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Abstract

The mapping of protein networks and the establishment of the functional relationships between expressed proteins and their effects on cellular processes represents a great challenge for functional or interaction proteomics. The combination of surface plasmon resonance (SPR)-based technology with mass spectrometry (MS) has created a unique analytical tool for functional proteomics investigations. Proteins are affinity purified, quantified and characterised in terms of their interactions, while the mass spectrometer identifies and structurally characterises the biomolecules. Recent developments have led to a closer integration of these key technologies, providing a combined approach which enables identification of proteins selected on the basis of their functional binding criteria. In addition to a historical overview of this field, some recent detailed examples of combined SPR-MS approaches will be reviewed in a number of key application areas, including ligand fishing, peptide sequence and post-translational modification analysis by SPR-MS/MS and enzyme inhibitor screening.

INTRODUCTION

One major aim of the proteomics field is to identify and quantify the proteins expressed in specific cells or tissues and to analyse changes in these expression patterns in response to physiological and pathological stimuli. Biological functions of most macromolecules, however, depend on their ability to interact with other molecules. Insights into protein interaction networks, therefore, are crucial for understanding biological processes and may provide opportunities to discover new drugs and develop personalised medicine. High expectations on the output of proteomics research have driven the advancement of existing technologies and the development of new approaches. Usually, experimental strategies are based on the concerted use of two or more technologies, as seen in the integrated use of separation methods, like two-dimensional gel electrophoresis or liquid chromatography, with protein identification techniques, such as mass spectrometry (MS) fingerprinting, in conjunction with bioinformatics. This paper describes the recent developments and advantages of another integrated approach based on the combination of surface plasmon resonance (SPR) technology and MS.

Compatible technologies for proteomics applications

Protein interaction analysis systems (Biacore AB) use the phenomenon of SPR to detect and quantify molecular interactions in real time and without the use of labels. They do this by monitoring the change in mass on a sensor surface, which results from the interaction between solution-borne and surface-immobilised binding partners. A comprehensive review of the technology on which these systems are based, and their applications, has been written by Franklin and McWhirter. By following the time course of the association and dissociation processes, a thorough characterisation of the interaction strength and kinetics can be made. The target proteins can also be affinity purified from crude biological mixes such as cell lysates and extracts, consuming only a few microlitres of sample. The proteins can be
quantified in terms of concentration and interaction rates, based on how they are captured and released from the sensor surface. As SPR detection is label free and non-destructive, the biomolecules that are selectively retained on the sensor surface can subsequently be recovered and analysed by MS. This combination became especially useful when rapid developments in biomolecular MS allowed the identification and structural characterisation of femtomolar amounts of proteins, since these amounts can typically be captured on a square millimetre of a sensor surface. The ability to identify and structurally characterise biomolecules that are captured on sensor surfaces opens up a completely new range of applications. One obvious application is to utilise the SPR-MS combination to fish or screen for the unknown interaction partners to a protein immobilised on the sensor surface. This has been demonstrated by Kikuchi et al., for example, who found three novel p53 binding proteins.

More generally, SPR-MS can be used to screen for any type of interaction partner from a complex mixture of biomolecules. Interaction partners can be candidate drug compounds, DNA-binding proteins, biomarkers for specific diseases, enzyme inhibitors found in plant extracts or specific post-translationally modified protein variants that are responsible for a certain interaction. Besides fishing from a complex mix of biomolecules, epitopes can also be screened by subjecting a known interaction partner to limited proteolysis and only capturing the peptide containing the epitope on the sensor surface, allowing the peptide to subsequently be identified by MS. This approach will certainly change the nature of conventional SPR-based epitope mapping, which has been applied since the early 1990s. In this conventional epitope mapping strategy, the interactions of various monoclonal antibodies (mAbs) with an antigen were studied simultaneously to determine which of the mAbs competed for the same interaction site. Quality control (QC) applications also benefit from the SPR-MS tandem, since SPR systems can efficiently fish for unwanted substances, such as by-products in therapeutic proteins or toxins and hormones in the food products, while MS confirms the final identity of the affinity-purified substance (which is often a requirement). A specific example is QC of the expression of recombinant proteins. For this type of QC experiment, only small-scale cultures are required to express sufficient protein for interaction analysis. In such cases, the sensor surface can be appropriately modified, for example to capture histidine- or glutathionine-S-transferase (GST)-tagged proteins. Following capture on the sensor surface, known interaction partners can be used to study the functionality and folding state of the recombinant protein. The final confirmation of the expressed protein structure is then obtained by MS analysis after recovering the captured protein from the sensor chip.

Early SPR-MS applications

Early work on integrating SPR and MS has been focused on direct MS analysis of biomolecules that have been captured on the sensor surface using matrix-assisted laser desorption/ionisation (MALDI) MS. A greater flexibility in experimental design is obtained when the affinity-captured biomolecules are eluted from the sensor surface and subsequently processed for MS analysis. The elution approach allows the combination of interaction analysis with electrospray ionisation (ESI) MS and proteolytic digestion of proteins, a process which is often required for protein identification. Another advantage of the elution approach is that the sensor surface can be reused, enabling multiple capture/recovery cycles to increase the overall yield for MS analysis. This multi-cycle approach is especially relevant when the quantity of molecules captured on the sensor surface is below 100 femtomol.
which may often be the case when fishing for low-abundant proteins or when working with low-affinity interactions. It should be noted, however, that successful MS analyses have been performed with less than 50 femtomol bound to the sensor surface.23–25

SURFACE PLASMON RESONANCE
Since the introduction of protein analysis systems for the characterisation of reversible interactions of biomolecules, the technology has matured into tools that are routinely and widely used in many fields where molecular recognition events are of interest. The SPR phenomenon has been known for over 30 years and the theory is fairly well developed.26

SPR occurs in thin conducting films at an interface between media of different refractive index. In Biacore systems, the media are the glass of the sensor chip and the sample solution and the conducting film is a gold layer on the sensor chip surface. Under conditions of total internal reflection, the light leaks an evanescent wave field across the interface into the medium of lower refractive index. At a certain combination of angle of incidence and energy (wavelength), the incident light excites plasmons (electron charge density waves) in the gold film. As a result, a characteristic absorption of energy via the evanescent wave field occurs and SPR is seen as a dip in the intensity of the reflected light at a specific incident angle (the SPR angle). This angle varies as a function of the refractive index of the medium near the sensor surface, which changes when biomolecules attach to the surface. During interaction analysis, the changes in the SPR angle over time are displayed in a plot called a sensorgram. The unit for the SPR signal is the resonance unit (RU) and for ‘average’ proteins, one RU corresponds approximately to 1 picogram of material bound per square millimetre of surface area.27 The principles of SPR detection are illustrated in Figure 1.

SPR-MS IN LIGAND FISHING
The automated Biacore® 3000 system features fully dedicated software and hardware for the recovery of proteins after affinity purification on the sensor surface for subsequent MS analysis. The following description of the SPR-MS workflow and examples are based on experiments performed with this system, which has four flow cells over a single sensor surface. Each flow cell has a surface area of about 1 mm². In SPR-MS experiments, it is important to capture enough material for MS analysis, so that three or four flow cells are usually used.

The standard ligand fishing experiment is performed by immobilising a bait molecule on the sensor surface, followed by injection of a sample containing the potential binding partner (target). After sample injection, the flow cell is washed briefly with an MS-compatible buffer. This buffer is defined as one which neither interferes with MS analysis nor disrupts the interaction between the bait and target molecules. Prior to recovery of the analyte, the fluidic system is washed with two wash solutions followed by equilibration with the MS-compatible buffer. During this step, the flow cell area is closed while the MS-compatible buffer is in the flow cells. A recovery solution is then incubated in the flow cell, eluted from the system and either deposited on a MALDI target or into a vial. Throughout the course of such an experiment, SPR detection is used to monitor and quantify the immobilisation of the bait protein and the capture and recovery of the analyte. Furthermore, the recovered sample can be automatically prepared for MS analysis, including, for example, enzymatic digestion.

To demonstrate the feasibility of integrating SPR analysis with MS in a typical ligand fishing experiment, a model system was chosen, consisting of calmodulin and the calmodulin-binding domain (CBD) of myosin light chain kinase. Calmodulin is a ubiquitous regulatory protein involved in a number...
of signal transduction pathways. Myosin light chain kinase is one of calmodulin’s interaction partners, to which it binds in the presence of calcium ions. CBD, a 20-mer fragment of the kinase responsible for the interaction with calmodulin, was immobilised on the sensor surface by covalent coupling. The goal of the experiment was to capture calmodulin from a crude bovine brain extract on the chip, followed by the recovery and identification of the captured material. Figure 2 presents a typical sensorgram (plot of SPR response versus time) obtained after the injection of brain extract over a CBD-derivatised chip, wash of the fluidic system and recovery of the bound material.

The abrupt changes in response immediately before and after the injection are due to bulk refractive index changes during switches between the running buffer and a user-specified MS-compatible buffer in order to minimise the contamination of the recovered material with the components of the running buffer. In the example shown, 50 mM NH₄HCO₃, 2 mM CaCl₂ was used as the MS-compatible buffer. Before and after the recovery solution was incubated in the flow cells, air was passed over the flow cells for 30 seconds to prevent
contamination of the recovery solutions with other solutions used in the ligand fishing experiment. When air flows through the flow cells, the SPR signal is temporarily distorted, as seen in the sensorgram. Between brain extract injection and recovery of the chip-bound material, the fluidic system around the sensor chip was washed with 50 mM NaOH.

The amount of recovered material was about 1,300 RU per flow cell, corresponding to a total of approximately 5.2 ng or 300 femtomol for a 17 kDa protein such as calmodulin. The actual amount delivered to the MALDI target can be slightly lower, however, due to possible losses in the flow system on the way from the chip to the target.

ON-TARGET DIGESTION AND IDENTIFICATION OF THE PROTEIN INCLUDING POST-TRANSLATIONAL MODIFICATIONS

In this experiment, calmodulin was eluted from the sensor chip with 50 mM NH₄HCO₃ buffer supplemented with 2 mM EGTA (to chelate the Ca²⁺ ions necessary for calmodulin binding to CBD). The recovery procedure delivers the eluate in a small volume (2 µl); in this case, it was programmed to deposit this material onto a 0.8 mm spot of a Bruker AnchorChip target mounted on a Biacore MALDI holder. This was followed by the transfer of 1 µl of trypsin solution in 60 per cent acetonitrile to the same position. Preliminary experiments showed that it takes 15–17 minutes for a drop of such composition to dry at room temperature, so program execution after trypsin addition was halted for 20 minutes. After this time, the sample was acidified and mixed with the matrix by transferring 1 µl each of 0.5 per cent trifluoroacetic acid (TFA) and 0.5 µl of α-cyano-4-hydroxycinnamic acid (HCCA) dissolved in ethanol/acetone (2:1) to the spot. The whole process was automated using Biacore 3000 control software.

After drying, the sample was analysed by MALDI-time-of-flight-MS using an Autoflex mass spectrometer (Bruker Daltonics). Figure 3 shows the resulting MS spectrum and the results of a database query using the MSDB database via the
Mascot search engine (Matrix Science), leading to the identification of bovine calmodulin. The database query included an option of acetylated N-terminus (as is the case with calmodulin), which helped to identify two of the peaks (1,563.7 and 3,389.7 Da). Bovine calmodulin was identified as the top match, with a Mowse score of 137. An additional peak (2,401.1 Da) was identified by matching the mass spectrum with the in silico digest containing another known modification of calmodulin, trimethylation of Lys 115. The overall sequence coverage was 95 per cent.

Tandem MS/MS is a powerful tool which complements peptide mass data with amino acid sequence information. Besides making protein identification much more confident, this allows detection of post-translationally modified amino acids and identification of the type of modification. In a recent publication by Zhukov et al., it was demonstrated that the two post-translational modifications of calmodulin could also be identified by subjecting the peptides with molecular masses 2,401.1 and 3,389.7 to MS/MS analysis. Besides identifying the type and position of the post-translational modifications, the full sequence of these peptides could be derived.

One attraction of the combined SPR–MS approach is that it enables completely novel, high information content assays to be designed. Borch and Roepstorff recently described a novel strategy for identifying enzyme inhibitors that interact directly with their enzyme targets. In this approach, the SPR instrument was used for detection of enzyme–inhibitor interactions, an on–chip assay of enzyme activity (incubation with model substrates and MS analysis of the products) and isolation of the inhibitor candidate from the chip. The design of this assay is shown schematically in Figure 4.

Compounds that were found to inhibit enzyme activity could thus be recovered from the sensor surface and characterised by MS. The value of this novel approach was demonstrated using both trypsin and carboxypeptidase B (CPB) as test systems. For the CPB system, the second step in the enzyme inhibitor screening (step B in Figure 4) was combined with ligand fishing by injecting plant extracts instead of pure inhibitors. Inhibitors that were fished from plant extract were subsequently subjected to MS analysis.
(Step C in Figure 4) and compared with the mass spectra of pure inhibitors. In this step, it was found that the C-terminal glycines of CPB inhibitors were cleaved upon the interaction with CBD. This was a novel discovery, as C-terminal glycines are not known as substrates for CPB.

OUTLOOK

By combining SPR with MS, a new and versatile tool has been created for finding new interaction partners and performing detailed characterisation of their function and structure. The examples described in this review clearly demonstrate that SPR and MS are highly compatible and complementary when used in combination. Real-time monitoring of the affinity purification process on the sensor surface offers easy optimisation and evaluation of the interaction process. Moreover, once binding partners are found, interaction characteristics can be studied in great detail using SPR technology, which enables high-resolution affinity and kinetic analysis. The automation of the whole process, including the on-target processing of the recovered material for MS analysis, leads to minimised sample losses and more rapid throughput, without the need for extensive user intervention.

The combination of SPR with MS offers unique possibilities for characterisation of interactions, for example by employing on-chip reactions such a chemical labelling and enzymatic cleavage, to offer more insight into protein function. The combination of SPR with high-resolution MS
techniques, such as tandem MS–MS, will also enable the role of post-translational modifications in protein function to be studied in the context of proteomics approaches. As exemplified by the enzyme inhibitor study, the SPR–MS combination may also provide a useful tool for the screening and characterisation of potentially useful agonists and antagonist compounds aimed at novel proteins identified in proteomics programmes. The versatility and power of these combined technologies may yet provide a new twist to the ‘lab on a chip’ concept, offering an important additional weapon to the proteomics armoury.

As a result of the constant technical improvements to both SPR (higher sensitivity, throughput and control over lower volumes in the microfluidic systems) and MS (higher sensitivity and tandem MS capabilities), it can be anticipated that these two technologies will continue to adapt to each other, ultimately resulting in a fully optimised SPR–MS solution. As a result, it can be expected that SPR–MS will increasingly be used in the expanding field of proteomics for quantitative and qualitative functional analysis.

References


