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Pseudo-Random Sequence Modifications for Ion Mobility Orthogonal Time of Flight Mass Spectrometry

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Abstract

Due to the inherently low duty cycle of ion mobility spectrometry (IMS) experiments that sample from continuous ion sources, a range of experimental advances have been developed to maximize ion utilization efficiency. The use of ion trapping mechanisms prior to the ion mobility drift tube has demonstrated significant gains over discrete sampling from continuous sources; however, these technologies have traditionally relied upon a signal averaging to attain analytically relevant signal-to-noise ratios (SNR). Multiplexed (MP) techniques based upon the Hadamard transform offer an alternative experimental approach by which ion utilization efficiency can be elevated to ~ 50 %. Recently, our research group demonstrated a unique multiplexed ion mobility time-of-flight (MP-IMS-TOF) approach that incorporates ion trapping and can extend ion utilization efficiency beyond 50 %. However, the spectral reconstruction of the multiplexed signal using this experiment approach requires the use of sample-specific weighing designs. Though general weighing designs have been shown to significantly enhance ion utilization efficiency using this MP technique, such weighing designs cannot be applied to all samples. By modifying both the ion funnel trap and the pseudo random sequence (PRS) used for the MP experiment we have eliminated the need for complex weighing matrices. For both simple and complex mixtures SNR enhancements of up to 13 were routinely observed as compared to the SA-IMS-TOF experiment. In addition, this new class of PRS provides a two fold enhancement in ion throughput compared to the traditional HT-IMS experiment.

INTRODUCTION

Ion mobility spectrometry (IMS) is a post-ionization gas phase technique that uses weak uniform electric fields to rapidly separate ions. The fundamental principle enabling this separation is based upon the drag force exerted on an ion as it traverses a drift cell filled with a homogenous neutral drift gas.^{1, 2} Due to the speed at which ions can be separated, IMS has traditionally been used as a rapid screening tool for narcotics,^{3, 4} explosives,^{5, 6} and chemical warfare agents.^{3, 7-9} More recently, its has been applied to address the challenges associated with analysis of complex biological systems.¹⁰⁻¹⁷ Combining IMS with traditional separation schemes such as capillary liquid chromatography-mass spectrometry (LC-MS), allows complex biological samples to be separated in multiple dimensions, which results in an increase in the depth of coverage. The higher peak capacity achieved by multidimensional techniques such as LC-IM-MS allows the use of a larger number of criteria for screening, identifying, and differentiating the wide range of existing biological states.

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A traditional broadband IMS experiment is initiated by admitting a discrete packet of ions into a drift tube. Assuming the drift tube contains a uniform electric field, homogeneous neutral gas, and the ion packet is unaffected by Coulombic repulsion, the distribution of the recorded ion signal is determined by:

$$w^2 = t_g^2 + \frac{16 \ln 2}{q} \cdot \frac{kT_d^2}{V} \quad (1)$$

where, w is the width of the peak profile measured at half height; t_g , the ion gate pulse width; k , Boltzmann's constant; T , the absolute temperature; t_d , the centroid of the ion drift time; q , the charge on the ion; and V , the voltage applied across the drift region.¹⁸ Stated more succinctly, the distribution of the recorded ion signal is a function of the initial ion gate pulse width and thermal diffusion. The resolving power (R_p) of an IMS instrument is determined by dividing the drift time of an ion population (t_d) by the peak width at half height (w).¹⁸ After optimizing voltage, temperature, and pressure, the IMS resolving power may be maximized by reducing the ion gate pulse width. Unfortunately, sensitivity is often sacrificed by minimizing ion gate pulse width, because fewer ions are delivered to the detector following each ion gate pulse. Practically, the balance between resolving power and sensitivity is attained when the width of the admitted ion packet is 0.1-1 % of the total IMS experiment time.^{19, 20} When sampling from continuous ion sources (e.g., electrospray ionization, radioactive ionization, atmospheric chemical ionization, and electron impact), the nature of the IMS experiment severely restricts the instrumental duty cycle and overall ion utilization efficiency. Despite the limitations imposed by sampling from continuous sources, IMS represents a complementary analysis approach that is directly compatible with the effluent of liquid and gas chromatographic separations.^{12, 15, 21-24} Pulsed ionization sources, most notably matrix assisted laser desorption ionization MALDI, explicitly address the issue of ion utilization efficiency by combining sample ionization with the ion gating event. However, coupling pulsed ionization sources with multidimensional on-line separations has yet to be realized in conjunction with IMS systems.

To address the low ion utilization efficiency of an IMS instrument that samples from continuous sources, ion trapping has been employed to accumulate ions prior to ion gating.²⁵⁻²⁹ Both 3D and linear quadrupole ion traps have been used as ion accumulation and IMS gating mechanisms. Though enhanced levels of IMS sensitivity have been demonstrated using these devices, the operating pressures necessary for effective trapping and ejection limit the range of experimental conditions in which they may be applied.

An alternative ion trapping approach that uses an ion funnel trap (IFT) and is capable of operating at higher pressure has recently been applied to IMS. This work by Clowers *et al.* has demonstrated the ability of a refined IFT to accumulate, store, and eject ions with charge densities that exceed the levels of ion current produced by a continuous ion source.³⁰ Though ion trapping has been shown to enhance the overall ion utilization efficiency of the IMS experiment, the charge capacity of an ion trap restricts the gains that may be realized. Given the incoming ion current of 1 nA and a trap charge capacity of 10^7 charges, such a trap is filled to its capacity in 1.5 ms, which constitutes ~2.5 % duty cycle for a typical ~ 60 ms-long IMS separation.

Whether ion trapping or pulsed ionization techniques are used, both types of IMS experiments have relied upon signal averaging (SA) to maximize signal-to-noise ratio (SNR). For any given SA experiment each subsequent measurement cannot be made until the previous experimental cycle is completed. Consequently, each subsequent ion gating event cannot be initiated until the slowest moving ion within the system reaches the detector. In the event an ion packet were released into the drift cell prior to ions from the previous gating event arriving at the detector, spectral overlap would be observed in the recorded signal. Therefore, the experimental

frequency and throughput of the IMS experiment is determined by the slowest moving ion in the system.

Multiplexing techniques have been applied to IMS to maximize both throughput and sensitivity. By modulating the pulsing frequency between two Bradbury-Nielsen ion gates, Knorr *et al.* demonstrated the throughput advantage afforded by Fourier transform IMS.³¹ Additionally, the Fourier transform as applied to IMS demonstrated a modest improvement in resolution and SNR.³²⁻³⁴ More recently, a 50% duty cycle was attained in a phase-resolved IMS experiment.³⁵ The Hadamard transform is another multiplexing technique that also promises a 50% duty cycle.³⁶ Instead of using sinusoidal modulation of the ion gate, as in the case of phase resolved IMS, the Hadamard transform IMS (HT-IMS) experiment uses a square ion gate pulse to modulate an ion beam. First reported by Clowers *et. al.*³⁷ and independently verified by Szumlas *et. al.*,³⁸ HT-IMS demonstrated up to a 10-fold improvement in SNR compared to the SA-IMS experiment. In both initial implementations of the HT-IMS technique, ions were modulated using a Bradbury-Nielsen gate, which accorded a pseudo-random pulsing sequence (PRS) derived from Simplex matrices.

Fundamentally, a PRS derived from a Simplex matrix has a duty cycle of ~50 %; however, Belov *et al.*, developed a novel multiplexing (MP) technique for IMS that can enhance ion utilization efficiency beyond 50%. This technique incorporated ion trapping prior to each ion gate pulse,³⁹ and used a modified PRS designed to integrate ion trapping into the multiplexed (MP) experiment to enhance ion utilization efficiency. Furthermore, ion utilization factors could be extended beyond the limit determined by the Simplex PRS because ions that traditionally would be neutralized during a HT-IMS experiment were trapped. While the ion utilization efficiency was increased using this approach, the filling rate of the ion funnel trap (IFT) was dependent on the intensity of the incoming ion beam. Because the modified PRS allowed ions to be accumulated for varying lengths of time (see Theoretical Background Section in ref. ³⁹), the charge density of the ion packets exiting the IFT also varied. Accordingly, a large variation was observed in the recorded ion signal, which introduced pseudo-noise after performing the inverse transform. To counter transformed signal artifacts introduced by ion trapping, an advanced weighting scheme was utilized to account for the varying ion accumulation times.³⁹ Since the rate at which the IFT accumulated ions depended strongly on sample concentration, in theory, a unique weighting scheme would be required for each sample. Fortunately, generalized weighting schemes could be used for similar samples; however, in order to reproducibly transform all MP signals arising from MP-IMS experiments that utilize ion trapping, *a priori* knowledge of the sample was necessary.

In order to circumvent the need for complex weighting schemes and minimize pseudo-noise resulting from the inverse transform of the MP signal, we have modified the previously used PRS and trapping sequence to normalize intensities of injection ion populations.³⁹ By using fixed IFT accumulation times prior to each ion gating event, this new PRS sequence improves the robustness and applicability of the MP experiment applied to IMS. With its implementation, a wider range of samples may be probed using the MP-IMS-TOF system with significantly increased levels of ion utilization efficiency and throughput compared to the signal averaging experiment.

EXPERIMENTAL

Electrospray ionization and ion funnel trap

As the configuration and operating details of the electrospray ionization source and ion funnel trap have been previously reported,^{11, 30, 40, 41} only a brief description is presented here. Ions were generated by applying a potential (2.25 kV greater than the inlet capillary) to a stainless steel union that connected a fused silica capillary (150 μm O.D. 50 μm I.D., Polymicro

Technologies, Phoenix, AZ) with a syringe pump and an etched silica micro-emitter.⁴² Each sample solution was infused at a rate of 250 nL/min, using a syringe pump (Harvard Apparatus, Holliston, MA). Electrosprayed ions were sampled into the vacuum chamber, which housed the ion funnel trap, through a 64 mm long $\times$ 0.43 mm inner diameter heated capillary held at $\sim 150^\circ\text{C}$. The ion funnel trap positioned directly behind the exit of the heated capillary was comprised of 75 brass plates, each separated by Teflon spacers. Both the brass plates and Teflon spacers were 0.5 mm thick.

The five regions of the IFT highlighted in Figure 1 are the: 1) IFT trap entrance that follows the heated capillary, 2) jet disruptor,⁴³ 3) converging electrode region for ion focusing, 4) ion trapping chamber, and 5) IFT exit and beginning of the IMS drift cell. Each electrode within the IFT was connected to a resistor series and controlled by a 9-channel power supply (Spectrum Solutions, Inc., Russellton, PA). Aside from the trapping chamber with a DC gradient of $\sim 1.5\text{ V/cm}$, the gradient applied across the IFT was $\sim 25\text{ V/cm}$. Alternating electrodes throughout the IFT were also attached to independent capacitor networks that carried two 180° out-of-phase RF potentials. The effective potential established by the DC and RF voltages served to radially confine ions and direct ion populations towards the IFT exit. Compared to previously implementations of the electrodynamic ion funnel,^{44,45} the current configuration adds to the radial confining capabilities of the funnel by introducing a series of DC-only mesh grids to axially confine ions.³⁰ These DC-only grids that surround the IFT trapping chamber were constructed using high-transmission nickel mesh at 20 lines per inch (Precision E-forming, LLC, Cortland, NY) and were pulsed in accordance with the PRS described in greater detail below. The grid located at the entrance of the trapping region serves to selectively admit ions for storage, while the trapping and exit grids serve to gate ions into the IFT in a manner similar to the dual or Tyndall gate designs described elsewhere.^{3, 19}

Ion mobility-time-of-flight mass spectrometer

The IMS experiment was initiated by ejecting trapped ion population from the IFT. The duration of this pulse was typically 200 μs and was accomplished by lowering the potential on the trapping and exit grids to match the electric field gradient within the IFT. The design of the IMS drift tube consisted of four modular segments, each of which contained 21 copper electrodes (80 mm outer diameter $\times$ 55 mm inner diameter $\times$ 0.5 mm thick); each electrode was separated by 10 mm Teflon spacers. By using a series of high precision 1 M Ω resistors (Riedon, Alhambra, CA) to connect the drift rings, an electric field of $\sim 16\text{ V/cm}$ was created throughout the 88 cm drift tube. The pressures within the IFT and IMS drift tube were monitored by two capacitance manometers (MKS Instruments, Willmington, MA) and maintained at ~ 4 Torr using a 4.4 L/s roughing pump (Edwards Vacuum, Willmington, MA). An 80-mm long conventional electrodynamic ion funnel was located at the end of the IMS drift cell and used to focus the mobility-separated ion populations into the time-of-flight (TOF) mass spectrometer. The spacing of the brass electrodes was similar to the IFT electrodes; however, the taper of the rear IF electrodes decreased linearly from 51 mm to 2.5 mm. A custom power supply containing both DC and RF sources was used to apply a DC electric field gradient that matched the IMS drift tube, in addition to a RF voltage of 115 V_{p-p} that resonated at 500 kHz. Following the DC-only conductance limit held at 35 V, ions entered a differentially pumped region held at 300 mTorr that housed a segmented RF-only quadrupole. This quadrupole was composed of two 11 mm segments biased at 30 and 22 V, respectively. An additional 2.5 mm conductance limit that operated at 15 V was located immediately behind the quadrupole exit. Once through the quadrupole conductance limit, the ion populations entered the collisional quadrupole of a commercial quadrupole TOF mass spectrometer (Q-Star Pulsar, MDS Sciex, Concord, Canada). The RF/DC quadrupole of this instrument was operated in the broadband mode to transmit a wide m/z range of ions into the TOF pulsing region operated at $\sim 7\text{ kHz}$.

The TOF instrument was optimized to acquire a m/z range of 50-2000 with a mass resolving power of >6000 .

Multiplexed sequence generation and signal acquisition. Data were acquired using a custom built software package and a time-to-digital converter (TDC) (Ortec-9353, 10 GHz TDC, Oak Ridge, TN). The ion accumulation and release events for the IFT were synchronized with the TOF pulser, using a PCI-6711 timing card (National Instruments, Austin, TX). This card was also used to output the PRS used for the MP-IMS-TOF experiment. The initial PRS were generated using MLSRS and primitive binary polynomials outlined by Harwit and Sloane.³⁶ After construction, the initial PRS was zero-filled by a user-defined value to account for ion accumulation in the IFT and to minimize the detrimental effects of thermal diffusion upon signal reconstruction.³⁹

Chemicals and materials

Leucine enkephaline, kemptide, bradykinin, fibrinopeptide A, and angiotensin I peptides were obtained from Sigma-Aldrich (St. Louis, MO) and prepared at concentrations that ranged from 1 nM to 5 μ M in an electrospray solution consisting of a 1:1 water:methanol mixture that contained 0.1 % formic acid by volume.. In addition to the standard peptide mixtures used to evaluate the performance of the MP experiment, tryptic digests of bovine serum albumin (BSA; Pierce Biotechnology, Rockford, IL) were prepared at concentrations that ranged from 1 nM to 1 μ M, using the same electrospray solution.

RESULTS AND DISCUSSION

Pseudo-Random Sequence Description

While optimal weighing or measurement designs use Hadamard matrices comprised of I 's and $-I$'s, experiments that modulate signals in a binary fashion (i.e., “on” and “off”) are best suited to Simplex matrices made of I 's and O 's.⁴⁶ Since IMS modulates ion beams in a binary fashion, pulsing sequences used for HT-IMS originate from Simplex matrices. An elegant and computationally effective algorithm for generating Simplex sequence employs a maximal length shift-register approach (MLSRS) applied to primitive binary polynomials.³⁶ The PRS correspond to the first row of a Simplex matrix and are of length

$$N=2^m - 1 \quad (2)$$

where m is an integer. For example, the first and last rows of the Simplex matrix constructed from a 5-bit primitive binary polynomial using MLSRS are:

$$\begin{aligned} S_{31} \text{ row } 1 &= [0000100101100111110001101110101] \\ &\vdots \\ S_{31} \text{ row } 31 &= [1010111011000111110011010010000] \end{aligned} \quad (3)$$

These two rows illustrate that the PRS duty cycle is $\sim 50\%$ with $2^{m-1}-1$ elements that equal O and 2^{m-1} elements set to I . Additionally, the number of successive I 's and O 's are also determined by the length of the PRS—a characteristic that has direct implications to IMS. For a PRS or Simplex sequence of length $N=2^m-1$, the number of successive O 's is equal to $m-1$ and the number of successive I 's equal to m . For a conventional HT-IMS experiment that uses a 5-bit PRS with 31 elements, the maximum length of time the ion beam is admitted to the drift tube is equal to 5 elements, and the maximum time the ion beam is obstructed is equal to 4 elements. Note that during an actual experiment only the first row of the Simplex matrix is used to modulate the ion gate. The remaining rows are required for signal reconstruction using the inverse transform.

Equation (1) shows that ions distribute throughout the IMS cell based on the width of the ion gate release and thermal diffusion. In order to eliminate the detrimental effects of thermal diffusion upon signal reconstruction and invoke ion accumulation, Belov *et al.* extended the PRS length to temporally separate each ion gating event,³⁹ which was accomplished by separating each element of the original PRS by additional elements set to zero. For example, given a zero-filling factor of 10, each “1” element in the initial PRS sequence (or modulation bin) is represented as 1000000000 in the extended sequence, while each “0” element comprises 0000000000. Importantly, the duration of each sub-modulation bin is equal to the duration of each TOF mass spectrum. While the traditional HT-IMS experiment provides a $\sqrt{N}/2$ SNR gain and a throughput advantage equal to 2^{m-2} , the throughput advantage of the modified PRS is 2^{m-1} or greater by a factor of 2.

The pulsing profile of the extended 5-bit PRS zero-filled by a factor of 20 is shown in Figure 2a. The solid trace corresponds to the waveform used to modulate the entrance of ions into the IMS drift tube. While the use of this experimental configuration allows high ion utilization efficiencies to be achieved, well-defined weighting factors are required during reconstruction to account for the varying ion accumulation times and the relationship of ion accumulation to trapping efficiency throughout the MP experiment. As precise determination of these weighing factors proves to be somewhat difficult in high throughput experiments with complex proteomic samples, we circumvent this issue by accumulating ions between adjacent releases in the PRS for identical periods to deliberately reduce the experimental duty cycle to 50%. This reduction gives rise to an increased robustness in the signal reconstruction procedure, which makes it independent of the ion source.

To ensure that each IMS gating event releases equivalent ion populations, accumulation periods prior to ion packet releases are now controlled. Figure 2b illustrates a waveform that controls the state of the IFT entrance grid (see Figure 1) and is complementary to the waveform shown in Figure 2a. In the case of a 5-bit extended PRS zero-filled by a factor of 10, each pulse from the extended sequence injects an ion population accumulated for 10 PRS elements (sub-modulation bins), which is the shortest interval between two adjacent releases. The histogram in Figure 2c compares the number of ion release events as a function of ion accumulation time, using the new normalized and previous adjustable³⁹ accumulation periods within the same extended 5-bit PRS. As the normalized PRS is derived from the initial implementation,³⁹ the total number of pulses for each experiment remains the same. The most important benefit afforded by the extended PRS with fixed accumulation periods is that the resulting MP ion signal can be routinely reconstructed from any encoded signals without the use of complex sample-dependent weighing designs.

Multiplexed Transform

A zoomed subset of the raw IMS-MS data obtained for a tryptically digested BSA sample prior to the inverse transform of the MP signal is shown in Figure 3a, and the corresponding inverse transformed spectrum is provided in Figure 3b. These results were obtained by assembling the results of the inverse Hadamard transform applied to each individual m/z value extracted into the IMS domain.³⁹ The abscissa of the mass spectrum is oriented vertically, and the corresponding IMS spectrum appears below each contour plot. Closer examination of the contour plot shown in Figure 3a reveals three distinct isotopic distributions in the m/z dimension that give rise to a range of peaks distributed throughout the IMS drift time axis. For this particular experimental configuration (TOF pulsing frequency of ~ 10 kHz) the total IMS experiment time was 62 ms, which corresponds to a 5-bit PRS that is zero-filled by a factor of 20. Because the data in Figure 3a originate from the extended 5-bit PRS, there are 16 distinct ion mobility gate releases that when transformed produce the contour plot shown in Figure 3b. From a throughput perspective, a 5-bit PRS provides a 16-fold enhancement in the duty cycle

of the IMS experiment. The difference in the IMS intensity scales between the two data sets highlights an increase in SNR in the transformed data. Theoretically, the peak intensities within a standard transformed spectrum do not deviate from the signal intensities observed in the raw data set because the standard inverse matrix contains a series of weighted coefficients related to the number of pulses within the PRS.³⁶ When transformed properly, the resultant reduction in the level of noise provides an enhancement in the SNR. However, we intentionally omitted the use of these weighted coefficients to graphically emphasize the change in SNR following inverse transformation.

MP and SA SNR Comparisons

As noted earlier (Pseudo-Random Sequence Description) the degree of zero-filling combined with the operating frequency of the TOF-MS determines the length of time used to accumulate ions within the IFT prior to each gating release. For a 5-bit PRS sequence zero-filled by 20 elements and a TOF pusher frequency of 10 kHz, each trapping event is equal to 2 ms. Therefore, to be technically correct and relate SNR gains to the Fellgett advantage, comparisons between MP and SA experiments must be made on the basis of comparing observations obtained using the same techniques; that is, observations obtained using a MP-IMS-TOF experiment which utilizes a 2 ms IFT accumulation time must be compared with those obtained with SA-IMS-TOF experiment that also uses a 2 ms IFT accumulation time. Comparison with a conventional SA-IMS-TOF experiment that does not utilize an IFT would neglect the contribution of the ion accumulation event to SNR and bias results towards the MP experiment. Conversely, comparisons using extended accumulation times may provide a higher SNR for the SA experiment; however, such comparisons to a MP experiment that did not use equivalent accumulation periods would not allow the gains provided solely by the MP experiment to be isolated and reported. Unless stated otherwise, comparisons between the MP and SA experiments were made using equivalent accumulation times within the IFT.

The mass spectra obtained in MP and SA analyses of three standard peptides (50 nM each) are shown in Figure 4. The noise for these spectra was determined by fitting each data set to a Poisson distribution, because a time-to-digital converter was used for signal acquisition. To calculate the SNR for a given analyte, the signal intensity of the most intense isotopic peak was divided by the standard deviation of the Poisson distribution. For the three peptides distributions shown in Figure 4, the SNR gain of a 5-bit MP-IMS-TOF compared to the SA-IMS-TOF experiment ranged from 7 to 13. The spectra shown in Figure 4 are effectively a comparison of a single ion gate release to the total ion signal generated from the 16 ion gate releases found within the 5-bit MP-IMS-TOF experiment. As mentioned previously, extended accumulation times can increase the SNR observed for a SA-IMS-TOF experiment. While MP and SA that use different IFT accumulation times are tenuous the relative practical improvements may be assessed. As such, comparisons were made between the zero-filled 5-bit MP-IMS-TOF experiment and a SA-IMS-TOF experiment for which ions were accumulated for the length of the IMS experiment (i.e., 62 ms). In addition to a slightly greater SNR, the ion utilization, trapping efficiency, and lack of space charge discrimination favor the use of the MP-IMS-TOF experiment over the SA-IMS-TOF experiment that used extended accumulation times. This observation is based on the space charge capacity of the system and the rate at which ions enter the IFT.³⁰

Effect of Concentration on SNR

A single MP experiment using a 5 bit PRS zero-filled by 20 elements requires ~62 ms to complete, and may be repeated in a fashion similar to SA to improve SNR. Assuming noise is random within a given experiment, the SNR for any signal averaging process scales as the square root of the number of measurements.⁴⁷ Consequently, given enough measurements, the SNR for the two different techniques measuring the same low signals should eventually

converge. However, the practicality and analytical sensitivity of a specific technique is directly dictated by the length of time required to achieve a given SNR.

To illustrate the benefits of the MP experiment, the SNR for two different concentrations of neurotensin as a function of total experiment time are shown in Figure 5 for both MP-IMS-TOF and SA-IMS-TOF experiments. The maximum SNR gains over the SA experiment were 3 and 8 for 100 nM and 5 nM concentrations, respectively. Given the throughput enhancement of the MP-IMS-TOF experiment, gains in SNR are most pronounced for smaller concentrations, as signal averaging experiments are more prone to ion statistical limitations.

The trends observed in Figure 5 also applied to more complex systems. Figure 6 compares SNR between the MP and SA experiments for a single peptide ⁴³⁷KVPQVSTPTLVEVSR⁴⁵¹ from a 60 nM solution of tryptically digested BSA, derived using the extended 5-bit PRS. This plot not only illustrates the SNR gain achieved with MP, but also emphasizes the added advantage of throughput enabled by this new technique. The SNR gain for an acquisition time of ~18 s (300 averages) demonstrated a 12-fold advantage for the MP-IMS-TOF experiment. To achieve the same level of SNR, the SA-IMS experiment required 16 times more averages. This factor is directly equivalent to the additional number of ion gate pulses released into the drift cell per MP experiment.

MP Protein Sequence Coverage

While Figures 3 through 6 have demonstrated the SNR gains due to multiplexing for individual ion species, these gains do not explicitly represent the benefits of multiplexing for a complex system. As a result, multiplexing was examined with respect to the number peptide identifications and protein coverage at a given analysis time, using BSA as a model complex system. Figure 7 shows the levels of coverage observed by infusing a 60 nM solution of tryptically digested BSA at a flow rate of 250 nL/min for both MP and SA experiments. The level of coverage for the MP-IMS-TOF (5-bit PRS zero-filled by 20) and SA-IMS-TOF techniques are plotted as a function of both total acquisition time and number of IMS averages. The maximum coverages were $64 \pm 4\%$ and $38 \pm 4\%$ for MP and SA, respectively. Again, it should be noted that the SA-IMS experiment in this figure, as well as in Figure 6, utilized a 2 ms accumulation time because extended accumulation times can induce space charge effects that adversely affect protein sequence coverage.³⁰ Though not shown in Figure 7, a practical comparison between the MP-IMS-TOF and the SA-IMS-TOF experiments were made using an IFT accumulation time of 16 ms. This IFT accumulation time was previously found to provide the optimum BSA sequence coverage without inducing space charge effects in the IFT.³⁰ The percent BSA coverage for the SA-IMS-TOF experiment using a 16 ms accumulation time and an 18.6 ms acquisition time was $33 \pm 4\%$. The MP-IMS-TOF approach exceeded the optimum SA-IMS-TOF levels by approximately a factor of 2. While the MP technique provided a significantly larger degree of coverage, perhaps most impressive was that the results for the MP-IMS-TOF were obtained in a third of the time compared to the SA technique. The general trend of protein coverage as a function of the acquisition time indicates that the SA-IMS-TOF experiment requires a significantly longer time to reach the levels observed in the MP experiment. On average, the same coverage was obtained at the acquisition time ratio comparable to the SNR difference for the two experiments, positioning MP-IMS-TOF as a robust high-sensitive high-throughput approach for proteomics applications.

CONCLUSIONS

Previous developments of an IFT combined with an IMS drift cell have demonstrated a marked increase in ion utilization efficiency compared to a traditional IMS experiment. However, this approach remains inherently limited by instrumental duty cycle. We have developed a MP-IMS-TOF instrument that is characterized by a fixed accumulation periods throughout the

encoding sequence and, therefore, is capable of deciphering arbitrary input signals without the use of complex weighing schemes. For the chemical systems examined, the SNR for the MP-IMS-TOF technique was greater in all instances than the SNR for the SA-IMS-TOF experiment. Because the MP-IMS-TOF results were derived from a 5-bit extended PRS, the theoretical SNR gain compared to the SA-IMS-TOF experiment was ~ 3 ,³⁶ which matched the experimental data at increased analyte concentrations (See Figure 5). However, at lower analyte concentrations the observed SNR gains increased by more than a factor of 10. We attribute this improvement to factors related to the two dimensional nature of the IM-TOF-MS technique and the increased throughput of the extended PRS compared to the conventional Simplex sequences. Because any given ion signal recorded using the current configuration has the added dimensionality of m/z , interfering species that otherwise would contribute to the noise measurement are drastically minimized. Further, the Fellgett advantage applies to the dimension in which signal modulation was applied. In the case of the IMS-TOF, the MP signal modulation occurs in the IMS dimension, while the signal acquisition is performed in the TOF domain. Compared to previous experiments using HT-IMS,^{37, 38} our multiplexing scheme generates twice as many ion pulses as those derived from the conventional Simplex sequence. It is these unique features that allow the measured SNR gains for the current system to exceed those predicted by theory. The multiplexing not only results in significant SNR gains for individual analytes, but also increases the level of information content derived from more complex system. We have shown that the MP-IMS-TOF platform provides better protein coverage than the SA-IMS-TOF instrument in only a fraction of the analysis time required by the conventional method. Further, these results demonstrate that MP-IMS-TOF technology has the potential to become a robust, high-sensitivity, high-throughput analysis tool for proteomics and biomedical applications.

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Figure 1.

Ion Funnel Trap (IFT) Schematic consisting of five distinct sections: 1.) ion funnel entrance; 2.) jet disruptor; 3.) converging region; 4.) ion trapping chamber; 5.) IFT exit and beginning of ion mobility drift cell. Ions are admitted to the trapping chamber by lowering the potential applied to the entrance grid and trapped in region #4 by controlling the DC potentials of the trapping and exit grids.

Figure 2. (a) Modified pseudo-random sequence used to modulate the ion funnel trapping and exit grids. (b) Complementary waveform used to control the ion funnel trap (IFT) entrance grid and normalize the number of ions introduced into the IMS drift cell. c.) Comparison of the number of pulses between the modified PRS with and without normalized trapping times as a function of ion accumulation time.

Figure 3.

Multiplexed Experiment Schema acquired using a 5-bit PRS zero-filled by 20 while normalizing ion accumulation time. (a) represents the raw IM-TOF data set prior to applying the inverse transform, the result of which is illustrated in (b). The mass spectrum from both (a) and (b) are located to the left and right of the respective contour plots. The IMS dimension is shown below each contour. To illustrate the SNR improvement provided by the MP experiment no weighting was used during the inverse transform which gave rise to the intensity differential between raw and transformed IMS spectra. The absence of reconstruction artifacts in (b) demonstrate the utility of normalized the accumulation time within the ion funnel trap.

Figure 4.

SNR Comparison in the m/z dimension between the multiplexed and signal averaged experiment for three peptides each at 50 nM. Because each MP ion gate release originated from a 2 ms accumulation time the SA equivalent was used for comparison.

Figure 5.

SNR Comparison of Neurotensin $[M+3H]^+3$ between the SA and MP experiments as a function of acquisition time for two concentrations, 100 nM (a) and 5 nM (b). For both concentrations the SNR for the MP experiment always exceeded that of the SA-IMS experiment with the most pronounced gains observed for the 5 nM sample.

Figure 6.

SNR and throughput advantage of the MP approach for the BSA tryptic peptide $^{437}\text{KVPQVSTPTLVEVSR}^{451} [\text{M}+3\text{H}]^{3+}$ compared to the SA experiment. Because the MP data were acquired using a modified 5 bit PRS with 16 ion gate releases, in order for the SA experiment to attain an equivalent SNR the experiment must be run 16 time longer. Combined with effective IFT modulation the modified PRS allows for an enhanced throughput and analytical sensitivity compared to both the traditional HT- and SA-IMS-TOF experiments.

Figure 7.

Percent coverage observed for a 60 nM tryptically digested BSA sample for the SA- and MP-IMS-TOF experiments. Coverage is plotted as a function of both the number of averages and total acquisition time. Approximately 30 seconds were required to attain the maximum coverage for the digested BSA sample using the MP-IMS-TOF experiment; whereas the SA-IMS-TOF experiment using a 2 ms accumulation time did not reach the levels of the coverage observed for the MP experiment even after 100 s.