A Simple and Flexible Infusion Platform for Automated Native Mass Spectrometry Analysis

Running Title: An Automated Native Mass Spectrometry System

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ABSTRACT: High throughput native mass spectrometry analysis of proteins and protein complexes has been enabled by recent development of infusion and liquid chromatography (LC) systems, which often include complete LC pumps without fully utilizing their gradient flows. We demonstrated a lower-cost infusion cart for native mass spectrometry applications using a single isocratic solvent pump that can operate at both nano- and high-flow configurations (0.05-150 µL/min) for both infusion and online buffer exchange experiments. The platform is controlled via open-source software and can potentially be expanded for customized experimental designs, offering a lower cost alternative to labs with limited budget and/or needs in student training.

INTRODUCTION

Native mass spectrometry (MS) is a valuable analytical technique that enables the direct detection of noncovalent interactions in the gas phase, with applications spanning detection of distinct proteoforms, ligand binding, and protein-protein interactions[1]. However, the typical native MS workflow is time consuming, requiring manual sample clean-up and infusion of samples onto the mass spectrometer (see these references published in the past year[2-6]). Implementation of more automated systems to increase throughput have been developed to eliminate the requirement of manual injection by coupling with autosampler[7] or NanoMate[8] devices. Bypassing the need for manual sample clean-up procedures using online buffer exchange (OBE) through size exclusion chromatography (SEC)[9-12], affinity chromatography[13], or filter-based clean-up[14] further increases throughput.

Implementation of this automated pipeline typically includes the use of a high pressure liquid chromatography (HPLC) system for high flow (1-2500 µL/min flow rate), and a separate HPLC system for nano flow (0.05-1.5 µL/min flow rate) to encompass a wide range of flow rates and applications. These robust, off-the-shelf systems are unlikely to be replaced in industry settings. However, infusion and OBE applications often do not require a solvent gradient, thus a full binary solvent HPLC is not necessary. Herein, we demonstrate an infusion cart platform that reduced cost compared to the previous design[7] for automated native MS by using a single pump that can operate at both nano- and high-flows (0.05-150 µL/min), cutting down cost to about 1/5th of the cost required to implement both flow systems separately. It also used open-source software for easy transferability to other MS systems and expansion to more sophisticated workflows. The simplified platform is attractive to labs with limited budget. In addition, the open, customizable platform offers great opportunities for student hands-on training in academic settings. We demonstrated the successful analysis of several protein standards on this system for both flow injection and OBE analysis and provided detailed information for implementing this system. We also highlight some key troubleshooting conducted that was valuable for training purposes.

METHODS

For detailed materials and cart construction information, refer to the Supporting Information.

Infusion MS

Samples were offline buffer exchanged and manually static sprayed onto a Waters Synapt G2-Si q-TOF (Supporting Information). To test the automated infusion cart (Figure 1), samples were stored in the Pal autosampler cooled to 4 °C, with a 5 µL sample loop, and 7 µL injected to account for dead volume on either side of the sample loop. Note that any commercial autosampler could be used. Sample queues and communication between the VICI pump and the mass spectrometer were achieved using an in-house control and communication software, LCMSNet[7], available open source on github (https://github.com/PNNL-Comp-Mass-Spec/LCMSNet). Solvent was 100 mM ammonium acetate, with PEEK tubing (I.D. 381 µm) from the pump to the valve flowing at 0.01-0.3 µL/min, and fused silica line (I.D. 20 µm) directly infusing samples with an HF-etched tip (I.D. 20 µm). Mass spectra were acquired between 400 and 8000 m/z, with the specified MS tune methods (Table S1).

Online buffer exchange (OBE) MS

OBE was completed using a size exclusion column (NativePac OBE-1, 50 x 2.1 mm, ThermoScientific) following the configuration shown in Figure 1. PEEK tubing (I.D. 381 µm), trimmed to 5 cm in length, was placed on either side of the column. The column was pre-equilibrated following the method described in Supporting Information. Extended fused silica line (I.D. 20 µm, length 1 m) was included immediately prior to the emitter to reduce current and was necessary for stable spray at these higher flow rates, as discussed elsewhere[11]. MS method parameters, and the optimized valve diversion method used to divert salt and other contaminants present in the samples to waste can be found in Table S2 and S3, respectively. Since the optimized time to divert sample to waste occurred while protein was still flowing through the extended silica tubing, the syringe was loaded with 100 mM ammonium acetate and set at 100 µL/min

to push sample to the detector while pump flow was diverted to waste.

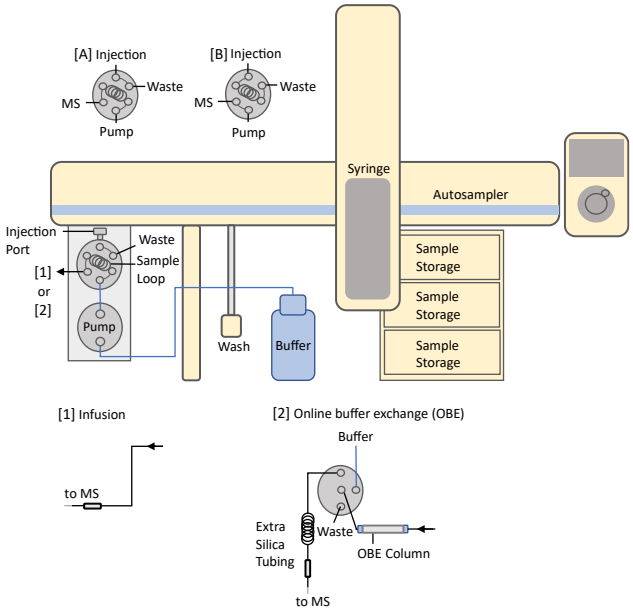

FIGURE 1 Infusion cart configuration includes a Pal autosampler, a pump connected to a valve in [B] configuration during sample injection into the sample loop, and [A] configuration during [1] direct infusion of sample onto the MS, or [2] loading of sample onto the OBE column for online sample clean-up prior to infusion onto the MS.

RESULTS AND DISCUSSION

To benchmark the performance for infusion MS, sample runs assessing limits of detection (LOD), extent of sample carry-over, and limits of flow rate were completed. LOD and extent of carry-over were evaluated by infusing carbonic anhydrase (CA) at 0.3 µL/min onto a Waters Synapt G2-Si q-TOF (Figure 2A). Approximately 4-fold higher sample quantity was required using the cart compared to the conventional manual pulled glass capillaries to overcome LOD when set to a signal-to-noise (S/N) threshold of 3. The extent of carry-over did not exceed S/N = 3 until CA concentrations of 0.5 µM (75 ng) and higher were infused, with an average % carryover of 4.5 %. Carryover was reduced to 0.13 % when a blank run was included between sample runs of 5 µM. Thus, the recommended working concentration using this system is 0.5-5 µM to overcome LOD and reduce carryover. Instrument clogging at the injection port due to buffer salts in the waste line was found to be the main source of instrument downtime. We performed daily cleaning using 0.015 % (w/v) DDM in ammonium acetate, followed by dIH_2O, to manually clean the injection valve, bypassing the lines going into the mass spectrometer. This could be implemented as an automated shutdown method if desired by replacing one of the ammonium acetate washes with the DDM-containing ammonium acetate wash. Although we found the remainder of the system not to require any additional cleaning, a second valve could be added to the configuration to toggle between running and wash solvents. For some applications, such as when studying native protein-protein interactions (PPIs), operating at low flow rates is desired to maximize sensitivity and minimize destruction of native protein complexes during desolvation[15, 16]. To evaluate the lower limits for flow rate for the system, 150 ng of CA was infused at 0.01, 0.05, and 0.3 µL/min and data were summed across the 150-, 30-, and 5-minute runs, respectively. If operating at lower flow rates and acquiring data for shorter time periods, a higher flow blank run after each sample run could be used to more rapidly flush dead volume in the system, or alternative systems such as the nanoMate[8] could be used. The results demonstrate maintenance of LOD at each of the flow rates tested (Figure 2B), with consistent spray (Figure S3) and resolution (Figure 2C) maintained down to 0.05 µL/min.

To assess the ability to maintain native PPIs during sample infusion with the cart, concanavalin A (ConA) was infused at 0.1 µL/min. A sample mass spectrum obtained from cart infusion was compared to data obtained using conventional manual static spray and demonstrated the ability to maintain ConA tetrameric species during cart infusion (Figure 2D). The fraction of peak area corresponding to tetrameric ConA was not statistically significantly different when comparing the infusion cart operated at 0.1 µL/min to the static spray results ($p = 0.077$, $p = 0.756$, and $p = 0.288$ for monomer, dimer, and tetramer, respectively, using a student two-tailed paired t-test, Figure 2E). However, the charge states shifted to higher values with the infusion cart method, presumably due to different flow rates and desolvation processes, as described by others[17]. Overall, the infusion cart yielded similar data to our manual method for stoichiometry analysis.

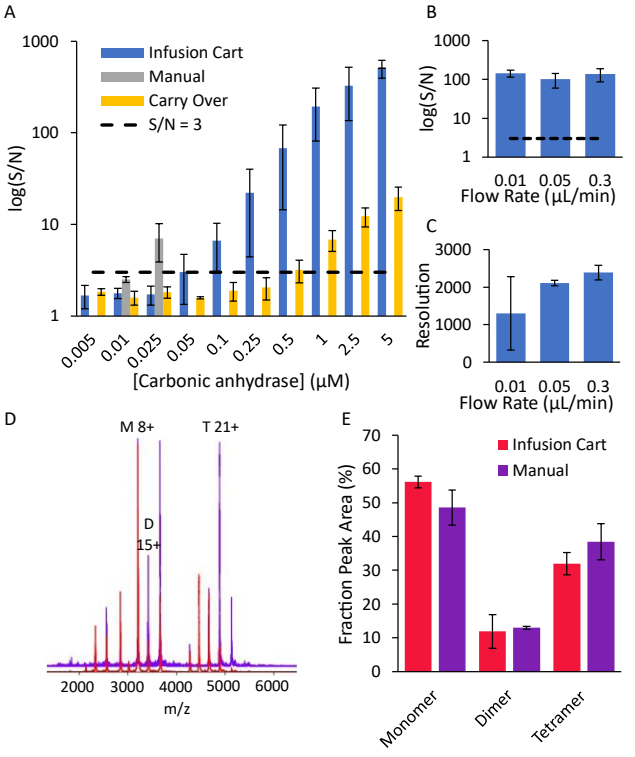

FIGURE 2 Assessment of infusion cart performance by evaluating A) LOD and sample carry-over by infusing varying concentrations of CA, B) limits of flow rate and C) mass spectral peak resolution by infusing 1 µM CA at 0.01, 0.05, and 0.3 µL/min (n = 3). Maintenance of PPIs was compared to the manual pulled glass capillary method using 0.5 µM ConA with example mass spectra obtained using each method overlaid in D). E) The fraction of peak area (%) associated with monomer (M), dimer (D), and tetramer (T) was assessed (n = 3).

Improvements in throughput have been accomplished previously using OBE[9-11]. We adopted this method using the same infusion cart set-up simply by adding a column in-line. The PEEK tubing was trimmed to minimize peak broadening

observed due to sample dilution (Figure S4). Within the recommended specifications of the pump, this configuration can be operated at flow rates up to ~150 µL/min (Table S4), reducing total MS data acquisition time to 2 minutes, with protein samples separated from salts and other buffer contaminants (Figure 3A). Working sample concentrations were ~10-fold higher (10-25 µM) than with the infusion set-up at lower flowrate. The ability to divert the flow of sample to waste prior to salt infusion onto the MS was shown to be successful, although some cytochrome C (Cyt C, ~12 kDa) could not be separated from eluted salt due to the resolution limit of the column. When executing the valve diversion method, a delayed start of data acquisition on the order of a few seconds was observed, likely due to a delay between reading and executing commands in the MassLynx software. This resulted in higher MW proteins to partially elute prior to the start of data acquisition at 150 µL/min. Thus, the flowrate was reduced to 100 µL/min to capture protein elution across all MW tested (Figure 3B). We tested Herceptin in original formulation solution and β-galactosidase (β-Gal) tetramer (~465 kDa) in Tris buffer with $MgCl_2$. Both were successfully desalted online in our system and yielded well resolved spectra (Figure 3C and 3D).

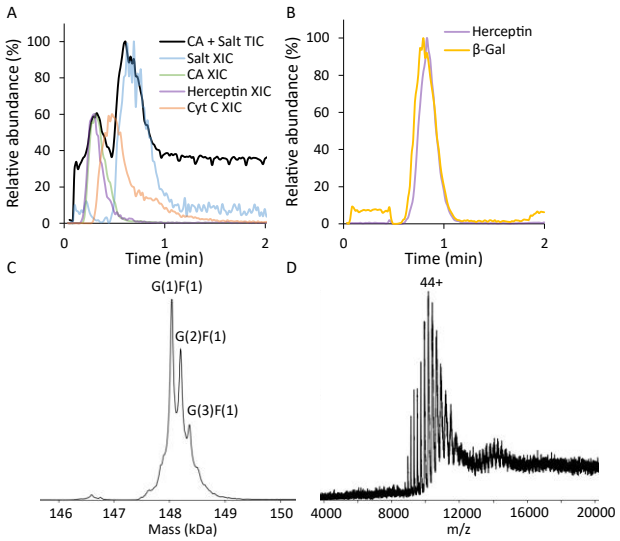

FIGURE 3 A) Total ion chromatogram (TIC) of injection of CA spiked with 200 µM sodium acetate (salt) without valve diversion to waste overlaid with extracted ion chromatograms (XIC) of CA, salt, and offline buffer exchanged Cyt C and Herceptin at 150 µL/min. Protein XIC traces were normalized to 60 % relative abundance to match protein relative abundance in the CA spiked with salt TIC for comparison purposes. B) XIC of proteins without offline buffer exchange and after OBE and valve diversion to waste at 100 µL/min. Example of C) a charge deconvoluted mass spectrum of Herceptin showing intact proteoforms corresponding to mass shifts of up to 3 galactose (G) and 1 fucose (F), and D) a m/z spectrum of β-Gal injected with OBE.

CONCLUSIONS

The work presented here demonstrated the ability to construct an automated native infusion cart at significantly lower cost compared to a previous design by replacing a full binary HPLC with a simplified flow pump. Additionally, the versatility of the accessible flow rate range from the pump and the open-source control software allows for facile implementation to other MS platforms and expansion to custom workflows, especially for research laboratories with limited budget and/or needs in technical training.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional materials, methods, cart construction, LCMSNet interface screenshots, and infusion cart performance figures (PDF)

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Author Contributions

The manuscript was written by SMT and edited by all. SMT and CCB contributed equally to figure generation and conducting experiments. DO assisted in cart construction and offered valuable input during troubleshooting. BCG helped with interfacing LCMSNet with cart and wrote corresponding SI section. MZ conceptualized work, acquired funding and managed the project. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

1. Tamara, S.; den Boer, M. A.; Heck, A. J. R. High-Resolution Native Mass Spectrometry. *Chem. Rev.* **2022**, *122* (8), 7269-7326. DOI: 10.1021/acs.chemrev.1c00212.

2. Du, Y.; Zhao, F.; Xing, J.; Liu, Z.; Cui, M. Stabilization of Labile Lysozyme-Ligand Interactions in Native Electrospray Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2023**, *34* (3), 366-373. DOI: 10.1021/jasms.2c00238.

3. Borotto, N. B.; Richards, T. K. Rapid Online Oxidation of Proteins and Peptides via Electrospray-Accelerated Ozonation. *J. Am. Soc. Mass Spectrom.* **2022**, *33* (11), 2078-2086. DOI: 10.1021/jasms.2c00182.

4. Schrader, R. L.; Walker, T. E.; Russell, D. H. Modified Ion Source for the Improved Collisional Activation of Protein Complexes. *J. Am. Soc. Mass Spectrom.* **2023**. DOI: 10.1021/jasms.3c00071.

5. Phetsanthad, A.; Li, G.; Jeon, C. K.; Ruotolo, B. T.; Li, L. Comparing Selected-Ion Collision Induced Unfolding with All Ion Unfolding Methods for Comprehensive Protein Conformational Characterization. *J. Am. Soc. Mass Spectrom.* **2022**, *33* (6), 944-951. DOI: 10.1021/jasms.2c00004.

6. Kostelic, M. M.; Hsieh, C.-C.; Sanders, H. M.; Zak, C. K.; Ryan, J. P.; Baker, E. S.; Aspinwall, C. A.; Marty, M. T. Surface Modified Nano-Electrospray Needles Improve Sensitivity for Native Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2022**, *33* (6), 1031-1037. DOI: 10.1021/jasms.2c00087.

7. Orton, D. J.; Tfaily, M. M.; Moore, R. J.; LaMarche, B. L.; Zheng, X.; Fillmore, T. L.; Chu, R. K.; Weitz, K. K.; Monroe, M. E.; Kelly, R. T.; Smith, R. D.; Baker, E. S. A Customizable Flow Injection System for Automated, High Throughput, and Time Sensitive Ion Mobility

Spectrometry and Mass Spectrometry Measurements. *Anal. Chem.* **2018**, *90* (1), 737-744. DOI: 10.1021/acs.analchem.7b02986.

8. Zhang, S.; Van Pelt, C. K.; Wilson, D. B. Quantitative Determination of Noncovalent Binding Interactions Using Automated Nanoelectrospray Mass Spectrometry. *Anal. Chem.* **2003**, *75* (13), 3010-3018. DOI: 10.1021/ac034089d.

9. Deslignière, E.; Ley, M.; Bourguet, M.; Ehkirch, A.; Botzanowski, T.; Erb, S.; Hernandez-Alba, O.; Cianférani, S. Pushing the limits of native MS: Online SEC-native MS for structural biology applications. *Int. J. Mass Spectrom.* **2021**, *461*, 116502. DOI: 10.1016/j.ijms.2020.116502.

10. Hecht, E. S.; Obiorah, E. C.; Liu, X.; Morrison, L.; Shion, H.; Lauber, M. Microflow size exclusion chromatography to preserve micromolar affinity complexes and achieve subunit separations for native state mass spectrometry. *J. Chromatogr. A.* **2022**, *1685*, 463638. DOI: 10.1016/j.chroma.2022.463638.

11. VanAernum, Z. L.; Busch, F.; Jones, B. J.; Jia, M.; Chen, Z.; Boyken, S. E.; Sahasrabuddhe, A.; Baker, D.; Wysocki, V. H. Rapid online buffer exchange for screening of proteins, protein complexes and cell lysates by native mass spectrometry. *Nat. Protoc.* **2020**, *15* (3), 1132-1157. DOI: 10.1038/s41596-019-0281-0.

12. Ren, C.; Bailey, A. O.; VanderPorten, E.; Oh, A.; Phung, W.; Mulvihill, M. M.; Harris, S. F.; Liu, Y.; Han, G.; Sandoval, W. Quantitative Determination of Protein-Ligand Affinity by Size Exclusion Chromatography Directly Coupled to High-Resolution Native Mass Spectrometry. *Anal. Chem.* **2019**, *91* (1), 903-911. DOI: 10.1021/acs.analchem.8b03829.

13. Busch, F.; VanAernum, Z. L.; Lai, S. M.; Gopalan, V.; Wysocki, V. H. Analysis of Tagged Proteins Using Tandem Affinity-Buffer Exchange Chromatography Online with Native Mass Spectrometry. *Biochemistry* **2021**, *60* (24), 1876-1884. DOI: 10.1021/acs.biochem.1c00138.

14. Park, H.-M.; Winton, V. J.; Drader, J. J.; Manalili Wheeler, S.; Lazar, G. A.; Kelleher, N. L.; Liu, Y.; Tran, J. C.; Compton, P. D. Novel Interface for High-Throughput Analysis of Biotherapeutics by Electrospray Mass Spectrometry. *Anal. Chem.* **2020**, *92* (2), 2186-2193. DOI: 10.1021/acs.analchem.9b04826.

15. Wilm, M. S.; Mann, M. Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? *Int. J. Mass Spectrom. Ion Processes* **1994**, *136* (2), 167-180. DOI: 10.1016/0168-1176(94)04024-9.

16. Wilm, M.; Mann, M. Analytical Properties of the Nanoelectrospray Ion Source. *Anal. Chem.* **1996**, *68* (1), 1-8. DOI: 10.1021/ac9509519.

17. Jecklin, M. C.; Touboul, D.; Boveet, C.; Wortmann, A.; Zenobi, R. Which electrospray-based ionization method best reflects protein-ligand interactions found in solution? A comparison of ESI, nanoESI, and ESSI for the determination of dissociation constants with mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2008**, *19* (3), 332-343. DOI: 10.1016/j.jasms.2007.11.007.

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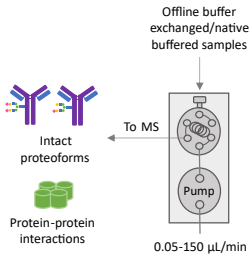

Infusion cart set-up depicting pump, and its operating range (µL/min), and the types of samples that can be analyzed, including noncovalent interactions.