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Surface Plasmon Resonance

Applications in Understanding Receptor–Ligand Interaction

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Abstract

During last decade there has been significant progress in the development of analytical techniques for evaluation of receptor–ligand interaction. Surface plasmon resonance (SPR)-based optical biosensors are now being used extensively to define the kinetics of wide variety of macromolecular interactions and high- and low-affinity small molecule interactions. The experimental design data analysis methods are evolving along with widespread applications in ligand fishing, microbiology, virology, host–pathogen interaction, epitope mapping, and protein–, cell–, membrane–, nucleic acid–protein interactions. SPR-based biosensors have strong impact on basic and applied research significantly. This brief review describes the SPR technology and few of its applications in relation to receptor–ligand interaction that has brought significant change in the methodology, analysis, interpretation, and application of the SPR technology.

Index Entries: Surface plasmon resonance (SPR); optical biosensor; receptor–ligand interaction; Biacore; ligand fishing.

Introduction

Surface plasmon resonance (SPR) technique is an optical method for measuring the refractive index of very thin layers of material adsorbed on a metal. A fraction of the light energy incident at a sharply defined angle can interact with the delocalized electrons in the metal film (plasmon) thus reducing the reflected light intensity. The SPR technique is based on the fact that, at certain conditions, surface plasmons on a metallic film can be excited by photons, thereby transforming a photon into a surface plasmon and it depend on the refractive index of the adsorbate *(1)*. The most common geometrical setup (the Kretschmann configuration) of SPR can be seen in

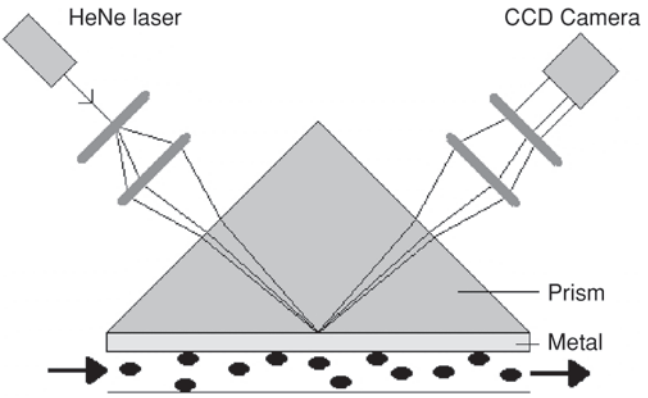

Fig. 1. The most common geometrical setup (the Kretschmann configuration) of SPR. The incoming light is located on the opposite side of the metallic slab than the adsorbate.

the Fig. 1. The incoming light is generally located on the opposite side of the metallic film than the adsorbate because photons cannot excite surface plasmons on the surface being hit. The photons induce an evanescent light field into the metallic film. Normally no transport of photons takes place through this field, but photons incident at a certain angle are able to pass through the field and to excite surface plasmons on the adsorbate side of the metallic film. Whenever a plasmon is excited, one photon disappears producing a dip in reflected light at that specific angle. The angle, which is dependent on refractive index of the adsorbate, is measured with a charged couple device (CCD) chip. In the case of protein adsorption, the difference between the refractive index of the buffer (i.e., water) and the refractive index of the adsorbate can be easily converted into mass and thickness of the adsorbate as all proteins have almost identical refractive indices.

The precise angle of incidence at which this occurs is determined by a number of factors, but in the Biacore system the principal determinant is the refractive index close to the backside of the metal film, to which target molecules are immobilized and addressed by ligands in a mobile phase running along a flow cell. If binding occurs to the immobilized target, the local refractive index changes, leading to a change in SPR angle, which is monitored in real time by detecting changes in the intensity of the reflected light, producing a sensorgram. The rates of change of the SPR signal can be analyzed to yield apparent rate constants for the association and dissociation phases of the reaction. The ratio of these values gives the apparent equilibrium constant (affinity). The size of the change in SPR signal is directly proportional to the mass being immobilized and thus can be interpreted crudely in terms of the stoichiometry of the interaction. Signals are easily obtained from very low quantities (less than a microgram) of material. Because the SPR signal depends only on binding to the immobilized template, it is also possible to study binding events from molecules in

extracts, i.e., it is not necessary to have highly purified components. The advantages of the Kretschmann configuration are that it is not necessary to shine light through the adsorbate and that it is easy to build (relative to other possible SPR configurations). The surface plasmons typically extend in the order of approx 200–300 nm into the medium, thus "sensing" a refractive index change of this volume. From these data, mass and thickness of adsorbed layers can be deduced. Conformational changes in the internal structure of the layers (binding behavior of proteins and cells) can also be measured.

For better understanding of SPR, it is important to understand the phenomenon of total internal reflection (TIR) which occurs at an interface between non-absorbing media. When a light beam propagates in a medium of higher refractive index and meets an interface at a medium of lower refractive index at an angle of incidence above a critical angle, the light is totally reflected at the interface and propagates back into the high refractive index medium *(2)*. Although the fully reflected beam does not lose any net energy across the TIR interface, the light beam leaks an electrical field intensity (called evanescent field wave) into the low refractive index medium. The amplitude of this evanescent field wave decreases exponentially with distance from the interface, decaying over a distance of about one light wavelength from the surface. If the TIR interface is coated with a layer of suitable conducting material, such as a metal, of a suitable thickness, the polarized component of the evanescent field wave may penetrate the metal layer and excite electromagnetic surface plasmon wave propagating within the conductor surface that is in contact with the low refractive index medium. For a nonmagnetic metal, like gold, this surface plasmon wave will also be polarized and, owing to its electromagnetic and surface propagation nature, will create an enhanced evanescent wave. This evanescent wave has electric field components directed in all spatial orientation during penetration into the low refractive index medium. Because the electric field penetrates a short distance in to the lower refractive index medium, the conditions for SPR are sensitive to the refractive index at the gold surface. For a given wavelength of incident light, SPR is seen as a dip in intensity of reflected polarized light at a specific angle of incidence. Biacore system works on this principle and developed SPR based biosensor system for several applications. The range of kinetic constants that can be measured with precision with Biacore are 10^3–$10^7/M{\cdot}s$ for the on rate (k_a) and 10^{-5} to $10^{-1}/s$ for the off rate (k_d) *(3)*.

SPR-Based Biosensor

In the 1980s, SPR and related techniques exploiting evanescent waves were applied to study biological and chemical interaction *(4–6)*. Now SPR biosensors have become an established method for measuring molecular interactions. The best indicator of optical biosensor technology is the growing number of commercially available instruments. Several companies

Table 1
Commercial Biosensors

Company	Website
Biacore AB	http://www.biacore.com
Affinity Sensors	http://www.affinity-sensors.com
Windsor Scientific Limited	http://www.windsor-ltd.co.uk
BuoTul AG	http://www.biotul.com
Nippon Laser and Electronics Lab	http://www.nle-lab.co.jp
Texas Instruments	http://www.ti.com/spr
GWC Instruments	http://www.gwcinstruments.com
Jandratek GmbH	http://www.jandratek.de
IBIS	http://www.ibis-spr.nl
Applied Biosystems	http://www.appliedbiosystems.com

currently manufacture a variety of instruments based on SPR-based biosensors. Few of them are listed in Table 1. SPR-based instruments permit the analysis of biomolecular interaction in real time without labeling requirements. Typical biological system examined using these instruments include antibody–antigen, ligand–receptor, and protein–nucleic acid interactions *(7,8)*. Biomolecular interaction analysis is not limited to proteins. Interactions between DNA–DNA, DNA–protein, lipid–protein, and complex biomolecules can be studied. Biomolecular interaction analysis can be used in several ways. SPR-based biosensor is one of the method. SPR biosensor-based instruments can be used for several qualitative and quantitative applications. Qualitative applications range from orphan ligand fishing *(9,10)*, small molecule and drug screening, molecular assembly, epitope mapping *(11,12)*, specificity analysis, and small-scale affinity flow purification. As a quantitative tool, SPR biosensors can be used to determine reaction kinetics (k_a, k_d) and affinity constants, equilibrium constants (k_D) for molecular interactions, estimation of functionally active concentration, thermodynamics ($\Delta H^{\text{vant Hoff}}$), stoichiometry, and mechanisms of receptor–ligand interaction. Biacore AB (Uppsala, Sweden) released the first commercial instrument based on SPR technology *(13)*. To date, the majority of publications reporting receptor–ligand studies based on optical biosensor technology used Biacore systems. Affinity Sensors (Franklin, MA) manufactures the IAsys line of instruments, which uses a cuvet system. IBIS system can be configured as a flow- or cuvet-based instrument. Nippon Laser and Electronics Lab's (Hokkaido, Japan) SPR-CELLIA system can be configured for either whole cells or macromolecules in two parallel flow paths *(14)*. More recently, Applied Biosystems has also launched Affinity Sensor instrument based on SPR technology.

Sensor Surface and Immobilization Chemistry

One of the most important parts of the biosensor is the surface where the molecules interact. The commercial availability of robust and repro-

ducible surfaces makes biosensors convenient to use. Several different surface chemistries are now available for different types of biological applications. The most common is carboxy-methylated dextran coupled with different functional groups to make it suitable for immobilization of any ligand. Surfaces currently available from Biacore are CM5 (carboxymethylated dextran), SA (streptavidin), NTA (nickel chelation), HPA (hydrophobic monolayer), L1 (lipophilic dextran), B1 (low charge carboxymethylated dextran), C1 (flat carboxymethylated), F1 (short dextran), and J1 (unmodified gold surface) *(15)*. CM5 chip is suitable for routine analysis. It permits immobilization of globular protein or any other biological material either by amine coupling, thiol coupling, or aldehyde coupling. The net charge of carboxy-methylated dextran is negatively charged allowing concentration of ligand on the chip surface and, hence, better immobilization. However, it is difficult to immobilize strong negatively charged proteins on a dextran surface. SA and NTA chips are basically used for conjugation of biotinylated protein and His-tagged protein, respectively. NTA surface has the inherent problem of leaching of the immobilized ligand leading to dripping base line. Consequently, it is not preferred for kinetic analysis. But there are software and analysis programs available that take corrective measures toward such problems. HPA is used for membrane immobilization and creates hybrid lipid bilayer. L1 surface is used for capture of liposomes. B1 surface is preferred to reduce nonspecific binding, F1 for immobilization of large analytes (direct immobilization of virus). J1 offers freedom to have user-defined surface for any specialized application.

Data Generation and Analysis

Kinetic analysis is a sensitive experimental procedure and is strongly affected by several experimental factors *(16)*. Poorly designed experiments with irrelevant controls result in the failure to fit the binding kinetics data to simple interaction models *(17)*. It is suggested to have a low-capacity surface or a minimum amount of ligand immobilization for kinetic analysis. This is particularly important for minimizing mass transport effects, aggregation, and crowding *(18)*. However, measurement of reaction on low-capacity surface leads to lower signal-to-noise ratio. Amine coupling, a commonly used immobilization procedure, is a random process that affects the functionality of the ligand as the orientation of the ligand can not be defined. After immobilization, if analyte binding sites are not exposed, poor signal is evident. In such cases, orienting the immobilization of the ligand to flow cells by selective ligand capture can improve signals. Carefully selecting procedures for regeneration of the immobilized ligand after passage of each analyte also improves signals *(19)*. To accurately interpret rate constants of binding reactions, it is important to minimize mass transport. These effects occur when the binding rate of analyte to the ligand is faster than diffusion of analyte to the surface. This leads to analyte concentration gradients within the flow cell during the association phase *(20)*.

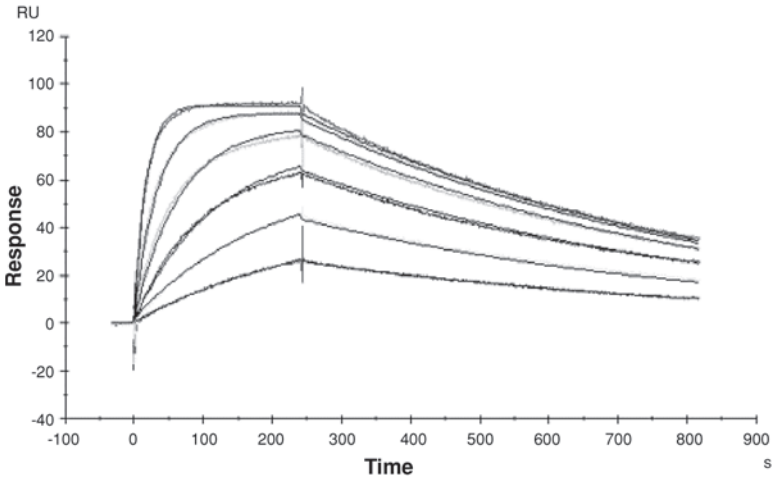

Fig. 2. Experimental data showing 1:1 global fit.

During the dissociation phase, the analyte concentration over the reaction surface does not immediately drop to zero owing to rebinding events, resulting in a slower apparent dissociation rate. Low surface capacity and high flow rates can minimize mass transport effects. In fact, a benefit of mass transport is that it may be used to determine the active concentration of analyte. This can be done by measuring analyte-binding rates at different flow rates *(21)*. A good fit of kinetic data can be confirmed by low chi^2 value (Fig. 2). The curve fitting using any software can be misleading when analyte concentrations are entered incorrectly (Fig. 3A) or wrong magnitude of concentration is entered (Fig. 3B). Myszka and coworkers *(22)* first proposed a two-compartment model demonstrating estimates of the intrinsic reaction rate constants when mass transport influences the binding reaction. The association and dissociation phase data for all the analyte injections were generally fit globally to a simple bimolecular reaction model, i.e., A + B = AB *(18)*. Global analyses are available in BIAevaluation software and CLAMP *(23)* and are the definitive method for determining kinetic constants from SPR experiments *(24)*. BIAevaluation 3.0 and CLAMP99 software packages differ mainly in the statistical assessment of simple bimolecular, heterogenous ligand and mass-transport limited interactions *(25)*. Use of low surface capacity (50-250 RU for CM5 chip, Biacore AB) minimizes experimental artifacts; therefore, the data collected on these surfaces often fits simple models *(26,27)*.

Membrane Receptor Screening

During the last decade there has been significant progress in the development of analytical techniques for the screening of ligand binding to membranes and membrane receptors *(28)*. In order to better understand the binding mechanism of molecules with membrane receptors, the ligand–

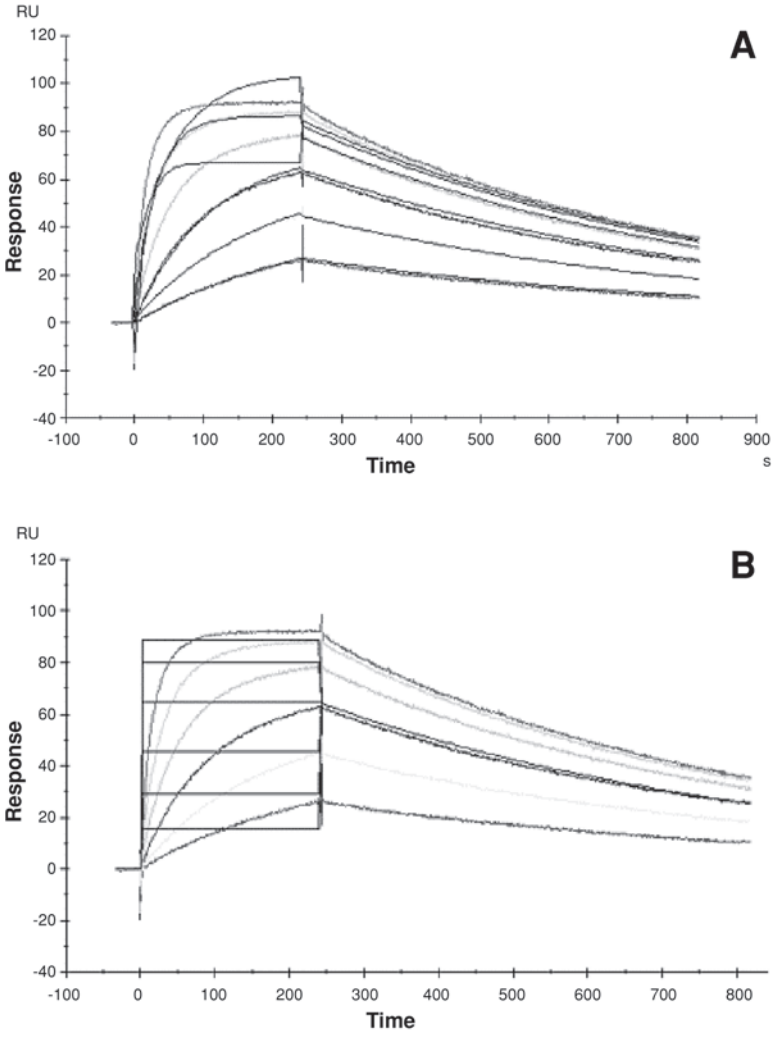

Fig. 3. Example of poor curve fit owing to **(A)** misplaced samples or incorrectly entered concentrations. If the wrong analyte concentration values are entered for the sensorgrams, a poor fit will be obtained. Only two deviant curves with an acceptable fit and the remaining curves do not fit properly. **(B)** Wrong order of magnitude. If concentrations are entered in BIAevaluation software with an incorrect or omitted order of magnitude suffix (e.g., entering 10 instead of 10 n for 10 n*M*), the software may be unable to find a fit for the curves.

receptor interaction must be probed directly in vivo or in reconstituted membrane systems *(29)*. SPR has been employed to determine the kinetics of cholera toxin binding to gangliosides inserted in supported lipid monolayer *(30,31)* and captured vesicles *(32,33)*. Cooper and coworkers *(34)* reported a vesicle capture sensor chip for kinetic analysis of membrane-bound receptors. Ellson and coworkers *(35)* used SPR together with polymer-supported lipid bilayers to study membrane bound subunits of

neutrophils and their role in production of reactive oxygen species. Using SPR, they proved that the addition of $p40_{phox}$, binding partner of $p67_{phox}$ (membrane bound subunit of neutrophil) to the minimal core complex stimulates reactive oxygen species formation. Sensor surface with high protein density is an essential requirement for biosensor studies of G protein-coupled receptor and other membrane proteins. Method for rapid immobilization and reconstitution of G protein-coupled receptor on CM-Dextran surface is known *(36)*. Antigen–antibody interaction on supported lipid monolayer *(37)* and polyvalent binding to membrane-bound receptors has also been studied using SPR *(32,33)*.

Protein–Cell Interaction

Biacore has been used to study the receptor–ligand interaction specifically with purified interacting partners. But information regarding the interaction of an immobilized protein with a whole cell is limited. Ravanat and coworkers *(38)* have established that Biacore can be used to monitor the specific interaction of platelets with surface bound proteins. Response to vascular injury by formation of a blood clot requires the adhesion of platelets to connective tissue and to each other as part of the coagulation process. Initial adhesion on exposed vessel walls involves interaction of the platelet surface glycoprotein complex GPIb-V-IX with von Willebrand factor adsorbed from plasma. This interaction is positively regulated by shear stress so that binding is stronger at higher sheer rates such as those occurring where laminar blood flow is disrupted at sites of vascular injury or in stenotic arteries. Sheer rates in Biacore flow cells are of the same order of magnitude, i.e., 80–8000/s. Monitoring of cell adsorption to ligand-coated surfaces in real time has been demonstrated using red blood cells and various ligands and Biacore's SPR technology *(39)*. Application of whole cells in SPR studies holds considerable potential as a cell sorting technique wherever pure cell population are in a viable, unaltered state. It is also possible to regenerate such surfaces to allow multiple analysis on the same sensor chip. Cell binding generally requires bulk transport of the cells to the interaction surface and generation of sufficient binding avidity to overcome hydrodynamic resistive forces. The major concern while using whole cell suspension for SPR analysis is blockage of microfludic systems. However, Quinn and his coworkers *(39)* were able to use physiological concentrations of erythrocytes for SPR analysis without any blockage. Cell binding activity becomes increasing erratic at high cell concentrations, which is mostly the result of turbulent fluid dynamic effects within the flow cells. Regeneration of chip surface used for whole cell interaction is very critical. Use of strong chemicals may cause lysis of the cell leading to irrecoverable damage of the sensor surface. High hydrodynamic shear force generated by high flow rates is the initial step for regeneration of such chip surface. Quinn and O'Kennedy *(40)* also developed methodology and applied SPR-based biosensors for the detection of cell–ligand interaction. We have used SPR (Biacore) in characterization and functional evaluation of in vitro

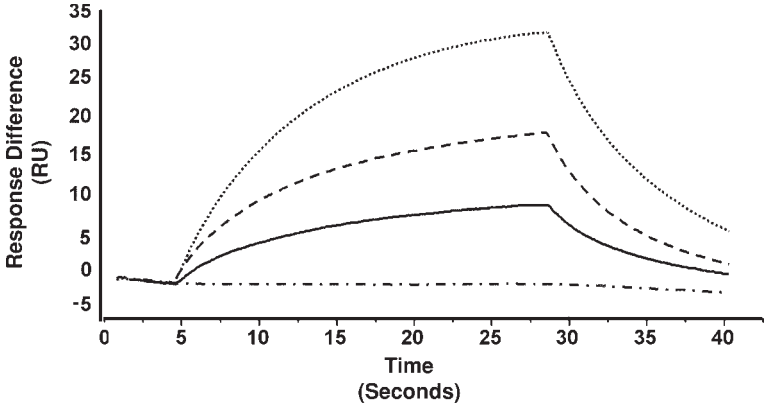

Fig. 4. SPR-based study to assess binding of erythrocytes to PfF2. In vitro refolded recombinant PfF2 was immobilized on Ni-NTA Chip (Biacore). Normal erythrocytes of different hematocrits [0.025% (– – –), 0.0125% (——) and 0.05% (· · · · ·)] and neuraminidase-treated human erythrocytes at 0.05% hematocrit (– · –) were injected to test their binding to immobilized PfF2.

refolded proteins *(41)*. The erythrocytic development of *Plasmodium falciparum* relies on parasite invasion of host erythrocytes, a process mediated in part by the interaction of erythrocyte binding antigen 175 (EBA-175) with the erythrocyte receptor glycophorin A (GA). The binding domain of EBA-175 that interacts with glycophorin A is a approx 330 residues module called F2. Studies have shown that F2 recognizes both sialic acids and the protein backbone on glycophorin A *(42)*. We have produced recombinant *P. falciparum* region II (PfF2) expressed in *Escherichia coli* and evaluated binding to normal human erythrocytes and neuraminidase treated erythrocytes to refolded PfF2 immobilized on Ni-NTA chip *(41)*. We injected different hematocrits (0.05, 0.025, and 0.0125%) of normal erythrocytes at constant flow rate and observed binding of erythrocytes to immobilized PfF2 where as neuraminidase treated erythrocytes did not bind (Fig. 4). Recently, by using SPR-based methods, we have studied residues involved in the *Plasmodium vivax* domain that binds Duffy antigen during red cell invasion *(43)*. We have also studied binding kinetics of glycosylated and deglycosylated receptor binding recombinant proteins of malaria parasite (Pattnaik et al., unpublished data).

Orphan Ligand Fishing

One of the most challenging areas of drug target discovery is the search for novel receptor–ligand pairs. Database mining techniques in conjunction with other computation methods are able to identify many novel sequences of putative receptors, but the ability to similarly identify the receptor's natural ligand is not possible by these methods. There are many receptors of predicated biological functions that have no known ligand, commonly referred to as orphans. Ligand fishing is a process by which

these receptors are screened against a multitude of compounds or cell/tissue extracts to identify putative ligands for the receptor *(44)*. This process is not limited to orphan receptors and ligands. Similar techniques can be applied to novel proteins that have no known binding partners. SPR technology has been applied successfully for ligand fishing *(45,46)*. The only drawback of this methodology is that once a sample has been identified as having a possible ligand, classical biochemical techniques need to be applied to identify the new ligand. Biacore 3000 is an excellent system for ligand fishing application. This offers analyte recovery for subsequent analysis and identification. Direct binding assays have the advantages of speed, automation, and the possibility of tremendous diversity in interacting molecules. However, such methods fail to measure receptor activation and are unable to distinguish between agonist and antagonists. On the other hand, cellular activation assays reveal more information about receptor–ligand interaction, receptor deactivation, and signal transduction.

Drug Discovery

The drug discovery process involves four main stages: target characterization, lead optimization, bioavailability study, and clinical trials. The speed, automation, and high data resolution of SPR-based biosensors make these instruments ideal as drug discovery tools. SPR biosensors provide a reliable method to assess the quality of targets that are destined for further applications. This technology has been used for characterization of target molecules and biopharmaceuticals *(47,48)*. Drug discovery biosensor assays currently have been developed that include the screening of compound libraries for binding to target proteins. HIV-1 protease inhibitors have been characterized by using SPR analysis *(49,50)*. Interaction of thrombin and thrombin inhibitors are also studied using biosensors to evaluate drug target interactions *(51)*. Catimel and coworkers *(9)* also reported use of cuvet-based optical mirror biosensor for micropreparative ligand fishing. Recent advances in data processing have made it possible to detect routinely the binding of low-molecular mass analytes (<500 Da). SPR has also been used for characterizing both high- and low-affinity small-molecule interactions *(52)*. An excellent example of using SPR biosensors in small-molecule screening was reported by Markgren and coworkers *(50)*. SPR-based technology has also been used for adsorption, distribution, metabolism, and excretion (ADME) assessment which includes a compound's ability to bind to serum carrier proteins, as well as permeate across membrane fractions *(53)*. The first step in determining a drug's efficacy is examining its bioavailability to permeate across membrane barriers, such as intestinal walls. Traditionally, in vitro methodologies were followed for quantitating the fractions of drugs absorbed. The same is now possible by using SPR-based biosensors. Danelian and coworkers *(54)* evaluated direct interaction of 27 drugs and a liposome surface and correlated fractions absorbed in human intestine. However, the general ranking of drugs based

on biosensor response relies on the assumption that the refractive index increments of drugs are equal. But, it depends on chemical composition and structure; hence, correction factors for small molecules may be necessary when ranking drugs based solely on binding response *(55)*. Implementation of SPR-based biosensors in the metabolism and ADME is in its infancy. Assays are being developed to monitor a drug candidate's binding potential to bind cytochrome P450 and glutathione-*S*-transferases *(55)*. Now, efforts are being directed toward development of screens or array slides for their possible use with SPR-based systems *(56)*.

Problems With SPR-Based Biosensors

Although the SPR technique is very popular and has led to high-profile discoveries and better understanding in receptor–ligand interaction, like any other analytical techniques, the SPR method has its own limitations. Often, signal-to-noise ratio in analysis of small molecules makes it very difficult to differentiate between true signal and noise. As SPR-based systems measure change in angle of reflected light that is proportional to the mass of the analytes, small molecules generate poor signal or weak sensogram. Several commercial SPR-based biosensors use carboxy-methylated dextran as the base matrix for immobilization of either of the interacting partners. Such matrix is very good for immobilization of proteins with pI ranging from 4.0 to 8.0. However, it becomes very difficult to immobilize proteins (using amine coupling) that are very acidic in nature. Although different types of immobilization chemistry are available, immobilization of molecules that does not affect the biological functionality is very difficult to attain. The best approach to avoid issues related to alteration of functional property owing to immobilization is to use monoclonal antibodies for directional or positional immobilization/capture of interacting partners. This alternative poses an extratechnical limitation, i.e., development of target-specific monoclonal antibodies against either of the interacting partners. SPR-based biosensors are known to require less sample volume and offer quick results. However, every assay requires rigorous standardization, which in turn uses up a considerable amount of precious ligand/analyte/receptor. Nevertheless, the analyte needs to be of high purity to offer reliable data. Several proteins that have pI of more than 8.0 have nonspecific interaction with dextran matrix as a result of charge phenomena. Neutralization of nonspecific signal requires incorporating inhibitors or other competitive molecules, which complicates data analysis. SPR-based methods are very good for analysis of 1:1, 1:2, rate limiting, and heterogeneous ligand bindings. However, it is difficult to interpret complex biological interactions using such systems. Viscosity of a sample is also of great concern. Viscous samples may clog flow cells, thereby permanently damaging the component. Drift in base line owing to gradual loss of immobilized molecules caused by improper immobilization, use of strong regeneration conditions, or use of unstable molecule create lots of problems

during data analysis. SPR-based commercial biosensors (Biacore and IAsys) are being used increasingly, but the high operation cost of the method and system make it unaffordable to many researchers.

Although commercial biosensors are easy to operate, actually interpreting binding kinetics is not always straightforward. The majority of published biosensor data does not fit the simple bimolecular interaction binding model (A + B = AB), which raises questions about the validity of the biosensor analysis *(17)*. There are a number of experimental artifacts that complicate biosensor analysis, including surface-imposed heterogeneity, mass transport, aggregation, avidity, crowding, matrix effects, and nonspecific binding *(16,18)*. There are certain important factors that need to be kept under consideration while working with SPR-based biosensors including the good quality of reagents, cleanliness of instrument, control of nonspecific binding, stability of base line, minimization of avidity effect, use of reference or control surface to deduce bulk refractive index change, use of proper washing steps and regeneration conditions, use of correct flow rate to avoid mass transfer limited binding, proper immobilization based on surface capacity, use of correct reference for data subtraction and zeroing, and use of multiples or replicates for each assay. Other than SPR-based methods several other methods are also known for study of receptor–ligand interaction like calorimetry, enzyme-linked immunosorbent assay (ELISA), radio-immuno assay, etc., but many either use a static assay model or do not offer real time kinetic data. The classical method is to measure change in density based on a calorimetric assay. However, such a system fails to offer signal when the rate of kinetic interaction between two molecules is too fast. ELISA-based methods are routinely used in several laboratories for saturation kinetic studies, but SPR-based methods offer real-time data and are also suitable for analysis of very weak/slow to very fast/strong interactions.

It is evident that advances in commercial biosensors based on SPR technology have made available a powerful means to examine and characterize the interactions of biological macromolecules with binding surfaces. It is now routine that, by analysis of the kinetic and equilibrium aspects of the observed experimental adsorption isotherms, rate and affinity constants can be determined. However, in order to properly interpret kinetic experiments, data must be collected under the appropriate conditions. Careful experimental design and data-processing methods can dramatically improve the quality of biosensor data making it possible to fit responses to simple models. Proper utilization of SPR-based biosensor technology will evolve as one of the most powerful biophysical tools for evaluation of receptor–ligand interactions.

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