MINI-REVIEW

Microfluidic devices for protein analysis using intact and top-down mass spectrometry

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Abstract
Top-down mass spectrometry (TD-MS) provides unique information on compositions, structures, and functions of proteins or protein complexes and has been recognized as a powerful approach to complement conventional tools in protein analysis. Employing microfluidic chips in TD-MS workflows offers unique advantages including flexible integration and automation of multiple sample treatment functions, lowered sample consumption, compatibility with high-sensitive ionization and higher throughput. Here we reviewed the reported microchips developed for TD-MS in aspects of design of ionization, separation and mixing modules, and fabrication approaches; we also attempted to summarize the design considerations that may inspire development of microchips with better performance and wider applications.

KEYWORDS
mass spectrometry, microfluidic chip, native mass spectrometry, protein structures, proteomics, top-down

1 | INTRODUCTION

Characterization structures of proteins and protein complexes is essential for understanding their biological functions and revealing their underlying physiological mechanisms. As a technique that directly measures the masses of biomolecules or their fragments, mass spectrometry (MS) can provide unique structural information that complements those from conventional biophysical tools such as X-ray crystallography, cryogenic electron microscopy and nuclear magnetic resonance. The covalent structures (including amino acid sequences and post-translational modifications [PTMs]) and protein assembly/disassembly can be revealed by MS through their unique masses or mass differences. In analyses of protein conformations or binding interfaces, which do
not result in intrinsic mass shift upon conversion between different states, MS-based methods encode the conformational or binding features into mass shifts induced by labeling reactions including various chemical labeling, fast oxidation, crosslinking, and hydrogen/deuterium exchange (HDX).

MS has been demonstrated as an indispensable tool for characterizing protein structures, conformational changes, interactions with small molecules or proteins (protein-protein interaction [PPI]), and proteomics. To determine/confirm the sequences of the detected species or access the local structural information at specific sites, these analyses often involve the measurement of fragments released from the intact proteins. The conventional bottom-up approach enzymatically digests proteins in solution and analyzes the resulting peptides following chromatographic separation. Despite its wide application, this approach loses information on connections between the detected peptides or protein subunits and is therefore unable to access structural information at the intact level. Moreover, it cannot distinguish peptides originating from co-existing conformational or binding states. In contrast, the top-down (TD) approach measures intact protein or protein complexes followed by tandem MS, that is, fragmenting the mass-selected precursor ions using gas phase dissociation reactions, allowing access to intact structural information prior to analyzing local structural features. Since gas-phase dissociation of proteins predominantly produces a series of terminal fragments and the local structural information can be revealed by comparing adjacent fragments that differ by very short segments, TD-MS often provides higher sequence resolution than bottom-up, which can reach the single residue level in many cases, when measuring small proteins or terminal portions of large proteins. Species in different conformational or binding states can be separated in solution by chromatography or electrophoresis in modes compatible with native-like conditions or in the gas phase by ion mobility or a mass filter (upon proper labeling) before mass analysis. Combining the TD strategy with native MS, a method capable of preserving crucial non-covalent interactions throughout the mass analysis and direct measurement of intact protein complexes, allows the identification of subunits or functional sites via cleaving covalent bonds (an approach termed as “native TD”) and characterization of stoichiometry and topology of protein assemblies via stripping off the non-covalent subunits (termed as “complex down” by some researchers). TD-MS also allows structural characterization in a conformation-specific manner when combined with labeling methods such as HDX, a method that reveals conformation or binding interface by encoding solvent accessibility features into site-dependent deuteration rates.

MS measurement requires the analytes to be ionized prior to mass analysis and detection. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are two widely used ionization techniques for biomolecules. MALDI produces predominantly singly charged ions of the analytes and their fragments, therefore minimizing the spectra complexity and allowing straightforward mass determination using the conversion from the detected mass to charge ratios (m/z) of analytes. This feature makes MALDI well-suited for characterizing covalent structures (sequence and certain PTMs) of proteins. However, such charging feature requires a very high m/z detection range for analyzing large intact proteins, which poses challenges in terms of ion transmission and detection to commercial instruments. Its throughput is limited by the dependence on matrix addition and formation of solid-phase sample spots. In addition, adding the acidic matrices in the sample solution and the subsequence co-crystallization of the analyte–matrix mixture disrupts the non-covalent interaction, either intra- or intermolecular, resulting in the dissociation of protein complexes and denaturation of proteins. Covalent treatments such as crosslinking are therefore required in the reported cases of MALDI-based characterization of protein complexes. In contrast, ESI directly ionizes analytes from the solution phase, resulting in multiple charging of macromolecules and is compatible with the near physiological conditions. ESI is so far the only ionization method for native MS and the favored ionization method for TD-MS.

Although the TD-MS workflow does not include the complex steps related to proteolysis or peptide separation, various sample treatments need to be carried out before being subjected to MS analysis. In addition to the separation of proteoforms or proteins in different conformational or binding states, intact protein species may be deglycosylated to reduce the spectra complexity or cleaved into a small number of large fragments to improve the sequence coverage (the so-called “middle-down” approach). When the non-covalent interactions do not need to be precisely preserved in analysis, intact proteins are preferentially measured in formats of ions in increased charge states, which benefit the fragmentation efficiency. Charges can be increased by either manipulating the charged droplets during ESI using supercharging reagents (such as sulfolane and m-nitrobenzyl alcohol) or unfolding proteins before ionization using organic solvent or acid. When protein higher-order structures or dynamics are characterized with labeling or foot-printing methods, proteins need to be mixed with proper labeling or quenching reagents and incubated for certain periods.

As a class of tools suited for processing fluids at volume scales ranging from picoliter to microliter, microfluidics...
provides unique benefits for the treatment of intact
proteins. Compared with bulky devices, a miniaturized
microfluidic chip (microchip) may better satisfy the
requirements of TD-MS analyses. First, it allows flexible
integration and automation of multiple functions in a
workflow that can be online coupled with MS analysis,
while maintaining flow stability and minimizing distur-
bance. Second, it reduces the consumption of samples and
reagents by the lowered dimension of channels or cham-
bers and may reduce the analysis time by improving the
efficiency of mixing or reactions. In addition, the total flow
rate of the processed sample flow can be lowered to the sub-
microliter per minute scale and even picoliter per minute
scale. Since the ionization efficiency decreases with the
increasing size of analytes in ESI, and a lower infusion
flow rate generally leads to higher ionization efficiency,
lower flow rates are beneficial for the sensitivity of TD-MS.
Since the pioneering works of using a microchip
in MS analysis, microchips have been applied to a
variety of tasks including PPI identification in cell
lysates, monitoring dynamic hormone secretion, single-cell proteomics, detection of ligand binding, and fast bioreactor monitoring. So far, the functions integrated into microchips for MS analysis include sample preparation, separation, digestion, preconcentration, and desalting. In particular, a number of microchip-based devices and strategies for intact protein analysis have emerged (for an overview of applications of these microchips, see Figure 1). Here, we attempt to review the designs and fabrication approaches of reported microchips developed for TD-MS and summarize the design considerations that may inspire the development of microchips with better performance and wider applications. Although there have been microchips developed for MALDI-based MS analysis, due to the limited application of MALDI in this field as discussed earlier, such microchips are not included in the scope of this mini-review.

2 | SCHEMES OF MICROCHIPS FOR TD-MS

Most microchips designed for TD-MS analysis of proteins consist of sample treatment modules and an ESI interface module, which directly ionizes the samples that have experienced the treatments and subjects them to MS analysis in real time.

2.1 | Designs of ESI interface

The effectiveness of the ESI interface module determines whether a microchip can be applied to online TD-MS analysis or should be used as an offline reactor. An ideal ESI interface for TD-MS should provide not only efficient mass transfer but also higher ionization efficiency for intact protein species, compared to those designed for small molecules or peptides. In addition to the low flow rate as mentioned in the Introduction section, the
desolvation and ionization efficiencies needed for TD-MS favor an ESI emitter with a small inner diameter (I.D.) of orifice. The existing designs of ESI interfaces can be categorized into three types (Figure 2A).

The first type of design utilizes the opening of a part of the chip, such as an edge, a corner, or a tapered nozzle at the outlet of the flow channel as the ESI interface (Scheme 1 in Figure 2A). For example, for assessment of hemoglobin glycation in whole blood lysate, Redman et al. used a corner nozzle to ionize protein analytes separated by capillary electrophoresis (CE) in the chip. Li et al. developed a three-layered microfluidic chip that contains a corner nozzle, to study the dynamics of neurotransmitter release from cells. Taking the advantages of simplicity in structure and relatively easy fabrication, this type of interface has been widely used for analyzing a broad range of biomolecules and has been incorporated by some commercialized microchips. However, due to the limitation of the structure and material, it is difficult to decrease the orifice I.D., which limits the ionization efficiency and electrospray stability.

The second type employs an external emitter for ESI, which was achieved by inserting a piece of fused-silica capillary, a glass capillary with a pulled tip, or a commercial ESI emitter into the end of the flow channel (Schemes 2 and 3 in Figure 2A). The spray voltage is then applied to the external emitter for ionization. Baker et al. demonstrated a microchip scheme using an eductor, which consists of a standard ESI needle connected with a sample transfer capillary, and a tapered gas nozzle. The nozzle introduces a pressure differential when a pulse of nitrogen gas is applied, pulling droplets from the needle into the gas flow and facilitating MS detection. Since mounting an external emitter is less dependent on the geometry and material of the microchip, multiple emitters can be...
mounted to the same chip. For example, Xue et al. fabricated a glass microchip with multiple channels connected to fused-silica capillaries, achieved multi-channel parallel analysis of peptides, and demonstrated an increase in throughput using this device. The external emitter design allows a flexible selection of emitters with sufficiently small orifice I.D. to ensure ionization efficiency and spray stability. It also makes it feasible to replace emitters without disposing the chip per se upon clogging, which can be frequently encountered in TD-MS, especially when denaturing conditions or high protein concentrations are used. However, connecting the external emitter to the chip increases the void volume and the risk of leakage.

Integrated emitters can also be used for on-chip ESI. Microfabrication techniques allow flow channels and emitters to be simultaneously constructed during chip fabrication (Schemes 4–6 in Figure 2A). The integrated emitter minimizes the void volume between the flow channel and emitter while maintaining high ionization efficiency and spray stability. Dietze et al. coupled electrophoretic separations to MS using a glass microchip containing a monolithically integrated nano-ESI emitter (Scheme 4 in Figure 2A). This strategy also allows the construction of multi-channel emitters. Yu et al. developed a microchip-based multi-channel ionization method that extracts natural compounds from complex matrices. The chips with integrated multi-nozzle array have been used as the standard consumable in commercial nano-ESI robots to provide a large size of samples with high throughput (Scheme 5 in Figure 2A). The geometry of the integrated emitter can be flexibly adjusted to adapt the design of the microchip without introducing concerns about increasing the void volume. However, integrated ESI interfaces are more difficult to manufacture, compared to other types of ESI interfaces, which makes them less cost-effective. When an integrated emitter is fixed to the chip, it is difficult to replace the emitter when it is clogged or emitters with different specifications are needed. To solve this problem, Li et al. developed a hybrid external-integrated scheme, where a finger-tight union, rather than an emitter, was integrated into the microchip (Scheme 6 in Figure 2A). This configuration allows flexible selection and convenient replacement of emitters while minimizing the void volume.

2.2 Schemes of sample treatment module

Separating protein analytes and mixing them with proper reagents for denaturation or labeling/foot-printing reactions are the two most frequently needed functions in TD-MS. Accordingly, various separation modules and micromixing modules were developed for TD-MS microchips (Figure 2B). While the former types of modules are mainly used for TD proteomics or proteoform profiling, the latter also aims at protein structural analysis (Figure 1).

2.2.1 Designs of separation module

As the well-established methods to separate intact proteins based on their differing hydrophobicities/hydrophilicities, sizes, charging behaviors, or specific binding interactions, liquid chromatography (LC) and CE in modes of zone electrophoresis, isoelectric focusing, and electrochromatography have been used for front-end separation for TD-MS analyses (Schemes 7 and 8 in Figure 2B). TD-MS microchips for TD proteomics primarily adopt these two principles for separating various proteins. Three types of LC columns, that is, open-tube packed bed, and monolithic bed have been employed for LC separation. Chen et al. integrated an 8-cm-long C18 LC column in a silicon multi-nozzle emitter array (MEA) microchip with an internal swept volume of < 15 nl, and well separated intact human plasma proteins including human serum albumin (HSA), hemoglobin A (HbA), and apolipoprotein A-I (apoA-I). Mao et al. developed an MEA chip consisting of 24 identical units that are uniformly distributed in a circular array, where each unit contains a sample injection module, a 5-cm-long fritted LC channel, either unpacked or packed with beads of desired coatings, and a multi-nozzle nano-ESI emitter. This chip allows a detection limit of < 5 red blood cells in the analysis of hemoglobin and it digests directly from microliters of blood. Using similar microchips, they also achieved rapid and multi-dimensional monitoring of diabetes biomarkers in human blood samples through direct TD proteomics analysis. Compared with the packed column design, the monolithic column design omits particle packing and features higher pore interconnectivity and higher surface accessibility to the mobile phase, allowing higher flow rates while minimizing the backpressure. These features are favored by microchip designs, although achieved at the cost of less options for chip substrate materials and more sophisticated fabrication. Redman et al. demonstrated the separation of charge variants of intact monoclonal antibodies prior to online MS identification using a microchip that incorporates a 23-cm-long CE separation. An electroosmotic pump reservoir was integrated as well for sustaining ESI. These designs took the advantages of flexible channel configuration and flow manipulation by microfluidics, enabling efficient separation without the need of conventional LC or CE instrumentation. These separation modules are directly connected to the built-in
ESI interfaces, and the resulting miniaturized devices are readily mounted to commercial instruments.

Other techniques can also be employed to separate proteins in microchips. Field-flow fractionation (FFF) separates macromolecular analytes (such as protein aggregates) by applying a field (temperature, gradient, magnetic, centrifugal, cross flow, etc.), which is perpendicular to the direction of sample transport through a long and narrow channel. It is therefore readily compatible with the typical layouts of microchips. Utilizing the asymmetrical flow FFF variant of FFF, Kim et al. developed a chip-type device that separates proteins for online TD protein identification with MS. Driven by mechanical or electric forces, non-volatile salts or small molecules could exit through a dialysis-like membrane filter and be separated from protein analytes to eliminate their interference with protein signals in MS analysis and their contamination to the mass spectrometer.

### 2.2.2 Designs of micromixing module

A micromixer is virtually a necessary module for a TD-MS microchip due to the ubiquitous need of rapid mixing in TD-MS analysis. Efficient mixing is essential for not only the speed of analysis but also the feasibility of performing time-resolved reactions with accurate duration control and high time resolution, especially at low time scales. The advantage of online mixing over offline mixing is that the reaction time is equivalent to the effective mixing time, which can be precisely regulated via adjustment of channel volume and flow rate, without interference from the duration of sample injection or data acquisition. In contrast, in an offline mixing approach (either automated or manual), the reaction proceeds until the detection step, making it difficult to accurately control the effective reaction time at will. When proteins are unfolded under denaturing conditions to increase their charge states, they may also undergo aggregation over time, which hampers the MS detection and may clog the tubing. Online denaturation allows not only rapid detection prior to extensive aggregation but also flexible adjustment of the denaturing level. Efficient mixing also benefits rapid stabilization of the flow system upon change in flow parameters such as flow rate and mixing ratio. Due to the nature of fluid mechanics, it frequently takes a conventional mixing device (such as a mixing tee) longer than 30 min in response to change in flow parameters. Such delay can be greatly reduced by properly designed micromixers.

In general, the turbulent flow regime is preferred at the mixing site because it results in a much higher mixing efficiency than the laminar flow regime. Accordingly, a higher Reynolds number \( (Re) \) is preferred. However, the dimension of the microscale flow channel, low flow rates used in microfluidics and intrinsic viscosity of protein solutions usually result in a low \( Re \) and a laminar flow, posing challenges for efficient mixing. Carefully designed internal structures and geometries of micromixers can achieve excellent mixing within relatively short distances on a submillimeter scale. Also, a high mixing index could be achieved by keeping in a \( Re \) range of 0.025–120. In general, micromixers can be divided into two categories: active and passive. Active micromixers depend on external energy sources to disturb the fluids and cause a mixing effect, while passive micromixers are based on the structure of the microchannels to enhance molecular diffusion and chaotic advection for efficient mixing. Due to the concerns over structural complexity induced by the existing active micromixer designs, passive micromixers are preferentially used in microchips for protein analysis (Schemes 9–11 in Figure 2B).

Passive micromixers can be classified as two-dimensional (2D) and three-dimensional (3D) according to their differing structures. Passive micromixers have simple planar structures such as obstacles, unbalanced collisions, convergence–divergence channels, and spiral channels. 3D passive micromixers are dependent on complex spatial structures, which require delicate fabrication and can generate various vortices. Svejdal et al. described a microchip containing in-channel monolith mixing plugs capable of performing HDX reaction at subsecond time scales. They introduced monolith mixing plugs to generate tortuous flow paths to shorten the length needed for diffusion and substantially increased the mixing efficiency. Rob et al. introduced a microchip to measure dynamics in weakly structured regions of proteins using subsecond HDX. The rapid mixing module integrated in this chip mixes the fluid that passes through the channel around an inner mixing capillary with the fluid that exits that capillary through a notch, which forces the latter fluid into the narrow space between the capillary and channel walls. Li et al. developed a microchip platform containing passive herring-bone-shaped micromixers that connect multiple switchable reaction channels of different lengths (Scheme 9 in Figure 2B), which allows adjustment of mixing/reaction times over a wider range through not only adjusting flow rate but also selecting flow channels. For HDX reactions, although pH 2.5 and a low temperature are often regarded as the “quenching” conditions, they only minimize the reaction rate instead of quenching the reaction. Efficient mixing with the “quenching” solutions is essential for the accuracy of HDX analysis.
TABLE 1  Representative examples of microchip construction method and materials.

<table>
<thead>
<tr>
<th>Application</th>
<th>Fabrication method</th>
<th>Primary material</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteomics</td>
<td>Soft lithography</td>
<td>Polydimethylsiloxane (PDMS)</td>
<td>60,106–110</td>
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<tr>
<td>3D-printing</td>
<td></td>
<td>Polymethylmethacrylate (PMMA)</td>
<td>28,111,112</td>
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<tr>
<td>3D-printing</td>
<td></td>
<td>Polypropylene (PP)</td>
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<tr>
<td>Etching</td>
<td></td>
<td>Glass</td>
<td>70,113–116</td>
</tr>
<tr>
<td>Etching</td>
<td></td>
<td>Silicon</td>
<td>81,117,118</td>
</tr>
<tr>
<td>Structural analysis</td>
<td>Soft lithography</td>
<td>PDMS</td>
<td>35,105</td>
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<tr>
<td>Etching</td>
<td></td>
<td>PMMA</td>
<td>28</td>
</tr>
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3 | FABRICATION OF TD-MS MICROCHIPS

Materials commonly used for chip fabrication include silicon, glass, and synthetic polymers, such as polymethylmethacrylate and polydimethylsiloxane (PDMS). Key factors affecting material selection include chemical stability, biocompatibility, and hydrophobicity/hydrophilicity required by specific applications. Glass microchips provide advantages in terms of ideal optical performance and modest protein adsorption. However, it suffers from higher cost and difficulty in processing. PDMS microchips are widely used in a variety of applications due to their low cost and simple fabrication procedure. The lithography-based method is often the method of choice for PDMS processing. However, the properties of prepolymer bring significant drawbacks in terms of protein adsorption and difficulty in scaling up. 3D printing has been applied to construct microchips made of 3D-printable synthetic polymers. It exhibits high accuracy and precision in fabricating complex spatial structures, such as the integrated ESI interface, through programmable fabrication procedures. However, like lithography-based methods, 3D printing also suffers from limitations in construction scale and throughput. Table 1 summarizes the fabrication methods and materials used in representative works of developing microchips for MS analysis.

4 | CONCLUDING REMARKS: CONSIDERATIONS FOR DESIGNING TD-MS MICROCHIPS

Higher separation efficiency, lower sample consumption, and higher ionization efficiency are always pursued in developing devices for TD-MS. Issues including those related to the solubility, complexity, and high dynamic range of the proteome still need to be addressed in TD-MS method development. Integrating orthogonal separations in a chip may benefit resolving the heterogeneity of proteoforms, reducing the mass spectral complexity and lowering the dynamic range requirements in individual MS scans. CE-based separation is attractive in chip development because it operates at lower flow rates and lower pressures and can provide multiple MS-compatible separation modes. However, the reproducibility of CE-based separation still needs further improvement.

When the chip per se is designed to be reusable, its durability is largely affected by the durability of its ESI interface. Clogging inside the emitter, inside the microchannels or at the channel-emitter junction is frequently encountered in TD-MS due to factors such as high protein concentration (high viscosity) and protein aggregation. The risk of clogging cannot be eliminated regardless of pretreatment when denaturing conditions are needed. If an emitter is fixed to the chip (or made by extruding a part of the chip), the entire chip cannot fully function once the emitter gets clogged. Connecting the chip with a separate ESI source results in a large void volume. Integrating a finger-tight union on the chip provides a solution to make the emitter both replaceable and reusable. It also allows flexible selection of proper emitters.

The mixing module plays an essential role in regulating reactions needed for a TD-MS method. The effectiveness of the reaction time is dependent on the flow stability and whether the reactants get efficiently mixed. The reaction time is defined by the volume of the mixing channel (between the junction of individual reactant channels and the outlet of the mixing channel) that the mixture travels through and the flow rate of the mixture. Notably, the reaction time is not equivalent to the “response time,” that is, the duration from the onset of flow parameter change to the point when the flow equilibrium and setpoints are reached. The response time is affected by parameters including channel geometry and mixing efficiency and can be reduced via careful channel design.

Due to the rapid progress in the fields of microfluidics and protein MS, it is difficult to cover all aspects of these fields in this mini-review. Future development of microchips for intact and TD protein analysis using MS
may also be inspired by designs of separation and mixing modules currently employed for applications in other fields. Interested readers are referred to recent reviews for more detailed and comprehensive discussion on topics of history and development of microfluidics, fabrication and applications of microfluidic devices, interfaces of microfluidic and bottom-up proteomics, and sample processing for native MS and TD-MS analysis.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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