Analyzing Biomolecular Interactions

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Very little happens in any biological system unless two or more molecules come together to form a stable complex. When molecules interact through specific molecular contacts, all of the principles of thermodynamics, dynamics, and biomolecular structure and recognition come into play. As increasing numbers of new proteins and DNA sequences are entered into databases such as SWISSPROT or GenBank, rapid methods to accurately characterize these biointeractions are needed. One useful model to consider involves a target molecule (T) with a specific binding site (such as a particular region in a protein tertiary structure or a specific sequence of DNA) and a probe molecule (P) that can bind to that site. The simplest binding model corresponds to P + T → C, where C is the resulting complex. Probe molecules can vary from small metabolites or drugs to large transcription complexes, and their interactions with the target range from the highly specific (P binds a single site) to the nonspecific (P binds most sites in the target class, such as related DNA sequences). In interaction processes that are complicated, there can be multiple binding sites, cooperative interactions, and so forth (*1*).

In order to determine the equilibrium and/or kinetic constants for binding, all techniques must factor the concentrations of P and T into the concentrations of free P and T, on the left side of the equation, as well as the concentrations in C, on the right side of the equation. This evaluation can be achieved by various methods, including equilibrium dialysis, spectral measurements, gel shift, calorimetry, DNase I footprinting, and related techniques (*1*). Many of these methods require labeling of P or T with a fluorescent or radioactive tag. Fitting methods, based on models such as the one discussed here, are used to extract the desired physical quantities from the bound and unbound concentrations (*1*). The fitting process is an important separate subject, but it will not be discussed here.

A recent development in instruments that investigate biomolecular interactions is surface plasmon resonance (SPR) detection with a biospecific sensor chip (*2*). Commercial instruments (from Pharmacia, now BIACORE AB) became available in 1990 (*2*, *3*). In BIACORE technology, the sensor chip is created by applying a thin layer of gold (~50 nm) to a glass surface (see drawing, above left).

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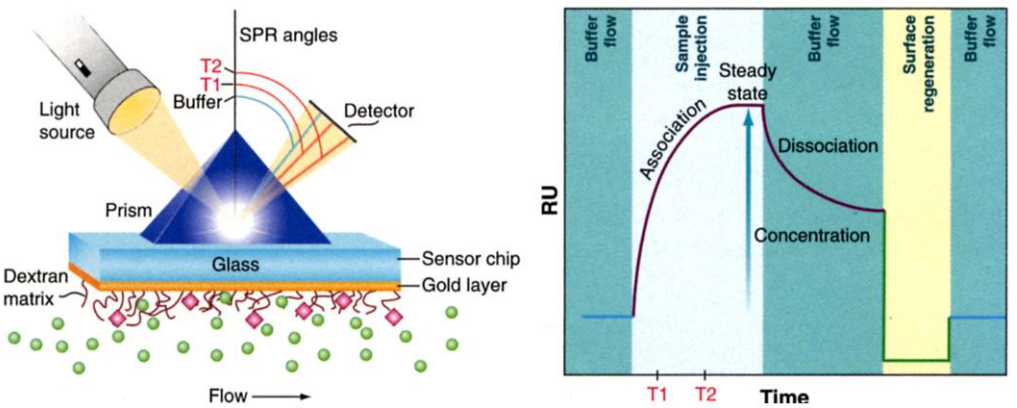
In the most common type of sensor chip, carboxymethyl-dextran is linked to the gold to give the interaction layer (~100 nm thick). One of the interacting molecules, either T or P, must be linked to this layer to create the biospecific recognition surface. To create this linkage, the chip is automatically docked into the instrument such that the gold-dextran surface forms one wall of a cell through which solution can flow. The dextran-carboxyl groups can then be activated by flow of appropriate reagents. For amine coupling, for example, proteins with amine groups can be injected onto the activated surface to form a covalent amide connector (see schematic graph, below right). Methods to attach a variety of molecules to the chip are available (*2*–*5*). A critical requirement of the linking process is that the binding properties of the attached molecule must not be perturbed very much. Because SPR responds to changes in refractive index (*2*, *3*, *6*) and, thus, to changes in mass, it is advantageous to attach the molecule with the lowest molecular weight to the surface. Attachment chemistry must be considered, however, and it may be necessary to link the heavier molecule to the surface and work with a smaller signal.

The SPR optical unit consists of a source for a light beam that passes through a prism and strikes the surface of a flow cell at an angle, such that the beam is totally reflected (see drawing). Under these conditions, an electromagnetic component of the beam, the evanescent wave, propagates into the aqueous layer and can interact with mobile electrons in the gold film at the surface of the glass. At a particular wavelength and incident angle, a surface plasmon wave of excited electrons (the plasmon resonance) is produced at the gold layer and is detected as a reduced intensity of the reflected light beam. In BIACORE instruments, monochromatic light in the shape of a wedge (a broad distribution of incident angles) is used (*2*, *3*). Each angle of the reflected beam strikes the instrument's detector at a different point and, thus, the detector continuously records the position of reduced light intensity and calculates the SPR angle from that figure. The optical device has no moving parts and the fixed geometry enhances stability and allows binding to be monitored in real time.



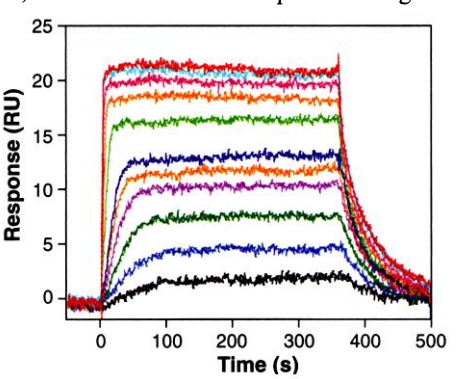
Illustrated SPR. At left, an SPR optical unit and a sensor chip detect the P molecules (green spheres) in the flow solution, which passes by the T (pink diamonds) linked to the dextran matrix. The blue SPR angle defines the position of the reduced-intensity beam. Time points T1 and T2, shown in the schematic sensorgram (right) correspond to the two red SPR angles, which shift as P binds to T over time. As the concentration of bound P increases (arrow), the RU response approaches saturation. The complex dissociates upon reintroduction of the buffer. As shown, the response to the injection solution will fall below the baseline if its refractive index is lower than that of the buffer.

The SPR angle is sensitive to the composition of the layer at the surface of the gold (*2*, *3*). A baseline SPR angle is first deter-

mined by washing buffer over the surface with a fixed amount of T attached (see drawing, blue angle). To this flow of buffer, some P is then added. The binding of P to T causes an increase in the refractive index at the surface, thereby changing the SPR angle because it is directly proportional to the amount of bound P. The red SPR angles in the drawing originate at the baseline angle (blue) but move away from the original position after the sample injection as the probe binds over time. No labeling of molecules is required in the SPR detection method, and the binding of probes with molecular weights greater than 200 daltons can usually be detected quite accurately.

With this BIACORE technology, the SPR angle change is reported as resonance units (RU), where 1000 RU correspond to an angle change of ~0.1°. For most proteins, binding of ~1 ng/mm^2 of protein at the dextran surface is required to cause a signal change of 1000 RU (*2*, *3*). The exact relation between RU and nanograms of material bound will vary with the refractive index of P (*6*). If the added molecule does not bind to a target or receptor, the SPR angle change in the sample and reference flow cells will be the same, and, after subtraction, will give a zero net RU response that indicates no binding occurred. Only bound P generates a positive SPR signal. That signal, recorded over time, produces a sensorgram, as shown here.

In a typical sensorgram, a baseline signal with no change in RU over time is followed by sample injection, which produces the association phase where RU increases with time. If the reaction rates are fast enough, it is possible to reach a steady state region, where the rates of association and dissociation are equal. Resuming buffer flow causes the complex to dissociate, and the kinetics of the dissociation can be recorded (*2*, *3*). At a desired time, a regeneration solution can be injected to remove remaining P bound to the surface, and the original RU value is re-established (see schematic). Thus, both kinetics and the equilibrium constants can be determined from a single experiment (*7*, *8*). BIACORE sensor chips are available with four flow channels that allow simultaneous monitoring of a blank and three target molecules.



Set of sensorgrams. Experimental results for the binding of a small molecule to DNA show that as the concentrations of the molecule increase (from bottom to top), the saturation (at ~22 RU) of the DNA sites is approached and the association reaction rate increases (*7*, *8*). The complex dissociates when buffer flow is restarted at 360 s.

The affinities of interest are usually quite strong in biological systems, and, with many of the binding methods available, most P will remain bound until sites on T have been saturated. Below this saturation level, it often becomes difficult to accurately determine the low concentration of free P. But with the SPR method, the concentration of unbound P is the same as the concentration of P in the flow solution, which eliminates the need to determine experimentally the concentration of free P.

Shown in the composite sensorgram is the data from the interaction of a small probe compound of less than 400 daltons with the minor groove of an immobilized hairpin DNA duplex that has a molecular weight of ~7000 daltons (*4*, *5*). Despite the low molecular weight of P with respect to T, the signal-to-noise ratio is adequate for fitting of the results to different binding models (*1*). A particularly interesting feature of this interaction that became clear in fitting the BIACORE results (see the composite) is that the compound binds in the DNA minor groove as a stacked dimer with an unusual binding mode that was unexpected. The dimer was easily detected in the BIACORE experiment because the SPR response depends on the mass of the binding component. At saturation, the RU value for a dimer will be twice that for a monomer and, thus, the SPR response is an excellent method for evaluating stoichiometry of P molecules that bind to T upon complex formation (*4*, *5*). Because the probe binds strongly to the DNA sequence and has relatively weak spectroscopic signals, these results would have been difficult to collect with other methods.

The test compound used in the sensorgram is one of several hundred candidates being screened by this method for possible activity against parasitic organisms. The synthesis and screening efforts, led by D. Boykin at Georgia State University and R. Tidwell at the University of North Carolina, have found an orally active agent that has excellent activity against parasitic diseases.

After a potential drug is identified in a screen such as the one illustrated here, questions about such details as its transport and method of cell uptake can also be investigated by SPR methods. Because the SPR method works on the surface of a sensor chip, a relatively new and exciting application of the SPR-biosensor surface method involves the creation of artificial membranes directly on an appropriately modified chip. This is a powerful tool for the research on membrane transport and interactions. As an example, immobilized liposomes with SPR detection have recently been used to evaluate the efficiency of intestinal uptake of several drugs (*9*).

An equally important question for drug delivery involves transport of potential drugs in blood after they are absorbed. Serum albumin is a protein that interacts with many drugs and is involved in their transport to cells (*10*); BIACORE technology has been used to investigate this transport by measuring the binding of drugs to serum albumin. After the protein is linked to a BIACORE sensor chip, many compounds can be passed over the chip and evaluated for interactions with serum albumin (or other proteins) in a short time period.

A different area of investigation by BIACORE technology, which has taken on increased importance with the sequencing of the human genome, involves use of SPR with probe molecules to detect single nucleotide polymorphisms (SNPs). Two reports have recently shown the power of SPR-biosensor methods to specifically detect SNPs through highly selective binding of probe molecules to GG or GT mismatched base pairs in DNA (*11*, *12*).

These examples show that, in addition to providing fundamental information about molecular interactions, the method is useful in a broad variety of applications. Rich and Myszka have provided very useful yearly compilations of the SPR literature (*13*), and the BIACORE Web site (www.biacore.com) contains an extensive list of references. Though still in the early stages, use of SPR biosensors is certain to bring many exciting new applications and discoveries in the future.

References and Notes

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