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Automated Removal of Phospholipids from Membrane Proteins for H/D Exchange Mass Spectrometry Workflows

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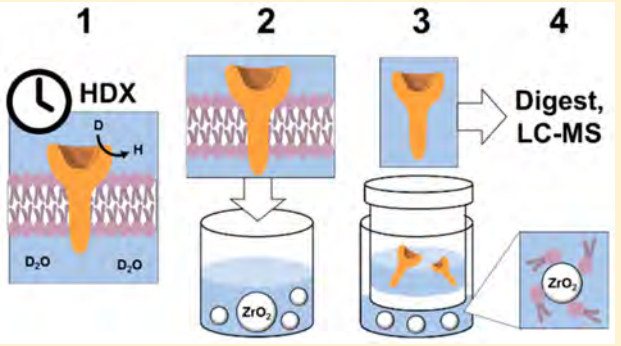
Supporting Information

ABSTRACT: Membrane proteins are currently the most common targets for pharmaceuticals. However, characterization of their structural dynamics by hydrogen/deuterium exchange mass spectrometry (HDX-MS) is sparse due to insufficient automated methods to handle full-length membrane proteins in lipid bilayers. Additionally, membrane lipids used to mimic the membrane environment and to solubilize membrane proteins can impair chromatography performance and cause ion suppression in the mass spectrometer. The workflow discussed herein advances HDX-MS capabilities and other MS applications for membrane proteins by providing a fully automated method for HDX-MS analysis based on a phospholipid removal scheme compatible with robotic handling. Phospholipids were depleted from protein samples by the addition of zirconium oxide beads, which were subsequently removed by inline filtration using syringeless nanofilters. To demonstrate this method, single-pass transmembrane protein FcγRIIa (CD32a) expressed into liposomes was used. Successful depletion of phospholipids ensured optimal liquid-chromatography−mass-spectrometry performance, and measurement of peptides from the transmembrane domain of FcγRIIa indicated phospholipids associated with this region were either not present or did not shield the transmembrane domain from digestion by pepsin. Furthermore, amino acid sequence coverage provided by this method was suitable to enable future measurement of structural dynamics of ectodomain, transmembrane domain, and endodomain of FcγRIIa. Moreover, this method is the first to enable fully automated HDX-MS on full-length transmembrane proteins in lipid bilayers, a notable advancement to facilitate understanding of membrane proteins, development of pharmaceuticals, and characterization for regulatory agencies.



The majority of drug targets are membrane proteins (MPs),[1] indicating the high demand and significance of proper structural characterization of this important class of pharmaceutical targets by hydrogen/deuterium exchange mass spectrometry (HDX-MS). However, MPs have proven to be difficult targets for HDX-MS due to their tendency to precipitate and aggregate in aqueous environments.

A satisfactory and successful solubilization method of native MPs has involved their insertion into artificial membrane constructs comprising bicelles,[1] liposomes,[2] or nanodiscs.[3,4] Whereas such membranes created in vitro can best simulate the native conformation and structural dynamics found in the cellular environment, they present technical challenges for analytical instrumentation. Membrane lipids can impair chromatography performance and cause ion suppression in the mass spectrometer from the overwhelming abundance of lipid molecules relative to protein molecules in lipid bilayer preparations.

Currently, HDX-MS analysis of MPs embedded in lipid bilayers is performed manually.[5,6] Because HDX-MS is essentially a kinetic study of hydrogen exchange rates,[7,8] variability in exchange, digestion, and other sample handling times can greatly increase measurement error. Poor repeatability and reproducibility of manually performed HDX can obscure subtle differences in structural dynamics between protein conditions. Moreover, the sheer number of replicates and time points needed to provide strong statistical significance is labor-intensive. When performed manually, the enormity of the task may require an unfortunate reduction of replicates or time-point measurements. Thus the automated, robust workflow for HDX-MS analysis of MPs provided herein can facilitate studies of MPs through the elimination of the arduous labor required by manual workflows.

EXPERIMENTAL SECTION

Materials. Full-length FcγRIIa (0.05 μg/μL) in liposomes was purchased from Abnova Corporation (Taipei, Taiwan). Abnova Corporation expressed FcγRIIa in vivo in the wheat germ expression system with proprietary liposome technology to produce FcγRIIa in liposomes. During production, wheat

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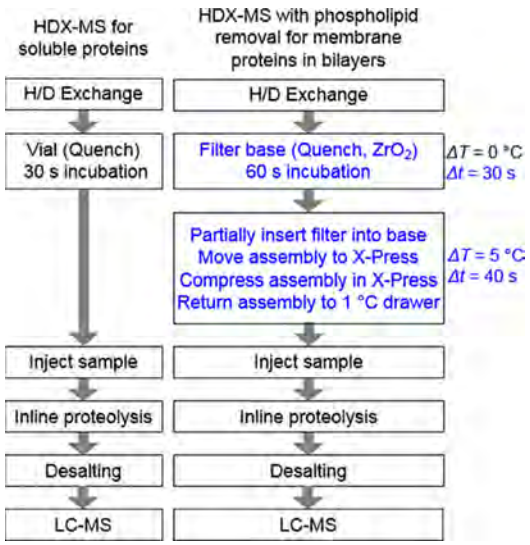
embryos were thoroughly washed to remove endosperm contaminants. Wheat germ expression systems produce properly folded proteins with disulfide bonds; however, this system does not provide glycosylation, and phosphorylation is poor.[9] Pierce tris(2-carboxyethyl)phosphine HCl (TCEP-HCl) was purchased from Thermo Fisher Scientific (Waltham, MA). Zirconium(IV) oxide 5 μm powder was purchased from Sigma-Aldrich (St. Louis, MO). Nanofilter vials (part no. 25535) with 0.2 μm pore size and poly(ether sulfone) (PES) membranes were purchased from Thomson Instrument Company (Oceanside, CA). A calibrated Thermo-Fisher model Orion 3-Star Benchtop pH meter (Thermo Scientific, Pittsburgh, PA) coupled to a catalog no. 13-620-223A double-junction refillable glass pH electrode (Fisher Scientific, Pittsburgh, PA) was used to measure pH.

Sample Handling and Automated Phospholipid Removal. FcγRIIa was provided in 25 mmol/L Tris-HCl, pH 8.0, and 2% glycerol at 0.05 μg/μL. Denaturation, membrane removal, inline proteolysis, desalting, and analytical separation were performed by a fully automated HDX PAL robot (LEAP Technologies, Carrboro, NC). Experiments to study effects of ZrO_2 were conducted with H_2O. Because the primary application of this method of phospholipid removal from transmembrane proteins in lipid bilayers was developed for HDX-MS workflows, the sample handling procedure mimicked a complete HDX-MS workflow in all other respects, as shown in Scheme 1. PAL robot transferred 25 pmol of FcγRIIa in 17.5 μL to an exchange vial containing 13 μL of 25 mmol/L Tris-HCl, pH 8.0, in H_2O. Next, 27 μL of sample was aspirated from the exchange reaction vial and added to the base of a filter system rather than a traditional quench vial. Filter bases contained 18 μL of 6 mol/L guanidine HCl, 0.1 mol/L sodium phosphate (pH 2.5), 0.4 mol/L TCEP-HCl, and 0.4 mg ZrO_2 at 1 °C. During an actual HDX experiment, the low pH and low temperature would quench labeling and reduce back-exchange of deuterons with protons in subsequent steps. TCEP was included to reduce the two disulfide bonds in FcγRIIa.

Scheme 1. Comparison of Automated HDX-MS Workflows with and without Phospholipid Removal Steps[a]



[a]Differences between workflows, including changes in temperature (ΔT) and time (Δt), are indicated in blue. All steps after H/D exchange are maintained at pH 2.5 for both workflows, and temperature was held at 1 °C, with the exception of transfer syringe, unless otherwise noted. The increment in time for all phospholipid removal and filtration steps is 70 s.

The notable addition to this workflow is 0.4 mg ZrO_2 per reaction to bind phospholipids. Sample was mixed twice by syringe and given an incubation time of 1 min, as previously reported,[5] prior to dilution with 20 μL of H_2O for a final volume of 65 μL. Dilution prior to filtration allows less material to be used for reactions prior to dilution and greater sample recovery after filtration due to the higher ratio of injection volume to filter dead volume. The dilution step does not affect pH. Next, a nanofilter cartridge, stored in the exchange drawer, was moved by the PAL robot arm to a position above the filter base and slightly pressed into the filter base. The whole filter assembly was then moved to the vial well of a LEAP X-Press module, and X-Press compressed the filter assembly to separate denatured protein from phospholipids bound to ZrO_2 particles. Mechanical operations for filtration increase sample handling by 40 s, pH 2.5 is conserved during processing, and the supernatant temperature is largely determined by the transfer syringe temperature. Changes in temperature and time indicated in Scheme 1 can be used to estimate the increase in back exchange within a user's apparatus for a particular protein. More information about the X-Press, including videos of operation, is available at leapwiki.com/mediawiki/index.php?title=LEAP_X-Press_Filtration_Station. 50 μL of filtered sample was injected into the sample loop, passed through an immobilized pepsin column (Enzymate BEH, 2.1 mm × 30 mm, 5 μm, Waters, Milford, MA) for 3 min, and collected on C18 guard column (1.0 mm, 5 μm, Grace Discovery Sciences), which was then washed for 30 s to remove additional salts. Additionally, an adjustable back pressure regulator (P-880, IDEX Health and Science, Oak Harbor, WA) was added to waste valve after trap column to increase back pressure for pepsin column to 100 bar.

Liquid Chromatography–Mass Spectrometry. Peptides from C18 guard column were eluted into a C18 Hypersil GOLD analytical column (1.0 mm × 5 cm, 1.9 μm, Thermo Scientific) at a flow rate of 50 μL/min from Dionex Ultimate 3000 HPLC with water containing 0.1% formic acid (v/v) as solvent A and acetonitrile containing 0.1% formic acid (v/v) as solvent B. Peptides were eluted using the following gradient: 5 to 15% solvent B in 1 min, 15 to 50% solvent B in 7 min, 50 to 95% solvent B in 0.5 min, 95% solvent B for 1.5 min, and 95 to 5% solvent B in 0.5 min. The temperature of all LC connection lines and valves in the HDX PAL compartment was maintained at 1 °C. Peptides were analyzed using a Thermo LTQ Velos Orbitrap Elite (Thermo Fisher, San Jose, CA) using the following settings: spray voltage, 3.7 kV; sheath gas flow rate, 25; capillary temperature, 270 °C; and resolution, 60 000. MS data were acquired by the Orbitrap to accurately measure precursor ion masses; product ions were then obtained by collision-induced dissociation and measured in the LTQ Velos ion-trap. Data-dependent acquisition was performed for the six most abundant ions per full scan. To increase the number of identifications, dynamic exclusion was used to exclude an ion for 40 s after the ion was detected twice within a 30 s interval.

To reduce carryover between protein samples, the following washing steps were performed. First, after each sample injection, the protease column was washed with 50 μL of 1.5 mol/L guanidine-HCl in water containing 0.1% formic acid. Second, after each chromatographic separation, a short blank was run to wash the C18 Hypersil GOLD analytical column.

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Additionally, after each protein sample, a full PAL-Orbitrap system blank was run twice using H_2O in place of protein. These washing steps removed all carryover, as determined by MS/MS analyses of blank runs. During the course of this study, changes in peak widths, retention times, and intensities were not observed.

Peptide Mapping. LC−MS/MS data of pepsin digests were processed with Genedata Expressionist 11.0 (Lexington, MA) for peptide identification. Expressionist 11.0 parameters were: enzyme, none; MS tolerance, 10 ppm; MS/MS tolerance, 0.5 Da; variable modification, oxidation. Peptides with scores ≥25 were manually confirmed by fragment ion assignment.

RESULTS AND DISCUSSION

Phospholipid Depletion by Zirconium Oxide. ZrO_2 was previously shown to bind to the phosphate headgroup of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM) for use as a solid-phase extraction for LC−MS applications.[5,10,11] ZrO_2 is also well suited for HDX because its zeta potential is optimal at low pH.[12] An alternative approach could be to pack ZrO_2 particles into a column for the inline removal of phospholipids prior to proteolysis; however, ZrO_2 would need to be regenerated after several samples to maintain efficient binding capacity. To circumvent column regeneration and ensure that every sample is exposed to active ZrO_2, ZrO_2 is added in solution.

When developing methods for HDX-MS, the exposure time and quantity of added ZrO_2 are carefully considered. Using an excess of ZrO_2 reduces the duration of exposure required to sufficiently deplete phospholipids. Short wait times provide the benefit of reduced back exchange. Likewise, smaller reaction volumes are used to ensure more efficient mixing of ZrO_2 with sample by using few PAL syringe mixing strokes, as additional mixing strokes increase sample contact with room-temperature syringe and overall duration. Both factors can contribute to back exchange. We were able to use the same number of mixing strokes as for a conventional method at these volumes. We recognize that a cold syringe, available in some robotic systems, and an additional cold drawer for storing filters can further reduce the back exchange.

To assess the amount of phospholipids removed from protein samples following ZrO_2 treatment, MS/MS scans were screened for common phospholipid precursors and confirmed by fragmentation data. Because wheat germ was the expression system used for liposome production, abundant phospholipids previously identified in wheat germ were used for our screening.[13] The results of ZrO_2 depletion of phospholipids from liposomes derived from wheat germ are presented in Table 1.

To remove the ZrO_2 particles, syringeless nanofilters were used. PES was selected for filter membrane material because of its low binding capacity for protein. Despite the low-binding nature of PES, initial trials using Whatman Mini-UniPrep filter cartridges determined that thick PES filter membranes bound an appreciable amount of protein; its 50 μL of dead volume required unreasonably large sample volumes. Although we succeeded in reducing protein binding on standard size filter membranes by first blocking with lysozyme and then washing three times to remove unbound lysozyme before sample treatment, the introduction of additional proteins into sample processing and added steps for blocking were counter-

Table 1. Intensities of Phospholipid Cations after Treatment with ZrO_2 Particles[a]

abbreviation	ion m/z	adduct	formula	intensity
LPC(18:1)	522.356	$[M + H]^+$	$C_{26}H_{52}O_7NP$	31.7
LPC(18:0)	524.371	$[M + H]^+$	$C_{26}H_{54}O_7NP$	9.9
LPC(16:0)	496.340	$[M + H]^+$	$C_{24}H_{50}O_7NP$	2.2
LPC(18:3)	518.324	$[M + H]^+$	$C_{26}H_{48}O_7NP$	ND
LPC(18:2)	520.340	$[M + H]^+$	$C_{26}H_{50}O_7NP$	ND
LPE(18:2)	478.293	$[M + H]^+$	$C_{23}H_{44}O_7NP$	ND
LPE(16:0)	454.293	$[M + H]^+$	$C_{21}H_{44}O_7NP$	ND
PE(18:3/18:2)	738.506	$[M + H]^+$	$C_{41}H_{72}O_8NP$	ND
PE(16:0/18:3)	714.507	$[M + H]^+$	$C_{39}H_{72}O_8NP$	ND
PE(18:2/18:2)	740.524	$[M + H]^+$	$C_{41}H_{74}O_8NP$	ND
PE(16:0/18:2)	716.523	$[M + H]^+$	$C_{39}H_{74}O_8NP$	ND
PE(18:1/18:2)	742.539	$[M + H]^+$	$C_{41}H_{76}O_8NP$	ND
PA(18:2/18:2)	714.507	$[M + NH_4]^+$	$C_{39}H_{69}O_8P$	ND
PA(16:0/18:2)	690.507	$[M + NH_4]^+$	$C_{37}H_{69}O_8P$	ND
PA(18:1/18:2)	716.523	$[M + NH_4]^+$	$C_{39}H_{71}O_8P$	ND

[a]Lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), and phosphatidic acid (PA). Signal presented as 1×10^4 intensity units. Phospholipids with no MS/MS data are listed as not detected (ND).

productive to the development of a robust automated method. Therefore, standard filters were replaced with Thomson nanofilters with thin PES filter membranes and only 8 μL of dead volume. We mention our experience with larger filters to stress the importance of using nanofilters to permit MP concentrations within the range typical for soluble proteins for the analysis by HDX-MS. This is particularly important because preparations of MPs expressed or reconstituted into lipid bilayers are typically of low yield relative to preparations for soluble proteins.

A soluble protein, PP2Cα,[14] was compared with and without filtration and ZrO_2 treatment (Figure S-1). Within the measurement uncertainties, both conditions produced the same result, confirming that the addition of ZrO_2 and filtration do not negatively impact the signal intensity or the sequence coverage of proteins in general. However, ZrO_2 may bind phosphoproteins,[15] similar to TiO_2, a potential disadvantage of this method. For HDX-MS studies on phosphorylated proteins, protein loss could be mitigated by performing in-solution digests; only phosphopeptides from protein digests would be bound by ZrO_2, allowing the measurement of all other portions of the protein.

Sequence Coverage of FcγRIIa. Full-length FcγRIIa expressed into liposomes was selected as a model protein to demonstrate this method. FcγRIIa is a transmembrane protein with three distinct domains: ectodomain, transmembrane domain, and endodomain. A 66% sequence coverage of full-length FcγRIIa was obtained with coverage in all domains and mapped onto structure PDB 3RY6[16] (Figure 1). Measurement of peptides from the transmembrane domain indicated that phospholipids associated with this region either were not present or did not shield the transmembrane domain from digestion by pepsin. More significant is the ability of this method to facilitate the measurement of structural perturbations of the endodomain, which contains an immune receptor tyrosine activating motif (ITAM) responsible for the function of FcγRIIa.[17] Whereas FcγRIIa and FcγRIIb both have similar ectodomains enabling them to bind Fc with the same affinity, they differ in cytosolic motifs, with FcγRIIb containing an

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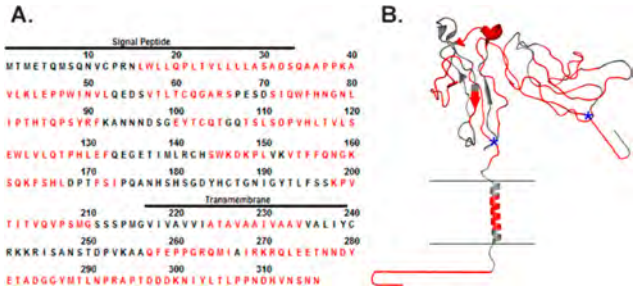

Figure 1. Sequence coverage of FcγRIIa. (A) Amino acid sequence coverage of FcγRIIa with regions with detected peptides shown in red and regions without detected peptides shown in black. (B) Sequence coverage mapped onto PDB 3RY6 FcγRIIa structure. FcγRIIa termini not included in crystal structure were appended as representations, joined using blue asterisks to PDB 3RY6 structure. Representation of C-terminus is shown in lipid bilayer for reference.

immune receptor tyrosine inhibitory motif (ITIM).[17] Therefore, structural studies using truncated FcγRIIa containing only ectodomain would be similar to that for FcγRIIb; however, studies using full-length FcγRIIa/b would permit the measurement of structural changes unique to ITAM/ITIM in the cytosol as it relates to receptor function.

CONCLUSIONS

The robust, fully automated method described herein may advance the field of structural characterization of MPs and be of high value in developing pharmaceuticals targeting MPs. Beyond HDX-MS, our automated method for phospholipid removal could be adapted for other MS applications. For example, proteomics of extracellular vesicles is a growing field in precision medicine, but yield of extracellular vesicles and their protein contents is low. Our method to remove phospholipids could be used for protein enrichment to increase the number of protein identifications by MS to supplement methods being developed for proteomics on extracellular vesicles.[18] The adaptable nature of our method for various applications suggests the fecundity of this technique, and, coupled to the easy integration and relatively low cost, this automated workflow could have a profound impact on MP studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00429.

Figure S-1. Effect of nanofilters and ZrO2 on amino acid sequence coverage of soluble protein PP2Cα. (PDF)

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Author Contributions

K.W.A., E.S.G., and J.W.H. designed experiments. K.W.A. conducted experiments, analyzed data, and wrote the manuscript. All authors reviewed results and approved the manuscript.

Notes

Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment identified are necessarily the best available for the purpose.
The authors declare no competing financial interest.

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