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Exploring the SPR methodology for
monitoring of critical attributes in toxicity
testing and bioproduction

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1. Scope of the thesis

Analysis of biological components is central in bioprocess monitoring, process control, product quality control and cell based toxicity assaying. One of these themes that is pursued in this thesis is the use of biosensors for monitoring of molecular markers, exploiting the natural selectivity of biomolecules. Another is the use of glycoconjugates to monitor the activity of biomolecules in a flu vaccine process is studied and were the sensor is based on the concept of weak affinity giving fast response time for the sensor.

A third theme is monitoring of cell cultures used for toxicity testing different protein markers is of interest.

When developing biosensor surfaces for new antigens commercial preparations of antibodies are often used. In this work we have chosen to look at lactate dehydrogenase (LDH) and describe the preparation and characterisation of antibody used in biosensor surface development.

The design of a sensor surface is important for the characteristics of a sensor. By binding antibodies in an oriented manner to the surface a better control of the properties of the antibodies is achieved. The demonstrated method also has the advantage of in situ purification and provides a flexible platform for antibody evaluation and sensor development.

In one sentence this thesis explores the possibility of utilizing recognition elements of a biosensor surface. In particular, surface plasmon resonance (SPR) is used as the primary biosensing tool, however most findings in are relevant for other biosensors.

Moreover, the thesis approaches existing bioanalytical impediments, such as purity and accessibility of the recognition elements on the sensor surface and preparation strategies to achieve this.

2. Introduction

2.1. *Bioanalysis in toxicity tests and bioprocesses*

Measurement and control on fermentations and cell cultures are fundamental both for successful production of bioproducts and for use in cell cultures used for toxicity testing (Mandenius et al., 2011). The focus on *in-vitro* cell cultures as an alternative to animal models for toxicity testing has increased the need for novel analytical methods. At the production of biopharmaceuticals the United States Federal Drug Administration (FDA) initiative for process analytical technology (PAT) was created to increase the innovation pace in the pharmaceutical industry. Older legislation was believed to make the industry reluctant to incorporate new technology and methods, thus trapping industry in old-fashioned and expensive technology. With a deeper understanding and a tighter monitoring of critical quality attributes during production would make it easier to make process changes and also facilitate faster batch release (United States Federal Drug Administration (FDA)).

Although small scale cell cultures and large scale bioproduction are very different in a lot of aspects, when it comes to analysis they also have some things in common. Both applications demand sensors that can work in complex media, have a high sensitivity and can work with small volumes. In the case of bioprocess monitoring a fast response is important for control purposes. In both cases there is a need for monitoring a big number of analytes of different kinds. The analytes of interest may be small molecules with origin in the cells metabolism or macromolecules excreted or leaked from the cells.

Nature is full of examples of the most extraordinary sensitive and selective recognition events ranging from cells able to react to chemical gradients to protein recognition. By utilising recognition strategies adapted from nature in connection with a transducer is a popular construction often termed a biosensor. Most biosensors use reactions or recognition event at a surface. This fact makes the design of recognition surfaces crucial for the performance of a biosensor. Moreover the specificity, development as well as the purity and characterisation of analytical elements are pivotal.

A common choice in biosensor development is the use of antibodies as recognition element. Thus there is a need for platforms for screening and evaluation of different antibodies. In

addition to antibodies, also other recognition elements and measurement strategies could be part of the toolbox for rational design of biosensors.

The aim of this work is to explore different possibilities for design of biosensor recognition surfaces. In addition natural constrains and possibilities such as purity, stability and orientation of recognition elements are approached. The work has been carried out using SPR-technology, however the conclusions is not specific to SPR technology but can be translated to other transducers as well.

3. Biosensor systems

A biosensor is a device composed of two parts, a biological recognition element and a transducer that translates the biological signal into an electrical signal. The biological recognition element is often composed of biomolecules but can be bio-membranes, organelles or whole cells. Transducers can broadly be divided into electrochemical, capacitive, optical, acoustic and even magnetic and thermal transducers are reported (Lowe, 2007).

An alternative to biosensors are bioassays, although intrinsic an assay lacking a transducer they can share recognition technology with biosensors. ELISA is the golden standard for bioassays in many fields ranging from clinical assays and research to bioprocess monitoring (Mattiasson et al. 2009). However ELISA assays are laborious and require extensive incubation and washing steps giving a delay in response signal (Bracewell et al., 2001). The use of pipetting robots or the set-up of flow-injection ELISA assays partly bypass these problems and may in the long perspective transform bioassays into biosensors.

Different transducer technologies may rely on fundamentally different physical principles or phenomena but still have a few things in common. Fluidic systems are used to minimize the volume of sample used for the analyte. The small volume is also a consequence of the intrinsic small size of several transducer systems where a small size of the analyte handling system is a requirement for a fast response and precise measurement. Another common technology is the use of gold covered surfaces functionalized with thiolated alkanes in self-assembled monolayers (SAM). A gold surface is covered with a SAM of thiolated alkanes where the thiols bind to the gold surface. If a fraction of the molecules forming the layer contains a reactive group, this group in turn can be used for coupling of other molecules. In this way the surface can be modified with molecules, proteins or DNA as recognition elements. An alternative is to immobilise a spacer or matrix element to modify the environment or reduce unspecific binding. The resulting surface often contains a reactive group such as a carboxylic acid useful for binding of ligand molecules (Johansson et al., 1991). SPR, QCM and capacitive sensors utilise gold surfaces in most configurations. Electrochemical sensors are more diverse although also often using this technology (Lowe, 2007).

In the work presented here the sensing surface is arranged with a SAM on gold, with the affinity ligand either adsorbed to the surface or covalently coupled to the surface. By binding carboxylated dextran to the SAM a 3D environment is created for immobilisation of the proteins (Löfås et al., 1990). This has the advantage that a more native environment is created for the proteins in the hydrogel, reducing steric hindrance of the proteins. The carboxylation of the dextran matrix is exploited to attract the proteins designated for immobilisation by electrostatic interaction. This concentration to the surface requires the buffer containing the protein of interest to have a pH below the pI of the protein, rendering a net positive charge of the protein (Johansson et al. 1991).

Today there are several mature equipments for biosensor applications. Probably SPR and QCM are the most used techniques used commercially exposed with designs that are continuously improved. SPR based sensors are the most commonly implemented in industrial applications (Thillaivinayagalingam et al., 2010).

QCM is an acoustic sensor based on a crystal of piezoelectric material. Although SiO₂ is not the strongest piezoelectric material, it is the most common due to availability and ease of modification. When put under an alternating voltage the sensor starts to oscillate. If the thickness of the crystal is much smaller than its other dimensions, the resonance frequency of the sensor depends on the crystals thickness. Thus a QCM sensor is sensitive to mass changes on its surface (Araya-Kleinstaubler & Lowe, 2007; Becker & Cooper, 2011). The QCM technique is an alternative to the SPR results provided in this thesis.

Electrochemical sensors are extensively described with the most well known example represented by the blood glucose sensors (Newman & Turner, 2005). More complex constructs are reported with a defined alignment of oxidising enzyme and electron shuffling components (Teller & Willner, 2010). Related sensors are based on potentiometric sensors such as enzyme field effect transistors (ENFET), impedance and capacitive sensors use an electrode with the above described gold alkylethioles recognition layer layout. Analyte binding induces a displacement of counterions over the surface which alters the capacitance of the surface (Lindholm-Sethson et al., 2010; Mattiasson et al., 2009; Arya et al., 2007).

Nanoparticles can be used for biosensing applications using different transducer methods. Intrinsic plasmonic properties of the nanoparticles is utilised in localised SPR sensors and enhancement of Raman spectroscopy. By using particle-particle spacers sensitive to the environment, plasmonic gold nanoparticles can be brought in close contact with a concomitant

change in absorption (Aili et al., 2009). In other cases functionalised nanoparticles have been used as enhancers in other technologies. Other metal nanoparticles and particles of latex have also been reported (Haick, 2007).

SPR is like QCM a mass sensitive sensor but based on an optical transducer. The transducer is basically a refractometer measuring the refractive index close to a gold surface. When a light beam is totally reflected in a prism an evanescent field is formed at the surface of the prism. When a thin gold surface is brought within this evanescent field it will couple with the free electrons at the gold surface. The incident angle of the incoming light determines the size of the evanescent field created. At a certain angle a standing wave will occur at the gold surface and energy will be drained from the light beam. Hence, at resonance the intensity of the reflected light will be lower. The resonance angle will change if the refractive index change close to the surface. Water has a RI of 1.3 and proteins have a RI close to 1.5, thus SPR is a useful tool for studying biomolecular binding to the surface (Liedberg et al., 1993; Fagerstam et al., 1992).

The instrument employed in this work used a Kretschman configuration. The set up is shown in Figure 1 with a lightbeam entering the prisma, reflect at the prism and the angle where resonance occur is detected by a sensor array (Homola, 2008).

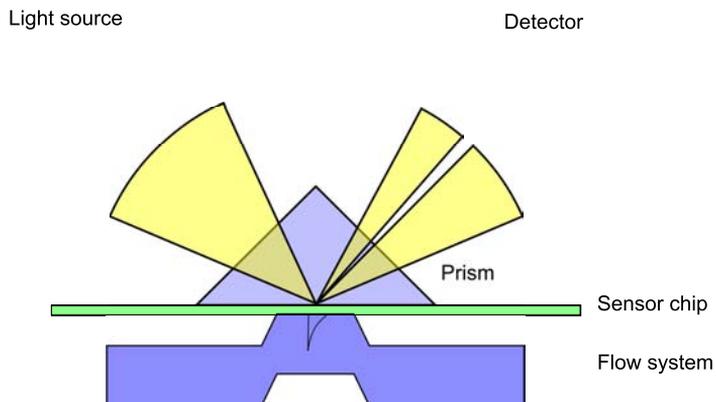


Figure 1: SPR setup used during this work.

There are several commercial devices for SPR measurements ranging from exclusive imaging equipment to simple single use totally integrated devices. Most systems have a fluidic handling system, a sensing surface and control and data handling software. The fluidic system may differ but most systems have in common that they are miniaturised to minimize sample

consumption. The high level of maturity of SPR systems makes them interesting for biosensor development as there are both advanced systems suitable for development and simpler systems suitable for sensor implementation.

4. Analytes in this thesis

The versatility of biosensor assay methodology provides a high flexibility for a huge variety of analytes that can bind the surface bound recognition element. In this thesis a limited number of analytes is studied which are applicable in cell culture monitoring.

4.1. Biomarkers

A biomarker is a feature that can be measured objectively in order to characterise or evaluate the physiological state of a biological entity. In several cases this is a molecule that can be used to diagnose or follow the progress or recovery from a disease. In clinical samples like blood or urine there are several challenges. The sample matrix containing the analyte is complex medium including everything from cells to biomolecules and ions. This requires that care is taken against unspecific binding at the sensor surface. Another problem is the fact that markers might not be specific for a special organ making them unfeasible for clinical diagnosis but useful in vitro where a well defined system is used.

4.1.1. LDH

Lactate dehydrogenase is a well described enzyme with a long use as a biomarker. Two different subunits, the M and the H, are combined in a tetramer. The LDH-H4 tetramer is found in heart cells and in erythrocytes while the LDH-M4 tetramere is found in skeletal muscular and liver tissue (Kopperschlager & Kirchberger, 1996; Read et al., 2001). Most other tissues have a combination of all isoforms giving a tissue specific fingerprint even though this fingerprint may differ between different species (O'Carra & Mulcahy, 1990). The evaluation of blood samples is thus ambiguous and LDH is today replaced in heart and liver diagnosis for more specific biomarkers. But may still find a use in clinic in cancer diagnostics (Kayser et al., 2010). LDH is a good marker of membrane integrity. There are reports indicating increasing levels of LDH during ischemia (Kotoh et al., 2011).

4.1.2. Albumin

Albumin is an abundant protein in blood plasma constituting 75% of total blood protein. The protein is important for maintaining the osmotic pressure that keeps the fluid in the blood from perfusing into the tissue. Additional functionalities are binding and transport of lipids, hormones and drugs and scavenging of oxidising agents (Francis, 2010, Quinlan et al., 2005).

Albumin is secreted by the liver and is thus a useful marker of hepatocyte function (De Bartolo et al., 2006). At inflammation albumin synthesis is decreased while acute phase proteins increase (Nilsson-Ehle et al., 2003).

4.1.3. Troponin

Troponin T (TnT) is a 38 kDa protein and a part of the tropomyosin complex responsible for contraction in muscles. The protein has three different isoforms where one is specific to cardiomyocytes (cTnT). Clinically troponin assays have replaced older enzymatic assays of less specific enzymes such as LDH, ALAT and ASAT (Nilsson-Ehle et al., 2003). In vitro cTnT assays have been used for toxicity assays (Andersson et al., 2010). cTnT is a membrane integrity marker with a fast release of the cytosolic pool of the protein followed by a slower release due to dissociation of cTnT from tropomyosin complex.

4.2. Analytes in vaccine production

Vaccine is still predominantly produced by virus propagation in fertilized eggs. Today several attempts are made to replace this old technology by cell derived protein based vaccines. A typical vaccine against seasonal flue consists of several antigenic components including HA as the most important one but also to some extent NA. Monitoring of the amount and quality of the different components are important during production and purification (Smith et al., 2009).

4.2.1. Hemagglutinin

Presently, influenza-A vaccines dominate production of vaccines. The two surface antigens that determine the specificity of the influenza virus are hemagglutinin (HA) and neuroamidase. Thus, assay methods for these, in particular HA, have high priority. HA is a trimer protein consisting of a membrane binding region inserted in the membrane of the virus and a more globular domain protruding from the virus surface. The globular domain contain sialyl acid binding regions specific to glycans in the human respiratory tract. Upon binding to glycans at cells in the respiratory tract the virus is internalized into the cells endosome where the low pH initializes a conformation change HA. The conformation change results in a protruding fusion peptide which is inserted in the endosome membrane. In this way the virus avoids the harsh environment of the endosome and gets access to the cell (Lodish et al., 2000).

HA is the primary target for neutralizing antibodies against influenza. Sterical hinderance of the sialyl acid binding region is a common target for those antibodies, this region is thus important for vaccine efficacy (Das et al., 2010).

5. Approaches in this thesis

In this thesis two approaches for improving the utility of a biosensor surface are employed: (1) to exploit the possibility of transient interactions for analyte recognition, and (2) orientation of the recognition element on the sensor surface. The prerequisites for these approaches are discussed below.

5.1. Exploiting transient interactions

Weak affinity or transient interactions are usually defined as interactions with a $K_d > 10^{-6}$ M. Weak interactions are very common in nature often occurring in connection with multivalency. Examples are the action of T-cell during immune response, white blood cells rolling on the surface of endothelial cells during inflammation and HA binding to sialic acid during invasion of their host cells (Ohlson, 2008).

In this thesis the possibility of using transient interactions in biosensing has been applied with glycoprotein interactions. The importance of glycosylation in mammalian proteins is shown by the fact that most proteins are glycosylated. Glycosylation has a key role in protein folding, for secreted proteins the half life in serum is determined by their glycosylation, it also have an important function in cell-cell recognition and hence in immune responses. Also pathogens take advantage of glycosylation to bind and invade cells, e.g. influenza hemagglutinin binding of sialic acid. During industrial production of proteins one important quality attribute is the glycosylation pattern of the product (Hossler et al., 2009). In this work glycostructures have been used as ligands for glycobinding proteins ensuring their function.

The utilisation of transient interactions in biosensors has the advantage of fast responses. Fast responses from sensors are necessary for control applications of bioprocesses where delay time in sensors is detrimental. The binding event to the surface ligand occurs at equilibrium and allows for continuous measurement (Ohlson et al., 2000). The drawback is that the sensitivity is lower since no accumulation of binding occurs at the surface. However, nature is full of transient interactions and this provides a useful source of systems for ligand screening.

5.2. Amplifying a surface biosensor interaction by orientation of the recognition element

The importance of a well characterized sensor surface is well recognized. There are several methods described for immobilization of antibodies or other molecules with defined orientation including anti-IGG-antibodies, biotin/streptavidin or His/Ni²⁺ capture and careful control immobilization conditions (Homola, 2008; Dutra et al., 2007; Rusmini et al., 2007, Pei et al., 2010). Traditionally, different chemical and physical methods have been used to immobilize antibodies and other molecules to surfaces, most of them resulting in a random orientation of the molecules of interest. This results in steric hindrance of analyte-binding and hence a loss of sensitivity and also impairment of kinetic evaluation of the binding event.

5.3. Exploiting the Protein G interaction

Proteins that can selectively capture the recognition element to be used on the biosensor surface is usually employed in purification preceding the biosensor surface preparation. In this thesis, this was done with LDH. This work combines purification with immobilization and orientation of the antibody ligand directly on the sensor surface. By capturing antibodies directly from crude solutions the purification steps are made redundant without any tagging of the antibody ligand. This strategy also encompasses the advantage of antibody orientation without the use of any tags or labels.

6. Summary of papers

6.1. Paper I - Glycoconjugates as transient ligands

Glycoconjugates were developed to exploit transient interactions with the vaccine component HA. The use of transient interaction has the advantage of fast measurement cycles. The assay not only gives a measure of the concentration of HA but can also indicate protein aggregation as aggregated proteins have several binding sites for the ligand which result in enhanced avidity.

6.2. Paper II - Assaying LDH

LDH is an appropriate target for toxicity testing. The second paper describes development of a possible SPR based biosensor for measurement of LDH. The importance of using well-defined and characterised components in ligand design is highlighted. The procedure of antibody selection is also highlighted.

6.3. Paper III – Ligand orientation by protein G

The paper describes a platform where covalently bound protein G is used to capture and orient antibodies to a dextran sensor surface. The protein G platform can be used for development of biosensors and screening and evaluation of antibodies. In addition the platform orients the captured antibodies giving them a higher binding efficiency compared with directly randomly coupled antibodies. Crosslinking of the captured antibodies demonstrated where the antibodies are first captured before covalently cross-linked to the surface.

7. Conclusions and future perspective

This thesis describes three methods of designing biosensors for meeting monitoring needs of cell culture and in pharmaceutical production. Different strategies of affinity ligand based approaches are demonstrated ranging from standard antibody immobilisation to in situ purification and orientation. In addition novel glycoconjugate ligands are demonstrated that utilise transient interactions for sensing. The importance of a well designed surface for interaction analysis is recognised throughout the work. This is exemplified by the protein G surface that has the possibility to capture antibody ligands from complex solutions while at the same time orienting them. The other example pursued in the thesis is the fine-tuning of the glycostructure fraction of the glyco-protein ligand used for HA detection.

In future, the need for well defined surfaces for biosensors will continue to grow. When applied to process monitoring applications the use of novel sensing ligands can be important for quantifying new analytical targets. Tools like the orienting protein G might also be a powerful platform in the development of new biosensors.

There are several trends that drive the development of biosensors. One important factor is the process analytical technology (PAT) initiative from the United States Food and Drug Administration, focusing on the development of novel methods for monitoring and controlling (bio)pharmaceutical production. Another trend that will impact biosensors favourably is the miniaturisation and decreasing prices of sensors and transducers making it possible to implement technology in industrial environments that was previous exclusive for research laboratories. Overall, this is promising for a further spread of biosensor technology and the development and implementation of biosensors in future.

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