BIAcore: a microchip-based system for analyzing the formation of macromolecular complexes

Structure 15 April 1995, **3**:331–333

The formation of macromolecular complexes, for example the complex between an antibody and an antigen, is essential to many biological processes. In order to gain a better insight into the biological processes involved, the formation of complexes is often measured and their interaction kinetics and affinities analyzed. Considerable effort has been expended in developing techniques to measure the formation of complexes, the most promising being electronic microchip-based biosensors. Such analytical systems have come into increasing use recently for the characterization of interactions between biological macromolecules and ligands. One such commercially available system, called BIAcore (Pharmacia Biosensor, Uppsala, Sweden), uses biosensor-based technology and offers a method for rapid analysis of interaction kinetics and affinities [1,2]. Such systems have the advantage of replacing tedious binding assays utilizing radiolabelled or fluorescent components and potentially unstable cell lines, and can be used where biological activity assays are not available.

The BIAcore system contains a sensor microchip, a laser light source emitting polarized light, an automated fluid-handling system [4], and a diode-array position-sensitive detector (Fig. 1). The sensor chip consists of a glass support, an overlaid gold film and a carboxymethylated dextran matrix [5] to which biomolecules can be coupled (Fig. 1). To study interactions between biomolecules, this system uses a surface plasmon resonance (SPR)-based assay, an optical technique that measures changes in the refractive index at the sensor chip surface. When plane-polarized light is incident on the sensor chip at an angle greater than a defined critical angle, and the intensity of the reflected light is monitored using a position-sensitive detector, SPR is observed as a decrease in light intensity for a specific angle of incidence (the SPR angle) [1,2]. The angle at which the decrease in light intensity occurs is proportional to the refractive index at the sensor chip; this is in turn proportional to surface mass changes. To study interactions between two molecules, one of the molecules is covalently immobilized on the sensor chip and the second molecule is injected over the chip surface. Interactions between the two molecules result in mass changes at the chip surface, which translate to refractive index changes and changes in the SPR angle. Changes in SPR angles are monitored as resonance units (RU, where 1000 RU corresponds to a 0.10° change in SPR angle, a 1.0 ng mm^{-2} change in surface mass, and a bulk refractive index change of 0.001) [1]. RU value shifts may occur either in response to specific binding to the immobilized molecule, or in response to differences in buffer compositions. Non-specific RU value changes can be distinguished from a specific response by carrying out blank injections, of buffer only or buffer plus a non-specific control protein, over the chip surface.

The coupling of biomolecules to the sensor chip surface is accomplished using amine, thiol or aldehyde-based immobilizations [6,7]. When a defined orientation of a protein molecule is required, cysteine residues can be introduced at specific sites in the protein to allow oriented coupling. A knowledge of the isoelectric point (pI) of the sample to be immobilized is useful before attempting coupling to the sensor chip. It is necessary to prepare the sample of macromolecule to be coupled in a buffer with a pH value below the pI, in order to enhance electrostatic interactions with carboxyl groups on the sensor chip. Various parameters in addition to pH can influence protein immobilization levels. These include the ionic strength of the buffer, the protein concentration and the duration of the coupling reaction. For proteins that are refractive to coupling using the methods described above, an alternative immobilization strategy involves biotinylation followed by binding to commercially available streptavidin-coated sensor chips.

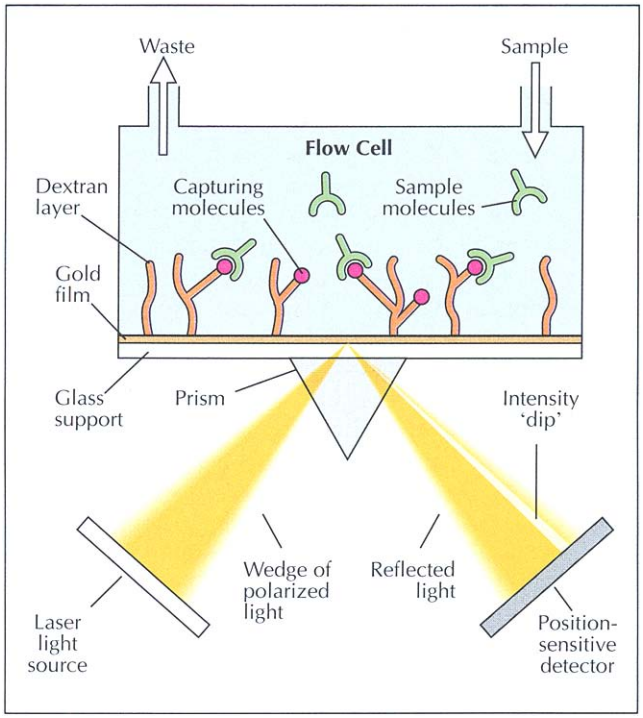

Fig. 1. The principle of operation of the optical biosensor in BIAcore. (Adapted from [27].)

 ISSN 0969-2126

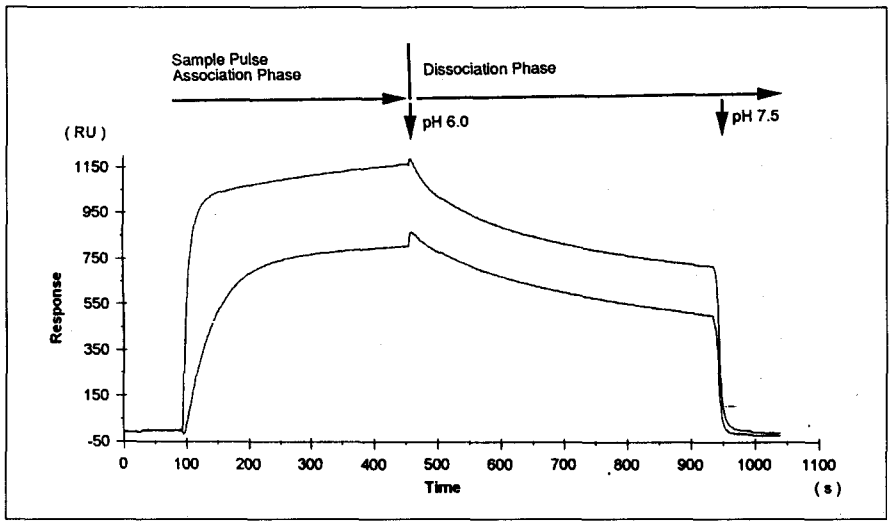

Fig. 2. Representative sensorgram data showing the binding of IgG to the neonatal Fc receptor (FcRn) immobilized on a biosensor chip. The figure illustrates difference sensorgrams for identical injections onto flowcells of a sensor chip that either have FcRn coupled or have a 'blank' coupling ($RU_{FcRn\ flowcell}$–$RU_{blank\ flowcell}$), in order to remove RU contributions due to buffer substances and salts in the injected samples. Two concentrations of IgG were injected during a sample pulse of about 6 min at a flow rate of 5 μl min^{-1}, corresponding to the association phase. Dissociation was subsequently monitored in buffer at pH 6.0 or at pH 7.5. At pH 7.5 the dissociation rate of the complex is very rapid, as visualized by the steep change in RU.

The data derived from the BIAcore system are in the form of sensorgrams, which are plots of RU *versus* time. Progress of association and dissociation events between molecules can thus be followed in real time. As an example, Fig. 2 shows representative sensorgrams derived for the binding of two concentrations of IgG to a soluble form of the neonatal constant framework of antibody Fc receptor (FcRn) [8], which was immobilized on a sensor chip. The data are shown as sensorgrams resulting from the difference in RU between identical injections onto the flow cells of a sensor chip, to which were coupled either FcRn or buffer. The end of the flow of sample through the cell (association phase) is at about 450 s. The complex dissociates in two steps: the first dissociation step is in a buffer of pH 6.0, and the second dissociation step, initiated at about 950 s, is in buffer of pH 7.5. This experiment illustrates the marked instability of the FcRn–IgG interaction at pH 7.5, a well-known feature of this receptor [9] that is important physiologically for the efficient unidirectional transport of IgG from the gut (pH 6.0–6.5) into the blood (pH 7.0–7.5). Visual inspection of the sensorgrams illustrates that the dissociation rate of the FcRn–IgG complex is significantly faster at pH 7.5 than at pH 6.0. Estimates of association and dissociation rate constants can be obtained from sensorgrams, using either linearized transformations of primary data [10] or non-linear curve-fitting methods [11]. Use of the recently developed BIAevaluation 2.0 software package enables rapid non-linear analysis of BIAcore-generated data using the Marquardt–Levenberg curve-fitting routine [12]. Affinity constants from experiments in which the interaction reaches equilibrium during the sample injection can also be determined by a method similar to Scatchard analysis (see BIAcore methods manual or [13]). Equilibrium analyses are especially useful when the association and/or dissociation kinetics are too rapid to be measured accurately, as for the dissociation rate of the FcRn–IgG complex at pH 7.5 (Fig. 2).

A look at the literature reveals diverse uses of BIAcore-biosensor technology, including identification of specific protein–protein interactions in complex systems [14–17], mapping of binding sites [18–20], identification of ligands for receptors from crude cellular supernatants [21], and investigations of kinetic parameters for previously characterized interactions [22]. From the perspective of a structural biologist, the BIAcore system offers a convenient tool with which to dissect intermolecular interfaces using proteins engineered with site-directed mutations. Cunningham and Wells [23] have carried out a comprehensive analysis of the energetic importance of 31 residues buried at the interface between human growth hormone (hGH) and the extracellular region of its receptor (hGHbp), deduced from the crystal structure of the complex [24]. Each of the 31 side chains in hGH was changed to alanine and the kinetics and affinities of interaction with hGHbp were measured using BIAcore assays. The study revealed that as few as a quarter of the side chains, clustered near the center of the interface, accounted for most of the binding energy [23]. These results, and more recent studies with alanine mutants of hGHbp [25], suggest a differentiation between a structural epitope (those residues at the intermolecular interface determined from a structural analysis) and a functional epitope (those contact residues that provide the majority of the stabilization energy for the complex). The conclusion from the structural and functional analysis of the hGH–hGHbp complex was that only a small and complementary subset of the total contact residues was required for maintaining binding affinity [25]. Further studies with other systems should verify the generality of these conclusions, but the authors [25] suggest that these results may also be characteristic of other protein–protein interfaces.

Crystal structure characterization can reveal higher-order structures that may turn out to be either functionally important, or an artifact of crystallization conditions. Further experiments using BIAcore technology can be designed to test the relevance of such structures. We have tested the biological relevance of an Fc receptor dimer that was crystallographically observed [26], by designing a receptor whose ability to dimerize was impaired when

coupled via a cysteine residue that was introduced at the dimer interface. Ligand binding by such a dimerization-impaired receptor was compared with ligand binding by a dimerization-competent receptor. It was deduced that the crystallographically observed Fc receptor dimer was important for high-affinity ligand binding (M Raghavan, YP Wang and PJ Bjorkman, unpublished data).

In combination with recombinant DNA technology for generating mutant proteins, biosensor-based functional assays are becoming essential tools in the structural biology laboratory. BIAcore is one such system that can be used conveniently to test structural predictions of possible contact sites, to examine contributions of individual residues at intermolecular interfaces to the overall binding energy, and to demonstrate the validity of proposed functional models. The diversity of applications of such systems will no doubt continue to expand in the coming months.

Acknowledgements: We thank Shirley Demer of Pharmacia Biosensor for technical advice and critical reading of this manuscript. Some of the work described here was supported by the Howard Hughes Medical Institute (PJB) and by a post-doctoral fellowship from the Cancer Research Institute (MR).

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