Chapter 13

SPR in Drug Discovery: Searching Bioactive Compounds in Plant Extracts

Maria Minunni and Anna Rita Bilia

Summary

Biosensors represent an interesting tool in the search of bioactive compounds. In particular, optical sensors based on Surface Plasmon Resonance transduction (SPR) allow monitoring of biomolecular interaction in real time and without any labelling of the interactants. The biosensor analysis can be applied to both pure compounds or to complex mixtures (e.g. plant extract). The SPR detection principle is here presented and the application to the analysis of plant extracts (i.e. of *Chelidonium majus* L.) as a paradigmatic example for the search of bioactive compounds able to interact with DNA, is also discussed.

Key words: Plant extract, DNA, Biomolecule immobilization, Affinity sensor

1. Introduction

Currently, the bottleneck of drug discovery has shifted from the generation of compound libraries to the identification of biologically active lead structures *(1, 2)*. Identification of biologically active molecules involves the use of bioassays intended not only at measuring their extent of interaction with specific receptors or binding proteins *(3)*, but also at evaluating their bioactivity *(4)*, bioavailability *(5)*, and pharmacokinetic behaviour *(6)*.

Behind conventional approaches, biosensor technology could play an important role in selecting compound exhibiting biological activity towards selected target receptors.

Biosensors represent new analytical devices which appear to be an analyst's dream: they are able to give rapid analysis responses; to operate directly on complex matrices, in many

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cases; to be selective and sensitive enough for the required application; to be portable and sometimes also disposable; and, to have fast analysis times *(1)*. Biosensors have mainly been applied for analytical purposes in environmental chemistry, clinical practice, and analysis of food, but recently several examples dealing with natural compounds have also been reported in the literature.

In this chapter, SPR-based biosensor technology is described and the protocol to immobilize proteins on a chip is given. In particular, using streptavidin as immobilized protein and how to further immobilize biotinylated nucleic acid sequences for investigating the ability of selected plant extracts to interact with the immobilized nucleic acid double strand is also reported. The application is suitable for evaluating the ability of the extracted compound to interact with DNA and thus with potential interest as antitumour agents (i.e. natural alkaloids).

1.1. Biosensors

As defined by IUPAC in 1999, a biosensor is an integrated device able to give qualitative and quantitative or semiquantitative specific information through the use of a biological element of recognition in close spatial contact with a transducer. The biological element is responsible for the biological recognition of the target analyte and thus for the sensor specificity. The biomolecule is immobilized on a physical transducer that translates the biorecognition event into a useful electrical signal **(Fig. 1)**. Biosensors can be divided mainly into two categories: catalytic and affinity biosensors *(7)*.

Catalytic biosensors involve a catalytic event in which a substrate is converted into a product.

Catalysis occurs at the transducer interface and substrate depletion or product formation is measured by a transducer. The well-known enzyme-based sensor for glucose, widely used in clinical practice for glycaemia measurements and marketed by many different companies belongs to this category.

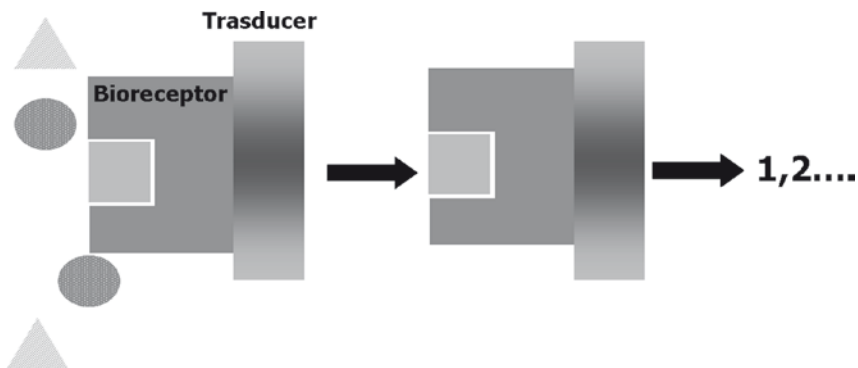

Fig. 1. Biosensor scheme.

In affinity biosensors, recognition of the analyte in solution by the immobilized biological element is based on an affinity reaction i.e. an antigen–antibody binding, nucleic acid hybridization, or receptor–ligand interaction. On the basis of these different interactions, the affinity biosensors can be divided into immunosensors, DNA sensors, aptasensors *(8)*, etc.

The transduction principles can be various and the choice is based on different considerations, such as the electrochemical or optical behaviour of the analyte, the molecular weight, the binding properties, etc.

Transduction can require the use of labels or can eventually be label-free. Moreover, the sensor can be single or multi-use, with this meaning that the chip is disposable or it can be used for more than one analysis. Portable or bench instrumentation is also available.

Relatively to drug discovery, different transduction principles have been reported *(9, 10)*. However, the most applied one is optical detection and in particular Surface Plasmon Resonance (SPR) *(11)*.

1.2. Optical Detection: Surface Plasmon Resonance-Based Sensing

SPR is a widely used optical method for biospecific interaction analysis since it is possible to monitor the affinity interactions in real time. Surface Plasmon is a charge density oscillation that may occur at the interface between two media with dielectric constants of opposite sign, such as a metal and a dielectric. Metal used are mainly gold (Au) and silver (Ag).

Surface plasmon can be excited by a *p*-polarized optical wave. Light is directed through a transparent optical substrate and the intensity of the resulting reflected light is measured with a detector. At certain incident angle of the light, part of the energy will couple into a surface plasmon wave travelling at the interface Au/sample. This angle will be called resonant angle. This coupling (resonance phenomenon) is observed as a sharp attenuation in reflectivity (in total reflection conditions) **(Fig. 2)**.

There are different parameters influencing the resonance angle at which the coupling occurs: among others, the refractive index of the media involved in the phenomenon (air, glass, and solution). So a change in the refractive index of the media involved (i.e. the solution flowing on the metal layer/glass) would be recorded as a change in the resonant angle at which the minimum in reflectivity occurs. It is possible thus to follow interface phenomena (i.e. changing the refractive index in the solution flowing on the glass/metal surface) by monitoring the shift in the resonant angle (as also the shift in the reflectivity minimum). This optical principle can be easily used for biosensor development. As mentioned above, a biosensor relies on the immobilization of a biological molecule on the transduction surface; in the case of SPR transduction, the molecule can be

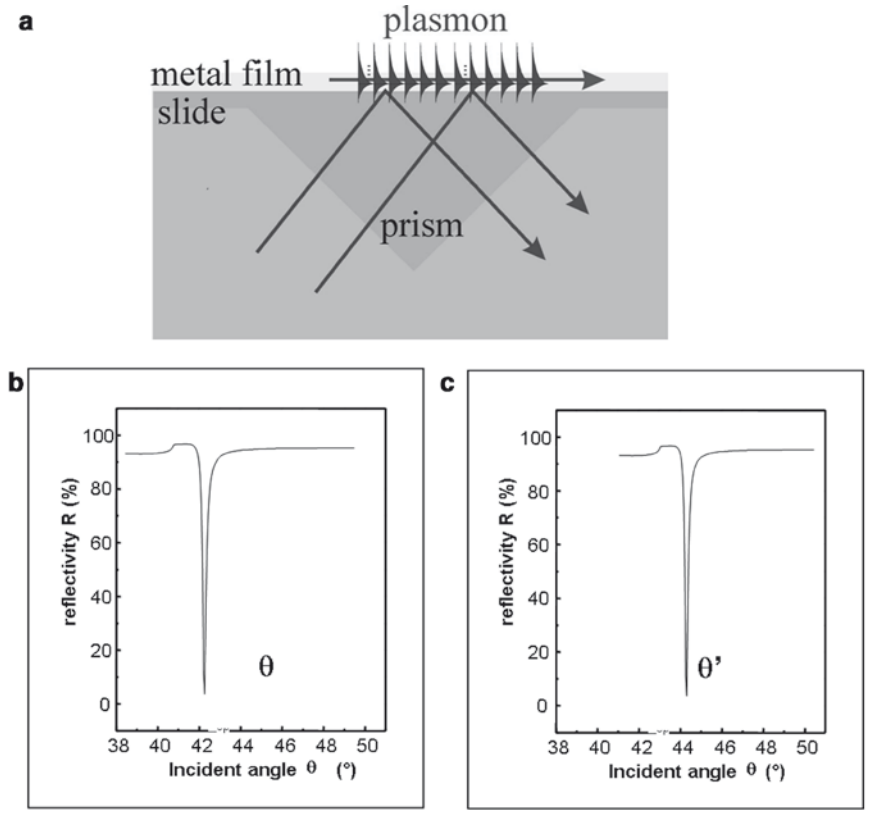

Fig. 2. Surface Plasmon Resonance (SPR) optical transduction: (**a**) SPR theory. At certain incident angle of the light, part of the energy will couple into a surface plasmon wave travelling at the interface Au/sample. This coupling is observed in (**b**) as a sharp attenuation in reflectivity; here a minimum in the reflectivity recorded when SPR occurs at the metal interface (the minimum is observed at the resonant angle) is shown at the resonant angle. When an interface phenomenon occurs (i.e. binding between a receptor and an analyte at the surface), a shift in the angle at which the minimum occurs (from θ to θ) can be observed in (**c**).

immobilized directly on the metal surface by physical adsorption or by optimized immobilization chemistries. The immobilization step as well as the subsequent interaction of the immobilized molecule with an analyte/interactant in solution can be followed in real time without the use of any label. In fact, the interface changes induced by the flowing solution induce a shift in the resonant angle. This event can be displayed in function of time generating what is called a sensorgram. A sensorgram is thus a way to represent interface phenomena in function of time. In particular, on SPR sensorgrams, changes over time in arbitrary units, such as Resonant Units (RU) is reported. Alternatively, the signal is expressed as Reflectivity %.

From the Sensorgram important information relative to interaction between the immobilized molecule and its ligand, added in solution, can be obtained.

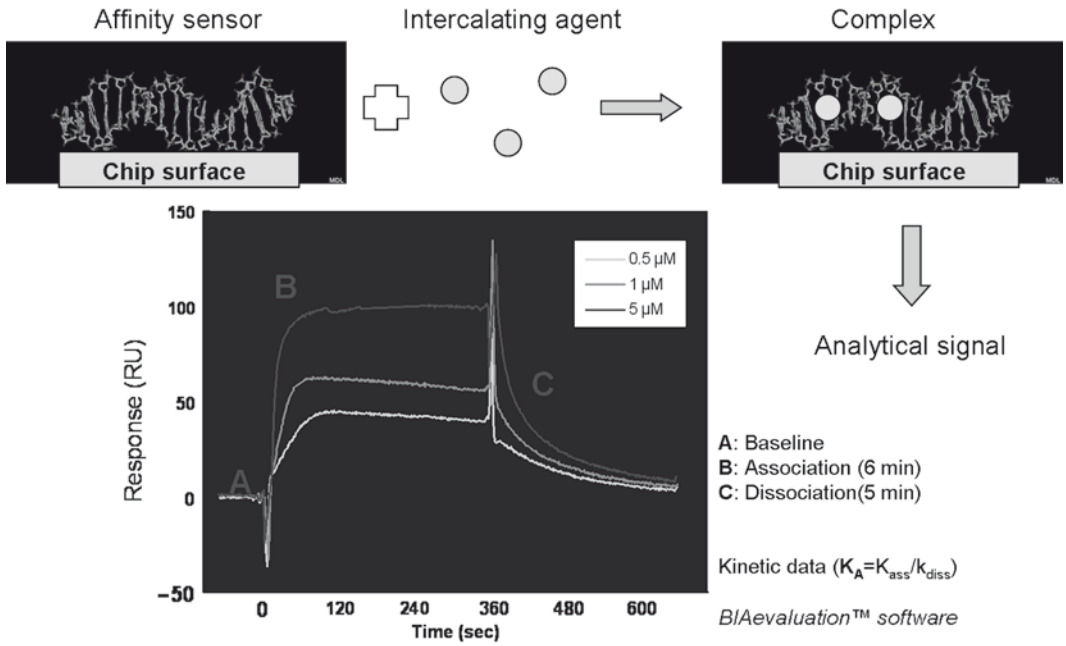

Fig. 3. Sensorgram reports the association and dissociation phases of the interaction between a binder agent (i.e. sanguinarine) and the DNA immobilized on the surface. Three different concentrations of the solution are tested.

In the Sensorgram reported in **Fig. 3**, different steps can be identified. We could consider a system with the immobilized analyte and the buffer solution flowing on the surface. As base line, the signal corresponding to the presence of buffer only is taken. After the addition of the interacting analyte, a complex is formed at the transducer surface, which is revealed by the shift in the recorded signal. The first part of the signal corresponds to the association phase and could eventually reach a plateau. When the sample containing the analyte is replaced by buffer at the end of the injection, dissociation is observed. The rates of association and dissociation are important parameters in the evaluation of the interaction. In other words, we can see how fast the complex is formed and how stable it is. Quantitative information about the analyte present in solution is also obtained by this technology: how much compound/analyte is in the sample? The concentration level can be in the range of submicromolar-nanomolar level, depending on the molecular mass of the ligand.

These findings are strategic when focused on drug development. In other words, one can evaluate if a molecule under study has the ability to bind a target receptor and eventually how fast is this interaction, how strong by calculating its affinity constant (K_A), how specific by testing chemically related molecules as well as negative control to test any unspecific binding.

Thinking more generally, one could have answers to the following questions: Does anything bind from this crude extract?

Is the interaction specific? Is sample activity reduced during purification? How much analyte/product is in the sample? How does the activity of a preparation compare with the last batch?

In terms dealing with drug discovery, this could be referred to a plant extract in natural compound screening. For example, this approach can be applied for the analysis of bioactive constituents with application in drug and herbal drug screening *(12)* or as new analytical tool in the Quality Control of Herbal Drugs, Herbal Drug Preparations, and Herbal Medicinal Products. In particular here is reported as paradigmatic example the case of the protocol applied using SPR sensing for herbal extract screening of some fractions obtained from an ethanolic extract of the fresh plant material of *Chelidonium majus* L. (great celandine). This plant contains benzo[*c*]phenanthridinium alkaloids, having multiple biological and pharmaceutical effects such as anti-inflammatory and anti-microbial activity, inhibition of SH-enzymes and microtubule assembly *(13, 14)* and, in particular, these alkaloids possess the ability to interact with DNA *(15)*.

For SPR-based sensing, commercially available bench-top instruments are perfectly suitable for laboratory use. Some have high degree of automation, contributing significantly to very good analytical performance. More recently, portable devices based on SPR have also been marketed demonstrating that the technology is also suitable for in-field measurements. In the last few years, the SPR transduction principle has been commercialized in an array format, which push a step forward this technology as interesting tool for screening purposes since it is possible to monitor simultaneously hundreds of interactions *(16)*.

2. Materials

2.1. Immobilization Step

1. 1-mM ethanolic solution of 11-mercaptoundecanol (Aldrich, 2 mg of the thiol in 10 mL of ethanol) is freshly prepared before use.
2. 600-mM epichlorohydrin in a 1:1 mixture of 400-mM NaOH.
3. Activating solution: 50 mM N-hydroxysuccinimide (NHS).
4. 200 mM N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC) in water.
5. Basic dextran solution: Add 3 g of dextran T500 (Amersham Biosciences) in 10 mL of 100 mM NaOH.
6. 1-M bromoacetic acid (Sigma) in 2-M NaOH.
7. Blocking solution: 1-M ethanolamine hydrochloride in water, pH 8.6.

8. Oligonucleotides (Amersham Biosciences-Uppsala, Sweden) are received, lyophilized, and then diluted with H_2O in stock solutions, divided into aliquots, at a concentration of 100 μM. The stock solutions are stored at –20°C. Freshly prepared diluted solution (concentration 1 μM) in hybridization buffer are used for the immobilization on the chip.

The base sequences of the 5′-biotinylated probe (25-mer) and of the complementary oligonucleotide (25-mer) are as follows:

Probe: 5′-biotin-GGCCATCGTTGAAGATGCCTCTGCC-3′;

Complementary sequence: 5′-GCAGAGGCATCTTCAACG-ATGGCC-3′

Prepare freshly diluted solution of complementary target (concentration 1 μM) in hybridization buffer (concentration 1 μM) and inject into the instrument, after the probe immobilization, to obtain a double helix to further expose to the plant extracts.

9. Streptavidin (protein from *Staphylococcus aureus*) (Sigma); the protein is diluted in 10-mM acetate buffer, pH 5.0 (concentration 1,000 μg/mL), divided into aliquots, and stored at –20 °C. Before immobilization, it is diluted down to 200 μg/mL streptavidin in the same buffer.
10. Immobilization buffer: 0.01 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 0.15 M NaCl, 3 mM EDTA, and 0.005% polyoxyethylene sorbitan monolaurate (Tween 20), pH 7.4.
11. Binding buffer: 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20, and 0.1% BSA, pH 7.4.
12. Regeneration solution: HCl 1 mM, 30 s.

2.2. Samples for Testing

2.2.1. Pure Compounds

1. Doxorubicin (Doxorubicin hydrochloride – Sigma, Aldrich) and sanguinarine (sanguinarine nitrate – City Chemical Corporation, New York, NY), are well-known intercalator *(17–19)* and are used here as standard compound. This means that before testing plant extract with unknown DNA binding activity, the system must be checked with compounds of well-documented activity, to demonstrate its reliability avoiding, possibly, false negative results.
2. Doxorubicin and sanguinarine 1 mg/mL in DMSO.

2.2.2. Plant Extract and Fractions

1. *C. majus* L. (great celandine) grows in summer and can be collected in fields.
2. Ethanol (Merck) at analytical grade is used for extraction.
3. 150 g of whole-fresh plant material of great celandine is used to obtain a crude EtOH extract by percolation. Deposit the plant in an ordinary paper filter, into a glass fennel in contact with a flask. Then add solvent (1,000 mL) to extract the compounds.

4. The extract is concentrated on rotavapour under reduced pressure. Evaporate a portion of the extract to dryness to obtain a dry residue. Re-suspend 2 g of it in $CHCl_3$ (5 mL). Then filter the solution, using a paper filter, to remove the insoluble part. Place the chloroform solution on the top of a silica column and fractionated by gravity column chromatography (Sigel CC fractionation). Elute using mixtures of $CHCl_3$–MeOH (from 100:1 to 1:100), flow for elution: flow, 3 mL/min, to obtain 100 fractions of 20 mL. Fractions 1–35 are obtained from $CHCl_3$ 100%; fractions 36–40 are obtained by $CHCl_3$–MeOH 96:4; fractions 41–60 from $CHCl_3$–MeOH 9:1; fractions 61–73 from $CHCl_3$–MeOH 4:1; fractions 74–84 from $CHCl_3$–MeOH 70:30; fractions 85–96 from the mixture of $CHCl_3$–MeOH 1:1, and the last three fractions from 100% methanol. Fractions are examined by thin layer chromatography (TLC) to select and collect those containing the compound of interest i.e. alkaloids. Eight main fractions *(1–8)* can be obtained **(Fig. 4)**.
5. These eight main fractions *(1–8)* are evaporated to dryness on rotavapour under reduced pressure to be analysed by biosensor analysis.

2.2.3. Thin Layer Chromatography of Purified Fractions from Great Celandine

1. Prepare Silica gel 60 F254 aluminium sheets (20 × 20 cm) (Merck).
2. Deposit a drop of the crude extract and fractions on the sheet.
3. Elute with $CHCl_3$–MeOH (9:1 and 1:1) using a light at 365 nm as detector.

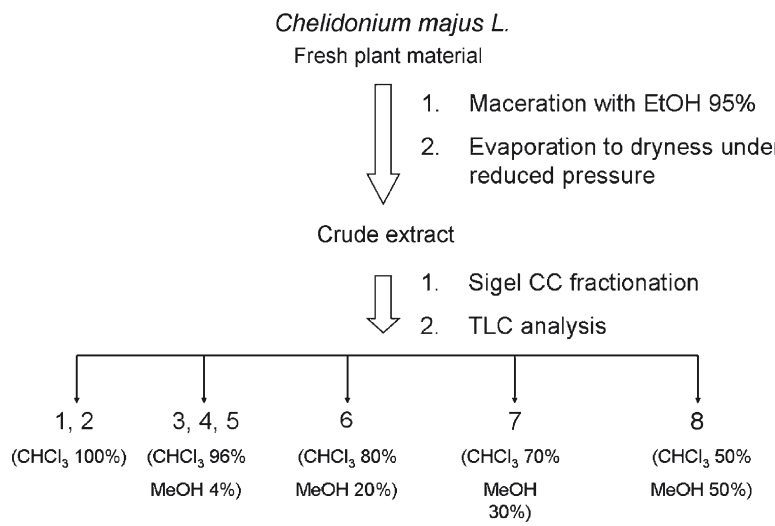

Fig. 4. Plant extracts fractionation by solid phase extraction on silica gel column.

2.3. Instrumentation

SPR measurements are performed using the BIACORE X ™ instrument (Biacore AB, Sweden) and carboxylated dextran-coated chips (CM5 chip, Biacore AB). The SPR signal is expressed in RU.

3. Methods

3.1. Immobilization of Biotinylated DNA Probe onto Streptavidin-Modified Chip Surfaces

To assure a strong surface anchoring of the DNA probe, the interaction occurring between streptavidin and biotin $K_A = 10^{15}\ M^{-1}$ is used. Thus, on commercial thiol/dextran-modified chip purchased by Biacore AB (Chip CM5), streptavidin is first immobilized to form a layer for biotin attachment.

1. Immobilization of streptavidin onto SPR Chips: The instrumentation operates in flow conditions and under a fine temperature control. The flow and the temperature can be varied. The immobilization procedure is performed at a constant flow rate of 5 μL/min and a temperature of 25°C. Immobilization buffer is used as running buffer. For the immobilization of the protein streptavidin, the dextran surface of the CM5 chip is further modified with 35 μL of streptavidin solution after treatment with 35 μL of activating solution. The remaining carboxylated sites on dextran are blocked with 35 μL of 1 M ethanolamine. After the blockings step, the DNA immobilization is performed.
2. Immobilization of the DNA probe: The biotinylated probe (1 μM) is injected (35 μL at a flow rate of 5 μL/min corresponds to 7 min of contact time between the solution and the surface). After the injection, the excess of unbound probe is removed by the running buffer and the relative signal in RU recorded. For a probe of 20 bases in length, the immobilization is considered successful when a signal of at least 500 RU is recorded.

After immobilizing the probe, a dsDNA stand, to be used as receptor, is obtained by adding to the surface the probe's corresponding complementary sequence.

3. The complementary sequence in hybridization buffer is thus injected (50 μL) and the formation of the double helix (dsDNA) can be followed in real time. The amount of hybrid formed is given by the corresponding RU recorded after the end of injection and washing off of the excess of the unbound oligo by the running buffer.

The instrument provides two flow cells: the probe is immobilized only on one of the two. In the other flow cell, where the probe is not immobilized, there is only the streptavidin attached to the chip. This second flow cell is referred as control cell,

meaning that this latter is used for checking the specificity of the interaction between the DNA and the testing compound/sample. If the interaction with the surface in flow cell one (where the DNA is immobilized) is specific, then no signal should be recorded on the control cell.

3.2. Binding Measurements

1. After the immobilization of small oligonucleotides onto the SPR chip, the interaction between the immobilized receptor and the samples in binding buffer is monitored with an association time of 6 min followed by 5 min of dissociation. Solutions of each compound are injected in the system and association and dissociation phase of the interaction were recorded **(Fig. 5)**. The flow cell without DNA is used as control cell to verify the absence of non-specific adsorption of the compounds on the sensor chip.
2. Binding interactions are monitored at a constant flow rate of 5 μL/min at a temperature of 25°C. The different compounds can be monitored with both in the association and dissociation phase of the interaction with the DNA. In particular, here the used association time is 6 min while the dissociation phase is monitored for 5 min.

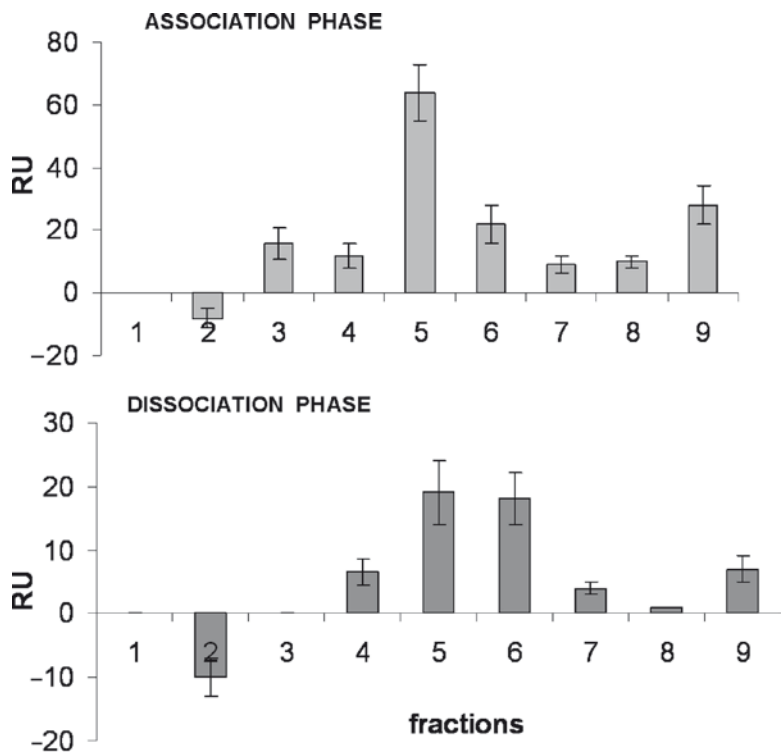

Fig. 5. Results obtained with the eight tested fraction; the signal reported are referred to the association and dissociation phase of the interaction.

3. The surface of the chip can be regenerated to remove the sample and obtain the receptor; however, the solution will also destroy the dsDNA, leaving only ssDNA probe on the surface. To restore the dsDNA, an injection of the complementary target should be done (*see* **Subheading 2.1**, **item 12**) and then the surface is ready for another binding measurement.
4. Negative controls can be tested to prove the specificity of the interaction, for example, compound that are well known that do not interact with DNA, i.e. emodine, chrysophanol.
5. The response of the sensor to blank solutions has to be tested to make sure that the signal is generated by the interaction between the compounds under study and the receptor (i.e. dsDNA on the surface) and not by a matrix effect. The blank solution should contain the same concentration of BSA that is present in the binding buffer.
6. The affinity constants (K_A) of the interaction between the immobilized DNA and pure compounds are estimated by the use of the dedicated BIAevaluation software™ (Biacore AB, Uppsala, Sweden). A variety of methods is available for measuring affinity of biomolecular interactions. SPR-detection can be used more generally than fluorescence or absorbance-based methods since it is sensitive to changes in mass (influencing the refractive index of the media) and no labelling of the reagents is required. The theoretical introduction (*see* **Note 8**) is limited to the description of one to one interactions between independent interaction sites, used for calculating the constant in this application. However, the software available with the instrumentation offers different possibilities for data fitting.

4. Notes

1. The NHS and EDAC solution must be prepared immediately before the use to avoid loss of activity.
2. The regenerating solution removes both the interactant and the target DNA strand, restores initial conditions (*see* **Subheading 1.2**, **step 3**) before performing a new cycle.
3. Pure sample solutions diluted in DMSO cannot be directly applied to the system, but should be diluted to have maximum 5–10% presence of organic solvent when tested with the biosensor (*20*).
4. For the dried crude extract (*Chelidonium*) the estimated detection limit (DL) is below 5 ppm (5 μg/mL) (*12*).

5. Affinity constant for the interaction with dsDNA for Doxorubicin (Molecular Weight 579.99) and sanguinarine (Molecular Weight 394.22) results respectively in $9 \pm 4 \times 10^5$ and $9 \pm 3 \times 10^5$ (M^{-1}) (*12*).
6. The association and dissociation phases of the interaction can be clearly distinguished, as shown in the sensorgram reported in **Fig. 3**. In the association phase, the ligand interacts with the DNA forming a complex; in the dissociation phase, the complex resulting from the interaction starts to dissociate, while the chip is washed with flowing buffer (phase C). The results are reported as resonance shift ΔRU, which is the difference between the final value of resonance units at the end of the association or dissociation phase and the initial value with buffer.
7. In the dissociation phase, some compounds in the fractions can be washed off very quickly by the flowing buffer while others remain bound to DNA. This is indicative somehow of the "strength" of the binding between the active compounds and immobilized DNA.
8. General consideration on measuring affinity and rate constants (*20*).

In the present application, one should refer to the fact that molecular interactions are characterized in part by the extent of complex formation, described by an equilibrium constant. If two interacting molecular species A and B that can form a complex are mixed in solutions, complex formation will proceed until an equilibrium is eventually reached:

$$A + B \leftrightarrow AB.$$

According to the law of Mass Action, the position of the equilibrium is dependent on the concentration of A, B, and AB, respectively, but can always be described by the equilibrium constant K. Depending on the "direction" in which the reaction is written, K is either expressed as an association equilibrium constant K_A (unit M^{-1}) or a dissociation equilibrium constant K_D (unit M). These constants are given as the inverse of each other:

$$\frac{[AB]}{[A][B]} = K_A \text{ and } \frac{[A][B]}{[AB]} = K_D$$

Affinity constants do not characterize the reaction completely: rates of association and dissociation are equally important. The equilibrium constant does not contain any information on the time span that will elapse before reaching the equilibrium. The rates of association and dissociations are described by association and dissociation rate constants respectively:

Association

$$A + B \rightarrow AB$$

The increase in concentration of AB complexes over time can be written as:

$$\frac{d[B]}{dt} = k_{ass}[A][B],$$

where k_{ass} is the association rate constant (unit M^{-1} s^{-1}).
Dissociation

$$AB \rightarrow A + B$$

The decrease in concentration of AB complexes over time can be written as:

$$\frac{d[AB]}{dt} = -k_{diss}[AB],$$

where k_{diss} is the dissociation rate constant (unit s^{-1}).

The dissociation rate constant k_{diss} indicates the fraction of AB complexes that dissociate per second. Values for k_{diss} typically range from 10^{-5} to 10^{-2} s^{-1}, corresponding to half-lives for biological complexes from a few minutes to several hours.

The observed net rate of formation or dissociation of AB complexes when approaching equilibrium from either excess of A, B, or AB is the sum of the two rate expressions

$$\frac{d[AB]}{dt} = k_{ass}[A][B] - k_{diss}[AB]$$

At equilibrium, there is a balance between association and dissociation and the net rate of complex formation will be zero:

$$\frac{d[AB]}{dt} = 0, \quad \text{i.e.} k_{ass}[A][B] = k_{diss}[AB]$$

Rearranging this expression shows that the equilibrium constant K is the ration of the association and dissociation rate constants:

$$\frac{k_{ass}}{k_{diss}} = \frac{[AB]}{[A][B]} = K_A$$

$$\frac{k_{diss}}{k_{ass}} = \frac{[A][B]}{[AB]} = K_D$$

The approach on which is based the work illustrated in this chapter is based on the Biacore developed theory.

To apply the general rate equation to the formation of AB complexes, the progress of the reaction must be monitored in some fashion. This can be done by a series of time-resolved determinations of the concentration either of AB complexes or of reactant A or B.

Generally, using subscripts 0 and t to denote time: $[B]_t = [B]_0 - [AB]_t$ and substituting in the general rate equation for formation of AB gives:

$$\frac{d[AB]}{dt} = k_{ass}[A]_t\left([B]_0 - [AB]_t\right) - k_{diss}[AB]_t$$

In real-time analysis, such as the one provided by Biacore instrumentation in the case study reported here, complex formation is monitored directly as the change in the response over time. The concentration of free analyte is kept constant through a continuous flow of fresh analyte solution passed on the sensor surface. Denoting the analyte as A, (concentration C) and the ligand as B gives,

$$\frac{dR}{dt} = k_{ass}C\left(R_{max} - R_t\right) - k_{diss}R_t,$$

where C is the concentration of the analyte free in solution; R_{max} is the total amount of binding sites of the immobilized ligand B, expressed as SPR response in RU; $R_{max} - R_t$ is the amount of remaining free binding sites at time t, expressed as SPR response in RU; dR/dt is the rate of formation of surface complexes expressed as SPR response in RU/s, i.e. the derivative of the response curve.

In accordance with the general rate equation, the observed response curve will be the sum of the association term and the dissociation term. During sample injection, complexes will continue forming until eventually, when the two terms balance each other, equilibrium is reached. Note that only the association term $k_{ass}C(R_{max} - R_t)$ is dependent on the concentration of the free analyte.

Dissociation is observed at a rate depending on the amount of complex formed when the sample is replaced by buffer at the end of the injection.

Rearranging the rate equation shows more clearly that the derivative of the binding curve is linearly related to the response. In principles, therefore, rate constant can be derived from a plot of dR/dt against R.

$$\frac{dR}{dt} = k_{ass}CR_{max} - \left(k_{ass}C + k_{diss}\right)R_t$$

Sometimes it could be difficult to estimate R_{max}, i.e. biological interaction can take several minutes or even hours to reach equilibrium, but this can be circumvented. From the rearranged rate equation, it can be seen that the slope (k_{ass} C + k_{diss}) of the line obtained by plotting dR/dt against R is itself linearly related to the concentration of the free analyte concentration C.

By plotting the slopes of the dR/dt vs. R lines as a function of analyte concentration C, a new line is obtained, with slope k_{ass} and intercept of the abscissa k_{diss}.

$$\text{Slope}\left(\mathrm{d}R / \mathrm{d}t \text{ vs. } R\right) = k_{ass}C + k_{diss} \quad \text{in other words: y} = k_{ass}x + q.$$

Relatively to the dissociation phase, we know that when the sample pulsed has passed on the sensor surface and is replaced by buffer, the concentration of the free analyte drops suddenly to zero. The derivative of the response curve then reflects the dissociation rate:

$$\frac{\mathrm{d}R}{\mathrm{d}t} = -k_{diss}R_t.$$

This is of course valid only under the assumption that re-association of released analyte is negligible, i.e. analyte is efficiently removed by the buffer flow. Integrating with respect to time gives:

$$\ln\frac{R_{t_1}}{R_{t_n}} = k_{diss}\left(t_n - t_1\right),$$

where R_{t_n} is the response at time n and R_{t_1} is the response at an arbitrary starting time 1. The dissociation rate constant k_{diss} can thus be obtained by plotting the log of the drop in response against time interval.

An important point to be considered is that association and dissociation reaction in surface-bound system are ultimately governed by mass transport of the analyte between surface and bulk solution. In order to have real and not apparent constant values it is important that the interaction does not occur in mass transport limited conditions.

9. The Biacore X™ instrument has two cells. The two cells can be modified with different receptors to have a working and a reference cell. Otherwise, one of the cells can be kept with only carboxylated dextran to check the effect of a "blank" to eliminate the signal due to eventual non-specific adsorption of the target onto the medication layers.

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