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Subzero Celsius separations in three-zone temperature controlled hydrogen deuterium exchange mass spectrometry

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Abstract

Hydrogen deuterium exchange mass spectrometry (HDX MS) reports on the conformational landscape of proteins by monitoring the exchange between backbone amide hydrogen atoms and deuterium in the solvent. To maintain the label for analysis, quench conditions of low temperature and pH are required during the chromatography step performed after protease digestion but before mass spectrometry. Separation at 0 °C is often chosen as this is the temperature where the most deuterium can be recovered without freezing of the typical water and acetonitrile mobile phases. Several recent reports of separations at subzero Celsius emphasize the promise for retaining more deuterium and using a much longer chromatographic gradient or direct infusion time. Here we present the construction and validation of a modified Waters nanoACQUITY HDX manager with a third temperature-controlled zone for peptide separations at subzero temperatures. A new Peltier-cooled door replaces the door of a traditional main cooling chamber and the separations and trapping column are routed through the door housing. To prevent freezing, 35% methanol is introduced post online digestion. No new pumps are required and online digestion is performed as in the past. Subzero separations, using conventional HPLC column geometry of 3 µm particles in a 1x50mm column, did not result in major changes to chromatographic efficiency when lowering the temperature from 0 to –20 °C. There were significant increases in deuterium recovery for both model peptides and biologically relevant protein systems. Given the higher levels of deuterium recovery, expanded gradient programs can be used to allow for higher chromatographic peak capacity and therefore the analysis of larger and more complex proteins and systems.

Keywords

HDX MS; subzero chromatography; low temperature separation; deuterium recovery

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1. Introduction

Hydrogen deuterium exchange mass spectrometry (HDX MS) has been used as a method of protein biophysical analysis for more than 25 years [1, 2]. It has become widely accepted over the past 10 years with the availability of commercial hardware [3] and multiple software solutions [4] that have enabled ease of analysis. Concomitantly, the current state of the field is expanding in terms of participant labs, speed, the desire to investigate large protein machines and multi-protein systems, and examining proteins in more native-like environments.

HDX relies on the natural phenomenon of backbone amide hydrogen exchange with solvent hydrogens [5]. This reaction is spontaneous in the forward and reverse directions and both base and acid catalyzed. Classic HDX MS experiments begin with labeling the analyte protein with deuterium and end with LC/MS analysis to locate the incorporated deuterium. In order to maintain the deuterium label during LC/MS analyses, chromatographic separation must be done rapidly, at low temperature (typically 0 °C), and at the pH minimum for exchange (2.5). Under these conditions, the half-life range for back exchange is on the order of 30 to 120 minutes [2]. Generally, separation is performed in 10–20 minutes (or faster) in order to minimize the loss of the deuterium label [6, 7]. Fast separations at 0 °C with traditional RP-HPLC are usually of low efficiency, but much higher efficiency separations can be achieved using UPLC [3, 8].

In the recent past, separations at < 0 °C, hereafter referred to as subzero, have been explored in order to limit the reversion of deuterium to hydrogen [9–13]. The intrinsic rate of exchange, k_{ex} , is slowed by reducing the temperature [5]. Fig. 1A shows the average exchange rates [k_{ex} (min⁻¹)] for the backbone amide hydrogens of poly-DL-alanine (PDLA) at temperatures from 0 to –20 °C at pH 2.5, as calculated according to [14]. The rate of exchange is 7.5-fold slower at –20 °C than at 0 °C and 66-fold slower at –20 °C than at 20 °C (calculation not shown). Recently, Venable et al. [9] showed this theory in practice when they demonstrated that reversion of deuterium to hydrogen in the analysis step can be greatly reduced by lowering the LC analysis temperature to –30 °C but preventing freezing with solvent modifiers (ethylene glycol and methanol) in the mobile phase. The increase in deuterium recovery afforded at subzero temperatures has also been employed to extend the ECD/ETD data accumulation window for top-down analyses of labeled proteins [10, 12, 13].

Lowering the temperature of the separation means increased deuterium recovery, which is useful for all HDX MS experiments. However, for experiments with high chromatographic complexity (*i.e.*, large proteins, protein complexes, etc.), lower temperature could also allow the chromatographic gradient to be extended in order to increase peak capacity without compromising levels of deuterium loss; the deuterium loss would be the same in a very cold long gradient as it would be in a warmer, short gradient. Fig. 1B shows that, from a theoretical point of view, a 1 hour separation at –20 °C has the same deuterium recovery as 10 minute separation at 0 °C. This theoretical calculation applies to PLDA, but could be imagined for other sequences. Deuterium recovery in a long, very cold separation that is no worse than separations at 0 °C would allow for a shallower gradient and, in principle, better separations for complex samples. The total cycle time would be longer, but the peak capacity

would be higher, and therefore the boundaries of what could be analyzed by HDX MS could be vastly expanded. However, as was also recently demonstrated [9, 11], lowering the temperature to -20°C or -30°C is not trivial because the backpressure realized when pumping solvents at subzero temperatures is too high for most currently available solvent pumps. One workaround [9, 11] was to use a small trap-like column (contained in a small commercial freezer with limited fine temperature control) that creates very low backpressure.

Here we present the construction and exploration of a system wherein temperature can be independently controlled in three zones: digestion, trapping and separation. This device is hereafter referred to as the SZ-nanoACQUITY where SZ stands for “subzero”. In the new third cooling zone, the trapping and separations column can be maintained at subzero temperatures. We describe separations at various subzero Celsius temperatures, ranging from 0°C to -20°C , in the SZ-nanoACQUITY which has a maximum operating pressure of 15,000 psi. The SZ-nanoACQUITY allows for online pepsin digestion – before antifreeze solvent addition for subzero separations – and the use of conventional HPLC column geometry. Unlike previous implementations of subzero chromatography, this is a unified device. The door of an existing nanoACQUITY HDX manager is simply replaced and the SZ-nanoACQUITY is a completely self-contained three temperature zone device with all requirements housed within in a small footprint and controlled by current software and hardware.

2. Materials and methods

2.1. Chemicals and materials

Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA). MassPREP digestion standards as well as trap and separation columns were obtained from The Waters Corporation (Milford, MA).

2.2. SZ-nanoACQUITY: door modification

The modified nanoACQUITY HDX manager door was designed and fabricated at Waters (Milford, MA). Whenever possible, existing Waters components were utilized in the construction of the custom door. A 121 watt cold plate (CP-121), pulse width modulated temperature controller (TC-48-20), and power supply (PS-24-12.5) were purchased from TE Technology (Traverse City, MI). Semi rigid closed cell PVC insulation, ” thick Lexan Polycarbonate sheet, and ” thick acrylic sheet were purchased from McMaster-Carr (Chicago, IL). The cold plate and Lexan sheet were attached to provide an independently controllable cold surface on the inside of a commercially available HDX Manager. The PVC insulation was machined in such a way to provide a thermally isolated chamber separate from the main chamber of the HDX Manager. An acrylic cover was machined and attached using thumb screws for easy access to the subzero chamber.

2.3. Sample preparation

2.3.1. Highly deuterated peptide mixtures—Highly deuterated peptides (Waters MassPREP Digestion Standard, Phosphorylase b) were prepared by dissolving 1 nanomole lyophilized peptides into 1 mL H₂O or D₂O that was adjusted to pH/pD 2.5 with HCl/DCl. Peptides were allowed to equilibrate at 37 °C for two hours before use. The labeled peptide mixture was diluted to 6.5 μM with methanol (final solution of 35% methanol) immediately prior to injection into the LC system.

2.3.2. Deuterium exchange reactions—Deuterium labeling was initiated with an 18-fold dilution of 40 picomoles of full length BTK with a buffer containing 99.9% D₂O, 20 mM Tris pD 8.00, 150mM NaCl, 10% glycerol. At specific time points (10 sec, 10 min, 100 min) the labeling reaction was quenched with the addition of an equal volume of quench buffer (150 mM potassium phosphate, 100% H₂O, pH 2.47). Quenched samples were immediately injected in the SZ-nanoACQUITY for online pepsin digestion using a Waters Enzymate column.

2.4. Theoretical calculations of deuterium incorporation

Theoretical calculations of deuterium incorporation were performed using HXPep (courtesy of Zhongqi Zhang, Amgen, and based on Bai et al. 1993 [14, 15]). We note that these calculations are only semi-quantitative estimates because they rely on NMR data acquired in H₂O or D₂O and may not be directly applicable to the aqueous/organic mixtures used here.

2.5. Chromatography and mass spectrometry

2.5.1. Direct injection chromatography—In direct injection experiments, 16 picomole aliquots of tryptic peptides were injected directly onto an Atlantis T3 3 μm 1.0 x 50 mm column (Waters Corp.) and eluted into the mass spectrometer with a 3–28% gradient of solvent B [where solvent A was water:methanol (65:35) and solvent B was acetonitrile] over 20 min at a flow rate of 70 μL/min. 0.1% formic acid was present in all mobile phases.

2.5.2. Single pump trapping chromatography—For peptide trapping/desalting experiments, quenched deuterium labeling samples were trapped and desalted on a VanGuard Pre-Column trap (2.1 mm x 5 mm, Acquity UPLC BEH C18, 3.5 μm) for 3 min. The trap was placed in-line with an Atlantis T3 3 μm 1.0 x 50 mm column (Waters Corp.) and eluted into the mass spectrometer using a 3–28% gradient of solvent B as above over 10 min at a flow rate of 70 μL/min. 0.1% formic acid was present in all mobile phases.

2.5.3. Mass spectrometry—Mass spectrometry was performed with a Waters SynaptG2si mass spectrometer using ESI (+) resolution HDMS^E mode. Instrument settings were: 3.2kV cone and 40 V capillary voltages with source and desolvation temperatures of 80 and 175 °C, respectively, and a desolvation gas flow of 600 L/hour. Mass spectra were acquired using either a 0.4 or 1.0 sec scan time.

2.6. Data analysis

All peptides were identified using PLGS 3.0 and deuteration levels were calculated using DynamX 3.0. Relative deuterium amounts for each peptide were calculated by subtracting

the average mass of the undeuterated control sample from that of the deuterium-labeled sample for isotopic distributions corresponding to the +1, +2, +3, or +4 charge state of each peptide. The data for BTK were not corrected for back exchange and are therefore reported as relative values [2, 16].

3. Results and discussion

3.1. HDX manager modification

The current version of commercial HDX managers is similar in construction to our initial build [3]. In these units, there are two temperature controlled zones. The first zone is the main chamber that houses both injection and trapping valves as well as the trapping and separation column; this chamber is normally maintained at 0 °C (Fig. 2A). The second zone is independent of the first and houses the pepsin column near a small resistive heater that maintains the chamber at digestion temperatures averaging 10–15 °C. To obtain a third cooling zone for subzero separations, we modified a commercial HDX manager door to create a space where both the trapping and separation columns could be cooled to subzero temperatures. Fig. 2A shows the new door with the large Peltier cooling units and indicates where the column and trap are housed. Temperature validation with multi-sensor thermocouples was performed (data not shown) to be certain that temperature could be controlled and maintained in the device. This new set up was able to easily cool a column and trap efficiently to –20 °C and the temperature of this zone can be maintained without altering the temperature of the main cooling chamber. Modifications to the flow path (Fig. 2B), but with no additional pumps, maintain traditional online pepsin digestion of quenched deuterium labeled samples. With a two pump UPLC platform with M-class ACQUITY pumps, pepsin digestion is driven by the “A” side of the auxiliary solvent manager (ASM) while the “B” side of the ASM pump is set to isocratically deliver methanol (as an antifreeze agent) through a tee post pepsin column. The solvent composition of eluent from the pepsin column becomes 35% methanol before it arrives at the subzero temperature region (gray in Fig. 2B).

3.2. Subzero chromatography

Assured that the modified SZ-nanoACQUITY door performed adequately as an isolated third temperature zone, we moved to validate the chromatographic performance of this system. Traditional UPLC separations in an HDX experiment employ 1.7–1.8 µm particles packed into either a 1x50 or 1x100 mm column. Using these geometries, separations at 0 °C yield system pressures that range from 7,000 to 15,000 psi at typical flow rates (50–120 µL/min). As we intended to lower the temperature to –20 °C, we needed to either alter the column geometry or alter the size of the packing material (or both) to accommodate a pressure limit of 15,000 for the hardware and fittings used. Previous work [9, 11] with subzero HDX separations reduced both the geometry as well as increasing the particle size to overcome the pressure limitations of their respective pumping systems: a 2.1x10mm 3 µm C18 (proZap) operating at ~5,000 psi [9] or a 2x30mm 5 µm C4 (Phenomenex) [11] operating at ~2,000 psi. We chose to maintain traditional column geometry at 1x50 mm and to increase the particle size of the packing material to 3 µm. We classify this separation, therefore, as HPLC above 10,000 psi rather than UHPLC or UPLC [17–19]. Concerned that

returning to 3 μm particles (from 1.7 μm UPLC particles that have been used for HDX MS nearly 10 years [3]) would compromise the chromatographic efficiency, especially at the subzero temperatures, we used a phosphorylase b standard to vet the integrity of the separations. This standard is a trypsin digestion of phosphorylase b and therefore no online digestion was needed to create peptides. We bypassed the flow path of the pepsin column (direct injection flow path not shown) to bring 16 picomoles the phosphorylase b peptides directly onto the reversed-phase column (with mobile phase A containing 35% methanol) for separation at three different temperatures (0 $^{\circ}\text{C}$, -10 $^{\circ}\text{C}$, and -20 $^{\circ}\text{C}$). Fig. 3A shows the 20-minute gradient separations of the unlabeled (no deuterium) digestion standard at 0, -10, and -20 $^{\circ}\text{C}$. The median chromatographic peak width at each temperature was found to be ~13.7 sec, ~14.3 sec, and ~19.8 sec for the near baseline separations at -0, -10, and -20 $^{\circ}\text{C}$, respectively [compare to typical peakwidths of 4.5–6.5 secs in traditional HDX UPLC separations]. The backpressure for each separation (0 $^{\circ}\text{C}$: ~9,100 psi; -10 $^{\circ}\text{C}$: ~10,200 psi; -20 $^{\circ}\text{C}$: ~12,000 psi) did not eclipse the pressure limit (15,000 psi) of the system. Chromatographic performance and peak shape, despite not being as crisp and well defined as with smaller particle size separations, was not greatly altered as temperature decreased from 0 $^{\circ}\text{C}$ to -20 $^{\circ}\text{C}$. Even though there were apparent differences in the chromatograms (with expected shifts in later retention time with decreasing temperatures), the elution order of the peptides was maintained and there were consistent peptides identified in each separation (see Fig. S1).

3.3. Subzero deuterium recovery

With confidence that we had not severely compromised chromatographic performance by increasing particle size, adding 35% methanol to a typical water:acetonitrile gradient, or moving to subzero temperatures, we proceeded to monitor deuterium recovery using a maximally deuterated version of the phosphorylase b digestion standard used above. The details of the preparation of this deuterated digest are found in the Materials & Methods.

For the 20 minute separation used here, we calculated the expected theoretical change deuterium recovery as a function of temperature for each of the tryptic peptides in the mixture following the method described in Refs [14, 15] (see Materials and Methods section 2.4). The results are shown in Figure S1A. The average theoretical increase in deuterium recovery when lowering the temperature from 0 $^{\circ}\text{C}$ to -20 $^{\circ}\text{C}$ was 1.1 Da ($D_{-20^{\circ}\text{C}} - D_{0.0^{\circ}\text{C}}$) with a range from 2.3 to 0.17 Da dependent on peptide. Lowering the temperature only to -10 $^{\circ}\text{C}$, the average theoretical increase in deuterium recovery in the same set of peptides was calculated to be 0.65 Da ($D_{-10^{\circ}\text{C}} - D_{0.0^{\circ}\text{C}}$) with a range from 1.3 to 0.11 Da dependent on peptide. The theoretical average difference between -10 $^{\circ}\text{C}$ and -20 $^{\circ}\text{C}$ was 0.41 Da ($D_{-20^{\circ}\text{C}} - D_{-10^{\circ}\text{C}}$) with a range from 0.98 to 0.050 Da.

With theoretical predictions in hand, we experimentally measured the deuterium recovery (Fig. 3B). Both the deuterium recovery (top panel) and the difference between experimental measurement and theoretical calculation (bottom panel) for a subset of the 30 coincident peptides are shown. Recall that these peptides were fully deuterated prior to injection. It is clear from these measurements that more deuterium was experimentally recovered at lower temperatures, as expected. The average increase in deuterium recovery (across all 30

peptides) when temperature was reduced from 0 °C to –20 °C was found to be 1.9 Da ($D_{-20^{\circ}\text{C}} - D_{0.0^{\circ}\text{C}}$) with a range from 5.5 to 0.056 Da dependent on peptide (see Fig. S1A for a presentation of all the data). Lowering the temperature to –10 °C gave an average increase in deuterium recovery in the same set of peptides of 1.1 Da ($D_{-10^{\circ}\text{C}} - D_{0.0^{\circ}\text{C}}$) with a range from 3.3 to 0.2 Da dependent on peptide. The average difference between –10 °C and –20 °C was 0.82 Da ($D_{-20^{\circ}\text{C}} - D_{-10^{\circ}\text{C}}$) with a range from 4.0 to –1.34 Da..

The bottom panel of Fig. 3B shows the relative difference in measured versus theoretical deuterium incorporation for the subset of the coincident peptides. Note that the theoretically expected value is different from the number of backbone amide hydrogens (green lines in the top panel of Figure 3B) because it considers the observed retention time. In most, but not all, cases experimental data are in fair agreement with the theory. A few peptides did not have experimental deuterium recovery that aligned with theoretical calculations. In peptides 372–387 and 726–740, there was more deuterium recovered in the –20 °C separation over what theory predicts. One complication of HDX studies where the analysis temperature is very low is that deuterium that was incorporated into certain sidechains will not have enough time to revert to hydrogen. Ordinarily, with all H₂O in the quench, digestion and separations, deuterium in sidechains easily reverts to hydrogen [2]. There was evidence for a lack of sidechain reversion with the –20 °C separation as 6 of the 30 coincident peptides (this number drops to 1 at –10 °C and 0 at 0 °C) incorporated more than the theoretically allowed maximum number of deuterons (see Fig. S1A). In these fully deuterated digest tests (Fig. 3) the mobile phase was 65% H₂O, 35% methanol with 0.1% formic acid. The very short residence times in the fluidics lines was less than a peptide may spend in H₂O when there is digestion and desalting. In traditional online digestion and desalting, there could be upwards of 4 min spent in an H₂O-rich environment. Therefore, in this subzero analysis, there may not have been enough time for deuterium to revert to hydrogen in all instances, particularly at arginine sidechains which exchange slower than other sidechain positions. This effect at lower temperatures must only be considered for samples that follow a direct injection flow path. Deuterium labeled samples that undergo online digestion and trapping, as described below, are less likely to see deuterium recoveries over 100% due to the fact they have additional time in H₂O-rich environments. In separate experiments (Fig. S2) where the deuterium labeled phosphorylase b digest was analyzed with a union in place of the pepsin column, and a 3 min desalting time bathed these peptides in H₂O solvent at 0 °C for much longer, no evidence for peptides with more than the theoretically allowed amount of deuterium was found in 10, 20, and 60 min gradients. The 10 minute gradient at 0 °C in these tests resulted in an average 77% deuterium recovery, the level of deuterium recovery typical for a commercial system (data not shown). The same sample after the same desalting step but with a 1 hour gradient at –20 °C had an average deuterium recovery of 82% for the same cohort of peptides (Fig. S2B). These measured values for deuterium recovery are in good agreement with what theory predicts for PDLA: 75 % for a 10 min separation 0 °C and 80% for a 1 hour separation at –20 °C (Fig 1B). There was no evidence for a decrease in deuterium recovery for the longer separation at –20 °C; the label was preserved even with a long, shallow gradient.

3.4. Subzero separation after online pepsin digestion

Having validated that our SZ-nanoACQUITY system performed well when a mixture of deuterated peptides was injected, we moved to using a real (*i.e.*, a not a QC standard) protein to investigate how the system would behave with online digestion, trapping and separation. Here we chose to use BTK, a non-receptor tyrosine kinase (70 kDa of unique sequence, 665 amino acids) for which we have previously acquired significant HDX data [20–23]. BTK digests and responds well; there are and copious amounts of digestion and deuterium recovery data for it using a regular HDX nanoACQUITY system.

Unlike the analyses of the maximally deuterated peptides, typical time-point labeling (10 sec, 10 min, and 100 min in deuterium) samples of BTK were prepared, quenched, and loaded in the SZ-nanoACQUITY for online pepsin digestion. The peptides eluting from the digestion column underwent a desalting step using a standard size peptide trap prior to chromatographic separation. Both trapping and separation were maintained at 0 °C or –20 °C using the modified door of the SZ-nanoACQUITY (Fig. 2). Fig. 4 shows a comparison of the analysis of BTK deuteration at 0 °C versus –20 °C. All other parameters besides trapping/separation temperature (protein lot, mobile phase, column size and chemistry, MS analysis method, data processing, etc., as described in the Materials & Methods) remained the same. The quality of the chromatographic separations and mass spectral analyses at both temperatures (Fig. 4A) were comparable. The TICs show similar features with slight shifts to longer retention times at –20 °C, as expected. The BPI traces have a similar field of peptides, albeit with some variability in the early eluting species but similar overall median chromatographic peak widths between the separations at both temperatures. The median chromatographic peak width at each temperature was found to be ~14.7 sec at 0 °C and ~15.1 sec at –20 °C. As with previous separations at these temperatures (Fig. 3), the system pressure maximum for each separation did not eclipse the pressure limit of the system (0 °C: ~10,000 psi; –20 °C: ~12,000 psi). Very clean and sharp peaks were found in EIC's. The apex retention time for the peptide shown here (BTK peptide 243–268, with average *m/z* for the undeuterated sample of 785.12 Da) was 5.04 min at 0 °C and 5.64 min at –20 °C, a small change of ~30 seconds. The EIC's and very well-resolved isotope clusters (Fig. 4A, right) were highly similar to the commercial nanoACQUITY with 1.7 μ m separations at 0 °C (data not shown, but see also [20–22] for previous work with BTK).

3.5 Comparison of deuterium recovery at 0 and –20 C from labeled proteins

Peptides identified from online digestion of BTK with the SZ-nanoACQUITY resulted in just under 70% sequence coverage for separations at both temperatures (Fig. S3). This coverage is less than what can be achieved using the commercial nanoACQUITY system (~84%, data not shown, see also [20–22]) and is mostly as a result of spectral overlap of labeled peptide ions complicating final analysis of deuterium incorporation. Together, there were a total of 69 peptides there were coincident between the two temperatures and which were used to compare deuterium recoveries at both temperatures. Fig. 4B shows example deuterium incorporation curves for 4 selected peptides from the digestion of BTK, in increasing order of elution (i–iv). All 69 peptides are shown in Fig. S4 and raw deuterium uptake data are shown in tabular format in Fig. S5. In nearly all peptides, and certainly the

four shown in Fig. 4B, the deuterium recovery was greater in -20°C separations for at all time points measured, with the exception of peptide 94–111 which did not show an increase in recovery above 0.5 Da at any time point.

A difference plot of the deuterium incorporated at -20°C versus 0°C ($D_{-20^{\circ}\text{C}} - D_{0.0^{\circ}\text{C}}$) at each labeling time for each of the coincident peptides is shown in Fig. 4C (calculations shown in Fig. S5). The bulk of the improvement in deuterium recovery for the separation of BTK peptides as a function of temperature is evident at the earlier retention times with minimal to no improvements near the end of this 10 minute gradient window. In the experiment described above with maximally deuterated phosphorylase b peptides, wherein only trapping was used and no peptic digestion, the largest increase in deuterium recovery (1.6 Da) was also found at the start of the gradient, but there were comparable, significant savings (between 1.0 and 1.2 Da) at the end of the gradient as well (Fig. S2A). Here we chose a 10 min gradient to study BTK in the SZ-nanoACQUITY because the prior results for BTK that were all acquired with the commercial nanoACQUITY HDX system using a 10 minute gradient. For BTK in a commercial nanoACQUITY, a 10 minute gradient is more than adequate to resolve all species, bearing in mind that those separations are UPLC not HPLC as with the SZ-nanoACQUITY. The deuterium recovery realized for BTK using the SZ-nanoACQUITY was a clear advantage for BTK peptides that had higher recovery (Fig 4C) and this should also be true to any other relatively simple protein HDX study utilizing the SZ-nanoACQUITY with -20°C trapping and separation. The greatest savings in deuterium, nearly 2 Da for some peptides, was most pronounced for later exchange time points (100 minutes versus 10 seconds) as more heavily deuterated peptides have more to lose. We cannot, however, disregard the effect that the peptide sequence has on deuterium loss. As stated above there is a small possibility that the sidechain of arginine retains deuterium despite exposure to H_2O , especially at subzero temperatures. It is not completely clear if this is the case for the differences in recovery seen for BTK (Fig. S5), as well as with phosphorylase b (Fig. S2). From a simple count of the number of arginine residues in each peptide, there is not a direct correlation between the number of arginine residues and increased recovery for all peptides.

4. Conclusions

The newly constructed SZ-nanoACQUITY just described is a step forward in hardware evolution that serves to expand the capability of a commercial HDX manager to meet the growing need for peak capacity and improved deuterium recovery within the HDX MS community. With such a system, it will be possible to analyze large and complex samples using long gradients and still achieve comparable deuterium recovery to that found at present with shorter gradients and less peak capacity.

The move from 0°C to -20°C did not result in major changes to chromatographic efficiency but provided more deuterium recovery. A 10 minute gradient at 0°C resulted in an average of 77% deuterium recovery for phosphorylase b tryptic peptide separations while after the same sample analyzed with a 1 hour gradient at -20°C yielded an average of 82% deuterium recovery (Fig. S2B). Clearly, because there is not a decrease in deuterium

recovery for long separations at low temperatures, valuable information about protein conformation during labeling is not lost with a long, shallow gradient.

A complication that we will next overcome is that separations to date had to be performed with larger particles than desired. While sub 2- μ m particles will yield substantial improvements in chromatographic efficiency and even better peak capacity, the back-pressure that results is outside the bounds of commercial pumping systems. Recently the Jorgenson group published in this journal on the development of a 45,000 psi ultrahigh pressure LC system that is capable of well-resolved gradient separations of complex mixtures of peptides [24]. Using such pumping strategies that can operate outside of currently available commercial system pressure limits, it would be possible to use sub 2- μ m particles and the added peak capacity that is provided to realize high efficient separations for complex HDX samples.

In closing it is worth mentioning that all analyses here were performed without enabling the ion mobility separation (IMS) capabilities of the G2si mass spectrometer. It is certainly the case that adding IMS separations [25, 26] will compensate in part for sub-optimal chromatographic resolution and peak capacity, thereby diminishing difficulties we encountered with mass spectral overlap due to poor peak capacity with larger particle size.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Highlights

- HDX MS subzero separations via an addition to widely available hardware
- There were no major changes to chromatographic efficiency at -10°C and -20°C
- Significant improvements in deuterium recovery were achieved at subzero temperatures

Figure 1.

Theoretical calculations for backbone amide hydrogen exchange at various temperatures and times. (A) The intrinsic rate of exchange [k_{ex} (min⁻¹)] for backbone amide hydrogens in poly-DL-alanine (PDLA) was calculated at temperatures from 0 °C to -20 °C at pH 2.5 using the parameters described by [14]. (B) Theoretical recovery of deuterium from a single maximally deuterated backbone amide of PDLA versus LC retention time, for various temperatures (at pH 2.5). Data were calculated from the rates shown in panel A. The k_{ex} at each temperature is shown in the inset. The values on the far right are the percent deuterium remaining after 1 hour.

Figure 2.

Customized SZ-nanoACQUITY to allow subzero temperature separations. (A) Schematic showing the of the new subzero-capable door. The angled view from the back/inside (left) and view from the front (right) are shown. The approximate position of the rest of the HDX manager, which includes the main temperature house and the pepsin column, are shown as a light blue/gray box. Key components of the door: 1, temperature control panel; 2, heat sinks and Peltier unit; 3, aluminum plate for thermal mass; 4, insulation; 5, -20 °C chamber; 6, separation column. (B) Flow path diagram showing the three temperature zones. 1. 0 °C zone (blue) of HDX manager main chamber, which houses the injection and trapping valves; 2. Pepsin column chamber (green), located in the wall of the HDX manager; 3. modified subzero cooling door (gray), which houses the trap and analytical columns. The solvents flowing through each path are indicated, including a tee (center) to introduce methanol post-digestion but pre-separation. Each pump (ASM, BSM) can deliver two solvents, independently (ASM) or mixed (BSM), as shown.

Figure 3.

Separation efficiency, reproducibility, and deuterium recovery using the SZ-nanoACQUITY.

(A) Separation of a mixture of phosphorylase B tryptic peptides at three temperatures, as shown: 0 °C (black trace), -10 °C (blue trace), and -20 °C (red trace). The y-axis of the chromatograms is base-peak intensity. (B) Selected peptide deuterium recovery (all peptides shown in Fig. S1). Top panel: deuterium recovery for selected coincident peptides, in order of retention time (left to right), at the three temperatures, as shown: 0 °C (black bar), -10 °C (blue bar), and -20 °C (red bar). The green lines and accompanying numbers indicate the number of backbone amide hydrogens in each peptide, calculated from the number of amino acids minus prolines minus one for the N-terminal residue. Bottom panel: difference in deuteration (experimental minus theory) measured for each peptide, at each temperature.

Figure 4.

Deuterium incorporation in peptic peptides of BTK with LC analyses at both 0 °C and -20 °C. (A) LC and MS examination including total ion chromatograms (TIC, left), base peak intensity (BPI, left-center), extracted ion chromatograms (EIC, right-center), and mass spectra of a representative peptide (MS, right) are shown for separations at 0 °C (top, black) and -20 °C (bottom, red). The peptide shown in the EIC and MS is that covering BTK residues 243–268 and the increase in deuterium above undeuterated () at the centroid mass value is shown. (B) Deuterium incorporation plots for selected peptides of BTK (i–iv); all data shown in Fig S4 . (C) Difference plot comparing deuterium incorporation at 0 and -20 °C ($D_{-20^{\circ}\text{C}} - D_{0^{\circ}\text{C}}$) for coincident peptides (all data shown in Fig. S5). Each peptide is a vertical bar. Peptides are shown in increasing elution order, from left to right between 2

and 10 min, and at three deuterium labeling times (10 sec, 10 min, 100 min) from top to bottom using the color scale shown. Peptides for which uptake graphs are shown in B (i–iv) are identified with a dashed box.