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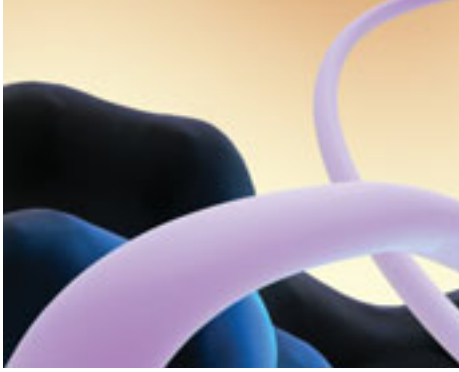
Biomolecular Interaction

Analysis and MS



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Surface plasmon resonance coupled with MS is a practical tool for identifying and characterizing protein interactions.

Biomolecular interaction analysis (BIA), a biosensor technology that detects binding events between two or more biomolecules, has become one of the most important and versatile approaches for understanding the function of proteins. BIA relies on the phenomenon of surface plasmon resonance (SPR), which occurs when monochromatic, p-polarized light is reflected on a metal-coated interface between two media (*1*). The intensity of the reflected light is reduced at a specific incident angle, producing a dip in intensity within the reflected wedge of light. The incident SPR angle depends on the refractive index of the material near the surface on the nonilluminated side. Hence, the binding of analytes in solution to ligands immobilized near the metal-coated interface can be followed by measuring changes of the incident angle during association and dissociation.

In a BIA experiment, molecules of interest or orphan receptors are immobilized on sensor chips; analytes are passed over the sensor chip through a microfluidics system, and the binding activity is monitored in real time. The microfluidics are operated automatically by micropumps and an autosampler, and thus the samples are screened automatically at high reproducibility. As the analytes and receptors bind, the refractive index in the vicinity of the surface is altered, and the SPR angle is correspondingly shifted (Figure 1a). This signal, measured in resonance units (RU), directly correlates with the amount of protein interacting near the surface (1000 RU = 1 ng bound protein/mm^2). When the flow is changed to buffer without analyte, the resulting dissociation of analyte and receptor causes a shift of the SPR angle toward the initial values. Results from BIA are presented as a sensorgram, which is a plot of changes in the resonance signal as a function of time (Figure 1b).

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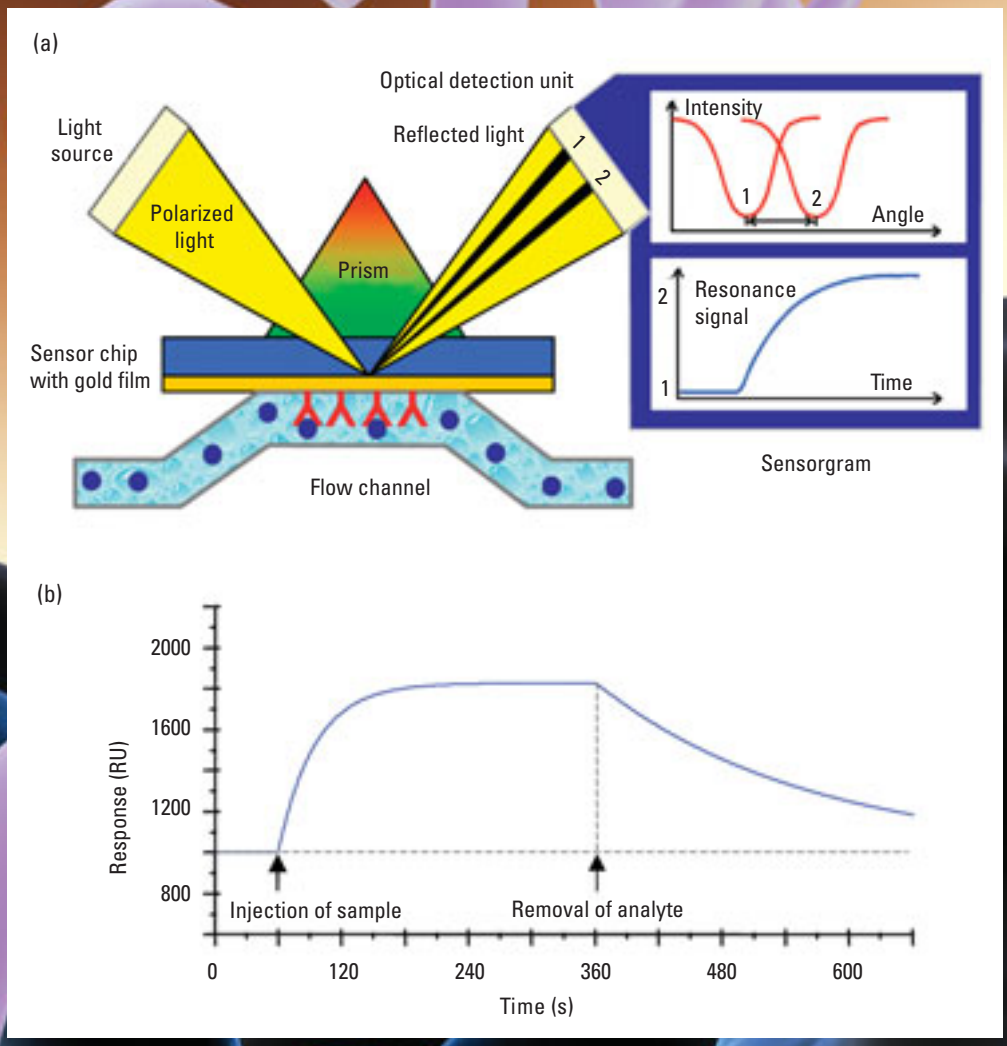


FIGURE 1. Basics.

(a) An increased sample concentration on the sensor chip surface causes a corresponding increase in the refractive index, which alters the SPR angle. The angle is monitored as a change in the detector position for the reflected intensity dip (from 1 to 2). (b) A sensorgram is a plot of the SPR signal versus time. The increase in resonance units from the initial baseline represents the association of molecules in the test solution with the surface-bound target molecule during sample injection. The plateau represents the steady-state phase of the interaction where binding is balanced by dissociation from the complex. The decrease in resonance units from the plateau represents the dissociation from the surface after removing the analyte from the running buffer.

librium constants for complex formation, can be derived from BIA. Other applications include discovering novel molecular interactions and screening small molecules for potential therapeutics. The ability to detect and quantify specific interactions in complex fluids, cell lysates, conditioned media, and a variety of other sources makes SPR biosensors suitable for ligand screening (*3*–*6*). In many academic and industrial research laboratories, SPR biosensors have become standard biophysical tools (*7*, *8*). With SPR, quick measurements can be taken in real time, and the fact that very small amounts of proteins are needed for analysis has made it a popular technique.

BIA only shows that an interaction has taken place, and the identity of the binding molecule(s) might not be known. Identifying the binding molecule is usually a time- and resource-consuming process, requiring a scale-up of starting material, followed by large-volume affinity chromatography. To overcome this limitation, BIA/MS methods were developed to detect, isolate, identify, and characterize the components. In this article, we will summarize BIA/MS principles and methodologies and highlight recent examples of experimental design and applications.

Commercial SPR instruments use different sensor surfaces and sample delivery systems. Biacore places the sensor surface on a removable microchip optically coupled to the instrument, where the sample is supplied via an integrated computer-controlled microfluidic system that provides a constant flow of analyte or buffer. IASYS places the sensor surface on a stirred microcuvette with integrated optics; IBIS, SPR670, and Cellia instruments can be configured as cuvette- or flow-cell-based systems. Different systems and configurations have been reviewed (*2*). Because all the work published to date has been performed with the Biacore system, we will refer to this system as BIA in this article.

Sensor surfaces can be functionalized to detect a wide variety of molecules and are used in direct- or affinity-capture modes. Quantitative information, such as kinetic parameters and equi-

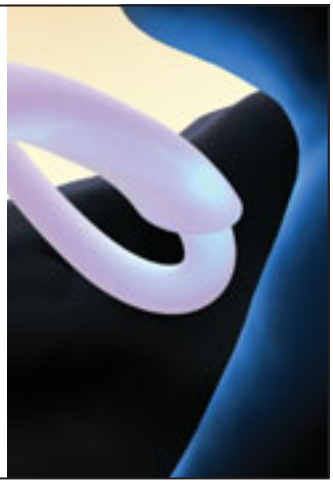
Why combine SPR and MS to study protein interactions?

One advantage of SPR biosensors versus a simple affinity column is the possibility of comparing, quantifying, and directly subtracting the binding on a reference flow cell from that on a specific flow cell, thereby eliminating false positives caused by nonspecific binding. Moreover, the ability to detect subtle differences in binding kinetics and binding affinity between target molecules and their ligands makes it possible to identify the most effective agonists and antagonists and to optimize ligand selectivity. In many cases, a slower dissociation curve in the sensorgram is already a good indication of an increase in the affinity of recognition, which allows a rapid screening of different analytes.

MS is the ideal complement to BIA because of its sensitivity and speed. BIA/MS makes it possible to link protein detection, capture, and kinetic analysis with the measurement of protein and peptide masses and protein identification. These measurements might even be possible for transient or unstable complexes that might be otherwise inaccessible by conventional techniques (*9*). Continuous improvements in the sensitivity of protein MS have increasingly facilitated the identification of protein complexes of both biological and therapeutic interest.

MS instruments and expertise are likely to become more widespread, perhaps even standard, in molecular biology laboratories. The improvements in hardware, automation, searching algorithms, and increased integration between cell biology and MS will continue to facilitate the large-scale identification of protein complexes (*10*). MS is also a unique tool for studying posttranslational modifications of proteins. One question that can be addressed by SPR combined with MS is the modulation of the affinity and specificity of protein interactions in the presence of diverse posttranslational modifications.

BIA/MS makes it possible to link protein detection, capture, and kinetic analysis with the measurement of protein and peptide masses and protein identification.



On-chip MALDI

In this method, the sensor chip used in the SPR analysis of the interaction is removed from the biosensor with the retained molecules still present on its surface, and MALDI-TOF-MS is then used to determine the molecular mass of proteins retained on the sensor chip surface (*11*, *12*). Although this method is easy and requires no special device or modification of BIA and only a slight modification of the target for the mass spectrometer, it has several limitations. The amount of analyte captured on the surface is sometimes too small for analysis, and the system does not allow multiple runs to be performed to accumulate more material. Because the matrix is applied directly on the sensor chip, it frequently cannot be reused.

The determination of the intact molecular mass by MALDI-TOF analysis cannot be considered a general method for identifying proteins. The mass accuracy of MALDI-TOF mass spectrometers is 10–50 ppm for small polypeptides, which allows for direct identification. For proteins >30 kDa, however, the mass deviation frequently rises to >0.1%, making it impossible to obtain unambiguous identification. For example, if the Swiss-Prot database is searched for protein sequences corresponding to a molecular mass of 30 kDa with a mass accuracy of 0.1%, >600 possible candidates are obtained. Furthermore, proteins are often modified posttranslationally (e.g., enzymatic cleavage, glycosylation, phosphorylation, acetylation, methylation), and all such modifications lead to a change in the molecular mass, which might not be apparent from the database information.

However, searching protein databases with intact masses can be a useful validation tool when the mass of the protein is known or can be predicted. In their "multitoxin BIA/MS analysis", Nelson and co-workers used BIA/MS as an alternative to ELISA to detect bacterial toxins in food. SPR detection of the binding of toxins to antibodies immobilized on the sensor chip was followed by identification of the bound toxins by MALDI-TOF-MS, based on their known mass (*13*). The advantages of this approach over conventional immunological methods are the speed of analysis and the ability to discriminate between specific binding of the targeted analyte and nonspecific interactions of other compounds with the sensor chip surface. Natsume and co-workers described a method for the on-chip purification of recombinant proteins expressed as glutathione-*S*-transferase fusions (*14*). In this case, determining the protein's molecular mass by MALDI-TOF-MS is useful for validating the purification and presence of nonspecific binding prior to using the sensor chip for interaction studies.

Microrecovery for MS

Sönksen and colleagues reported a novel microrecovery method to elute bound molecules from the sensor chip that provides flexibility in choosing the analytical procedure for processing and identifying the bound molecule (*15*). To minimize sample loss during the procedure, they delivered a small volume (3 μL) of elution solvent separated from the system buffer by two air bubbles. With this "sandwich" elution, they recovered subpicomole levels of bound molecules without dispersion for subsequent MS analysis. These data also proved that the sensitivity of MALDI-TOF-MS is adequate for analyzing molecules captured on the sensor chip. However, for identification, proteins bound to the sensor chip must be enzymatically digested after elution, a process that can lead to critical sample loss and therefore insufficient signal intensity for MS or MS/MS. To increase the sample amount, microrecovery procedures can be repeated because the elution procedure is not destructive to the sensor chip. As a result, it is also possible to compare different ligands by using the same surface.

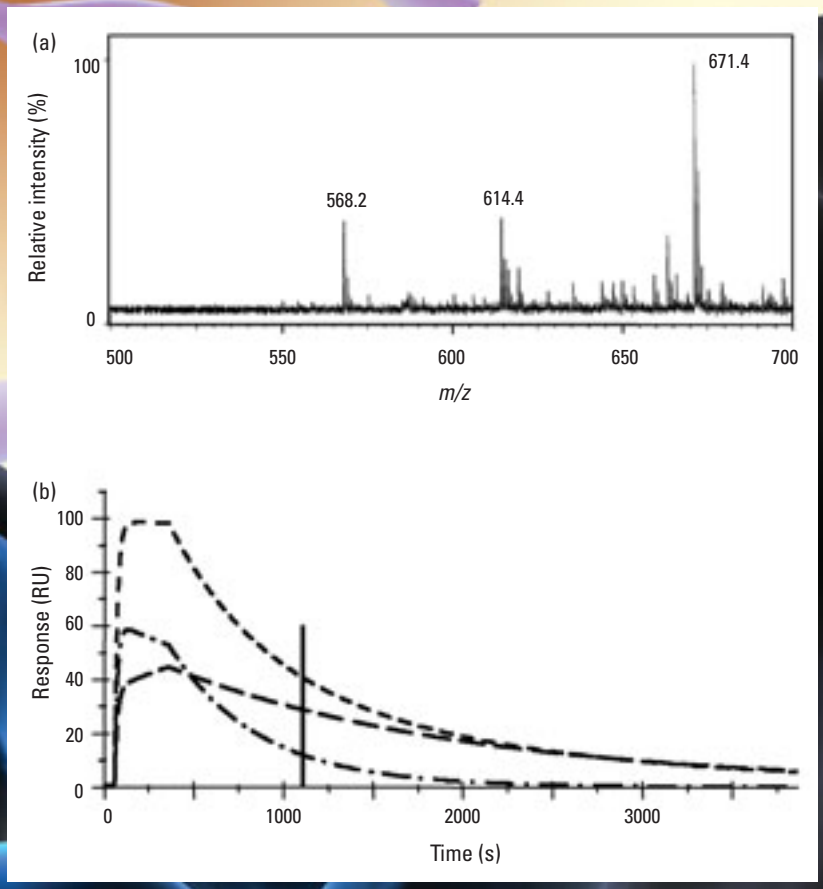


FIGURE 2. Competition experiment with simultaneous binding of saquinavir and indinavir to a sensor surface with immobilized HIV-1 protease.

(a) MALDI-TOF spectrum showing saquinavir (670 Da) and indinavir (613 Da) eluted from the surface. A standard curve (not shown) for the relationship between the peak intensities and different concentrations of the two components showed that the peak intensity ratio of 0.4 should reflect an indinavir/saquinavir concentration of 0.4. (b) Simulated sensorgram for the binding and dissociation of saquinavir and indinavir to the HIV-1 protease sensor chip. The vertical line indicates the time at which the ratio between the two inhibitors should be 0.4. Dashed line, saquinavir plus indinavir; long dashed line, saquinavir; dotted and dashed line, indinavir. (Adapted from Ref. 15.)

This technology has been applied to the study of interactions such as antibody–antigen and DNA–protein interactions. Traditionally, BIA has been used mainly for the analysis of rather large molecules (>5000 Da), because the BIA signal is mass dependent and sensitivity increases as the molecular mass of the analyte increases. On the other hand, the sensitivity of MALDI and electrospray ionization MS (ESI-MS) in general decreases with increasing mass. The improvements recently introduced in methodology and instrumentation for this approach have enhanced the overall sensitivity and allowed the detection of small molecules (<200 Da), as was shown in the study of binding of low-molecular-weight inhibitors to HIV-1 protease (*16*).

Femtomole amounts of two drug candidates in mixtures, saquinavir (670 Da) and indinavir (613 Da), were captured on a sensor chip to which HIV-1 protease was immobilized. After elution from the sensor chip, structural information was obtained for the inhibitors by postsource decay in combination with MALDI-MS or MS/MS using nano-ESI. Moreover, the possibility of analyzing the interaction with heterogeneous ligands was investigated by co-injecting the two inhibitors followed by MALDI-TOF-MS of the fraction eluted at a definite time point of the dissociation phase. In BIA experiments of interaction with two or more analytes, the resulting sensorgram is very difficult to analyze because many variables must be taken into account, including the different kinetic constants as well as concentrations and masses of the different analytes. The MS analysis allows measurement of the peak intensity ratio (Figure 2a) and estimation of the contribution of each inhibitor to the dissociation phase. The measured value of the peak intensity ratio after a definite interval from the beginning of the dissociation can be compared with that obtained in a simulated sensorgram (Figure 2b). According to the simulation, an indinavir/saquinavir ratio of ~0.4 is reached 1200 s after the start of the injection, which is in good agreement with the time interval that actually occurred between injection of the sample mixture and elution. This comparison confirms the estimated relative binding affinities of the two inhibitors.

On-chip digestion for on-line BIA/MS/MS sequencing

Natsume developed another very elegant approach in which a small volume of solution containing a proteolytic enzyme is delivered to the sensor flow cells (*9*). The flow is then stopped to allow digestion to take place "on-chip", without dilution or loss of sample, while the detector monitors in real time the mass decrease caused by proteolytic release of peptides from the bound protein. Finally, the mixture of peptides generated is eluted and trapped on a reversed-phase capillary column fitted to the outlet port of the flow cells. The column is subsequently connected to a microelectrospray interface column of a nano-LC/MS/MS system, and the mixture is further separated and analyzed by tandem MS. The validity of the system was demonstrated by analyzing different model interactions, for example, His-tagged proteins captured on nickel-chelating nitrilotriacetic acid (NTA) chips.

To test a real-life application, lysates from cells expressing low levels of calmodulin were injected onto a sensor chip containing an immobilized peptide that displayed a calmodulin binding sequence. The detection of a specific and Ca^{2+}-dependent binding by calmodulin also proved that the affinity capture in BIA can be used to pick out the true ligand, even from a crude mixture, and that the small amount of material binding to the chip is enough to give a proper identification (*17*). One of the clear advantages of microrecovery is that it allows subsequent handling of the sample for identification. Thus, subsequent tryptic digestion followed by peptide mass mapping by MALDI-TOF-MS or sequencing by ESI-MS/MS is possible.

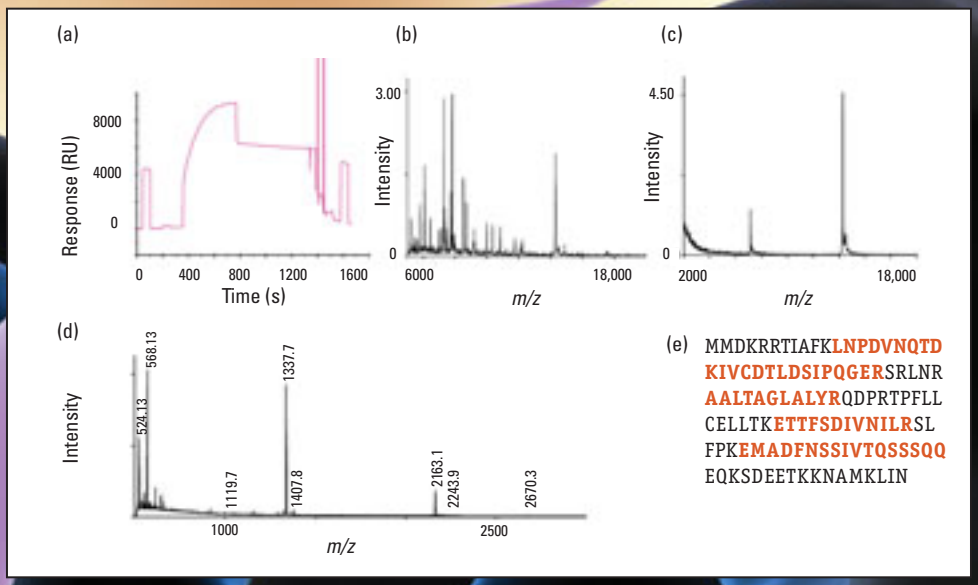


FIGURE 3. Recovery and MALDI-TOF analysis of recombinant 6×His-tagged ParR protein captured on an NTA sensor chip directly from a cell lysate.

(a) Sensorgram of binding and recovery from lysate of *E. coli* expressing 6×His-tagged ParR. MALDI-TOF spectra of the (b) lysate and (c) protein recovered from the sensor chip. (d) MALDI-TOF spectrum of the protein after recovery from the sensor chip and digestion with trypsin. (e) Sequence coverage obtained from the peptide mass map. Matching peptides are in red.

Identification by peptide mass mapping

ParR proteins are DNA binding proteins that bind repeats of a defined DNA sequence. Binding leads to pairing and partitioning of low-copy-number plasmids containing binding repeats (*18*). Recombinant 14-kDa ParR protein containing an N-terminal 6×His-tag was expressed in *E. coli*. Diluted cell lysates (Figure 3b) were injected directly onto the BIA system containing NTA sensor chips on which Ni^{2+} was chelated. As the sensorgram in Figure 3a shows, ~5.6 kRU corresponding to 5.6 ng or 400 fmol per flow cell are bound. After washing, the bound proteins were almost quantitatively eluted with 5% formic acid by using an automatic sandwich elution method on the Biacore 3000.

When the recovered proteins were analyzed by MALDI-TOF, only signals from the expected intact 6×His-tagged proteins were present in the spectra (Figure 3c). To further verify that the proteins recovered from the sensor chips were indeed ParR, the micro-recovered proteins from a single sandwich elution were digested with trypsin and analyzed by MALDI-TOF (Figure 3d). When the Mascot server was used to search the Swiss-Prot database with the obtained peptide masses (www.matrix-science.com), the only hit with a significant score was ParR. The sequence coverage of the digested eluant to ParR is 57% (Figure 3e). Thus, it was demonstrated that eluates from Biacore sensor chips can be used for verification and identification of proteins isolated directly from cell lysates.

The cell lysate was further tested for binding to the specific double-stranded ParC DNA, which was linked to a streptavidin-derivatized chip via a biotin tag. The cell lysate showed binding, but dissociation was spontaneous when the analyte was removed from the solution (Figure 4a). However, when the His-tagged ParR was isolated from the cell lysate by affinity chromatography on a Ni–NTA sepharose column, it showed binding with a slow off-rate, which allowed recovery and subsequent MS analysis (Figure 4b). It thus seems that the cell lysate contained components that prevented stable binding of ParR to its target DNA.

Enzyme–inhibitor interactions and binding epitopes

A modification of the sandwich method was used to study how the glycosylation of the fungal enzyme polygalacturonase (PG) would affect the formation of the complex with its plant-inhibiting protein, PGIP (*19*). Solutions of native PG and PG treated with the glycanase *endo*-H were passed over a surface of immobilized PGIP. The sensorgram for the interaction showed that the affinity of the deglycosylated enzyme for the inhibitor

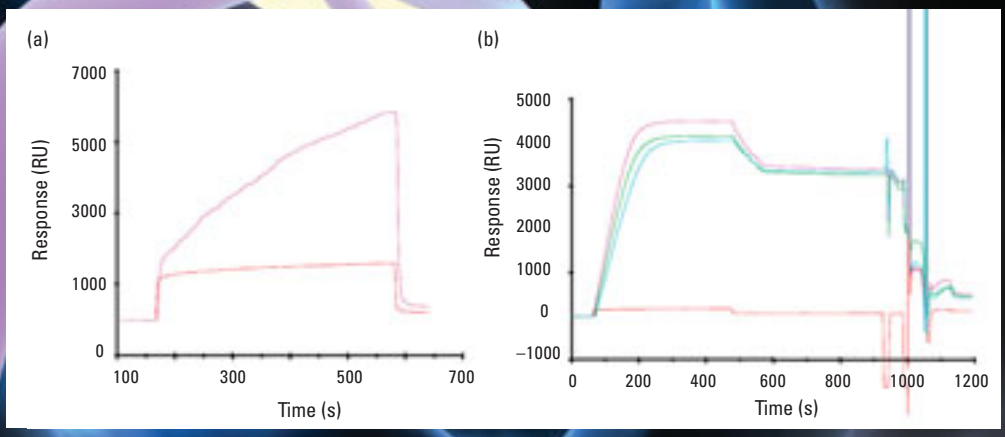


FIGURE 4. Par binding.

(a) Sensorgram for binding components in the cell lysate to a ParR-specific double-stranded DNA linked to a streptavidin-derivatized chip via a biotin tag. (b) Binding of the His-tagged Par isolated from the cell lysate by affinity chromatography.

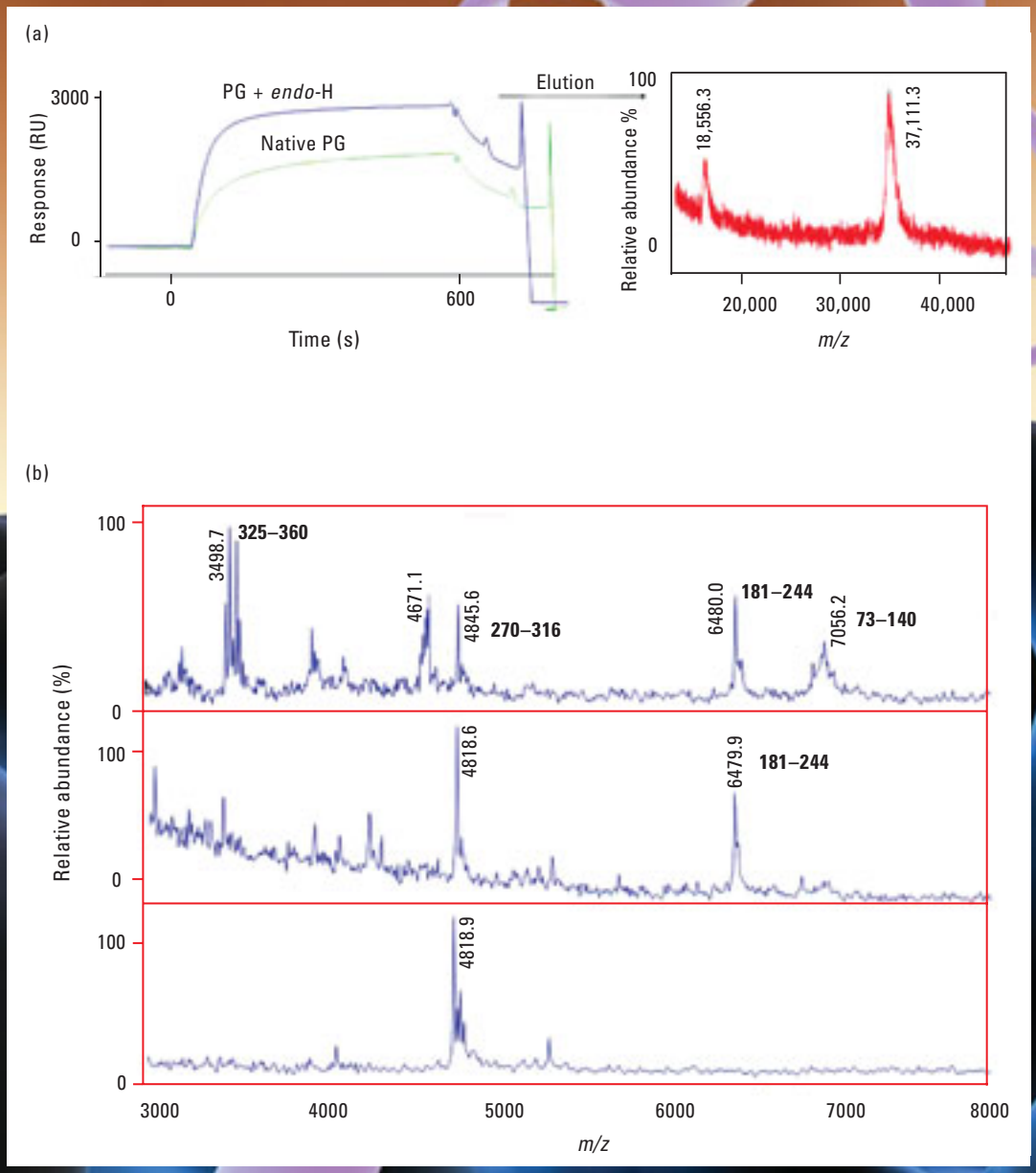


FIGURE 5. PG and PGIP.

(a) (left) Sensorgrams obtained for the interaction between the immobilized PG inhibitor PGIP-2 and either the native PG or the enzyme deglycosylated with *endo*-H. (right) The deglycosylated enzyme retained on the sensor surface is eluted and analyzed by MALDI-TOF. (b) MALDI spectra of (top) the crude peptide mixture derived by treating the native enzyme with the endoproteinase Lys-C, (middle) recovered material after passing it over the sensor surface immobilized with PGIP, and (bottom) recovered material after passing it over a reference cell as a control. The peptide at *m/z* 6479.9 contains the binding epitope. (Adapted with permission from Ref. 19.)

was even higher than that of the glycosylated form, and the mass spectrum obtained after one cycle of elution (~40 fmol of eluted protein) confirmed that the interacting molecule was the deglycosylated form of the enzyme (Figure 5a). The observed mass (37,110 Da) is in good agreement with the presence of four GlcNAc residues attached at the four potential N-glycosylation sites, as expected from the specificity of the glycanase *endo*-H. This example shows that BIA/MS might be a valid tool for characterizing protein complexes in which protein interaction is modulated by posttranslational modifications.

BIA/MS was subsequently used to identify the domain of the enzyme recognized by PGIP (*19*). The affinity capture of an interacting peptide from a mixture of fragments obtained by limited proteolysis of the native form of the enzyme proved to be sensitive enough to characterize the interacting peptide eluted from the sensor surface (Figure 5b). One peptide with *m/z* 6479.9 was specifically recovered from the flow cell containing immobilized PGIP, whereas it was absent in the fraction eluted from a blank surface used as a control. Several residues in the interacting fragment were subjected to directed mutagenesis. Subsequent BIA of the engineered forms of the enzyme confirmed that the peptide identified in the BIA/

MS experiment contained many residues critical for the complex formation (*20*).

Possibilities

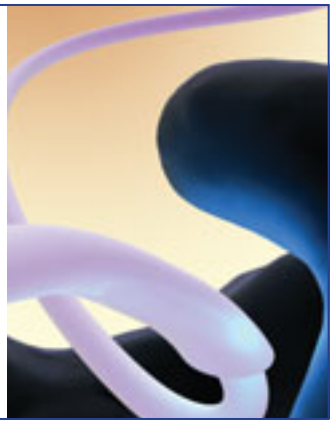
The different examples of the coupling of BIA and MS show that this approach has the potential to address many different types of biological questions, allowing quantitative and qualitative binding assessments in a variety of biological samples. Small amounts of material can be rapidly detected and identified, and nonspecifically interacting components can easily be ascertained. Applications of the technique can be envisaged with different scopes and experimental setups. In some cases, using an autosampler is key to large-scale operations, for example, the screening of multiple samples to identify unknown interacting partners in a proteomics strategy. In other cases, the system can be operated manually for more subtle control of the parameters, as illustrated by the detailed characterization of structural requirements for complex formation.

Currently, the major limitation of BIA/MS is the analysis of interactions with fast off-rates ($>10^{-2}s^{-1}$). The bound analyte dissociates rapidly from the sensor chip, and even if the elution is performed immediately, it is often difficult to recover a sufficient amount for subsequent MS analysis. The use of high densities of immobilization, for example, by using all the flow cells at the same time, can minimize this limitation. Software has been developed to perform multiple cycles of capture and elution in an automated fashion. In addition, it is now possible to perform automated liquid handling and deposition of the recovered analyte directly onto a MALDI target. These new features allow automated high-throughput BIA/MS.

Another possibility is discovering if the interacting protein is posttranslationally modified, which determines the interaction with other molecules and is one of the most interesting challenges in proteomics (*21*). Unfortunately, this information cannot be obtained in a simple BIA experiment. A further potential advantage of the method is the possibility of performing on-chip reactions, which could encompass phosphorylation, oxidation, deglycosylation, and enzymatic cleavage. In this way, BIA/MS becomes a versatile platform where one can play with all the tools of analytical biochemistry and protein chemistry as well as identify and verify posttranslational modifications required for specific interactions.

Benedetta Mattei was a postdoctoral fellow at the University of Southern Denmark and is now an assistant professor at the University of Rome. Mattei's research interests include biomolecular interactions and structure–function relationships of proteins involved in plant pathogen recognition. Peter Roepstorff heads the protein research group at the University of Southern Denmark. His research interests include the uses of MS to characterize posttranslational modifications of proteins and protein–ligand interactions. Jonas Borch is a Ph.D. student in the protein research group at the University of Southern Denmark. He is developing combinations of SPR and MS to study protein–ligand interactions, especially interactions regulated by posttranslational modifications and protein–nucleic acid interactions. Address correspondence about this article to Roepstorff at the Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark (roe@bmb.sdu.dk).

BIA/MS has the potential to characterize posttranslational modifications such as phosphorylation, oxidation, deglycosylation, and enzymatic cleavage.



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