L_j \right) I_{(N-1)N}
$$

(2.30)

For the electric fields at the 0:th and $N$:th layers we have:

$$
\begin{bmatrix}
E^+_0 \\
E^-_0
\end{bmatrix} = \begin{bmatrix}
E^{\text{incoming}}_0 \\
E^{\text{reflected}}_0
\end{bmatrix}
$$

$$
\begin{bmatrix}
E^+_N \\
E^-_N
\end{bmatrix} = \begin{bmatrix}
E^{\text{transmitted}}_N \\
0
\end{bmatrix}
$$

(2.31)

From the definition of the complex Fresnel reflection and transmission coefficients we then find:

$$
\tau = \frac{E^+_0}{E^-_0}
$$

(2.32)

$$
\tau = \frac{E^+_N}{E^-_N}
$$

(2.33)

By substitution of EQN. 2.32 into EQN. 2.26 the reflection and transmission coefficients of the layer stack can be expressed in terms of the scattering matrix elements:

$$
\frac{M_{21}}{M_{11}}
$$

$L$ is sometimes also called the propagation matrix and $I$ the transmission matrix.
We need now to determine the scattering matrix, $M$, for the layer stack. The layer matrices, $L_j$, describe the phase shift undergone upon propagation through a uniform film and have the form:

$$L_j = \begin{bmatrix} e^{-i\varphi_j} & 0 \\ 0 & e^{i\varphi_j} \end{bmatrix}$$

In which the film phase thickness, $\varphi_j$, is given by:

$$\varphi_j = \frac{2\pi d_j}{\lambda} N_j \cos \phi_j$$

Where $N_j$ is the complex refractive index. Turning then to the interface matrices, we start by expanding the Eqn. 2.28. For two adjacent layers $i$ and $j$ we then have:

$$E_i^+ = I_{11} E_j^+ + I_{12} E_j^-$$
$$E_i^- = I_{21} E_j^+ + I_{22} E_j^-$$

When the light is incident from layer $i$, we have $E_j^- = 0$. By direct comparison with Eqn. 2.32 we immediately find the first two coefficients:

$$I_{11} = \frac{1}{\tau_{ij}}$$
$$I_{21} = \frac{r_{ij}}{\tau_{ij}}$$

Consider next the case where $E_i^+ = 0$ (light is incident from layer $j$) we then have:

$$E_i^- = \tau_{ji} E_j^-$$
$$E_j^+ = r_{ji} E_j^-$$

By combination of Eqn. 2.36 with Eqn. 2.38 it is possible to find expressions for the remaining two coefficients:

$$I_{12} = -\frac{r_{ji}}{\tau_{ij}}$$
$$I_{22} = \tau_{ji} - \frac{r_{ij} r_{ji}}{\tau_{ij}}$$

From the Fresnel reflection formulae we have the relations: $r_{ji} = -r_{ij}$, and $\tau_{ji} = \frac{1-r_{ij}^2}{\tau_{ij}}$. The complete interface matrix then has the form:

$$I_{ij} = \frac{1}{\tau_{ij}} \begin{bmatrix} 1 & r_{ij} \\ r_{ij} & 1 \end{bmatrix}$$

Since the general expression Eqn. 2.26 is valid for both p- and s-polarized light we have so far omitted the polarization notation. The Fresnel reflection and transmis-
Figure 2.4: Four layer model of an SPR biosensor. In the most simple case an SPR sensor can be modeled as a four layer stratified medium where the constituents are a glass prism, coated with a thin metal film, to which the organic sensing layer is adsorbed. The semi-infinite ambient layer is typically aqueous buffer.

The intensity reflectance and transmittances of the layer stack are found as the square modulus of the coefficients in Eqn. 2.33.

\[ r_{ij} = \frac{\bar{n}_j \cos \phi_i - \bar{n}_i \cos \phi_j}{\bar{n}_j \cos \phi_i + \bar{n}_i \cos \phi_j} \]
\[ \tau_{ij} = \frac{2\bar{n}_j \cos \phi_i}{\bar{n}_j \cos \phi_i + \bar{n}_i \cos \phi_j} \] (2.41)

The intensity reflectance and transmittances of the layer stack are found as the square modulus of the coefficients in Eqn. 2.33.

Implementation

As the number of layers increase, the matrix algebra required to find the reflection coefficient of the stack quickly becomes tedious. However, the stratified medium matrix model as described v.s. can easily be implemented in a computer program, for instance MATLAB. A special case which is somewhat useful in SPR biosensing concerns a stack consisting of two thin film layers on top of a glass prism and in an aqueous ambient (Figure 2.4). Some algebra will give an expression for the intensity reflection from such a layer stack:

\[ R = \left| \frac{r_{01}(1 + r_{12}r_{23}e^{2i\phi_2}) + e^{2i\phi_1}(r_{12} + r_{23}e^{2i\phi_2})}{1 + r_{01}e^{2i\phi_1}(r_{12} + r_{23}e^{2i\phi_2}) + r_{12}r_{23}e^{2i\phi_2}} \right|^2 \] (2.42)

In the above expression, which is valid for \( \bar{n}_j = n_j + ik_j \), the response upon introduction of an analyte can be modeled as a thickness increase or as an increase in refractive index of the adsorbate layer. The former is most suitable for immobilization of biomacromolecules to a two-dimensional organic linker layer, whereas the latter can be employed when a sensing matrix (for instance a hydrogel) is used. By eliminating all terms with \( r_{23} \) in Eqn. 2.42, an expression for the more simple
2.2 Stratified medium matrix model

Figure 2.5: Refractive indices of BK7 glass, SF10 glass, and complex refractive index of gold. Gold data was measured using spectroscopic ellipsometry, while optical data for glass was obtained from Schott.\textsuperscript{52}

A more general model of an SPR biosensor is given in Figure 2.6. In this case, an additional adhesion layer between the prism and the metal film has been included. Furthermore, the active part of the biosensor consists in this model of a biomolecular linker layer, a sensing layer and a response layer. Typically, the biomolecular linker layer serves as an attachment point for the sensing layer to which the molecules making up the response layer can adhere. Although an analytical expression for the reflectance of the layer stack in Figure 2.6 could be derived, the intensity reflectance is best found using a computer program.
2.3 Optical SP excitation

We saw in section 2.1 that for two semi-infinite dielectric - metal layers the momentum of incident plane wave light can never match the surface plasmon momentum. In 1968, Otto presented the ATR configuration for SP excitation through an air gap between a glass prism and a semi-infinite metal film.\cite{30} In the same year, Kretschmann and Raether devised a setup in which the metal film was evaporated directly on the prism.\cite{31} These two configurations are referred to as prism couplers and utilize the evanescent field of light reflected at the prism boundary to excite surface plasmons.

Prism coupler

In order to have resonance, the $x$-component of the incident light wavevector needs to match the SP wavevector. This resonance condition can be expressed:

$$k'_{SP} = k^\text{photon}_x = \frac{\omega}{c} \sqrt{\varepsilon_0} \sin \phi_0$$

in which $\varepsilon_0$ is the dispersion of the prism and $\phi_0$ the internal angle of incidence. In order to satisfy Eqn. 2.43 it is required that $n_{\text{prism}} \sin \phi_0 > n_a \sin \phi_a$ (the subscript $a$ denotes ambient), and the resonance condition can therefore only be fulfilled at values of $\phi_0$ greater than the critical angle of incidence, $\phi_c$. In Figure 2.7 the principal outlines for the experimental configurations (Otto and Kretschmann) used to fulfill this condition are shown. In general, when for instance an equilateral prism is employed, and when the angle of incidence deviates from the prism angle, the incident light will be refracted at the prism boundaries upon entry and exit.
Accordingly, the external angle of incidence will be different from the internal angle within the prism. For clarity, all angles considered here are always the internal angle of incidence and the refraction, and possible loss, of light at the prism boundaries are disregarded.

Among the prism couplers, the Kretschmann configuration is prevalent much thanks to its simplicity. The Otto configuration requires precise manufacture and positioning techniques in order to achieve perfectly parallel prism and metal surfaces with an exact and sub-micron wide gap in between. Direct evaporation of the metal film on a prism surface is on the contrary quite straightforward, but seldom used. Instead, an index matching oil or gel is used to optically couple gold coated glass slides to the prism. This allows for exchange of the functional gold film and reuse of the prism. In addition, because the light is incident from below, a flow cell could be docked to the sensor surface, making the Kretschmann configuration well suited for application within biosensing wherein samples are sequentially injected over the surface.

The reflectivity in the Otto and Kretschmann configuration can be calculated using the matrix model described in section 2.2. Figure 2.8 shows the reflectivity of TM-polarized light, \( R_{TM} \), in the Otto and Kretschmann setups respectively. For the Otto-configuration, the reflectivity curve for a 300 nm air gap displays a pronounced minimum at an angle of incidence of around 42° (\( \lambda = 633 \text{ nm} \)) corresponding to fulfillment of the resonance condition. When the gap is filled...
Figure 2.9: Calculated reflectivity curves for different gold film thicknesses in the Kretschmann configuration. The substrate is BK7 glass and the ambient is water ($n = 1.33$), $\lambda = 633$ nm. The optimal film thickness is close to 50 nm as indicated by the thicker curve.

with water the minimum shifts to about $72^\circ$ and the reflectivity becomes nearly zero. In the Kretschmann setup the minimum shifts in a similar fashion. A noteworthy observation is that the width of the resonance becomes larger at high angles. At angles below the critical angle of incidence, $\phi_c$, which is manifested as a ‘kink’ in the reflectivity at about $41^\circ$ and $61^\circ$ respectively, the reflectivity is still quite high, and the 50 nm gold film then acts as a mirror. The reflectivity at resonance becomes zero when the radiative damping of the SP equals the internal damping ($\Gamma_{\text{rad}} = \Gamma_i$).\textsuperscript{37} This happens only for a very precisely defined metal film thickness which is also a function of the wavelength. Figure 2.9 shows calculated reflectivity curves for 633 nm light for different gold films in the Kretschmann configuration. The optimal thickness is in this case close to 50 nm. This gold film thickness is suitable for most parts of the red spectrum. We will refer to the angle of incidence or the wavelength of the incident light that meets the resonance condition and gives a minimum in reflected intensity as the SPR angle ($\phi_{\text{spr}}$) or the SPR wavelength ($\lambda_{\text{spr}}$) respectively.

**Grating coupler**

Corrugated surfaces can also be used to couple light into surface plasmon modes, this configuration is referred to as a Grating Coupler (GC). GCs are attractive as large-volume low-cost devices because they are easily manufactured using established plastic moulding technology. The dispersion relation of a GC device is
given by Eqn. 2.44 \[ k_x = \frac{\omega}{c} \sqrt{\varepsilon_d} \sin \phi_d \pm u \frac{2\pi}{\Lambda} \] wherein \( \Lambda \) is the grating constant, \( u \) is an integer and the subscript \( d \) denotes the dielectric ambient. In this type of device, light is typically incident from above, through the medium, which could be troublesome in biosensors with complex samples, i.e. blood. In addition, in wavelength spectroscopy mode, the sensitivity of GC-SPR devices is lower than for ATR-based sensors. Several instances of GC-SPR sensors has been presented. For example, the Flexchip\textsuperscript{TM} instrument which is part of the Biacore line-up (now GE Healthcare) utilize a grating coupler in imaging SPR mode. Recently, an approach based on angular interrogation that enables measurements in parallel along a two-dimensional array was presented. Grating coupled SPR sensors have also been used in combination with electrochemical measurements.

2.4 Imaging surface plasmon resonance

If the optical detection unit of the SPR sensor is a Charge-Coupled Device (CCD) chip or any other type of array detector and the substrate is evenly illuminated with a large beam, the reflected light from the surface can be displayed as a two-dimensional image, in which each pixel depicts a part of the illuminated surface area. This type of sensor geometry is referred to as imaging surface plasmon resonance* or surface plasmon microscopy and was first suggested by Knoll et al. in 1988. Another way of achieving lateral resolution is to narrow the illuminating beam and scan the substrate. iSPR sensors can be based on intensity modulation in which case the wavelength and angle of incidence are fixed and the reflected intensities measured as functions of in plane position and/or time. The observed contrast in the SPR image is then obtained when different regions of the substrate have different refractive indices so that the resonance condition varies over the surface. Alternatively, angular or wavelength spectra can be acquired for each depicted region of the substrate and the SPR angle or wavelength can be calculated to render an SPR map of the sample.

As for all optical microscopy techniques, the limit in resolution for iSPR is governed by diffraction. This means that the highest attainable lateral resolution is in the \( \mu m \) range, of the same order of magnitude as the wavelength of the light. However, this only applies to the direction perpendicular to the plane of incidence. In the direction of SP propagation, the resolution is limited by the plasmon propagation length (\( L_{SP} \), Eqn. 2.22). Since \( L_{SP} \) is inversely proportional to the sum of the radiative and internal damping (\( \Gamma_{rad} \) and \( \Gamma_i \)) and these in turn depend on the wavelength (from the dispersion relation), the propagation length will be different for different wavelengths. Figure 2.10 illustrates this wavelength dependence.

*Sometimes the term “surface plasmon resonance imaging” (SPRI) is used
Surface plasmon resonance

Figure 2.10: Calculated propagation length ($L_{SP}$) and probe depth ($\delta_z$) for 50 nm gold on BK7 glass in aqueous buffer ($n = 1.33$) as a function of wavelength. The probe depth (which was calculated for the semi-infinite case only) is shown for both the ambient (◦) and the gold film (○). Note that the y-axes are logarithmic.

From the leftmost graph in Figure 2.10, it can be seen that the propagation length in an aqueous ambient is about an order of magnitude larger than the diffraction limit which means that $L_{SP}$ is the limiting factor in iSPR lateral resolution. It should be noted that the calculated values for the propagation length are valid only at resonance because we have used the assumption that $\Gamma_{rad} = \Gamma_{i} = k''_{\infty}$, which means that $L_{SP} = 1/[4 \cdot \Im(m \left(\frac{\omega}{c} \sqrt{\frac{\epsilon_m \epsilon_a}{\epsilon_m + \epsilon_a}}\right)]$, but this holds at resonance only. The probe depth (which in this case was calculated for a semi-infinite gold-dielectric system) is shown in the rightmost graph of Figure 2.10.

Choice of wavelength and incidence angle

In intensity mode iSPR the choice of wavelength and incidence angle have a large impact on the sensitivity and dynamic range. Figure 2.11 shows calculated reflectivity as a function of wavelength and incidence angle for Kretschmann based SPR sensors of two different prism materials. The dark streaks in the graphs correspond to fulfillment of the resonance condition. In order to obtain contrast in an SPR image, it is necessary to select angles and wavelengths that lie on one of the flanks of the resonance curve. As is seen in Figure 2.11, this can be attained with a multitude of combinations of wavelength and incidence angle. In most cases, one strive to obtain as low an angle of incidence as possible. This is because the oblique angle in a simple iSPR configuration distorts the image, thereby leading to a reduced lateral resolution. Since the SPR angle decreases with wavelength, it is then recommendable to work in the far red of the visible spectra. Most CCD detectors, however, are designed to be less sensitive in the infrared posing a restraint
Figure 2.11: Calculated reflectivity as a function of angle of incidence and wavelength. The reflectivity for two different glass substrates (BK7 and SF10) is shown, the gold film thickness is 50 nm and the ambient is water \((n = 1.33)\) in both cases. The darkest areas correspond to fulfillment of the resonance condition.

On the choice of wavelength*. The main reason for choosing shorter wavelengths lie, however, in the desire to have as short a propagation length as possible. The trade-off is then between propagation length and image distortion both of which affect the lateral resolution negatively. In the Kretschmann geometry, in the red and in aqueous ambient, propagation lengths lie in the 10 µm range (Figure 2.10), this resolution is quite acceptable for most applications within biosensing.

In terms of sensitivity† or contrast in SPR imaging, many considerations need to be taken into account. First of all, SPR is sensitive to both bulk refractive index change and changes in refractive index or thickness of adsorbed thin films. This

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*This is primarily due to an (often removable) filter in front of the CCD of most digital cameras and this problem can be circumvented through normalization.

†Here, we will only consider the theoretical instrumental sensitivity in terms of response to changes in optical properties. In real biosensing systems an additional contributor to the real sensitivity is the concentration dependent change in optical properties, \(\frac{\partial n}{\partial c}\), or \(\frac{\partial d}{\partial c}\).
means that there are at least two separate sources that can give rise to contrast in an SPR image; local differences in refractive index or adsorbed molecules. In most biosensing applications, the change in bulk refractive index is equal over the substrate and it is only molecular binding events that give rise to contrast. In this case, a relevant model system is the growth in thickness of a thin film adsorbed on the gold surface, or an increase in refractive index of an already present film. We define sensitivity as the slope of the calibration curve, which for the three cases described above renders:

\[ S_n = \frac{\partial \Delta R}{\partial n_a}, \quad (2.45) \]
\[ S_d = \frac{\partial \Delta R}{\partial d_f}, \quad (2.46) \]
\[ S_{nf} = \frac{\partial \Delta R}{\partial n_f}. \quad (2.47) \]

wherein, \( n_a \) denotes refractive index of the ambient (bulk), \( d_f \) and \( n_f \), thickness and refractive index of a surface film. Figure 2.12 shows calculated reflectivity curves for different thicknesses of a thin film with refractive index (\( n = 1.5 \)). These calculations were made at a wavelength of 750 nm. The SPR angle for the gold film in the absence of an organic film is in this case about 66.45°. We see from the difference curves in Figure 2.12B and C that the highest contrast is obtained at a slightly lower angle than \( \phi_{SPR} \), in this specific case at about 66.2° (thick line in the graphs). This is in agreement with theoretically derived findings by Yeatman et al. who found that the highest sensitivity (defined in terms of the slope of the SPR curve) is obtained when \( \Gamma_{rad} = \Gamma_i/2 \) and at an angle offset from the resonance by \( \Delta \phi = -w/\sqrt{3} \), wherein \( w \) is the width of the resonance dip. The slope (calculated as the numerical gradient of the curves in Figure 2.12C and shown in D) shows that the maximum change in reflected intensity is about 12%/nm and drops to about 6%/nm at an organic film thickness of 5 nm. This can give an appreciation of the dynamic range of the iSPR sensor in intensity mode. From the reflectivity maps in Figure 2.11 we see that the width of the resonance decrease with wavelength, thereby increasing the slope of the flanks. Therefore, we expect the sensitivity to increase with wavelength. On the other hand, there is a decrease in dynamic range when the slope increases, making a relinquishment in sensitivity beneficial for the dynamic range of the iSPR sensor.

Approximate analytical expressions for the sensitivity can be derived by applying the Cauchy-Lorentz distribution, which can accurately describe the reflectivity in the range close to SPR resonance. This is a commonly employed approach in other work. Another approach is to take the derivative of the expression Eqn. 2.42, or of the dispersion relation Eqn. 2.19. We will settle for a numerical calculation based on the Fresnel formalism (section 2.2), the result of which is presented in Figure 2.13. The dashed lines in the left graph of Figure 2.13 show
2.4 Imaging surface plasmon resonance

Figure 2.12: Reflectivity curves for different thicknesses of an organic adlayer (n = 1.5) in an aqueous ambient (n = 1.33). The wavelength, \( \lambda = 750 \text{ nm} \) in all cases. A) Calculated reflectivity for different thicknesses (0, 5, and 10 nm) of the organic film. The SPR angle, \( \phi_{SPR} = 66.45^\circ \) in the absence of an organic film. The reflectivity of TE-polarized light is also included (dashed line). B) Change in reflectivity as a function of incidence angle for growth of a 10 nm thick organic film, in 1 nm steps. The arrows indicate increasing thickness. C) Reflectivity change as a function of film thickness at different angles of incidence. D) Numerical gradient of the curves in C. Note the logarithmic y-axis.
Figure 2.13: Sensitivity for three different SPR response incentives; Change in refractive index of the semi-infinite bulk (×), change in refractive index within a 20 nm thick sensing layer, e.g. a hydrogel, (○), and growth of a thin organic film, n = 1.5, (◁). The left graph shows the optimal angle, which is the angle that gives the highest sensitivity, as a function of wavelength (solid lines) for the three different cases. The dashed lines represents $\phi_{spr}$. In the right graph the maximum sensitivity defined as the slope of the change in reflectivity is plotted as a function of wavelength. Note that the scale is logarithmic. The unit is either mRIU$^{-1}$ or nm$^{-1}$ depending on whether the monitored change is in refractive index or thickness. It is assumed that the optimal angle of incidence is chosen.
2.4 Imaging surface plasmon resonance

**Figure 2.14:** SPR images taken at four different AOIs (marked by ● on the angular axis in the upper graph) showing circa 2.1 nm thick lines of micro contact printed 1-mercaptohexadecanethiol (HDT) on gold. The image contrast shifts when the AOI pass the SPR angle as indicated in the upper graph. The critical angle of reflection ($\Theta_c$) has been indicated in the (unnormalized) SPR curves.

The SPR angle as a function of wavelength, these curves are similar to the maps in Figure 2.11. The solid lines show the optimal choice of incidence angle in order to maximize the sensitivity. In the right graph, the sensitivities of three different incentives for shifting the resonance condition have been plotted; bulk refractive index change, index change within an already present (non-dispersive) organic film, and thickness increase of a thin film. In all three cases, the sensitivity increase with wavelength.

An example on SPR imaging is given in Figure 2.14. In this case, a gold substrate has been patterned with a self assembled monolayer of an alkanethiolate using micro contact printing. In array biosensors, imaging SPR can be used in intensity mode to study molecular binding events in real time. Figure 2.15 shows an example of this, wherein a synthetic peptide with specificity to calmodulin is covalently immobilized to a sensorchip, and the subsequent interaction with calmodulin monitored. The shift in reflectivity is plotted as a function of time.

**Imaging SPR in spectral mode**

There is a fundamental limit to the dynamic range of intensity modulated SPR sensors since shifts larger than the width of the resonance can not be detected. One way of increasing the dynamic range is to acquire angular or wavelength reflectivity spectra. With iSPR it is then possible to determine $\lambda_{spr}$ or $\phi_{spr}$ with lateral resolution. Angular spectral interrogation mode is a very common implementation in commercial SPR sensors. However, in imaging mode, one concern with angular
interrogation is the variation in image distortion which needs to be corrected for. This can be accomplished, with some effort, by image post-processing or through clever optical design. By fixing the incidence angle and scanning the wavelength, correcting for distortion becomes much simpler. The sensitivity in wavelength spectral mode can be defined as $\partial \lambda_{spr} / \partial n$, or $\partial \lambda_{spr} / \partial d$, depending on whether the incentive is an increase in refractive index or in thickness, and can be calculated in the same way as described v.s. Figure 2.16 (right graph) shows the calculated sensitivity as a function of wavelength for two different cases, either a bulk index change or addition of a thin film. As can be seen, the sensitivity increases with wavelength. Hence, it is generally better from a sensitivity point of view to work at longer wavelengths, particularly so for bulk index sensing. For addition of a thin film, however, the wavelength dependence can also be seen but is in this case much smaller. One consequence of this is that for sensor applications in which one want to discriminate between an (often unspecific) change in bulk refractive index to a change in film thickness (as is the case in many biosensor applications) it might be beneficial to design sensors to work in the shorter wavelength regime. The values in Figure 2.16 are valid in aqueous ambient. In air, the sensitivity is generally higher and the wavelength dependence more pronounced.

The left graph in Figure 2.16 shows calculated reflectivity curves in wavelength interrogation mode. The drop in reflectivity at shorter wavelengths is caused by electron excitation related absorption. At these energies, the gold film can no longer be considered free electron like and the surface plasmon resonance condition can not be fulfilled.
Figure 2.16: Calculated reflectivity curves and sensitivity for SPR in wavelength interrogation mode. The left graph shows reflectivity curves at an angle of incidence of 72°, for a 50 nm thick gold film in aqueous ambient (n = 1.33), the arrow indicates the shift upon increasing the bulk refractive index or for addition of a thin film. The reflectivity of TE-polarized light is also shown (dotted curve). In the right graph, the wavelength dependence of the sensitivity defined in terms of shift in λ_{spr} is shown. The curves show both bulk refractive index sensitivity (×) and the thin film sensitivity (n = 1.5, ◁).

An example where imaging SPR in wavelength interrogation mode is employed in detection of biointeraction events is given in Figure 2.17. The reflectivity curves in Figure 2.17 shows how λ_{SPR} shifts towards longer wavelengths upon binding of biomolecules. The z-value of each pixel in the surface maps corresponds to a λ_{SPR} value calculated from measured reflectivity curves.

Sensitivity in affinity biosensors

In the previous section, the instrumental sensitivity was discussed in terms of \( \frac{\partial \Delta R}{\partial n} \) and \( \frac{\partial \lambda}{\partial n} \). In affinity biosensors, the measured change in refractive index is caused by a change in the concentration of an analyte present over the surface. The induced refractive index shift is given by:

\[
\Delta n = \left( \frac{\partial n}{\partial c} \right) \frac{\Delta \Gamma}{d}
\]

in which \( \left( \frac{\partial n}{\partial c} \right) \) is the refractive index increment, d is the thickness of the sensing layer and \( \Delta \Gamma \) is the change in surface concentration of the analyte molecules. For proteins, a typical value for \( \left( \frac{\partial n}{\partial c} \right) \) is 0.18 ml g\(^{-1}\).66
**Figure 2.17:** Interactions on a biosensor array chip monitored by imaging SPR. A self-assembled monolayer was patterned with biotin using micro-contact printing (○). The surface was then incubated with Neutravidin (×), a biotin-tagged peptide (■) and exposed to a Fragment Antigen Binding (Fab) part of an antibody with specificity towards the peptide (◀). The white markers in the first panel indicate the regions of interest from which the line profiles (a), and the SPR curves (b and c) where acquired. (Data for this Figure part of the work in Paper VI).
2.5 Imaging SPR and electrochemistry

Electrochemistry primarily deals with the study of chemical reactions that takes place at the interface of an electrode and an electrolyte. Because of the high sensitivity to optical changes in the immediate vicinity of metal surfaces, SPR is a particularly suited technique for the study of electrochemical processes at the electrode/electrolyte boundary. For this reason, SPR has been combined with electrochemistry in a number of applications, for example in studies of monolayers of redox-proteins\textsuperscript{67} and for detection of enzymatic reactions.\textsuperscript{68,69} Ertl and coworkers\textsuperscript{70–72} employed imaging surface plasmon resonance in a novel study of spatiotemporal pattern formation on electrodes. They also studied the formation of stationary concentration patterns on electrode surfaces\textsuperscript{73} and suggested potential future applications regarding tailoring of electrode patterns.

When electrochemistry is combined with imaging surface plasmon resonance, the substrate is used as an SPR sensor surface and as a working electrode simultaneously. Electrochemical reactions taking place at the electrode interface can then be studied with spatial resolution (\textbf{Paper II}). \textbf{Figure 2.18A} shows a section view of a typical experimental setup wherein the sensor surface can be used either as working or as a bipolar electrode. An open cuvette is tightly pressed towards the sensor surface which is connected to the electroanalytical equipment* using a contact pad. Reference and counter electrodes are then immersed in the electrolyte solution. Elaborate descriptions of typical experimental setups and details of electroanalysis can be found in textbook literature.\textsuperscript{74,75}

In electrochemical SPR, several phenomena can affect the optical properties and induce an SPR signal. First and foremost, when a redox reaction takes place, the different forms (reduced/oxidized) can have different refractive indices, in which case a refractive index change takes place over the electrode surface as the reaction proceeds in either direction. Secondly, when a potential is applied to the surface in an ionic solution, the composition of the Electric Double Layer (EDL) consisting of a surface layer of ions and a diffuse layer of counter-ions is changed at the interface. The thickness of the EDL is typically a few nanometers\textsuperscript{†}, at least an order of magnitude less than the probe depth of SPR. Even so, the presence of a charged double layer can give rise to significant SPR signals. In this case, the SPR condition is influenced by the structuring of ions at the surface. There is also an additional contribution, by the presence of an electric field at the surface/electrolyte interface, which affects the optical properties of the gold film. The latter effect is observed in particular when the surface charge is positive and there is a deficiency in electron density.\textsuperscript{76} The presence of the EDL can influence both the position and the width of the resonance curve, because both the real and

*For instance a potentiostat, wherein the potential of the working electrode (vs. a reference electrode) can be controlled and the resulting current measured.

†Given by the Debye screening length.
imaginary part of the dielectric constant will be altered by the interfacial electric field.\textsuperscript{77} Furthermore, in particular at sustained large positive potentials a layer of gold oxide with significantly different optical properties will form that might change the SPR condition.\textsuperscript{78}

The relation between redox species concentration and applied potential at an interface is given by the Nernst equation (\textit{Eqn. 2.49})

\begin{equation}
E = E^0 - \frac{RT}{zF} \ln \left( \frac{c}{1-c} \right)
\end{equation}

wherein $E$ is the potential at the working electrode, $E^0$ the formal potential, $z$ the number of electrons transferred in the redox reaction and $c$ is the fraction of molecules in either redox form ($c = \frac{[\text{Red}]}{[\text{Red}] + [\text{Ox}]}$). The SPR response to changes in redox species concentration, $\partial R/\partial c$, can be subdivided into two components, the refractive index increment, $\partial n/\partial c$ and the sensitivity to changes in refractive index, $\partial R/\partial n$. \textbf{Figure 2.18D} shows measured SPR signal (solid squares) over the working electrode/sensor surface as a function of applied potential in a three electrode setup. The solid line is the calculated response from the Nernst equation. The calculation was performed using a value of $\partial R/\partial n$ from \textbf{Figure 2.13} and $\partial n/\partial c$ from literature.\textsuperscript{79} The Cyclic Voltammetry (CV) measurement shows how the current varies with the potential. From the CV it can be seen that the shift in SPR response coincides with the formal potential, which is the potential between the two peaks in the CV. The dependence of the SPR response to applied potential in electrolyte solution in the absence of redox species is shown by the filled triangles in \textbf{Figure 2.18D}. When the surface charge is positive, attraction of negatively charged ions and the change in optical properties of the gold leads to a positive shift in the SPR signal. In the presence of redox species, however, the redox reaction induced shift in bulk refractive index appears to be the dominating factor.

On bipolar electrodes, in which the surface functions as an anode and a cathode simultaneously, a spatial gradient in the potential drop across the electrode/electrolyte interface is setup. The potential drop in the gradient region can be visualized using imaging SPR (\textbf{Figure 2.18C}) (\textbf{Papers II} and \textbf{III}). Using the calibration curve in \textbf{Figure 2.18D}, the SPR response of the line profiles in \textbf{Figure 2.18C} can then be converted into surface potential.
Figure 2.18: A). Schematic view of the electrolytic cell used in combined imaging surface plasmon resonance and electric measurements. B). SPR curves in supporting electrolyte, 0.5 M KNO$_3$ (solid), and in a mixture of 0.5 M KNO$_3$ and 10+10 mM [Fe(CN)$_6$]$^{3-/4-}$ (dashed). The vertical lines mark the respective wavelengths at which the data in C and D was acquired. C). SPR response when an electric current is passed in the electrolyte between electrodes 1 and 2 and the sensor surface acts as a bipolar electrode. A gradient in the potential drop between the sensor surface and solution causes oxidation to occur on one side of the surface and reduction on the other. The slope of the gradient depends on the current passed through the solution. D). SPR response at different potentials when the sensor surface acts as a working electrode in a three electrode setup. In the presence of redox species (■) and in supporting electrolyte only (◆). The solid curve is a calculated fit based on the Nernst equation (see text). Also shown is a CV (dashed). The left axis in C and D applies to both figures, while the right axis is for the CV only. (Part of this figure to appear in Paper X)
Surface plasmon resonance
Cleverly designed surface chemistry is an important path to the introduction of functionality, e.g. specificity and selectivity in biosensors. Soft matter surface coatings such as polymers, organic molecular monolayers, biological membranes and biomacromolecules can for instance be used as linker layers and provide means of molecular immobilization in biosensor applications. The wide variety of available chemistries and the endless possibilities of synthesis of new compounds enables tailoring of desired surface properties.

In this chapter, an introduction to the various surface chemistries that were used in the papers included in this thesis (Figure 3.1) is given.

3.1 Self assembled monolayers

Self Assembled Monolayers (SAMs) has become a very important tool for functional surface modification in biosensors. Although studies of molecular film for-
Figure 3.1: Overview of the different soft matter surface modifications employed in studies within this thesis (not to scale). The substrates are in all cases glass slides coated with a thin layer of gold. The different surface chemistries are: I) Hydrophobic barriers of an ionic surfactant patterned onto a dextran hydrogel. II) Micro contact printed patterns of thiolates on gold. III) Composition gradient of thiolates on gold. IV) Ethyleneglycol-containing polymer array with variable thickness. V) Amphoteric polymer composition gradient. VI) Mixed thiolate monolayer on gold.
formation at interfaces and on metal surfaces had been of interest early on*, it was in the early 80's when Nuzzo and Allara reported on the adsorption of organic disulfides to gold surfaces that the path for applications of SAMs within the field of biosensors was cleared. Early on it was found that monolayers of \( \omega \)-substituted alkanethiolates on gold were of special interest, due to the high degree of ordering, potential for tailoring of surface properties, and ease of further functionalization of SAMs of such. A fairly recent review by Whitesides and coworkers covers the details of formation and gives several examples on applications of SAMs.

Monolayers of alkanethiolates form spontaneously upon immersion of a gold substrate in a thiol solution with a concentration in the micro- to millimolar regime. The initial adsorption step is generally very rapid, but the layer structure ill defined and the alkyl chains in a largely gauche conformation. Over time, however, a well organized crystalline like (all-trans) structure is adopted, as illustrated in Figure 3.2. On the Au(111) crystal plane, an overlayer with a \((\sqrt{3} \times \sqrt{3})R30^\circ\) configuration is formed. The crystalline structure of the alkyl chains is stabilized by intermolecular van der waals forces, the energy of which is about 1 kcal/mol for each CH\(_2\)-group. Since the distance between the adsorption sites of sulfuric atoms is about 5 Å, which is slightly more than the van der waal radii of the CH\(_2\) groups, a chain tilt with respect to the surface normal, \(\alpha = 30^\circ\), is induced. The strength of the Au-S bond is in the order of 40 kcal/mol, which is of the same magnitude as covalent interactions. All the same, the thiolate layer is of a very complex dynamic structure and reorganization and lateral diffusion seems to be frequently occurring. A large number of defects are embedded in SAMs on gold, these include crystal defects in the gold substrate such as terraces, islands, grain boundaries and impurities, but also defects in the monolayer itself due to phase transitions or vacancies in the overlayer structure.

SAMs of organosulfur compounds more complex than plain alkyl-derivatives are frequently used in real applications. Unfortunately, the incorporation of bulky tail-groups, e.g. carboxyls, amines, amino-acid derivatives etc., usually has a negative impact on the stability and structural organization of the monolayers. Therefore, the compounds providing the end-function are generally mixed with spacer thiols to form heterogeneous SAMs that can still be highly ordered. Care needs to be taken when optimizing concentrations and incubation times, particularly for mixed monolayers, as these parameters will influence the composition and organization of the monolayer. In some cases introduction of hydrogen bond donors can be used to improve the stability, an example is the inclusion of secondary amides in the thiol compound, which facilitates lateral hydrogen bonding. When the intended end-function of the SAM is chemical binding of biomacromolecules, the dilution of functional groups on the surface can serve a secondary purpose by reducing steric hindrance in the interaction between species much larger than the

*Langmuir pioneered this work in 1920 in studies of amphiphilic molecular layers.
Assembly from solution

Figure 3.2: Schematic overview of the self assembly of alkanethiols (1-mercaptohexadecanoic acid) from solution on gold (111). Assembly is a spontaneous process that proceeds in several steps. Adsorption occurs within minutes in dilute solutions while it takes longer for the highly organized crystalline (√3 × √3)R30° layer to form. The strength of the Au-S bond is in the order of magnitude of 40 kcal/mol and the alkyl chains are stabilized by lateral interactions of about 1 kcal/mol per methyl-group. Also shown is the approximate tilt-angle of the alkyl chains, α = 30°.

tail-group.

The presence of a self assembled monolayer on the surface of an electrode can be detected electrochemically, since densely packed, defect deficient SAMs of organic molecules can block electron transfer from solution to the substrate surface. Depending on the quality and characteristics of the monolayer the blocking properties will be different. Therefore, electrochemical methods, for example CV, is a very useful analytical technique when studying the properties of SAMs. Electrochemical analysis can be combined with imaging surface plasmon resonance, to enable laterally resolved measurements (Paper II).

Ethylene glycol containing thiolates

Of special interest in biosensor applications are SAMs containing oligo(ethylene glycol) (OEG) chains*. This is because OEG SAMs have been shown to suppress unspecific adsorption of proteins. The protein repellent properties of ethylene glycol derivatives can be attributed in part to their hydrophilic character. In addition, chains of more than a few EG-units can adopt a helical like conformation in SAMs, the occurrence of which has also been correlated with protein-resistance.

Within this thesis, SAMs of OEG containing thiolates have been used as a bio-inert anchoring layer for an ethylene glycol based hydrogel (Paper IV and V),

*With the general structure: \( \ldots - (O-(CH_2)_2)_n - \ldots \)
for specific protein immobilization (\textit{Paper III}), and as means of immobilization of synthetic polypeptides in an array format for biosensor purposes (\textit{Paper VI}).

### 3.2 Matrix surfaces

In covalent immobilization of biomacromolecules, it is in many cases important to ensure that the native state of the molecules is preserved. Immobilization on rigid structures, such as dense alkanethiolate monolayers, can sometimes cause proteins to loose their secondary structure or impede biointeractions sterically. Therefore, in biosensors for interaction analysis, controlled immobilization to more flexible three-dimensional structures is mostly preferred. In SPR sensors specifically, since the evanescent field extends a few hundred nanometers into the ambient, by using a three-dimensional matrix the sensor signal can be enhanced. For example, matrices based on dextran polymer have been very successfully employed in the Biacore line-up of SPR sensors.\textsuperscript{15,93} A very important characteristic of matrix surfaces for immobilization is a low degree of non-specific binding. One way of accomplishing this is to employ poly(ethylene glycol) (PEG) based polymers.\textsuperscript{94} PEG matrices have for instance been used in the suppression of biological fouling.\textsuperscript{95} In \textit{Paper IV} of this thesis, a PEG based matrix is employed as a template for immobilization in biointeraction studies.

### 3.3 Surface pattern techniques

**Micro contact printing**

Micro Contact Printing (\(\mu\text{CP}\)) is a soft lithographic technique developed to fulfil the need for a cheap and uncomplicated way of producing patterns of biomolecules on rough and non-planar surfaces. In soft lithography an elastomer is used either as a stamp, as is done in \(\mu\text{CP}\), to print a molecular pattern, or as a mould, which is the case in micro moulding in capillaries, replica moulding and micro transfer moulding, to create three-dimensional structures of polymers or patterns of biomolecules.\textsuperscript{96,97} With soft lithography, a wide variety of materials can be patterned on flat as well as rough surfaces. Micro contact printing is most suitable for patterning two-dimensional structures; for example protein layers\textsuperscript{98} or SAMs of alkane thiolates.\textsuperscript{99,100}

In micro contact printing, an elastomeric stamp is first submerged or covered with a solution (ink) of the molecule to be printed. The inked stamp is then brought into conformal contact with the surface to be patterned (\textit{Figure 3.3}). The ink concentration, ink transfer time and the surface contact time are important parameters that affect the quality of the pattern produced. These parameters need to be optimized for different types of inking solutions.
Poly(dimethylsiloxane) (PDMS) is the elastomer most commonly used in micro contact printing. Besides being elastic, it is optically transparent, highly biocompatible and durable, which makes it useful in many applications. Stamps are generally cast off a master mould fabricated using traditional photolithographic techniques. Many positive properties of PDMS are related to the low surface energy and chemical inertness, which assures that most molecules does not adhere irreversibly to the stamp. The elasticity and softness of the PDMS is the cause of some of the negative properties of the stamp. During curing, the stamp shrinks by about 1% and especially non polar organic solvents tend to swell the cured stamp. Transfer of residual PDMS to the substrate can also sometimes occur.

Sagging or pairing of the structures on the stamp can take place if the aspect ratio of the structures is too high or too low. When the molecule to be printed is polar or dissolved in water, it is often necessary to modify the stamp and render the PDMS surface more hydrophilic, this can be accomplished by a brief exposure of the stamp to an oxygen plasma. Gradient patterns has been made with PDMS stamps by means of varying the thickness of the elastomer.

In the papers included in this thesis, µCP has been used to create hydrophobic barriers for the confinement of tiny droplets of protein solution (Paper I), as means of achieving multi-component SAMs (Paper II) and in bio-functionalization of thiolate monolayers (Paper VI).

**Electrochemical patterning**

Although the S-Au interaction of adsorbed thiols on gold surfaces is very strong, it is not entirely irreversible. Even at room temperature, immersed in a solution with thiols, there appears to be a continuously ongoing replacement of thiols on the surface with those present in solution. Most alkane thiols can be reductively desorbed from gold surfaces at a potential of circa -1.0 V vs. Ag/AgCl. This process is fully reversible, as the thiols will reattach upon restoration of the surface.
3.3 Surface pattern techniques

potential. The exact potential required to remove the thiols from the gold surface is dependent on the choice of electrolyte as well as the length of the alkyl chain and the properties of any present tail-groups on the thiols. Therefore, different thiols will leave the surface at different potentials. This can in fact be used as means of patterning through selective desorption of thiols, as proposed by Tender et al.\textsuperscript{106,107} and Wolfbeis et al.\textsuperscript{108} They demonstrated how individual elements of an array of gold microelectrodes could be selectively functionalized with different thiols. In their method the surface needed to be patterned and divided into smaller elements that could be individually contacted. Bohn and coworkers, on the other hand, have devised a means of electrochemical patterning that allows for spatial patterns to be set up on electrodes,\textsuperscript{109–112} whereby for instance the thiol monolayer composition can be varied laterally. Their method is based on passing a current through a thin, thereby resistive, gold film, causing a potential gradient to be set up over the surface. This potential gradient could then be used to desorb alkanethiols from a previously formed monolayer. The empty regions of the surface was then backfilled with another thiol from solution, ultimately leading to a gradient mixture of two different thiols.

Within the scope of this thesis, a novel method for electrochemical patterning of conducting surfaces was explored (Paper III). In this approach, an electric field is induced in an electrolyte by passing a current between two feeder electrodes (Figure 3.4). When the electric field is large enough, reductions will be induced on one side of the surface and oxidations on the other as the surface becomes a bipolar electrode. The potential difference between a point along the surface and the solution will vary laterally and a potential gradient is thus induced. Various types of molecular gradients can be made with this method, since the redox reactions can be of virtually any type. For example, electrodeposition of metals, electropolymerization, or reductive desorption of thiols can all be facilely governed on a variety of substrates using the setup in Figure 3.4.

Ink-jet printing

Dosage of very small droplets of liquid can be readily accomplished using conventional ink-jet printing technology that has been available in for instance consumer desktop printers for years.\textsuperscript{113} Ink-jet printing is based on the application of a pressure pulse to a confined volume of liquid that allows a small part of the bulk liquid to be ejected through an orifice. In a piezodispenser, a piezoelectric material is used to generate the pressure pulse. Generally, the piezoelectric material is coated around a glass capillary so that when a voltage is applied the walls of the capillary will deform, resulting in the ejection of a liquid droplet from the nozzle. Ink-jet printing can be used to print proteins,\textsuperscript{114,115} thiol SAMs,\textsuperscript{116,117} DNA,\textsuperscript{118,119} and other biomolecules for immobilization on surfaces.

Under optimal conditions, a single nearly spherical droplet following a straight
trajectory will be dispensed. Upon impact with the substrate, the droplet momentum, which is originally directed towards the surface, will cause a radial flow parallel to the surface. This results in rapid spreading of the droplet, which forms a liquid disk. If the kinetic energy of the droplet is much larger than the surface energy, splashing will occur, and a number of smaller droplets will be formed. However, if the surface energy is high enough, the liquid disk will retract to form a sessile droplet with the shape of a spherical cap. The contact area of the sessile droplet is governed by the difference in surface energy of the substrate and the liquid. On hydrophilic, high energy surfaces, aqueous drops tend to spread over a larger area. This can be disadvantageous in micro array applications since it limits the maximum spot density and the risk of cross-contamination is increased. One way to circumvent this is the introduction of a hydrophobic grid, used to divide the hydrophilic substrate into smaller elements.

In molecular arrays involving chemical binding or biomolecular interactions, sufficient time needs to be given for the present species to interact. However, under normal conditions, nano-liter sized water droplets evaporate very quickly. Another related problem is the accumulation of solute at the rim as the droplet evaporates (Figure 3.5). The reason for this is that initially during evaporation, the contact radius of the spherical cap is pinned, leading to a net radial flow within the droplet. Unless immobilization of the solute to the substrate is rapid, some type of evaporation control is often necessary. This can be accomplished by increasing the vapor pressure of the environment through humidity control or by lowering the vapor pressure of the droplet through temperature control. Alternatively, the viscosity of the solvent can be increased by addition of i.e. glycerol or sucrose. Additives that act as hydrogen-bond donors can also significantly enhance the stability of piezodispensed proteins. In some instances, evap-

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*The coffee stain phenomenon.
3.3 Surface pattern techniques

Figure 3.5: Solute delivery using piezodispensation. Too rapid evaporation of the solvent causes accumulation of solute molecules at the droplet boundaries, something which can be prevented by control of the rate of evaporation.

oration might be beneficial, because the solute concentration increases when the solvent evaporates.
The instrumental platform for iSPR used herein was designed and built during the course of this work. Our design was based on the Kretschmann configuration, wherein surface plasmons are optically excited by means of an evanescent field. The instrument was built on an optical bench using standard mounts and fashioned mechanical components. The basic system, shown schematically in Figure 4.1, consists of the optical components, a light source with appropriate filters, a polarizer, a prism, and a CCD detector, and a rotational translation stage for control of the incidence angle. The system is controlled via an appropriate data acquisition software built within the NI LabVIEW environment. Moreover, some different flow cells and sample compartments were designed to allow for measurements in a confined liquid volume or under controlled flow. While the specific instrument built here was intended for laboratory use, the configuration is generic and adaptable for field use.

In this chapter, a brief overview of the components of the imaging SPR instrument is presented along with some justification and discussion of the general features.
Figure 4.1: Schematic overview and photograph of the optical configuration in the imaging SPR instrument.

4.1 Optical setup

Prism

A 25 mm equilateral glass prism (BK7) with a refractive index of \( \sim 1.51 \) was mainly used in this work. The prism material was chosen primarily to match the supply of gold covered glass substrates. Substrate gold surfaces are optically coupled to the prism using an index matching oil, as illustrated in Figure 4.2A. When the external incidence angle deviate from the prism angle (60°), refraction of the beam takes place at the prism boundaries as illustrated in Figure 4.2B. Because of the oblique angle the illuminated/imaged area on the sample is larger than the width of the incident beam and the image becomes compressed in the axis parallel to the plane of incidence. The use of a high refractive index prism (for instance SF-10, \( n \approx 1.72 \)) would lead to less distortion since the SPR angle becomes lower, and hence closer to the prism angle, with the dispersion of the prism. Because image distortion is a function of incidence angle, the image resolution will depend on the chosen angle of incidence and will vary during an angular SPR scan. While distortion leads to slightly lower resolution as well as a decrease in illuminating intensity, it can easily be corrected for as described v.i.

Light source

Different types of light sources can be employed in SPR sensing. In the present setup, two different broadband sources were put to use. Firstly, a Xenon discharge lamp (Oriel Inc.) used in conjunction with a set of interference filters can be used for fixed wavelength iSPR. Additionally, a Xenon lamp connected to a monochromator (Acton Research SpectraPro 300i) is used for iSPR in spectral
interrogation mode. Of common use in laboratory setups is the HeNe laser with a wavelength of 632 nm. In an imaging setup, a laser light source can be used with a beam-expander to increase the diameter of the light beam. In the present setup, light from the monochromator was collimated using a single condenser. The divergence of the collimated beam is a trade-off with the beam diameter, larger beam diameters inevitably leads to greater divergence.

Spectral resolution in a diffraction grating based monochromator partially depend on the groove distance which should be on the same order of magnitude as the reciprocal of the wavelength. The grating efficiency, the relative intensity of transmitted light, is generally optimized (blazed) for a particular wavelength range. Because the efficiency also depends on the polarization, care must be taken during SPR measurements when the TE-polarized beam is used for referencing. In the present setup, for the most part, a 300 g/mm grating blazed at 500 nm was used. While the efficiency at 600-800 nm is only about 50% for this particular grating, there is very little difference between the TE- and TM-polarized beams which justifies the via media choice of grating.

Detector

A monochromatic 12 bit 1 MPixel CCD camera (Retiga EXi, QImaging) was used to acquire SPR images. In intensity mode SPR, an important parameter is the bit depth of the detector. When a 12 bit A/D converter is used, in theory, the minimal resolvable shift in reflectivity is around 0.025%. In practice, however, the intensity resolution is governed by noise which is nearly an order of magnitude larger and also depend on the gain setting of the camera. Since both the incident light intensity and the detector sensitivity varies with wavelength, the intensity resolution will also be wavelength dependent.
The lateral resolution* of the images in the direction perpendicular to the plane of incidence depends solely on the magnifying optics, while in the direction parallel to the plane of incidence the resolution is dependent on the angle of incidence. In the perpendicular direction, the resolution is 2.2 µm/pixel, and the parallel resolution at an internal angle of incidence of 72.5° is around 8 µm/pixel. The parallel resolution could be well improved by tilting the CCD detector such that the detector plane becomes largely parallel with the substrate surface. In this manner, the depth of focus could also be increased. The specific camera used here is, however, not suited for this type of modification. Alternatively, the use of a tilt-shift imaging optics of the type commonly employed in architectural photography or a similar optical configuration could be used to the same effect. In any case, the image resolution can easily be calculated at any angle of incidence and distortion correction is straightforward to implement.

4.2 Fluidics and cell for electrochemistry

There are virtually no limitations to the design of flow systems or cuvettes for the imaging SPR system, as long as the substrate forms one side or the base of the liquid cell. However, some important considerations need to be taken into account when designing a flow system for Biomolecular Interaction Analysis (BIA) in an array format. Typically, it is desired to keep the total volume of the liquid handling system minimal, in order to reduce sample consumption. Additionally, in array applications, one prerequisite is that the analyte can be reproducibly delivered in equal concentration and roughly simultaneously over the entire array. This is best achieved by keeping the physical dimensions of the sample compartment low. The flow system and cuvette predominately used in this work are presented schematically in Figure 4.3. The volume of the sample compartment in the liquid flow cell is circa three microliters. Therefore, with a flow speed of 30 µL per minute the liquid is fully replaced in around six seconds which is sufficient for most array biosensor applications. In addition, a different cuvette for electrochemistry can be seen in Figure 2.18A. The electrochemical cuvette also includes a contact for the surface as well as room for the immersion of counter and reference electrodes. A modified version of this cuvette was used in the electrochemical bipolar patterning in Paper III.

4.3 Interface

While the user interface is of minor importance in an experimental setup for laboratory use, the physical interface between the computer and the devices that

*Note that we differentiate between resolution, which is the minimum resolvable response or the limit of detection, and lateral resolution, which is the resolution in the SPR images.
4.4 Data processing and analysis

Data processing is a very important step in any sensor system. In particular, in indirect measurement methods, data analysis is a crucial step affecting both instrumental resolution and sensitivity.

The two modes of operation which are employed in the iSPR sensor described

Figure 4.3: A) Cuvette for electrochemistry. B) Flow cell for sample delivery during e.g. biological interaction analysis. To scale.
**Figure 4.4:** Simplified flowchart over the software controlled operation of the imaging SPR instrument. The most critical steps are the synchronized loops. The frame grabber is synchronized to the monochromator in order to acquire correct spectra. In an image sensorogram, the time of acquire for each image is also stored.
4.5 Performance

in this work require slightly different data processing. In intensity or kinetic mode, the signal is the reflected intensity as a function of time. The sample frequency can be up to 10 Hz which is significantly faster than any expected signal response caused by biomolecular interactions. Therefore, a convenient way of reducing noise is to take the average over a certain time interval, in the present case this was mostly done over 1 s. Analogously, CCD detector noise can be reduced to some extent by lowering gain and increasing exposure time or through pixel binning. An even more convenient route to noise reduction is to increase the size of the Region of Interest (ROI), averaging the signal over a larger detector area. It has also been shown recently, that incorporating multiple areas for referencing leads to lower noise levels in intensity mode iSPR.\textsuperscript{126}

In spectral mode, the resulting data is an image stack, consisting of a large number of images acquired at different frequencies of the incident light, typically at a sampling frequency of 1 nm\textsuperscript{−1}. These spectra are always normalized using TE-polarized light as reference. Before normalization, because the TE- and TM-polarized spectra can not be acquired in parallel, the data needs to be interpolated to be equidistant. The appearance of raw and normalized spectra and the data processing step is shown in Figure 4.5. There exists numerous methods of determining the SPR wavelength from a reflectivity spectrum, each of which may have different qualities.\textsuperscript{127,128} Herein, a weighted centroid algorithm (Eqn. 4.1) was used.\textsuperscript{129,130}

\[
\lambda_{SPR} = \frac{\sum_i \lambda_i \cdot (L - R_i)^2}{\sum_i (L - R_i)^2}
\]  

(4.1)

An important parameter in the weighted centroid algorithm is the threshold value, $L$, which defines a reflectivity, $R$, at which all values above are neglected. Because of the asymmetric shape of the SPR reflectivity curve, the weighted centroid algorithm introduces a slight deviation in the SPR wavelength from the true value. Typically, it is the shift in $\lambda_{SPR}$ that is of interest rather than the absolute value, in which case subtraction of two images is performed. In that case, the deviation is canceled and of no importance. Even in absolute measurements, the deviation can be neglected most of the time, particularly if the threshold is cleverly chosen to reduce the asymmetry.\textsuperscript{127}

4.5 Performance

The performance, in terms of resolution and sensitivity of the iSPR instrument in any given measurement is highly dependent on the experimental circumstances. While the instrumental sensitivity can be maximized, in accordance with Figure 2.13, by proper choice of a combination of the wavelength and the angle of incidence, the resolution will depend also on other experimental specific parameters, such as $\left(\frac{\partial n}{\partial c}\right)$, unspecific binding, temperature drift, fluctuations in the light source and camera noise.
Figure 4.5: Data processing to determine the SPR wavelength from a stack of images acquired during a spectral scan. A) Raw spectra. The TE-polarized spectrum is used for normalization. B) Normalized spectrum (—). All values below the threshold (×) are used as input in a weighted centroid algorithm to determine $\lambda_{SPR}$. Each pixel in the image stack is processed individually.
The limit of detection was determined for the experimental conditions in Paper VI. Herein, the angle of incidence was chosen in order to yield a reasonable image resolution with limited distortion, and the wavelength was set to achieve the highest sensitivity. By averaging the reflected intensity over $\sim 2200$ pixels for $\sim 20$ samples acquired during 5 seconds, the limit of quantification was assessed to be in the order of $3 \times 10^{-5}$ RIU. This value is expected in intensity modulated SPR and comparable to the resolutions of other similar, imaging SPR configurations, reported in literature, but about two orders of magnitude inferior to the non-imaging Biacore instruments. Figure 4.6 shows normalized SPR curves acquired during eight consecutive wavelength scans over an OEG SAM surface patterned with spots of neutravidin (surface pattern procedure more fully described in Paper VI). The standard deviation of the mean SPR wavelength, determined using Eqn. 4.1, when treating the curves from the SAM and the protein separately is $\sigma \approx 0.08$ nm. However, by taking the mean of the difference between $\lambda_{SPR}$ of a protein spot and a SAM spot for each of the scans, a standard deviation of $\sigma \approx 0.02$ nm is obtained. Under the experimental conditions used in Figure 4.6 ($\partial \lambda / \partial n \approx 3$ nm mRIU$^{-1}$), this corresponds to a resolution of $2 \times 10^{-5}$ RIU.
Since the launch of the first Biacore instrument in 1990, SPR has proven a tremendously useful tool in the field of biological interaction analysis. The search query “surface plasmon resonance” currently* renders more than 11 000 hits in ISI Web of Knowledge, out of which 1200 were published in 2008, and the rate of appearance of new papers is anticipated to increase steadily.

On the one hand, iSPR is very useful as a transducer in label free detection of analytes, in which the tendency is towards the development of more sensitive and higher-throughput instrumentation. One might envision future SPR appliances as being low-cost, robust, portable, sensitive, and fast devices. However, because SPR is a label-free method, specificity relies on the affinity of the sensing surface towards the analyte, and might be limited by the background response. Even if single molecular binding events could be resolved, one must be able to ensure that it is the analyte that is being detected. Therefore, the development of novel interfaces for affinity sensors will continue to be important, even more so when the

*August 17th, 2008
instrumental resolution is improved.

There are many potential applications of imaging surface plasmon resonance related to the field of biomimetics. For example, the behavior of eukaryotic cells, which are in the size range 10-100 µm, are studied with a variety of techniques. In particular, cell/substrate contact investigations are often performed using fluorescent labeling and total internal reflection microscopy. In an interesting work, Giebel et al. have used imaging SPR in ex vivo studies of the movement and adhesion of living cells on aluminium substrates. Using imaging SPR, it is further possible to quantify the cell-substrate distance in real time. The variety of available tools for tailoring of surface properties of SPR substrates should also enable many interesting studies of cell behavioral patterns.

The interaction of higher organisms with soft surfaces can also be studied with imaging SPR. For example, barnacles, which can be a great nuisance in marine biofouling, undergo a larval stage during which they adhere very strongly to surfaces. Figure 5.1 shows a sequence of SPR images acquired while barnacle larvae are ‘walking’ over the sensor surface, in this case an amine-terminated self-assembled monolayer, leaving footprints of a protein adhesive. Using this technique, the response of the larvae to different surface coatings can be investigated by monitoring their behavior in real time, and the potential of anti-fouling surface coatings can be assessed. The grafted polymer matrices described herein are particularly interesting model surfaces in such future studies. In addition, the ‘bipolar patterning’ approach of gradient manufacturing presented in Paper III offers an alternate, versatile route to the development of new functional materials.

There are some modifications to the imaging SPR instrument employed here that could increase the resolution. For example, increasing the power of the light source, or the efficiency of the monochromator would lower the noise, signal drift could be tackled by temperature control, and the use of a trapezoidal prism would lower the distortion. Further improvements in sensitivity could be reached if the phase shift of the reflected light was also evaluated, as is done in ellipsometry. Future alterations to the experimental setup are likely to include one or several of these improvements.
Figure 5.1: Use of imaging SPR to study deposition of footprints from cyprid larvae. As the cyprid walks over the SPR surface, its antenules (black arrows in photo) leaves behind a temporary protein adhesive, a 'footprint'. The white arrows in the sequential SPR images highlight the footprint deposition. The photograph shows a cyprid larva of the barnacle *Balanus amphitrite*. Work in progress. (The inset photo is published by permission from Inter-Research Science Center. ©Inter-Research 2007, Mar.Ecol.Prog.Ser. Vol. 340: 1-8, 2007.)
Future perspectives


