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Biomolecular interactions by Surface Plasmon Resonance technology

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Summary. - The Surface Plasmon Resonance (SPR) technique makes it possible to measure biomolecular interactions in real-time with a high degree of sensitivity and without the need of label. The information obtained is both qualitative and quantitative and it is possible to obtain the kinetic parameters of the interaction. This new technology has been used to study a diverse set of interaction partners of biological interest, such as protein-protein, protein-lipids, protein- nucleic acids or protein and low molecular weight molecules such as drugs, substrates and cofactors. In addition to basic biomedical research, the SPR biosensor has recently been used in food analysis, proteomics, immunogenicity and drug discovery.

Key words: biomolecular interaction, kinetic constants, surface plasmon resonance, protein-protein interaction.

Riassunto (*Studio delle interazioni biomolecolari attraverso la tecnica della Risonanza Plasmonica di Superficie*). - La tecnica basata sul principio della Risonanza Plasmonica di Superficie (SPR) consente di misurare in tempo reale le interazioni biomolecolari, con alta sensibilità ed in assenza di traccianti, ed è in grado di fornire i parametri quantitativi sia di tipo cinetico che energetico. Questa nuova tecnologia è applicabile allo studio di qualsiasi tipo d'interazione tra molecole d'interesse biologico, del tipo proteina-proteina, lipidi-proteina, acidi nucleici-proteina, o proteina con altre molecole di basso peso molecolare quali farmaci, substrati e cofattori. Recentemente, in aggiunta alle ricerche di base in campo biomedico, il biosensore SPR è stato applicato in numerose aree come analisi degli alimenti, proteomica, immunogenicità e ricerca di nuovi farmaci.

Parole chiave: interazioni biomolecolari, costanti cinetiche, risonanza plasmonica di superficie.

Introduction

A major challenge in cell and molecular biology research is to understand the relationship between the structural features of biological macromolecules and their function. An understanding of the mechanisms that control cellular processes requires knowledge of how protein activities, interactions and complex formation are regulated at specific sites and times in the cell. Information on how macromolecules assemble into complexes and break down over time is required to define biomolecular binding [1].

Development of Surface Plasmon Resonance (SPR) biosensor has made kinetic analysis of most biomolecular interactions routinely accessible and permits the real-time analysis of reactions without the use of labels. Since SPR detection is independent of the chemical nature of the sample being analyzed, in principle all types of molecules such as proteins, lipids, nucleic acids and small molecules such as drugs, substrates and cofactors can be used to monitor biomolecular interactions. The increasing numbers of studies that apply this technology indicate its usefulness in terms of both the variety of ligands and analytes employed and the sample environments in which tissue extracts, cell lysate, serum and milk and a variety of buffer can be used [2].

Since the first Biacore system, BIAcore AB (Biospecific Interaction Analysis) (Pharmacia Biosensor, Uppsala) introduced in 1990, a growing number of commercially available instruments based on biosensor technology, including several BIACORE instruments with different characteristic, have become available, some of them dedicated to specific applications.

The principal interest in the use of SPR for biomedical research is that it provides a platform for monitoring molecular interactions and defining the characteristics of proteins in terms of their specificity of interaction with other molecules; the association and dissociation rates at which they interact and their

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affinity, how tightly they bind to each other. In addition, when the binding properties of different components are compared or when the effects of point mutation, deletion or posttranslational modification are studied, a visual assessment of binding curves can be sufficient to interpret the data that describe an interaction.

The principle underlying Surface Plasmon Resonance detection

The SPR biosensors, including the Biacore systems, use a highly specialized optical technique to monitor changes in the refractive index in the vicinity of a surface. This SPR phenomenon occurs when, in conditions of total internal reflection, polarized light strikes a conducting gold layer at the interface (*sensor surface*) between a solid support phase and a liquid phase, two media of different refractive index: the glass of a sensor surface (high refractive index) and a buffer (low refractive index). The experimental procedure involves immobilizing one reactant (*ligand*) on a surface and monitoring its interaction with a second component (*analyte*) in solution. Essentially, SPR detects changes in mass in the aqueous layer close to the sensor chip surface by measuring changes in the refractive index.

A Biacore instrument comprises an SPR detector, a sensor chip and an integrated liquid handling system for the exact transport of the sample to the adsorption and detection spot. The sensor chip consists of a glass coated with a thin layer of gold, usually modified with a carboxymethylated dextran layer, which forms a hydrophilic environment for the attached molecules, preserving them in a non-denaturated state. The integrated microfluid system allows the molecules in the test solution - the analyte - to pass over the sensor surface in a continuous, pulse-free and controlled flow that maintains constant analyte concentrations at the sensor chip surface. The flow cells are designed for samples of as little as 5 µl. When the analyte binds to a target molecule bound to sensor chip, the mass increases and when it dissociates the mass falls. This produces changes in the refractive index close to the surface, which are detected as changes in the SPR signals expressed in arbitrary or resonance units (RUs). A sensorgram is obtained by monitoring the changes of resonance signal (RU) as a function of time (s) (Fig.1 A). Initially the buffer flows over the sensor surface (Fig. 1 A, base line). When the sample containing the analyte is injected, the upward slope of the curve indicates the to association of the analyte with the immobilized ligand (Fig.1 A, complex formation). After injection, the bound analyte dissociates in the buffer flow (Fig. 1 A, dissociation). Before performing

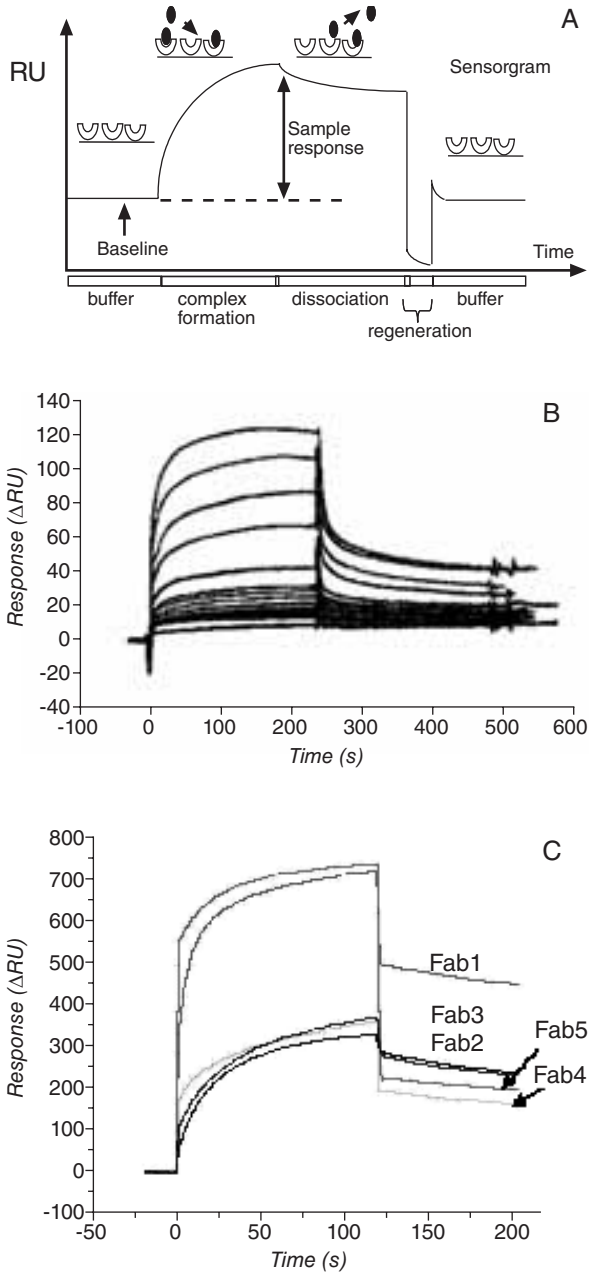

Fig. 1. - **A)** The progress of an interaction monitored as a sensorgram is shown. Base line represents a continuous flow of buffer. An increase in signal (association) is observed when the analyte binds during the injection to the ligand immobilized on the surface of the sensor chip. At the end of the injection the sample is replaced by a continuous flow of the buffer and the decrease in signal (dissociation) reflects the dissociation of the analyte from the surface-bound complex. A regeneration solution is injected to dissociate the remaining analyte (regeneration). **B)** Sensorgrams of the binding of β-dystroglycan-peptide (amino acids 821-895) to Grb2 immobilized on the sensor chip. Individual SPR profiles were obtained at increasing concentrations of β-dystroglycan-peptide in the range of 0.01 -17 µM from the bottom to the top. Association and dissociation phases started at 0 and at 240 s. **C)** Sensorgrams of the binding of monoclonal recombinant antibodies, (Fab1, Fab2, Fab3, Fab4 and Fab5) to its protein antigen immobilized on the sensor chip. Fab concentration was 2 µM. Differences in association and dissociation rates within the tested Fabs can be visually assessed.

a new injection, a regeneration solution is injected to dissociate the remaining analyte until the base line is reached (Fig. 1 A, regeneration). Monitored response units are directly related to the analyte bound to the surface layer. In general, changes in the refractive angle produced by changes in the mass concentration at the surface layer are independent of the chemical nature of the sample being analyzed, and are practically the same for all proteins and peptides and are similar for glycoproteins, nucleic acids and lipids.

The detector comes with an operating program that allows evaluation of the data analysis.

Sensor chips

Different types of sensor chip are available to immobilize different ligands. In our studies using SPR analysis of protein-protein or DNA-protein interactions we have used three types of sensor chips: CM5, SA and NTA.

The CM5 sensor chip is the most versatile and widely used for direct immobilization of a ligand. It is coated with a layer of carboxymethylated dextran and, through covalent derivatization, it is possible to immobilize a peptide/protein-ligand via amine coupling ($-NH_2$), coupling by thiol-disulphide exchange (-SH) or aldehyde coupling (-CHO).

The SA sensor chip, with streptavidin immobilized to carboxymethylated dextran, is used to capture biotinylated peptides and proteins. It is also ideal for the capture of large biotinylated DNA fragments and for the study of nucleic acid interactions [3].

The NTA sensor chip is a dextran surface matrix with immobilized nitrilotria-cetic acid that captures ligands via metal chelation, providing a means for capturing polyhistidine (H_6)-tagged ligands. The advantage of the NTA sensor chip is the possibility to control the sterical orientation of the ligand for optimal site exposure and the potential to regenerate the NTA surface and to use it many times, even with different His-tagged proteins.

The correct steric orientation of a ligand can also be obtained by coupling, on the surface of CM5 sensor chip, monoclonal antibodies directed against tags such as GST, His or FLAG, or other specific antibodies. In fact, SPR analysis of biomolecular interactions is often performed with recombinant tag-fused proteins that can be captured by the anti-tag mAbs immobilized on the sensor chips.

Because sensor chips provide high chemical stability and low non-specific binding, the sensor chip surface can be regenerated for numerous cycles (100 on average) depending on the nature of the immobilized ligands.

The recent availability of hydrophobic (HPA) and lipophylic (L1) sensor chips is making it possible to analyze protein interactions with lipid surface and membrane-associated proteins [4].

Applications

SPR biosensors have been used to study a wide range of biomolecular interactions, providing both qualitative (identification, site specificity, epitope mapping) and quantitative (kinetics, affinity and concentration analysis) information [2, 3, 5]. In this short review we will briefly summarize some SPR application and data on protein-protein interactions that we have obtained using the BiacoreX and BIAevaluation software version 3.1, which permits global analysis of entire data sets. This highly sensitive and semi-automatic biosensor instrument is characterized by two-cells sensor chips. It allows detection of the response of an analyte injected simultaneously over two flow cells. It is possible to immobilize a small amount of ligand on the flow cell 1 (Fc1) and use the flow cell 2 (Fc2) as reference surface. Since systematic artifacts associated with the injection are essentially equal in the reaction and reference flow cells, by subtracting the reference surface data from the reaction surface data the quality of the reaction surface data is improved.

Protein-protein interaction

Molecular analysis of protein-protein interfaces by site-directed mutagenesis and SPR analysis

SPR analysis is a suitable technique to investigate the effects of amino acid substitutions on protein-binding properties and to characterize the interface between two interacting proteins, such as the dystroglycan subunits [6]. Dystroglycan, a central component of the dystrophin-associated protein complex, plays an important role in muscle stability and in neuromuscular disorders [7]. It is formed by two subunits, a and b, derived by enzymatic cleavage from a precursor polypeptide. α-Dystroglycan binds extracellular matrix proteins and interacts non-covalently with β-dystroglycan, a transmembrane protein, which binds dystrophin and other cytosolic proteins, including Grb2, inside the cell. It is likely that the interaction between α and β subunits acts as the major factor affecting the stability of the entire complex. The non-covalent binding occurs between the C-terminal region of the α-dystroglycan and the N-terminal ectodomain of the β-dystroglycan subunits [8-

10]. By comparing sensorgrams of the binding a) of wild-type or mutated β-dystroglycan ectodomain to α-dystroglycan C-terminal region immobilized on the sensor chip, or b) between wild-type or mutated α-dystroglycan C-terminal to β-dystroglycan ectodomain, we found that specific aromatic residues play a crucial role in inter-subunit interactions [6].

Amino acid mutations are often associated with genetic disorders and one objective is to elucidate how mutations affect the functional properties of a protein, encoded by a particular gene, possibly causing distinct specific diseases. SPR analysis has been used to study the effects of activating missense mutations of fibroblast growth factor receptor 2 (FGFR2) on FGFR2 ligand binding affinity and specificity [11, 12]. FGFR2 mutations are responsible for a variety of craniosynostosis syndromes. In particular in a recent study, the pathogenic FGFR2 mutations associated with Apert syndrome and Pfeiffer syndrome were found to produce distinct changes in FGFR2 ligand binding affinity and specificity, which correlate with the craniofacial and limb phenotypes observed in patients carrying these mutations [12].

Kinetic analysis of the interaction between β-dystroglycan and growth factor receptor 2 (Grb2)

Using the BIAcore X instrument we have quantified the interaction between the cytoplasmic domain of β-dystroglycan and Grb2 and compared the K_D of the interaction determined by SPR analysis with the values obtained using different experimental approaches such as fluorescence and solid-phase assay [13]. Grb2 is an adaptor molecule involved in signal transduction and cytoskeleton organization. It is composed of one SH2 (Src homology 2) domain, which binds phosphotyrosyl peptides, flanked by two SH3 domains. Grb2 binds to the cytoplasmic domain of β-dystroglycan, a central component of the dystrophin-associated protein complex [7]. The β-dystroglycan cytoplasmic region contains several proline-rich consensus sequences for binding with the Grb2-SH3-domains. Therefore, we performed SPR analysis: 1) to determine which of the two Grb2-SH3 domains, the N-terminal or the C-terminal, binds to β-dystroglycan and 2) to identify the binding sites on β-dystroglycan by comparing the kinetics parameters of β-dystroglycan fragments containing one (amino acids 876-895) or two (amino acids 821-895) proline-rich consensus sequences for the Grb2-SH3 interaction. Fig. 1 B shows sensorgrams of β-dystroglycan fragment (a.a. 821-895), at increasing concentrations ranging between 0.01-17 mM, which was allowed to flow over the sensor surface with GST-Grb2 immobilized to one cell and GST to the reference cell of the sensor chip. To obtain the kinetic parameters, sensorgrams may be modelled using one of several binding models provided with BIAevaluation software 3.0. This allows calculation of the association rate constant (k_{on}) and the dissociation rate constant (k_{off}). The ratio of k_{off} to k_{on} can be used to estimate the equilibrium constant K_D. Analysis of the data from SPR revealed a high-affinity interaction ($K_D \approx 240$ nM) between Grb2 and β-dystroglycan similar to that obtained by solid-phase binding assay and in solution by fluorescence [13]. In addition, we showed that both Grb2-SH3 domains bind β-dystroglycan, but the N-terminal binds with higher affinity than the C-terminal SH3 domain. We also identified the Grb2 binding site on a proline-rich sequence contained in amino acids 876-895 of β-dystroglycan and found that this region binds to Grb2 with the same affinity of the whole cytoplasmic domain of β-dystroglycan [13].

Antigen-antibody interaction

The SPR analysis has been widely used to characterize antibodies by identifying binding antigens, mapping binding epitopes, and measuring kinetic and affinity parameters. Fig. 1 C shows the responses of different Fabs, produced against the antigen A. Sensorgrams of Fabs2, 3 and 4 are very similar. Fab1 and Fab5 show both a higher association but they differ in their dissociation slopes. The different profiles of the Fab1 and Fab5 binding response indicate that these two Fabs bind the antigen with different kinetics. By using SPR analysis it is easy to perform epitope mapping by injecting after the first Fab, over the Fab/antigen complex, a second Fab. If the response does not change that means the two Fabs compete for antigen binding. In contrast the signal increases when two Fabs bind different epitopes (see refs in [5]).

Conclusion

SPR biosensors have been widely used to study the kinetics and affinity of molecular interactions; for identification of novel binding partners by direct capture of proteins from cell or tissue extract on SPR surfaces followed by mass spectrometry; to characterize antibodies and other proteins intended for therapeutic purposes [2, 3, 5]. The increasing number of published papers is showing how SPR technology is making a significant impact in food analysis, pharmaceutical discovery and basic research [3].

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