

Surface plasmon resonance and its use in biomolecular interaction analysis (BIA)

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Since the advent of surface plasmon resonance (SPR)-based interaction analysis techniques in 1990 the field has grown rapidly. So far, more than 220 publications and hundreds of laboratories have reported useful applications for this label-free real-time binding approach. Milestones passed during the past year include the direct detection of low molecular mass (200 Da) binding events and applications in several new fields as disparate as chaperonins, cellular adhesion, molecular biology, transcription and small-molecule screening.

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Introduction

Functional analysis is the description of a biospecific interaction in relation to time. It is quite clear that the real-time measuring technique spectrophotometry has had a tremendous impact on our understanding and description of enzymes' kinetic properties. However, substances that participate in signaling and the control of biological systems but lack catalytic properties have not yielded functional information as easily. Functional analysis requires a detailed description of the physicochemical parameters of the interaction, such as association and dissociation rate constants, affinity and *in vivo* concentration. Surface plasmon resonance (SPR) has several intrinsic features that allow for label-free, real-time monitoring of interactions. It is important to realize that SPR is a quantum-mechanical detection phenomenon, and for the technique to be useful for interaction analysis it must be integrated with bioselective interfaces and optimized sample-presentation modules.

The basic experimental protocol for examining the binding interaction between two biomolecules can be summarized as a four-step process: (a) immobilize or capture one of the binding partners; (b) inject the second binding partner, and record a real-time interaction curve; (c) perform step (b) using a series of concentrations of the second binding partner; and (d) choose an appropriate kinetic model and fit raw data to extract rate-constant estimates.

The first integrated technology that applied SPR to functional analysis was BIAcore®, available from Pharmacia Biosensor (Uppsala, Sweden) [1]. Other suppliers of integrated technologies have existed for several years but these less sophisticated systems have yet to make a significant impact. The following review is

focused on summarizing the rapidly expanding field of biomolecular interaction analysis (BIA).

The concept of real-time BIA was borne within the tradition of method development for biological research and a background in biosensor development. The basic technical concept can be summarized as the detection of biospecific adsorption of an analyte in continuous flow to an immobilized ligand at the same spot and at the same time as it occurs [2].

SPR is sensitive to refractive index changes on a suitable metal surface and can be used to measure the adsorption of proteins in a label-free manner [3]. The surface must be chemically modified to allow covalent coupling of the ligand and to ensure that it exhibits very low non-specific binding of proteins [4,5]. A carboxylated dextran surface coupled to a thin gold surface for the detection system gives the surface such properties. To transport samples and reagents to the adsorption and detection spot, an integrated liquid handling system was developed [6]. These three components — SPR, biocompatible sensor chips, and integrated fluidics — together enable researchers to quantify protein–ligand interactions (Fig. 1). The binding and dissociation of the analyte to the immobilized ligand are followed in real-time and presented in a 'sensorgram' of response versus time (Fig. 2). The response signal from the SPR detector is proportional to the mass of protein/surface area [7].

Immunology

Immunologists were among the first to take advantage of SPR in studying interactions between antigens and antibodies. More recently, they have expanded this

Abbreviations

BIA—biomolecular interaction analysis; **GST**—glutathione S-transferase; **SAM**—S-adenosylmethionine; **SH2**—Src homology 2; **SH3**—Src homology 3; **SPR**—surface plasmon resonance.

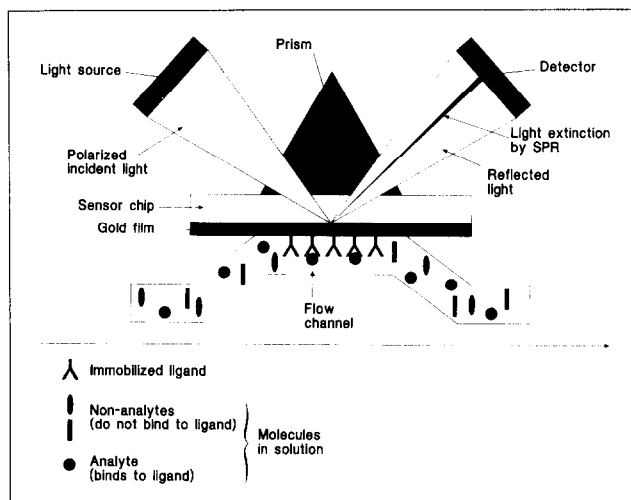


Fig. 1. The figure shows the configuration of an SPR detector, sensor chip and integrated fluidic cartridge with flow channel for continuous flow of buffer, samples and reagents in BIAcore®. The biospecific adsorption of an analyte on the immobilized ligand is measured at the same spot and at the same time as it occurs. The detector features a polarized wedge-shaped light beam from a light-emitting diode which is reflected in the gold film on the sensor chip and detected on a diode array. SPR occurs in the gold film and can be detected as extinction of light in the reflected beam for a special angle of incidence. This SPR angle varies with refractive index changes on and in the vicinity of the surface as a result of adsorption of molecules on the ligand immobilized to the dextran layer on the sensor chip.

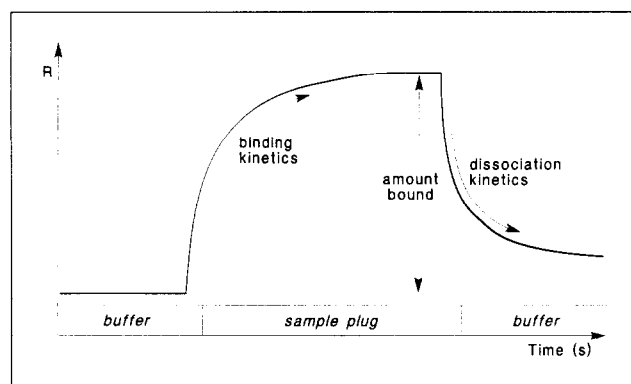


Fig. 2. Response (R) is followed with time in a sensorgram, and is proportional to the mass of adsorbed protein [13]. Analyte in the sample plug is adsorbed on the immobilized ligand, and from the adsorption profile the association rate constant, k_{on} , can be determined. After the sample pulse has passed, the biospecific complex so formed will dissociate, and from the logarithmic decay the dissociation rate constant, k_{off} , can be calculated.

approach in order to understand a variety of other important problems.

Bjorkman and colleagues [8] have used SPR to explore how maternal immunoglobulins are transferred from milk to the circulation of newborn rats. This unidirectional transfer is attributable to the pH-dependent interaction of immunoglobulins with a receptor (the Fc receptor) on intestinal epithelial cells. This binding was thought to be regulated by the deprotonation of histidine residues on the Fc receptor because

the immunoglobulins are being transferred from milk (at pH 6.0) to the blood of the neonate (at pH 7.0). The combination of site-directed mutagenesis with SPR experiments performed at pH 6.0 and 7.0 allowed these workers to exclude all histidine residues in the Fc receptor, and to implicate a cluster of histidine residues within the immunoglobulin itself as the molecular switch responsible for transfer.

Margulies and colleagues [9] have used SPR to study the interaction of major histocompatibility complex (MHC) molecules with the T-cell receptor (TCR) and with peptide antigens. An immobilized TCR bound specifically only to complexes of the appropriate peptide antigen and MHC combination, and not to 'empty' MHC molecules or to MHC-peptide combinations other than those from which the T cell had originally been derived (Fig. 3). The apparent affinity constants derived for these interactions $K_d \sim 10^{-7}$ M and the relatively swift dissociation rates of the TCR-peptide-MHC complexes ($t_{1/2} \sim 30$ s) support a biologically attractive model, in which activation of a T cell would require the simultaneous engagement of many TCRs on a single T-cell surface by multiple peptide-MHC complexes on the surface of an antigen-presenting cell.

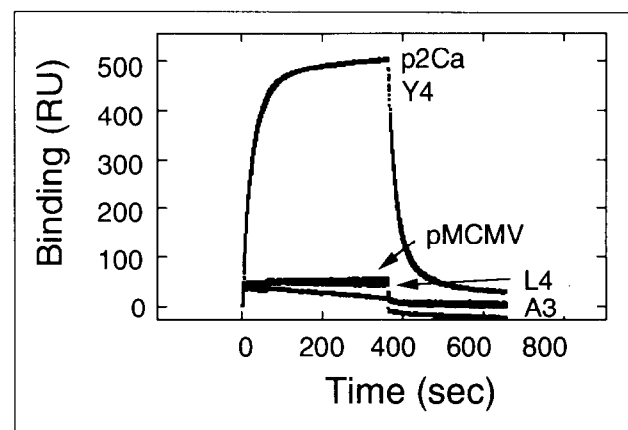


Fig. 3. Sensorgrams demonstrating the specificity of T-cell receptor (TCR) binding to peptide-MHC combinations. A soluble TCR derived from a T cell specific for the peptide p2CL (sequence LSPFPFDL) bound by the class I MHC molecule L^d was initially coupled to the sensor surface. Subsequently, various combinations of soluble MHC (sH-2L d) plus peptides were injected. A response was only obtained when the proper combination of MHC and the peptide was injected. sH-2L d (at $1.0 \mu\text{M}$) complexed with the indicated peptides (at $250 \mu\text{M}$) injected over a 2C cell TCR surface at $5 \mu\text{l min}^{-1}$ and $100 \mu\text{l min}^{-1}$ during dissociation. Figure courtesy of D Margulies (National Institutes of Health).

The binding of peptide antigens to MHC molecules was, in contrast, found to be extremely stable. For example, the $t_{1/2}$ for the dissociation of a complex between the MHC class I K^b molecule and a peptide derived from ovalbumin (residues 257–264) was ~ 1000 min [10]. By measuring the binding of a panel of ovalbumin peptide analogues containing substitutions throughout the sequence, these studies also demonstrated that in addition to interactions with the so-called anchor residues of peptide antigens, interactions between the class I molecule

and other residues within the antigen contribute significantly to the stability of the complex.

Chaperones

The study of molecular chaperones comprises essentially the investigation of protein-protein interactions, and so it is natural that SPR is now being used to elucidate the mechanism by which this important class of biomolecules mediates the folding of proteins. The group led by Andreas Pluckhoun have used BIAcore to examine the interaction of the GroEL chaperone (the *Escherichia coli* member of the heat shock protein 60 family) with different forms of the protein β -lactamase. In the cell, β -lactamase is initially synthesized as a pre-protein, including a hydrophobic N-terminal signal sequence that is cleaved during the maturation process. Interestingly, BIAcore data indicated that GroEL bound tightly to a synthetic peptide corresponding to the pre-sequence, but not to an amphipathic peptide derived from the mature protein [11]. This result supports longstanding suggestions that molecular chaperones mediate the folding of their substrates by binding to and stabilizing hydrophobic stretches of amino acids that would otherwise form nuclei for aggregation and misfolding [12,13].

BIAcore has also been instrumental in resolving a controversy about the ATP-dependent reaction cycle of GroEL. In 1993, Hartl and colleagues [14] described a detailed model whereby protein substrates, in the course of folding, underwent steps of binding and release by GroEL. This on-and-off process was thought to be regulated by the interaction of GroEL with a single oligomer of the cofactor GroES. More recently, this model of the GroEL cycle was challenged by Lorimer and colleagues [15], who proposed an alternative model that invoked an obligatory step in the cycle in which GroEL simultaneously bound two GroES oligomers.

The two contrasting models could be tested directly using BIAcore. According to the more recent model, a complex formed on the sensor surface between GroEL and GroES should require the injection of free GroES before dissociation can occur. When these experiments were performed [16•], however, the rate of complex dissociation was found to be independent of the presence of GroES and to neatly parallel the measured rate of ATP hydrolysis by GroEL (Fig. 4), findings which established the original model of Hartl and colleagues [14] as the correct one.

Molecular biology

Several groups have used SPR to study the mechanisms of gene expression. The *E. coli* methionine repressor, MetJ, is a DNA-binding protein whose affinity for operator DNA is known to be enhanced 100-fold in the presence of S-adenosylmethionine (SAM). On the basis of crystallographic studies, SAM has been

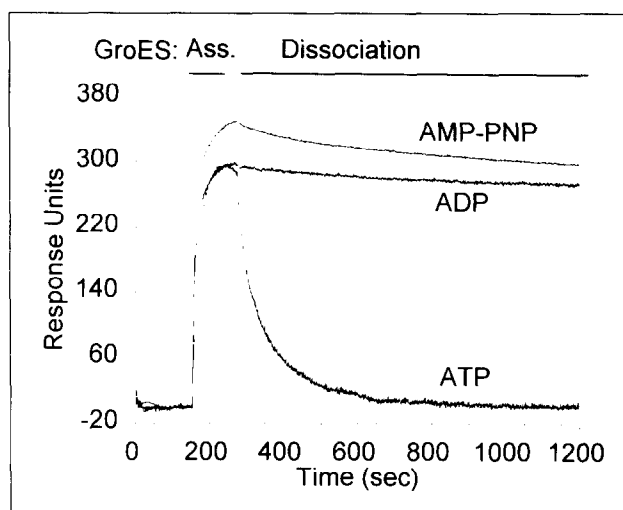


Fig. 4. An example of the use of SPR to study the function of molecular chaperones. At $t=150$ s, GroES analyte was injected over a GroEL sensor surface. After the injection, the dissociation phase was continued in the presence of ATP, ADP, or the non-hydrolyzable ATP analog, AMP-PNP. According to one model for GroEL function, the dissociation of the complex should not occur unless free GroES is injected during the dissociation phase [15]. However, dissociation of the complex was found to be dependent only upon the presence of hydrolyzable ATP. The rate of dissociation matches the measured rate of ATP hydrolysis by GroEL (~ 0.04 ATP s^{-1} subunit $^{-1}$), supporting the model proposed by the Hartl group [14]. Figure courtesy of M Hayer-Hartl (Rockefeller Research Laboratories).

proposed to control an 'electric genetic switch' through an electrostatic potential between the positive charge of the SAM sulfur atom and the negative phosphates of the DNA. To test this model, MetJ-DNA binding studies were performed on BIAcore in the presence of SAM analogues in which the positive sulfur atom was located at different distances from the DNA phosphates. The hierarchy of induced affinities supported the electric genetic switch theory [17].

SPR has also been used to evaluate the affinities of mutants of the transcription factor Zif268 to DNA that has been shown to select for modified sequence-binding specificity using a phage display system. The goal of these studies was to identify useful therapeutic agents that would be capable of specifically binding to and modulating transcription from precisely defined DNA sequences.

Each of the 11 proteins studied in detail was found to have a higher affinity for the DNA on which it was selected. These changes in selectivity were found to be governed by differences in the rate of protein-DNA dissociation (k_{off}). The authors noted the advantage of real-time measurements derived using SPR over data derived from gel-shift assays, which are not ideally suited to the measurement of half-lives of DNA-protein complexes.

Ptashne and co-workers have used SPR to compare two models for transcriptional activation. According to the existing paradigm, gene activation involves a

multi-step assembly process, various steps of which are mediated by DNA-binding proteins called activators. This process has been thought to be highly complex, with various components undergoing crucial conformation changes in order for transcription to occur [18,19]. An alternative theory, and a simpler one, is that recruitment of the RNA polymerase holoenzyme to DNA by an activator suffices for gene activation [20]. To test the two theories, the Ptashne group [21••] took advantage of the observation that a mutant form of the RNA polymerase holoenzyme can bind to fragments of the transcriptional activator Gal4 containing only the DNA-binding portion and not the classical activation region. Using transcriptional activation assays and BIAcore experiments, it was found that these Gal4 fragments act as effective transcriptional activators simply by recruiting the RNA polymerase to the gene.

Signal transduction

BIAcore technology lends itself well to the study of the emerging field of signal transduction because of the number of different binding interactions involved that do not directly involve enzymatic activity. The technology has been, and still is, most frequently applied to the study of the interactions of the Src homology 2 (SH2) domains with their phosphotyrosine-containing ligands. However, significant contributions have also been made to unraveling the specificity requirements of receptor–ligand interactions, and the assembly and function of an *E. coli* chemotactic mechanism [22,23].

The past year has brought new contributions to the study of specificity elements involved in the SH2 domain/phosphotyrosine peptide interactions, as well as to the affinity and specificity of the Src homology 3 (SH3) domain interactions with polyproline-containing ligands. SH2 domains are thought to activate biochemical signaling pathways by their direct interaction with phosphotyrosine (pY)-containing ligands. Early SPR studies determined the range of affinities of these interactions as being very broad and, on occasion, surprisingly high. These studies were performed, without fail, using glutathione S-transferase (GST)–SH2 domain fusion proteins and short pY-containing peptides. Ladbury *et al.* [24•] showed this year that the GST dimerization that can occur appears to produce a significant avidity effect during the binding interactions. These authors have also determined equilibrium affinity constants on BIAcore using non-fusion proteins that agree well with measurements taken using isothermal titration calorimetry [24•]. These new values are an order of magnitude lower in affinity than previously determined values.

Of particular interest is a study performed in Tony Pawson's laboratory which showed that the substrate specificity of the Src SH2 domain can be completely transformed to that seen for the Grb2 SH2 domain by simply changing one amino acid residue in a structural loop that lines the binding pocket [25]. This study is an

elegant example of how useful BIAcore technology can be in connecting structural information with function.

Two other papers have defined the substrate specificity of the Lck SH2 domain for peptide ligands, helping to determine exactly which elements are responsible for high-affinity interactions [26,27]. SH3 domains are small polypeptide segments that are responsible for protein–protein interactions triggered by extracellular signals. These domains are known to interact with short sequences within a protein that are rich in proline and are often found within the same protein as SH2 domains.

Recently, Schlessinger *et al.* [28] have determined, in the case of the adaptor protein Grb2, that binding of each of these two domains to their ligands is independent of occupancy of the other. These authors also compared the affinities determined by isothermal titration calorimetry and BIAcore of the Grb2 for the polyproline substrate, and demonstrated a 15-fold difference between the values recorded using the two methods. However, they predict that the higher affinity measured using BIAcore is a more realistic representation of the interaction *in vivo* as the matrix may provide an environment more suited to the conformation that the peptide must adopt in order to be recognized by the protein domain.

SH3 domains are known to recognize a surprisingly narrow range of substrates, as shown in an interesting study by Rickles *et al.* [29], in which they determined the specificity of Src family member SH3 domains using phage display libraries. They then confirmed the selection process and obtained apparent dissociation constants through binding studies performed with immobilized peptide and mobile SH3 domains. Thus BIAcore can be very effective at obtaining specificity and affinity information that is quite useful when trying to sort out the numerous possible interactions within a signal-transduction cascade.

Cell adhesion and low-affinity binding

Intense interest has been shown recently in the factors that govern cell–cell interactions. These interactions range from the long-lasting, typical of the structurally important cadherins, to the very transient, as seen in the mucins and other proteins of the immunoglobulin superfamily involved in leukocyte interactions.

A group in Oxford has used SPR technology to determine the affinities and mechanisms of transient cell-adhesion molecules through characterization of CD2, a T-lymphocyte cell-surface protein, and its interactions with its ligands in both human- and rat-derived systems. One of the first myths that they dispelled was that the glycosylation pattern of CD2 does not appear to be necessary for structural integrity or binding to a ligand [30]. This was accomplished by isolating protein that was deglycosylated or singly glycosylated at each site and comparing antibody recognition on BIAcore. They also compared the X-ray crystal structures of the wild-type protein with the protein with only one

N-acetylglucosamine at each site and determined that no significant differences occurred in the backbone of the CD2.

The Oxford group also developed some elegant techniques utilizing high flow rates and careful experimental controls to determine low binding affinity of CD2 to its ligands [31]. By changing the data collection rate of BIAcore, they were able to determine k_{off} rates in the 4–6 s⁻¹ range, which is much faster than had previously been accomplished. They determined directly the equilibrium binding constants and showed that the rat CD2–rat CD48 interaction has a K_d of 60–90 μM , and the human CD2–CD58 interaction a K_d of 6–22 μM . From these values and the k_{off} rates, they were able to calculate an approximate k_{on} value of $4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [31].

Finally, in another paper, the same group determined the binding topology of the CD2–CD48 interaction through multiple single-site mutation analysis [32]. Again, they analyzed the interactions through the use of BIAcore and established that the topology is head-to-head, instead of the three-layer packing model that had been proposed previously. An interesting aspect of the experiments described in this paper is that they used the recently discovered avidity effects seen when binding GST-fusion proteins to a ligand on the surface to increase the signal of CD2 binding to mutant CD48. Thus it is apparent that BIA technology can be used not only to obtain high-affinity binding constants but, with careful experimental design, to monitor low-affinity interactions.

Screening for new ligands

One of the most powerful applications of SPR is the detection of specific interactions within complex mixtures. As such, it is ideally suited to the analysis of steps in a purification process. An SPR-based binding assay is generally rapid compared with an activity assay (for example, the measurement of cell activation), and unlike DNA-homology-based cloning approaches it identifies proteins involved in specific interactions, not merely proteins related by sequence. In an elegant use of this approach, Stitt and coworkers [33•] were able to identify two previously discovered proteins, the anti-coagulation factor Protein S and a related protein, Gas6, as ligands for the Tyro 3/Axl family of receptor tyrosine kinases.

New methodologies

Several innovations have greatly expanded the scope of interactions that may be explored using SPR technology. The first of these is the development of so-called multispot sensing protocols, in which the interaction of the analyte with receptor is measured in a series of flow cells with different amounts of immobilized receptor. Using these protocols in combination with

BIAcore 2000, it has been possible to overcome the original molecular mass limits of SPR, in order to study interactions involving fluid-phase ligands as small as 180 Da [34•]. The same protocols have also been used to extend the range of interactions that may be studied to those with $K_d < 50 \mu\text{M}$.

A second innovation is an expansion of the methods for coupling substrate proteins to sensor surfaces. Gershon and Khilko [35•] have recently described a procedure for binding the nickel chelator nitrilotriacetic acid (NTA) to the sensor surface, which can then be used to immobilize hexahistidine-tagged proteins. An important advantage of this approach is that immobilization does not involve modification of the bound protein, which can lead to inactivation. In the case of the vaccinia virus poly(A) polymerase studied by Gershon and Khilko, 99% of the hexahistidine-tagged protein was functional for ligand binding, compared with only 40% when the protein was chemically crosslinked to the surface. Another advantage of this approach is that it allows for facile analysis of proteins expressed in *E. coli* with a his-tag. In many cases, purification of the protein can essentially be performed *in situ* by injection of crude *E. coli* lysates containing the tagged protein over NTA-modified surfaces (A Szabo, unpublished data).

Finally, in a boon to molecular biologists eager to know the minimum time necessary to perform a given enzymatic manipulation of DNA, Nilsson and co-workers [36] have demonstrated that it is possible to perform and follow in real time the progress of a variety of reactions on DNA molecules immobilized on the sensor surface. In their experiments they initially immobilized a biotinylated oligonucleotide to a streptavidin-coated surface and annealed it to a complementary oligonucleotide. Upon subsequent injection of a partially overlapping double-stranded oligonucleotide plus ligase, an increase in signal was observed corresponding to a ligation that was 64% efficient after an injection time of 20 min. Further changes in response were used to follow reactions corresponding to DNA synthesis by DNA polymerase and restriction endonuclease digestion.

Conclusions

The past year has seen an explosion in the use of SPR for interaction analysis. The technique has been effective in a wide variety of application areas and this seems to be continuing for new areas of research such as apoptosis, carbohydrate research, gene therapy and screening combinatorial libraries for function inhibitors. One can think of SPR as an interaction workbench that embodies the flexibility inherent in label-free detection with the precise low-noise data critical for extraction of complex information. Indeed, the small revolution that is occurring in the analysis of data using global fitting methods and numerical analysis will extend the set of possible mathematical descriptions available with which to understand the biological implications of a set

of sensorgrams [37,38]. New ways to present binding partners actively and homogeneously will improve the interpretation of results. Advancement in robotics, sensitivity and reagents will offer drug-screening programs direct binding confirmation of their primary 'endpoint' assays. Structural information and functional assays such as BIA will make possible true rational design of both inhibitors and proteins with desired therapeutic roles.

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