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Tag and capture flow hydrogen exchange mass spectrometry with a fluororous-immobilized probe

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Abstract

Analysis of complex mixtures of proteins by hydrogen exchange (HX) mass spectrometry (MS) is limited by one's ability to resolve the protein(s) of interest from the proteins that are not of interest. One strategy to overcome this problem is to tag the target protein(s) to allow for rapid removal from the mixture for subsequent analysis. Here we illustrate a new solution involving fluororous conjugation of a retrievable probe. The appended fluororous tag allows for facile immobilization on a fluororous surface. When a target protein is passed over the immobilized probe molecule it can be efficiently captured and then exposed to a flowing stream of deuterated buffer for hydrogen exchange. The utility of this method is illustrated for a model system of the Elongin BC protein complex bound to a peptide from HIV Vif. Efficient capture is demonstrated and deuteration when immobilized was identical to deuteration in conventional solution-phase hydrogen exchange MS. Protein captured from a crude bacterial cell lysate could also be deuterated without need for separate purification steps before HX MS. The advantages and disadvantages of the method are discussed in light of miniaturization and automation.

Graphical abstract

Keywords

Elongin BC; Vif; affinity; fluororous immobilization

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Introduction

Hydrogen exchange monitored by mass spectrometry (HX MS) is an important tool in structural biology¹. HX MS has the ability, among other things, to monitor protein conformation and detect conformational changes. The HX MS methodology has seen tremendous advances over the last decade including innovations in mass analysis, chromatography, sample handling, and data analysis (e.g. Refs.²⁻⁶). Further advances in HX MS methodology will allow HX MS to become even faster, simpler, and amenable to studying more complex protein systems. To this end, we desired to develop a method to conduct HX on a surface using a tag/capture system. Conducting HX of a protein on a surface requires that the protein of interest be captured on the surface, retained during D₂O incubation, and released after quenching for mass analysis.

Tag/capture systems provide several advantages to the HX MS methodology including: (1) the ability to capture/isolate a desired protein system from a complex mixture for analysis, (2) avoiding traditional D₂O labeling dilutions, and (3) providing a platform for complete sample handling and deuterium labeling automation. The principle of tag/capture in HX MS has been demonstrated before using biotin/streptavidin⁷⁻⁹ or with antibodies¹⁰. There are improvements that could be made on these prior implementations. In the ideal tag/capture scenario, the binding between the tag and the captured molecule is extremely rapid, very high affinity and able to hold together under HX quench conditions (i.e., pH 2.5). A major limitation with many tag/capture systems is that while they have high affinity at neutral pH, they do not tolerate the low pH conditions required for the HX quenching step (e.g., GST-affinity and Ni-NTA/6xHis tag/purifications systems). While biotin/streptavidin has some affinity at lower pH, binding between antibodies and antigens is not maintained in quench conditions. The ability of the tag/capture system to tolerate HX quenching conditions and be compatible with multiple capture and release cycles would be clear advantages of an ideal system.

Conducting HX of proteins on a surface would also potentially avoid conventional sample dilution techniques and allow for automation. If the molecule to be labeled with deuterium were immobilized, it would be possible to conduct the HX reaction in a flow chamber (e.g. Refs.^{11,12}) by flowing deuterated buffer over the captured/immobilized protein for isotope exchange. An entire platform that included flow HX could be designed and built onto a micro-fluidic chip or a multi-valve setup where sample loading, washing, the HX reaction, and elution of proteins being analyzed could then be controlled by computer. One could then image single-step isolation of proteins or protein complexes from complex mixtures, such as *E. coli* or mammalian cell lysates, followed by automated flow HX exchange.

One system that may fulfill many requirements of an ideal tag/capture system for HX is based on the self associative properties of perfluorinated compounds [Refs.¹³⁻¹⁶ and references therein]. The chemical properties of perfluorinated hydrocarbons differ significantly from hydrocarbons because of the fundamental differences between C-F and C-H bonds. These differences lead to several unique properties of perfluorinated carbons: chemical inertness over a wide range of conditions¹⁷, and poor solubility in most aqueous and hydrocarbon solvents. Perfluorocarbons will dissolve in perfluorinated solvents, and are

thus said to be fluorophilic. This phenomenon is known as the fluorous effect and results in a high affinity between fluorinated substances^{15,17-19}. The fluorous effect can be attributed to the differences in the C-F and C-H bond polarizabilities. Cohesive London Dispersion forces, which arise from temporary induced dipoles, are greater in hydrocarbons because C-H bonds are more easily polarized than C-F bonds¹⁸. The fluorous effect can be explained more generally by the thermodynamic theory of non-electrolyte solutions for two non-polar molecules [see¹⁸⁻²¹ for theory and review of fluorous chemistry]. Following the seminal publication by Horváth and Rábai demonstrating the utility of perfluorocarbon solvents and the fluorous effect in chemical synthesis²², the fluorous effect has been utilized in other fields, including fluorous-conjugated small-molecule immobilization on fluorous surfaces such as small-molecule microarrays^{23,24}. The low background of protein capture by fluorous microarrays suggested that fluorous chemistry may be useful as a tag/capture HX MS system.

The concept of using a fluorous tag/capture system is illustrated in FIGURE 1. This system requires a chemical probe (red in Fig 1) specific for the target protein that is to be analyzed with HX MS. The probe can be a small molecule, peptide, and/or protein that associates with the target protein in solution. Covalently attached to the probe is a fluorous tag (either C₈F₁₇ or C₆F₁₃ tag) which tightly associates with perfluorinated hydrocarbon surfaces. The fluorous surface that was chosen for the work described here was fluorous coated silica beads packed into a column format. Other fluorous substrates/surfaces could also be used. To capture the protein to be studied, the target is flowed over the fluorous probe loaded surface. After successful capture, the captured target protein is washed and then labeled by flowing D₂O over the surface (through the column) for the desired labeling time. After labeling, the HX reaction is quenched by flowing acidic buffer over the beads. The target protein is released from the probe due to denaturation and flows out of the column for further analysis. The mass of the eluted, deuterated protein is determined directly or digested with pepsin and the deuterated peptides analyzed.

A model system was needed to test the fluorous-based capture system for HX applications. We previously²⁵ characterized the interaction between the HIV-1 accessory protein Vif and the cellular heterodimeric Elongin BC complex and chose this as the model system (FIGURE 1 inset). A short peptide from Vif is sufficient for tight (250 nM) binding to the Elongin BC complex. The Vif₁₃₈₋₁₆₁ peptide was synthesized using standard solid phase peptide synthesis and two C₆F₁₃ fluorous tags were attached to the N-terminus an amine reactive, succinimide-containing fluorous tag. To ensure that the fluorous tags did not interfere with Vif₁₃₈₋₁₆₁:Elongin BC binding, a spacer was utilized to tether the peptide to the fluorous tags. The fluorous-Vif₁₃₈₋₁₆₁ peptide was immobilized onto fluorous silica, Elongin BC captured onto the fluorous substrate, deuterated buffer was introduced and exchange characterized. We demonstrate the utility of this system for HX MS and various aspects of using this system for successful tag/capture experiments.

Experimental Section

Synthesis of fluorous-Vif₁₃₈₋₁₆₁

Synthesis was conducted using standard solid phase peptide synthesis. Briefly, the fluorous Vif peptide (FluorFmoc-PEG₂-GHNVGSLQYLALALITPKKIKK-NH₂) was synthesized on a 100 μmol scale using standard 9-fluorenylmethoxycarbonyl (Fmoc) peptide chemistry on NovaPEG Rink amide resin using a CEM Liberty 9008005 microwave peptide synthesizer. Subsequent transformations were conducted using manual peptide synthesis techniques. After removal of the N-terminal Fmoc group, the resin was suspended in DMF and coupled with Fmoc-NH-(CH₂)₃-PEG₂-HNCOCH₂OCH₂CO₂H (Novabiochem 851031, 2 equiv.) using HCTU (2 equiv.) and DIPEA (5 equiv.). After subsequent Fmoc deprotection (20% Piperidine/DMF), the resin was suspended in dichloromethane and fluorous tagged using N-[2,7-Bis(1H,1H,2H,2H-perfluorooctyl)-9-fluorenylmethoxycarbonyloxy] succinimide (Fluorous Technologies Incorporated, Pittsburgh, PA, F026005, 2 equiv.) and DIPEA (5 equiv.). The peptide was then cleaved from the resin using 95% TFA, 2.5% TIS, 2.5% H₂O and precipitated in ethanol to afford the crude, fluorous tagged, C-terminal amidated peptide. The peptide was purified on a Varian ProStar HPLC using a Dynamax 21.4 × 250 mm 300 Å C18 column eluted with water-acetonitrile containing 0.1% TFA as the mobile phase and UV monitoring at 220 nm. The fractions were collected and the solvent removed on a lyophilizer to produce 126 mg of the desired peptide as a dry powder.

Preparation of fluorous flash[®] packed column

An Alltech[®] (Grace, Deerfield, IL) analytical in-line guard column (3 mm × 22 mm id) was dry packed with ~ 7 mg of FluoroFlash[®] (40 μm, 60 Å pore diameter, 0.7 pore volume) (Fluorous Technologies Incorporated, Pittsburgh, PA) silica gel. The void volume, V_{void}, (value used to determine column volume equivalents) was calculated using the diameter (d), length of the column (L), and pore volume (V_{pore}) according to Equation 1 and determined to be 108 μL.

$$V_{\text{void}} = \left(\frac{\pi d^2 V_{\text{pore}} L}{4000} \right) \quad \text{Equation 1}$$

The packed fluorous column was washed with 20 column volumes of DMSO, distilled water, and wash buffer (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol at pH 7.0). All wash steps and column incubations were conducted with a 250 μL Hamilton syringe (Reno, NV) unless otherwise stated. The fluorous silica was passivated by flowing 1 mL of 1.46 mg/mL bovine serum albumin (BSA) (Biorad, Hercules, CA) through the packed column. Unbound BSA was removed by washing with 20 column volumes of wash buffer. Non-specific binding of Elongin BC was tested by flowing 500 pmols of protein (100 μL of 5 μM) through the fluorous column pre- and post-BSA passivation using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 μL/min. The flow-through and a 2 column volume wash was collected and mass analyzed as described above.

Elongin BC expression and purification

Preparation of recombinant Elongin BC complex and the Vif₁₃₅₋₁₅₈ peptide was conducted as previously reported²⁵. For the preparation of the *E. coli* lysate, a 2 g pellet of *E. coli* (BL21 DE3 pLysS) cells was re-suspended in lysis buffer containing 20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.0, and supplemented with PMSF and lysozyme. Re-suspended cells were lysed by sonication and the soluble protein fraction isolated by centrifugation at 30,000 g for 40 minutes. The soluble protein fraction (supernatant) was separated from the insoluble fraction and used as described below.

Immobilization of fluoros-Vif₁₃₈₋₁₆₁ and capture of Elongin BC

All fluoros immobilizations and captures were conducted at 25 °C. To immobilize fluoros-Vif₁₃₈₋₁₆₁ onto the fluoros silica, 125 nmols of the peptide (250 µL of 500 µM) were passed through the fluoros column using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 µL/min. To test the specific binding of fluoros-Vif₁₃₈₋₁₆₁ to fluoros silica, the non-fluoros tagged Vif₁₃₅₋₁₅₈ peptide was incubated with a packed fluoros column and washed as described for fluoros-Vif₁₃₈₋₁₆₁. Unbound fluoros-Vif₁₃₈₋₁₆₁ was removed by washing with 800-1000 column volumes of 20% methanol using a Shimadzu SCL-10A VP HPLC, followed by 20 column volumes of wash buffer. To capture purified Elongin BC or Elongin BC from an *E. coli* lysate, 500 pmols of purified protein (100 µL of 5 µM) or 100 µLs of lysate were flowed through a fluoros-Vif₁₃₈₋₁₆₁ loaded column using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 µL/min. The fluoros column was washed with 50 column volumes of wash buffer. Captured Elongin BC was eluted from the fluoros column by rapidly flowing 15 column volumes of quench buffer (0.8% formic acid, 0.8 M guanidine hydrochloride, pH 2.2) and mass analyzed as described above.

Hydrogen exchange

Solution-based HX reactions were conducted by incubating Elongin BC (4.00 µM) with a 2.5 molar excess of the fluoros-Vif₁₃₈₋₁₆₁ (10 µM) at 25 °C for 30 minutes. Equilibrated samples were then labeled by a 10-fold dilution in D₂O buffer (20 mM, HEPES, 1 mM DTT, 150 mM NaCl). After the desired labeling times, samples were quenched to pH 2.0 by addition of a 4:1 ratio (v/v) of quench buffer (0.8 M GdnHCl, 0.8% FA, pH 2.0, 0 °C) to each protein sample. The pH of the quenched samples had to be below the typical pH 2.6 value as no Elongin C (only Elongin B) elution was observed using the fluoros system at pH 2.6. This can likely be attributed to the strong interaction between the Vif peptide and Elongin C that remained intact at pH 2.6 and required the lower pH for complete dissociation. For fluoros-based HX experiments, the Elongin BC complex was captured onto a fluoros-Vif₁₃₈₋₁₆₁ loaded column and labeling was initiated by rapid manual flushing (~ 3 mL/min) of the column with 250 µL of D₂O buffer (see above). After initial flushing of the fluoros column with D₂O buffer, a continuous flow of D₂O buffer was maintained through the column using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 µL/min. The actual reported labeling times in the work presented accounted for the initial D₂O buffer flushing and flow using the syringe pump. HX reactions were then quenched (see above for quench buffer) by disconnecting the flow of D₂O buffer and then

rapidly flowing (~ 3 mL/min) 15 column volumes of quench buffer through the column. Column eluent from the quenching step was collected in 1.6 mL microcentrifuge tubes and subjected to LC-MS analysis.

LC-MS analysis

Samples were injected into a Shimadzu SCL-10A VP HPLC flowing water containing 0.05% formic acid, pH 2.6 at 50 μ L/min coupled to a Waters LCT premier mass spectrometer with a standard electrospray interface. Protein samples were trapped and desalted using an Alltech analytical in-line guard column, packed with POROS 20-R2 reversed-phase media (PerSeptive Biosystems) and eluted directly into the mass spectrometer with a gradient of 15-98% acetonitrile (containing 0.05% formic acid, pH 2.6) in 5 minutes. For samples containing deuterium, the injector, column and all associated tubing were kept at 0 $^{\circ}$ C to minimize back exchange. No correction for back exchange was made and all values reported are relative²⁶.

Results and Discussion

Selective capture of the Elongin BC complex with fluoros-Vif₁₃₈₋₁₆₁

Several controls were conducted prior to testing the system shown in FIGURE 1. We first ensured that the fluoros tags attached to the Vif₁₃₈₋₁₆₁ peptide did not interfere with Elongin BC binding and then examined and dealt with non-specific binding of Elongin BC to the fluoros silica support. To ascertain if the fluoros tagged Vif peptide had altered binding to the Elongin BC complex, a HX MS based binding assay²⁵ was conducted (see Supporting Information Figure S1). The results showed that solution hydrogen exchange of Elongin C was the same with or without the attachment of the fluoros tag to Vif₁₃₈₋₁₆₁, verifying that the fluoros tags on the Vif₁₃₈₋₁₆₁ peptide did not interfere with association. We did detect non-specific binding of Elongin BC to the fluoros silica but this could be eliminated by passivating the fluoros support with bovine serum albumin (see Supporting Information Figure S2). Having established that fluoros-Vif₁₃₈₋₁₆₁ bound to Elongin BC in solution and could be used as a fluoros probe, the next step was to determine if immobilized fluoros-Vif₁₃₈₋₁₆₁ packed into a column could be used to capture Elongin BC.

The ability of fluoros-Vif₁₃₈₋₁₆₁ to bind to fluoros silica and capture Elongin BC was tested by loading 125 nmols of fluoros-tagged peptide through a column packed with fluoros silica particles, washing extensively, and then flowing purified Elongin BC through the column. In principle, if the fluoros-Vif₁₃₈₋₁₆₁ bound to the fluoros silica and Elongin BC was passed through the column, no Elongin BC should be present in the column flow-through as Elongin BC should specifically bind to the Vif peptide attached via fluoros to the fluoros silica. A non-fluoros tagged Vif peptide was used as a negative control; this peptide should not associate with the fluoros silica and flow directly through the column. No retention of Elongin BC should be observed upon incubation with fluoros silica that had been treated with the non-fluoros tagged peptide. The results of these capture experiments are shown in FIGURE 2. When a non-fluoros tagged Vif peptide was used (FIGURE 2A), the MS of the fluoros column flow-through was the same as the input meaning that no Elongin BC was captured by the fluoros column. [Note: in these and

subsequent experiments, we did not test the efficiency of capture by quantifying the resulting MS signal, determine the eluted yield of protein after the quench step, or push the limits of sensitivity of the method by using small quantities of protein. Our goal was simply to qualitatively observe capture or release. The maximum relative intensity of each mass spectrum, therefore, is determined by the base-peak]. In contrast (FIGURE 2B), when fluoros-Vif₁₃₈₋₁₆₁ was passed over the fluoros silica in the column and Elongin BC was introduced, there was no Elongin BC in the column flow-through, presumably because the Vif probe had captured it in the column. To verify capture and release, a low-pH, denaturing HX quench solution (see Experimental Procedures) was passed through the column to denature and elute bound Elongin BC. The expected signal (FIGURE 2B,iii) for Elongin BC verified that indeed it had bound to the fluoros-Vif peptide and had been eluted by the low pH conditions. These results demonstrate that capture of a fluoros-tagged probe on a fluoros coated silica particle can be accomplished and that both binding and elution of a target protein to the probe can be accomplished.

Hydrogen exchange of Elongin BC on fluoros silica

Confident that the Elongin BC complex could be captured by the fluoros-Vif₁₃₈₋₁₆₁ peptide immobilized onto fluoros silica packed into a column format, the next step was to see if HX MS analysis of Elongin BC could be conducted using the fluoros capture system and if deuteration results were similar to those that were observed using a solution-based HX MS protocol. The conventional solution HX of Elongin BC bound to the Vif peptide was done first. The Elongin BC complex was incubated with a 2.5-fold molar excess of fluoros-Vif₁₃₈₋₁₆₁ in solution and samples were labeled and analyzed (see Experimental Procedures). A representative mass spectrum from the solution-based HX MS analysis of Elongin BC (Supporting Information Figure S3A) shows that, as expected, signals for Elongin BC and the Vif peptide were both present in the mass spectrum. Our next step was to perform the HX labeling experiments using the fluoros scheme described in FIGURE 1. The Elongin BC complex was captured in the fluoros column (as described in FIGURE 2) and deuterium labeling was initiated by first very rapidly flowing D₂O buffer through the column followed by somewhat slower flow of the same D₂O buffer for the desired labeling time. The shortest labeling time conducted in these experiments was 2 minutes. Reproducibly controlling the timing of D₂O flow into the fluoros column was challenging and limited our ability to monitor shorter labeling times (<1 minute); automation and better control of the flow with designed-for-purpose mixing devices would likely eliminate these issues. Unlike conventional solution-based HX MS, there was no effective dilution of the protein in the labeling step, similar to other dilution-free methods¹¹ and the level of deuterium that the peptide/proteins experienced (in the work here, 99.9% D₂O was used) was also not diluted. The HX quench step was conducted post labeling by rapidly flowing quench solution through the column. As described in FIGURE 1, the quench step served two purposes: (1) quench the amide HX reaction, and (2) denature and elute the Elongin BC complex from the fluoros surface. Eluent from the column upon quenching was manually collected in a microcentrifuge tube and subjected to LC-MS analysis. A representative mass spectrum from the fluoros-based HX MS analysis of Elongin BC (Supporting Information FIGURE S3B) shows an obvious advantage of the capture system: the only signals in the

final mass spectrum are those from Elongin BC and no signals from the Vif peptide are present.

To determine if capture of Elongin BC in some way altered the structure of the protein or if other undesirable interactions in the fluoruous silica column altered exchange into Elongin BC, we compared the deuteration of Elongin BC in solution and using the fluoruous capture system. FIGURE 3. Both the appearance of the mass spectra (panels *i*) and the deuterium uptake curves panels *ii*) illustrate that HX of Elongin BC in the fluoruous capture system was very similar to conventional solution exchange (bear in mind that exchange in solution was with an effective solution concentration after dilution of 90% D₂O/10% H₂O whereas that in the capture flow experiment was 99.9% D₂O). Based on these results, we were confident that immobilization onto a fluoruous surface did not alter Elongin BC conformation(s) and that the fluoruous based platform could be utilized for HX MS applications.

Selective capture of Elongin BC from an *E. coli* lysate

The results presented above illustrate the utility of a fluoruous capture system for HX MS applications. One limitation of conducting HX MS using conventional solution based techniques is that the protein system of interest needs to be isolated and purified prior to biophysical analysis. Protein expression and purification using conventional techniques can be challenging and include multiple rounds of purification to isolate pure recombinant protein. Protein purification can also be time consuming and large delicate, multi-protein complexes may not be easily purified with multiple steps, some of which can be harsh. A method that would allow for the selective capture and immediate HX analysis of a protein or protein complex/system (similar to that in TAP tagging approaches²⁷) would be valuable. An advantage of using the fluoruous-based tag/capture flow HX system is that the target protein might be selectively captured from either purified protein or a complex mixture such as an *E. coli* or mammalian cell lysate, without significant background protein contamination.

To demonstrate the ability of the fluoruous-based system to selectively capture a target protein from a complex mixture, an *E. coli* lysate was produced from cells expressing Elongin BC [as in Ref. ²⁵, see Experimental Procedures]. A mass spectrum of the lysate (FIGURE 4A,i) illustrates the complex nature of the lysate and the multitude of *E. coli* proteins present. The lysate was then directly applied to a fluoruous-Vif₁₃₈₋₁₆₁-loaded fluoruous column (as described above) and the unbound *E. coli* proteins removed by washing the column with 50 column volumes of wash buffer. After washing the column, 250 uL of wash buffer was then flowed through the column, collected, and analyzed by MS. The mass spectrum (FIGURE 4A,ii) shows that extensive washing removed all unbound proteins from the fluoruous column. To elute captured Elongin BC, quench buffer was flowed through the fluoruous column and the eluent collected and analyzed by MS. The resulting spectrum (FIGURE 4A,iii) shows the charge envelopes for both Elongins and significantly fewer contaminant peaks than the spectrum in of the entire lysate (panel *i*). These results show that Elongin BC can be selectively captured from the *E. coli* lysate. The next step was to demonstrate that HX could then be performed on the protein captured directly from lysate.

We repeated capture of Elongin BC with fresh lysate (as just described). Before elution the captured complex was subjected to HX as described above for purified Elongin BC (FIGURE 3). HX of Elongin BC captured from *E. coli* lysate was measured and the results for two charge states are shown in FIGURE 4B. The relative deuterium level of Elongin BC after 2 minutes of labeling was 24.8 for Elongin C and 28.6 for Elongin B, slightly less than that observed above (FIGURE 3) where Elongin C was 28.0 in solution and 31.5 with fluororous silica and Elongin B was 32 in solution and 34.5 with fluororous silica. These results show that HX MS can be conducted on a protein complex captured from a complex mixture (i.e., *E. coli* lysate) using this fluororous tag/capture system. One caveat is that using this strategy, a new capture must be performed for each desired labeling time. One could image a scheme in which parallel beds of fluororous silica were simultaneously exposed to lysate, then each bed independently exposed to deuterium for the desired time before elution and MS analysis.

Towards automation of tag/capture flow HX with fluororous

Automation of all the steps required for the work just described is well within reach and work on these aspects is ongoing. While we have only reported the concept using analysis of undigested proteins (as in FIGURE 5A), one obvious expansion is the digestion of proteins eluted from the fluororous column. Initial experiments have been performed using the system shown in FIGURE 5B and will be reported elsewhere. The system depicted in FIGURE 5 contains a 6-way switching valve which controls solutions that flow through the fluororous column. The fluororous column can be loaded with probe, washed, and loaded with target protein mixture, exposed to D₂O and quench buffer all through the 6-way valve because the outlet of this valve is directly connected to the fluororous column. The eluent from the fluororous column flows into a standard Rheodyne® injection valve which contains a trap for intact protein capture. This valve is submerged in ice to minimize HX back exchange. Peptide level HX experiments could be conducted with the modified system depicted in FIGURE 5B where a pepsin column is placed after the fluororous column. After protein capture and washing, the 4-way valve between the fluororous and pepsin columns would be switched so that protein eluent from the fluororous column is digested and subsequently separated in the second injection valve system shown. Initial experiments with the system shown in FIGURE 5B indicate that proteins can be captured; digested, and analyzed. Peptide-level MS analysis of HX in solution versus fluororous capture HX are nearly identical (data not shown), however, further peptide characterization using the Vif:Elongin BC system and other protein systems is required for complete characterization and validation of the automated design depicted in both FIGURE 5A and B. Miniaturization, refinement and optimization of protein loads can all be imagined and would further implement automation in this methodology.

Conclusions

The work presented illustrates that fluororous chemistry can be utilized in tag/capture HX MS applications. The fluororous system has the possibility to alleviate several issues with conventional in-solution labeling including the need to fully purify a desired protein system, manual labeling, and sample dilution during D₂O addition. A significant advantage is the

stability of fluororous interactions at the quench pH required in HX MS experiments. The fluororous-tagged species does not elute upon adjusting the pH to quench conditions and therefore does not enter the LC-MS system; thus, interference can be reduced. Fluororous tags can be successfully attached to peptides and immobilized on fluororous silica. The immobilized fluororous-Vif₁₃₈₋₁₆₁ in this work was successful at capturing Elongin BC from a purified solution and from an *E. coli* lysate. This concept could be utilized for any protein:ligand interaction including protein:protein, protein:small molecule, or protein:oligonucleotide interactions provided a fluororous-labeled probe molecule is available. Attachment of the fluororous moiety to a peptide during synthesis, as described in this work, is a straightforward strategy for probes that are small and can be made synthetically. One should not be confined to the exact strategy described here, but rather to the principle. For larger proteins, covalent attachment of a fluororous moiety via sulfhydryl coupling (or other type of attachment) to a cysteine (or other inserted residue) engineered at either terminal of a protein could be imagined. The fluororous tag could be attached to each member of a protein complex (e.g. protein complex containing A, B and C) in turn in order to prevent the tagged member (e.g. protein A) from entering the LC-MS system during study the other members (e.g., proteins B and C).

There are several important limitations of the fluororous technique. Proper passivation steps need be taken in order to eliminate non-specific interactions with the fluororous silica. We found that treatment with BSA was sufficient to eliminate most all non-specific binding. To work well, the protein:ligand interaction has to be of moderate to high affinity (we estimate 1 μ M) to retain the desired protein on the surface for HX analysis. The K_d of the Elongin BC with this region of Vif is ~ 250 nM²⁵, allowing efficient capture. We do not know the upper limit for the K_d for this system, although that could be determined in future research. An additional parameter that could be optimized for the described fluororous-based system is the ratio of the fluororous probe to target protein. Only one ratio of fluororous probe to target protein (*i.e.* Vif peptide:Elongin BC) was tested in these experiments. Further work could be conducted to optimize the fluororous probe:target protein ratios to optimize capture efficiency while using the minimal amount of fluororous probe. One important concern when conducting HX on a surface is that the surface itself does not alter protein conformation. The similar deuteration results of Elongin BC in solution as compared to when immobilized on the fluororous column show that with the right length of linker, impact due to proximity to the surface can be eliminated.

Despite several putative limitations, HX MS analysis of recombinant Elongin BC using the fluororous capture system illustrates that HX on a surface support is possible. As was illustrated using the capture of protein from *E. coli* lysate, such a system could significantly reduce the steps required for analysis of proteins overexpressed in liquid culture or *in vivo* (FIGURE S4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The fluorine capture flow HX MS workflow. A fluorine labeled probe (1) is created, immobilized on a fluorine-coated surface (2), the target protein captured (3), deuterium buffer passed over (4), exchange quenched by lowering the pH (5), and the eluted protein analyzed by LC-MS (6). The immobilized probe can be re-equilibrated with pH 7 buffer and reused (purple line connecting step 5 and step 2). The inset shows a more detailed view of the probe (Vif₁₃₈₋₁₆₁) and the captured protein (Elongin BC) used as the model system in this work. See also Ref. ²⁵.

Figure 2. Selective capture of recombinant Elongin BC. Passivated fluorous columns (see Figure S2) were loaded with a (A) non-fluorous Vif peptide or (B) fluorous-Vif₁₃₈₋₁₆₁. Mass spectra of 500 pmoles of Elongin BC before (i) and after (ii) flowing through each column. The quenched sample from the fluorous-Vif₁₃₈₋₁₆₁ loaded column is shown in panel B, iii. In all spectra, the charge envelope for Elongin B is indicated by the blue dots, and Elongin C by red dots.

Figure 3. HX MS analysis of Elongin BC captured on fluorosilica. (A) Deuteration of the +9 charge state of Elongin C and (B) the +11 charge state of Elongin B using the fluorosilica capture system (i) compared to solution based experiments (ii). Deuterium uptake curves for Elongin B and C are shown in (iii). Deuteration using the fluorosilica system is highlighted in red and solution based results in black. These deuterium levels were not corrected for back exchange. The average of three determinations is shown.

Figure 4.

Fluorous-based capture of Elongin BC from a complex mixture before HX MS. (A) (i) ESI mass spectra of an *E. coli* lysate containing Elongin BC before loading onto the column. The lysate shown in (i) was passed through a fluorous-Vif₁₃₈₋₁₆₁ loaded fluorous column, the column washed extensively, and bound proteins eluted by quenching. ESI mass spectra were obtained from the wash solution (ii) and after elution (iii). The charge envelope for Elongin B is indicated by blue dots and Elongin C by red dots. (B) HX analysis of Elongin BC from an *E. coli* lysate. The process illustrated in panel A was repeated and a deuterium labeling step was introduced. Representative mass spectra are shown in (i) for the +11 charge state of Elongin B and (ii) for the +9 charge state of Elongin C for the undeuterated (UN) and after 2 minutes of deuterium labeling.

Figure 5. Designs for semi-automated flow HX MS using fluororous capture. (A) A valve setup for intact protein HX analysis. (B) A valve setup for peptide-level HX analysis using the fluororous capture system. (C) ESI mass spectrum of 500 pmols of Elongin BC captured using the valve setup shown in Panel A.