Chapter 12

Surface Plasmon Resonance Biosensorics in Urine Proteomics

Peter B. Luppa, Jochen Metzger, and Heike Schneider

Abstract

Surface plasmon resonance (SPR) is a novel biophysical detection method. In combination with sophisticated surface chemistries and sensing instrumentations, SPR biosensors are approved as tools for molecular interaction studies. SPR plays also a role in interaction proteomics. Once being detected in urine, SPR helps to unravel the functions of new proteins. Due to its outstanding analytical characteristics, SPR also moves more and more into the realm of quantitative analyses in the clinical laboratory. Complex urine determinations of proteins and/or metabolites will bring the SPR biosensor both to the core lab and to point-of-care-testing.

This review delineates first the optical phenomena of SPR near to the gold surface, and also the main features of bioconjugation chemistry on a solid-state surface. Then the kinetic calculation of molecular interaction analysis using SPR is introduced. In order to portray the capability of the method, new applications in urine proteomics and proteinuria diagnostics are finally described in detail.

Key words: Surface plasmon resonance, Biosensor, Kinetic analysis, Bioconjugation, Protein array, Proteinuria

1. Introduction

1.1. Biosensors: Definition and Applications

1.1.1. Terms and Definitions

In the following chapter, we will stringently use the word *analyte* for an interesting protein, peptide or other macromolecule, which should be analyzed in a biological sample in terms of mass concentration or biochemical function by use of an SPR biosensor. In contrast, the term *ligand* will be used as natural interaction partner for the *analyte*. The *ligand* molecule is attached to the biosensor surface and enables the biospecific interaction with the *analyte*.

A biosensor integrates a *surface-immobilized biological element*, enabling a reversible biospecific interaction with the analyte,

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and a *signal transducer*. Compared to conventional analytical instruments, biosensors are characterized by an integrated structure of these two pivot components. The biological element is an attached layer of macromolecules qualified for biorecognition, such as enzymes, receptors, peptides, single-stranded DNA or RNA. Even whole cells or other complex systems are applicable for the biological element (1–7).

In general, there are two different devices: biocatalytic and bioaffinity-based biosensors.

- The *biocatalytic biosensor* uses enzymes as the biological compound, catalyzing a signaling biochemical reaction. Probably the most successful commercialization of biosensors is the near patient measurement of capillary glucose using various handheld systems with disposable reagent cartridges (8).
- The *bioaffinity-based biosensor*, designed to monitor the specific binding event with a ligand molecule, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies, or antibody-related substances for biomolecular recognition.

Many devices are connected with a flow-through cell, enabling a flow-injection analysis mode of operation. Biosensors combine high analytical specificity with the processing power of modern electronics to achieve highly sensitive detection systems. Up to now, they are already extensively used as diagnostic tools in point-of-care testing (9).

1.1.2. Measuring Principles

The general biosensor design is depicted in Fig. 1 (2). There are four types of biosensor detection modes: *electrochemical* (potentiometric, amperometric, or conductometric/capacitative), *microgravimetric*, *thermometric*, and *optical*. The latter detection mode is most widespread in use. All biosensor types may either be run direct (nonlabeled) or indirect (labeled). The direct sensors are able to detect the physical changes during the biospecific interaction, whereas the indirect sensors use signal-generating labels, which allow more sensitive and versatile detection modes in many cases. Although the latter are highly sensitive due to the analytical characteristics of the label applied, the concept of a direct sensor device is still fascinating. When using an optical detection system, such a biosensor holds multiple advantages due to its simplicity and makes the device progressive and future directed.

1.2. Surface Plasmon Resonance

Biosensors applying the surface plasmon resonance (SPR) principle for signaling feature the advantages of a label-free optical detection mode. The following advantages compared to other bioanalytical methods are worth mentioning:

- Molecular interaction analysis without modification of the analyte molecules.

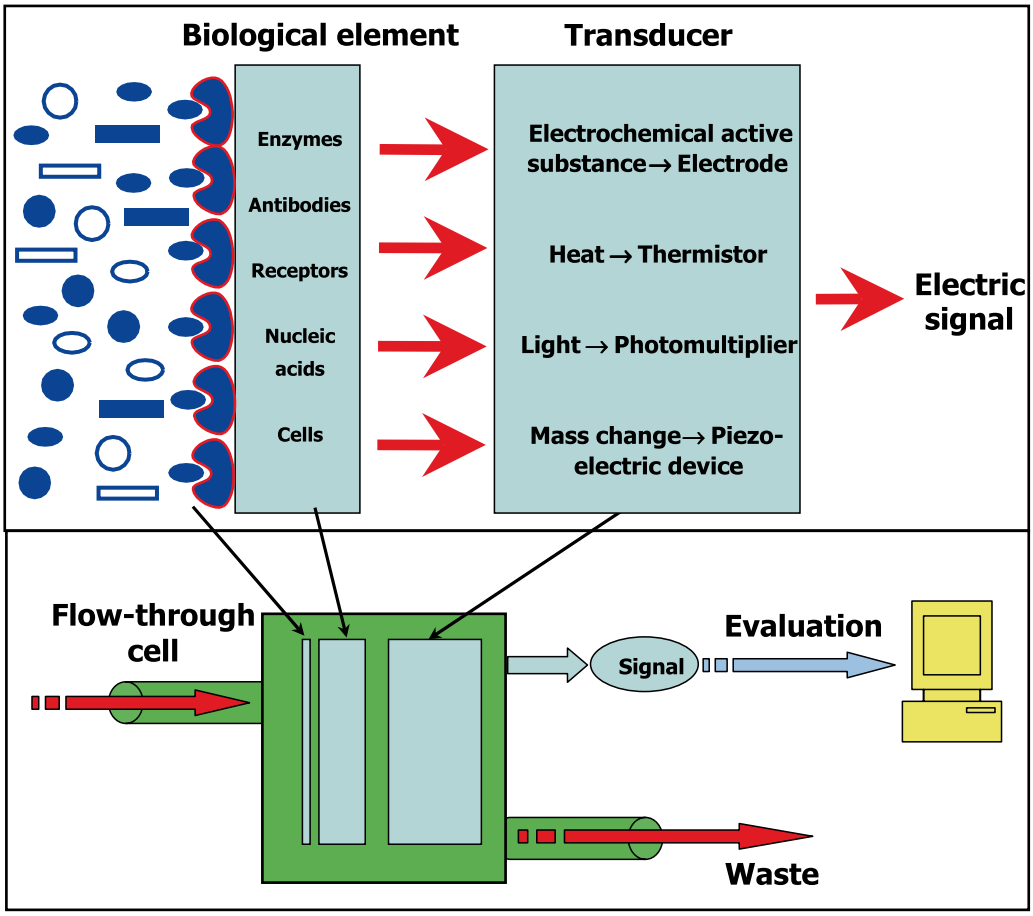


Fig. 1. General biosensor design depicting the intimate integration of biochemical recognition at the solid-state surface and the signal transduction.

- Universal approach to assay development due to the possibility to use native interaction partners without being influenced by the physicochemical properties of a label.
- Measurement of kinetic parameters without need of complex competition or displacement assays

1.2.1. General Application Fields

Applications of optical biosensors in life science comprise food, veterinary, pharmaceutical, and environment investigations, as well as numerous clinical applications in research and diagnostics. In the latter fields, the SPR technology was proven to be useful in a variety of tasks such as ligand fishing, high-throughput small molecule screening, epitope mapping, and others (9–12). Analytes are proteins in general, particularly enzymes, peptides, antibodies, and receptors, as well as oligonucleotides. But also

viruses, whole cells, and other complex systems are investigated. Worth mentioning are in particular the improvements in mass spectrometry-coupled biosensor technology.

In the last 3 years, more than 1,000 research articles were published each year with an increase of about 10% per annum. Most manuscripts are dealing with kinetic and equilibrium analysis formats to determine reaction rate constants and interaction affinities. But also the number of applications in analyte concentration quantification, notably in clinical diagnostics, is increasing. Closer information in this context is presented by the review surveys 2002–2005 by Rich and Myszka (13–16).

1.2.2. SPR Optical Phenomenon

SPR is a quantum optical phenomenon arising in thin metal films under conditions of total internal reflection of incoming monochromatic and p-polarized (i.e., the electric vector component is parallel to the plane of incidence) light at the interface of two transparent media of different refractive indices (RI) (17). At a certain angle, the incident light is completely reflected even though an electromagnetic field component of the light, the so-called evanescent field, penetrates the substance with a low RI (Fig. 2). That exponential decaying evanescence wave is then produced and penetrates through the metal film leading to the oscillation of free electrons. Surface plasmons are electromagnetic surface density fluctuations of the oscillating electrons at the metal-liquid interface and are addressed quantum mechanically as quasi particle. The evanescent wave has components in all spacial orientations. It penetrates and "interrogates" even thicker biological layers up to 300 nm, regardless of a potential sample turbidity. In response to the dissipation of energy, there is a sharp dip in the intensity of reflected light. The corresponding light angle is called SPR angle. Its position depends on the amount of surface-bound molecules and is detected by measuring the reflected light intensity. The SPR angle is highly sensitive to changes in the RI of a thin layer adjacent to the metal surface. These changes

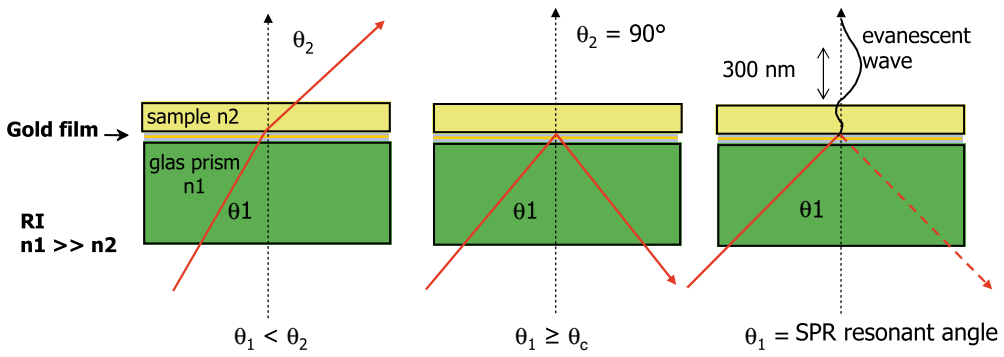


Fig. 2. Optical principle of SPR technology.

correspond to the evanescent wave probing distance when a volume, defined by the size of the illuminated area and the penetration depth, is investigated. For example, when biomolecules interact with already immobilized molecules in this volume, an increase in the surface concentration occurs and the resonant angle shifts to greater values. The magnitude of the angular shift, defined as the SPR response depends on the mean RI change due to the biomolecular interaction in the probed volume.

Gold (Au) is most practical to be used as metal film. It produces a strong, easy to measure SPR signal in the near infrared region. On one side, Au is resistant to oxidation and other contaminants. On the other side, this metal (in case when freshly spattered on a glass or plastic support) is reactive to a series of organic compounds in order to be immobilized onto the metal surface. The SPR generating surface is usually composed of approximately 50 nm thick layer of Au deposited. Other metals are applicable for the generation of plasmons but are not as practical. Indium is very expensive, copper, and aluminium have a too expanded SPR response, and silver is susceptible to oxidation.

In general, there are three configurations of biosensor devices that are suited to generate SPR resonance:

- Prism-based systems (see Fig. 3, configuration applied for most Biacore systems),
- Grating-based systems and
- Optical waveguide-based SPR systems.

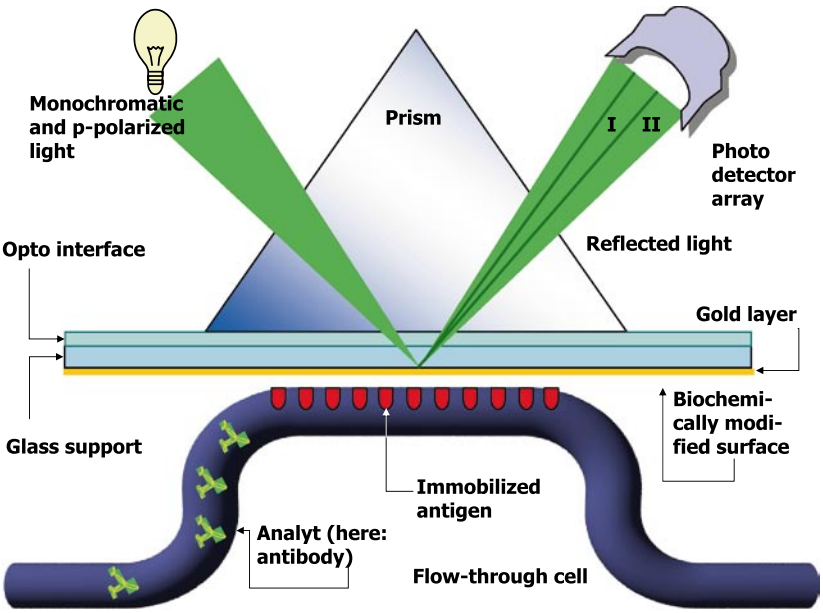


Fig. 3. Design of a typical SPR biosensor showing how the biological interactions occur in the flow cell on the functionalized solid-state surface of the sensor.

In a typical biosensor run, one of the interaction partners is coupled to the sensor surface, whereas the dissolved opponent passes over the surface under continuous flow conditions. The SPR signal as a measure of the SPR angle is generated by a change in mass concentration over the biosensor chip as the soluble macromolecular analyte binds to or dissociates from the immobilized ligand. There is a linear relationship between the amount of bound material and the shift of the SPR angle. Scanning mirror biosensors measure the SPR angle shift in millidegree as a response unit to quantify the binding of macromolecules to the sensor surface. The response also depends on the refractive index of the bulk solution.

Binding is expressed in an increase of arbitrary resonance units (RU) and plotted in form of a sensorgram (Fig. 4). The sensorgram is the transformation of the detector array output to the RU as a function of time. The absolute value of the signal is not relevant to biosensing. It is the change in signal that is important. The response of 1,000 RU corresponds to a shift of 120 millidegree in the resonant angle position, which represents a protein concentration of about 1.0 ng/mm^2 or in a bulk RI change of about 10^{-3}.

Recently, the development of grating- and waveguide-based systems enables protein arrays, which use the "SPR imaging technique" (18) or a multichannel SPR sensor based on spectroscopy of plasmons and wavelength division multiplexing of sensing channels (19). SPR spectroscopic imaging was also established as "SPR wavelength interrogation-based sensor" (20).

The detection principle limits the size of the analytes in solution, which are bound to the chip surface. If the molecular weight of the compound is below 200 Da, the change in refractive index during the biospecific interaction is too low to be detected directly. Therefore, SPR proved to be more sensitive for larger analytes

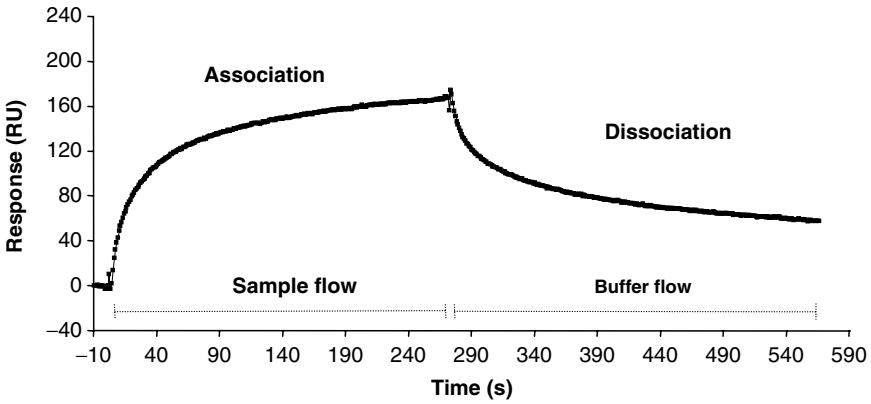


Fig. 4. Typical SPR sensorgram showing association of analyte with the ligand during sample injection and dissociation of ligand-bound analyte after the subsequent switch to running buffer.

than for small molecules. This is an evident disadvantage of the SPR technology, which is, however, controllable by skilled scientists. The penetration depth of the evanescent wave of 200–300 nm also determines the size of macromolecules or particles that can be studied. Particles larger than 400 nm cannot be measured totally. As a result, the signal is not linear related to the amount of bound particles. Under these circumstances, a quantitative or kinetic analysis cannot be performed – but it is possible to study the binding qualitatively.

1.3. Real-Time Biospecific Interaction Analysis Using SPR Sensorics

Biomolecular interactions can be directly monitored using so-called evanescent field sensors. Conventionally, biomolecular interactions are studied using techniques as immunoassays (ELISA or RIA), equilibrium dialysis, affinity chromatography, and spectroscopy. SPR gives two main advantages over these techniques: The binding events are monitored in real-time and it is not necessary to label the interacting biomolecules. Thus, a kinetic evaluation of these interactions is possible. In principle, absolute reaction rate constants can be measured.

1.3.1. Kinetic Measurements Using Biosensor Sensorgrams

The fundamentals for the kinetic evaluation of SPR biosensor data were compiled in the 1990s (21–23). Various commercially or freely available evaluation programs are able to calculate the association and dissociation data (see below). The evaluation algorithms perform a nonlinear regression analysis of the measured sensorgrams.

The analysis of binding events can follow the respective interaction models:

- 1:1 binding;
- 1:1 binding with mass transfer;
- Bivalent analyte binding;
- Heterogeneous analyte or ligand binding and
- Two-state reaction with conformational change.

The most appropriate model will be chosen by a fitting analysis based on the residual plots with low χ^2 values, which were obtained from analysis of the sensorgrams, the χ^2 value being a standard statistical measure of the closeness of fit.

Exemplarily, this is depicted for a Langmuir 1:1-binding: If a soluble analyte molecule A reacts with a ligand B (e.g., f_{ab} fragment with an immobilized epitope), this reaction is calculated as a kinetic pseudo-first-order. The rate constant of the complex formation is described by the association constant k_a, the rate of the dissociation being defined by the dissociation rate constant k_d.

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{AB}$$

These rate constants are defined as follows:

$$\mathrm{d[AB]} / \mathrm{d}t = k_a[A][B]$$

$$-\mathrm{d[AB]}/\mathrm{d}t = k_d[\mathrm{AB}]$$

The net formation rate of the complex formation (*association phase*) in the SPR sensorgram is defined as sum of the complex formation and simultaneous dissociation:

$$\mathrm{d[AB]}/\mathrm{d}t = k_a[A][B] - k_d[\mathrm{AB}]$$

where

- [AB] is the amount of formed complex, corresponding to the measured parameter R.
- $[A]$ is the concentration C of the analyte protein in the sample solution.
- $[B]$ is the current available amount of nonbound ligand $(R_{max} - R)$.

For the *association kinetics*, it follows from the above that:

$$\mathrm{d}R/\mathrm{d}t = k_a C(R_{max} - R) - k_d R$$

This can be transformed into:

$$\mathrm{d}R/\mathrm{d}t = k_a C R_{max} - (k_a C + k_d)R \qquad (1)$$

In a plot of $\mathrm{d}R/\mathrm{d}t$ versus R, both k_a and k_d can be determined from a single association sensorgram in case R_{max} is known. If this is not the case, the determination of sensorgrams at various analyte concentrations is advisable.

The transformation of Eq. 1 under the assumption that C is constant produces:

$$R = k_a C R_{max} / (k_a C + k_d) \times \left(1 - \mathrm{e}^{-(k_a C + k_d)t}\right) \qquad (2)$$

Equation 2 can be used for a nonlinear regression analysis of the sensorgrams cohort.

Dissociation kinetics: After the injection phase, when the surface is rinsed with buffer and merely the complex dissociation process takes place, the following equation is valid:

$$-\mathrm{d}R/\mathrm{d}t = k_d R$$

This zero-order reaction can be described by

$$R_t = R_0 \times \mathrm{e}^{-k_d(t - t_0)} \quad \text{or} \quad \ln R_0 / R_t = k_d(t - t_0) \qquad (3)$$

Equation 3 can now also be used for nonlinear regression analysis of the dissociation phases of the sensorgrams cohort in the $\mathrm{d}R/\mathrm{d}t$ versus R plot according to the transformation to:

$$dR/dt = k_d R_0 \times e^{-k_d(t-t_0)}$$

1.3.2. Implications from the Kinetic Measurements

According to these formulas, it is possible to determine the rate constants for association and dissociation from the measured binding curves. The premise hereof is simply the knowledge of the molar concentration of the analyte. The kinetic evaluation process can be done by use of an adequate software programme. Examples are:

- BiaEvaluation (Biacore, http://www.biacore.com) (24)
- SPRevaluation (patented by Maureen D. O'Connor-McCourt) (25) or
- SCRUBBER-2 (http://www.cores.utah.edu/interaction/software.html)

There are, however, only few interactions recorded on SPR biosensors, which can be correctly described by the simple 1:1 interaction model. Several other more complicated binding mechanisms are possible:

- Simple bivalent
- Two-state or three-state conformational change
- Surface heterogeneity

Such kinetic reactions of higher order are best described by multiple rate equations. But the prerequisite should be considered that fitting data concerning the association and dissociation phase are to be collected for a concentration series of the analyte. This so-called global fitting analysis discriminates between the above mentioned reaction types.

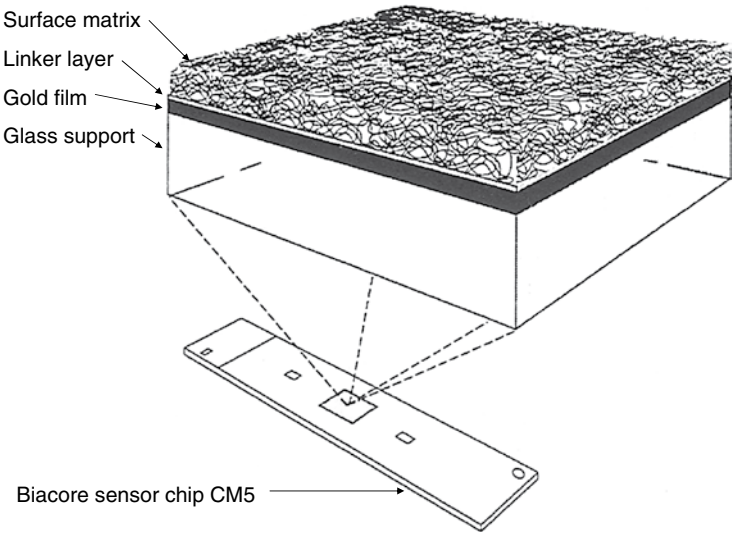
There is also a series of other factors affecting the binding response. One part are simple artifacts, and most important are, however, avidity effects, mass transport, or rebinding effects (22).

Mass transport phenomena can be reduced by working at low densities of immobilized binding partners on the sensor surface. The mass transport coefficient k_m, given in Fig. 5, is dependent on the diffusion coefficient of the analyte, the dimensions of the flow cell and the flow rate. By incorporating a mass-transport step into the mathematical binding model, it enables to calculate reaction rate constants (24).

Under steady-state conditions (association of the analyte with the surface is balanced by the simultaneous dissociation of the surface-bound complex), the so-called equilibrium analysis is able to derive the thermodynamically relevant association constant:

$$dR/dt = k_a C(R_{max} - R_{eq}) - k_d R_{eq} = 0 \quad \text{at equilibrium.}$$

The equation gives:



Courtesy of Biacore, part of GE Healthcare

Fig. 5. Interactions at the biosensor surface under special consideration of mass transfer phenomena.

$$k_a C(R_{max} - R_{eq}) = k_d R_{eq}.$$

This can be transformed to:

$$k_a / k_d = \text{Association constant } K_A = R_{eq} / C(R_{max} - R_{eq})$$

or

$$R_{eq} / C = K_A R_{max} - K_A R_{eq}$$

The Scatchard plot R_{eq}/C versus R_{eq} gives a straight line from which R_{max} and K_A can be calculated.

1.4. Technical Aspects

1.4.1. Flow Cell and Microfluidics

Apart from cuvette systems, e.g., applied in the IAsys device, SPR measurements are usually performed in flow injection analysis (FIA) systems. Biacore devices use such flow cells on the biosensor surface for interaction analysis. These flow cells are formed when a microfluidic flow system is brought into contact with a sensor surface on which one interacting partner is immobilized. The second interaction partner is delivered in the solution by being pumped into the FIA cell.

Several flow system designs have evolved:

- Serial flow cell systems
- Independent flow cell systems
- Hydrodynamic addressing flow cell systems

One major advantage of the flow cell design is that there are at least two flow cells (FC) of which one may act as a reference cell when being loaded with a reference ligand in order to subtract unspecific binding phenomena. In the clinical laboratory, this is an imperative prerequisite for analysis when applying (un)diluted serum or urine samples. Thus, the gross signal can be subtracted from the reference signal to gain a specific sensorgram.

There are, however, problems when using such FIA systems. Often, an interdiffusion of sample and of flow buffer is encountered during the transport process from the inlet to the interior of the flow cell. To minimize this problem, Biacore designed an integrated μ-fluidic cartridge (IFC) system that brings the sample loops and valves close to the reaction site on the biosensor surface. This IFC consists of a series of precision cast channels built-in a silicon polymer plate, forming sample loops and flow channels. The unit is controlled by a series of pneumatically driven diaphragmatic valves directing the solvent flow through a chosen loop to the solid-state surface. The material properties of the silicon polymer and the precision of the docking mechanism prevent leakages from the channels.

The traditional flow cell technology, which uses a small number of channels to flow the analyte sequentially in a single direction, limits flexibility, throughput, and optimization of experimental conditions. The *Flexchip* from Biacore uses the "single pass, multi-target flow cell system" for an array format. Potential ligands are spotted onto a gold-coated surface of an array chip. A sealed window with inlet/outlet valves is then positioned over the array to form a flow cell and inserted in the instrument. A solution containing the interacting analyte is injected over the array and interactions on each spot are detected in real time via grating-coupled SPR. Up to 400 interactions can be monitored in a single experiment, which is beneficial for proteomics applications (26).

Also, the new *ProteOn XPR36* from Bio-Rad Laboratories expands the flow cell approach to SPR by providing multiple flow channels. A multichannel module forms six channels on the sensor chip surface, and up to six different ligand samples can be immobilized in each of the channels. A second set of channels is superposed orthogonally, creating a crisscross pattern of two sets of flow channels. This results in a 6×6 interaction array. The response at each interaction spot generates 36 sensorgrams corresponding to six analytes interacting with six ligands.

The ProteOn XPR36 utilizes a synchronized sequential scanning illumination system coupled with an imaging device to detect the SPR response for each point on the sensor chip. The measurement uses a total of 78 spots, 36 of which represent the interaction data. The additional 42 spots are called interspots and are used for normalization.

1.4.2. The Biosensor Surface

One of the most striking advantages of SPR analysis is the wide range of different proteins that can be measured in a quantitative way without major nonspecific binding effects. The SPR response is a measure of the RI changes of a chip surface, which occur during binding of an analyte protein to a specific surface matrix. But also the sample bulk solution itself plays a role in the RI change. The instrument and the experimental design, however, can eliminate this signal.

Therefore, it can be stated that the SPR response primarily reflects mass concentration changes of biomolecules on the sensor chip in the flow cell. By use of radiolabeled proteins, Stenberg et al. were able to correlate closely the absolute surface concentration of protein to the SPR response in a classical paper (27). They applied antibodies, transferrin, and human chymotrypsinogen as radiolabeled proteins. Responses were linear correlated over the range of 2–50 ng/mm^2 surface concentrations. Interestingly, there was no significant difference in specific responses for the different proteins tested. This close agreement in RU response makes the SPR sensor a very appropriate tool for urinary protein determinations. The smallest response, which can be reliably measured in different SPR devices, is around 10 RU, corresponding to a potential lower limit of detection of 10 pg/mm^2.

In the commonly used biosensor chip formats, a surface matrix of noncrosslinked carboxymethyl dextran (CMD) is applied for covalent attachment of ligands (28). The main reasons for its broad application are:

- High ligand binding capacity
- Resistance to alkaline and acidic treatment
- Provision of a hydrophilic environment
- Applicability to a wide range of coupling chemistries

It should be stated, however, that partially due to the hydrogel-like nature of the CMD matrix, nonspecific binding phenomena and mass-transport limitations may occur, which lead to misrepresentation of true binding kinetics. Therefore, it is sometimes more preferable to use planar surfaces such as self-assembling monolayer (SAM) composed of long chain *n*-alkylthiols or polyethylene glycol block copolymers as functionalized surface coatings. Another advantage for the use of a SAM rather than a CMD layer is the possibility to construct biosensor supports with various reactive groups allowing immobilization of combinations of two or more ligands.

1.4.2.1. CMD-Coated Biosensor Surface

The commercially available CMD-coated biosensor supports (e.g., CM5 sensor chips from Biacore (Fig. 6)) are sufficiently eligible for the majority of biomolecular interaction analyses. In cases with special demands on CMD chain length, molecular structure or carboxymethyl composition, dextran coatings can be efficiently

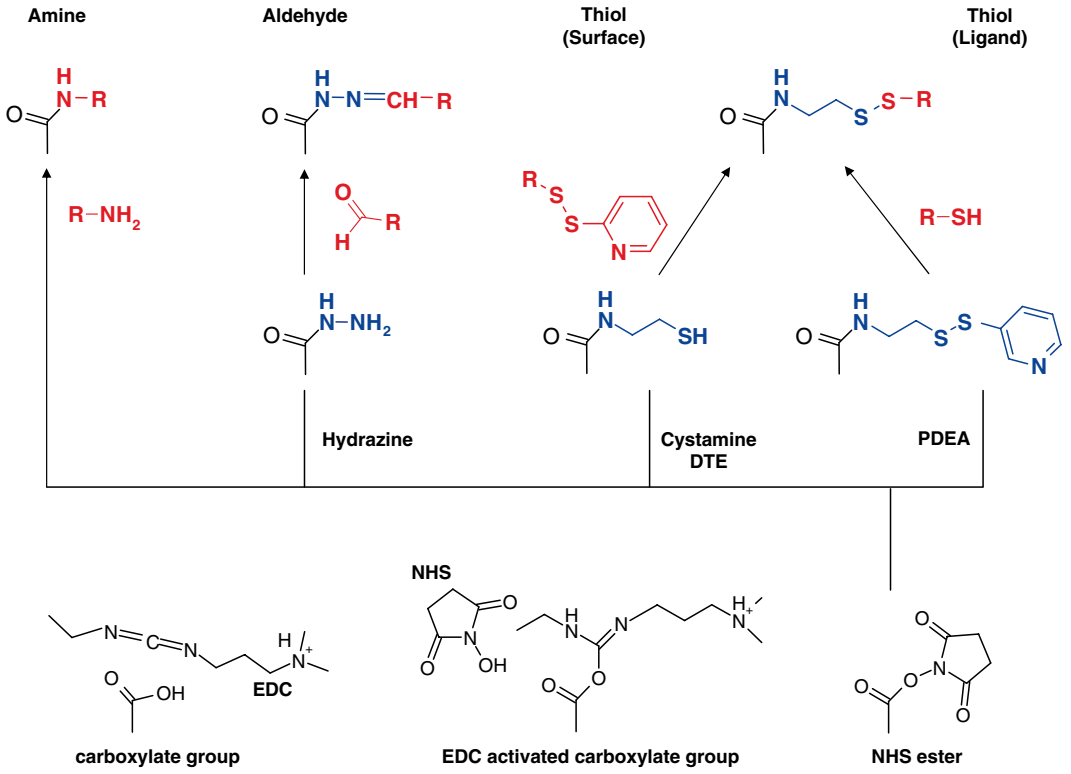


Fig. 6. Design of a SPR biosensor chip.

prepared after formation of a 16-mercapto-hexadecane-1-ol linker layer on the gold surface followed by an epichlorohydrin treatment. The introduced epoxide functions readily react with the dextran, which can subsequently be carboxymethylated to different degrees of substitution using bromoacetic acid (29).

1.4.2.2. SAM-Coated Biosensor Surface

SAM formation is initiated by the interaction of sulfur of *n*-alkylthiol head groups with gold atoms. After initial chemisorption, which takes only a few minutes, a higher degree of conformational order is reached after several hours by the subsequent reorganization of the hydrophobic *n*-alkyl chains. This late crystallization-like process is driven by lateral interchain interactions, such as van der Waals, steric, repulsive, and electrostatic forces, and leads to a densely packed rigid arrangement of the molecules on the gold surface (for review see (30)).

Due to these slow rearrangement processes, it is necessary to expose the gold-coated surfaces to the *n*-alkylthiols for an extended period of time. There are other factors, however, that affect the stability of the SAM, like total *n*-alkylthiol concentra-

tion and alkyl chain length. Generally, ethanol solutions containing 1 mM of 6- to 16-C long *n*-alkylthiols at incubation times of more than 12 h are recommended for optimal results in SAM formation.

1.4.2.3. PEG-Coated Biosensor Surfaces

Polyethylene glycol block copolymers are starting materials for the preparation of high-density grafted polymer brushes. Due to their resistance to nonspecific adsorption, they play an important role as surface modifiers of silica substrates, i.e., for the production of medical implants and nonbiofouling devices. Preparation and functionalization rely on a complex and sophisticated chemistry due to the fact that polymerisation is initiated directly on the surface. This restricts their usefulness in SPR biosensorics to certain special applications. A method that combines self-assembly of *n*-alkanethiols on gold and surface-confined atom transfer radical polymerization of poly(2-vinylpyridine) to determine thermodynamic adsorption properties of fibronectin adhesion-promoting peptide is given by Li et al. (31).

1.4.3. The Optointerface

Another pivotal component for the generation of the SPR signal at the chip surface is the optointerface enabling an optimal optical coupling. The gold film is usually deposited on a thin glass slide, which is tightly coupled to the glass (or moulded plastics) prism by use of an optically matched soft polymer. This coupling technique is comparable to the use of immersion oils for enhancing the optical resolution in light microscopy. Instead of oily liquids, however, the soft polymer is applied (see also Fig. 3) (21).

2. Materials

2.1. Commercially Available Systems

The majority of researchers use Biacore SPR systems. Also, in literature, the vast majority of work is performed using Biacore platforms (13–16). In the last 2 years, however, the quote of mentionable manufacturers increased significantly. Table 1 shows a comprehensive (but surely not complete) survey of commercially available SPR biosensor devices.

Because of the large number of applications, in particular for medical laboratory diagnostics, we focus this survey on Biacore platforms and chips, but many aspects may be applied also to other available SPR instruments.

2.2. Buffer Solutions

All buffers for biosensor measurements should be sterile-filtered using 0.2 µm FP CA-S filters (Whatman) and exhaustively helium-degassed before application.

Table 1
Survey of commercially available SPR Biosensor instrumentations (status as of December, 2006)

Biosensor type	Company	Website
Autolab Esprit/Springle	Eco Chemie BV, Utrecht, The Netherlands	www.ecochemie.nl
Biacore 2000, 3000, A100, C, X, Q, J, T100, Flexchip[a]	Biacore AB, Uppsala, Schweden (newly acquired by GE Healthcare)	www.biacore.com
BIOS-1	Artificial Sensing Instruments, Zürich, Switzerland	www.microvacuum.com
Biosuplar	Analytical µ-Systems GmbH, Sinzing, Germany	www.biosuplar.de
FT-SPR 100	GWC Technologies, Madison, WI, USA	www.gwctechnologies.com
IAsys[b]	NeoSensors, Sedgefield, UK	www.neosensors.com
MultiSPRinter	Toyobo Co., Ltd., Fukui, Japan	www.toyobo.co.jp
OWLS	MicroVacuum Ltd, Budapest, Hungary	www.microvacuum.com
Plasmonic	Hofmann Sensorsysteme, Wallenfels, Germany	www.plasmonic.de
ProteOn XPR36	Bio-Rad Laboratories, Hercules, CA, USA	www.biorad.com
SensiQ	Nomadics, Inc., Oklahoma City, OK, USA	www.nomadics.com
SPR 670	Rikei Corporation, Tokyo, Japan	www.rikei.com
SPRi Array	GenOptics, Orsay Cedex, France	www.genoptics-spr.com
Spreeta TSPR2KXY Biosensor	Texas Instruments, Dallas, TX, USA	www.spreeta.com
SR7000 Surface Plasmon Resonance Instrument	Reichert Analytical Instruments, Depew, NY, USA	www.reichert.com
VirChip	Vir A/S, Taastrup, Denmark	www.vir.dk

[a]Formerly "ABI 8500 Affinity Chip Analyzer" from Applied Biosystems, Foster City, CA, USA
[b]Formerly product of Affinity Sensors

For Biacore sensor systems, HEPES-buffered saline (HBS) is preferably used as general running buffer.

HBS-N: 10 mM HEPES pH7.4, 0.15 M NaCl.

HBS-EP: 10 mM HEPES pH7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% v/v surfactant P20.

HBS-P: 10 mM HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v surfactant P20.

These buffers may be modified individually in order to suppress possibly occurring nonspecific binding phenomena. These modifications include the addition of various compounds such as bovine serum albumin, human serum albumin, Tween 20, etc. at different concentrations.

Ligand immobilizations in a biosensor system are best performed by using buffer systems with low ionic strength to favor covalent binding of the ligand to the chemically preactivated biosensor matrix by electrostatic attraction. Additionally, the pH of the buffer should be below the isoelectric point of the ligand. Recommended buffers are 10 mM formiate for pH 3.0–4.5; 10 mM acetate for pH 4.0–5.5; 5 mM malate or 20 mM 2-(*N*-morpholino)-ethanesulfonic acid for pH 5.5–6.5; 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for pH 6.5–8.0, and 10 mM borate for pH 8.0–10.0.

2.3. Preparation of Functionalized Chip Surfaces

2.3.1. Preparation of a CMD Biosensor Surface

For the preparation of CMD biosensor surfaces, the gold slides have first to be coated with hydroxyalkylthiols, such as 11-mercaptoundecan-1-ol or 16-mercaptohexadecan-1-ol. Adsorption of the alkylthiol compounds on the gold is readily achieved by contacting the slides with a 1 mM ethanolic solution for 18–20 h. This and all subsequent reactions are best performed at room temperature. After extensive washing with 96% ethanol and distilled water to remove unbound material, the gold surface is treated with a 0.6 M solution of epichlorohydrin in a 1:1 mixture of 0.4 M sodium hydroxide and bis-2-methoxyethyl ether for 4 h. After an additional washing with distilled water and 96% v/v ethanol, the slides are incubated for 20 h in a 30% (wt/vol) basic dextran solution (Sigma), which was prepared in 100 mM sodium hydroxide. For the substitution with carboxymethyl groups, the CMD-coated slides are finally incubated with 1 M bromoacetic acid in 2 M sodium hydroxide for 16 h. After rinsing with 96% ethanol and drying under a stream of argon, the CMD-coated slides are mounted onto a chip carrier and inserted into the biosensor flow system.

2.3.2. Preparation of n-Alkylthiol-Based SAM

Functionalized SAM surfaces are prepared by immersing gold slides in a 1 mM ethanolic solution of the appropriate *n*-alkylthiols for 18–20 h at room temperature. SAM-coated gold surfaces are subsequently rinsed with 96% v/v ethanol, dried under an argon stream, assembled onto a chip carrier, and inserted into the biosensor flow system.

3. Methods

3.1. Biochemical Immobilization Strategies

The choice of the appropriate immobilization technique depends on the chemical properties of the ligand. Due to the requirement to retain analyte-binding activity over a series of measurements and postanalytical regeneration steps, covalent coupling is generally favored over noncovalent capturing. However, there are certain instances, where affinity tags and adaptor proteins are more amenable for ligand binding.

- First, in the case of biomolecules with a low isoelectric point and high electrostatic repulsion from the hydrophilic biosensor surface, such as nucleic acids, polysaccharides, and acidic proteins, conjugation or fusion to affinity tags often represents the only viable option to achieve stable attachment under physiological salt and pH conditions.
- Secondly, affinity-mediated capturing instead of rigid chemical coupling to the biosensor surfaces better preserves full analyte binding activity of labile proteins. In this context, it is worth mentioning that for affinity capturing, the protein structure can be better maintained by the addition of stabilizing agents and cosolvents, whereas this is in most cases not applicable for covalent coupling due to the risk of chemical side reactions.
- Thirdly, affinity tag-mediated ligand immobilization allows site-directed orientation, which often improves the accessibility of the analyte to its binding regions. The latter property is important in particular for the immobilization of synthetic peptides or other small molecules where reactive groups are lacking or reserved for biological function.

3.1.1. Strategies for Covalent Ligand Immobilization

CMD and SAM biosensor surfaces both allow the application of multiple chemistries for ligand immobilization. Covalent attachment of natural or synthetic ligands can be achieved by derivatization of the functionalized biosensor surface, for example, with amine- or thiol-reactive cross-linker containing *N*-hydroxysuccinimidyl or maleimidyl groups. The standard chemistries commonly used for the coupling of amino acid or carbohydrate moieties are summarized in Fig. 7.

A central role in all chemical coupling reactions play the water-soluble amine-reactive cross-linker *N*-ethyl-*N*′-(3-diethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). A combination of these two reagents can be used for rapid transformation of carboxylate into active ester intermediates, which in turn are susceptible to the attack of primary amines and other nucleophilic groups. Since all proteins contain amine functions at

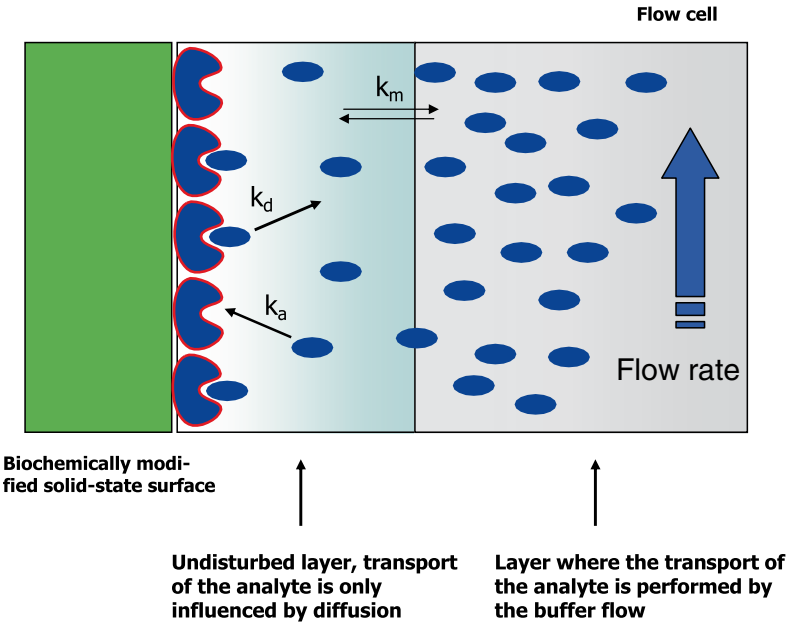


Fig. 7. Overview of approved immobilization chemistries for covalent ligand coupling on SPR biosensor matrices (abbreviation: *PDEA* poly(2-(diethylamino) ethyl methacrylate)).

their *N*-terminus and in ε-position of lysine side chains amine linkage is recommended as first choice for the covalent attachment of proteins. However, the carbodiimide reaction requires uncharged amino groups. Hence, for ligands with an isoelectric point less than 3.5, other coupling chemistries have to be applied. One alternative method is coupling by thiol-disulfide exchange. The introduction of disulfides can either be performed on the chip by injection of cysteamin over an EDC/NHS-activated surface followed by dithioerythritol-mediated reduction of free sulfhydryls or on the ligand by thiolation of carboxylate or amine functions with pyridyl disulfide containing heterobifunctional cross-linker, such as 2-(2-pyridinyl-dithio)ethaneamine or 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio) toluene.

In situations where polysaccharides and glycoproteins serve as ligands, immobilization can be carried out by selective periodate oxidation of *cis*-diols and conjugation of the resulting aldehyde groups on a hydrazide preactivated support. One representative example for the use of the aldehyde chemistry in protein attachment is the oriented immobilization of IgGs via their Fc-localized carbohydrate moieties (32).

3.1.2. Affinity-Based Strategies for Ligand Binding

The major challenge in the development of an affinity-based coupling strategy is the selection of an appropriate combination of affinity tag and surface immobilized capture agent. Especially,

Table 2
Affinity-tags and capture molecules for SPR biosensor applications

Tag	Capture	Mode	Comments
Fc portion of Igs	Protein G Protein A Protein M	Fc-receptor binding	– Oriented immobilization of mAbs, i.e., for epitope mapping – Selection of Fc-receptor depends on Ig isotype
Fc portion of Igs	Anti-human Fc RAMFc	Anti-Ig binding	– Oriented immobilization of mAbs – Recommended for the capture of Fc fusion proteins – Anti-Immunoglobulins must be affinity pure
Biotin	SA Avidin Neutravidin	Biotinylation/ SA-binding	– Extremely stable affinity interaction (10^{15}/M) – Coupling of oligonucleotides and peptides – Small molecules require the introduction of a linker sequence – Other Biotin/SA-Systems are available, i.e., on the basis of SA-binding peptides
Poly-His	Ni^{2+}-Chelate	Poly-amino acid fusion/Nickel-binding	– Short recognition motif – Weak binding may lead to gradual loss of ligand – Binding is affected by EDTA, imidazole and acids – Anti-His mAbs can be used as an alternative capture reagent
GST	anti-GST mAbs	Protein fusion/ Ab-binding	– GST allows simple one-step purification of fusion proteins prior SPR analysis – High affinity anti-GST mAbs are available that resist harsh regeneration conditions
hAGT	Benzylguanine	Protein fusion/ substrate linkage	– Highly stable due to the formation of a thioether bond – No further purification of fusion proteins needed

hAGT O6-alkylguanine-DNA-alkyltransferase, *EDTA* ethylene diamine tetraacetic acid, *GST* glutathione-S-transferase, *Ig* immunoglobulin, *mAb* monoclonal antibody, *RAMFc* rabbit anti-mouse Fc, *SA* strepavidin

the ability to mediate tight interaction under a broad variety of experimental conditions is of particular importance in this context. The decision which affinity labels should be applied crucially depends on the chemical and physical characteristics of the ligand. Table 2 outlines the most accepted affinity methods for biosensor applications. With exception of antigen capturing by surface-immobilized specific antibodies, these capturing methods require

modification of the target protein by either chemical reaction, i.e., with biotinylation reagents, or by genetic fusion to small affinity protein domains or polyamino acid sequences.

To immobilize capture antibodies in a site-directed manner on the biosensor support, proteins are available, which specifically react with the Fc portion of immunoglobulins. These include the staphylococcal Fc-receptor proteins A and G as well as anti-immunoglobulins from animals being immunized with species-specific Fc-fragments, most typically of mouse or human origin. It should also be mentioned that, besides capturing of xenoantibodies, antiimmunoglobulins can be applied for the immobilization of recombinant Fc fusion proteins.

The *streptavidin-biotin system* provides a convenient method for the capture of DNA oligonucleotides and peptides since the conjugation of biotin can be performed directly during automated synthesis. Another important area of application concerns immobilization of acidic proteins, polysaccharides, and glycoconjugates due to the fact that the streptavidin–biotin interaction can overcome electrostatic repulsion of these anionic compounds from the biosensor surface. A variety of biotinylation reagents directed against different functional groups are commercially available. The most important are NHS-biotin for primary amines, biotin hydrazide for carboxylic acids and aldehydes, iodoacetyl-biotin, and biotin maleimide for thiol compounds, and photoactivatable biotin derivatives for nucleic acids.

In recent years, a series of *fusion tags* have been developed for applications such as protein purification and microarray preparation. Some of them were found to be very useful in biosensor applications. The most widely used among these are capture of polyhistidine-tagged proteins on nickel coated surfaces and binding of gluthatione-S-transferase (GST)-fused proteins to surface-bound GST-specific monoclonal antibodies.

These affinity tag-mediated immobilization techniques have the collective attribute to allow oriented immobilization under mild conditions so that antigenicity and biological activity is retained. They suffer, however, from the drawback that the interaction is not stable enough and results in a drift of the baseline signal and in a lack of resistance to the harsh regeneration conditions necessary for efficient reconstitution of the biosensor surface after analysis. A new affinity capture strategy – based on the irreversible reaction of the human DNA repair enzyme O_6-alkylguanine-DNA-alkyltransferase (hAGT) with O_6-benzylguanine (BG) – has recently been developed (33): It enables both covalent as well as site-directed attachment of proteins. For immobilization of hAGT-fusion proteins, a BG derivative with an aminopolyethylene glycol substitution is previously coupled via EDC/NHS-chemistry to a carboxylated biosensor matrix. hAGT reacts with the BG head group to form a stable thioether linkage

after injection of the hAGT-fusion protein into the biosensor flow system. Due to the high specificity of the hAGT–BG interaction, hAGT-fusion proteins can be immobilized directly from cell culture lysates without the need of prior purification.

3.1.3. Analyte Measurement and Chip Regeneration

In a typical analytical run, a solution with the analyte is injected under continuous flow conditions over the biosensor surface. This period defines the association phase. It is followed by a switch to running buffer for the detection of analyte dissociation. At the end of the dissociation phase, the ligand surface is treated with regeneration solutions containing, for example, acids, bases, or organic solvents.

It is generally recommended for biosensor measurements to simultaneously evaluate binding of the analyte to an irrelevant reference surface. This allows a satisfying compensation of nonspecific binding, bulk refractive index background, and matrix effects. Ideally, the reference surface should be prepared by coupling of a control antigen with similar biochemical properties.

For the reliable determination of binding affinities and rate constants, the binding reactions must be conducted under optimal experimental conditions. It is worth mentioning that there are a number of sources of error that have to be considered in the design of the experimental protocol in SPR biosensor analysis. The major causes are nonspecific binding, matrix effects, mass transport limitations, rebinding of the analyte, and gradual loss of ligand binding activity, the latter being caused mainly by the accumulation of bound material on the chip surface due to incomplete regeneration (see also Subheading 1.3.2).

Nonspecific binding can be prevented depending on its chemical nature either by an increase of the ionic strength or by the addition of suitable additives to the analyte dilution and running buffer. Recommended blocking agents include nonionic surfactants, such as Tween-20, and irrelevant proteins, such as bovine or human serum albumin. The addition of soluble CMD to the buffers is an effective way to reduce nonspecific binding in cases where compounds in the sample exhibit affinity to a dextran hydrogel matrix.

Matrix effects arise from changes in the extension of the surface matrix in response to differences in the pH or ionic strength of sample and running buffer. They can be minimized by adjusting the composition of the running buffer and the analyte solution.

In order to prevent *mass transport limitations*, it is advisable to perform kinetic measurements at low ligand immobilization levels and relatively high flow rates. Conversely, low flow rates and high surface binding capacities were found to be more appropriate for concentration measurements.

Rebinding occurs in situations when the analyte binds with only low affinity to the immobilized ligand. Rebinding can be assumed when an increase in the flow rate results in higher dissociation rates. Another indication of rebinding phenomena is when a slight dissociation is visible in the sensorgrams at the end of the analyte injection phase. Rebinding can be prevented by the use of higher flow rates, by immobilization of ligand at low densities, or by injection of soluble ligand in the dissociation phase.

After each cycle of measurement, the sensor chip has to be regenerated in such a way that all bound materials from the sample are completely removed. Although there are some general guidelines available in literature, for example, removal of antibodies by repeated short injections of hydrochloric acid or glycine buffer at pH 2.5, the optimum regeneration conditions have to be evaluated empirically in most cases. An adequate strategy to find the best regeneration conditions on the basis of six multivariate stock solutions is presented by Andersson et al. (34). During this phase of method optimization, it is important to ascertain that the rate of analyte binding is stable over a sufficient number of sample injections and does not decrease due to ligand inactivation. Alternatively to generally used regeneration methods, a new approach for the collection of kinetic binding data is the so-called "kinetic titration" (35). This method involves sequentially injecting an analyte concentration series without regeneration.

3.2. Exemplified: SPR Measurements and Kinetic Evaluation

3.2.1. General Recommendations for Chemical Coupling Reactions

HBS as running buffer and a constant flow rate between 2 and 5 µl/min is generally recommended for all chemical coupling strategies. Since efficient ligand immobilization to the biosensor matrix requires electrostatic preconcentration, dilution buffers should be adjusted to a pH below the pI of the ligand molecule.

3.2.1.1. Amine Coupling

The carboxyl groups of CMD or SAM biosensor chips are activated in one flow cell (FC) by a 35 µl-injection of an aqueous solution of 200 mM EDC and 50 mM NHS. After ligand immobilization, nonconverted active ester groups are blocked by a 35 µl-pulse of a 1.0 M ethanolamine hydrochloride solution, pH 8.5.

3.2.1.2. Aldehyde Coupling

After activation of the CMD or SAM biosensor surface with EDC and NHS (see Subheading "Amine Coupling"), 35 µl of 5 mM hydrazine or carbohydrazine in distilled water are injected to generate reactive hydrazine groups. Next, nonconverted NHS-esters are inactivated with a 1.0 M ethanolamine solution, pH 8.5. After injection of the aldehyde, containing ligand, the reactive hydrazide groups on the biosensor surface spontaneously react with the

aldehyde functions to yield hydrazone bonds. These can be reduced with sodium cyanoborohydride to form an imine bond, which is in contrast to the hydrazone bond completely stable under acidic conditions. For complete reduction of hydrazone bonds, a volume of 40 µl of 0.1 M sodium cyanoborohydride in 0.1 M acetate buffer, pH 4.0, is injected over the biosensor surface at a flow rate of 2 µl/min.

3.2.1.3. Ligand Thiol Coupling

In the ligand thiol coupling procedure, the ligand of interest contains free thiols to bind to reactive disulfide groups on the biosensor surface. After activation of the CMD or SAM biosensor surface with EDC and NHS, sulfhydryl-reactive groups are introduced on the biosensor surface by injection of 20 µl of 80 mM 2-(2-pyridinyldithio)-ethaneamine hydrochloride in 0.1 M borate buffer, pH 8.5. This is immediately followed by the addition of the ligand at a concentration of 10–200 µg/ml. For maximum coupling efficiency, it is recommended to dissolve the ligand in dilution buffers with moderate acidic pH (4.0–5.0). Finally, residual disulfide groups are blocked on the biosensor surface by a 20 µl-injection of 50 mM l-cysteine and 1 M NaCl in 0.1 M formate buffer, pH 4.3.

3.2.1.4. Surface Thiol Coupling

In the surface thiol coupling technique, the ligand of interest is modified to contain active disulfide groups, which in turn can be coupled to thiol groups on the biosensor surface. Firstly, the CMD or SAM biosensor is activated by EDC and NHS injection. This is followed by a 15 µl-injection of 40 mM cysteamine in 0.1 M borate buffer, pH 8.0, to introduce disulfide groups on the biosensor surface. Subsequently, the cystamine disulfides are reduced to thiols by injection of 15 µl of 0.1 M dithioerythritol or dithiothreitol. Next, the ligand of interest, which was prepared to contain reactive disulfide groups by exposure to the sulfhydryl-containing cross-linker poly(2-(diethylamino) ethyl methacrylate (PDEA) or *N*-succinimidyl 3-(2-pyridyldithio)propionate, is injected over the thiolated biosensor matrix at a concentration of 10–200 µg/ml. After the end of the ligand coupling reaction, nonconverted thiol groups are blocked on the biosensor surface by a 20 µl-pulse of 20 mM PDEA and 1 M NaCl in 0.1 M formate buffer, pH 4.3.

3.2.2. Selected Examples for Surface Ligand Capturing

3.2.2.1. Immobilization of Capture Antibodies

For covalent immobilization of capture antibodies, such as rabbit anti-mouse IgG, goat anti-human IgG, goat anti-GST (Biacore), or the His-tag specific Penta-His antibody (Qiagen, Hilden, Germany), a volume of 70 µl of a 30 µg/ml antibody solution in 10 mM sodium acetate, pH 5.0, is injected over an EDC/NHS-activated CMD or SAM biosensor surface. After deactivation of remaining ester groups with 1.0 M ethanolamine hydrochloride, pH 8.5, the high density antibody coated biosensor chips can be

tethered in the specific FC with monoclonal antibodies or recombinant fusion proteins at amounts in the range of 1–100 µg. In contrast, the reference FC can be loaded with an irrelevant antibody or antigen control, allowing its use for the elimination of matrix effects in the resulting Fc2-Fc1 sensorgram.

3.2.2.2. Immobilization of hAGT-Fusion Proteins

For covalent attachment of the benzylguanine derivative BG-PEG-NH_2 as an amine-terminated hAGT substrate onto the solid state of a CMD or SAM biosensor surface, a 3.1 µM solution in 1 ml HBS buffer is prepared. After subsequent centrifugation at 20,000 × *g* for 20 min to remove all insoluble material from solution, a 70 µl-aliquot is applied over the EDC/NHS-activated biosensor surface resulting in an approximately 2,000 RU stable increase of the SPR baseline signal. All nonconverted NHS-activated ester groups are then blocked by a 35 µl-pulse of 1.0 M ethanolamine hydrochloride, pH 8.5. After an at least 15 min period of buffer flow, the hAGT-fusion protein can be immobilized by a 35 µl-injection of 100 µg/ml of purified protein at a flow rate of 1 µl/min. Due to the extraordinarily high specificity of the hAGT–BG interaction, however, it is also possible to alternatively inject whole cell extracts from the bacterial expression system at a total protein concentration of 10 mg/ml. hAGT without a fusion partner can be immobilized in the reference FC to allow the subtraction of matrix effects in a Fc2-Fc1 sensorgram.

3.2.3. Kinetic Measurements of Immobilized Ligand and Its Receptor in Solution

Kinetic analysis is exemplarily described for the interaction of the sexual hormone binding globulin (SHBG) to 17α- and 1α-aminoalkyl dihydrotestosterone (DHT) derivatives (36). Measurements were performed on the BIAcore X device at 25°C. Commercially available B1-chips that contain a CMD matrix with a very low-degree of carboxymethylation were chosen to ensure a low steroid surface density in the amine coupling step. For immobilization of the steroidal ligands in one flow cell, the carboxyl groups of the dextran layer were activated with a 35 µl-injection of a 0.2 M EDC/0.05 M NHS solution followed by a 30 µl-injection of a mixture of 100 µM aminoalkyl DHT derivative and 200 µM of ethanolamine in 100 mM borate buffer (pH 9.0) at a flow-rate of 5 µl/min. After the signal reached approx. 200–350 RU, the nonconverted active ester groups were blocked by a 35 µl-pulse of 1.0 M ethanolamine hydrochloride (pH 8.5). The second flow cell was treated in a similar fashion but without the addition of the steroid/ethanolamine mixture so as to allow its use as a reference surface. In order to obtain a homogeneous analyte preparation, affinity-purified SHBG was *N*-deglycosylated using PNGase F and subjected to size-exclusion chromatography. 45 µl-aliquots of SHBG were injected at concentrations of 50, 75, 100, 150, 200, 300, and 400 nM into the biosensor flow system. The total sensorgram recording times were 1,050 s with an association

phase of 450 s and a dissociation phase of 600 s. At the end of the dissociation phase, 100 mM H_3PO_4 was added for regenerating the chip surface.

Analysis of the sensorgram data for the calculation of kinetic rate constants was done with the BIAevaluation program from Biacore AB. The evaluation algorithms perform a nonlinear regression analysis of the measured sensorgrams. The most appropriate model describing the SHBG–steroid interaction was determined to be the bivalent binding model. This was evaluated by a global fitting analysis based on the residual plots with low χ^2 values. The χ^2 value is a standard statistical measure of the closeness of fit and can be obtained from analyzing the sensorgrams. The bivalent binding mode is supported by the structure of SHBG as a homodimer. The association constants derived from the biosensor studies were found to be in good agreement with those determined by Scatchard analysis under equilibrium conditions.

4. Notes

1. SPR is a useful completion of protein analysis in proteome research. This chapter will portray the capability of the SPR method by the description of new applications in urine proteomics and proteinuria diagnostics. The applications are due to the main advantage of SPR: The ability to study interactions of proteins and peptides in their native forms with sizes ranging a few hundreds of daltons to large complexes such as cells (37).
2. It is estimated that the urine proteome contains more than 1,500 proteins. Provided most of proteins are digested to peptides on their way to excretion, one will find more than 8,000 peptides in urine (38). State-of-the-art peptide analysis methods are still unable to cover and characterize all peptides in urine samples from patients with end-stage nephritic syndrome in a manageable time at low costs. To cover as much proteins and peptides as possible, one has to combine different separation or capture approaches such as 2D-PAGE, capillary electrophoresis, free flow electrophoresis, or different selecting surfaces for liquid chromatography and chip technology (SELDI). This has to be combined with the detection methods of mass spectrometry or specific dye labeling (39). A worthwhile task for SPR biosensorics is the identification and kinetic description of protein/ligand binding events. A promising approach offers the highly selective SPR-analysis combined with mass spectrometry (MS), called biospecific interaction analysis/MS (BIA/MS). This approach is

not only able to identify the captured protein or peptide but also determines the respective binding affinities. A fully automated system for ligand fishing SPR combined to MALDI-TOF MS was recently established. This system is able to perform ligand capture, recovery, deposition onto a MALDI target, and the sample preparation including tryptic digestion (40). Apart from the fact that SPR is used to analyze cell extracts or other body fluids, there are already a series of promising examples of urine proteomics applications. Nedelkov et al. studied protein–protein interactions using BIA/MS. The glomerular filtration marker cystatin C was isolated from urine samples by its interaction with papain. Whereas no novel protein–protein interactions could be discovered, some general aspects of urine SPR analysis were elucidated in this publication (41). For example, the signal-to-noise ratio in urine samples was found to be favorably compared to serum/plasma samples. Human urine samples from apparently healthy subjects are characterized by low protein concentrations but might have high salt load. Requisite sample dilutions for the SPR analysis with buffers used (e.g., HBS-EP at pH 7.4) bring the salt content to an appropriate level and prevent degradation of small proteins in urine by adjusting the pH value. Proteins are usually degraded in urine with low pH values below 6.5. Additionally, unspecific binding to the chip is prevented by a tenfold dilution (42).

3. For comprehensive proteinuria diagnostics, it is valuable to use various techniques that cover as many proteins and peptides as possible. In this context, the combination BIA/MS helps to detect novel disease specific markers or complete a marker profile as mentioned above. But for diagnosis in routine clinical chemistry laboratories, these methods are too laborious even if data interpretation is automated. To make a laboratory diagnosis, one has to select a limited number of proteins for urine analysis. Most SPR protein analyses are carried out in individual reactions rather than multiplex systems, but there are some publications with parallel or sequential multityping. The novel Flexchip and ProteON XPR36 devices (see Subheading 2.3.2) will facilitate these multiarray approaches.

   Nedelkov and Nelson (6) characterized the tubular dysfunction markers retinol-binding protein, urinary protein 1, β2-microglobulin, and cystatin C simultaneously by applying BIA/MS (42). A fundamental benefit of using MALDI-TOF is that the amount of truncated forms of proteins may be quantified. Many proteins are truncated in particular when cells in the renal tubular system are damaged. A fundamental

drawback of the BIA/MS system is that the sensor chip can only be used singular, caused by the irreversible shuttering through the matrix. Furthermore, the technique is costly and extremely complex.

Another approach is to use a sequential SPR analysis. Its feasibility was demonstrated with model substances and two clinical chemistry parameter in human urine samples. Chung et al. (43) analyzed human chorionic gonadotrophin (hCG) and albumin (hA) in urine by coupling anti-hCG and anti-hA to the SPR surface. The combination of hCG and hA is useful to monitor a pregnancy at risk, in particular for women with type I diabetes. The concentration range for sequential analysis of hCG and hA was 415–46,100 mIU/ml and 20–200 µg/ml, respectively. The SPR assay uses secondary detection antibodies in a sandwich system for signal generation.

Urinary N-telopeptide (NTx), a biochemical marker for bone resorption, is used to monitor the therapeutic management of primary osteoporosis. Lung et al. (44) established a SPR-based assay to quantify NTx, which is a degradation product from bony type I collagen and excreted in urine. The authors compared the SPR assay with conventional ELISA and found similar results in identifying subjects with significantly increased bone loss. Advantageous for the SPR-based assay is the fact that it works faster than the ELISA and that the SPR method is reusable manifold.

4. Due to the fact that SPR biosensorics can provide affinity and kinetic data, unique features such as protein–peptide interaction analysis will be fruitful also for urine proteomics. Therefore, the urine matrix poses no special challenge for SPR. The integration of SPR into protein array technology will accelerate discovery in existing applications and will also play a significant role in driving the development of urine proteomics (45). Moreover, chip data processing has the potential to create a new quality in the field of proteinuria diagnostics. What is emerging by applying the SPR technology is the discovery and characterization of new diagnostic protein markers in their native state in urine.

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