Chapter 5

Surface Plasmon Resonance Biosensing

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Summary

Surface plasmon resonance (SPR) biosensors belong to label-free optical biosensing technologies. The SPR method is based on optical measurement of refractive index changes associated with the binding of analyte molecules in a sample to biorecognize molecules immobilized on the SPR sensor. Since late 1990's, SPR biosensors have become the main tool for the study of biomolecular interactions both in life science and pharmaceutical research. In addition, they have been increasingly applied in the detection of chemical and biological substances in important areas such as medical diagnostics, environmental monitoring, food safety and security. This chapter reviews the main principles of SPR biosensor technology and discusses applications of this technology for rapid, sensitive and specific detection of chemical and biological analytes.

Key words: Optical biosensors, Affinity biosensing, Biorecognition elements, Detection of chemical and biological species, Bioassays.

1. Introduction

Optical affinity biosensors based on surface plasmon resonance (SPR) present one of the most advanced label-free optical sensing technologies *(1–3)*. Their ability to monitor the interaction between a molecule immobilized on the surface of the sensor and the interacting molecular partner in a solution have made SPR sensors a very powerful tool for biomolecular interaction analysis and biomolecular research in general *(4)*. In recent years, SPR biosensors have been increasingly used also for the detection of chemical and biological substances related to medical diagnostics, environmental monitoring, food safety and security *(5)*.

Avraham Rasooly and Keith E. Herold (eds.), *Methods in Molecular Biology: Biosensors and Biodetection, Vol. 503*

DOI: 10.1007/978-1-60327-567-5_5

This chapter deals with SPR biosensor technology and its application in the quantification of chemical and biological species.

1.1. Principles of SPR Biosensing

SPR affinity biosensors are devices that consist of three main subsystems: sensor hardware (optical reader), biorecognition element, and sample preparation and delivery system (**Fig. 1**).

In the optical reader of an SPR sensor, a light wave excites a special mode of electromagnetic field – surface plasmon (SP). Surface plasmon propagates along a thin metal film and its field probes the medium adjacent to the metal surface. Any change in the refractive index in the proximity of the metal surface results in a change in the velocity of the surface plasmon. This change in the propagation can be determined from the characteristics of the light wave coupled to the surface plasmon. Biorecognition elements specific to analyte molecules are immobilized on the surface of the metal. If a liquid sample is brought in contact with the sensor surface, molecules of analyte are captured by biorecognition molecules (**Fig. 2**). The binding gives rise to a refractive index change close to the sensor surface, which can be measured by the optical reader. The liquid sample is brought to the sensor surface using a sample preparation and a delivery system.

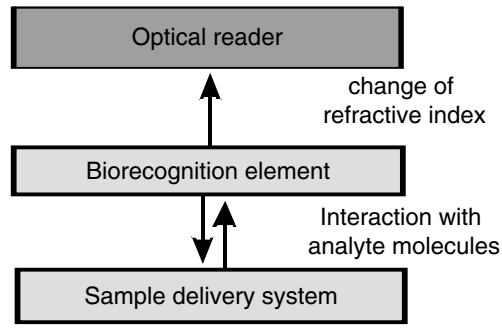

Fig. 1. Principal components of SPR affinity biosensor.

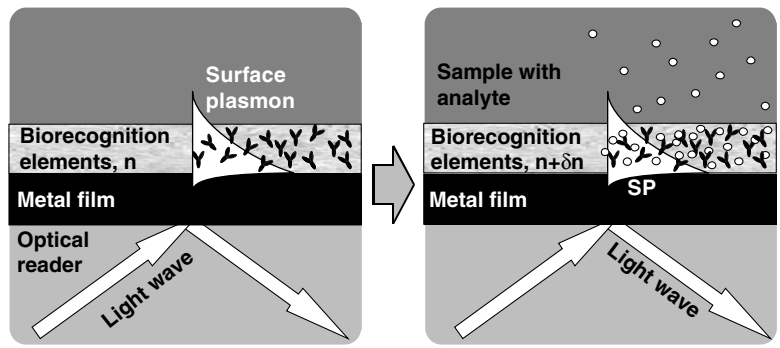

Fig. 2. Principle of operation of SPR affinity biosensors.

The following sections present the underlying principles and characteristics of the main parts of an SPR affinity biosensor.

1.2. Optical Reader

Nowadays, numerous SPR readers are available as commercial systems or research prototypes. Although their designs may differ considerably, the underlying physical principles of the SPR method remain the same. Therefore, the first part of this section is dedicated to fundamentals of surface plasmons and SPR sensing while the following part illuminates possible differences among the SPR sensing platforms and gives examples of the most frequent SPR sensing platforms.

1.2.1. Surface Plasmons and Their Optical Interrogation

A surface plasmon is an electromagnetic wave, which propagates along an interface between a dielectric and a metal and is characterized by the propagation constant and electromagnetic field distribution *(6)*. The field of a surface plasmon is transverse-magnetic (TM) polarized (the vector of magnetic intensity is perpendicular to the plane of incidence) and evanescently decays into both the media while the major part of the field is located in the dielectric. The propagation constant of SP β_{SP} is determined by the optical constants of the surrounding media (permittivity ε_M of the metal and the refractive index of the dielectric n_D) as follows:

$$\beta_{SP} = \frac{\omega}{c} n_{ef} = \frac{\omega}{c} \sqrt{\frac{\varepsilon_M n_D^2}{\varepsilon_M + n_D^2}}, \quad (1)$$

where ω is the angular frequency, c is the speed of light in vacuum and n_{ef} denotes the effective refractive index of the surface plasmon. Although several metals can support surface plasmons at optical frequencies, all the main existing SPR (bio)sensors use gold due to its excellent chemical stability. The electro-magnetic field of the SP is strongly localized at the metal surface and its penetration depth into the dielectric L_{pd} (*see* **Note 1**) is typically 150–400 nm depending on the operating wavelength **(Fig. 3)**.

A change in the refractive index of the dielectric within a distance h from the metal surface produces a change in the effective refractive index of the surface plasmon n_{ef}. The magnitude of the change depends on the thickness of the layer h within which the refractive index change occurs, operating wavelength, and a refractive index distribution. If the thickness of the layer is much higher than that of the penetration depth L_{pd} of the SP field, the change in the effective refractive index of the surface plasmon can be calculated as

$$\Delta n_{ef} = \frac{n_{ef}^3}{n_D^3} (\Delta n_D)_{h >> L_{pd}}. \quad (2)$$

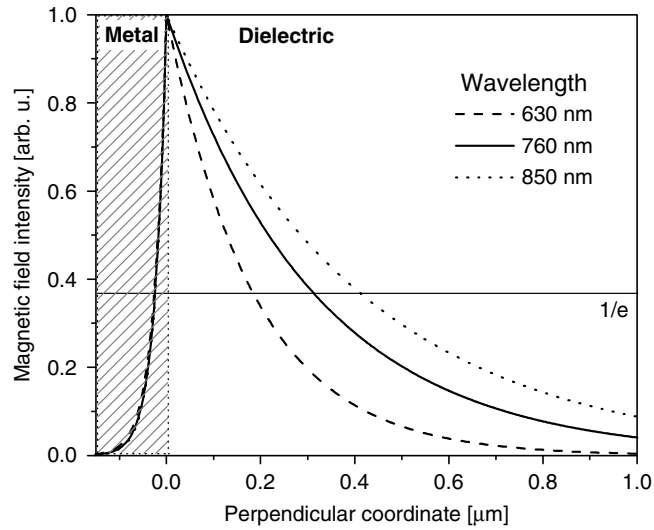

Fig. 3. Distribution of electromagnetic field of a SP at the gold–water interface for three different wavelengths.

The change in the effective refractive index of a surface plasmon due to a refractive index change within a thin layer $h \ll L_{pd}$ can be estimated using the perturbation theory *(5)* as

$$\Delta n_{ef} = \frac{2h}{L_{pd}} \frac{n_{ef}^3}{n_D^3} (\Delta n_D)_{h \ll L_{pd}}. \qquad (3)$$

If the refractive index change is produced by the binding of specific molecules at the sensor surface, the change of refractive index Δn_b b occurring within a layer of thickness h can be expressed as

$$\Delta n_b = \left(\frac{dn}{dc}\right)_{vol} \Delta c_b = \left(\frac{dn}{dc}\right)_{vol} \frac{\Delta\Gamma}{h}, \qquad (4)$$

where $(dn/dc)_{vol}$ is the refractive index increment, Δc_b is the wt/vol concentration of bound molecules within the sensitive layer with the thickness h and $\Delta\Gamma$ is the corresponding surface concentration (mass per surface area). The linear relation between the refractive index change and the surface concentration of bound molecular mass in **Eq. 4** is often referred to as de Feijter formula *(7)*. The refractive index increment $(dn/dc)_{vol}$ is a well-characterized property for most of the biochemical species and ranges typically from 0.1 to 0.3 cm^3g^{-1} *(8)*. Proteins and nucleic acids typically exhibit $(dn/dc)_{vol} = 0.18\,cm^3g^{-1}$ with variability less than 8%. As follows from **Eqs. 2–4**, a change in the effective index of the surface plasmon due to the capture of analyte can be expressed as

$$\Delta n_{ef} = K\Delta\Gamma, \tag{5}$$

where K is a constant.

The optical reader of the SPR sensor measures changes in a characteristic of a plasmon-coupled light wave resulting from changes in the effective refractive index of a surface plasmon. Several coupling mechanism are used to couple a light wave to a surface plasmon. The most common mechanisms include the attenuated total reflection in prism couplers and diffraction on diffraction grating couplers *(6)*.

Most SPR sensors are based on the Kretschmann configuration of the attenuated total reflection method. In this geometry, a light wave passes through a high refractive index prism and is totally reflected at the prism base covered with a thin gold film (**Fig. 4**). Light evanescently tunnels through the thin metal film and can excite an SP at the outer boundary of the gold if the incident light wave and SP are closely phase-matched. The phase-matching condition can be written as

$$n_p \sin\theta = n_{ef}, \tag{6}$$

where n_p is the refractive index of the coupling prism and θ is the angle of incidence on the metal film (in the prism).

The coupling of incident light wave to an SP is accompanied by a transfer of energy and results in a drop of the intensity of the reflected light wave. As the coupling occurs only within a narrow range of angles of incidence (or wavelengths), the excitation of SP produces a narrow dip in the angular (or wavelength) spectrum of the reflected light. Based on these characteristics, the reflected light wave is measured in the SPR sensor hardware.

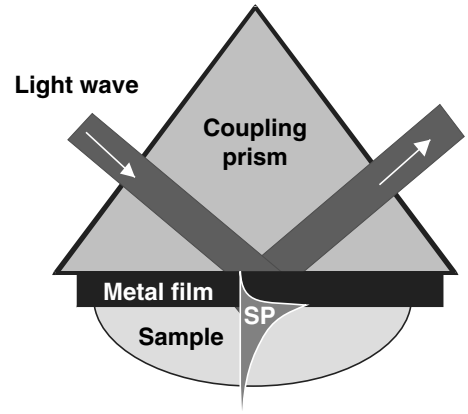

Fig. 4. Excitation of surface plasmons in the Kretschmann geometry of the attenuated total reflection method.

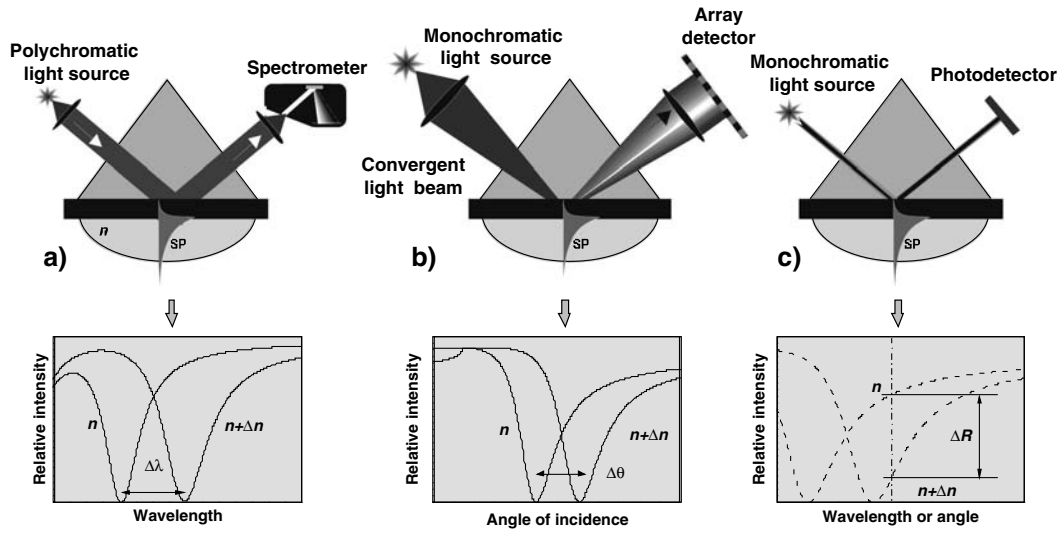

Fig. 5. SPR sensors based on modulation of (**a**) wavelength, (**b**) angle of incidence, and (**c**) light intensity.

SPR sensors are classified as: (a) SPR sensors with wavelength modulation (angle of incidence is fixed and the coupling wavelength serves as a sensor output), (b) SPR sensors with angular modulation (coupling wavelength is fixed and the coupling angle of incidence serves as a sensor output), (c) SPR sensors with intensity modulation (both the angle of incidence and the wavelength of incident light are fixed at nearly resonant values and the light intensity serves as a sensor output) **(Fig. 5)**.

A change in the refractive index at the metal surface results in a change in the effective refractive index of an SP and a change in the coupling condition *(6)*. Consequently, the change in the refractive index produces a change in the position of the SPR dip in the spectrum of reflected light (SPR sensors with angular or wavelength modulation) or a change in the intensity of reflected light (SPR sensors with intensity modulation) **(Fig. 5)**. The relation between the refractive index change and the sensor output depends on the spatial distribution of the refractive index change and design of the SPR reader (coupling method, modulation method, and operating wavelength). **Table 1** shows sensor responses to changes in the bulk refractive index of the sample and changes in the refractive index at the surface of the metal. Changes in the bulk refractive index are given in refractive index units (RIU) and changes in the surface refractive index are expressed in an equivalent amount of captured protein. It can be concluded from **Table 1** that the change of the sensor response induced by the adsorption of 10 pg × mm^{-2} of a protein is comparable to the change induced by the change of the bulk refractive index above the sensor surface of 10^{-5} RIU. This relation depends on the resonant wavelength and the method of

Table 1
Comparison of responses of prism-based SPR sensors with different modulation approaches

	Wavelength modulation (nm)	Angular modulation (deg)	Intensity modulation (%)
Bulk change 10^{-5} RIU	0.055	1×10^{-3}	0.12
Surface change 10 pg × mm^{-2}	0.063	1.15×10^{-3}	0.14

$(dn/dc)_{vol} = 0.18\,cm^3g^{-1}$ operating wavelength – 750 nm

coupling and the presented comparison applies to the operating wavelength of 750 nm. High-performance SPR sensors used for the most demanding applications are able to resolve changes in the surface concentration as small as 0.1 pg mm^{-2}. This suggests (**Table 1**) that the optical system of the sensor has to resolve changes of resonant wavelength smaller than 0.5 pm or the resonant angle of incidence smaller than 10^{-5} degree.

1.2.2. Implementations of SPR Readers

Since the first commercial SPR sensors appeared on the market in 1990, numerous SPR platforms have been introduced by several companies. The most widely used SPR sensors are sensors developed by Biacore (since 2006 Biacore has been a part of GE Healthcare) which offer a wide range of SPR sensors for biomolecular interaction research. Their product portfolio includes, for instance, high-resolution angular-modulation systems with four sensing channels and automated flow delivery, and sample handling, such as Biacore 3000 and Biacore T100. Biacore Flexchip, IBIS–iSPR (Ibis, The Netherlands) and Proteomic Processor (Lumera Corporation, USA) represent array SPR sensors based on intensity modulation (SPR imaging) with several hundreds of sensing channels. Autolab ESPRIT (Eco Chemie B.V., Netherlands) system is also based on angular modulation and fast scanning and sample delivery is realized by direct autosampling in two measuring cuvettes. The Multiskop system (Optrel GbR, Germany) can be used either in the angular scanning mode for accurate measurements or with a quadrant-cell photodiode for real-time kinetic measurements. Examples of research prototypes include β-SPR (Sensia, Spain) which is an angular modulation based system, with two independent sensing channels and Plasmon IV (Institute of Photonics and Electronics, Czech Republic), a four-channel SPR sensor with wavelength modulation.

An SPR reader comprises four key subsystems (**Fig. 6**) – light source module, sensor head with coupling optics, detector module, and data processing unit.

The choice of a light source depends on the modulation method used and operating range required. In SPR sensors with angular and intensity modulations, monochromatic light or quasi monochromatic light is used and therefore laser diodes or narrowband light emitting diodes are the light sources of choice. In SPR sensors with wavelength modulation, polychromatic light sources such as halogen lamps are most commonly employed. The light source needs to be stable in terms of emitted intensity and spectral distribution for optimum performance of the SPR sensor, (*see* **Note 2**).

The sensor head performs multiple functions and therefore comprises several subsystems. Illuminating optics deliver light beams of appropriate spatial distribution into the SPR coupling element (parallel beam in intensity and wavelength modulation based SPR sensors and wedge-shaped beam in angular modulation based sensors). The coupling optics typically consist of a coupling prism and an SPR sensor chip which is optically matched to the prism (*see* **Note 3**). As SP is a TM electromagnetic wave, polarization of light coupling to the SP is controlled with polarizing optics which is, typically, a linear polarizer. Light reflected from the SPR chip passes through the coupler and is collected at the detector module. Signals from individual channels are separated either by optical means in the detector module or by software means in the data processing unit (*see* **Note 4**). As the temperature fluctuations can induce changes in the refractive index of samples and thus interfere with SPR biosensing,

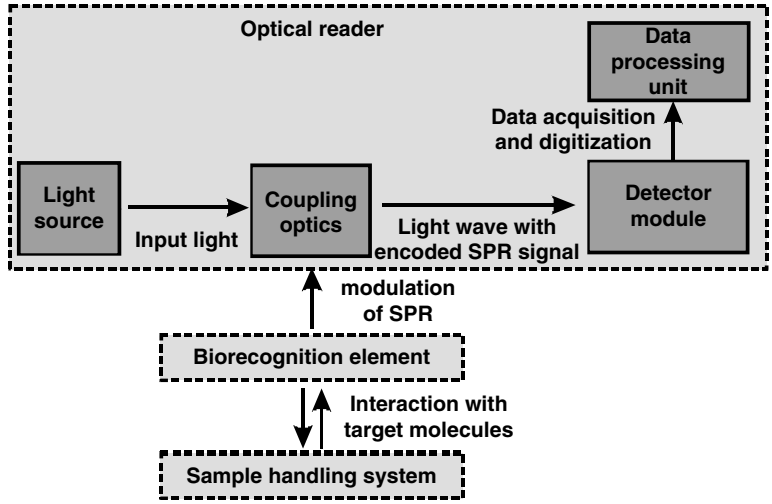

Fig. 6. Scheme of an SPR biosensor and main subsystems of optical SPR reader.

precise control of temperature at the sensor surface is required for reliable results. High-performance SPR sensors are therefore equipped with temperature stabilization of the coupling optics and the analyzed sample.

The detector module measures the intensity of light transmitted through the sensor head or its spectral or angular distribution. In the intensity modulation-based sensors, array detectors such as 2D CCD cameras are frequently used to allow simultaneous measurements in multiple areas of the sensing surface (*see* **Note 5**). In angular modulation-based sensors, the angular spectrum is usually spread across an array detector and different sensing channels (if more than one channel is present) are measured with one or more detector lines. SPR sensors with wavelength modulation are usually equipped with a separate spectrometer for each sensing channel. For more details, see **Fig. 5**.

In the process of detection of light, the detector module produces a set of intensity values (the number of intensities for an individual channel depends on the type of detector). The data corresponding to individual sensing channels are processed separately. In SPR sensors based on angular or wavelength modulation, spectra for individual sensing channels are extracted and an appropriate algorithm locates the position of the SPR dip *(9)*. In SPR sensors based on intensity modulation, the sensor output is usually defined as an averaged intensity over the area of the detector corresponding to a sensing channel. The sensor output can be calibrated to a change in the refractive index, or surface concentration of bound molecules.

1.3. Biorecognition Element

In SPR affinity biosensors, the biomolecular recognition element is immobilized on the solid surface of the SPR sensor reader and captures analyte molecules. The choice of biorecognition elements (BRE) and immobilization method presents an important step in the development of an SPR biosensor and has a direct impact on the key performance characteristics of the developed SPR biosensor system such as specificity and limit of detection *(10)*.

Numerous molecules can serve as biomolecular recognition elements. The key factors for their selection include high affinity to the target analyte, stability of biological activity, specificity, and availability of functional groups for their direct immobilization. Antibodies remain by far the most frequently used biorecognition elements *(11)*. However, development of high-quality antibodies is still a rather expensive and laborious process. Other molecules that have been commonly employed in SPR sensors include antibody fragments, antigens, engineered affibodies, peptides, oligonucleotides and molecular imprinted polymers *(12, 13)*.

Surface chemistries used for immobilization of biorecognition elements on the SPR reader are required to provide a high density of biorecognition elements on the surface while maintaining their

biological activity. Given the complexity and variability of biorecognition elements, there is no universal immobilization method. The choice of immobilization chemistry is therefore made based on the properties of a specific biorecognition element *(5)*. Non-specific binding of non-target molecules in complex samples such as bodily fluids, food samples, etc. presents another challenge for surface chemistry of SPR biosensors.

Since the beginning of SPR biosensors research in early 1990's various approaches to the immobilization of biomolecules on the SPR sensor surface have been developed. These include procedures based on physical adsorption via hydrophobic and electrostatic interactions *(14)*, strong covalent binding *(15)*, or attachment of tagged proteins by a site-specific non-covalent interaction between the tag and a capture molecule. The attachment to the surface can be mediated by a molecular recognition event such as biotin–avidin interaction *(16)* or DNA hybridization *(17)*. The covalent attachment requires the presence of reactive groups on the support (usually electrophilic groups such as aldehydes or succinimidyl esters) able to react with nucleophilic groups (amino, thiol, hydroxyl) on the ligand molecules *(15, 18)*. One of the most remarkable techniques in surface chemistry is the spontaneous self-organization of *n*-alkylthiols or disulfides on gold surfaces **(Fig. 7)**. Self-assembled monolayers (SAMs) have been employed in many immobilization methods for spatially controlled attachment of biomolecular recognition elements *(19)*. Three-dimensional matrices that contain binding sites in a structured 3D environment (e.g. hydrogels such as carboxymethyl dextran) present an interesting alternative for the immobilization of biomolecular recognition elements *(15)*. This approach offers a high number of binding sites for covalent attachment of biomolecular recognition elements and relatively non-fouling background. However, the binding of analyte molecules to the immobilized elements may be slowed down due to the diffusion of the analyte through the hydrogel matrix *(20–22)*.

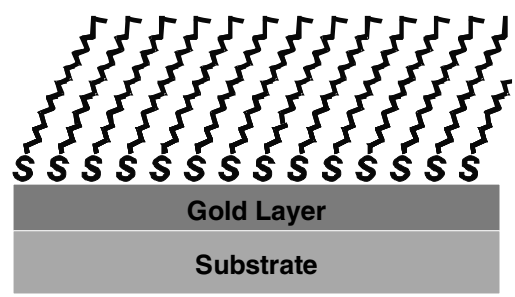

Fig. 7. Scheme of *n*-dodecanethiolate self-assembled on gold substrate. The assembly is held together by bonds between the sulfur head groups and the gold surface and van der Waals and hydrophobic interactions between neighboring hydrocarbon chains.

1.4. Sample Delivery System

A liquid sample is brought to the sensor surface using a sample preparation and delivery system. In principle, the sample is either injected into a cuvette that is interfaced with a sensing surface and the interaction takes place in a fixed volume under no-flow conditions or the sample is continuously flowed through a special chamber (a flow cell unit) attached to the sensor surface *(23)*. In cuvette-based SPR sensors, the analyte contained in a fixed volume of liquid sample (cuvette volume is typically 10–200 µL) interacts with biorecognition elements on the sensing surface. This interaction may be affected by mass transport of the analyte from the sample volume to the sensing surface; therefore SPR sensors employing cuvettes usually allow stirring of the sample in the cuvette during the measurement. In flow cell-based SPR sensors, the measured sample flows through a flow cell (volume of a flow cell chamber is 10 nL–10 µL) at flow rates ranging from 1–100 µL/min (*see* **Note 6**). In the flow cell, the analyte molecules diffuse to the sensor surface and interact with biorecognition elements on the sensing surface. In order to provide a constant flow of sample of a desired flow rate, flow cells are connected with peristaltic or syringe pumps. Advanced sample delivery systems offer complex and automated sample management *(24)*.

2. Materials

2.1. Immobilization of Biorecognition Elements

1. The buffer solutions used for immobilization of biorecognition elements include: PB (10 mM phosphate buffer, pH 7.0 at 20°C), PBS (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 at 20°C), PBM (10 mM phosphate buffer, 15 mM $MgCl_2$, pH 7.2 at 20°C); PBNa (10 mM phosphate buffer, 0.75 M NaCl, 2.7 mM KCl, pH 8.0 at 20°C), PBST (10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, pH 7.4), Tris–HCl (10 mM Tris, 50 mM NaCl, pH 8.0 at 20°C), and SA (1–10 mM sodium acetate, pH 5.0 at 20°C). Phosphate buffers can be prepared using standard procedure from stock monobasic and dibasic sodium and potassium phosphate solutions with a ratio corresponding to a desired pH. When appropriate, pH of the buffer may be adjusted with HCl or NaOH.
2. Alkylthiols (AT), i.e. C_{11}-chained tetra(ethylene glycol)- terminated alkanethiol (AT-EG4), C_{11}-chained tetra(ethylene glycol) -COOH- terminated alkanethiol (AT-EG4–COOH), and C_{16}-chained biotin-terminated (AT-BAT), and C_{11}-chained NH_2-terminated thiols (AT–NH2) can be obtained from Prochimia, Poland (www.prochimia.com). 11-Amino-1-undecanethiol, hydrochloride (AT-NH2) can be obtained from Dojindo Laboratories, Japan (www.dojindo.com).

3. Reagents for activation of carboxylic groups *N*-hydroxysuccinimide (NHS, catalog number: 56480), *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, catalog number: E7750), *N,N,N*′,*N*′-Tetramethyl-O-(*N*-succinimidyl) uronium tetrafluoroborate (TSTU, catalog number: 85972), and 4-(dimethylamino)-pyridin (DMAP) are available from Sigma-Aldrich, USA (www.sigma.com). Trifluoroacetic acid, ammonium hydroxide solution, acetic acid and triethylmaine (all of these reagents should be reagent grade or better) are available, for instance, from Sigma-Aldrich, USA (www.sigma.com).
4. Standard solvents such as absolute ethanol, anhydrous dioxane, and anhydrous dimethylformamide (DMF) can be obtained from Sigma-Aldrich, USA.
5. Tween 20, streptavidin, caseine, ethanolamine, and bovine serum albumin can be obtained from Sigma-Aldrich, USA.

3. Methods

3.1. Immobilization of Biorecognition Elements

Immobilization of biorecognition elements on the gold surface of the SPR reader can be performed either in the SPR system using the microfluidic channels or outside the SPR instrument on the whole surface of the sensor chip. In the first approach, the whole functionalization process or its final steps involving delivery of biorecognition elements to a selected sensing channel are performed with the sensor microfluidics. This approach makes it possible to observe the SPR sensor response to the immobilization of biorecognition elements and determine the surface concentration of the immobilized molecules. Alternatively, a spatially-resolved functionalization of the SPR chip by biorecognition elements can be achieved by techniques such as microspotting, in which small droplets of a solution containing biorecognition elements are delivered on the reactive sensor surface. This approach is particularly useful for producing SPR arrays with a large number of sensing channels which cannot be individually addressed by microfluidic devices.

In this chapter, we present protocols for four selected immobilization methods for in situ attachment of oligonucleotides and proteins on the gold surface of an SPR chip.

3.1.1. Immobilization of Antibodies (Proteins) via Covalent Attachment to COOH/OEG Self-Assembled Monolayer (SAM) (15, 25, 26)

Suitable for immobilization of proteins and other molecules containing primary amines.

Advantages: Stability of immobilized biorecognition element layer under a wide range of environmental conditions (pH, temperature) and good resistance to non-specific binding of molecules from complex media such as serum, urine, and food matrix.

Disadvantages: Immobilization level of biorecognition elements depends on accessibility of primary amines for covalent binding to *N*-hydroxysuccinimidyl esters created on SAM, not-oriented immobilization.

1. Prepare the 1 mM stock solutions of AT-EG4–COOH and AT-EG4 thiols in absolute ethanol. Seal the tubes well with parafilm. Store them at a temperature of –20°C before use (*see* **Note 7**).
2. Rinse the gold sensor surface with water and absolute ethanol. Dry with nitrogen stream. Clean the gold sensor surface in UV ozone cleaner for 20 min, wash it with water (*see* **Note 8**) and dry with nitrogen stream (*see* **Note 9**).
3. Immerse the clean sensor chip in solution mixed of AT-EG4–COOH and AT-EG4 thiols mixed in the ratio of 3:7 at a room temperature with a total thiol concentration of 200 µM. Use PP tube or glass Petri dish. Add trifluoroacetic acid to the final concentration of 2%. Seal the tube or dish or with parafilm. After 10 min of incubation at 40°C, store the chip in this solution in a dark place at a room temperature overnight. Immersed chip can be stored for up to 2 weeks.
4. Rinse the chip with ethanolic solution of ammonium hydroxide (10% v/v), ethanol, water and ethanol and dry with nitrogen stream (see Note 10).
5. Immerse the sensor chip in a solution of TSTU in argon-deaerated DMF (2 mg/mL), add DMAP (0.02 M) and shake it for 1 h under inert gas. Alternatively, expose the chip to freshly prepared water solution of NHS (0.05 M) and EDC (0.2 M) for 10 min. Alternatively, dissolve NHS in anhydrous dioxane (2.02 mg/mL) and EDC in water (100 mg/mL), mix them in the ratio of 49:1 and incubate the sensor chip in this solution for 1 h at room temperature. Storage in inert gas is recommended.
6. Rinse the sensor chip with ethanol and water, dry it with nitrogen and mount it into the SPR instrument immediately.
7. Flow 10 mM sodium acetate buffer, pH 5.0 (SA) at 25°C at higher flow rate (e.g. 50 µL/min, depending on the sensor fluidics, **Note 11**) through the sensor flow cell for a few minutes.
8. Inject antibody or protein in SA buffer at a concentration of 5–50 µg/mL and let it flow through the flow cell for 15 min at 30 µL/min. (Optimal flow rate can differ depending on the design of the used flow cell; optimal concentration can differ depending on immobilized ligand reactivity with surface-bound *N*-hydroxysuccinimidyl esters.) Replace the solution with SA buffer. For proteins sensitive to low pH, use PB buffer (10 mM phosphate, pH 7.0) instead of SA.

9. Inject 1 M ethanolamine, pH 8.0 at 20°C or Tris buffer for 10 min to deactivate the residual N-hydroxysuccinimidyl esters. Flow 10 mM phosphate buffer, 0.75 mM NaCl, pH 8.5 (PBNa) along the functionalized surface for 5 min to remove all non-covalently bound biorecognition elements.
10. Switch to appropriate detection/assay buffer such as 10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS). Inject suitable blocking agent (e.g. bovine serum albumin, caseine, dry milk) in PBS at a concentration of 0.2–10 mg/mL to block the sensor surface. The concentration of the blocking agent dependas on the complexity of the analyte solution; in general, the more complex the solution, the higher should be the concentration of the blocking agent.

3.1.2. Immobilization of Biotinylated Ligands to Streptavidin Coupled via Covalent Attachment to COOH/OEG Self-Assembled Monolayer (26, 27)

Suitable for immobilization of biotin-tagged ligands (proteins, antibodies, oligonucleotides)

Advantages: Site-specific immobilization, high level of immobilized biotinylated biorecognition elements, stability of immobilized biorecognition element layer under a wide range of environmental conditions (pH, temperature), and good resistance to non-specific binding of molecules from complex media such as serum, urine, and food matrix.

Disadvantages: Biotinylation of biorecognition elements required before immobilization.

1. Prepare 1 mM stock solutions of AT-EG4-COOH and AT-EG4 thiols in absolute ethanol. Seal the tubes well with parafilm. Store them at a temperature of –20°C before use (*see* **Note 7**).
2. Rinse the gold sensor surface with water and absolute ethanol. Dry it with nitrogen stream. Clean the gold sensor surface in UV ozone cleaner for 20 min, wash it with water (*see* **Note 8**) and dry it with nitrogen stream (*see* **Note 9**).
3. Immerse the clean sensor chip into solution of AT-EG4-COOH and AT-EG4 thiols mixed in the ratio of 3:7 at room temperature with a total thiol concentration of 200 µM. Use PP tube or glass Petri dish. Add trifluoroacetic acid to the final concentration of 2%. Seal the tube or dish with parafilm. After 10 min of incubation at 40°C, store the chip in this solution in a dark place at room temperature overnight. The immersed chip can be stored for up to 2 weeks.
4. Rinse the chip with ethanolic solution of ammonium hydroxide (10% v/v), ethanol, water and ethanol and dry it with a stream of nitrogen (*see* **Note 10**).
5. Immerse the sensor chip in a solution of TSTU in argon-deaerated DMF (2 mg/mL), add DMAP (0.02 M) and shake it for 1 h under inert gas. Alternatively, expose the chip to

freshly prepared water solution of NHS (0.05 M) and EDC (0.2 M) for 10 min. Alternatively, dissolve NHS in anhydrous dioxane (2.02 mg/mL) and EDC in water (100 mg/mL), mix them in the ratio of 49:1 and incubate the sensor chip in this solution for 1 h at room temperature. Storage in inert gas is recommended.

6. Rinse the sensor chip with ethanol and water, dry it with nitrogen and mount it in the SPR instrument immediately.
7. Flow 10 mM sodium acetate buffer, pH 5.0 (SA) at 25°C at a higher flow rate (e.g. 50 µL/min, depending on the sensor fluidics, **Note 11**) through the sensor flow cell for a few minutes.
8. Inject streptavidin in SA buffer at concentration of 25 µg/ml and let it flow through the flow-cell for 15 min at 30 µl/min. (Optimal flow rate can differ depending on design of the used flow cell.) Replace the solution with SA buffer.
9. Inject 1 M ethanolamine, pH 8.0 at 20°C or Tris buffer for 10 min to deactivate the residual N-hydroxysuccinimidyl esters. Flow 10 mM phosphate buffer, 0.75 mM NaCl, pH 8.5 (PBNa) along the streptavidin-coated surface for 5 min to remove all non-covalently bound streptavidin. Flush the sensor surface with SA; at this point the streptavidin immobilization level can be determined.
10. Switch to 10 mM phosphate buffer, 15 mM $MgCl_2$ buffer, pH 7.2 (PBM) (oligonucleotide immobilization) or to 10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) (protein immobilization). Inject solution of biotinylated oligonucleotide probes with hexa(ethyleneglycol) spacer at concentration of 50 nM in PBM or biotinylated antibodies at concentration of 10 µg/mL in PBS until a plateau is reached (typically 10 min at a flow rate of 30 µL/min). Replace the solution with PBM buffer or PBS buffer, respectively. When a stable level is reached, the immobilization of biorecognition element on the sensor surface is completed and the chip is ready for use.

A typical sensorgram illustrating this protocol is shown in **Fig. 8** which shows sensor response to the immobilization of biotinylated oligonucleotides.

3.1.3. Immobilization of Biotinylated Ligands via BAT/OEG Method (16, 17, 28)

Suitable for immobilization of biotin-tagged ligands (proteins, antibodies, oligonucleotides).

Advantages: Site-specific immobilization, high level of immobilized biotinylated biorecognition elements, stability of immobilized biorecognition element layer under a wide range of environmental conditions (pH, temperature), good resistance to non-specific binding of molecules from complex media such as serum, urine, and food matrix.

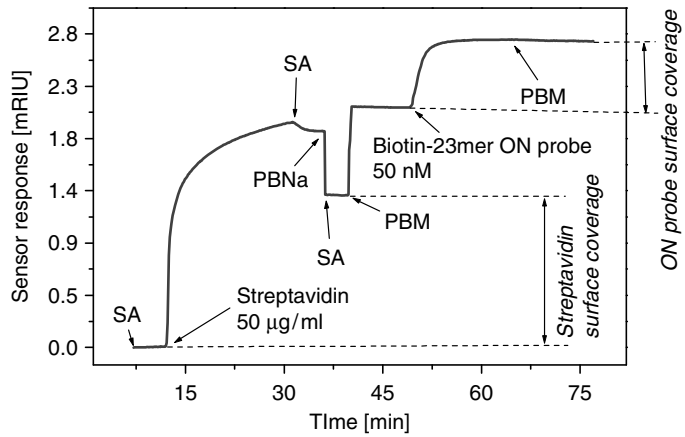

Fig. 8. A typical sensorgram corresponding to immobilization of biotinylated oligonucleotides on the SPR chip via COOH/OEG method. The resulting amounts of bound streptavidin and oligonucleotide (ON) are indicated. Arrows indicate injections of solutions.

Disadvantages: Biotinylation of biorecognition elements required before immobilization.

1. Prepare the 5 mM stock solution of four(ethylene glycol) thiol (AT-EG4) and 1 mM stock solution of biotin-terminated four(ethylene glycol) thiol (AT-EG4-BAT) in absolute ethanol. Seal the tubes well with parafilm. Store them at a temperature of –20°C before use (*see* **Note 7**).
2. Rinse the gold sensor surface with absolute ethanol. Dry it with nitrogen stream. Clean the gold sensor surface in UV ozone cleaner for 20 min, wash it with water and dry it with nitrogen stream (*see* **Note 9**).
3. Immerse the clean sensor chip in solution mixed of AT-EG4-BAT and AT-EG4 thiols mixed in the ratio of 1:9 at a room temperature with a total thiol concentration of 100 µM. The stock AT solution is typically used (*see* **Subheading 2.1**, **item 2**). In a glass Petri dish, combine absolute ethanol (14,860 µl), AT-EG4-BAT (50 µl, 1 mM) and AT-EG4 (90 µl, 5 mM). Use PP tube or glass Petri dish. Immerse chip immediately in this thiol solution. Seal the tube/dish well with parafilm. Store overnight or for up to 2 weeks at room temperature.
4. Rinse the chip with absolute ethanol, blow it dry with a stream of nitrogen and mount it in the SPR instrument immediately.
5. Inject streptavidin in SA buffer at a concentration of 5 µg/mL and let it flow through the flow cell for 15 min at 30 µL/min. (Optimal flow rate can differ depending on the design of the used flow cell.) Replace the solution with SA buffer.
6. Switch to 10 mM phosphate buffer, 15 mM $MgCl_2$ buffer, pH 7.2 (PBM) (oligonucleotide immobilization) or to 10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS)

(protein immobilization). Inject solution of biotinylated oligonucleotide probes with hexa(ethyleneglycol) spacer at concentration of 50 nM in PBM or biotinylated antibodies at concentration of 5 µg/mL in PBS until a plateau is reached (typically 10 min at a flow rate of 30 µL/min). Replace the solution with PBM buffer or PBS buffer, respectively. When a stable level is reached, the immobilization of the biorecognition element on the sensor surface is completed and the chip is ready for use.

3.1.4. Immobilization of Proteins via Physical Adsorption to Positively Charged Surface of SAM (25)

Suitable for immobilization of a wide range of biorecognition elements (proteins, antibodies, oligonucleotides), particularly suitable for biomolecules containing negatively charged groups/epitopes.

Advantages: Simple procedure, very high level of immobilized biorecognition elements.

Disadvantages: Low resistance to non-specific binding from complex media, surface blocking is required.

1. Prepare 1 mM stock solution of AT-NH2 thiols in absolute ethanol. Seal the tubes well with parafilm. Store it at a temperature of –20°C before use.
2. Rinse the gold sensor surface with water and absolute ethanol. Dry it with nitrogen stream. Clean the gold sensor surface in UV ozone cleaner for 20 min, wash it with water (*see* **Note 8**) and dry with nitrogen stream (*see* **Note 9**).
3. Immerse the clean sensor chip into a solution of AT-NH2 with a total thiol concentration of 200 µM and at room temperature. Use PP tube or glass Petri dish. Add triethylamine to the final concentration of 3%. Seal the tube or dish with parafilm. Store the chip in this solution in a dark place at room temperature overnight. The immersed chip can be stored for up to 2 weeks.
4. Rinse the chip with ethanolic solution of acetic acid (10% v/v), ethanol, water and ethanol and dry it with nitrogen stream (*see* **Note 10**). Mount it in the SPR instrument immediately.
5. Flow 10 mM phosphate buffer, pH 7.0 (PB) at 25°C at higher flow rate (e.g. 50 µL/min, depending on the sensor fluidics, **Note 11**) through the sensor flow cell for a few minutes.
6. Inject antibody or protein in PB buffer at a concentration of 2–20 µg/mL and let it flow through the flow cell for 15 min at 50 µL/min. (Optimal flow rate can differ depending on design of the used flow cell.) Replace the solution with PB buffer.
7. Flow 10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, 0.05% Tween, pH 7.4 (PBST) along the functionalized surface for 15 min to remove all weakly bound biorecognition elements. Flush the sensor surface with PB; at this point the biorecognition element immobilization level can be determined.

8. Switch to 10 mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS). Inject suitable blocking agent (e.g. bovine serum albumin, caseine, dry milk) in PBS at a concentration of 0.2–10 mg/mL to block the sensor surface. The used concentration of blocking agent is dependent on the complexity of analyte solution; in general, the higher complexity of analyte solution, the higher concentration and the more careful selection of blocking agent are necessary.

In typical biosensing experiments, one of the sensing channels is functionalized with a biorecognition element against the specific target while the other channel (reference) is coated with molecules which are structurally similar to the used biorecognition element but are expected to have no interaction with the sample. Response of the reference channel is then subtracted from the sensing channel to reduce the effect of interferences.

3.2. Detection of Chemical and Biological Analytes

3.2.1. Direct Binding Assay

Direct detection is usually preferred in applications when binding of the analyte in the concentration range of interest provides a sufficient sensor response. Direct detection is typically well suited for medium and large analytes (>5,000 Da) for which detection limits in the ng/mL or sub-ng/mL range can be achieved *(5, 29, 30)*.

In the direct detection format analyte molecules in a sample bind to the biorecognition elements immobilized on the sensor surface (e.g. antibody, **Fig. 9**), and the sensor response is proportional to the concentration of the captured analyte acquired.

In the direct detection format, the flow cell of the SPR sensor is initially loaded with buffer (both the sensing and reference channels) and when the baseline is established, a sample is injected and incubated with the sensor surface for a certain period of time (*see* **Note 12**). Subsequently, the sample is replaced with the buffer. Typical responses of the SPR sensor are shown in **Fig. 10**.

The response of the sensor to the analyte present in the sample can be quantified by the following metrics:

- Slope of the sensor response immediately after the injection; the slope is determined using data from a fixed time interval (*see* **Note 13**)

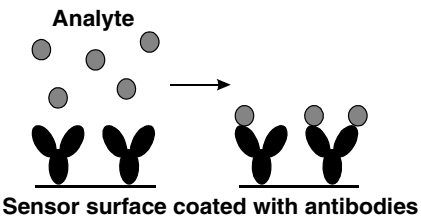

Fig. 9. Detection formats used in SPR biosensors: direct detection.

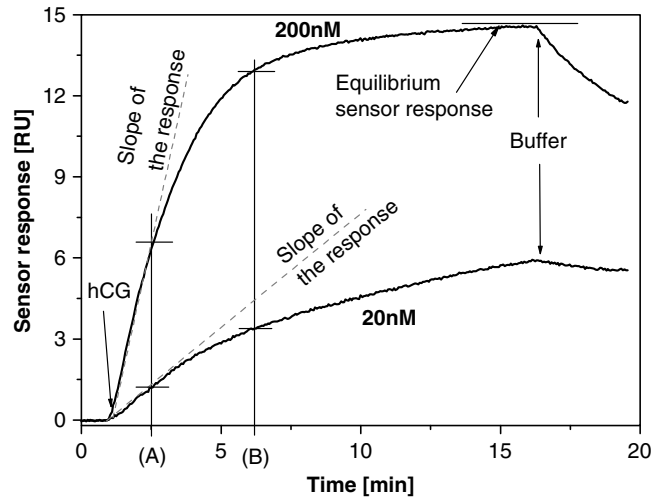

Fig. 10. Typical sensorgram showing response of the sensor to direct detection of two concentrations of hCG hormone. The slopes of sensor response after the injection are plotted. The initial slope of the sensor response (time A), typical example of a fixed-time measurement (time B) and the equilibrium sensor responses are denoted. Arrows indicate injections of solutions.

- Equilibrium sensor response, i.e. sensor response when the association and dissociation processes are in equilibrium and sensor response no longer increases with time
- Fixed-time response, i.e. response of the sensor observed in a fixed time after the sample was injected (*see* **Note 13**)

If the appropriate calibration curve is available (**Fig. 11**), the sensor response (the slope, the equilibrium response or the fixed-time response, respectively) can be translated to the concentration of analyte.

The ability of the sensor to detect the analyte is characterized by the limit of detection (LOD). LOD is defined as a concentration of the analyte that produces a sensor response equal to three standard deviations of the sensor response for blank sample *(31);* the LOD for this model oligonucleotide system was approximately 100 pM (**Fig. 11**).

3.2.2. Sandwich Assay

The lowest detection limits of the direct SPR sensors can be improved by using a sandwich assay. A sandwich assay is generally used to improve the limit of detection beyond that available in the direct detection format and improve specificity of the detection. This approach has also been demonstrated to be suitable for detection in complex matrices *(29)*. In the sandwich assay detection format, the primary biorecognition element immobilized on the sensor surface captures the analyte from the sample

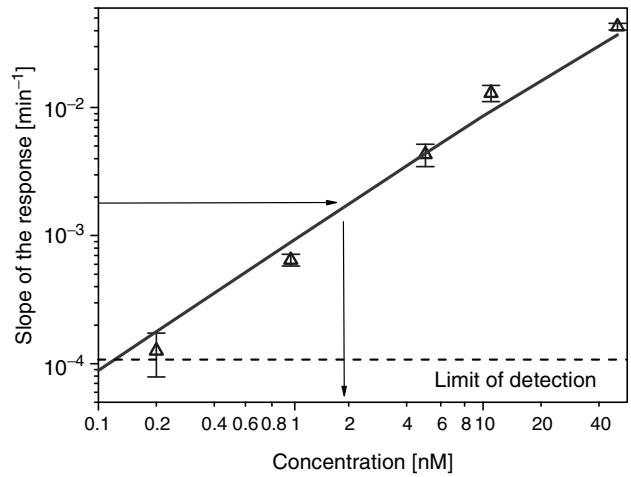

Fig. 11. Determination of concentration of analyte from the calibration curve and the limit of detection. The calibration curve is quantified by the slope of the sensor response.

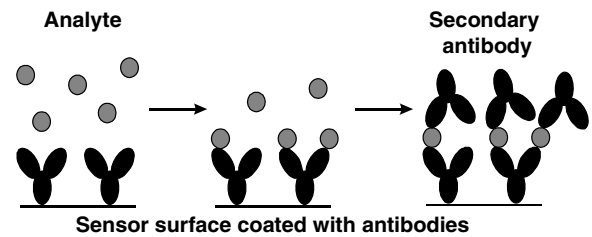

Fig. 12. Detection formats used in SPR affinity biosensors: sandwich assay.

and the subsequently-injected secondary biorecognition element binds to the previously captured analyte (**Fig. 12**).

3.2.3. Binding Inhibition Assay

Competitive assays are commonly used for detections of low concentrations of small analytes, whose binding to surface-immobilized recognition molecules does not produce a measurable sensor response. The binding inhibition assay has been successfully used for small chemicals such as pesticides and toxins with detection limits below 0.1 ng/mL *(32, 33)*. In the binding inhibition assay (**Fig. 13**) analyte molecules or their derivatives are immobilized on the sensor surface. A known quantity of biorecognition elements is incubated with the sample and allowed to bind to the analyte in a sample. The mixture is then flowed over the sensor surface and the unreacted biorecognition elements are captured at the sensor surface. As the concentration of the analyte increases, the concentration of free epitopes on the biorecognition elements decreases and therefore the sensor response is inversely proportional to the concentration of the analyte.

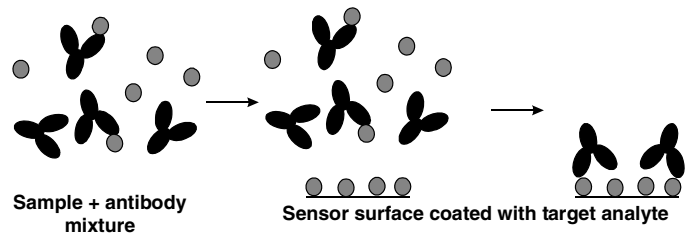

Fig. 13. Detection formats used in SPR affinity biosensors: binding inhibition assay.

4. Notes

1. Penetration depth is defined as the distance from the interface, where the amplitude of the field decreases by a factor of $1/e \approx 0.37$.
2. In SPR systems, the stability of the wavelength spectrum significantly influences the performance of the sensor. Therefore, special attention needs to be paid to the light source condition:
   - The spectrum is influenced by the changes of temperature. Therefore, the light source module requires typically 20–60 min to stabilize or warm up before the experiment.
   - The spectrum can change or become unstable when the lifetime of the light source is exceeded.
3. The coupling prism and the sensor chip are typically made of material with identical or very close refractive indices (e.g. the standard optical glass NBK7). The chip must be optically matched to the coupling prism using a layer of refractive index matching material (e.g. refractive index matching liquid or polymer).
4. Signals can be directed into different detectors such as spectrometers, or line CCD arrays, or different portions of a single 2D array detector. This is often achieved using standard imaging optics, or an array of lenses aligned with the array of sensing channels.
5. The approach based on the intensity measurement is usually referred to as SPR imaging. A 2D array of sensing channels is illuminated with monochromatic light under the angle of incidence close to the resonance and the reflected light is projected on a CCD camera. The spatial distribution of light intensity can be mapped on the spatial distribution of sensing channels.
6. The choice of a flow rate depends mostly on the geometry of the fluidics. Faster flow rates can slightly improve the mass transport when very fast binding needs to be monitored;

slower flow rates may reduce consumption of sample when the binding is too slow even for a relatively high concentration of analyte.

7. Instead of four (ethylene glycol), general oligo(ethylene glycol) thiol may be used. However, shorter OEG groups are likely to lead to coatings with a lower resistance to non-specific binding.
8. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ cm and total organic content of less than five parts per billion.
9. Alternatively, different cleaning procedures can be used (such as plasma cleaner, piranha solution, etc.) unless the sensor chip is stored since production in clean and inert atmosphere. To clean the gold sensor surface in Piranha solution, use 3:1 mixture of sulfuric acid and 30% hydrogen peroxide in a glass or teflon vial. The solution must be mixed before cleaning or directly applied to the gold surface, applying the sulfuric acid first, followed by the peroxide. Piranha solutions are extremely energetic and may result in explosion or skin burns if not handled with extreme caution.
10. During the manipulations with the coated gold sensor chip, it is necessary to avoid touching the sensor area with the tweezers. It could destroy the molecular coatings or contaminate the sensor surface.
11. Air bubbles are strongly undesirable in all running buffers. Buffer solutions should be degassed before preparation of all solutions and injection to the sensor. In order to eliminate the risk of air bubbles, certain SPR sensor systems incorporate inline degassers or bubble traps.
12. The time of incubation depends on details of the specific application and factors such as affinity between the biorecognition element and analyte, concentration of analyte, desired limit of detection and operating range of the sensor, etc.
13. The time of detection should be adjusted based on the required detection limits and stability of the sensor output. While, in general, longer detection times yield better limits of detection, at long detection times the limits of detection may be limited by the stability of sensor output.

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