MnESI-MS Platform for Sensitive and Robust Analysis of Intact Antibodies

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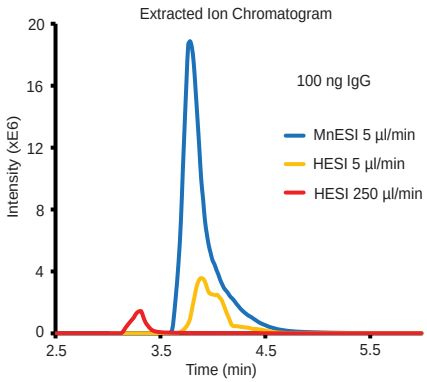
Goal

To establish a microflow-nanospray ESI-MS (MnESI-MS) platform for mass spectrometry analysis of intact monoclonal antibody (mAb), that achieves high sensitivity, high quantitation accuracy, and high robustness, using Newomics® MnESI sources, M3 multinozzle emitters, and flow splitting kits (as needed for high-flow HPLC/UPLC systems).

Key Words

Microflow-nanospray ESI-MS (MnESI-MS), Ion source, M3 emitter, Multinozzle, Intact protein MS, Monoclonal Antibody (mAb), Glycoform, Quantitation, Limit of Quantitation, Microflow LC-MS, High-flow LC-MS, Flow Splitting



Introduction

LC-MS analysis of intact proteins (top-down approach) can provide whole molecular quantification and high-level sequence and structure information, including catabolism or other modifications to proteins. Recent advances in sample preparation techniques and high-resolution MS instruments have led to the increased application of quantitative bioanalysis of large protein therapeutics using top-down approach [1-6]. Currently high-flow LC-MS has been widely used for analysis of intact proteins. Due to the low ionization efficiency at high (analytical) flow rates, the sensitivity is poor with a typical limit of quantitation (LOQ) in the range of ~100 ng/ml. The Newomics award-winning silicon multinozzle emitters (M3 emitters) split the incoming microflow eluent into multiple nanoflows at each nozzle, thereby significantly enhancing the ionization efficiency and reducing the matrix effects for ESI-MS ([7-10] and Newomics Application Notes [11-15]). In this Application Note, we demonstrate a new microflow-nanospray ESI-MS platform for LC-MS quantification of intact mAb. We obtained over 10-fold improvement in sensitivity and achieved a LOQ of 10 ng/mL with NIST mAb. Furthermore, we achieved a CV of less than 5% which outperformed the high-flow LC-MS method with the same amount of injection material. Our MnESI-MS platform is amenable for a microflow LC system, as well as a high-flow LC system when interfaced with a Newomics® flow splitting kit. The new LC-MnESI-MS method will facilitate rapid characterization and quantitation of therapeutic antibodies.

Methods

1. Sample Preparation

The IgG monoclonal antibody was purchased from NIST (SRM 8671). Bovine Serum Albumin (BSA, Cat. # A3059-10G) and HPLC-grade water and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (FA) was purchased from Fisher Scientific (Cat. # A117-50). Aliquoted antibody stored at -80°C freezer was thawed at room temperature and serially diluted into solutions of 0.1% FA or 50 ng/µl BSA in 0.1% FA before LC-MS analysis.

2. MnESI-MS platform for LC-MS analysis

For sensitivity comparison, 100 ng neat IgG mAb was injected using a 1 µl full loop injection method. For IgG dilution analysis, 5 µl IgG of various concentration in 50 ng/µl BSA solution was injected using a user defined program. The microflow LC was performed with a 0.3 mm ID BEH C4 column (50 x 0.3 mm, Waters Cat. #186009260 for neat IgG analysis; 100 x 0.3 mm, Waters Cat. #186009261 for IgG dilution analysis using BSA as carrier protein). The high-flow LC was performed with a 2.1 mm ID BEH C4 column (50 x 2.1 mm, Waters Cat. # 186004495). All columns have the same chemistry with 300 Å pore size and 1.7 µm bead size. The column temperature was set at 70 °C. The LC gradients were listed in **Table 1** and **Table 2**. Mobile phase A was 3% ACN with 0.2% FA; Mobile phase B was 97% ACN with 0.2% FA.

Table 1. LC gradient for sensitivity comparison using 100 ng neat IgG.

Time (min)	% B
0	20
0.2	20
5	45
5.8	60
6.2	90
10	90
10.1	20
15	20

Table 2. LC gradient for IgG dilution analysis using BSA as carrier protein.

Time (min)	% B
0	20
2.2	20
2.5	28
7.4	38
7.6	90
10.8	90
10.9	20
14	20

Mass spectra were acquired on a Thermo Fisher Orbitrap Q Exactive Plus mass spectrometer interfaced with an UltiMate 3000 RSLC nano UPLC system (Thermo Fisher Scientific). MnESI ion source was either directly connected to the microflow LC column or to a high-flow column through a Newomics® flow splitter (**Figure 1**).

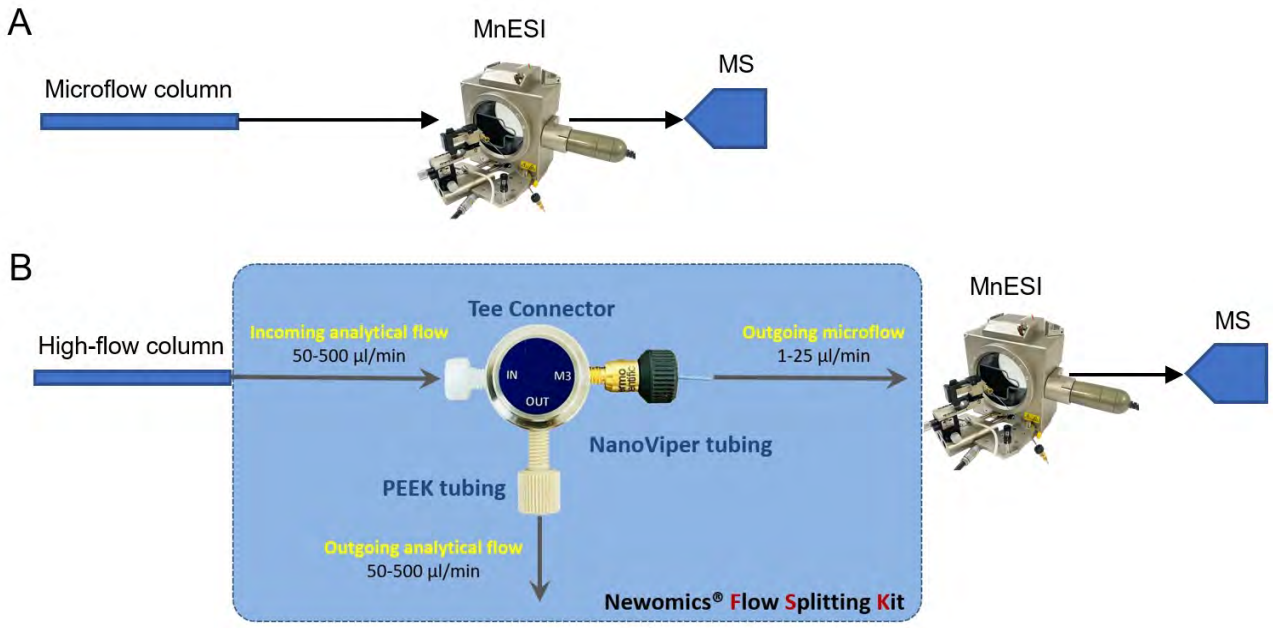


Figure 1. Workflows of the LC-MnESI-MS platform. A. Microflow LC-MS setup. A microflow LC column was connected to the MnESI-MS directly. B. High-flow LC-MS setup. A high-flow LC column was connected to MnESI-MS through a flow splitter. The majority (~90%) of the incoming flow was diverted to a UV detector or directly to waste.

We performed comparison of the LC-MS analysis among three platforms. The LC and MS source conditions were listed in **Table 3**. The following emitters were used in our studies: a Thermo Fisher high-flow needle insert assembly (HESI high-flow, single nozzle, 100 µm ID, catalog # Opton 53010), a Thermo Fisher low-flow needle insert assembly (HESI low-flow, single nozzle, 75 µm ID, catalog # Opton 53011), a Newomics® M3 emitter (8 nozzles, 10 µm ID, catalog # E8N10MU01), and a Newomics® M3 emitter (8 nozzles, 20 µm ID, catalog # E8N20MU01 for post column splitting analysis).

For M3 emitters, the electrospray voltage was optimized at ~3 kV with 2.4 mm from the ion transfer tube. The optimal source condition for MnESI at 20 µl/min for post-column splitting analysis was the same as those for MnESI at 5 µl/min. 1 unit of desolvation gas was used for M3 emitters.

For the HESI needle, the electrospray voltage was optimized at 3.8 kV at position A for 5 µl/min flow rate, and 3.5 kV at position C for 250 µl/min flow rate. The ion transfer tube temperature was set at 275 °C for microflow and 300 °C for high flow.

Table 3. LC and MS source conditions for the three platforms.

Platform	LC-MnESI-MS	Microflow LC-MS	High-flow LC-MS
Flow rate (µl/min)	5	5	250
Column ID (mm)	0.3	0.3	2.1
Ion Source	Newomics MnESI Source	Ion Max Source	Ion Max Source
Emitter/Sprayer	M3 emitter (10 µm ID nozzle x8)	HESI probe (75 µm ID)	HESI probe (100 µm ID)
Emitter/Probe Position	2.4 mm from ion transfer tube	Position A	Position C
Spray Voltage (kV)	3	3.8	3.5
Gas Flow	1 unit sheath gas	5 units sheath gas	35 units sheath gas, 10 units AUX gas (300 °C)
Capillary Temperature	275 °C	275 °C	300 °C

The shared MS conditions for the three platforms were listed in **Table 4**. For IgG serial dilution analysis using BSA as the carrier protein, a full MS scan from m/z 2,500 to 3,200 was acquired.

Table 4.Shared MS conditions

Full MS scan range m/z	1,800-4,200
Resolution	17,500
AGC target	3.0 X 10e6
Max injection time	150 ms
Microscans	10
S-lens RF level (%)	80
In source CID (eV)	80

3. Data Analysis

Data were analyzed using Xcalibur software and Biopharma Finder 2.0 software (Thermo Fisher Scientific). The quantification of the most abundant IgG proteoforms (G0FG1F) for the dilution curve was processed by Biopharma Finder 2.0 using the sliding window deconvolution algorithm with the ReSpect function. The window size was set at 0.1 min with 30% offset.

Results and Discussion

1. MnESI-MS platform significantly improves sensitivity for intact monoclonal antibody analysis

We performed a three-way comparison to assess the sensitivity performance of the MnESI-MS platform. We examined the sensitivity gain of MnESI over the conventional HESI platform using 100 ng on-column neat IgG mAb under the optimized conditions for each analysis. First, we experimented a wide range of flow rates (1 - 25 µl/min) for MnESI and found 5 µl/min had the best balance of sensitivity and robustness for antibody analysis. For example, 5 µl/min was about 1.2- and 1.5-fold more sensitive than 10 and 15 µl/min flow rate, respectively. Although reduction of the flow rate to 2 µl/min further increased the sensitivity, it sacrificed robustness and throughput. Therefore, we chose 5 µl/min as our default flow rate for MnESI and compared to HESI at the same flow rate or HESI at 250 µl/min with an analytical column.

Figure 2A shows the representative extracted ion chromatogram of the most abundant ion from each platform. We observed a slight charge envelope shift with the highest charge state of 51+ for microflow (MnESI and microflow HESI) compared to 49+ for high-flow HESI (**Figure 2B**), suggesting better ionization of the denatured IgG for microflow LC-MS. We demonstrated an average of 17.9-fold sensitivity gain of MnESI over high-flow HESI, and an average of 2.5-fold sensitivity gain of MnESI over microflow HESI using the peak area of the most abundant ion for quantification (**Figure 2C**).

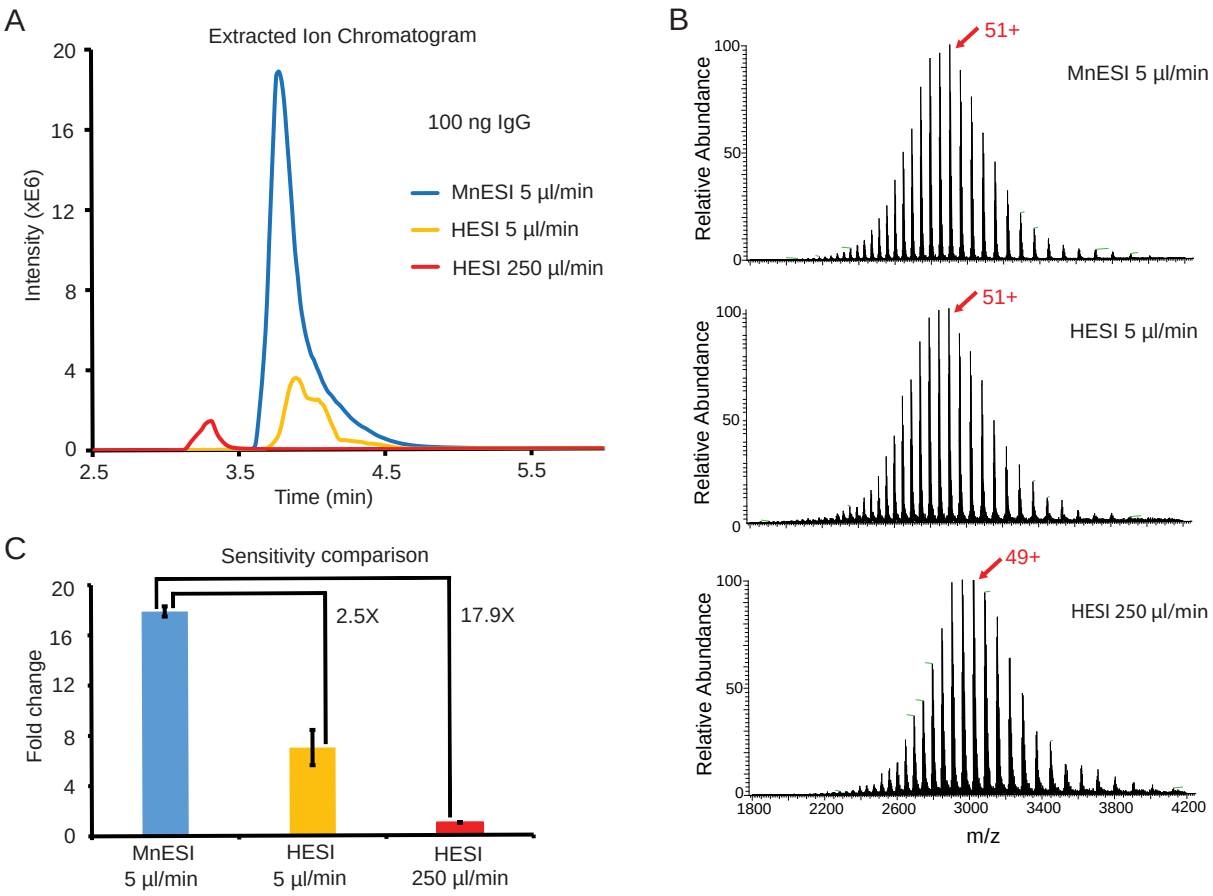


Figure 2. Sensitivity comparison of three platforms for intact antibody quantitation. A. Representative extracted ion chromatograms of the most abundant ion from the three platforms. B. Compared to MnESI and microflow HESI, full MS spectrum from high-flow HESI shows the charge envelope shifts towards the higher m/z, with the most abundant charge state shifted from 51+ to 49+. C. Sensitivity comparison of the three platforms. The peak area intensity from extracted ion chromatogram of the most abundant ion from 100 ng on column of neat IgG mAb was used to calculate the fold change.

2. MnESI-MS platform achieves a lower limit of quantitation and excellent robustness for intact monoclonal antibody analysis

We then examined whether the sensitivity gain of MnESI over the conventional HESI platform can be translated into the improvement in limit of detection and quantification. We performed serial dilutions of IgG using BSA as the carrier protein. We optimized the LC gradient to separate the IgG peak from that of BSA and used a narrow MS scan range to improve IgG detection. In addition, we chose the charge deconvolution method for quantification over the extracted ion chromatogram method because it mitigated the effect of IgG peak overlapping with that of BSA at their low concentration (**Figure 3**). Using this method, we were able to detect intact IgG down to 4 ng/ml by MnESI-MS. In addition to all five major glycoforms, some low abundant proteoforms of IgG were identified at 200 ng/ml (**Figure 3B**) [16].

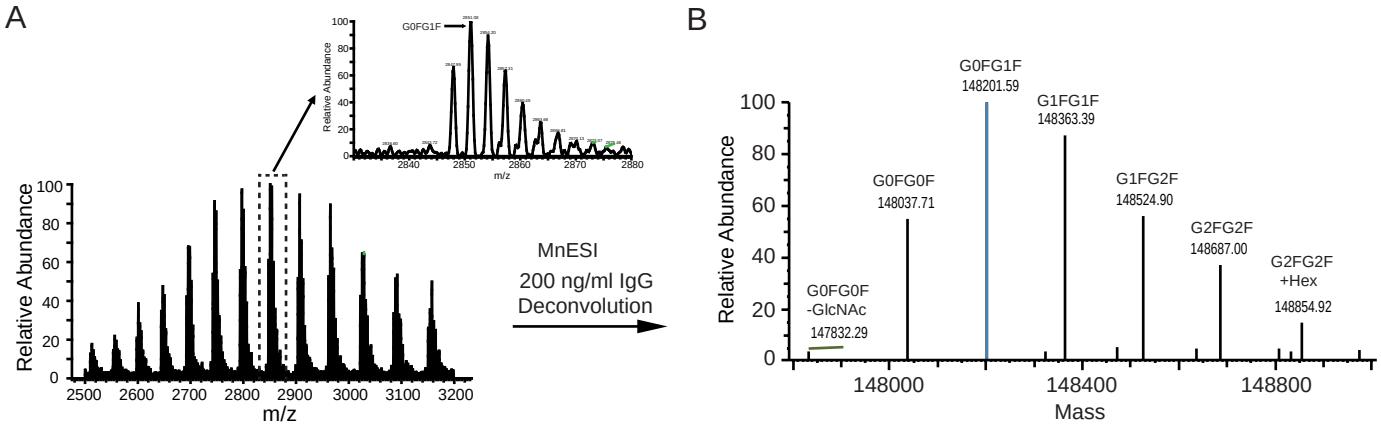


Figure 3. MS data processing and analysis method for IgG mAb dilution analysis. A. IgG mAb charge envelope from LC-MnESI-MS at 200 ng/ml. The insert shows a zoom-in view of the most abundant charge state (within the dashed box). B. After charge deconvolution, all five major glycoforms and a few low abundant pretoforms of IgG were identified, and the most abudant glycoform of IgG (G0FG1F) was used for quantification analysis.

We used the most abundant glycoform of IgG (G0FG1F) for quantification with the linear regression and $1/x^2$ weighting. A linear dynamic range of more than 3 orders of magnitude was achieved for MnESI at 5 µl/min flow rate, with a limit of quantification (LOQ) at 10 ng/ml (**Figure 4A**). CV values for the dilution curve were within 20% at low concentrations (< 100 ng/ml), with the overall lower CV at higher IgG concentration (**Figure 4B**). The quantitation accuracies were determined to be above 90% (**Figure 4C**).

In contrast, we achieved more than 2 orders of magnitude of the linear dynamic range for high-flow HESI, with a LOQ at 100 ng/ml (**Figure 4D**). The CVs from high-flow HESI were mostly within 10%, with overall lower CV at higher IgG concentrations (**Figure 4E**). The quantitation accuracies were mostly above 90% (**Figure 4F**). Compared to HESI at 250 µl/min, MnESI at 5 µl/min exhibited a 10-fold lower LOQ (**Figure 4A** and **4D**), which is consistent to the 10-fold sensitivity gain from the neat IgG analysis (**Figure 2**).

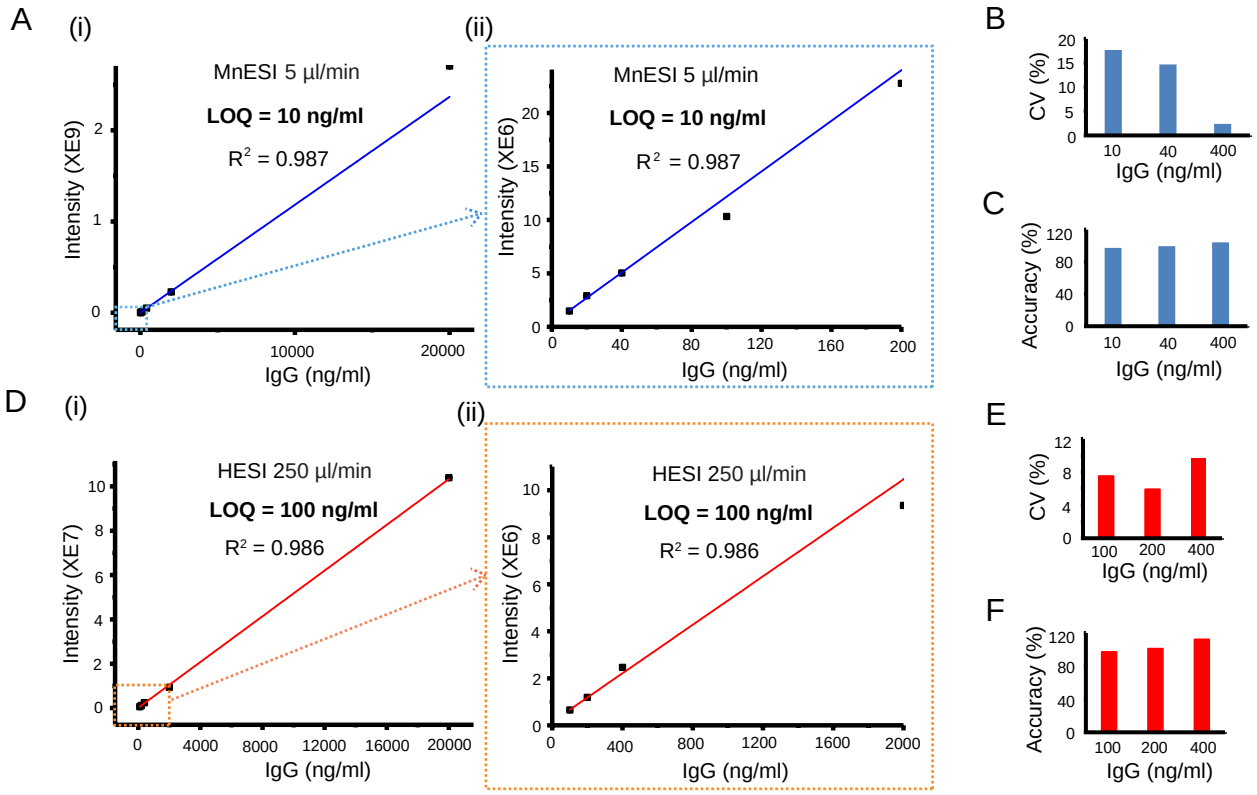


Figure 4. Dynamic range and robustness comparison for two platforms. The calibration curve was generated using the most abundant isoform (G0FG1F) from the charge deconvolution. A. MnESI platform. (i). The calibration curve from LC-MnESI-MS. (ii). A zoom-in from the boxed area in A (i) for low concentrations. B and C. CV and accuracy from MnESI platform. D. HESI platform. (i). The calibration curve from high-flow LC-MS (HESI at 250 µl/min). D (ii). A zoom-in from the boxed area in B (i) for low concentrations. E and F. CV and accuracy from the HESI platform. A linear regression with $1/x^2$ weighting was applied to the data. A 10-fold improvement in LOQ was achieved for LC-MnESI-MS compared to high-flow LC-MS using HESI.

We also performed the robustness assessment of MnESI-MS for intact mAb analysis. A CV of 1.8% was achieved from the 100 consecutive LC-MS runs, without any significant decrease in signal intensity when using EIC peak area for quantification at 10 µl/min flow rate (**Figure 5**). Additionally, we did not observe any significant degradation of this emitter after 100 runs. The lifetime of the emitter will be explored in future studies.

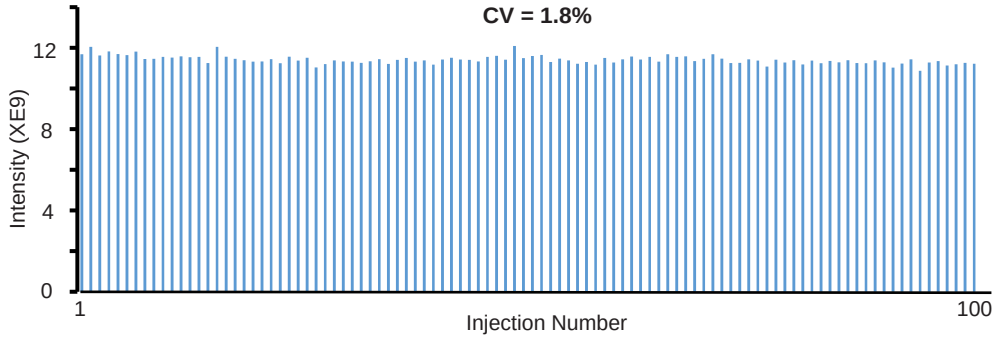


Figure 5. Robustness analysis of the LC-MnESI-MS platform. EIC peak area from 100 consecutive runs of 100 ng IgG on a single emitter was used for quantification. The flow rate was 10 µl/min. The CV was 1.8%.

3. MnESI-MS platform seamlessly integrates with the high-flow LC system for intact monoclonal antibody analysis

Since the traditional workflow for intact MS analysis of IgG is high-flow LC-MS by HESI, we evaluated the performance of coupling MnESI with a high-flow LC system and 2.1mm ID column using a Newomics® post-column splitting kit (**Figure 1**). A T-splitter was used to split the analytical flow of 200 µl/min from the LC column down to a microflow of 20 µl/min that is delivered to a 20 µm-ID, 8-nozzle M3 emitter for spray into MS. A representative extracted ion chromatogram of the most abundant ion from both platforms is shown in **Figure 6A**. Compared to the conventional high-flow method without splitting, this new method by flow splitting to M3 emitters achieved slightly better sensitivity (~1.3 fold), with only 10% of IgG delivered to MS (**Figure 6B**). Our MnESI-MS workflow with post-column splitting allows simultaneous detection of mAb by UV and MS without the need to change the LC system. In addition, majority of the IgG injected onto the high-flow column was diverted to UV detector or waste rather than going into the mass spectrometer, minimizing the contamination of ion cone and C-trap of the QE instrument. This will significantly improve the assay robustness for intact antibody quantitation.

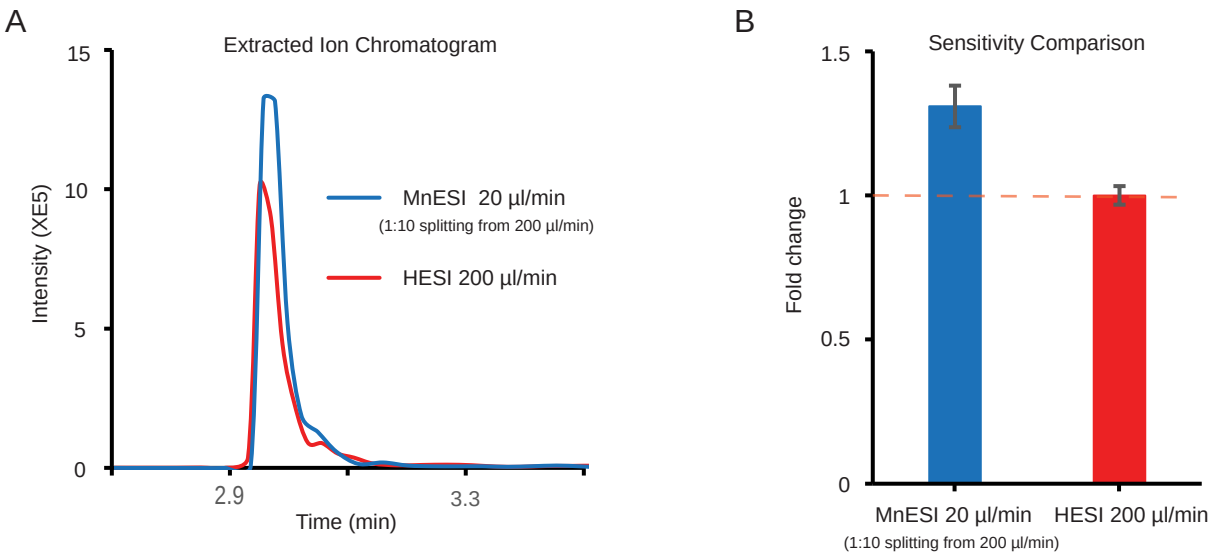


Figure 6. Integration of MnESI-MS platform with the high-flow LC-MS workflow using a post-column splitting kit. A. Representative extracted ion chromatogram of the most abundant ion from LC-MnESI-MS at 20 µl/min (after 1:10 splitting from 200 µl/min) and HESI at 200 µl/min (high-flow LC-MS). B. Sensitivity comparison of LC-MnESI-MS at 20 µl/min (after 1:10 splitting from 200 µl/min) and HESI at 200 µl/min (high-flow LC-MS). The same amount of starting IgG (100 ng on column at 20 ng/µl concentration) was injected for both workflows. A 20 µm- ID, 8-nozzle M3 emitter was used in the analysis for MnESI-MS.

Conclusions

We have established a new MnESI-MS platform for sensitive and robust LC-MS analysis of intact monoclonal antibodies. By interfacing to an Orbitrap Q Exactive Plus mass spectrometer, Newomics® MnESI platforms have achieved high sensitivity and high robustness for quantification of intact mAb. We demonstrate the following significant advantages of our LC-MnESI-MS platform using M3 emitters over the traditional high-flow LC-HESI-MS platform:

1. More than 10-fold sensitivity gain and ~10-fold lower LOQ (10 ng/mL).
2. Robust performance for microflow LC-MS with a CV of < 2% over 100 injections.
3. Compatibility with high-flow LC systems with the post-column flow splitting, showing increases in sensitivity and robustness (due to reduced MS contamination).

References

1. Donnelly, D.P. et al. (2019) Best practices and benchmarks for intact protein analysis for top-down mass spectrometry. Nat Methods 16 (7), 587-594.
2. Kang, L. et al. (2017) Simultaneous Catabolite Identification and Quantitation of Large Therapeutic Protein at the Intact Level by Immunoaffinity Capture Liquid Chromatography-High-Resolution Mass Spectrometry. Anal Chem 89 (11), 6065-6075.
3. Kang, L. et al. (2020) LC-MS bioanalysis of intact proteins and peptides. Biomed Chromatogr 34 (1), e4633.
4. Kellie, J.F. et al. (2020) Intact Protein Mass Spectrometry for Therapeutic Protein Quantitation, Pharmacokinetics, and Biotransforma tion in Preclinical and Clinical Studies: An Industry Perspective. J Am Soc Mass Spectrom.
5. Qiu, X. et al. (2018) Quantitation of intact monoclonal antibody in biological samples: comparison of different data processing strategies. Bioanalysis 10 (13), 1055-1067.
6. Williams, J.P. et al. (2020) Top-Down Characterization of Denatured Proteins and Native Protein Complexes Using Electron Capture Dissociation Implemented within a Modified Ion Mobility-Mass Spectrometer. Anal Chem 92 (5), 3674-3681.
7. Kim, W. et al. (2007) Microfabricated monolithic multinozzle emitters for nanoelectrospray mass spectrometry. Anal Chem 79 (10), 3703-7.
8. Mao, P. et al. (2013) Multinozzle emitter array chips for small-volume proteomics. Anal Chem 85 (2), 816-9.
9. Mao, P. et al. (2011) Multinozzle emitter arrays for nanoelectrospray mass spectrometry. Anal Chem 83 (15), 6082-9.
10. Chen, Y. et al. (2018) Quantitation of Intact Proteins in Human Plasma Using Top-Down Parallel Reaction Monitoring-MS. Anal Chem 90 (18), 10650-10653.
11. Newomics Application Note 1: https://www.newomics.com/wp-content/uploads/2019/05/Newomics-Application-Note-1.pdf
12. Newomics Application Note 2: https://www.newomics.com/wp-content/uploads/2019/05/Newomics-Application-Note-2.pdf
13. Newomics Application Note 3: https://www.newomics.com/wp-content/uploads/2019/12/Newomics-Application-Note-3_shotgun-lipidomics.pdf
14. Newomics Application Note 4: https://www.newomics.com/wp-content/uploads/2020/02/Newomics-Application-Note-4_Native-MS.pdf
15. Newomics Application Note 5: https://www.newomics.com/wp-content/uploads/2020/11/Application-Note-5-Native-Protein-Complexes.pdf
16. ACS Book series: "State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization"; Volume 2, Biopharmaceutical Characterization: the NIST mAb Case Study; Chapter 1, Determination of the NISTmAb Primary Structure.

Ordering Information

Product	Catalog #
MnESI Source for Thermo Scientific New Generation Mass Spectrometers	IS-T01
MnESI Source for Thermo Scientific Legacy Mass Spectrometers	IS-T02
MnESI Source for Bruker Mass Spectrometers	IS-B01
M3 Emitter, 10 µm ID, 8-nozzle	E8N10MU01
M3 Emitter, 20 µm ID, 8-nozzle	E8N20MU01
M3 Emitter, 20 µm ID, 5-nozzle	E5N20MU01
Flow Splitting Kit for Microflow LC-MS, 1-5 µl/min	FSK-01
Flow Splitting Kit for Microflow LC-MS, 5-15 µl/min	FSK-02
Flow Splitting Kit for Microflow LC-MS, 10-25 µl/min	FSK-03
Flow Splitting Kit for Microflow LC-MS, custom	FSK-10

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