Review

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Biosensor chip mass spectrometry: A chip-based proteomics approach

Rapid advances in genomic sequencing, bioinformatics, and analytical instrumentation have created the field of proteomics, which at present is based largely on two-dimensional electrophoresis (2-DE) separation of complex protein mixtures and identification of individual proteins using mass spectrometry. These analyses provide a wealth of data, which upon further evaluation leads to many questions regarding the structure and function of the proteins. The challenge of answering these questions create a need for high-specificity approaches that may be used in the analysis of biomolecular recognition events and interacting partners, and thereby places great demands on general protein characterization instrumentation and the types of analyses they need to perform. Over the past five years we have been actively involved in interfacing two general, instrumental techniques, surface plasmon resonance-biomolecular interaction analysis (SPR-BIA) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, into a single concerted approach for use in the functional and structural characterization of proteins. Reviewed here is the recent progress made using biomolecular interaction analysis - mass spectrometry (BIA-MS) in the detailed characterization of proteins and protein-protein interactions and the development of biosensor chip mass spectrometry (BCMS) as a new chip-based proteomics approach.

Keywords: Biosensor / Chip / Biomolecular interactions / Surface plasmon resonance / Matrix-assisted laser desorption/ionization time of flight / Review EL 3826

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Abbreviations: BCMS, biosensor chip mass spectrometry; **BIA-MS**, biomolecular interaction analysis-mass spectrometry; **EDC-NHS**, *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide; **FC**, flow cell; **HBS-EP**, HEPES-NaCl-EDTA-surfactant P20 buffer; **IL-1α**, interleukin-1α; **SPR-BIA**, surface plasmon resonance – biomolecular interaction analysis

1 Introduction

A number of landmark developments in the biological, computer, and analytical sciences have all but revolutionized conventional approaches to protein characterization and, in turn, collectively become the foundation for the relatively new field of proteomics. Large-scale sequencing efforts have resulted, or will soon result, in the complete genome sequencing of a number of select organisms. Once completed and cataloged into databases, these genomes represent libraries that can be translated into all of the potential proteins contained within their respective organisms. The constant improvements in computer technologies, bioinformatics, and access to data *via* the World Wide Web form a link between the virtual world of the genome libraries and the real world of the protein characterization laboratory. Empirical data generated in these laboratories, using, *e.g.*, 2-DE and mass spectrometry, can be utilized to correlate proteins under study with corresponding genes, thereby expediting protein/gene discovery and broadening the understanding of the underlying biomolecular pathways and interactions critical to defined cellular states. Of key importance within the protein characterization laboratory has been the rapid pro-

Proteomics and 2-DE

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gression of analytical instrumentation capable of providing this defining information with high sensitivity and accuracy.

Over the past decade, two analytical approaches have found ever-increasing use in protein characterization: surface plasmon resonance – biomolecular interaction analysis (SPR-BIA) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. SPR-BIA is an optical (nondestructive), chip-based technique able to monitor biomolecular interactions as they occur between an immobilized receptor and a solution-borne ligand. The technique has become a valuable tool for the functional characterization of proteins and is broadly used for determining the kinetic and affinity parameters involved in biomolecular interactions [1–4]. On the other hand, MALDI-TOF is used in the structural characterization of proteins, with analyses ranging from sequence verification through an accurate mass determination to protein identification *via* peptide mass mapping combined with database search [5–8]. When considering their compatibility (chip-based SPR detection format receptive to mass analysis) and complementarity (the analysis of protein function and structure) it can be surmised that a multidimensional analytical approach is created by bringing SPR-BIA and MALDI-TOF together to form a single concerted analysis. Accordingly, we have devoted much effort to combining SPR-BIA with MALDI-TOF to form an analysis termed biomolecular interaction analysis-mass spectrometry (BIA-MS) [9–14]. The centerpiece of the BIA-MS analysis is a small (1×1 cm) sensor chip, compatible with, and functional during, both SPR-BIA and MALDI-TOF analyses. The chips are generally gold-coated glass substrates, chemically modified to allow immobilization of a variety of molecules. During BIA-MS, the binding of a solution-borne analyte to a surface (on-chip) immobilized receptor is monitored in real time by SPR. As the SPR detection is nondestructive, the analyte selectively retained on the chip during SPR-BIA can be subsequently analyzed directly from the chip using MALDI-TOF-MS. MALDI-TOF confirms the identity of the affinity-retained analyte on the sensor chip (*via* its unique molecular mass) and readily detects multiple affinity-retrieved analytes and nonspecifically retained components (information not readily attainable from SPR-BIA alone).

In the initial development stages, BIA-MS demonstrated a highly sensitive concerted analysis, with detection limits at or below 20 fmole [9]. Dynamic range issues were also investigated and the ability to perform the concerted analysis over a mass range of up to ~ 150 kDa was shown, with multiple analytes retained from complex mixtures at the 10–100 fmole level [11]. Also demonstrated was the ability to maintain the spatial resolution of multiple interaction sites, *i.e.* flow cells (FC), 0.5×2 mm, present on a single chip throughout both SPR-BIA and MALDI-TOF, thereby enabling up to four separate interactions to be characterized on a single sensor chip [9]. Further studies showed BIA-MS to be a valuable tool in applications such as the detection of analytes affinity-retrieved from natural biological fluids ("ligand fishing") [9, 14], the analysis of sequential binding events [11, 12], the recognition of impurities retained during SPR-BIA [10], and the study of heterogeneous (competitive) binding systems [14]. However, BIA-MS can enter into new arenas of proteome investigations by incorporating additional molecular biology and/or protein chemistry techniques into the analysis. Reviewed here are the use of BIA-MS in two separate applications where (i) epitope-tagging techniques, and (ii) postcapture (on-chip) proteolysis are used in the detailed characterization and/or identification of trace-level protein retrieved directly from complex biological mixtures.

2 Epitope tagging with BIA-MS

We have recently shown that BIA-MS can be used with gene-tagging techniques for protein identification [13]. A general overview of the approach is shown in Fig. 1. Using various tagging approaches, tags are fused or inserted into nominally unknown genes for the purpose of tracking proteins throughout expression and for selectively isolating protein or its tag-containing proteolytic fragment(s) from the expression system. During BIA-MS, SPR-BIA is used (using a highly selective tag-specific receptor immobilized on the sensor chip surface) to affinity-isolate, detect, and precisely quantify the tagged polypeptide(s) retrieved (on-chip) from the expression system. Following SPR-BIA, the masses of the tagged polypeptides are accurately determined *via* MALDI-TOF analysis performed directly from the sensor chip surface. These masses are then used in protein structural characterization, such as sequence verification and identification through sequence database search.

In one example [13], a 91-base oligonucleotide coding for a polypeptide containing three repeats of the epitope HTTPHH was fused into the glutathione-*S*-transferase (GST) coding region present in a pGE-5X-3 cloning vector. The vector was transformed into *Escherichia coli*, and the cells were grown and induced for ~ 2 h before pelleting and lysis. The lysate was then treated with trypsin to produce epitope-tagged peptides, the lengths of which varied depending on the extent of cleavage. BIA-MS was performed on the trypsinized lysate during which the epitope-tagged peptides were selectively retrieved from the lysate using a surface-immobilized HTTPHH-specific monoclonal antibody (mAb7G8) and subsequently ana-

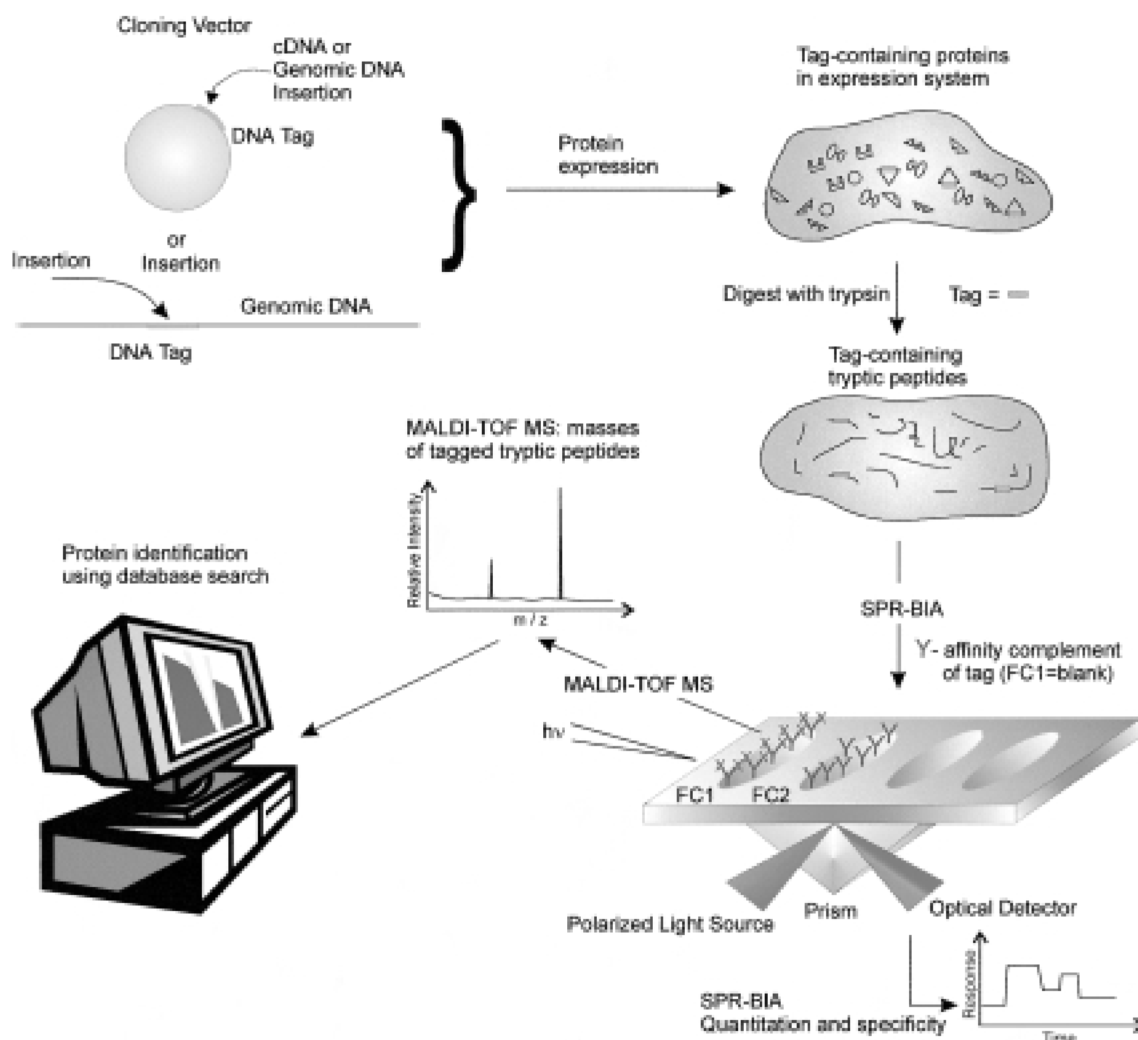

Figure 1. Overview of BIA-MS of epitope-tagged proteins. Tags are fused or inserted into genes that are expressed in a certain system (*e.g., E. coli*). Following expression, the whole system is digested with trypsin. The resulting tag-containing tryptic peptides are affinity-retrieved on a sensor chip through an interaction with a tag-specific receptor (*e.g.*, antibody). Detection and quantitation of the retrieved peptides is achieved utilizing SPR-BIA. The subsequent MALDI-TOF-MS analysis yields the masses of the tagged peptides, which are then used for protein characterization (*e.g.*, identification through protein sequence database search).

lyzed by MALDI-TOF. Control experiments were performed during both SPR-BIA and MALDI-TOF to accurately quantify the amount of peptide retained from the lysate and to unambiguously identify specifically retained peptides (for a complete experiment description see [13]).

Figure 2a shows the activation and derivatization of the two flow cells (FC1 and FC2) on a CM5 sensor chip with mAb7G8. A response change of ~ 8000 RU was observed by SPR-BIA, corresponding to an antibody density of ~ 8 ng/flow cell (for proteins, 1000 RU ~ 1 ng/mm^2, each flow cell's area is 1 mm^2) and indicating approximately 50 fmole of IgG ($M_{r\ IgG}$ ~ 150 000) present on the surface of each of the flow cells. After the serial derivatization, FC1 was addressed with trypsin in order to deactivate the antibody and create a control flow cell. Figure 2b shows sensorgrams resulting from the serial routing of trypsinized *E. coli* lysate across the surface of the flow cells. Three sensorgrams are shown, one for each of the two flow cells, and one for the real-time background subtraction of the signal from the control flow cell from that of the active flow cell (FC2–FC1). Changes in response of 30 and 10 RU are observed for the active (FC2) and control (FC1) flow cells, indicating the retention of 30 and 10 pg of material, respectively. The background-subtracted sensorgram shows a response change of 20 RU, indicating the retention of ~ 20 pg of material unique to FC2. Figure 2c shows MALDI-TOF spectra taken directly

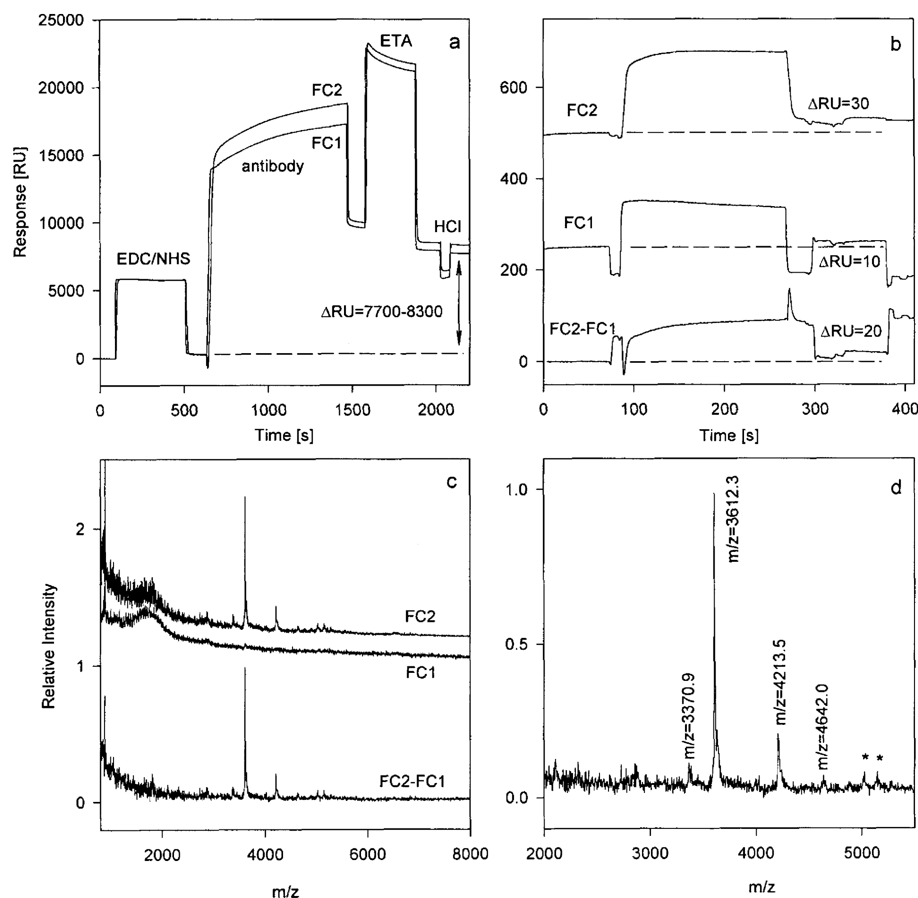

Figure 2. BIA-MS of epitope-tagged peptides. (a) SPR-BIA of mAb7G8 antibody immobilization. HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20) was utilized as a running buffer at a flow rate of 10 μL/min; ETA, ethanolamine. (b) SPR-BIA sensorgrams resulting from the binding of unfractionated, trypsinized *E. coli* lysate routed serially across the surface of the active (FC2) and blank (FC1) flow cell; also shown is a real-time background-subtracted sensorgram (FC2–FC1). (c) MALDI-TOF spectra obtained from the targeting of the active (FC2) and control (FC1) flow cells. Also shown is the background-subtracted spectrum (FC2–FC1). (d) Expanded view of the peptide region of the background-subtracted MALDI-TOF spectrum; *, unidentified signals.

from the surface of the flow cells (FC2 and FC1), and the spectrum resulting from a background subtraction routine (FC2-FC1). Minor signals at *m/z* of ~ 2800 Da and ~ 6500 Da are observed to cancel out as a result of the subtraction, suggesting that the compounds responsible for these signals were retained through interactions with something other than the immobilized antibody (most probably with the carboxymethyl chip surface). The multimeric signal in the *m/z* range of ~ 1800 Da, due to residual detergent (present in the incubation buffer), is also observed to cancel out during the subtraction routine. Figure 2d shows an expanded view of the peptide region of the background-subtracted spectrum. Ion signals are observed at *m/z* = 3370.9, 3612.3, 4213.5, and 4642 Da. The mole amount of the peptides present on the biosensor chip can be estimated at 800 amole to 5.5 fmole, dependent on the distribution of the 20 pg of material within the flow cell between the ion signals. The data shown in Fig. 2d were sufficient to successfully identify the tag-containing protein as GST utilizing either the masses of the proteolytic peptides (from which the mass of the tag was subtracted) or the mass differences between the proteolytic peptides to fuel a protein sequence database search (for more details see [13]).

The aforementioned experiments were performed to investigate the use of BIA-MS as a new functional approach in isolating, detecting, and identifying epitope-tagged proteins. The results demonstrate the effectiveness of BIA-MS in analyzing compounds selectively retrieved from complex biological mixtures and in differentiating between targeted and nontargeted compounds. Moreover, the extreme sensitivity of the BIA-MS approach is clearly demonstrated. Detection of tagged species at the low- to sub-fmole levels is viewed as highly significant, especially when considering that they originated from an *E. coli* system and were retrieved using only a single affinity extraction step. Such sensitive and rapid analysis performed on a complex biological mixture, when combined with accurate mass determination of proteolytic fragments, suggest a viable approach for protein identification through a protein database search. This novel functional proteomics approach stands to find use in characterizing cDNA or genomic DNA libraries on large scales. In such a scenario, SPR-BIA is used to screen for functional proteins by observing interactions with surface-immobilized targets. Structural characterization (identification) then proceeds on library constituents deemed significant using the approach described here. An alternative use of the

BIA-MS identification approach is that of screening for genetic polymorphisms at the protein level. With proper placement of the epitope within selectively (PCR) amplified genes, and the judicious use of protease treatment, a single tag may be used to retrieve gene product covering 10–100 amino acid residues (*i.e.*, 30–300 bases), thereby making possible a single analysis capable of simultaneously screening for potential point mutations (recognized as discrete mass shifts in the protein) stemming from multiple polymorphisms present within a gene. Clearly, the example given here represents a first step in the development of a number of new and novel gene/protein characterization approaches using BIA-MS.

3 Biosensor chip mass spectrometry (BCMS)

3.1 Ligand fishing

A use for BIA-MS that promises to be of great importance in the bioanalytical laboratory is that of "ligand (analyte) fishing". In general, the process utilizes a known biomolecule as a functional hook to fish unknown binding components from complex biological systems. The two component techniques of BIA-MS each serve different, yet complementary roles in ligand fishing. SPR-BIA is used to recognize the presence of binding ligands (analytes), in particular biological systems, to effectively retrieve the analyte from the solution (*e.g.*, a complex biological mixture) in a single step and to provide a quantitative estimate of the amount of material bound. MALDI-TOF, on the other hand, fulfills the role of providing initial defining structural information on the retained ligands – that being the molecular mass of the compounds. Past uses of BIA-MS in ligand fishing have shown promise in systems (antibody-antigen) where a single molecular mass determination is sufficient to unambiguously identify the retained proteins [9]. However, incorporating postcapture enzymatic digestion of the retained ligands expands the BIA-MS approach to a full-scale functional proteomics tool, allowing rigorous characterization of the retained proteins. Thus, we have incorporated on-chip proteolytic digestion into BIA-MS, creating an approach termed BCMS. In brief, the process (Fig. 3) begins with BIA-MS, *e.g.*, selective retrieval of an analyte (the ligand) using an immobilized receptor and washing away of nonspecific compounds; however, the analytical capabilities are significantly enhanced by using enzymes immobilized on flow cells downstream of the capture flow cell. After capture, the analyte is routed into the enzymatically active flow cell(s) where the digestion occurs. With proper experimental design, BCMS is able to yield accurate molecular masses of both the native protein and the proteolytic fragments and these data are used in the rigorous characterization and/or identification of the protein.

Although the approach shown in Fig. 3 may seem obvious, and therefore trivial, failure to recognize and optimize

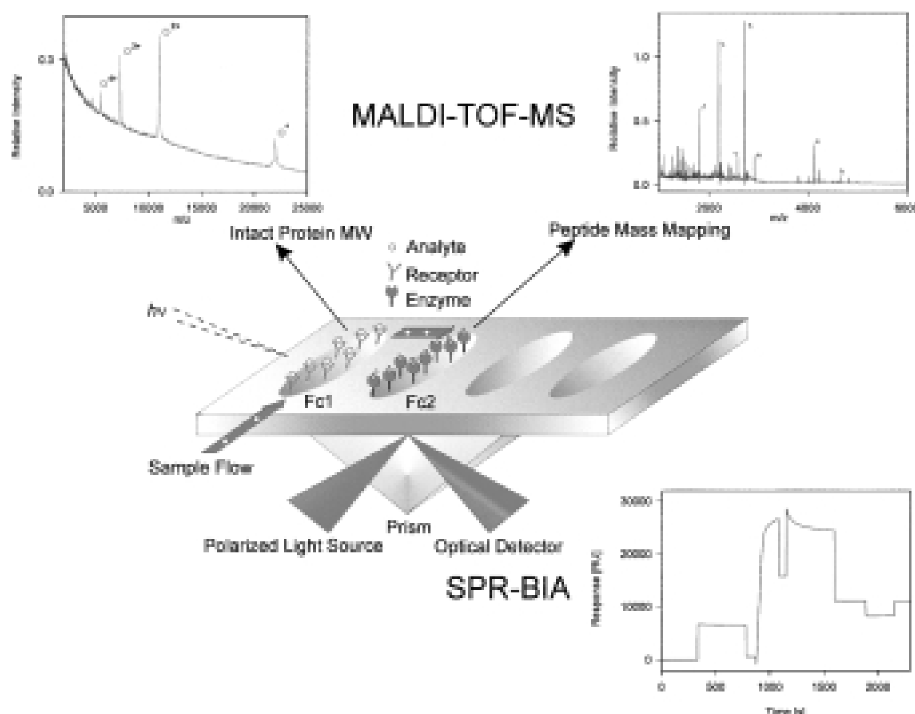

Figure 3. Overview of BIA-MS with on-chip incorporated proteolytic digestion (BCMS). A receptor is covalently immobilized on the surface of the first flow (FC1) cell of the sensor chip. A second flow cell (FC2) is derivatized with a proteolytic enzyme. The analyte (ligand)-containing solution is routed through FC1 where the component of interest is affinity-captured. Following washing of nonspecifically retained components, the ligand is eluted/routed from FC1 into FC2, where time for digestion is allowed. MALDI-TOF-MS analysis performed on the surface of FC2 yields accurate masses of the proteolytic peptide fragments that can be used for in-depth protein characterization. MALDI-TOF-MS performed on the surface of FC1 (lacking the subsequent proteolytic digestion) yields the mass of the intact protein.

a number of requirements will severely degrade performance of this chip-based technique. First of all, the flow cell on the chip surface is a two-dimensional element (with a ~ 1 mm^2 surface) to which only a limited amount of affinity receptor can be immobilized. The *N*-ethyl-*N′*-(dimethylaminopropyl)carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) immobilization protocol [1] used throughout the following experiments gives relatively high immobilization yields and functional (50–80%) efficiencies for antibodies, while introducing the least number of artifacts (*e.g.*, nonspecific binding) into the analysis. However, for general use, other surface chemistries must be explored when immobilizing a wide variety of biomolecular species (the immobilization of pepsin shown later is a clear example). Once surface immobilization conditions are optimized, capture efficiency is a product of, essentially, getting the analyte solution over the surface of the chip. To avoid deviations resulting from mass transfer, SPR-BIA kinetics experiments are typically performed at relatively high flow rates (> 20 µL/min), resulting in a decreased capture efficiency. Increased analyte capture efficiencies of 25–50% can be achieved using flow rates of 1–5 µL/min, and it is these flow rates that are preferred for BCMS. Once the analyte is captured, it is important to maintain control of the small amounts (and volumes) of analyte throughout subsequent transfers and operations. The advantage of the SPR-BIA instrument (Biacore, AB Uppsala, Sweden) is a low-volume microfluidics system capable of transferring sub-µL volumes of solution to different points on the chip with great precision, while viewing the transfers with SPR. A final requirement is that experimental artifacts are eliminated from the analysis. Specifically, artifacts arising from chemistries/enzymologies performed on the analyte can, if unchecked, dominate MALDI-TOF mass spectra, essentially suppressing signals from the analyte (especially when present in low amounts). To this end, surface-immobilized enzymes are used to provide high enzyme/substrate ratios and to eliminate autolysis fragments from mass spectra.

Given here is an example demonstrating the feasibility of this "lab-on-a-chip" approach to protein characterization using a human interleukin-1 alpha (IL-1α) antibody-antigen system. Because the overall intent of the analysis was to characterize ligands retained by an immobilized receptor, an initial accurate molecular mass determination of the native protein was viewed as essential. The molecular masses of intact proteins retained during BCMS can be determined with relative errors of ~ 0.1% by performing MALDI-TOF directly from the surface of the sensor chip. Even with this degree of error, the addition or subtraction of a single amino acid residue, or most post-translational modifications of the protein, can be readily recognized in proteins up to ~ 20 kDa in mass. Secondary

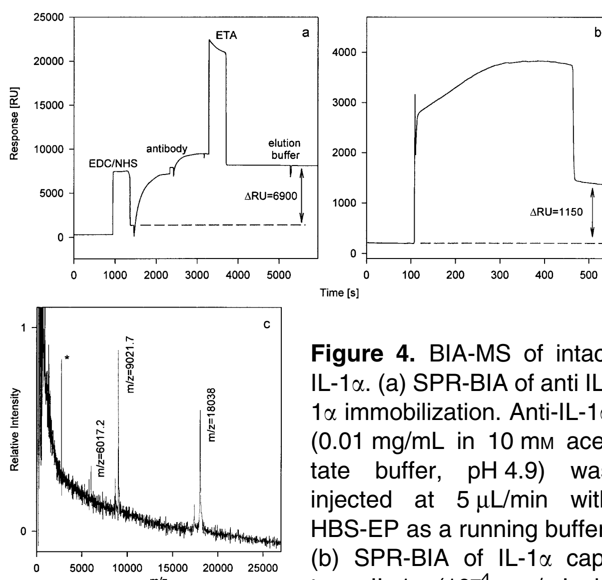

Figure 4. BIA-MS of intact IL-1α. (a) SPR-BIA of anti IL-1α immobilization. Anti-IL-1α (0.01 mg/mL in 10 mM acetate buffer, pH 4.9) was injected at 5 µL/min with HBS-EP as a running buffer. (b) SPR-BIA of IL-1α capture. IL-1α (10^{-4} mg/mL, in HBS-EP, containing 10 mg/mL HSA) was injected at 5 µL/min with 10 mM phosphate, pH 5.7, as a running buffer. (c) MALDI-TOF analysis of the sensor chip surface; *, unidentified signal.

information from MALDI-TOF that is valuable to the concerted analysis is the recognition of unexpected signals in the mass spectrum due to, *e.g.*, nonspecific binding. It is therefore wise to perform direct mass measurement of proteins retained during BCMS as an initial screen to search out variations in protein structure and nonspecific binding. Figure 4 illustrates the process as applied to the IL-1α system. Figure 4a shows the immobilization of anti-IL-1α antibody in FC1. The response change of 6900 RU indicates the immobilization of 6.9 ng of material, which corresponds to ~ 46 fmoles of antibody (M_r IgG ~ 150 000) immobilized (maximum valence ~ 92 fmole). Figure 4b shows the subsequent capture of IL-1α (10^{-4} mg/mL, in large excess (10 mg/mL) of human serum albumin, HSA). A response change of 1150 RU indicates the retention of ~ 1.15 ng of proteinaceous material (~ 60 fmole of IL-1α). Figure 4c shows a MALDI-TOF spectrum taken from the surface of this sensor chip. The observed *m/z* values of 18 038 Da, 9021.7 Da, and 6017.2 Da correspond to the singly, doubly, and triply charged ions of IL-1α and are in good agreement with the calculated mass of IL-1α ($M_{r\ \text{recombinant human IL-1}\alpha}$ = 18 047.5). A second, less intense, series of signals indicate the presence of a species at M_r 17 390, presumable due to a truncated version of the IL-1α (loss of six amino acids from the *N*-terminal). Neither signals for HSA nor the antibody were observed in spectra taken from the surface of the flow cell or regions on the chip bordering the flow cell.

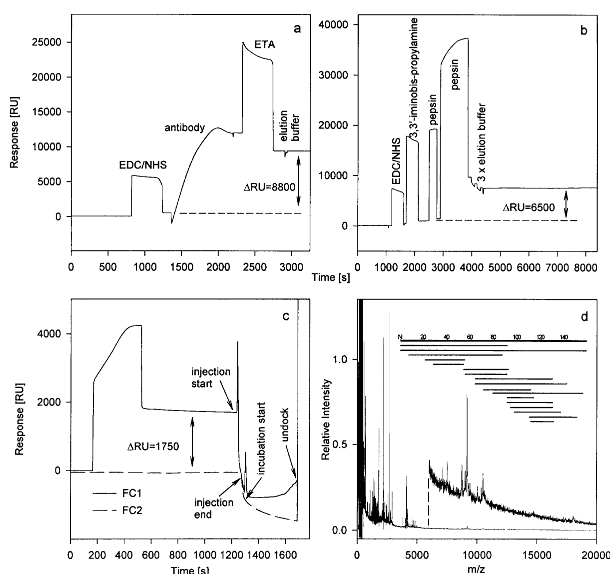

Figure 5. BCMS of IL-1α (a) SPR-BIA of anti IL-1α immobilization in FC1 (same as in Fig. 4). (b) Pepsin immobilization in FC2; 3,3′ iminobis-propylamine (10% v/v in water, pH ~ 12) served as an amino linker to which pepsin (0.5 mg/mL, with 0.2 mM EDC and 5.0 mg/mL with 0.2 mM EDC, both in 10 mM phosphate, pH 5.7) was consequently immobilized, utilizing 10 mM phosphate, pH 5.7, as a running buffer. (c) IL-1α capture in FC1, elution and digestion in the pepsin-active FC2. IL-1α (10^{-4} mg/mL, in HBS-EP, containing 10 mg/mL HSA) was injected at 5 μL/min with 10 mM phosphate, pH 5.7, as a running buffer. During capture, flow through the instrument was directed only through FC1. After the capture, the flow was directed through both flow cells and after a short rinsing period (at flow rate of 10 μL/min), the flow rate of the system was reduced to 1 μL/min. One μL of a pH 2.5 elution buffer was then injected over the flow cells and progress through the system was monitored in real-time using SPR. Once the sample-carrying elution buffer entered FC2, flow was stopped and approximately 6 min was given for digestion of the analyte. (d) The subsequent MALDI-TOF analysis yields masses of the IL-1α peptic fragments that are in good agreement with the IL-1α sequence (inset).

3.2 Ligand proteolysis

The next step in BCMS involves proteolysis of the ligand after capture. Because of the generally low amounts of ligand captured during SPR-BIA and the fact that elution from the chip to external receptacles for digestion/further analysis is certain to result in substantial losses to both the sample and digestion mixture, it is preferred to perform the digestion with minimal subsequent manipulations on the sensor chip itself. In past work, we have used enzymatically active mass spectrometer targets with great success in digesting proteins for MALDI-TOF characterization [15–17]. In BCMS, flow cells downstream of the capture flow cell may be enzymatically activated for the same purpose, as shown in Fig. 5. Figures 5a and b show the immobilization of anti-IL-1α antibody and pepsin in FC1 and FC2, respectively. The SPR responses observed in the two sensorgrams correspond to ~ 8.8 ng (~ 59 fmoles) of antibody immobilized in FC1 (~ 118 fmoles valence) and ~ 6.5 ng (~ 190 fmoles) of pepsin immobilized in FC2. Noted is the use of 3,3′-iminobis-propylamine in the surface activation procedure for the pepsin immobilization. Attempts to immobilize pepsin through the EDC/NHS protocol (using an amine-targeted linkage) failed to provide surfaces with enzymatic activity. The reason for this shortcoming is the low p*I* of the enzyme; the enzyme has few free amines for immobilization and is repelled by the negative charge of the carboxylate surface of the sensor chip. Switching to the amine surface resolves this problem and results in high densities of immobilized enzyme. Figure 5c shows the affinity capture of IL-1α in FC1, followed by elution, routine, and subsequent incubation in FC2. Details of the procedure are given in the figure caption. As there is a substantial dead volume preceding the first flow cell, and between the two flow cells, SPR detection is essential in tracking the location of the elution buffer throughout the microfluidics and on the surface of the chip. Furthermore, any changes occurring in the fluidics during the incubation period can be easily observed. The choice of elution buffer is also important because, in the two-flow cell arrangement shown in this example, it must be compatible with both elution and enzyme activity. For this reason, pepsin was chosen as digest enzyme rather than more specific enzymes that are active at pH ~ 7. The pH 2.5 citrate buffer is able to break the antibody/antigen interaction, and is optimal for pepsin digestion. It is also important to note that the entire process, from initial injection of analyte to introduction into the mass spectrometer, took ~ 30 min. Prolonging the capture/rinse/digest process ultimately results in less sample available for mass spectrometric analysis due to, *e.g.*, dissociation from the receptor (during extended rinsing – as observed in the SPR-BIA data) and loss of proteolytic fragments through absorption to the microfluidics or sensor chip upon complete drying of the digest mixture.

Using the SPR data quantitatively, the IL-1α was digested for 6 min using a 2:1 enzyme-to-substrate ratio (~ 190 fmole pepsin to 95 fmole IL-1α). Figure 5d shows a mass spectrum taken directly from the pepsin-activated flow cell present on the sensor chip. Multiple signals ranging up to the parent molecular mass of IL-1α are observed as a result of the digest. Figure 5d (inset) shows the alignment of fragments with the IL-1α sequence-cleaved *C*-terminal to F, L, E, and D. These sites were chosen based on

cleavage sites observed during our past work using mass spectrometer targets derivatized with pepsin in a manner identical to that used for FC2 [17]. Seventeen of the 25 observed signals (68%) are found to correlate (within 0.1% relative error) with the IL-1α and essentially verify the amino acid sequence of the protein with 100% coverage. Of the eight remaining signals, all but two could be correlated with pepsin cleavage if the cataloged, broad-range specificity of the pepsin was used (up to eight residues dependent on the reference); however, we feel more confident with using the cleavage sites observed during our aforementioned studies in which pepsin was prepared in the same manner as on the sensor chip.

This form of analysis, on-chip capture, processing, and digestion, followed by MALDI-TOF, leads to what we believe to be a concerted chip-based approach that can be used in functional analysis and identification of unknown proteins that bind specifically to immobilized receptors. Since the very process of fractionation used in BCMS is related to the function of an immobilized receptor (with respect to a solution-borne complement), immobilized (orphan) receptors can be used as "hooks" for "ligand fishing" functional complements from biological solutions under native (physiological) conditions. Once isolated, the analysis moves into identification of the retained ligands. MS provides a direct link between genome databases and protein identification *via in silico* digestion of theoretical proteins resulting from genetic code and matching of (real) MS data obtained on proteolytic fragments. Thus, it can be seen how BCMS can be employed to screen biological fluids in search of ligands exhibiting an affinity towards an immobilized receptor, and then identify the ligands using the proteome approaches to protein identification that have been established over the past five years. Such a functional proteomics process stands to complement function-related analyses such as yeast two-hybrid and phage-display systems.

3.3 Outlook

Certainly, much work must be performed in perfecting this chip-based approach for protein discovery. Primarily, the approach stands to be further optimized in terms of accuracy in the protein characterization. To circumvent the low specificity of the pepsin cleavage, high-specificity enzymes may be used. We have in other studies successfully immobilized high-specificity enzymes, *i.e.*, trypsin and Glu-C, on sensor chips. However, the low (or high) pHs generally needed to elute an analyte from a receptor are not optimal for their activity and, therefore, direct transfer from a capture to a digest flow cell using a single buffer is not ideal. We are currently undertaking efforts to alleviate this problem by using on-chip buffer exchange surfaces (recapture sites) so that a variety of proteases can be utilized in the analysis. Buffer exchange, as well as other operations such as reduction of disulfides, can be executed in flow cells downstream from the capture cell, and preceding the enzymatically active flow cell; this approach requires at least three flow cells on the sensor chip surface: capture, buffer exchange, and digestion. In the present work we were limited to two flow cells by the instrument configuration, but biosensors utilizing flour flow cells are commercially available, making the addition of buffer exchange surfaces possible. Protein identification also stands to benefit from the use of higher performance MALDI-TOF instrumentation. The instrumentation (continuous extraction MALDI-TOF) used during these studies limited the amount of mass spectrometric data that could be gathered to only mapping data of generally low mass accuracy. State-of-the-art instrumentation using delayed extraction technologies [18, 19] would significantly improve mass accuracy and, when coupled with in-source [20–22] and post-source [23–25] partial sequencing, would prove an ideal method for unequivocally identifying the captured proteins (even when low-specificity enzymes are used). Experiments are currently underway to evaluate the use of more involved experimental design and higher performance instrumentation in gaining improved data for protein identification.

4 Conclusions

The examples given here and in references elsewhere [9–14] clearly demonstrate a highly compatible and complementary match when SPR-BIA and MALDI-TOF are combined to form BIA-MS. The multiplexed detection and chip-based format are viewed as an ideal analytical platform for the investigation of both protein function and structure. Moreover, BIA-MS is exquisitely sensitive, generally able to detect and characterize proteins present in complex biological fluids at the low- to sub-fmole level. By incorporating tagging techniques and protein identification *via* proteolytic digestion/database searches into the analysis, BCMS moves one step closer to a fully integrated microarray system capable of total protein characterization. The constant technical improvement of both SPR-BIA (*e.g.*, higher sensitivity and lower volume microfluidics) and MALDI-TOF (*e.g.*, delayed ion extraction and tandem MS capabilities), combined with intelligent experimental design, will ultimately result in even higher performance BCMS. As a result, it can be expected that BCMS will find future use in the continually expanding field of proteomics.

This publication was supported in part by Grant No. 1 R43 CA82079-01 from the National Cancer Institute. Its

contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

Received November 25, 1999

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