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COMMENTARY

Electrospray Ionization and Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

EMERGING TECHNOLOGIES IN BIOMEDICAL SCIENCES

Ray Bakhtiar† and Randall W. Nelson†‡*

*Department of Chemistry and Chemical Biology, Stevens Institute of Technology, Hoboken, NJ 07030; and †Intrinsic Bioprobes, Inc., Tempe, AZ 85281, U.S.A.

ABSTRACT. Tremendous progress in biomedical sciences has been made possible in part by recent advances in bioanalytical methods, in particular biological mass spectrometry. Since the introduction of electrospray ionization mass spectrometry (ESI-MS) in 1984 and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) in 1988, the field of bioanalytical mass spectrometry has seen rapid growth. In concert with separation techniques such as capillary electrophoresis and high performance liquid chromatography, mass spectrometry allows characterization of a large array of small organic molecules, peptides, proteins, oligonucleotides, and RNA fragments. Thus, substantially more expedient and definitive determination of molecular weight is now possible by mass spectrometric analysis. In this commentary, general descriptions of ESI- and MALDI-MS are presented. Furthermore, several recent developments and applications in addressing difficult biological problems are discussed. BIOCHEM PHARMACOL **59**;8:891–905, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. proteomics; non-covalent complexes; sequencing; high throughput mass spectrometry; combinatorial chemistry; biomolecular interaction analysis

Some of the most difficult chemical, biological, and environmental problems require a combination of instrumental attributes such as speed, sensitivity, wide linear dynamic range, low detection limit, and capability of on-line analysis in conjunction with separation techniques. In recent years, new strategies in the ionization of macromolecules have presented significant improvements in biological MS§. Sensitive and rapid analytical MS techniques provide an incentive for the development of assays for use in situations where a small sample quantity does not permit detailed structural characterization by NMR spectroscopy and x-ray crystallography.

A valuable parameter for characterization of a biopolymer is the MW. MS has the potential to provide more accurate numbers than traditional analytical techniques, such as SDS–PAGE and gel shift assay. Traditional mass spectrometric methods, which proved useful for analyzing compounds of low MW, are of little use for biopolymers. The latter methods, e.g. electron impact ionization and chemical ionization, require relatively volatile samples. Indeed, biopolymers are usually polar and large; therefore, they cannot be vaporized without extensive, even catastrophic decomposition. The major breakthrough for analysis of biopolymers came with the introduction of ESI in 1984 and MALDI in 1988 [for an overview, see Ref. 1]. Both techniques are very sensitive and allow observation of intact biopolymers of 100,000 Da or higher. In this article, we would like to present a brief description of the ionization processes involved in ESI and MALDI as well as a number of examples that will demonstrate the capability of ESI- and MALDI-MS techniques.

Due to the rapid growth in modern MS technology, the newcomers to this field will have a quite different educational training (i.e. as clinical scientists, pharmacologists, biochemists, toxicologists, or synthetic chemists) than the veterans of this area. Therefore, this commentary is intended for non-specialists who submit samples on a regular basis to MS facilities for analysis, but have a limited knowledge of recent applications using ESI- and MALDI-MS methodologies. For more advanced utilities or detailed technical descriptions of the ionization processes and the equipment, we refer the reader to a number of excellent articles [2–36] and books [1, 37–41]. In addition, due to space limitations, we can present only what we perceive to be the most significant and recent applications.

ESI-MS

As the name might imply, ESI utilizes an electric field to yield a spray of fine droplets. In ESI, a dilute solution is sprayed from a fine needle, which carries a high potential

† Corresponding authors (and present address): [1] Ray Bakhtiar, Ph.D., DMPK Department, Novartis Pharma, Building 405, Room 229, 59 Route 10, East Hanover, NJ 07936. Tel. (973) 781-3562; FAX (973) 781-6076; E-mail: ray.bakhtiar@pharma.novartis.com or (2) Dr. Randall W. Nelson, Intrinsic Bioprobes, Inc., 2009 E. 5th Street, Ste. 11, Tempe, AZ 85281. Tel. (480) 804-1778; FAX (480) 804-0778; E-mail: ibi@inficad.com

§ *Abbreviations*: AUC, area under the curve; BIA, biomolecular interaction analysis; CE, capillary electrophoresis; ChTX, charybdotoxin; ESI, electrospray ionization; FC, flow cell; HTS, high throughput screening; IgG, immunoglobulin G; MALDI, matrix-assisted laser desorption ionization; 8-MOP, 8-methoxypsoralen; MS, mass spectrometry; RU, resonance unit; SPR, surface plasmon resonance; and TOF, time-of-flight.

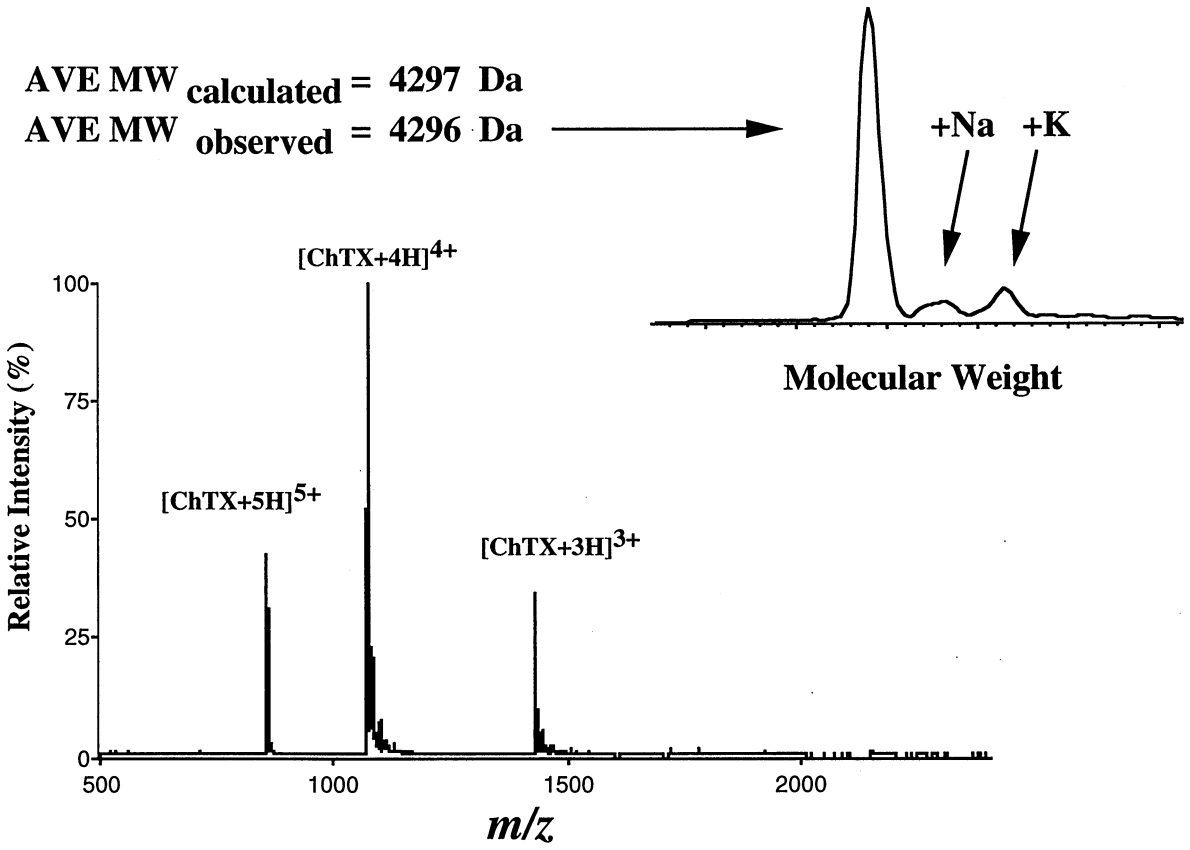

FIG. 1. Positive ion ESI mass spectrum of ChTX taken at pH 4 using a triple quadrupole MS. Ion peaks representing charge states 3+, 4+, and 5+ are dominant. The inset shows the deconvoluted spectrum, indicating the presence of sodium and potassium adducts. Presumably, solvents, pipets, transfer lines to the HPLC-MS, and reagents used during sample preparation and clean-up can contribute cations (i.e. Na^+, K^+) to the sample.

(about 4–5 kV). If the needle carries a positive potential, the droplets will have an excess of positive charges, usually protons. Evaporation of the volatile solvent (i.e. H_2O, CH_3OH, or CH_3CN) results in increased Coulombic repulsion between the positive charges, which causes fragmentation of the droplet, generating smaller droplets.

For example, Fig. 1 depicts the ESI mass spectrum of ChTX, a 37-residue globular polypeptide that is used as a template to deduce models for the external pore appearance of potassium channels. ChTX, a potassium channel blocker, has three disulfide bonds and is one of the toxins found in certain types of scorpions [42]. The ESI mass spectrum of ChTX displays signals corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$, and $[M + 5H]^{5+}$ (see Fig. 1). These three signals appear in different parts of the mass spectrum, because mass spectrometers measure the mass-to-charge ratio (m/z) of the ions. All three signals can be utilized to calculate the MW of this compound, which results in improved mass accuracy. Deconvolution of the spectrum using software available on all commercial ESI mass spectrometers yields the MW of the compound in question. In this case, the ChTX was determined to be 4296 Da, which is only 1 atomic mass unit (0.02%) lower than the calculated molecular mass (4297 Da) based on the amino acid composition.

There are two theories about ion formation in ESI. One theory suggests that ionized sample molecules are expelled from the droplets. Alternatively, it has been proposed that single ionized sample molecules remain after continuous solvent evaporation and droplet fragmentation. The ions generated by ESI carry multiple protons, provided the sample molecules have a MW of more than about 1000 atomic mass units. The characteristic feature of ESI that distinguishes it from other ionization techniques is that it generally imparts multiple charges to larger analyte molecules, and the extent of multiple charging increases nearly in proportion with MW. The resulting highly charged molecular ions are thus within the m/z range where conventional mass spectrometers function quite well. It is the multiple-charging phenomenon that allows assay of high-mass ions by mass analyzers with only a modest m/z range.

In ESI-MS, the sample may be ionized by protons yielding series of multiply-charged species. Thus, a series of $[M + nH]^{n+}$, $[M + (n + 1)H]^{(n+1)+}$, . . . (n = an integer corresponding to the charge state of the ion) signals might be observed. Assuming that a positive ion series represent different protonation states, then the m/z values of two successive peaks can be denoted as $(m/z)_1$ and $(m/z)_2$, and we can write

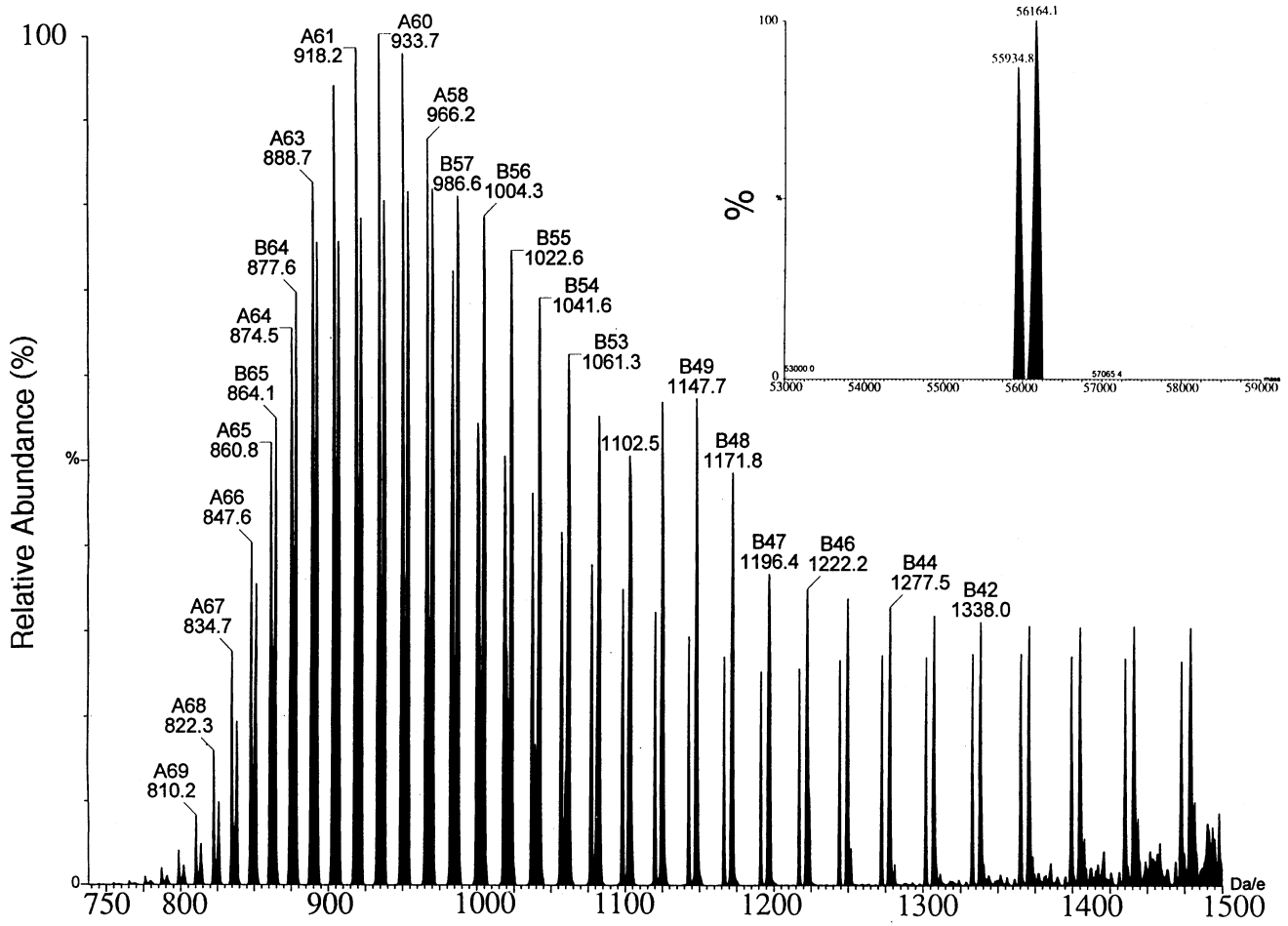

FIG. 2. Positive ion LC-ESI mass spectrum of cytochrome P450 2B1 incubated with NADPH (100 μM) and 8-MOP (100 μM). A series of multiply-charged molecular ions are observed, which are labeled with the protonation state (A*n* or B*n*) and the number of protons (*n*) attached to the protein molecule. The average MW of P450 2B1 prior (A*n* peak series; 55,934.8) and subsequent (B*n* peak series; 56,164.1) to incubation with 8-MOP yielded a difference of 237.9 ± 9.6, which is in reasonable agreement with the postulated covalent addition of an 8-MOP epoxide intermediate (232.2). The inset depicts the deconvoluted ESI spectrum. Reprinted with permission from *Biochemistry* 37: 13184–13193, 1998. Copyright (1998) American Chemical Society. [Ref. 43.]

$$(m/z)_1 = (M + n)/n$$

and

$$(m/z)_2 = (M + n + 1)/(n + 1)$$

The above equations can be solved simultaneously to obtain n and consequently M,

$$n = [(m/z)_2 - 1]/[(m/z)_1 - (m/z)_2]$$

The conversion of m/z to an actual mass of the species of interest is carried out by the mass spectrometer software. For example, suppose the mass (M) of an unknown protein is equal to 10,000 Da. If the ESI-MS of this protein yields a series of multiply-charged ions including one containing n = 20, then the signal would appear at m/z = (10,000 + 20)/20 = 501. An additional example is shown in Fig. 2, where LC-ESI-MS analysis of intact P450 2B1 that had been exposed to NADPH and 8-MOP yielded an envelope of multiply-charged ions with distributions of +38 to +72 [43]. Cytochrome P450 enzymes (~ 55 kDa) are a group of monooxygenase enzymes that oxygenate a wide range of substrates. Cytochrome P450 enzymes are of physiological significance, because they represent several key transformations in metabolism. In some cases, incubation of a specific compound with the P450 enzyme(s) in the presence or absence of the selective inhibitor can demonstrate the role of a particular P450 in a metabolic pathway [44, 45]. These heme-containing proteins are difficult to characterize since they are membrane-bound and thus relatively insoluble in an aqueous environment. In an elegant study, Koenigs and Trager [43] examined the mechanism-based inactivation of P450 2B1, a rat hepatic P450 isozyme, by several furanocoumarins (e.g. 8-MOP). Additional studies utilizing ESI-MS, which involved an adduct of a reactive electrophile of tienilic acid with P450 2C9 apoprotein, a human P450 isozyme, were also reported [46]. A comparison of ESI mass

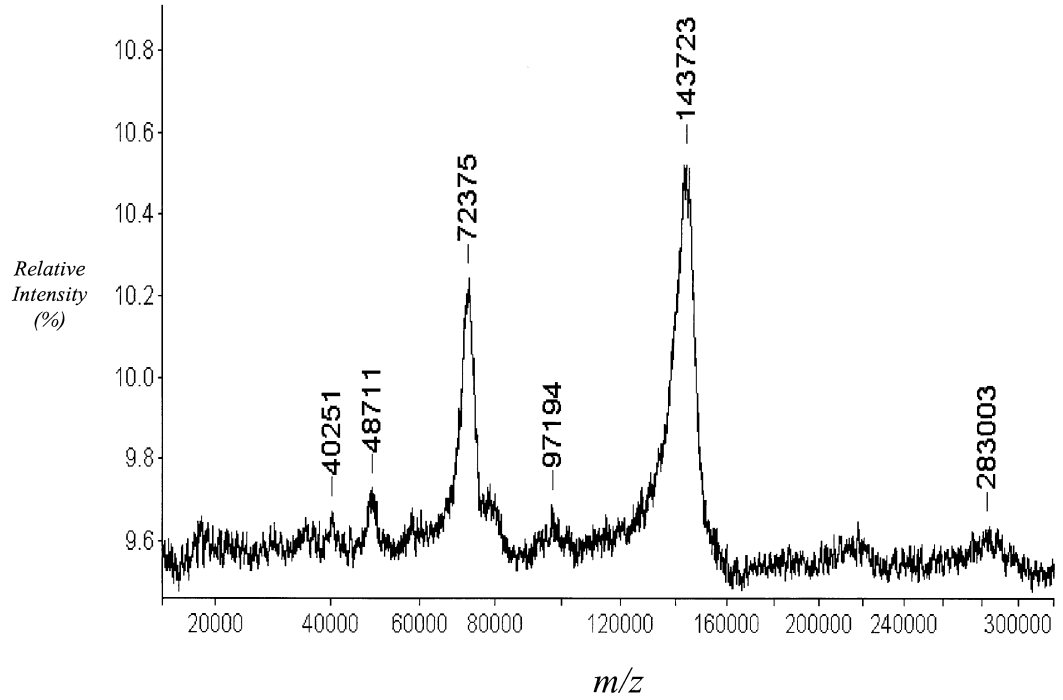

FIG. 3. MALDI-TOF mass spectrum of native myeloperoxidase (15 μM) in 5 mM phosphate buffer (pH 7.4). The signals at 143.7 and 72.3 kDa correspond to the singly ($[M + H]^+$) and doubly charged ($[M + 2H]^{2+}$) species, respectively. The spectrum confirms the high purity of the native enzyme subsequent to preparation. Reprinted with permission from *Biochemistry* 37: 17923–17930, 1998. Copyright (1998) American Chemical Society. [Ref. 56.]

spectra (Fig. 2) of P450 2B1 prior and subsequent to incubation with 8-MOP clearly indicates a mass difference in accord with the covalent addition of a furanoepoxide intermediate [43]. Similar studies that successfully utilized MS in the analysis of P450 adducts also have been reported by other laboratories [47–49].

Because of the "soft" nature of the ionization process, ESI-MS has provided valuable information on structurally specific biomolecular interactions, including DNA–drug, enzyme–inhibitor, DNA–protein, and protein–protein interactions. In addition, compounds including transition metal complexes, synthetic polymers, and molecular clusters have been examined using ESI-MS, which in the majority of cases yields *intact* molecular ions with little or no fragmentation. The attractive features of ESI have made it a topic of intense research activity, which has resulted in rapid acceptance and use in the pharmaceutical industry, biotechnology, and basic research.

MALDI–MS

MALDI-TOF has evolved over the past decade as one of the foremost mass spectrometric techniques for the analysis of biomolecules [50–55]. The approach is based on the ability to generate intact vapor-phase ions of large, thermally-labile biomolecules by desorption/ionization from a crystal comprised of small volatile (matrix) molecules. Pulsed laser radiation tuned to an absorption maximum of the matrix is used to initiate the desorption/ionization event and to simultaneously generate a packet of ions of different m/z values. These ions are accelerated to the same electrostatic potential and allowed to drift an equal distance before striking a detector. The mass of the ions is determined by equating the flight times of the ions to m/z. The technique possesses the virtues of high speed and sensitivity, detection of all ions in a single desorption/ionization event (no scanning), and a mass range of 1–1,000,000 Da. For example, Fig. 3 shows an MALDI-TOF mass spectrum of native myeloperoxidase in 5 mM phosphate buffer (pH 7.4) [56]. The signals at 143.7 and 72.3 kDa correspond to the singly and doubly charged species, respectively. Since the production of primarily singly charged ions ($z = 1$) is predominant, MALDI clearly demands high m/z mass analyzers such as TOF-MS. Because of the large kinetic energy differences of the ions generated at the surface of the sample target, a mass spectrum of poor resolution may result. However, there have been several technical advancements that can markedly enhance the resolution, and they are beyond the scope of this article [1, 21, 24]. Since for very high-mass measurements, sensitivity is frequently critical, the resolution-enhancing alternatives are not utilized, and lower resolution is accepted. Of equal importance is the ability to simultaneously analyze the masses of multiple components present in mixtures (often in the presence of buffer compounds), thereby allowing the detection of "known" analytes as well as "unknown" components present in the analytical fluid [1, 21, 24, 28, 50–55].

Even though the MALDI process is largely immune to the presence of buffer compounds and salts and is able to analyze samples containing multiple biomolecular species, limitations soon are reached when MALDI-TOF is used for the analysis of a trace component present in a complex biological mixture. The reasons behind the failure to observe signal from a trace-level compound present in a mixture can be many, and include such factors as, for example, a general preference in the MALDI process for compounds present in the mixture at high concentrations, variations in the chemical composition of biomolecules (having an effect on the ability to desorb and ionize the species), or simply the fact that the trace level compound is present in the mixture at a concentration below the detection limits normally encountered during MALDI-TOF analysis (generally subnanomolar). When such limitations are encountered, fractionation techniques are needed to selectively retrieve and concentrate the trace level compound from the biological mixture prior to MALDI-TOF analysis.

HIGH THROUGHPUT MASS SPECTROMETRY

The drug discovery process has been accelerated greatly by the advances in combinatorial chemistry for selection of new lead drug candidates. A central theme underlying this new technology is the ability to synthesize a repertoire of compounds with randomized structural variation. While the promise and opportunities are significant, combinatorial approaches pose several challenging tasks that must be met to realize the full potential of this technology. One of the challenges has been to develop fast and reliable HTS analytical methods to support the high throughput activities of medicinal and combinatorial chemistry. Such methods in analytical chemistry have the potential to initiate a paradigm transition in drug discovery from rather laborious and time-consuming steps to accelerated identification of novel drug candidates.

Among the many intriguing avenues, indeed, MS has become an indispensable analytical tool for the identification and quantitation of combinatorial libraries [57–71]. For example, automated multi-stage fast HPLC-MS and tandem MS have been utilized in rapid drug metabolite profiling [58, 66, 69]. A similar approach could be used in conjunction with affinity chromatography techniques to identify and rank high-affinity ligands in a single MS run [59–61, 67, 71]. In addition, MS has played a central role in so-called "cassette," "N-in-one," or "cocktail" dosing pharmacokinetic studies, in the early stages of drug discovery [63, 64, 69]. In these studies, structurally analogous classes of drug candidates are administered simultaneously to a laboratory animal, and, consequently, the estimated AUC values are calculated and compounds are prioritized. Automated sample preparation methods such as 96-well solid phase extraction modules, which are operated by robots, have been coupled successfully to HPLC-MS for HTS of drugs and their metabolites in biological fluids [65]. The "N-in-one" dosing approach requires smaller numbers of animals and is more efficient than the traditional single-dosing/single-assay studies. However, HPLC-MS method development and data analysis are increased, and possible drug–drug interactions could result in false positives and incorrect pharmacokinetic profiles. For example, inhibition of metabolism can occur when drugs compete for the same metabolic pathways, and thus elevated AUC levels may be observed. Thus, alternative approaches have been taken, which include single-dosing/multiple-analysis and "sample-pooling" analysis using HPLC-MS-based methodologies [63, 69, 72].

A complementary approach to genomic research, which has recently attracted growing attention by pharmaceutical companies, is proteomics [4, 18, 23]. The term "proteomics" originates from proteome, which refers to the systematic identification of the total protein complement of the genome. In proteome analysis, the protein profile of an organism or tissue is studied, and pertinent information (i.e. post-translational modifications, protein–protein interactions, and identification of individual protein markers in biological fluids upon multiple dosing of an experimental drug in a laboratory animal model) can be discerned for the identification of targets for potential drugs or diagnostic reagents. MS has earned a critical role as a fundamental tool for HTS of proteins and peptide mass fingerprinting for protein identification [4, 8, 23]. As a result, MS data have been used in conjunction with database searching algorithms [8] to yield critical information on the identity of 2-D gel spots in an automated fashion [18].

NON-COVALENT AND COVALENT INTERACTIONS

Some of the most difficult chemical and biological problems require a combination of instrumental attributes such as speed, sensitivity, wide linear dynamic range, low detection limit, and capability of on-line analysis in conjunction with separation techniques. Conventional analytical techniques such as radiochemical and immunochemical techniques commonly have been utilized for the detection of chemicals covalently bound to macromolecules. However, in the radiochemical method, differentiation between a chemical bound to the macromolecule versus its metabolite(s) is difficult. In the immunoassay-based methods, lack of epitope selectivity is considered a disadvantage. Alternatively, in recent years, new strategies in the ionization of macromolecules have presented significant improvements in biological MS of covalent and non-covalent complexes.

A growing body of literature has been devoted to the application of MS for detection of covalent modification of proteins by small molecules [73–93]. This is of particular interest due to its biochemical and physiological importance in understanding the alteration of cellular biochemistry, tumorigenesis, cell death, post-translational modifications, and mechanism-based enzyme inhibition. Sensitive and rapid analytical techniques such as HPLC-, CE-, or

MALDI-MS provide an incentive for the development of analytical assays for use in situations where a small sample quantity does not permit detailed structural characterization by NMR spectroscopy and x-ray crystallography. Since the mass spectrum of modified biopolymers affords little or no fragmentation, the technique can be useful in obtaining accurate measurement of the concentration of the specimen using an internal standard. Furthermore, the on-line mapping of protein adducts is an excellent tool to characterize the exact site of covalent adduction. Mass changes due to post-translational modifications (e.g. acetylation, farnesylation, phosphorylation, methylation, or glycosylation) [74, 81–83, 89, 91] of peptides and proteins are easily detected. Determination of the protein fragment of increased mass after digestion allows elucidation of the site of the post-translational modification, which plays a critical role in functional activities and signal transduction in all living organisms. For example, several reports have demonstrated the successful application of MS in the analysis of key hepatic enzymes and their covalent adducts with xenobiotics. These include metallothionein–chloroambucil [80, 87], human aldehyde dehydrogenase–disulfiram [85], and glutathione *S*-transferase–haloenol lactone complexes [86].

Since its introduction, ESI-MS (and to some extent MALDI-MS) has served as a powerful tool in providing evidence in support of the existence of non-covalently associated macromolecular complexes in the gas phase. Due to their "gentle" nature, ESI- and MALDI-MS have been utilized in the investigation of non-covalent complexes such as guest–host, antibody–antigen, protein aggregation, enzyme–substrate, and small molecule–DNA interactions in the gas phase [3, 11, 94–98]. The observation of both specific and nonspecific associations requires that the complex tolerate the interface conditions (i.e. heat, collisional activation due to electrical voltage) during the desolvation/desorption processes [94]. Detection of non-covalent complexes by MALDI is more challenging because of the acidic environment during the desorption process. Using alternative approaches such as switching to less acidic conditions, it may be possible to observe non-covalent complexes by MALDI [99]. However, it is important to point out that in an ideal case, MS data on non-covalent interactions should be confirmed by independent studies in the solution phase. For example, circular dichroism spectroscopy and ESI-MS yielded similar results on direct determination of solution binding constants for non-covalent complexes between bacterial cell wall peptide analogues and vancomycin group antibiotics [97]. ESI-MS was utilized at pH 8 to detect and characterize the multiplicity of insulin in stable non-covalent complexes with Zn(II) ions [96]. Other Zn–biopolymer complexes were detected successfully using ESI-MS [95] and MALDI-MS [98]. Clearly, beyond the applications to primary structure analysis, higher-order structures and specific non-covalent interaction studies remain an emerging and exciting area of MS.

BIA

Affinity isolation is undoubtedly the most specific of separation techniques, and when coupled with MALDI-TOF [50–55], it offers an extremely powerful method for the selective isolation and concentration of a desired ligand. In general practice, a receptor is immobilized onto a solid support (e.g. agarose, magnetic beads, pipettor tips, or mass spectrometer target) and used to selectively retrieve a complementary ligand from solution for structural characterization by MS. A number of examples have demonstrated the use of affinity isolation prior to MALDI-TOF for the detection [100–106] and quantitation [107–111] of trace levels of polypeptides present in natural biological carriers. Given that the analytes retrieved during the process are detected directly at unique m/z values, assays can be designed to evaluate expression media for the production of the correct construct [106], to screen biological systems for metabolites of a target species [110], and to assay for multiple species in a single analysis (using multiple immobilized receptors) [108, 109]. Other analyses involving affinity isolation prior to MALDI-TOF are geared towards characterization of the receptor rather than the ligand. Epitope mapping using MS is a clear example of receptor characterization. Using this method, monoclonal antibodies (generally immobilized) are probed with peptides that have been produced either synthetically or through proteolytic digestion of a larger antigenic polypeptide. The antibody will retain only the peptide fragments that contain the epitope, which, once eluted from the antibody, can be identified using MS. A number of examples demonstrating the use of MS in epitope mapping can be found in the literature [112–117], each describing slightly different methodologies to accomplish the mapping.

Although the aforementioned analyses using affinity isolation in combination with MS are, in themselves, quite impressive, the performance of affinity isolation/MALDI-TOF methods can be increased significantly when the affinity capture step is used analytically, in itself part of a separate analysis. Biosensor technology based on the non-destructive detection principle of SPR has found much use over the past 10 years, independent of MS, in the evaluation of affinity interactions [118–121]. Briefly, a sensor chip surface, composed of an affinity receptor-derivatized gold layer on a glass substrate, is monitored using SPR, while the chip surface is exposed to a complementary affinity ligand. Differences in surface concentration due to the receptor–ligand interaction result in a refractive index change across a dielectric junction formed at the sensor surface/substrate interface, which in turn varies the resonance angle at which light is absorbed into the junction. With proper calibration, the change in the SPR resonance angle can be equated accurately with the mass of material retained on the biosensor surface. In the most popular form of SPR-based biosensor, termed BIA, an arbitrary term of resonance units, RU, is used for quantitation, with 1000 RU corresponding to a surface concentration of 1 ng/mm^2. The data resulting

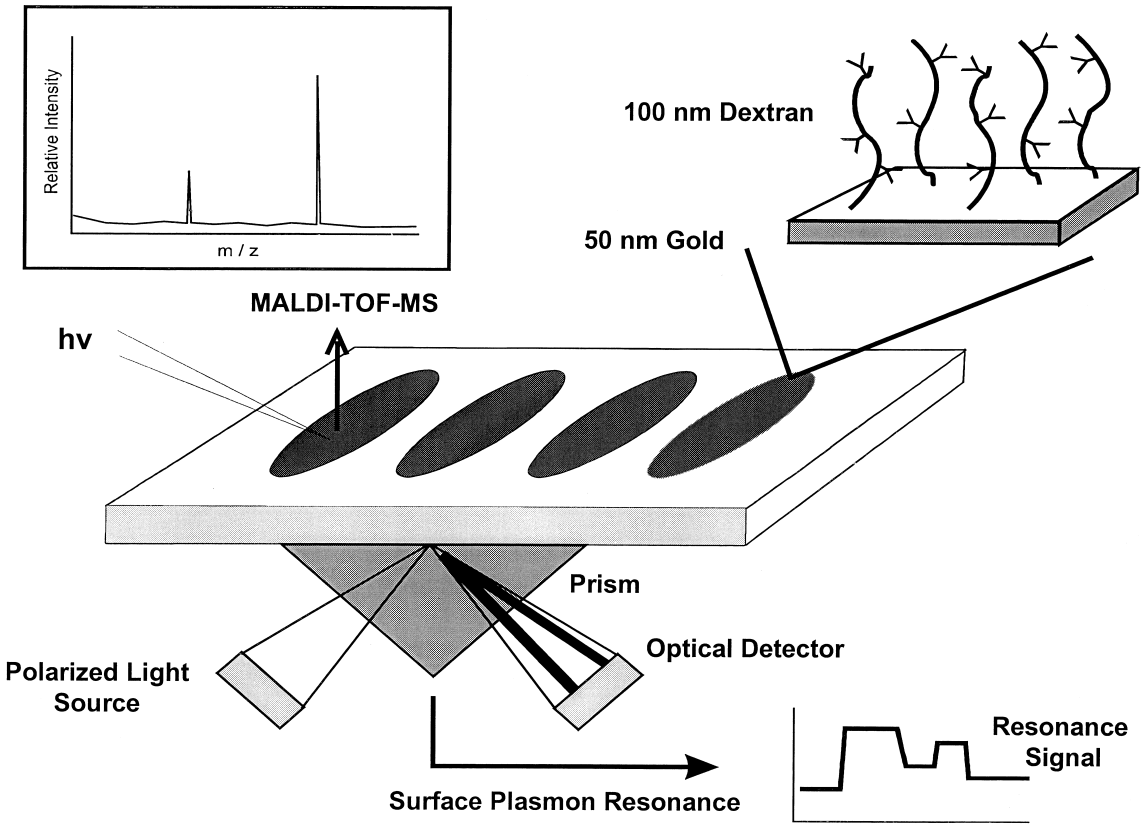

FIG. 4. Illustration of the combined SPR-based BIA/MS approach. Derivatized biosensor chips, having multiple (2–4) flow cells each, are used in the real-time SPR-BIA analysis of interactions between surface-bound receptors and solution-phase ligands. The sensor chips are removed from the biosensor after SPR-BIA, with ligands still retained within the flow cells, and prepared for MALDI-TOF by application of an appropriate matrix to the flow cells. The matrix solution disrupts the receptor–ligand interaction, liberating the ligand into solution for incorporation into the matrix crystals. With proper application of the matrix, the crystals settle onto the original location of the interaction, and spatial resolution between flow cells is preserved. The flow cells are targeted individually during MALDI-TOF, and the retained ligand(s) is detected at precise and characteristic *m/z* values.

from the SPR monitoring of the interaction are reported as a sensorgram showing the mass of ligand bound to the chip surface as a function of time. Sensorgram data, as a function of analyte concentration, then can be used to determine kinetic parameters and dissociation constants of the interaction.

After SPR-BIA interrogation of the interaction, ligand retained by the receptor is left on the surface of the sensor chip and is able to be analyzed with MALDI-TOF. The general concept of such BIA/MS is illustrated in Fig. 4. A microfluidics system is used to imprint multiple (2–4) interaction regions (FCs, flow cells) on the surface of an SPR-active sensor chip. Solutions are routed, using the same microfluidics, over the flow cells to chemically activate the sensor chip surface for subsequent immobilization of the receptor. SPR sensing is used throughout the process, and a final reading of the derivatization procedure yields the amount of receptor immobilized within the flow cell. The sensor chip then is used in the real-time SPR-BIA analysis of interactions between surface-bound receptor and solution-borne ligands. Shifts in the SPR response result from biospecific capture of ligands and are a direct measure of the amount of analyte retained on the surface of the sensor chip. Once SPR-BIA analyses are complete, the sensor chip is removed from the biosensor with the retained compounds still present within the flow cells and is prepared for MALDI-TOF by application of an appropriate matrix solution to the flow cells. The matrix solution is of a nature capable of disrupting the affinity interaction (e.g. acidified or containing a denaturant), and essentially liberates the ligand into solution for incorporation into the matrix crystals. With careful application of the matrix using, for example, a microdrop delivery system, ligands within a flow cell are liberated into solution, incorporated into the matrix crystals (upon drying of the solvent), and redeposited on the area of the same flow cell, thus preserving spatial resolution between the flow cells. MALDI-TOF then follows, with flow cells targeted individually and retained ligands detected as a function of *m/z*.

Given that component techniques of BIA/MS operate on mutually exclusive detection principles and are performed for different analytical purposes, the combination of SPR-BIA with MALDI-TOF forms an extremely powerful approach to the analysis of biomolecular recognition events. In its simplest form, SPR-BIA is used to determine

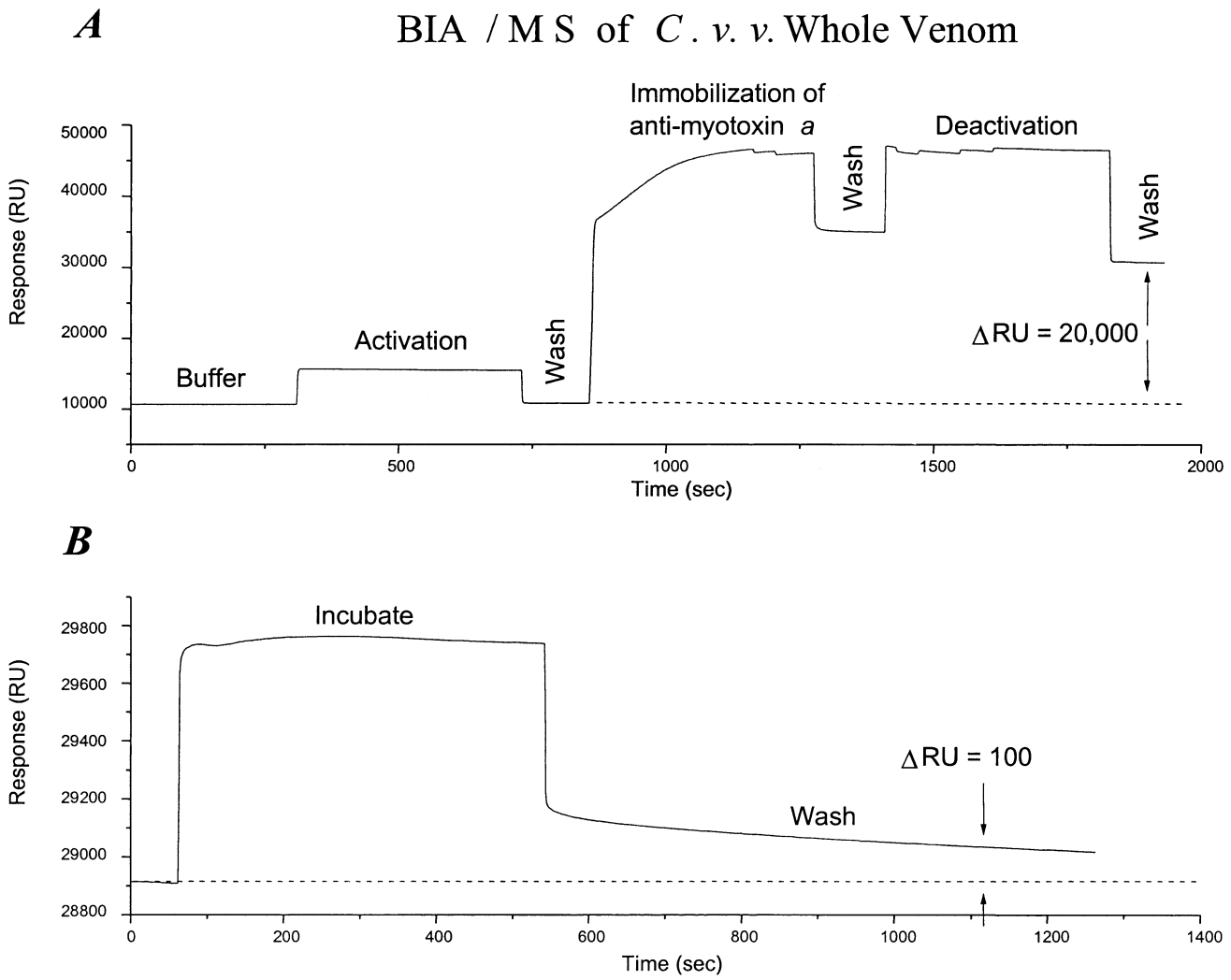

FIG. 5. (A) Sensorgram of the anti-myotoxin *a* IgG immobilization process applied to flow cell (FC) 1 of a Biacore CM5 chip. The response change of ΔRU ≈ 20,000 RU corresponds to ~ 150 fmol/mm^2 of anti-myotoxin *a* IgG covalently immobilized on the surface of the sensor. All four flow cells on the chip were derivatized using the same procedure and exhibited virtually identical sensorgrams. (B) Sensorgram showing ligand fishing of myotoxin *a* from the whole venom of the prairie rattlesnake (C. *v. viridis*). The sensorgram was obtained from FC 1 and shows a response change of ΔRU ≈ 100 RU indicating ~ 20 fmol of myotoxin *a* retained within the flow cell. All four flow cells showed the retention of approximately the same amount of toxin.

the association and dissociation kinetics of the interaction and ultimately derive a dissociation constant for the event. After SPR-BIA analysis, MALDI-TOF is used to confirm that the SPR-BIA data are valid, i.e. due only to known components of the interaction. In situations where more than just the targeted ligand is bound and detected on the biosensor chip, MALDI-TOF is used to determine the nature of the unknown components. Specifically, MALDI-TOF is used to identify the unknown components as being retained either through nonspecific interaction with the sensor chip, or through specific interaction with the immobilized receptor. In this latter analysis, MALDI-TOF is capable of immediately recognizing variants of the target ligand, mass-shifted due to point mutations or chemical modification, that would be in competition with the wild-type ligand in occupying the receptor. Such information is critical when considering that dissociation constants span many orders of magnitude in strength, and that variants present in solution at even trace levels will contribute to a binding curve given a modification that sufficiently increases their affinity towards a receptor. MALDI-TOF data taken directly from the biosensor chip are capable of supplying the data necessary (i.e. the number of components and their MWs) to begin an accurate fit of the binding curves derived during the SPR-BIA kinetic analysis. The analysis can be enhanced even further when semi-quantitative MALDI-TOF is used to determine the relative abundance of each component bound during SPR-BIA. The semi-quantitative MALDI-TOF data represent the relative quantity of each of the bound ligands present on the biosensor surface. This ratio can be used, with knowledge of the MWs of the ligands, to determine the relative mass amount of each of the bound ligands retained on the sensor chip. The combined mass amount, as deter-

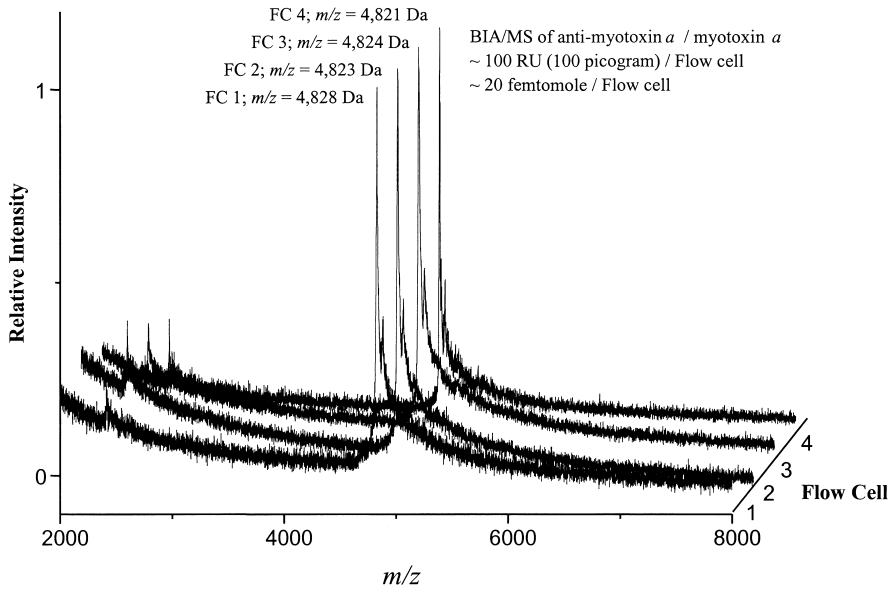

FIG. 6. MALDI-TOF spectra of myotoxin *a* isolated during "ligand fishing" of C. *v. viridis* whole venom using the anti-myotoxin *a* IgG-derivatized biosensor chip. Signal is observed in all four flow cells at *m/z* = 4823 ± 5 Da, consistent with the selective retention of myotoxin *a* from the venom.

mined using SPR-BIA, then can be dissected accurately into the contributions of the individual ligands. This information can be used to produce separate binding curves for the individual ligands, the sum of which equals the composite binding curve.

A different use for BIA/MS that promises to be more widely used in the bioanalytical laboratory is that of "ligand fishing." In general, the process is that of using a known biomolecule as a hook to fish unknown binding components from biological systems. The approach has been around for years; however, methods other than SPR-BIA or MALDI-TOF have been used for the detection and characterization of the unknown binding compounds. The two component techniques of BIA/MS each serve different, yet complementary roles in ligand fishing. SPR-BIA is used to quantitatively detect the presence of binding ligands in particular biological systems, while MALDI-TOF is used to provide structurally characterizing data on the retained compounds. We have already begun investigating the use of BIA/MS in such ligand fishing using model systems. Figures 5 and 6 show an example in which the biological system of interest was that of the whole venom from a prairie rattlesnake (*Crotalus viridis viridis*), known to contain myotoxin *a* as a minor component (approximately 5%, w/w). Briefly, a sensor chip was prepared by derivatizing four flow cells with anti-myotoxin *a* IgG. The derivatization process

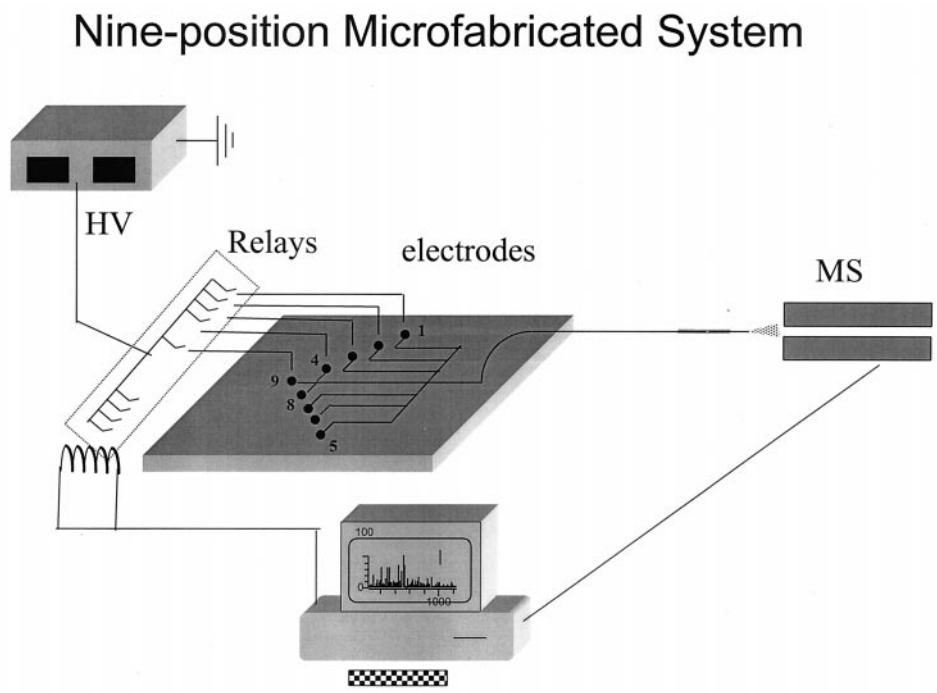

FIG. 7. Schematic diagram of a nine-position microfabricated device coupled to an ESI-MS using a micro-ESI ion source. This microfluid device was manufactured by utilizing photolithography/etching technology. The diameter of each reservoir is about 1 mm, and the sample flow can be controlled by a personal computer. The etched channels are 30 μm deep and 72–73 μm wide. The system has the potential for use in sequential automated analysis of proteins and their digests (i.e. obtained from 2-D gel electrophoresis) with low femtomole/microliter sensitivity. Reprinted with permission from *Anal Chem* 70: 3728–3734, 1998. Copyright (1998) American Chemical Society. [Ref. 132.]

for all four flow cells yielded sensorgrams virtually identical to that shown in Fig. 5A (FC 1; $\Delta RU = 20{,}000 \pm 700 \approx$ 20 ng of IgG/flow cell). A solution of whole venom from the prairie rattlesnake was circulated across the flow cells for ~ 8 min, after which the flow cells were rinsed for ~ 12 min (to remove any non-specified compounds). Figure 5B shows the sensorgram resulting from exposing FC 1 to the rattlesnake venom. A change in response of $\Delta RU \approx 100$ indicates ≈ 100 pg of material bound to the area of the flow cell. All four flow cells were exposed to the same incubation conditions and exhibited virtually the same sensorgram. Figure 6 shows four MALDI-TOF mass spectra resulting from targeting the individual flow cells of the sensor chip (FC 1–4). The major ion species present in each spectrum is observed at $m/z = 4823 \pm 5$ Da, consistent with the retention of myotoxin *a* (MW = 4821.8). Given both the SPR-BIA and MALDI-TOF data, it is estimated that ~ 20 fmol of myotoxin *a* was bound to each flow cell during the ligand fishing process.

Thus, there appear to be several prescribed reasons for combining SPR-BIA with MALDI-TOF. In recognition of the complementary nature of the two techniques, we have investigated the interfacing of SPR-BIA with MALDI-TOF [122–127]. To preserve system integrity and achieve the greatest analytical sensitivities, we have taken the approach depicted in Fig. 4, an approach in which ligands retained during SPR-BIA are analyzed directly from the sensor chip using MALDI-TOF. The approach has been shown to be a valuable tool in the detection of targeted analytes retrieved from natural biological fluids [122, 127], the evaluation of sequential binding events [123, 124], the determination of compounds nonspecifically retained during BIA analysis [126, 127], and the high-sensitivity analysis of affinity-tagged compounds present in complex biological mixtures [128]. These previous studies lay the foundation for what promises to be a high sensitivity, multiplexed protein analytical workstation. A small amount of a protein is selectively retrieved from complex biological mixtures and accurately detected/quantitated using SPR. The captured protein then is eluted from the receptor and routed through the microfluidics to a different flow cell on the sensor chip that has been derivatized with a protease [129]. Time is given for digestion, after which the resulting proteolytic fragments are analyzed using MALDI-TOF. Data resulting from the analysis of the proteolytic digest, with or without partial sequence information obtained from in-source or post-source decay methods, is then used to fuel a genomic or protein sequence database search capable of identifying the retained protein. We are now in the process of perfecting methods and devices capable of performing such identifying analyses using BIA/MS.*

* Nedelkov D, Tubbs KA and Nelson RW, Manuscript submitted for publication.

CONCLUDING REMARKS AND FUTURE PROSPECTS

MALDI- and ESI-MS are extremely useful tools for analysis of small molecules, peptides, proteins, glycoproteins, oligosaccharides, and oligonucleotides. Usually less than 1 pmol is sufficient for a high quality mass spectrum, which provides the MW of the sample as well as an indication of its purity. Since MALDI-TOF and quadrupole ESI mass spectrometers are easy to operate, they are rapidly becoming standard laboratory equipment in the biotechnology industry and academic laboratories. Currently, several vendors are marketing benchtop MS units ranging from $100,000 to $180,000. The future prospects of MS are exciting, with advancements in miniaturization (i.e. chip technology [124, 130] and microfabrication devices using photolithography/etching technology), database searching algorithms, rapid DNA sequencing, HTS, and automation/robotics technologies [131]. For example, Fig. 7 depicts a nine-position microfabricated device manufactured using photolithography/etching technology by Figeys and co-workers [132–134]. This system was coupled to a mass spectrometer and applied to automated sequential identification of proteins separated by high-resolution 2-D gel electrophoresis [132]. Detection limits in the low femtomoles per microliter range were reported. The technique has the potential to be utilized in determination of the precise sites of post-translational modifications in conjunction with tandem MS and sequence database searching software [135].

In this article, we did not discuss a number of other related topics such as the use of HPLC-ESI-MS and MS/MS in conjunction with on-line NMR spectroscopy [136, 137], quadrupole TOF-MS technology [138, 139], ultra-high resolution MS and its utility in protein folding studies [140], and several other exciting and promising developments in related areas [141–158]. The number of reports on the above subjects is growing in an exponential fashion, and key references have been provided for interested readers throughout the commentary.

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