Microprobe-Capture In-Emitter Elution: An Affinity Capture Technique to Directly Couple a Label-Free Optical Sensing Technology with Mass Spectrometry for Top-Down Protein Analysis

Ruben Yiqi Luo[1,2], Samuel Yang[1]

[1]Department of Pathology, School of Medicine, Stanford University, Stanford, CA, USA

[2]Clinical Laboratories, Stanford Health Care, Palo Alto, CA, USA

Corresponding Author: Ruben Yiqi Luo

Address: 3375 Hillview Ave, Palo Alto, CA 94304

Tel: 650-724-1318

Email: rubenluo@stanford.edu

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Abstract

Affinity capture of an analyte by a capture agent is one of the most effective sample preparation approaches for protein analytes. We describe a new affinity capture technique for top-down protein analysis, called microprobe-capture in-emitter elution (MPIE), which can directly couple a label-free optical sensing technology (next-generation biolayer interferometry, BLI) with MS. To implement MPIE, an analyte is first captured on the surface of a microprobe, and subsequently eluted from the microprobe inside an electrospray emitter. The capture process is monitored in real-time via BLI. When electrospray is established from the emitter to a mass spectrometer, the analyte is immediately ionized via electrospray ionization (ESI) for HR-MS analysis. By this means, BLI and HR-MS are directly coupled in the form of MPIE-ESI-MS. The performance of MPIE-ESI-MS was demonstrated by the analysis of β-amyloid 1-40 and transferrin using both standard samples and human specimens. In comparison to the conventional affinity capture techniques such as bead-based immunoprecipitation, MPIE innovates the affinity capture methodology by introducing real-time process monitoring and providing binding characteristics of analytes, offering more information-rich experimental results. Thus, MPIE is a valuable addition to the TD-MS sample preparation toolbox, and more applications of MPIE-ESI-MS in top-down protein analysis are expected.

Introduction

Top-down mass spectrometry (TD-MS), a mass spectrometry (MS) methodology to analyze proteins in their intact state without prior enzymatic digestion, can be used to elucidate post-

translational modifications and amino acid variations in proteoforms. It has been widely employed in biological research and started being accepted in clinical diagnostics.[1–3] While it is an ideal tool to analyze intact protein targets, sample preparation, i.e., the procedure to purify analytes and remove interferents from sample matrices, plays an essential role in determining the quality of data acquired during TD-MS analysis.[4,5] Among the sample preparation approaches for protein analytes, affinity capture of an analyte by a capture agent is one of the most effective options (also named immunoaffinity capture if the capture agent is an antibody). A well-known example is mass spectrometric immunoassay (MSIA), which uses an antibody immobilized on porous materials as the capture agent.[6] The challenges to the conventional affinity capture techniques lie in the lack of process monitoring. As no signal is generated during typical affinity capture processes, it may cost significant time and efforts to optimize experiment conditions, verify affinity capture performance, and troubleshoot experiment protocols when MS responses are weak or absent.

On the other hand, in the research field of biomolecular interactions, label-free optical sensing technologies have been employed as mainstream platforms to identify interacting partners and characterize biologics.[7] A label-free optical sensing technology senses the refractive index change or optical thickness change on a sensing surface caused by biomolecular interactions, achieving real-time measurement without employing a reporter molecule (enzyme, fluorophore, etc.). The real-time monitoring of biomolecular complex formation can provide interaction characteristics such as kinetic and affinity constants. Incorporating a label-free optical sensing technology with MS can be an ideal solution to overcome the challenges to the conventional affinity capture techniques.

In the past decade, label-free optical sensing technologies have advanced from complex fluidics-based platforms to simple dip-and-measure sensing microprobes. A novel technology of this kind is called thin-film interferometry (TFI) or next-generation biolayer interferometry (BLI), which utilizes quartz-glass BLI microprobes to measure biomolecular interactions taking place on the microprobe tips.[8,9] The dip-and-measure feature brings flexibility into experiment design: the microprobes loaded with biomolecules can be applied to various sample types for biomolecular interaction measurement and/or affinity capture. Thus, next-generation BLI can be a candidate technology to be incorporated with MS. Efforts were made previously to connect traditional BLI (using optical fiber BLI microprobes instead of quartz-glass ones) with MS, however the two technologies were not directly coupled and the analytes must be eluted before MS analysis.[10–12] Because only a tiny amount of analyte (one layer of molecules) could be captured on the surface of a microprobe, the "indirect coupling" required either multiple affinity capture-elution cycles or an additional step of concentration to mitigate the analyte dilution in elution liquid, resulting in elongated experimental procedures.

In this article, we describe a new affinity capture technique for top-down protein analysis, called microprobe-capture in-emitter elution (MPIE), which enables direct coupling of next-generation BLI with MS. To implement MPIE, an analyte is first captured on the surface of a microprobe, and subsequently eluted from the microprobe inside an electrospray emitter. The capture process is monitored in real-time via BLI. When electrospray is established from the emitter to a mass spectrometer, the analyte is immediately ionized via electrospray ionization (ESI) for HR-MS analysis. By this means, BLI and HR-MS are directly coupled in the form of MPIE-ESI-MS,

which can add significant value to MS, or more specifically, TD-MS since affinity capture is typically applied to protein analytes and enzymatic digestion is not included.

Materials and Methods

Materials and Samples

LC-MS grade water, acetonitrile, and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA). β-Amyloid (Aβ) 1-40 standard was purchased from Abcam (Cambridge, UK), and a biotinylated mouse monoclonal anti-Aβ IgG antibody (anti-Aβ Ab) from Biolegend (San Diego, CA). Human transferrin (Tf) standard (Tf purified from human serum) was purchased from Sigma-Aldrich (St. Louis, MI), and a mouse monoclonal anti-transferrin IgG antibody (anti-Tf Ab) from Sinobiological (Wayne, PA), and biotinylated using EZ-Link NHS-PEG4-Biotin from Thermo Fisher Scientific (Waltham, MA). Remnant cerebrospinal fluid (CSF) and serum samples from general patients were obtained from Stanford Health Care, following approved IRB protocols for the use of remnant patient specimens.

Sample Preparation

Aβ 1-40 and Tf were used as model protein targets to study the performance of MPIE-ESI-MS. The Aβ 1-40 standard was first dissolved in DMSO and then diluted in phosphate-buffered saline at pH 7.4 with 0.02% Tween 20, 0.05% sodium azide, and 0.2% BSA (PBST-B) to make a concentration series of Aβ 1-40 standard samples. The Tf standard was first dissolved in

phosphate-buffered saline at pH 7.4 with 0.02% Tween 20 and 0.05% sodium azide (PBST), and then diluted in PBST to make a concentration series of Tf standard samples. The capture agents anti-Aβ Ab and anti-Tf Ab were diluted in PBST-B to 10 μg/ml for use. The Aβ 1-40 standard samples were prepared right before use. For Aβ analysis, CSF samples were 1:1 diluted in PBST-B. For Tf analysis, CSF samples were 1:1 diluted in PBST and serum samples were 1:19 diluted in PBST.

MPIE-ESI-MS Experiment

An MPIE-ESI-MS experiment consists of two parts: BLI-based affinity capture and in-emitter elution ESI-MS. The BLI-based affinity capture was carried out in 3 steps: (1) a BLI microprobe pre-coated with streptavidin was dipped into a capture agent (anti-Aβ Ab or anti-Tf Ab) solution for 10 min to load the capture agent; (2) the microprobe was dipped into a sample for 10 min to capture the corresponding analyte (Aβ or Tf), and then rinsed in the running buffer (PBST-B for Aβ analysis or PBST for Tf analysis) for 1 min to remove non-specifically bound molecules. The in-emitter elution ESI-MS was carried out in 6 steps: (1) an electrospray emitter was filled with a sheath liquid (10 mM ammonium formate in water); (2) after affinity capture, the microprobe was rinsed in the sheath liquid for 10 s, inserted into the emitter through the regular open end, and settled in the tapered end by gravity; (3) the emitter was mounted to the ESI ion source; (4) the elution liquid-delivering capillary was inserted into the emitter through the regular open end and positioned right behind the microprobe; (5) a positive voltage was applied to the sheath liquid in the emitter to establish electrospray; (6) MS data acquisition was initiated, and injection of the elution liquid was started subsequently. The emitter was placed ~2 mm away from the

mass spectrometer inlet with the electrospray voltage set at 2.2 kV. The injection of the elution liquid was driven by 5 psi of pneumatic pressure. The parts described above are shown in Figure 1A. The procedures of running BLI-based affinity capture and setting up the in-emitter elution ESI-MS are illustrated in Figure 1B and Figure 1C, respectively.

The BLI-based affinity capture was implemented in a Gator Plus analyzer (Gator Bio, Palo Alto, CA). Cylindrical quartz-glass BLI microprobes with 1 mm diameter were used. The in-emitter elution ESI-MS was implemented in an EMASS-II ESI ion source which coupled an ECE-001 capillary electrophoresis (CE) instrument (CMP Scientific, Brooklyn, NY) with an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA). A glass electrospray emitter with 1.5 mm O.D. and 1.17 mm I.D. (CMP Scientific, Brooklyn, NY) was used for MPIE, which has a regular open end and a tapered open end (tip orifice diameter 20-30 μm). A fused-silica capillary (360 μm O.D., 50 μm I.D., 100 cm) was used to deliver an elution liquid (80% acetonitrile and 2% formic acid in water) into the emitter. was employed to carry out MS analysis.

The MS parameters for Aβ analysis were ion-transfer capillary temperature 320°C, S-lens RF level 50, and number of microscans 5. Full MS dd-MS^2 mode was applied: primary mass spectra acquired in positive polarity at resolution 140K; stepped normalized collision energy 20, 30, and 40 for fragmentation; secondary mass spectra acquired for the top 5 abundant precursor ions at resolution 70K. When analyzing CSF samples, targeted-SIM dd-MS^2 mode was used to increase the analytical sensitivity. The isolation window was set at m/z 4.0 and the number of microscans was increased to 10. The MS parameters for Tf analysis were ion-transfer capillary temperature

350°C, S-lens RF level 50, and number of microscans 10. Primary mass spectra were acquired in positive polarity at resolution 17.5K.

Data Analysis

The acquired data in each MPIE-ESI-MS experiment was viewed as a time trace of MS responses, and the elution time window of an analyte was identified by checking the molecular ions of the analyte at each time point. The data in the elution time window were selected for deconvolution using Biopharma Finder 4.1 (Thermo Fisher Scientific, San Jose, CA), employing the Xtract algorithm for Aβ analysis and the ReSpect algorithm for Tf analysis. MS peaks of analytes were displayed in deconvoluted spectra in their uncharged state, showing monoisotopic molecular masses through the Xtract algorithm and average molecular masses through the ReSpect algorithm.

Results and Discussion

The performance of MPIE-ESI-MS was demonstrated by the analysis of Aβ 1-40 and Tf, which are typical human proteins used as clinical diagnostic markers. Aβ 1-40 is a 40-amino acid small protein (or peptide) closely related to Alzheimer's disease.[13] Tf is a 679-amino acid glycosylated large protein that plays an essential role in iron metabolism, and its proteoforms are used to diagnose CSF leak.[14] A concentration series of Aβ 1-40 standard samples at 3.3, 1.1, 0.37, and 0.12 μg/ml were analyzed by MPIE-ESI-MS, as shown in Figure 2A. Similarly, a concentration series of Tf standard samples at 1.0, 0.50, 0.25, 0.13 μg/ml, and 0.063 μg/ml were analyzed by

MPIE-ESI-MS, as shown in Figure 3A. In both cases a blank sample (running buffer) was employed as both the reference for the label-free optical sensing measurement and the negative control for the MS analysis. Real-time monitoring of affinity capture process was implemented in the next-generation BLI analyzer, and the reference-subtracted time traces of label-free optical sensing responses (sensorgrams) were obtained. The sensorgrams showed the association phase (analyte-capturing phase) and dissociation phase (sensing surface rinsed in the running buffer). The intact-protein MS analysis of Aβ 1-40 standard samples showed a dominant MS peak at 4327.1 Da and that of Tf standard samples showed a dominant MS peak at 79554 Da in deconvoluted mass spectra. The blank samples revealed only noise in the raw mass spectra and no MS peak could be resolved after deconvolution. In the Aβ 1-40 analysis, the dominant analyte should be Aβ 1-40 as the measured molecular mass (monoisotopic mass) matched the theoretical molecular mass 4327.148 Da calculated from its primary structure. The assignment could be confirmed by MS^2 fragmentation: the MS^2 analysis of the +4 precursor ion of Aβ 1-40 (m/z 1083.3) in an Aβ 1-40 standard sample is shown in Figure 2B. In the deconvoluted MS^2 mass spectrum, 37 b-fragments and 9 y-fragments were identified by matching their masses to the predicted fragments derived from the amino acid sequence of Aβ 1-40, resulting in a sequence coverage of 95%. Moreover, a minor MS peak at 4349.1 Da was observed in some deconvoluted mass spectra, which was probably the sodium adduct of Aβ 1-40. In the Tf analysis, since it is known that a tetrasialo-Tf proteoform predominates in normal human serum and the Tf standard was purified from human serum, the dominant analyte was likely the major serum Tf proteoform as the measured molecular mass (average mass) matched the theoretical mass 79554.71 Da calculated from its primary structure with the reported N-glycans of the tetrasialo-Tf.[15,16] Note

that the eluted Tf molecules were denatured and iron-free since a protein-denaturing elution liquid was used.

In the past, limited capture capacity of label-free optical sensing devices was a major barrier that hindered the combination of label-free optical sensing technologies and MS. Because only a minute amount of analyte (one layer of molecules) could be captured on the surface of a microprobe, the captured analyte must be eluted into a small volume of solution to make enough concentration for MS analysis. Therefore, the use of the particular electrospray emitter was crucial to MPIE-ESI-MS: the captured analyte on a microprobe was eluted into a tiny space at the tapered end of the electrospray emitter, which has a calculated volume of about 0.8 μl based on the dimensions; the tiny space enforced restricted dispersion of eluted analyte, enhancing the analytical sensitivity of the subsequent MS analysis. As demonstrated in Figure 3A, the limit of detection for the Tf standard was at least 0.063 μg/ml, which corresponded to a saturation rate of anti-Tf Ab at 17% (calculation in supporting information). Provided that there was one layer of anti-Tf Ab molecules on the surface of a microprobe to capture Tf molecules and the maximum density for a typical protein on a label-free optical sensing surface is 1-2 ng/mm^2,[17] the amount of anti-Tf Ab on a microprobe (tip area 4 mm^2) should be within 40 fmol and the captured Tf molecules should be no more than 7 fmol, which was an very low quantity. However, the 7 fmol Tf molecules would make a concentration of about 8 nM after dispersed in the tapered end of the electrospray emitter, resulting in a decent analyte concentration for MS analysis. The feature of restricted dispersion of eluted analyte in MPIE-ESI-MS substantially brings up the concentration of the minute amount of analyte captured by a BLI microprobe, allowing for the successful coupling of a label-free optical sensing technology with MS with good analytical sensitivity.

A sensorgram not only indicates the amount of the analyte captured on a microprobe, but it can also be fitted to the Langmuir molecular interaction model to obtain the kinetic and affinity constants between the two binding partners (Langmuir model explained in supporting information).[18] Using the sensorgrams in Figure 2A and Figure 3A, the association kinetic constants (k_a) and dissociation kinetic constants (k_d) could be measured, and the affinity constant (dissociation equilibrium constant K_D) could be calculated from the measured kinetic constants. Regarding the binding pair of anti-Aβ Ab and Aβ 1-40, k_a was 7.4×10^5 $M^{-1}s^{-1}$, k_d was 4.9×10^{-3} s^{-1}, and K_D was 6.6×10^{-8} M. Regarding the binding pair of anti-Tf Ab and Tf, k_a was 2.8×10^5 $M^{-1}s^{-1}$, k_d was 1.1×10^{-3} s^{-1}, and K_D was 3.9×10^{-9} M. The affinity constants were in the regular range of mouse monoclonal antibodies, and consistent with the knowledge that antibodies bind more strongly to larger-sized antigens.

MPIE-ESI-MS was applied to human specimens to evaluate its applicability to clinical diagnostics. For Aβ 1-40 analysis, two normal CSF samples drawn from individuals without Alzheimer's disease were tested. Given the normal Aβ 1-40 concentration in CSF at the level of 10 ng/ml and the affinity constant (K_D) of the binding pair at 6.6×10^{-8} M,[19] the saturation of anti-Aβ Ab was around 3.4% (calculation in supporting information). As the low saturation rate of capture agent limited the amount of Aβ 1-40 captured on the microprobes, targeted-SIM dd-MS^2 mode was used to increase the analytical sensitivity of the MS analysis. Aβ 1-40 was successfully detected in the two normal CSF samples, as shown in Figure 2C. For Tf analysis, a serum sample and a CSF sample drawn from the same individual were tested. In Figure 3B, the deconvoluted mass spectrum of the serum sample showed a dominant MS peak at 79554 Da and

small MS peaks around 79554 Da. The MS peaks should be mainly serum Tf proteoforms and the dominant MS peak at 79554 Da was the major serum Tf proteoform, consistent with that measured from the Tf standard samples. In Figure 3C, the deconvoluted mass spectrum of the CSF sample showed a significant MS peak at 78008 Da and small MS peaks close to 78008 Da, in addition to the serum Tf proteoforms. The MS peaks around 78008 Da should be mainly brain Tf proteoforms, consistent with the reports that two types of Tf proteoforms (serum and brain) are present in CSF.[20,21] The test results of the human specimens demonstrated that MPIE-ESI-MS has the potential to be used as a clinical diagnostic tool.

It should be noted that the capture agents in MPIE are not limited to antibodies. Other biologics that bind to a specific target with sufficient affinity can be used, such as lectins or enzymes. It was demonstrated in the analysis of Aβ 1-40 and Tf that binding characteristics of the binding pair (capture agent and analyte) could be obtained together with the MS analysis of the analyte in a single MPIE-ESI-MS experiment. Combining the binding characteristics with the structural information obtained from MS analysis can add significant value to the study of unknown targets. In addition, as label-free optical sensing devices are equipped with optimal surface chemistry to mitigate non-specific binding of interferents,[17,22] the performance of affinity capture in MPIE is more advantageous than the conventional affinity capture techniques. Thus, MPIE-ESI-MS can be a useful tool for biological research.

Conclusion

It was demonstrated that the affinity capture technique MPIE can directly couple a label-free optical sensing technology (next-generation BLI) with MS. The employment of next-generation BLI brings unique advantages to top-down protein analysis: (1) BLI allows for effective step-by-step optimization of affinity capture conditions without requiring MS analysis; (2) real-time monitoring of affinity capture process provides an estimated amount of captured analyte for every sample, serving as a means of quality control; (3) captured analyte is eluted into a tiny space at the tapered end of the electrospray emitter to enhance the analytical sensitivity of MS analysis. In comparison to the conventional affinity capture techniques such as bead-based immunoprecipitation, MPIE innovates the affinity capture methodology by introducing real-time process monitoring and providing binding characteristics of analytes, offering more information-rich experimental results. Thus, MPIE is a valuable addition to the TD-MS sample preparation toolbox, and more applications of MPIE-ESI-MS in top-down protein analysis are expected.

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Figures

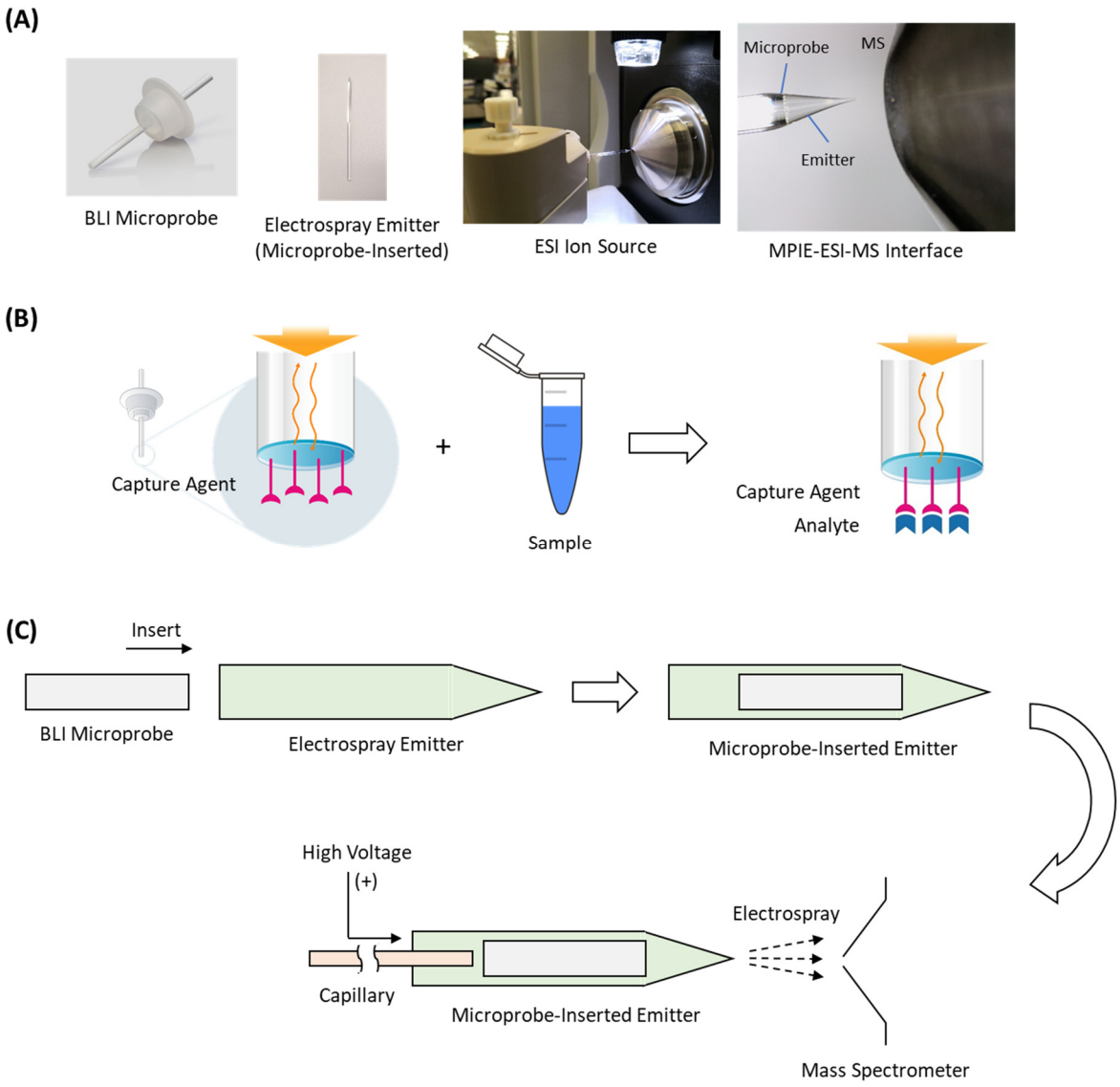

Figure 1. (A) Pictures of BLI microprobe, electrospray emitter, ESI ion source, and MPIE-ESI-MS interface under stereoscope. (B) Illustration of running BLI-based affinity capture. (C) Illustration of setting up the in-emitter elution ESI-MS.

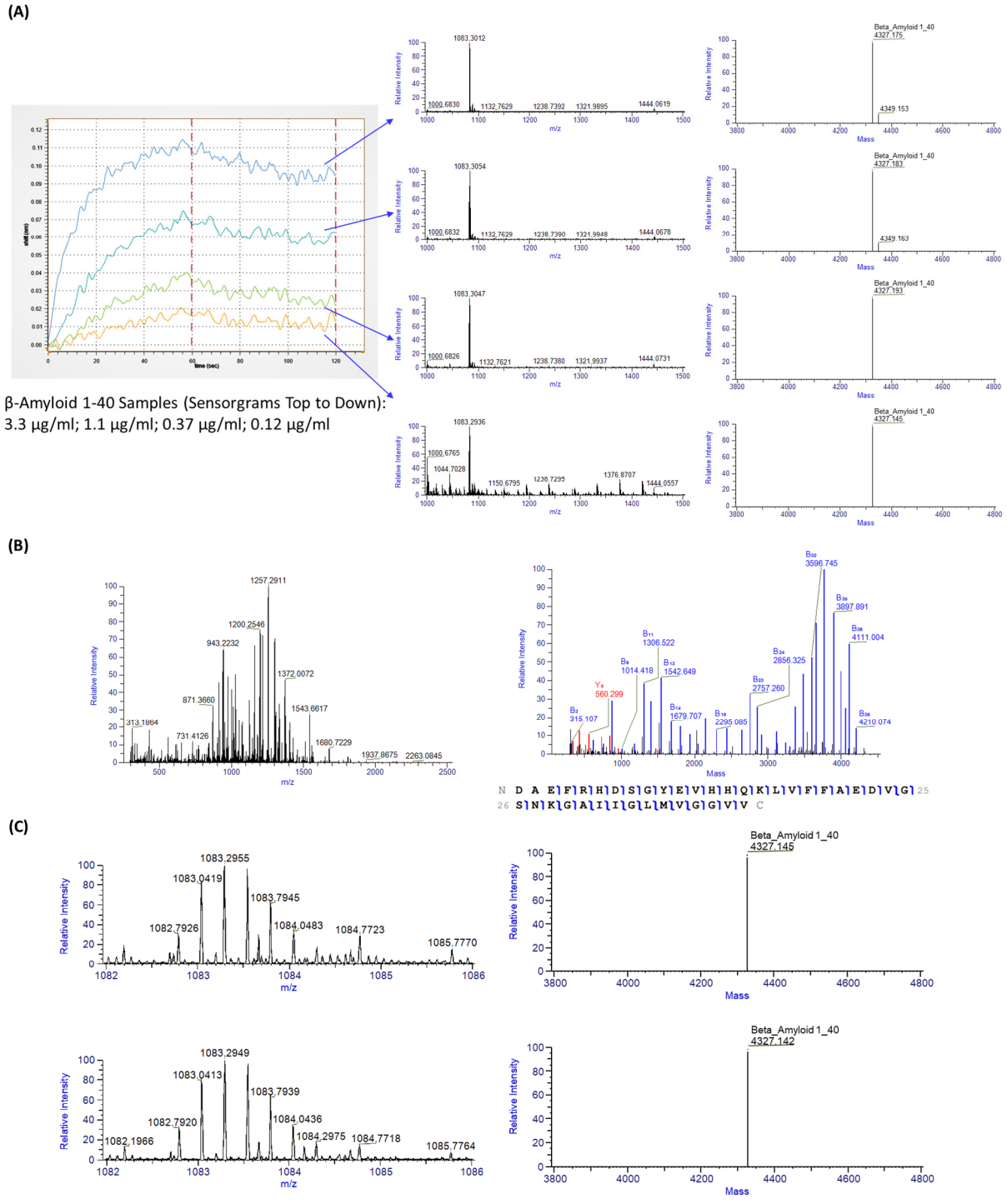

Figure 2. MPIE-ESI-MS analysis of Aβ 1-40. (A) A concentration series of Aβ 1-40 standard samples: sensorgrams obtained from the 4 microprobes measuring 4 samples at different concentrations (left); raw mass spectra and deconvoluted mass spectra of eluted Aβ 1-40

molecules from the 4 microprobes (right), showing Aβ 1-40 (4327.1 Da) and probably the sodium adduct of Aβ 1-40 (4349.1 Da). (B) MS^2 analysis of the +4 precursor ion of Aβ 1-40 (m/z 1083.3) in a Aβ 1-40 standard sample: raw MS^2 mass spectrum (left) and deconvoluted MS^2 mass spectrum (right) of Aβ 1-40 fragments, showing the identified fragments of Aβ 1-40. (C) Two normal CSF samples: raw mass spectra (left) and deconvoluted mass spectra (right) of eluted Aβ 1-40 molecules, demonstrating the capture and analysis of Aβ 1-40 in CSF.

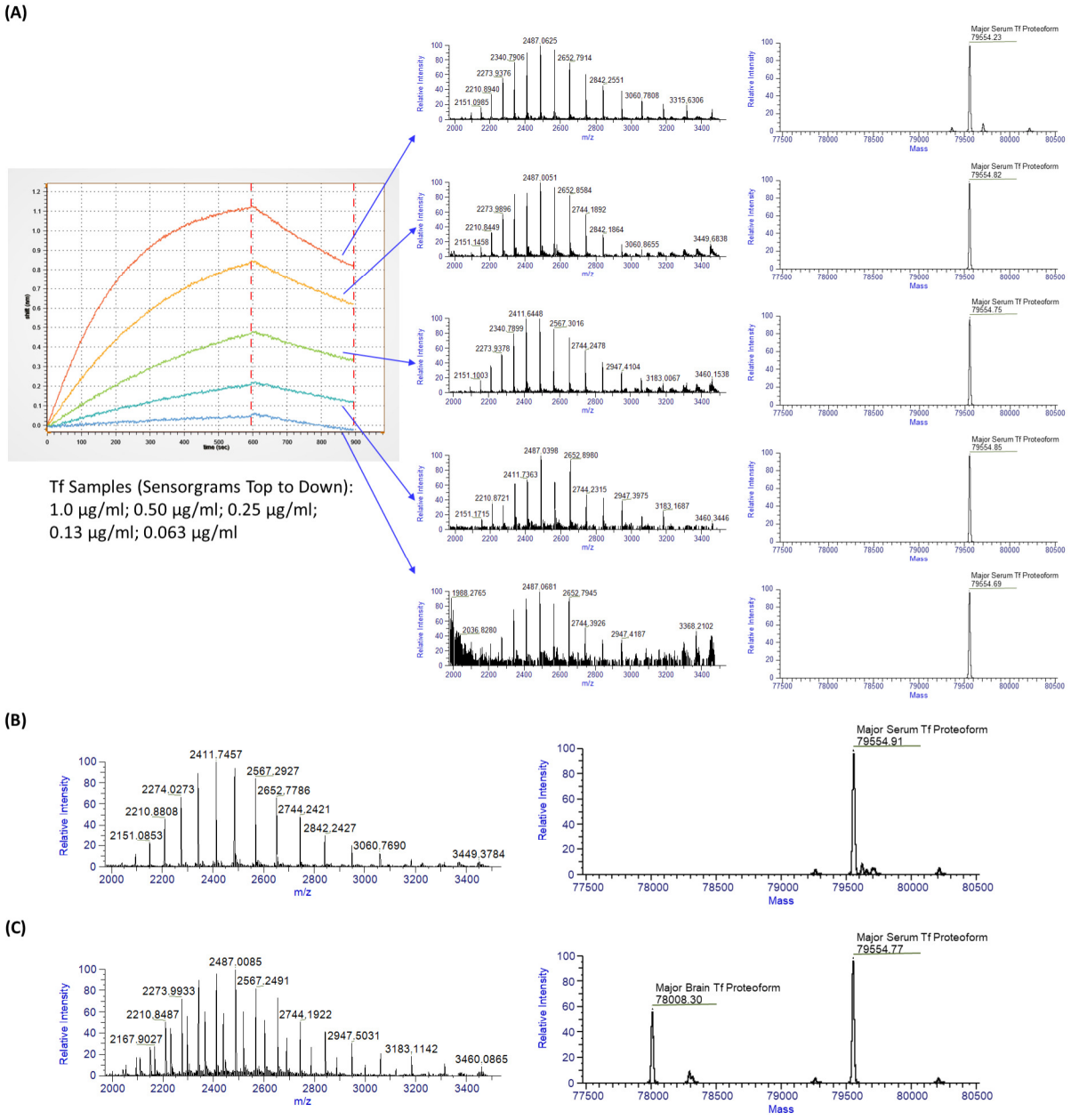

Figure 3. MPIE-ESI-MS analysis of Tf. (A) A concentration series of Tf standard samples: sensorgrams obtained from the 5 microprobes measuring 5 samples at different concentrations (left); MS raw mass spectra and deconvoluted mass spectra of eluted Tf molecules from the 5 microprobes (right), showing the major serum Tf proteoform (79554 Da). (B) A serum sample: raw mass spectrum (left) and deconvoluted mass spectrum (right) of eluted Tf molecules, showing the major (79554 Da) and minor serum Tf proteoforms. (C) A CSF sample: raw mass

spectrum (left) and deconvoluted mass spectrum (right) of eluted Tf molecules, showing the major (78008 Da) and minor brain Tf proteoforms in addition to the serum Tf proteoforms. The serum and CSF samples were drawn from the same individual.

Supporting Information

Langmuir molecular interaction model

In label-free analysis, a sensorgram shows the binding kinetics between two binding partners (analyte and capture agent), including association phase and dissociation phase. The binding kinetics can be described by the Langmuir molecular interaction model and the related equations. The kinetic and affinity constants can be obtained by fitting the sensorgram to the equations.

Langmuir Molecular Interaction Model

$$\text{Analyte + Capture Agent} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Complex}$$

Solution Microprobe Surface Microprobe Surface

Association Phase Kinetic Equation:

$$\frac{d[AB]}{dt} = k_1[A][B] - k_2[B]$$

$$K_D = \frac{k_2}{k_1}$$

Association Phase Kinetic Equation:

$$[AB] = \frac{[A][B]_0}{K_D + [A]}\left[1 - e^{-(k_1[A]+k_2)t}\right]$$

Dissociation Phase Kinetic Equation:

$$[AB] = [AB]_0 e^{-k_2 t}$$

k_1 – association kinetic constant, k_2 – dissociation kinetic constant, K_D – equilibrium constant, [A] – concentration of analyte in solution, [B] – density of unbound capture agent on microprobe surface, $[B]_0$ – density of all capture agent on microprobe surface, [AB] – density of complex on

microprobe surface, $[AB]_0$ – density of complex on microprobe surface at the starting point of dissociation phase

Saturation rate of capture agent on the surface of a microprobe

In label-free analysis, the density of the complex formed by an analyte and a capture agent can be calculated using the Langmuir molecular interaction model. At equilibrium, the density is described by this equation.

$$[AB] = \frac{[A][B]_0}{K_D + [A]}$$

The saturation rate of the capture agent can be calculated by this equation.

$$\frac{[AB]}{[B]_0} = \frac{[A]}{K_D + [A]}$$

In the MPIE-ESI-MS analysis of the 0.063 μg/ml Tf standard sample, given the concentration of analyte (Tf) at the limit of detection 0.063 μg/ml (0.79 nM) and the equilibrium constant K_D at 3.9×10^{-9} M, the saturation rate was 17%.

In the MPIE-ESI-MS analysis of Aβ 1-40 in normal CSF samples, given the concentration of analyte (Aβ 1-40) at the level of 10 ng/ml (2.3 nM) and the equilibrium constant K_D at 6.6×10^{-8} M, the saturation rate was 3.4%.