

Coupling Online Size Exclusion Chromatography with Charge Detection-Mass Spectrometry Using Hadamard Transform Multiplexing

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ABSTRACT: Charge detection mass spectrometry (CD-MS) is a powerful technique for the analysis of large, heterogeneous biomolecules. By directly measuring the charge states of individual ions, CD-MS can measure the masses from spectra where conventional deconvolution approaches fail due to the lack of isotopic resolution or distinguishable charge states. However, CD-MS is inherently slow because hundreds or thousands of spectra need to be collected to produce adequate ion statistics. The slower speed of CD-MS complicates efforts to couple it with online separation techniques, which limit the number of spectra that can be acquired during a chromatographic peak. Here, we present the application of Hadamard transform multiplexing to online size exclusion chromatography (SEC) coupled with Orbitrap CD-MS, with a goal of using SEC for separating complex mixtures prior to CD-MS analysis. We developed a microcontroller to deliver pulsed injections from a large sample loop onto a SEC for online CD-MS analysis. Data showed a series of peaks spaced according to the pseudo-random injection sequence, which were demultiplexed with a Hadamard transform algorithm. The demultiplexed data revealed improved CD-MS signals while preserving retention time information. This multiplexing approach provides a general solution to the inherent incompatibilities of online separations and CD-MS detection that will enable a range of applications.

INTRODUCTION

Analysis of large, heterogeneous biomolecules by native mass spectrometry (MS) is challenging because the charge states of ions often cannot be determined. Charge detection-mass spectrometry (CD-MS) solves this challenge by directly measuring the charge states of individual ions.¹⁻⁴ By avoiding the need to resolve charge states or isotopes, CD-MS has enabled characterization of very large assemblies such as protein complexes,³ intact virus capsids,⁵ vaccines,⁶ and gene therapies.^{7,8} Growing needs in biotechnology to characterize these heterogeneous assemblies has led to increased interest in CD-MS.

One requirement of CD-MS is that only a limited number of ions can be measured at a time, which requires acquisition of hundreds or thousands of spectra to provide enough detection events for mass measurements with acceptable signal to noise ratios (SNR). The need for these long acquisitions generally limits the coupling of CD-MS with online separation techniques, such as size exclusion chromatography (SEC), which typically only provides enough time for 50–200 scans during the elution of a chromatographic peak.

Progress has been made on speeding up CD-MS by refining instrumentation to enable the measurement of multiple ions simultaneously^{4,9} and improve charge state assignments with deconvolution algorithms.¹⁰ However, a recent study to evaluate the feasibility of online SEC-CD-MS produced deconvolved mass spectra that, while sufficient to measure accurate protein masses, had lower signal-to-noise ratios (SNR) and contained significantly more erroneous peaks than spectra produced from infusion experiments.¹¹ A second study using online buffer exchange^{12,13} with CD-MS for the analysis of IgM

assemblies was also able to produce sufficient mass distributions after careful optimization of MS conditions but was still limited to only several hundred ions per run.¹⁴ A third study explored the use of capillary electrophoresis (CE) and other fluidic devices with CD-MS and observed that short elution profiles often produced low-quality CD-MS spectra.¹⁵

Hadamard transform multiplexing provides a powerful strategy for improving throughput and SNR when scan/separation speeds are slower than detection speeds. It has been applied to a range of separation techniques—including gas chromatography (GC),^{16,17} liquid chromatography (LC),^{18,19} and capillary electrophoresis (CE)^{20,21}—and to enhance a range of analytical techniques—including optical spectroscopy,^{22,23} time-of-flight MS,²⁴⁻²⁶ and ion mobility spectrometry.²⁷⁻³¹ With conventional single-plex methods, a sample is injected, and the second sample must wait until the first injection is completed before it can be injected. With Hadamard transform multiplexing, multiple overlapping injections are performed in rapid succession as dictated by a pseudo-random binary sequence (PRBS). The resulting chromatogram contains multiple peaks for each analyte in the sample, which can be demultiplexed by multiplication with a matrix derived from the PRBS. This results in a single peak for each analyte with accurate retention times and improved SNR.

Here, we applied Hadamard transform multiplexing to size exclusion chromatography (SEC) coupled with CD-MS for the acquisition of spectra from multiple chromatographic peaks. We describe a simple multi-injection strategy using a standard LC pump and 6-port injection valve. We use an open-source Hadamard transform CD-MS demultiplexing algorithm built on our UniDec software platform to analyze the data.^{10,32,33} Finally,

we demonstrate the Hadamard transform SEC-CD-MS method on a mixture of model proteins and on a complex cell lysate mixture. Together, this experimental design provides a general solution to the problem of coupling CD-MS detection with isocratic separations that can be easily adopted on a range of platforms for a range of applications.

EXPERIMENTAL SECTION

Materials

β -galactosidase and GroEL were purchased from Sigma or expressed and purified as previously described.³⁴ All protein samples were buffer exchanged into 200 mM ammonium acetate (Sigma) using either Biospin P6 (BioRad) spin columns or a Superdex 6 Increase 10/300 (Cytiva) size exclusion column. Note: because multiple injections are co-eluting during Hadamard transform SEC, the salts from one injection may co-elute with the proteins in another, so it is not possible to do significant sample clean-up online using this approach. Protein samples were diluted to 0.1–0.5 mg/mL in 200 mM ammonium acetate prior to analysis.

To produce *E. coli* cell lysate, *E. coli* OverExpress C43 (DE3) cells were cultured in 1 L flasks of Terrific Broth (TB) media, as previously described.³⁵ Cells were then harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. Cells were lysed in 20 mM Tris, 100 mM NaCl, pH 7.4 with protease inhibitor using an LM20 microfluidizer (Microfluidics International Corporation) at 20,000 psi. The cell lysate was clarified by centrifugation at 20,000 $\times g$ for 25 minutes at 4 °C, and membranes were removed at 100,000 $\times g$ for 2 hours and 10 minutes at 4 °C. The supernatant with soluble proteins was diluted 10 $\times$ in 200 mM ammonium acetate and reconstituted in a 300 kDa molecular weight cutoff filter five times to isolate high-mass proteins.

Multi-injection Online SEC

A constant flow of 200 mM ammonium acetate was supplied by an Agilent 1100 quaternary pump at a flow rate of 0.2 mL/min. This flow was directed to a 6-port switching valve with either a 100 μ L or 500 μ L sample loop containing the protein sample (**Figure 1**). The valve was controlled by an Arduino Uno microcontroller, which was programmed with a PRBS to control injections. Code for the Arduino is provided at <https://github.com/michaelmarty/UniDec/blob/master/PublicScripts/Multiplexing/HTswitch2.ino>.

Injections were performed according to a PRBS with a length of $2^n - 1$ where $n = 1, 3, 5$, or 6 . The specific sequences used were obtained from Harwit and Sloane²³ and are “1110100”, “0000100101100111110001101110101”, and “0000010000110001010011110100011100100101101101101101101101111” for the 3-, 5-, and 6-bit sequences, respectively. A one-bit sequence is simply “1”, a standard single-plex chromatographic injection. The 3-bit sequence was chosen because it had a quick analysis time but contained enough injections to see the injection pattern. The 5-bit sequence was chosen because it was longer than the 3-bit, with more injections to demonstrate the effects of scaling the PRBS length. The longer 6-bit sequence was used only for complex lysate data, where the analysis benefitted from additional injections. When necessary, additional zeros were added to the end of the PRBS to ensure that all chromatographic peaks were observed prior to the end of data acquisition.

In addition to the PRBS, the two critical parameters to define the injection sequence were the injection time (how long the valve stayed in the *inject* position before being switched back to *load* during each cycle) and the cycle time (how long the code waited before proceeding to the next digit of the PRBS). At the beginning of each cycle, the PRBS is evaluated. If the digit is a 1, the valve is switched to *inject* for as long as specified by the injection time. After the injection time is done, it is switched back to *load* for the remainder of the cycle time. If the digit is a 0, the valve remains in *load* for the entire duration of the cycle time. After one cycle is completed, it proceeds to evaluate the next digit in the sequence and continues until the sequence is completed. Here, the injection time was set to 1.5 seconds (5 μ L injection), and the cycle time was 1 min. A trigger is sent from the Arduino to the MS to start acquisition at the start of the sequence, and the acquisition time on the MS is set to the total sequence length multiplied by the cycle time. No other coordination is performed to synchronize MS scans and LC injections.

After the loop, flow was directed to a Superose 6 Increase 5/150 column (Cytiva) and then to the heated electrospray ionization (HESI) source. Approximately 10 ft. of 0.005 in. PEEK “resistor tube” was placed between the grounding union and ESI needle to reduce spray current while spraying high ionic strength mobile phase at high flow rates.¹² All data was collected with at least three replicate injection sequences, and representative data is shown where applicable below. Details on collection of static nano-ESI data are provided in the Supporting Methods.

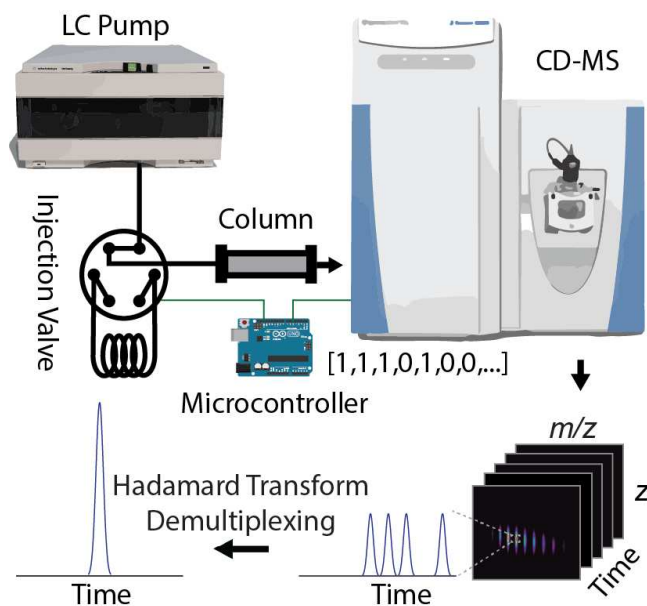


Figure 1. Schematic of Hadamard transform SEC-CD-MS workflow. 200 mM ammonium acetate is supplied by the LC pump at 200 μ L/minute. The sample is loaded into a 100 μ L or 500 μ L sample loop connected to a 6-port valve. The 6-port valve is shown in the sample loading position. Counterclockwise from the pump inlet is the outlet to the column, sample loop entrance, injection port, waste outlet, and sample loop exit. The valve is controlled by a microcontroller and switches the 6-port valve between inject and load positions according to the PRBS. CD-MS spectra are acquired by a Thermo Q-Exactive HF UHMR Orbitrap mass spectrometer.

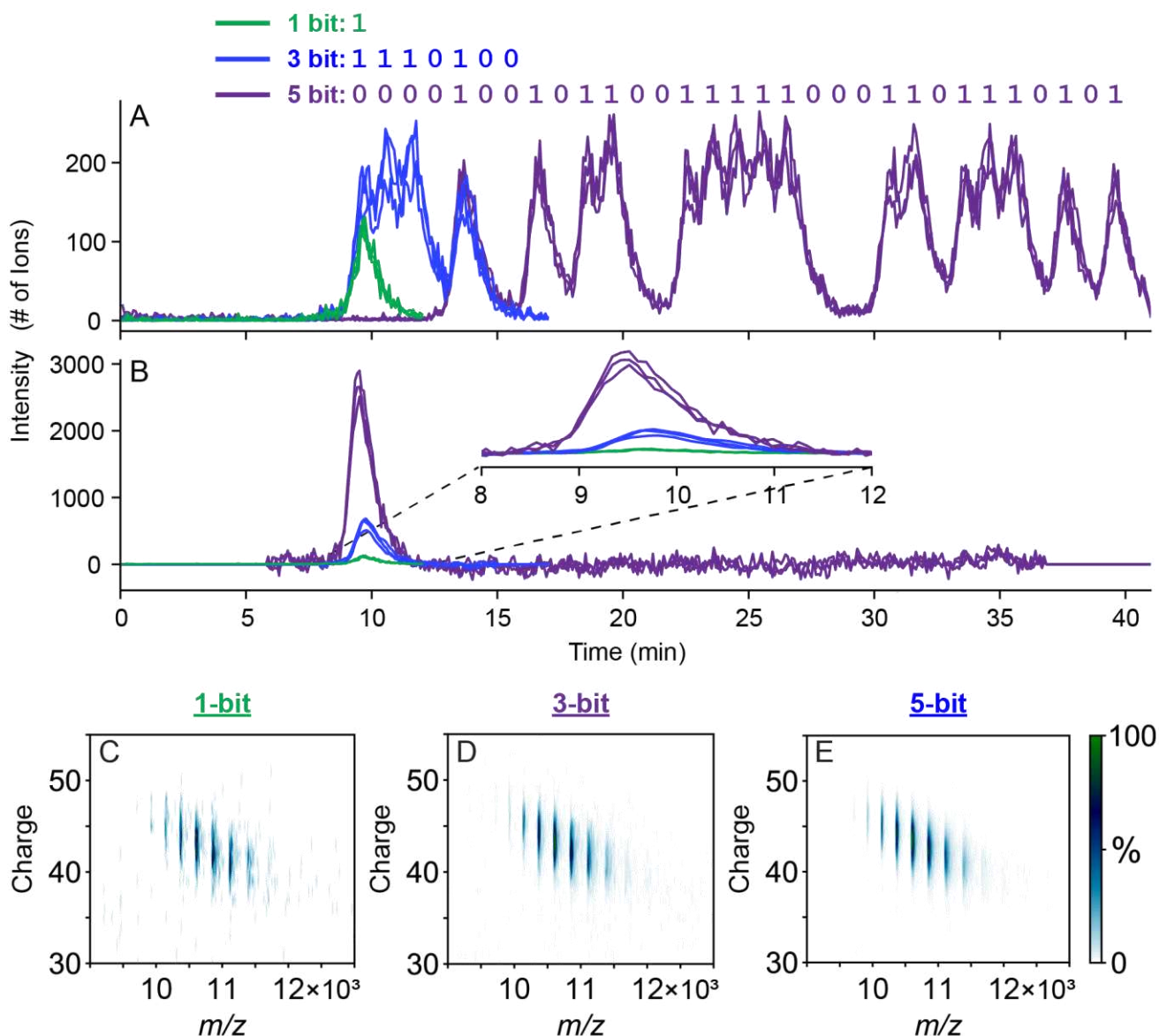


Figure 2. Comparison of β -galactosidase SEC-CD-MS chromatograms generated by 1- (green), 3- (blue), and 5-bit (purple) injection sequences before (A) and after (B) HT demultiplexing. Data from three replicates are shown for each series to demonstrate reproducibility. The raw m/z vs. charge distributions (C, D, E) produced by 1-bit (C), 3-bit (D), and 5-bit (E) injection sequences for β -galactosidase. Ion counts from this data are reported in **Table 1**.

Mass Spectrometry

All experiments were performed on a Thermo Fisher Scientific Q-Exactive HF Ultra High Mass Range (UHMR) Orbitrap mass spectrometer using the Direct Mass Technology (DMT) acquisition mode, which implements the STORI processing method.³⁶ Ions were generated by a HESI source using a spray voltage of 3.8 kV, a sheath gas setting of 20, and an auxiliary gas setting of 5. The m/z range was set to 3,000–12,000 m/z , and extended trapping settings of 25–100 V were used to maximize desolvation while minimizing fragmentation of ions. CD-MS spectra with DMT were collected with a resolution setting of 240,000, 1 microscan, and ion injection times were fixed between 50 and 200 ms to maintain ion populations of 10–100 ions per scan. All 1-, 3-, and 5-bit sequences were collected with 200 ms ion injection times. The

6-bit sequence for *E. coli* lysate was collected with 50 ms ion injection times, likely due to a higher concentration of protein in the sample. It should also be possible to use automatic injection control (AIC) acquisition features in the future,¹⁵ but further tuning will be necessary to adjust the AIC algorithm to respond on a chromatographic time scale.

Data Processing

Raw files containing CD-MS data were initially processed in STORIBOARD (Proteinaceous) and then further processed using a custom Hadamard transform algorithm implemented in the UniChromCD window of UniDec, which is described briefly here and in more detail in a companion manuscript.³³ Extracted ion chromatograms (EICs) were generated by defining an m/z range and charge state range for each ion population of interest. A kernel array was constructed based on the PRBS (including

Sequence	# of injections	Runtime (minutes)	Replicate	# of Ions	Ions/minute of runtime
1-bit	1	12	1	980	81.7
			2	914	76.2
			3	906	75.5
			Avg	933	77.8
			SD	40.6	3.4
3-bit	4	17	1	5,351	315
			2	5,213	307
			3	4,032	237
			Avg	4,865	286
			SD	725	42.6
5-bit	16	41	1	22,725	554
			2	20,559	501
			3	19,402	473
			Avg	20,895	509
			SD	1689	41.1

Table 1. The total number of injections, total run times, number of ions, and average number of ions/minute for 1-, 3-, and 5-bit injection sequences for β -galactosidase data presented in **Figures 2** and **3**. For 1-bit data, the retention time of 12-minutes defines the total sequence runtime. For 3- and 5-bit data, the runtime is defined by a 1-minute cycle time times the sequence length, including zero-padding.

zero pads when applicable) by substituting -1s for 0s in the sequence and infilling with 0s so that the array was the same size as the number of scans in the full chromatogram. The kernel array was then rotated to move the zero padded section to the beginning of the sequence and smoothed by convolution with a Gaussian function with a width (FWHM) of 2 scans. The modified kernel was then convolved with each EIC, and the resulting demultiplexed EICs were shifted back to the original time index. The compiled program and source code can be found at: <https://github.com/michaelmarty/UniDec>. Additional details on deconvolution are provided in the Supporting Methods.

RESULTS AND DISCUSSION

Comparison of Injection Sequence Lengths

To evaluate our Hadamard transform multiplexed injection strategy, we began by comparing injection sequences with different lengths. A 0.1 mg/mL ($\sim 0.2 \mu\text{M}$) solution of β -galactosidase was analyzed using 1-bit (1 injection), 3-bit (4 injections), and 5-bit (16 injections) sequences in triplicate. The 3- and 5-bit sequences were each padded with 10 zeros, resulting in 17- and 41-minute run times, respectively. The single injection (1-bit) chromatogram was recorded for 12 minutes, which was the minimum required to elute the sample.

The multiplexed total ion chromatograms (TICs) are similar in intensity and retention time to the 1-bit (single-plex) injection, as seen in **Figure 2A**. However, the areas of the demultiplexed peaks scale with the number of injections (**Figure 2B**), demonstrating improved signal for the multiplexed data. Importantly, even though the 5-bit PRBS used in this experiment starts with 4 zeros (i.e. no injection occurs for the first four minutes), the retention time is correctly

reported as 9.5 minutes in the demultiplexed chromatogram. Together, these data demonstrate the ability of the Hadamard transform algorithm to sum the areas of all individual peaks in the multiplexed chromatogram into a single peak at the correct retention time.

As shown in **Table 1**, the Hadamard transform multiplexed data yields a significantly higher number of total ions collected, which improves the quality of the data. Achieving the same ion count obtained from the 5-bit sequence with sequential 12-minute single injection runs would take 192 minutes (not including time between runs). In contrast, the 5-bit data shown here was acquired in only 41 minutes, yielding a higher number of ions collected per minute of instrument time (**Table 1**). Together, these data illustrate the significant throughput advantage of using multiplexed injection strategies for LC-CD-MS experiments.

Examining the CD-MS spectra (**Figure 2**) and the resulting raw and deconvolved mass distributions (**Figure S1**), all three sequences yield the correct mass peak, but the quality of the data improves with additional injections. The data from the 1-bit sequence is noisy, especially prior to deconvolution. In contrast, the 5-bit sequence produces a smoother mass distribution with reduced baseline noise, illustrating the benefits of higher ion counts in CD-MS data.

Comparing these results with conventional static nano-ESI infusion (**Figure S1**) showed the CD-MS results are similar. The higher flow ESI source used for SEC-CD-MS yielded peaks that were broader, likely due to more challenging desolvation, and at lower average charge states, likely due to charge stripping during desolvation. However, these differences are not intrinsic to the separation, and good data can be obtained with either method.

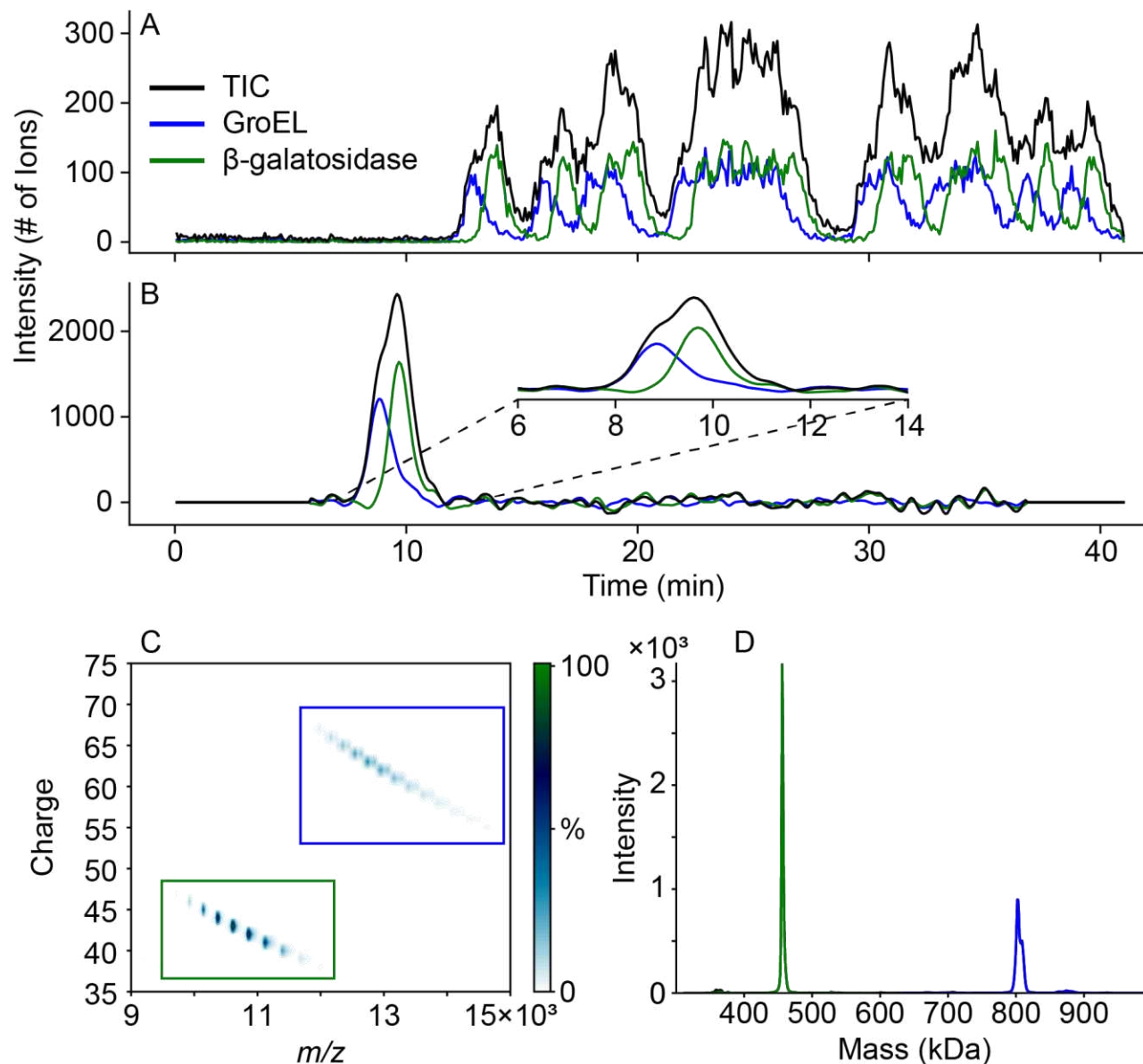


Figure 3. Hadamard transform SEC-CD-MS analysis of a mixture of β -galactosidase (green) and GroEL (blue) using the 5-bit injection sequence. (A) Raw TIC (black) with overlaid EICs, colored respectively. (B) Demultiplexed chromatogram with inset showing the two overlapping EIC peaks. (C) m/z vs. charge heatmap after deconvolution with blue and green rectangles showing the m/z and charge ranges selected for the EICs of GroEL and β -galactosidase, respectively. (D) Deconvolved mass spectra showing both protein mass distributions, with extracted distributions in their respective colors.

Separation of a Mixture of Proteins

Next, to test the ability to separate two proteins, a simple mixture containing β -galactosidase and GroEL was prepared and injected using the 5-bit sequence. Compared to the single protein sample shown above, the TIC (Figure 3A) shows broader features where the injection sequence pattern is not obvious. Extracted ion chromatograms (EICs) were created for each protein by selecting both an m/z and charge range that encompassed the full charge state distributions, as seen in Figure 3C. The resulting EICs (Figure 3A) reveal a notable shift between the two multiplexed chromatograms. Hadamard transform demultiplexing produced a TIC and EICs that were well defined and with minimal artifacts (Figure 3B). Although the resolving power of the short SEC column used here was not

sufficient to resolve the two species in the TIC, the EICs show clear separation and the expected elution order with the larger GroEL eluting first.

As with the isolated β -galactosidase data above, the mass distributions were similar between the SEC-CD-MS and conventional static infusion (Figure S2). After UniDec deconvolution to reduce the spread in the charge dimension, the deconvolved mass spectrum (Figure 3D) shows extracted mass assignments for both species. Somewhat broader peaks were observed from GroEL, likely due to poorer desolvation on the HESI source. Overall, these data demonstrate that Hadamard transform SEC-CD-MS can be used to analyze mixed samples and extract the chromatograms and mass distributions of isolated species.

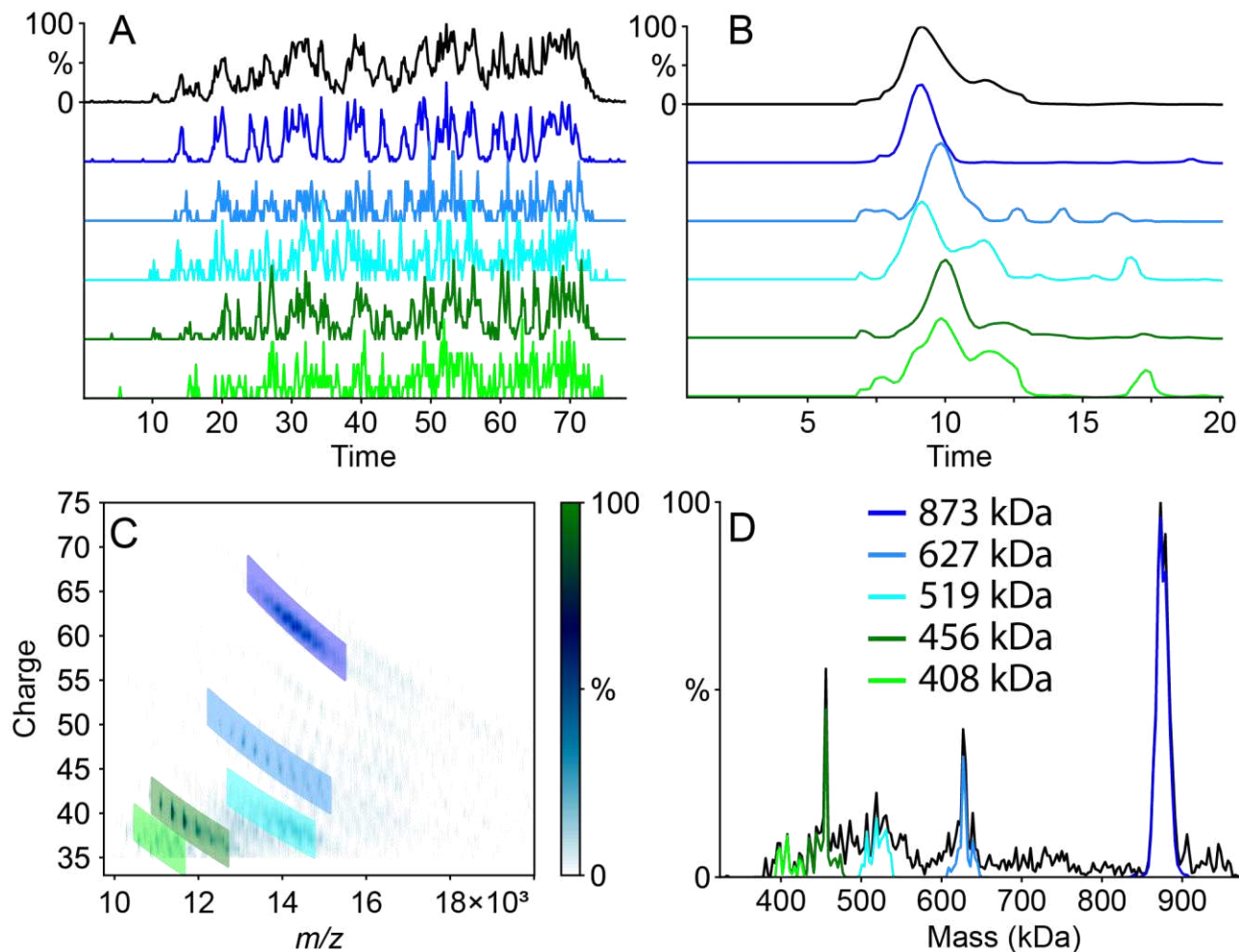


Figure 4. High-mass fraction of *E. coli* cell lysate. (A) Raw TIC (black) and EICs (colored) of the five most abundant observed proteins. (B) Demultiplexed TIC and EICs from each of the chromatograms shown in (A). (C) m/z vs. charge heatmap after deconvolution with areas selected for EIC extraction shown in shaded polygons. (D) Deconvolved mass spectra with colored traces indicating mass distributions from the selected regions from (C).

Separation of a Complex Mixture

Finally, we tested the performance of Hadamard transform SEC-CD-MS on a complex mixture of *E. coli* cell lysate. To deplete smaller species, we used a 300 kDa molecular weight cutoff filter. Here, 500 μ L of *E. coli* cell lysate was diluted 5 $\times$ to a total volume of 2500 μ L, then centrifuged back down to 500 μ L. This process was repeated a total of 5 times. However, initial experiments were still dominated by low mass proteins, so the instrument was tuned to further reject low mass ions by setting the injection flatapole, inter-flatapole lens, and bent flatapole to 9 V, 8 V, and 10 V, respectively, similar to the “voltage rollercoaster filtering” method described by McGee et. al.³⁷ This tuning resulted in a mass distribution spanning the 400 kDa to 1 MDa range seen in **Figure 4D**. To collect more scans on this complex mixture, it was injected using a 6-bit injection sequence (63 segments, 32 injections) for a total run time of 78 minutes including 15 zero pads.

Even after deconvolution, the m/z vs. charge distribution (**Figure 4C**) contains numerous species that were close in m/z and charge, which hindered our ability to cleanly select protein signals using the simple m/z and charge state range method in **Figure 3**. Thus, we developed a method to select protein signals along predicted m/z vs. charge curves. Using curve selection to

produce the EICs of the 5 most prominent features (**Figure 4A**), we further processed the demultiplexed chromatograms (**Figure 4B**) using a masked multiplex³¹ method to remove artifacts. Here, we observed that the masses generally correlated with the retention time, as expected. Attempts to extract less prominent features produced demultiplexed EICs with poor SNR and peak shapes, suggesting that even longer injection sequences or more precise extraction settings would be required to fully resolve lower abundance species in mixtures such as this.

Although it is not possible to confidently identify proteins from this mixture by intact mass alone, a few observations can be made about the potential of Hadamard transform SEC-CD-MS for the analysis of complex mixtures. First, even when five or fewer ions are observed in each retention time bin of an EIC, the Hadamard transform algorithm is still able to produce good quality chromatographic peaks that allow retention times to be accurately measured. Second, in this example both the lightest blue and light green EICs originate from relatively crowded areas of the m/z vs. charge distribution and may contain contributions from two or more proteins that could have different retention times. In **Figure 4B**, we observe what appear to be multimodal retention time distributions, demonstrating

that Hadamard transform demultiplexing can resolve complex chromatographic features that are not simple Gaussian distributions. However, these distributions could also be artifacts due to the low SNR for these two species. In any case, having the ability to isolate data in mass, charge, and/or retention time helps in analysis of these peaks that would be otherwise difficult to discern. Future efforts will employ higher resolution SEC columns, longer injection sequences, and additional prefractionation to probe the capacity of this method to characterize complex mixtures with increased dynamic range.

CONCLUSION

Performing CD-MS on a chromatographic time scale is challenging because many ions are needed to produce quality CD-MS spectra, but only a few ions can be collected in each scan. By stacking together multiple injections in short succession, Hadamard transform demultiplexing improves SEC-CD-MS by increasing SNR in both chromatographic and mass dimensions. It enhances the duty cycle relative to simply averaging repeated single-plex injections, enabling more efficient use of instrument time without sacrificing retention time resolution or information.

Hadamard transform multiplexing can be broadly applied to any chromatography system that uses isocratic gradients and that is capable of handling multiple injections, making it a generalized and versatile technique for generating high-quality LC-CD-MS data. For example, isocratic HIC has been applied to antibodies,³⁸ and CE-MS has been successfully used with proteins as large as GroEL,³⁹ both of which would be compatible with multiplexed injections and CD-MS data acquisition. The two primary limitations of the method are that it does not work with gradient separations and that it cannot be used for online sample clean up because contaminants from earlier injections could come out of the column at the same time as the analyte from later injections, thus contaminating subsequent injections.

Finally, we have presented here a simple and cheap design of a system using open-source software and hardware that can be readily adapted to different instrument systems. We expect that it will be relatively easy to implement in most labs. However, it should also be possible to translate on automated SEC-MS systems by reprogramming the autosampler to introduce multiple injections in a pre-defined sequence. Combining these advanced separation strategies with improved instrumental methods^{2,40,41} for collecting CD-MS data will improve the characterization of heterogeneous assemblies for a range of applications.

ASSOCIATED CONTENT

Supporting Information

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

Supplemental methods on static electrospray ionization CD-MS data collection of β -galactosidase and β -galactosidase/GroEL mixture; supplemental methods on deconvolution settings; supplemental figures comparing Hadamard transform SEC-CD-MS with static electrospray ionization CD-MS.

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CONFLICT OF INTEREST STATEMENT

MTM and JDS have submitted a provisional patent on the Hadamard transform SEC-CD-MS methodology.

NOTES

All raw data for this manuscript is available at MassIVE, with Hadamard transform SEC data at MSV000095091 (DOI: [10.25345/C5FN1139K](https://doi.org/10.25345/C5FN1139K)). Code is available for free download at: <https://github.com/michaelmarty/UniDec>. A video tutorial on the software is available at: <https://youtu.be/exrAdR-0iHs>.

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