Chip–based nanoelectrospray mass spectrometry for protein characterization

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In the last several years, significant progress has been made in the development of microfluidic-based analytical technologies for proteomic and drug discovery applications. Chip-based nanoelectrospray coupled to a mass spectrometer detector is one of the recently developed analytical microscale technologies. This technology offers unique advantages for automated nanoelectrospray including reduced sample consumption, improved detection sensitivity and enhanced data quality for proteomic studies. This review presents an overview and introduction of recent developments in chip devices coupled to electrospray mass spectrometers including the development of the automated nanoelectrospray ionization chip device for protein characterization. Applications using automated chip-based nanoelectrospray ionization technology in proteomic and bioanalytical studies are also extensively reviewed in the fields of high-throughput protein identification, protein post-translational modification studies, top-down proteomics, biomarker screening by pattern recognition, noncovalent protein–ligand binding for drug discovery and lipid analysis. Additionally, future trends in chip-based nanoelectrospray technology are discussed.

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With the completion of the Human Genome Project and the sequencing of numerous other organism genomes, characterization of the proteome has become the focus of many scientific laboratories. This focus evolved from the study of one gene, protein or pathway in an organism, towards the systematic identification and quantitation of all expressed cellular components, to comprehensively characterize all proteins present in a cell under specific conditions [1]. This is particularly important due to the evidence found for the poor abundance correlation between expressed proteins and those simply encoded at the messenger RNA (mRNA) level [2,3]. Focus has also been placed on studying dynamic protein regulation, including modification state, and protein–protein interactions in a given biologic compartment at a given time [4–6]. These new approaches to large-scale biology have triggered many analytical challenges and produced an increasing demand for rapid, low-cost, automated instrumentation, with high detection sensitivity that has the capability of analyzing trace amounts of samples. To face these challenges, developments in miniaturized analytical systems such as microfabricated chip devices have therefore become of increasing importance.

Compared with conventional instrumentation, the potential benefits of analytical microfluidic devices include reduced sample/reagent consumption, fast analysis speed and readiness for automation and system integration [7–11]. Several on-chip detection methods are being developed for microfluidic devices such as ultraviolet (UV) absorption, fluorescence and electrochemistry [12]. However, the analysis of proteomic samples present at low abundance often requires the use of mass spectrometry (MS) for the desired sensitivity and selectivity [12]. With a proper interface or built-in device, analytes from a microchip can be delivered to the external mass spectrometric detector. As a result, MS has gained increased popularity in chip-based analyses [13,14].

MS plays an essential role in proteomic analysis and research. Current technologies used for proteomic studies are based on a variety of separation techniques followed by identification

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of the separated proteins and proteolytic peptides using MS [1,15]. In recent years, it has been increasingly recognized that the key to proteomics relies not only on the instrument itself, but also on front-end analytical strategies and sample-handling techniques [16,17]. Electrospray facilitates the atmospheric pressure ionization of a liquid sample by creating highly charged droplets through which the processes of evaporation and droplet fission create gas-phase ions representative of the analytes in solution. Nanoelectrospray ionization (nano-ESI)/MS, introduced by Wilm and Mann [18,19], has become a widely used technique for many proteomics studies, due to its low flow rates with low sample consumption and improved detection limits. Unlike conventional ESI, in which pneumatically assisted nebulization is required, nano-ESI is typically an electrostatic means for aerosol generation and droplet dispersion [18,20,21]. As a result, nano-ESI significantly improves analyte desolvation, ionization and transfer efficiencies into the mass spectrometer [18]. Recent experiments indicate that the low flow rates of nano-ESI effect the fundamental ionization by reduction of matrix suppression observed in both infusion [22,23] and liquid chromatography (LC) [24] sample introductory methods. Ultra-low flow rates of 1–2 nl/min have been shown to improve overall transfer efficiency and yield higher sensitivity [25]. The low flow rates and enhanced sensitivity offered by nano-ESI/MS make it an ideal choice for chip–MS coupling. In addition, nano-ESI favors the generation of multiply protonated peptides and proteins, which in turn promotes more facile amide bond fragmentation when the ions are activated for dissociation. Furthermore, in applications such as drug screening, the soft nano-ESI process allows for a straightforward study of noncovalent binding interactions. However, disadvantages of nano-ESI include:

- Low sample throughput due to the tedious task of individually aligning each nano-ESI tip for optimal spray characteristics
- Potential sample-to-sample carryover
- Tip blockage if the nano-ESI tip is used to analyze multiple samples
- Poor reproducibility of the relative intensities of analytes between tips due to the variable shape of the spray tip and the variable capillary-sampling cone orifice distance for each repeat analysis

In this article, an overview of the recent developments in chip-based nano-ESI/MS technologies is provided. An emphasis is placed on the development of nano-ESI chip technologies and their extensive applications for protein characterization and proteomics-based drug discovery. Results generated from the past several years in the authors' and several other laboratories are discussed.

Current state of microchip device technologies for nano-ESI/MS

During the past 5 years, many different approaches were taken in the design and development of a nano-ESI interface for coupling microchip devices with MS, and interfacing on-chip sample preparation with MS analysis. Limbach and coworkers were the first to classify the chip-based nano-ESI/MS techniques as either off- or on-chip ion production, referring to how samples are introduced to the mass spectrometer [13]. Many research groups have taken the off-chip ion production approach, where the samples are transferred indirectly from the chip device into the ion source of the mass spectrometer. This approach was carried out by gluing or bonding a fused-silica capillary or a nanoelectrospray needle into a microchannel on a glass or polymer microchip [28–31]. A modified technique based on the above approaches attached a separate spraying capillary or a needle with a liquid junction interface at the chip outlet [32–36]. An alternative method using external micro-ion sprayers has been described by Henion [37–39]. The published data from the above approaches clearly demonstrates the potential utility of coupling microchip devices with MS. For example, Liu and coworkers demonstrated direct infusion of peptide samples at a rate of 5 s/sample using an array of 96 fused-silica capillaries [40]. Li and coworkers employed on-chip capillary electrophoresis (CE)/ESI/MS with a nano-ESI emitter for sequential injection, preconcentration and separation of tryptic digests. Throughput of up to 12 samples/h and a detection limit of 25 fmol was achieved [41]. Quantitative on-chip CE/MS analysis of carnitines in human plasma, and a chip-based P450 reactor for kinetic analysis of the P450 biotransformation of imipramine into desipramine, has also been successfully accomplished by Henion and coworkers [35,39]. However, an unfavorable characteristic of these devices is the increased dead volume at the chip device/transfer capillary interface. The dead volume can lead to loss of resolution in the upstream separation step and the transfer line itself can be a potential source of chemical noise due to background contamination.

The alternative approach to integrating chip devices with MS is to use on-chip ion production. During this process, ion production is directly integrated onto the microchip by fabrication of a nano-ESI emitter as an internal chip part. This approach is expected to reduce the dead volume and improve performance. The first report of a direct electrospray from the planar open end of a microchannel at the edge of a glass chip was by Karger [42], and a similar interface was also used by Ramsey [43]. In both cases, the devices provided a flat hydrophilic glass surface for the formation of an electrospray. However, these devices required an impractically high voltage to overcome the fluid surface tension to initiate and maintain stable spray. As a result, widespread use of this approach has not occurred.

In recent years, various constructions have been demonstrated for on-chip ion production from an integrated microchip. On-chip ESI devices have been fabricated from monolithic silicon substrate [44] and silicon dioxide [27]. Sharp integrated spray emitter devices have also been fabricated from a variety of polymers including parylene [45], poly(ethylene terephthalate) [46], polycarbonate [47], poly(methyl methacrylate) [48–50] and poly(dimethylsiloxane) [51–54]. Beyond fabrication, the investigators also demonstrated the performance of their devices in a variety of application fields such as drug quantitation, peptide sequencing and protein analysis.

Despite the considerable progress made for various chip devices, very few laboratories have developed a true miniaturized proteomic platform referred to as proteomics-on-a-chip [55], which is virtually a microfluidic device with integrated sample preparation, preconcentration and even multidimensional separation on-chip for direct MS analysis. Thibault and coworkers recently reported an integrated glass microsystem for analysis of in-gel digest samples [41]. The system included a large injection port (2.4 µl), a CE separation channel and a low dead volume-glued nano-ESI emitter for interfacing with ESI/MS. Sample preconcentration was achieved by packing C18 beads or immobilized metal affinity chromatography (IMAC) beads into the injection port for the enrichment of target peptides prior to CE separation and MS detection. The device was used for rapid identification of 72 proteins from human prostate cancer cells at a rate of 12 samples/h [41]. IMAC affinity capture and separation in the device of phosphopeptides from gel samples was also demonstrated [41]. This device is the first fully integrated chip-based proteomic platform for MS analysis, although the detection sensitivity of the device still needs to be improved for low-abundant proteins.

The ESI Chip™ device is a silicon-based on-chip nanoelectrospray microchip device developed by Advion BioSciences, Inc. using deep reactive ion etching (DRIE) technologies [44,201]. DRIE is used to form high-aspect ratio structures from the surface of a substrate with a high degree of anisotropy. DRIE methods allow multiple devices to be formed simultaneously, creating low-cost, highly reproducible devices. The ESI Chip is one of the more advanced designs consisting of 100 out-of-plane tips (nozzles) with an inner and outer diameter of 10 and 20 µm, respectively, for each nozzle (FIGURE 1). There is a 10-µm (internal diameter [i.d.]) channel through the wafer connecting each spray nozzle on one planar surface with the inlet on the opposite planar surface. This through-chip channel has a dead volume of only 25 pl [44,56]. The 10 × 10 array of nozzles on the ESI Chip offers the capability of 100 samples to be sequentially analyzed making high-throughput, automated nano-ESI/MS analysis possible (FIGURE 1). Another unique feature of the chip is the incorporation of the ESI ground potential or counter electrode into the spray nozzle. This is very different from conventional electrospray devices, which define the electric field by the potential difference between the spray device (fluid potential) and the mass spectrometer inlet or atmospheric pressure ionization (API) interface. In the ESI Chip, the electric field around the nozzle tip is formed from the potential difference between the microfabricated silicon substrate as an integrated counter electrode and the voltage applied to the fluid via the conductive pipette tip [201]. As the distance between the electrodes is only a few microns and constant, an extremely strong and stable electric field is reproducibly generated, essentially decoupling the ESI process from the inlet of the mass spectrometer [201].

The ESI Chip is supported and positioned in front of the ion-sampling orifice of the mass spectrometer through a robotic platform referred to as the NanoMate® (Advion BioSciences). The platform serves as a robotic liquid handing device that sequentially delivers samples from a 96-well plate through a conductive pipette tip to a nano-ESI nozzle in the ESI chip-based array and then initiates the nano-ESI spray process (FIGURE 1). There is a dedicated pipette tip and nozzle for each sample to ensure that the system provides a fully automated nano-ESI/MS infusion analysis without any carryover or cross contamination between samples [57,58].

Selected proteomic applications of the ESI Chip system

Currently, there are many proteomic applications of chip-based MS. In addition, the use of microchip devices with MS detection for pharmaceutical and environmental analyses is also increasing [9,13]. During the last 10 years, genomics benefited from the trend towards miniaturization. Proteomics now appears poised to benefit in a similar manner from the developments in miniaturization technologies. Ideally, all steps upstream from sample preparation and separation would be integrated onto a single microfabricated device for direct MS analysis, which will provide the potential for dramatic

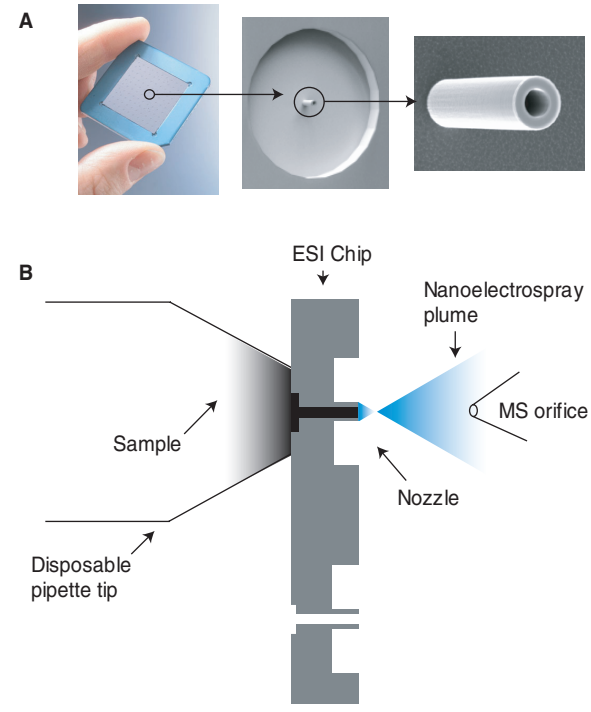

Figure 1. The ESI Chip system. (A) Sequential photographs of the ESI Chip demonstrating the array of microfabricated nanoelectrospray nozzles with successive enlargements of an individual nozzle. This final scanning electron micrograph shows a single nanoelectrospray emitter with dimensions of 10 µm internal diameter and 20 µm outer diameter. **(B)** Schematic representation for a cross-section of a portion of an ESI Chip. A disposable pipette tip, containing sample, presses and seals against the inlet side of the chip with the nanoelectrospray plume spraying towards the mass spectrometer ion orifice entrance. Nanoelectrospray is initiated by applying a head pressure and voltage to the sample in the pipette tip. Reproduced with permission from [57].
ESI: Electrospray ionization; MS: Mass spectrometry.

advancements in the field of proteomics. This application section highlights six selected proteomics application areas using nano-ESI chip-based MS.

Protein identification

Detection and identification of proteins from a biologic sample is the most basic task in proteomics. To tackle the current challenges in this field, sensitivity and high throughput is becoming increasingly important due to the dynamic nature of protein expression, protein–protein interactions and protein modifications, which require analysis as a function of time and cell state [17,59]. Current technologies for protein identification are based on a variety of separation techniques followed by identification of the separated proteins and proteolytic peptides using MS analysis [1,15]. 2D gel electrophoresis and 2D-LC, as well as the combination of the above approaches, are widely employed for upstream separation prior to MS analysis.

Initial experiments using an automated nano-ESI device coupled with ion trap MS for protein solution digests and bovine serum albumin (BSA) gel spots demonstrated the detection sensitivity, feasibility and reproducibility of the automated nano-ESI device. The detection sensitivity of 500 amol/µl of myoglobin digest and 150 fmol of BSA loaded on-gel were reported from infusion analysis [57]. Further results obtained for 2D gel spots of *Escherichia coli* and yeast crude cell extracts by the fully automated device indicate that the automated nano-ESI system is a valuable tool for high-throughput protein identification with 96 samples being fully analyzed in approximately a 6-h period [57]. An important advantage of nano-ESI tandem MS (MS/MS; MS^n) infusion is that it permits low-volume sample consumption and a flexible MS acquisition time. Throughout the infusion analysis, the mass spectrometer is operated in the data-dependant acquisition (DDA) mode with dynamic exclusion enabled, allowing extended time to acquire more scans in both MS and MS/MS modes. Thus, unlike online LC/MS/MS with narrow peak elution times for detection, this infusion approach provides sufficient acquisition time for selecting the low-abundance ions for MS/MS in DDA experiments and therefore improves detection sensitivity as well as protein sequence coverage. The extended, flexible acquisition time in DDA experiments offered by the infusion approach can partially compensate for its loss of concentration effect compared with the online LC/MS method. Additionally, as this approach is infusion-based nano-ESI MS, the front-end sample handling and preparation are particularly important compared with online LC/MS analysis. Recently, the detection sensitivity for BSA gel spots has been improved to 75 fmol loaded on-gel by modifying the procedures for in-gel digestion, extraction, and robotic ZipTipC18 cleanup. Two to three BSA tryptic peptides were consistently detected across five gel spots, each from 75 fmol loaded on-gel, yielding an average sequence coverage of 6.5% [UNPUBLISHED OBSERVATIONS]. Using the modified experimental protocol, 40 2D gel spots of *E. coli* crude extract that were located in two regions of the gel with relatively faint spots were selected for evaluation of the improved protocol (FIGURE 2). Following robotic ZipTipC18 cleanup and NanoMate/LCQ-Deca mass spectrometer analysis, all 40 spots were unambiguously identified as shown in TABLE 1. Several extremely faint spots (barely visible with colloidal Coomassie blue stain) such as spot numbers three, six, ten and 12 were identified with two to six peptides being detected (FIGURE 2). Recently, Rutherford and coworkers developed and reported a new approach for the identification of proteins in complex biologic matrices by combining three technologies [60]. Microwave power was used for decreasing enzymatic digestion time from overnight to 10 min, a robotic ESI chip-based nano-ESI analysis was employed for sample infusion, and finally a gas-phase fractionation (GPF) method [61] was employed to further deconvolute sample complexity [60]. In their experiments, 19 GPF segments, each with an increasing mass-to-charge (m/z) range covering 25 atomic mass units (amu), were acquired 1 min each by data-dependent MS/MS for a mixture of ten protein digests and serum samples. The results demonstrated that all ten proteins were identified with 20–70% sequence coverage in the digest mixture. More than 300 proteins were identified in a serum sample in just one single run. The results suggest that GPF using MS as a mass fractionation device identifies more proteins, while chip-based infusion make the GPF analysis possible by extending the acquisition time for each segment [60].

Traditionally, with the advantages of improved salt tolerance and higher throughput compared with nano-ESI/MS/MS, matrix-assisted laser desorption/ionization (MALDI)-MS has been the preferred screening method for protein identification. Most proteomics data available in the literature use MALDI-MS to identify proteins resolved on a 2D gel. However, the automated nano-ESI system with MS/MS analysis for 2D gel-separated proteins can generate high-quality MS/MS spectra for unambiguous protein identification, as well as perform *de novo* sequencing. The offline sample preparation process has been automated and is therefore essentially the same for both MALDI-MS and automated nano-ESI/MS/MS. The detection sensitivity provided in nano-ESI/MS/MS appears to be comparable with that of MALDI-MS, in which tens of femtomoles of in-gel protein are required. Therefore, the automated nano-ESI system is an effective high-throughput complement to the present MALDI-MS approach as a screening method for gel-based protein identification.

Effort has also focused on a new approach with ESI chip-based infusion analysis for offline-collected capillary LC (CapLC) fractions to achieve better detection sensitivity and higher sequence coverage for gel samples [62,63]. Online LC/MS/MS with either a CapLC column or a nano-LC column is widely used for analysis of complex protein samples. In CapLC, a 300 µm column is used with flow rates of 4–8 µl/min. The LC running times can be as short as 30 min. However, the technique suffers from relatively low detection sensitivity and poor sequence coverage due to high flow rates (requiring fast MS scanning) compared with nano-LC, where 75 µm columns at flow rates of 200 nl/min are used. However, the analysis times for nano-LC/MS can be as long as 2 h,

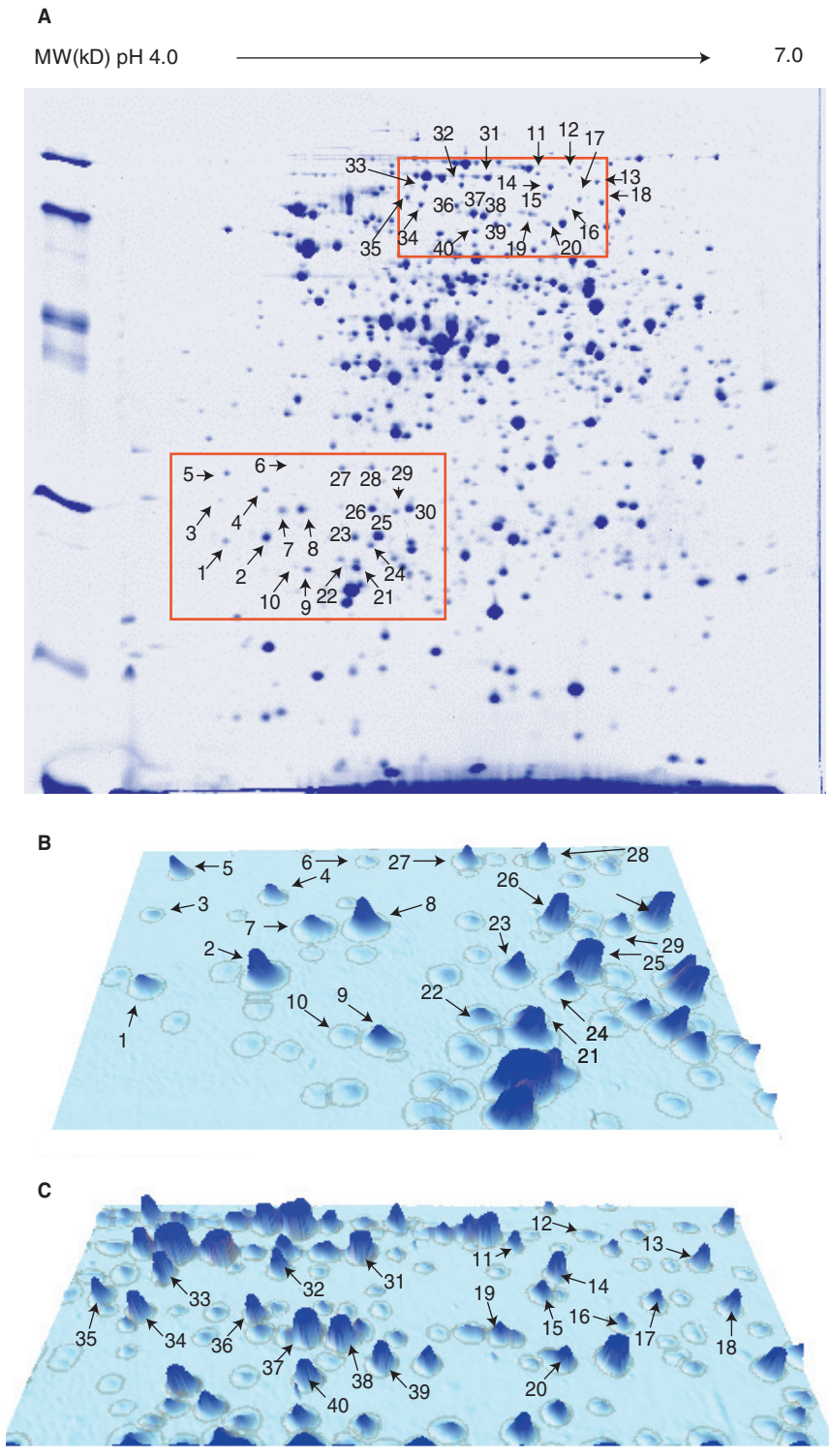

Figure 2. 2D electrophoresis gel of 80 µg of *Escherichia coli* crude cell extract. (A) The gel was colloidal Coomassie blue-stained and the gel regions and spots selected for identification are boxed and numbered. **(B)** Expanded view of gel spots in the low mass region with 3D image analyzed by Progenesis software. **(C)** Expanded view of gel spots in the high mass region with 3D image analyzed by Progenesis software.
MW: Molecular weight.

resulting in relatively low throughput for protein identification. In order to speed up the analysis while maintaining a good detection sensitivity and sequence coverage for protein identification, offline CapLC fractions were collected with an automated robotic collection system in a 96-well plate and only fractions of interest were directly interrogated using chip-based automated nano-ESI/MS/MS [62,63]. The chip-based infusion analysis of offline-collected CapLC fractions yielded consistently higher sequence coverage results than online CapLC/MS/MS for BSA digest and for a four-protein digest mixture (500 fmol each), but the results were comparable with the data from online nano-LC/MS/MS [62]. The comparison results were obtained between online nano-LC/MS/MS and offline CapLC fractionation followed by chip-based infusion nano-MS/MS analysis of a 60-kDa gel band of *E. coli* crude cell extract. Results from the two methods were surprisingly complementary. A total of 18 proteins were identified, with eight being identified by both approaches. Five proteins were uniquely detected by online nano-LC, suggesting that this method provided the best detection sensitivity through enrichment of low-abundant peptides with minimal ion suppression due to better separation. Interestingly, there were also five proteins detected uniquely by chip-based infusion analysis. This result suggests that infusion-based nano-ESI/MS/MS is a good complement to the online nano-LC/MS/MS method, being more likely to identify the low-abundance peptides (proteins) that co-eluted with other higher abundance peptides (proteins) and therefore failed to be detected by online nano-LC. In addition, chip-based infusion also required less overall MS analysis time and eliminates the need for spray optimization over a wide organic gradient as is required for online LC analysis. Consistent with the above findings, Lund and coworkers reported the complementary nature of online nano-LC/MS/MS and chip-based infusion when identifying proteins from a ribosomal fraction of yeast cell lysate [63]. Out of a total of 81 proteins identified, 47 were identified by both

Table 1. Automated chip-based nanoelectrospray ionization/mass spectrometry analysis for excised 2D gel spots of *Escherichia coli* crude cell extracts.

Spot number	Protein name	MW (kDa)	Isoelectric point	Number of peptides hit[§]	Sequence coverage (%)
1	NC_002655 ORF, hypothetical protein	20.99	4.52	3	17.3
2	Heat shock protein (Phage λ replication)	21.80	4.68	4	22.3
3	Phosphoglycolate phosphatase	27.39	4.58	4	22.6
4	Enhancing lycopene biosynthesis protein 2	22.98	4.68	2	12.9
5	3-mercaptopyruvate sulfurtransferase	30.81	4.56	6	23.1
6	NC_000913 ORF, hypothetical protein	25.87	4.71	2	8.8
7	FKBP-type peptidyl-prolyl cis-trans isomerase	20.85	4.86	3	17.4
8	FKBP-type peptidyl-prolyl cis-trans isomerase	20.85	4.86	3	17.4
9	FKBP-type 22-kDa peptidyl-prolyl cis-trans isomerase	22.22	4.85	3	18.5
10	Nitrate/nitrite response regulator	23.58	4.82	6	37.2
11	Tetrahydropteroyltriglutamate methyltransferase	84.67	5.61	3	4.8
12	DNA gyrase subunit B, GyrB	89.95	5.72	3	5.2
13	Malate synthase G	80.49	5.79	9	15.0
14	Formate acetyltrasferase 1	85.36	5.69	10	17.4
15	β-D-glucoside glucohydrolase	83.46	5.85	14	23.9
16	NC_000913 ORF, hypothetical protein	65.97	5.86	2	2.3
17	Theronine tRNA synthetase	74.07	5.80	6	12.0
	4-enzyme protein:3-hydroxyacyl-CoA dehydrogenase	79.57	5.84	1	2.1
18	4-enzyme protein:3-hydroxyacyl-CoA dehydrogenase	79.57	5.84	11	17.6
19	Methionine tRNA synthetase	76.26	5.56	6	11.4
20	L-glutamine:D-fructose-6-phosphatase	66.89	5.56	8	18.1
21	Elongation factor p	20.59	4.90	2	15.4
22	Peptide methionine sulfoxide reductase	23.32	4.99	2	12.7
23	Phosphoribosylaminoimidazole-succinocarboxamide synthetase	26.99	5.07	5	30.0
24	Hypothetical protein	24.35	5.09	3	22.0
25	Lysine-, argine-, orthine-binding protein	27.99	5.62	5	28.9
26	Histine-binding periplasmic protein	28.48	5.47	4	21.5
27	Hypothetical protein	32.46	4.98	4	20.6
28	Agmatinase	33.56	5.14	1	5.6
29	3-methyl-2-oxobutanoate hydroxymethyltransferase	28.23	5.15	1	4.9
30	Negative response regulator of gene	27.79	5.21	4	21.4
31	Hydroxyperoxidase II	84.16	5.54	4	9.0

Table 1. Automated chip-based nanoelectrospray ionization/mass spectrometry analysis for excised 2D gel spots of *Escherichia coli* crude cell extracts (cont.).

Spot number	Protein name	MW (kDa)	Isoelectric point	Number of peptides hit[§]	Sequence coverage (%)
32	Putative multimodular enzyme	82.42	5.34	5	10.3
33	Phosphate acetyl transferase	77.14	5.23	12	24.5
34	Glycine tRNA synthetase, β subunit	76.81	5.29	19	32.4
35	Putative GTP-binding factor	65.45	5.10	8	18.3
36	Heat shock protein	95.59	5.37	10	15.9
37	Tansketolase 1 isozyme	72.20	5.43	5	10.1
	Tansketolase 2 isozyme	73.04	5.43	1	2.0
38	Acetyl-CoA synthetase	72.09	5.50	12	24.7
39	L-glutamine:D-fructose-6-phosphatase	66.89	5.56	5	11.8
40	Aspartate tRNA synthetase	65.91	5.47	13	27.3

[§]MS/MS spectra were searched using BioWorks 3.1 against ecoli.fasta database with filter of xcorr versus charge state: (+1, +2, +3) > 1.50, 2.00, 2.50.
MW: Molecular weight; ORF: Open reading frame; tRNA: Transfer RNA.

approaches, 18 by the infusion approach exclusively and 16 by online nano-LC/MS/MS alone. Again, these results indicate that both analytical approaches are complementary in protein identification analysis.

Protein post-translational modification studies

Characterization of protein post-translational modifications (PTMs) is one of the most difficult challenges facing the proteomics field. Although MS has proven to be the most powerful method for mapping post-translationally modified proteins [64], the reality is that PTM analysis is far more complicated than simple protein identification. A number of practical issues lie at the root of this complication. First, proteins are often modified with a low stoichiometry and have a wide dynamic variation over time requiring a highly sensitive detection method. Second, identification of PTMs requires the isolation of the specific peptides containing the modified residues and to discern these PTM sites, high protein sequence coverage is necessary. Furthermore, peptides containing PTMs often undergo rapid degradation and may even have poor ionization efficiency in MS analysis. Finally, more than 200 different modifications are reported, making the potential for a multiply modified protein very likely, and consequently the protein would be even more complex [4]. In this section, focus will be placed on the application of chip-based nano-ESI/MS for the two most common and important PTMs: phosphorylation and glycosylation.

Protein phosphorylation

Protein phosphorylation is one of the few modifications described to date that has been shown to be reversible. Thus, it plays an important regulatory role in various cell activities including growth, differentiation, division and metabolism. Pinpointing the sites of phosphorylation in a protein is necessary in order to understand regulatory mechanisms [65,66]. MS has been proven the most efficient means to identify the precise phosphorylated residues in a protein mixture if the phosphorylated peptides can be selectively isolated from a protein digest by either affinity enrichment (such as IMAC) or MS-based selective detection (such as precursor ion scanning in the negative ionization mode for m/z 79 and neutral loss scanning for phosphopeptides). Several different approaches for mapping phosphorylation sites in protein digests were reported using ESI chip-based infusion on a variety of different mass spectrometers. Initial experiments were performed using chip-based nano-ESI in combination with precursor ion scanning on a QSTAR® Pulsar i quadrupole time-of-flight (QTOF) tandem mass spectrometer (Applied Biosystems) for the identification of phosphorylation sites of bovine β-casein digest. The standard phosphoprotein digest was analyzed with negative ionization precursor ion scanning of m/z 79 followed by positive ionization product ion scanning. In addition, the above digest was fortified with a synthetic tyrosine-phosphorylated angiotensin II peptide, and positive precursor ion scanning of m/z 216.04 was performed. The results demonstrated that using the chip-based nano-ESI coupled with precursor ion scanning, it was possible to map the phosphorylation sites of β-casein in a 50 fmol/µl digest (FIGURE 3A & B). Precursor ion scanning of m/z 216.04 provided high sensitivity and better selectivity for the identification of tyrosine-phosphorylated peptides (100 fmol/µl) in serine/threonine-phosphorylated complex digests (1 pmol/µl), as shown in FIGURE 3C. The chip-based infusion approach allows for extended analysis times to perform slow precursor ion scanning across a broad mass range for improved detection of phosphopeptides in the complex mixture (one scan requires 36 s at a dwell time of 30 ms with a step size of 1 Da across m/z 400–1700). It also permits the increased multiple channel averaging (MCA) scan numbers in the subsequent product ion scan of the phosphopeptides.

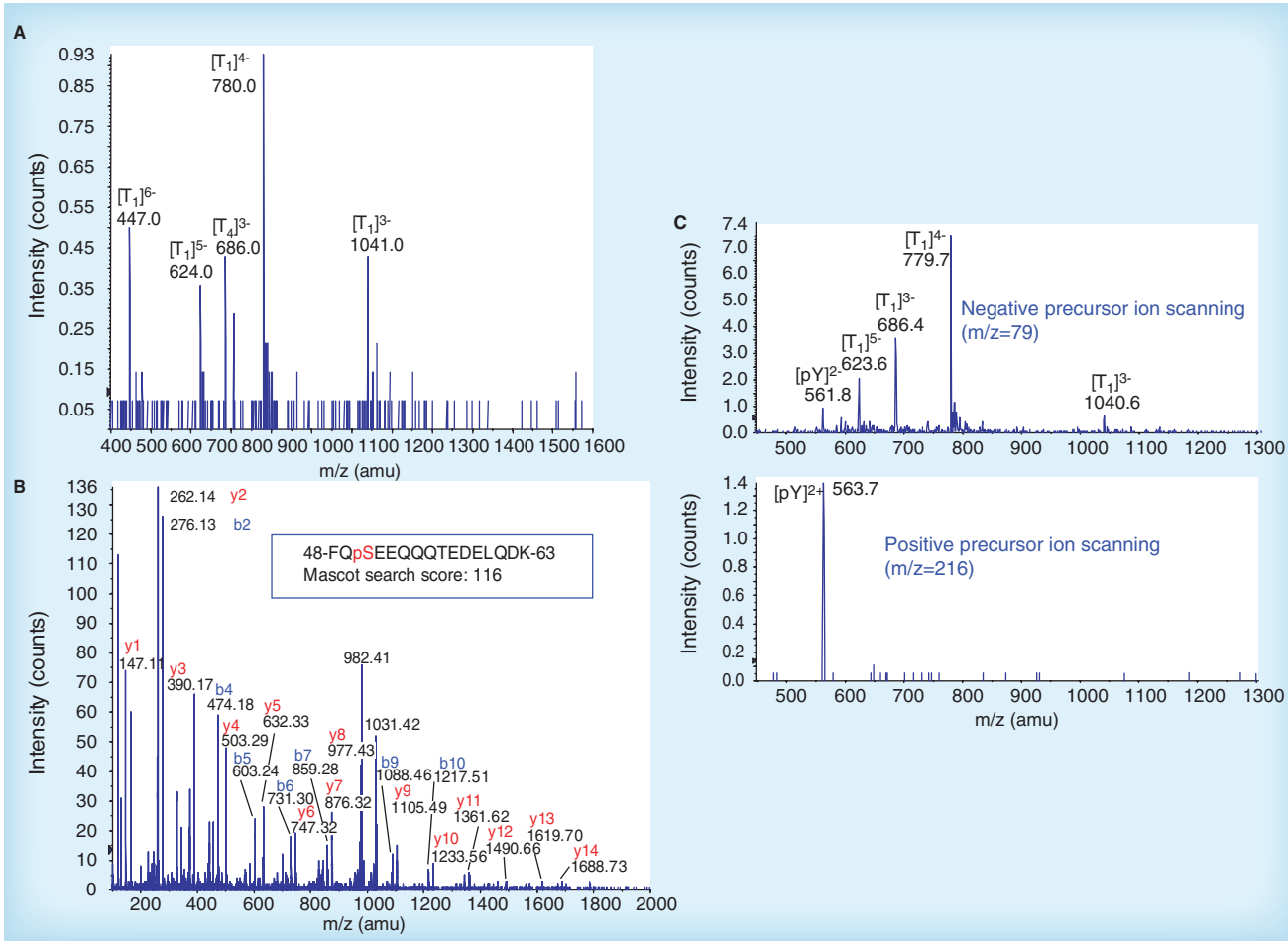

Figure 3. MS spectra of precursor ion scanning of m/z 79. (A) Spectrum of precursor ion scanning of m/z 79 in negative ionization mode for 50 fmol/µl β-casein digest showing that both T1 and T4 phosphopeptides were selectively detected. **(B)** MS/MS spectrum of the parent ion m/z 1031.4 (T4 ion) for 50 fmol/µl β-casein digest followed by a Mascot database search against the Swissprot database. **(C)** Comparison of MS spectra of precursor ion scanning of m/z 79 in negative ionization mode (top panel) and of m/z 216.04 in positive ionization mode (bottom panel) for 0.1 pmol/µl peptide containing a phosphorylated tyrosine residue in the presence of 1 pmol/µl β-casein digest.
amu: Atomic mass unit; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; m/z: Mass-to-charge ratio; pY: Represents tyrosine-phosphorylated angiotensin II.

Chelius and coworkers used the ESI chip-based nano-ESI system coupled with the linear ion trap LTQ mass spectrometer operated in DDA scanning mode with automatically triggered neutral-loss scanning for the mapping of phosphorylation sites in protein digests [67]. In all data-dependent MS/MS spectra acquired, the software would automatically look for a neutral loss of 98, 49 or 32.7 amu corresponding to a loss of phosphate for a singly, doubly or triply charged phosphopeptide, respectively. If observed, the software would automatically perform MS^3 on the ion generated from the neutral loss. In this manner the sequence of the phosphorylated parent was verified. The data were automatically analyzed using BioWorks 3.1 software. The results demonstrated that for α-casein, the phosphorylated peptides could be identified in MS/MS and MS^3 spectra at concentrations as low as 50 fmol/µl. For β-casein, all phosphorylation sites were identified in a single analysis at 1–5 fmol/µl [67]. FIGURE 4 shows that the same neutral loss scanning approach was used for a doubly phosphorylated peptide of T7 ion from an α-casein digest. A high-quality MS^4 spectrum can also be obtained manually on the basis of a second neutral-loss ion at m/z 866.6 (FIGURE 4). These results demonstrate that automated nanoelectrospray coupled to a linear ion trap offers a powerful and effective approach for rapid and accurate identification of phosphorylation sites including peptides containing multiple phosphates.

In a recent effort, chip-based infusion precursor ion scanning was employed for identifying and validating phosphopeptides from a complex sample of glutathione S-transferase (GST)–myosin fusion protein digest. Sequences of the phosphopeptides and identification of the phosphorylation sites were obtained by online CapLC/MS/MS and by collecting CapLC fractions followed by chip-based infusion MS/MS analysis [68]. Three phosphorylated residues (T230, S232 and S233) of the myosin fusion protein were unambiguously identified; furthermore, the results demonstrated the improved MS/MS spectra quality for phosphopeptides with increased

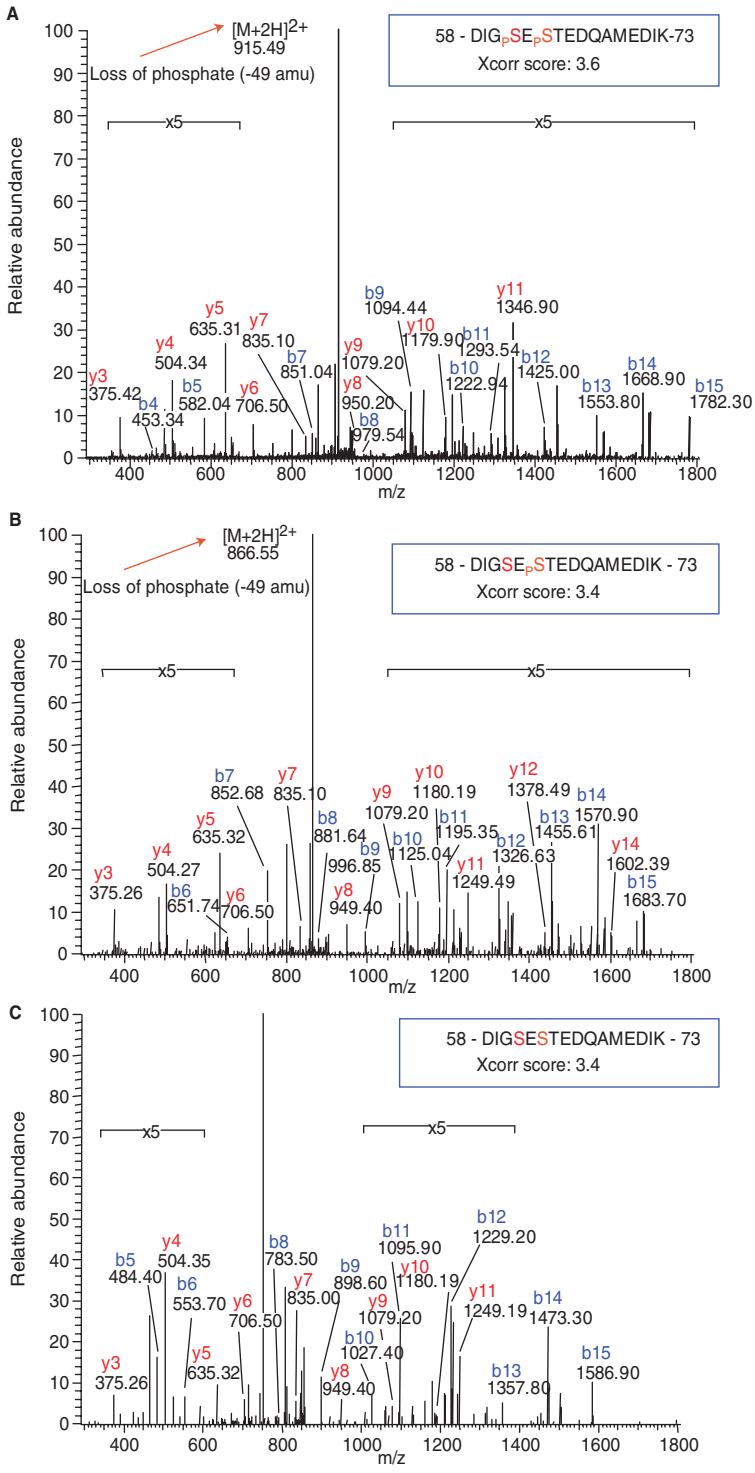

Figure 4. ESI chip-based nano-ESI/linear ion trap MS^n analysis for mapping the diphosphorylated T7 peptide of α-casein digest. (A) MS^2 spectrum derived by collision-induced dissociation of the $(M+2H)^{2+}$ precursor ion of the α-casein phosphorylated peptide, m/z = 964.6 and database searching by BioWorks 3.1 software. **(B)** MS^3 spectrum derived by DDA triggered neutral-loss scanning of the $(M+2H)^{2+}$ precursor ion of the α-casein phosphorylated peptide, m/z 964.6 → m/z 915.5. **(C)** MS^4 spectrum derived by collision-induced dissociation of the secondary neutral-loss ion, m/z = 964.6 → m/z 915.5 → m/z 866.0 and identification using BioWorks 3.1 software.
amu: Atomic mass unit; DDA: Data-dependant acquisition; ESI: Electrospray ionization; MS: Mass spectrometry; MS^n: Tandem mass spectrometry; m/z: Mass-to-charge ratio.

Xcorr scores achieved by chip-infusion nano-ESI analysis of the offline CapLC fractions [68]. The fractionation approach significantly improves the signal-to-noise (S/N) ratio for the MS/MS spectra by signal averaging the ion current from the target analyte permitted in extended infusion times compared with the few scans from the elution profile of a typical online chromatographic peak. To explore the feasibility of affinity capture of phosphopeptides followed by chip-based nano-ESI analysis, an in-house created monolithic IMAC column and ovalbumin digest were investigated [56]. After the column was charged with Fe(III), sample was loaded, washed and the captured peptides were eluted directly into a 96-well plate. The eluted sample was then subjected to nano-ESI/MS analysis using a 2000 Q Trap® linear ion trap mass spectrometer (Applied Biosystems). The full-scan MS spectra for a tryptic digest of 250 fmol/µl ovalbumin before and after affinity capture showed that the expected T30 phosphopeptide, doubly charged at m/z 1045, was selectively enriched. MS/MS was performed and a Mascot database search confirmed S345 as the site of phosphorylation [56]. The combined use of IMAC affinity capture and automated chip-based nano-ESI/MS/MS demonstrates one of many possible approaches for phosphorylation studies.

Protein glycosylation

Glycosylation is one of the most common PTMs. It is estimated that over 50% of all proteins are glycosylated [69]. Glycans in a protein can influence protein function by affecting its folding, solubility, stability and recognition of a binding partner. Alterations in protein glycosylation have often been associated with diseases. Characterization of the detailed structure of glycoproteins is very important in biomedical research and drug discovery. Structural elucidation of glycans is historically difficult as they are typically highly heterogenous and have poor ionization efficiency. Glycans are structurally branched in a nonlinear nature, with a variety of types of intersaccharide linkages. Generally, a comprehensive analysis of a glycoprotein consists of identification of glycosylated peptides, identification of

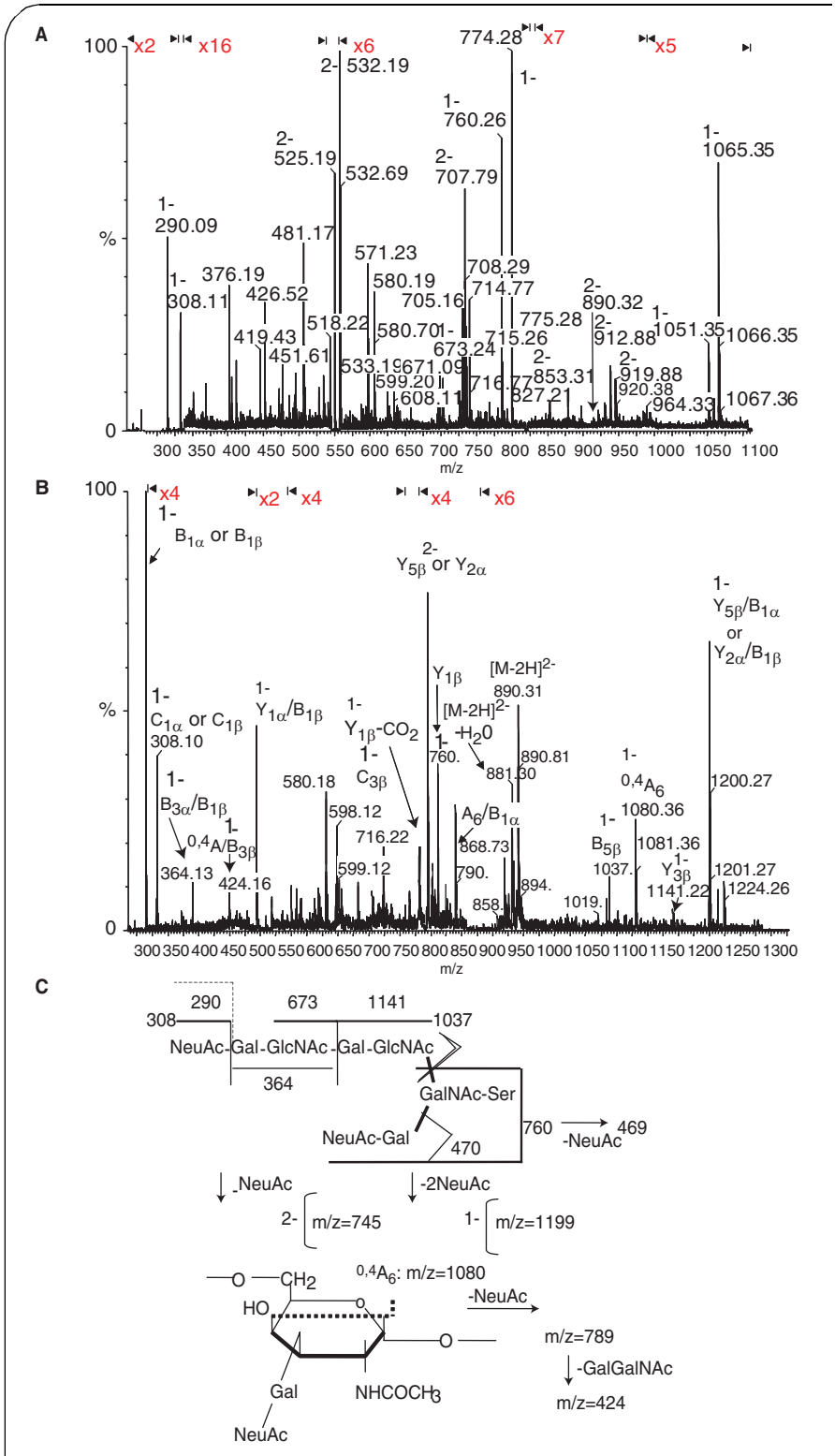

Figure 5. Automated electrospray ionization chip–based nanoelectrospray ionization/MS analysis of a complex mixture of O-glycosylated peptide mixture. (A) Full-scan MS for the sample extracted from urine of the BPy case with singly and doubly charged states assigned. **(B)** Tandem MS spectrum of NeuAc2Gal3GlcNAc2 GalNAc-Ser detected as a doubly charged ion at m/z 890.32, $[M-2H]^{2-}$. **(C)** Fragmentation scheme of NeuAc2Gal3GlcNAc2 GalNAc-Ser detected as a doubly charged ion at m/z 890.32. Reproduced with permission from [73].
BPy: N-acetyllactosamine deficiency; MS: Mass spectrometry; m/z: Mass-to-charge ratio.

the location of the glycosylation sites and elucidation of the glycan structure. Although ESI/MS/MS remains widely recognized as an effective means for oligosaccharide characterization (sequence, linkage and branching), the hydrophilic nature of glycans has often been cited as the cause of poor ESI sensitivity requiring either derivatization [70] or nanoelectrospray [71,72] to improve ionization efficiency. Nano-ESI offers improved ionization efficiency and low flow rates, providing extended analysis time for completing MS^n analyses on oligosaccharides [71]. Zamfir and coworkers first reported the implementation of an ESI chip-based nano-ESI system in combination with QTOF-MS/MS for mapping, sequencing and computer-assisted structure assignment of oligosaccharide amino acids in complex mixtures associated with two different glycosylation disorder diseases [73]. Urine samples from a patient diagnosed with hereditary N-acetyllactosamine deficiency (BPy) and a patient with a glycosylation congenital disorder (M5) were used to investigate the capabilities of automated nano-ESI for carbohydrate ionization and sequencing. The nano-ESI system and QTOF mass spectrometer were operated in negative ion mode for both MS and MS/MS analyses, and computerized assignment of the detected species was performed. The results confirmed a complex mixture of O-glycosylated amino acids and dipeptides in the BPy case and demonstrated the presence of mixtures of O- and N-linked glycans either as free oligosaccharides or attached to one or more amino acids in the M5 case [73]. Classical nano-ESI/MS/MS was found to be useful for improving the S/N ratio of low-abundance ions in complex mixtures, but it is often associated with spray instability. However, the ability of the ESI Chip device to provide a constant, extended electrospray signal creates reliable conditions for high sensitivity, which is particularly useful for detection and sequencing of minor glycopeptide components. For example, a doubly charged ion at m/z 890.32 in the MS spectrum for the BPy sample (FIGURE 5A) can be successfully fragmented (FIGURE 5B & C) and identified as O-glycosylated sialylated

serine (NeuAc2Gal3GlcNAc2 GalNAc-Ser) with direct chip-based infusion nano-ESI/MS/MS [73]. Previously, the only means possible for fragmentation of such an ion from a complex mixture was by online CE/ESI-QTOF-MS in a data-dependent analysis [74]. This work demonstrates that chip-based nano-ESI provides great potential for high-performance glycoscreening and structure elucidation as well as for discovery of novel carbohydrate variants in complex biologic mixtures. Recently, Chelius and coworkers reported another approach using the ESI Chip combined with MS^n. The linear ion trap enabled the detailed examination and mapping of glycosylation sites and glycan structures [75]. Bovine ribonuclease B (RNase B) digest containing high mannose-type N-glycosylation, without prior separation or modification of the isoforms, and two standard synthetic glycans were used to test feasibility. A linear ion trap LTQ mass spectrometer, operated in both positive and negative ionization modes, was used. In the MS spectrum of a 1 pmol/µl RNase B digest, a pattern of ions was observed at a spacing of 81 amu, which is a pattern typical of high mannose-type glycopeptides for doubly charged species [75]. The subsequent MS^n analysis for each of five selected potential glycopeptide ions led to complete sequencing of the high mannose glycan structure and unambiguous identification of the amino acid sequence of the RNase B glycopeptide. The sequence of the glycopeptides was achieved by performing MS^4 on m/z 475, identifying the site of glycosylation as residue N34 [75]. This work also demonstrates that the glycan structure can be obtained using MS/MS up to MS^5 with extended chip-based infusion without the release of free glycans from the complex tryptic digest. Furthermore the results indicate that the major benefit of chip-based nano-ESI is the allowance of sufficient spray time with stable, sensitive MS signal for complete acquisition of multiple MS/MS experiments in a single analysis.

Top-down nano-ESI/MS for proteome characterization

The bottom-up approach consists of sequencing tryptic peptides from proteolyzed intact protein samples, as described in the above sections, and has been the gold standard for proteome characterization using conventional mass spectrometers. The limitations of this method include increased sample complexity due to enzymatic digestion and the inability to achieve complete sequence coverage in most cases. In the past few years, a complementary technique to peptide-based proteomics known as top-down proteomics has been gaining popularity in characterizing the proteome. This approach involves high-resolution measurement of an intact molecular weight value and direct MS/MS analysis of intact protein ions primarily using Fourier transform ion cyclotron resonance (FTICR)/MS [76,77]. Particularly with the development of the gentle MS/MS process of electron capture dissociation (ECD) by McLafferty [78,79], the top-down approach offers informatic advantages in both protein identification with 100% sequence coverage and PTM characterization [80–82]. However, the intact proteins present significant challenges in sensitivity and throughput for analysis and automated front-end sample handling, as well as computer-assisted data reduction [77]. Recently, Kelleher has developed an automated system for data acquisition using nano-ESI/FTMS and a data retrieval software program coupled to a 2D separation platform for top-down proteomics [83,84]. The nano-ESI chip-based device in a fully automated robotic system was successfully integrated into the automated data detection and process system for high-throughput top-down analysis with improved detection sensitivity [85,86]. Typically, 10-µl fractions collected from an upstream 2D-LC separation were enough for more than 50 min of stable nanoelectrospray, providing ample time to acquire high-quality MS and MS/MS scans of two to four intact proteins per sample [87]. Using a general format of automated data detection and processing set, Patrie and coworkers were able to characterize up to six proteins in 45 min [85]. Meng and coworkers used the integrated system to demonstrate the top-down approach on a reasonably large scale for the *Saccharomyces cerevisiae* cell lysate. MS/MS data from approximately 210 proteins between 5 and 39 kDa were generated in a hybrid quadrupole FTMS. The subsequent database search resulted in characterization of 117 gene products harboring 31 PTMs [87]. Renfrow and coworkers also used the ESI chip device with FTICR/MS for successful identification and determination of aberrant O-glycosylation in the immunoglobulin A1 hinge region [88]. These results demonstrate that this chip-based device is becoming an attractive infusion-based nano-ESI ion source in the rapidly growing field of top-down proteomics.

Proteomic pattern recognition for biomarker screening

The field of MS-based biomarker discovery is at an early stage of evolution [89]. Study of protein expression profiles is becoming increasingly attractive in the areas of both functional and clinical proteomics, since protein expression profiles can be used for identifying novel therapeutic targets and diagnostic biomarkers in a variety of human diseases. In practice, biomarkers may be expressed as either single or multiple markers whose patterns of up- and downregulation may signal disease susceptibility, onset, progression, therapeutic response, drug efficacy or toxicity. Recently, several groups successfully developed a novel technology by combining surface-enhanced laser desorption/ionization (SELDI)-TOF serum profiling with bioinformatics analysis to move beyond single marker discovery to multimarker pattern analysis [90,91]. The serum biomarker patterns are being developed to correlate with several disease states such as ovarian, prostate and breast cancer [92–94]. In 2003, Schultz and coworkers applied chip-based nano-ESI/MS technology to a high-throughput serum analysis after which the data were analyzed using a bioinformatics algorithm. The result was the successful discrimination between the sera of ovarian cancer patients and those of healthy people [95]. A total of 275 serum samples obtained from 95 healthy individuals and 180 individuals affected with ovarian cancer were analyzed using automated chip-based infusion on a QSTAR mass spectrometer operated in TOF/MS positive ion mode. To first build a model with ProteomeQuest software (Correlogic Systems, Inc.), 57 control samples and 97 cancer

samples were randomly selected. The model identified features unique to the cancer serum samples compared with the controls. The remaining 121 samples (38 control and 83 cancer cases) were then analyzed based on the model. The model scores each sample based on a probability match to the features identified in the mass spectra of the control and cancer samples. The results were evaluated using two criteria: sensitivity, which is the percentage of positive samples identified by the model as true positive, and selectivity, which is the percentage of negative samples identified by the model as true negative. When considering all 175 serum samples, the model had a sensitivity of 87%, a selectivity of 84%, and successfully identified ten out of 11 Stage I ovarian cancer patients [95]. Recently, a modified approach using summed data generated in MCA mode improved the sensitivity of the model to 97% and the selectivity to 94%, and correctly identified eight out of ten Stage I cancer samples [96]. Despite the fact that bioinformatics software is able to differentiate control versus disease samples by spectral differences, these changes could not be identified by visual comparison of spectra. However, this approach may provide great potential as an additional complement to SELDI techniques for clinical biomarker tests.

Noncovalent binding interactions & drug screening

Noncovalent interactions between proteins, as well as between proteins and ligands, are critically important in cellular processes such as cell signaling and cell division. An important new technique to probe these noncovalent interactions is MS. To determine protein–protein or protein–ligand noncovalent interactions by MS, a soft ionization process, allowing the noncovalent interaction to remain intact, is required. Electrospray as well as nanoelectrospray have proven valuable in determining noncovalent interactions. For this application, nanoelectrospray offers several benefits over conventional electrospray including decreased sample consumption, increased tolerance to salts, and the formation of smaller droplets leading to increased ionization efficiency [18,97].

With the increasing costs associated with the development of novel drugs, there is pressure on the pharmaceutical industry to streamline research and development. This is achieved by screening a target protein against a combinatorial library. With the low sample consumption of nanoelectrospray, less protein is required to screen more ligands. By examining the results, the chemists and biologists gain a greater understanding of the structure activity relationship between the protein–ligand. Furthermore, by identifying ligands that bind selectively and with high affinity to target proteins, lower drug dosages can be prescribed to patients, which in turn will minimize the chances of adverse side effects.

The ESI Chip has recently been used to detect and screen for drug candidates by protein–ligand interactions [98]. Benkestock and coworkers coupled chip-based nano-ESI with a Q-TOF 1 mass spectrometer to assess the binding affinity of a subset of a combinatorial library to two different human fatty acid-binding proteins (FABP); heart (H)-FABP and adipose (A)-FABP. FIGURE 6A depicts the spectrum of H-FABP, while FIGURE 6B–E show the resulting mass spectra of H-FABP incubated individually with four different ligands. The spectra from FIGURE 6A & B are very similar, indicating that ligand 647 does not form a noncovalent interaction with H-FABP. However, in FIGURES 6C–E, ions corresponding to the protein–ligand complexes are observed, suggesting that each of these ligands forms a noncovalent interaction with H-FABP. In addition to simply observing whether or not the protein and ligand formed a noncovalent interaction, some quantitative information is also

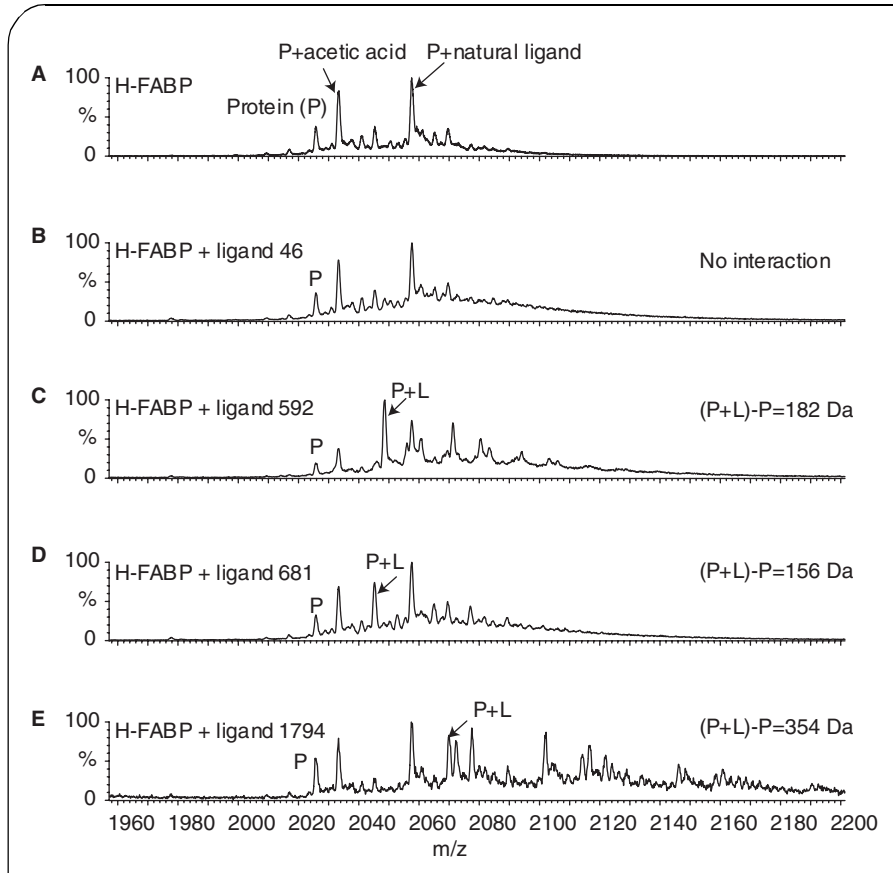

Figure 6. MS spectra of H-FABP. (A) H-FABP (23 µM) without ligand. **(B–E)** (blowup of charge state +8) Potential ligands to H-FABP. P = m/z of H-FABP and P+L = m/z of complex. (P+L)-P = molecular mass of the ligand. Reproduced with permission from [98].
H-FABP: Heart fatty acid-binding protein; m/z: Mass-to-charge ratio.

observed. For example, if one considers the relative intensity of 'P' versus 'P+L' for FIGURE 6C compared with FIGURE 6E, it becomes apparent that ligand 592 from FIGURE 6C has a higher binding affinity for H-FABP than ligand 1794 from FIGURE 6E. Furthermore, Benkestock and coworkers showed that when a cocktail of seven ligands, only one of which was known to have an affinity for H-FABP, was incubated with H-FABP, the ligand with the known affinity for H-FABP was easily identified [98]. Benkestock compared the results of the ligand screening by chip-based nano-ESI with results obtained from nuclear magnetic resonance (NMR) screening. The results for the 23 ligands screened against H- and A-FABP by NMR and MS are shown in TABLE 2. The two techniques demonstrated very good correlation for all ligands except for one, 2500. Interestingly, the MS results showed a selectivity difference between ligands 3081 and 3084 and the two FABP proteins. It is this ligand selectivity difference between two very similar proteins that is most interesting and crucial for the development of novel pharmaceuticals. The authors concluded that chip-based automated nano-ESI could be used as a primary screening method

Table 2. Comparison of ligand binding between nuclear magnetic resonance and mass spectrometry screening.

Ligand	$MW_{(theor.)}$ (Da)	H-FABP			A-FABP		
		MS hit	*ΔM (Da)*	*NMR hit*	*MS hit*	*ΔM (Da)*	*NMR hit*
293	200	N	-	N	N	-	N
592	182	Y§§	0	Y	Y§§	0	Y
596	153	N	-	N	N	-	N
601	174	N	-	N	N	-	N
647	123	N	-	N	N	-	N
663	147	N	-	N	N	-	N
681	155	Y	+1	Y	Y	0	Y
1379	154	N	-	N	N	-	N
1794	354	Y	0	Y	Y	0	Y
1892	138	Y	0	Y	Y	-2	Y
2281	163	N	-	N	N	-	N
2396	151	Y§	-	Y	Y§	-	Y
2497	172	Y	0	Y	Y	0	Y
2500	142	N	-	Y	N	-	Y
2506	172	Y	-1	Y	Y	-3	Y
3081	182	Y	0	Y	Y§§	-1	Y
3084	202	Y§§	-2	Y	y	-1	Y
3559	197	N	-	N	N	-	N
4033	183	Y§§	-1	Y	Y§§	-2	Y
4438	189	Y§§	0	Y	Y§§	-2	Y
4465	225	N	-	N	N	-	N
BVT.1960	166	Y	0	Y	Y	0	Y
BVT.1961	208	Y§§	0	Y	Y§§	0	Y
Comp. mixture	168	Y§§	+1	Y	Y§§	-3	Y

§Very weak binder.
§§Potentially strong binder.
ΔM = MW(theor.)–MW(exp.).
Reproduced with permission from [98].
A/H-FABP: Adipose/heart fatty acid-binding protein; MS: Mass spectrometry; MW: Molecular weight; N: No; NMR: Nucelar magnetic resonance; Y: Yes.

in the early phases of drug development. The authors estimated that a sample capacity of 2200 compounds/week could be screened individually [98]. This was 50-times faster than NMR for single compounds and approximately fivefold faster for screens of mixtures. The speed, in combination with low protein consumption, renders this approach very promising.

Robinson and coworkers used chip-based nanoelectrospray to screen the binding affinity of various synthetic ligands to the tetrameric plasma protein transthyretin (TTR), a 55-kDa multiprotein target [99]. It is known that TTR has two binding sites and that when ligands are bound to the tetramer, the complex is more stable than its apo- form and dissociation of the tetramer is significantly reduced [100,101]. The authors showed the resulting mass spectrum of TTR incubated with thyroxine. Ions corresponding to apo-TTR-tetramer, tetramer complexed with one thyroxine molecule and tetramer complexed with two thyroxine molecules were clearly observed. Furthermore, by changing the cone voltage, it was demonstrated that the ESI Chip could be used to access the stability of TTR in the presence of ligands. The authors also showed a high degree of nozzle-to-nozzle reproducibility using this chip-based approach. By analyzing TTR across five different nozzles and focusing on the most intense ion in the +15 charge state of the tetramer, the relative standard deviation was calculated as 5.3%. The authors also made some comparisons between the chip-based approach and the conventional pulled-capillary approach. It was found that although the chip-based approach had only half the sensitivity of the pulled-capillary method, the chip-based approach had a tenfold increase in signal stability compared with pulled-capillary. The authors conclude that measuring the ability of small molecules to stabilize protein complexes using the chip-based approach will enable high-throughput screening of multiprotein complexes.

The ESI Chip device holds an additional benefit in that the nozzles within a chip, as well as between chips, are identical to one another. A high degree of nozzle-to-nozzle reproducibility has been previously demonstrated [58,99,102]. This high reproducibility is absolutely imperative when performing quantitative studies, such as for the determination of dissociation constants as was reported recently using a Q-TOF micro-mass spectrometer [103]. The report investigated the well-characterized interactions between RNase A and the ligands cytidine 2′-monophosphate (2′-CMP) and cytidine triphosphate (CTP). FIGURE 7A shows the mass spectrum of 10 μM RNase A in 10 mM ammonium acetate, pH 6.8, and the resulting spectra of RNase A–ligand complexes are shown in FIGURE 7B–D. The three charge states from +8 to +6 of the protein and its complexes are observed. In these spectra, not only are the ions indicating the presence of free RNase A apparent, but a second set of ions, shifted to a higher m/z, is also present. This second set of ions corresponds to the RNase A–2′-CMP or RNase A–CTP complex, unambiguously indicating the presence of this noncovalent protein–ligand interaction. A similar scenario is observed in FIGURE 7D, where 10 μM of both 2′-CMP and CTP have been added to the RNase A solution, and once again the ion series corresponding to free RNase A is present, as well as an additional ion series corresponding to both protein–CMP and protein–CTP complexes shifted to higher m/z values. The peak areas from three charge states were summed for both free RNase A and RNase A–ligand complex(es) and used for quantitative noncovalent binding interaction studies. Two different means were used for determining dissociation constants (Kds) of RNase A–ligand complexes. The first was through a titration experiment where the protein concentration was held constant and the ligand concentration was systematically varied. Then, by plotting [RL]/[R] versus [Li]-[RL], according to the equation [RL]/[R] = 1/Kd ([Li] - [RL]) for a single binding site, the Kd can be determined from the inverse of the slope [103]. The second means was using a competitive binding experiment. By using an equimolar concentration of RNase A, 2′-CMP and CTP, the dissociation constants could be determined using the two equations, Kd_{RL1} = [R]([R]+[RL2])/[RL1] and Kd_{RL2} = [R]([R]+[RL1])/[RL2]. The experimentally determined Kds for RNase A–CMP and RNase A–CTP by both titration and competitive binding are consistent,

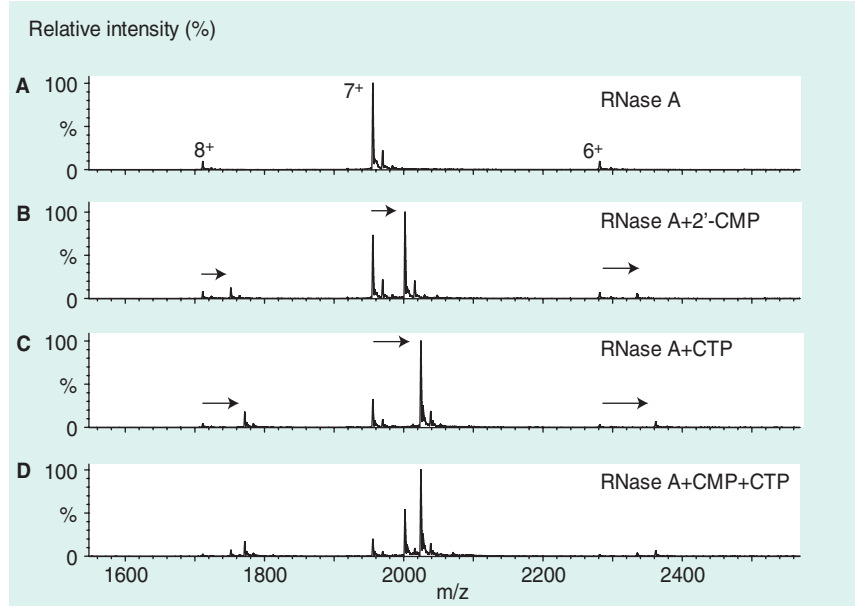

Figure 7. Representative nanoelectrospray mass spectra of the noncovalent interactions of RNase A with 2′-CMP and CTP obtained in 10 mM ammonium acetate, pH 6.8. Arrows indicate the mass shift at each charge state for complexes formed. **(A–D)** Mass spectra of **(A)** 10 μM RNase A, **(B)** 10 μM RNase A plus 10 μM 2′-CMP, **(C)** 10 μM RNase A plus 10 μM 2′-CTP, **(D)** 10 μM RNase A plus 10 μM 2′-CMP and 10 μM 2′-CTP. Reproduced with permission from [103].
CMP: Cytidine monophosphate; CTP: Cytidine triphosphate; RNase: Ribonuclease.

suggesting that nonspecific binding in this assay was minimized. Furthermore, these results obtained from chip-based nano-ESI/MS are comparable with those published from other conventional techniques such as calorimetry and circular dichroism [104-106]. It was demonstrated that the chip-based approach is in relatively good agreement with previously reported values.

In this report, the authors also investigated the noncovalent interactions between an endocellulase mutant protein, Cel6A D117Acd, and four oligosaccharide ligands. Dissociation constants were determined through both titration and competitive binding experiments. This was the first report of Kds for three endocellulase–ligand complexes. The only system whose dissociation constant had previously been determined was Cel6A D117Acd-cellotriose. The Kd of this complex, as determined by spectrofluorimetry, is 68.0 ± 6.6 µM [107], and determined by chip-based nano-ESI is 77.5 ± 9.2 µM. Again, the chip-based results were in very good agreement with those obtained by conventional techniques. The authors concluded that chip-based nanoelectrospray can be used to determine micromolar and submicromolar solution binding constants.

Not only is this chip-based nano-ESI system being deemed useful for drug discovery by screening protein–ligand and multiprotein–ligand complexes, but also through its quantitative and qualitative capabilities for pharmaceutical compounds. The value of the system in quantitative determination of noncovalent interactions has been discussed. However the quantitative utility of the system for drug discovery does not end there.

The automated chip-based infusion nano-ESI platform has also been employed in the quantitation of drug molecules from biologic matrices. Kapron and coworkers reported the quantitation of midazolam from fortified human plasma using alprazolam as an internal standard [102]. The intra- and interassay precision and accuracy were acceptable for rapid drug discovery analyses. Six different plasma lots provided variability and accuracy within 20% at the lower limit of quantitation and 15% at the upper limit of quantitation. Dethy and coworkers reported quantitation of verapamil and norverapamil from human plasma using gallopamil as an internal standard [108]. Acceptable precision and accuracy values were acquired over the range of 5–500 ng/ml. The chip-based system has also been used for Caco-2 analysis, an *in vitro* permeability test of a drug candidate across the intestine membrane used in the drug discovery process [109]. The results from an offline desalt step followed by chip-based automated nano-ESI/MS/MS were compared with those from an LC/MS/MS approach. The permeability and recovery data obtained from a parallel analysis of Caco-2 samples by conventional LC/MS/MS and by chip-based nano-ESI/MS/MS were in excellent agreement [109]. The results demonstrate that the chip-based approach is an alternative for rapid Caco-2 analysis.

In recent reports, the utility of infusion chip-based nano-ESI for the structure elucidation of metabolites has been highlighted. It is important that the pharmaceutical industry has knowledge of the metabolites formed *in vivo* to avoid a drug being removed from the market because of unexpected toxic metabolites that were overlooked in the development phase. Consequently, it is invaluable for pharmaceutical companies to have a means of identifying all metabolites formed by a particular drug. Hofgartner and coworkers have shown that by collecting LC fractions and then infusing each fraction with the chip-based nano-ESI system, data can be acquired for a longer duration and summed, improving the S/N ratios in the resulting mass spectra [110]. By infusing an LC fraction containing a metabolite of interest, Hofgartner and coworkers reported a 20-fold gain in sensitivity and increased structural information, compared with online LC/MS/MS results [110,111]. This gain is apparently attributed to infusion providing the time to optimize MS/MS conditions, followed by the ability to sum spectra over a 2 min analysis time. Hofgartner points out that nano-ESI also allows one to perform many different types of experiments such as MS, selective reaction monitoring, precursor scanning and MS^n, in positive or negative ionization mode. Miller and coworkers successfully demonstrated the potential of chip-based infusion with precursor ion scanning for metabolite identification [112]. Consequently, chip-based nano-ESI will aid this very challenging metabolite identification application.

Lipidomics

Lipidomics is an expanding research field, due in part to recent advances in technologies and novel applications of ESI/MS. Lipidomics focuses on quantitative and qualitative identification of lipid metabolism, biologically active lipids, biomembrane and lipid-mediated signaling transduction that regulates cellular homeostasis during health and disease. Several excellent and extensive reviews covering these developments and applications have recently been published [113,114]. ESI/MS has proven to be one of the most sensitive, discriminating and direct methods to assess alterations in the cellular lipidome [114]. This is mainly since ESI/MS can provide rapid and sensitive quantitative analysis of lipid classes, subclasses and individual molecular species without prior chromatographic separation or derivatization. Precursor ion or neutral loss scanning in a classic nano-ESI/MS/MS analysis has been demonstrated as particularly useful for qualitative and quantitative analysis of complex membrane lipid mixtures at the subpicomole level [115]. As current ESI/MS-based lipidomic analysis is mainly focused on the infusion approach, one can expect that chip-based infusion would be an ideal nano-ESI source in this relatively new and expanding research field. As a good example, Ejsing and coworkers recently developed a new approach toward shotgun lipidomics for high-throughput profiling of the molecular composition of phospholipids [116]. The described chip-based nano-ESI system was used to acquire automated acquisition for the profiling (both qualitative and quantitative) of the various phospholipids by multiple precursor ion scanning for the head groups and fatty acid groups in both negative and positive ion modes on a Q-STAR mass spectrometer. The subsequent identification and quantification of detected lipid species was performed by computer-assisted data analysis with the newly developed Lipid Profiler software. The spectra generated from multiple precursor ion scans showed that seven different glycerophospholipids were simultaneously detected [116]

and quantified in the automated analysis, with 27 min per run (1 min for initial TOF/MS, 25 min for multiple precursor ion scanning and 1 min for TOF/MS). Consistent TOF/MS signal intensity between the first and last minute of acquisition were achieved for ten runs of the same aliquot of sample, demonstrating that automated chip-based nano-ESI provides a constant infusion spray for multiple precursor ion analysis throughout an entire 27-min acquisition time with reproducibility suitable for high-throughput lipidomics studies [116].

Expert opinion & five-year view

The use of microfluidic devices with MS detection has now progressed to commercialization. Proteomic applications using chip-based devices coupled to MS is an active field motivated by the large-scale need to characterize low-abundance proteins with limited available quantity of sample. The studies outlined in this review demonstrate that automated chip-based nano-ESI is a promising technique, offering not only automation and high throughput, but also many other benefits to a wide variety of proteomic applications. These benefits include low sample consumption, ease of use, no sample-to-sample cross-contamination and extended acquisition times that yield improved MS data quality. The ongoing developments promise even greater future improvements. For example, the development of high-density (400 nozzles) ESI Chips can reduce the cost per sample. Design of an ESI Chip with a single inlet leading to multiple nozzle outlets allowing spray from a multiplex of nozzles simultaneously could provide nanoelectrospray benefits of lower ion suppression and higher sensitivity even at relatively high flow rates. The coupling of a nano-LC column to the ESI Chip or the coupling of a pipette tip, packed with stationary phase, to the ESI Chip offers online separation capability in an automated format [117]. In addition, as the reduction or absence of signal suppression was demonstrated by nano-ESI/MS at minimal flow rates below 20 nl/min [22,24], development of small i.d. spray nozzles or emitter devices would provide a true nano-ESI platform for MS analysis, which could be extremely useful in further improved detection sensitivity for a variety of proteomics applications.

Despite the substantial progress made in recent years, there remain many challenges in the field still requiring investigation. Thus far, major research has focused on the development of individual reaction, separation and detection platforms on a chip. Therefore, the integration of sample preparation and liquid delivery techniques to the chip making a true proteomics lab-on-a-chip platform will be a key and significant step forward. The glass-based microsystem device introduced by Thibault's laboratory is closest to fulfilling the requirements as an integrated device for proteomics study [41]. However, no product has yet been commercialized. Other developments in this field have already been presented including a chip with a built-in single LC column by Agilent Technologies [118], and placement of monolithic stationary phase within the 100 flow paths on the ESI Chip [119] and within the microchannel of the SU-8-based microsystem [120]. Development of multidimensional LC capabilities integrated into the chip will make the device more powerful in deconvoluting complex samples. An array of multiple integrated LC columns on a chip for either repeat or single use would be another direction for the technology but is a challenging task. Incorporating affinity techniques directly onto the chip through either an integrated LC column or by coating the surface of the chip device for improving separation would be extremely attractive for PTMs studies. These fully integrated, chip-based affinity techniques could also possibly lead to the development of microarray and microfluidic devices for ESI/MS analysis. In addition, the application of microfluidic devices in pharmaceutical analyses has great potential. Fast screening of drug candidates from combinatory chemistry, the assessment of drug metabolites, and the rapid quantitation of early ADME studies could potentially be conducted using chip-based ESI/MS.

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Key issues

- Nanoelectrospray offers many benefits over conventional electrospray including improved ionization efficiency, reduced matrix suppression and low sample consumption.
- However, using conventional pulled-capillaries, the nanoelectrospray technique suffers from shortcomings including tedious and intensive optimization processes requiring a skilled user, limited sample throughput and poor reproducibility between capillaries.
- Automated chip-based nanoelectrospray takes advantage of all the benefits of nanoelectrospray and offers a solution to all the shortcomings of the conventional pulled-capillary technique.
- Chip-based nanoelectrospray offers an easy-to-use, high-throughput means of performing nanoelectrospray with a high degree of nozzle-to-nozzle reproducibility to meet the needs of proteomic analyses.
- This infusion nanoelectrospray device provides sufficient time for data averaging, improving data quality and for providing the time required to perform tandem mass spectrometry, precursor ion scanning, neutral-loss scanning and other slower scanning techniques.
- Automated, infusion, chip-based nanoelectrospray can be used in a variety of proteomic applications such as protein identification, post-translational modification characterization, top-down proteomics, noncovalent interactions and lipid analysis.
- Integration of sample preparation and liquid chromatography on chip-based nanoelectrospray devices for future products.

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