CHAPTER THREE

Using hydrogen/deuterium exchange mass spectrometry to understand bacterial membrane efflux proteins

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Abstract

Bacterial multidrug efflux pumps play major roles in antibiotic and multidrug resistance as well as fulfilling many important physiological functions. These molecular machines are highly dynamic, with structural malleability at the core of their action, and elaborating their conformational features can provide insight into the mechanisms underpinning their activities and responses to transport substrates and inhibitors. Hydrogen/deuterium exchange mass spectrometry (HDX-MS) is a structural biology

Methods in Enzymology, Volume 724
ISSN 0076-6879, https://doi.org/10.1016/bs.mie.2025.08.026

assay used to monitor protein backbone dynamics and is capable of elucidating conformational signatures that define function and fold. In recent years, HDX-MS methods have advanced, making its use more amenable to the study of membrane proteins, yet experimental challenges remain. In this chapter, we provide background on HDX-MS, its limitations, and discussions around experimental design and optimization for studying efflux pump proteins, including its application to study membrane proteins in lipid environments. To demonstrate its utility, we provide an original case study on AcrB inhibition by an efflux pump inhibitor (MBX-3756), employing membrane-scaffold protein lipid nanodiscs, revealing perturbation of backbone motions distinguishing of hydrophobic-trap targeting inhibitory action. This chapter can serve as a guide for designing HDX-MS investigations on efflux pump proteins and membrane proteins in general.

1. Introduction

Hydrogen/Deuterium eXchange Mass Spectrometry (HDX-MS) is a powerful analytical technique to investigate protein conformational dynamics. HDX-MS involves the exchange of backbone amide hydrogens (1.01 Da) to isotopic deuterium (2.01 Da), and the extent of exchange is measured by MS (Weis, 2016). The exchange rate is dependent on the folded state of the protein (i.e. solvent accessibility and secondary protein structure), the intrinsic chemical properties of the amino acid sequence, pH and temperature (Masson et al., 2019). In a typical HDX-MS experiment, proteins are diluted in deuterated buffer (labeling) for a series of timepoints, and exchange is minimized by lowering the pH to ~2.5 and the temperature to 0 °C via a chilled acidic quench solution. By tightly controlling pH and temperature, the rate of HDX is predominately influenced by the presence of H-bonds in a protein structure, and to a lesser extent solvent accessibility (Hamuro, 2024).

For an unstructured polypeptide, it is possible to calculate the chemical HDX rate (K_{ch}) from pH, temperature, primary sequence and ionic strength of solution data (Bai et al., 1993), however for folded proteins, the situation becomes more complicated. Observed HDX exchange rates (K_{obs}) are influenced by the aforementioned factors, i.e. the presence of secondary structure elements. The slowing of K_{obs} is defined as the backbone H-bond protection factor (*pf*) between the experimental HDX rate in folded versus unfolded proteins (Vadas, Jenkins, Dornan, & Burke, 2017):

$$\mathrm{pf} = \frac{\mathrm{K_{ch}}}{\mathrm{K_{obs}}} \tag{1}$$

Exchange in folded forms relies on the innate structural fluctuations of proteins to expose the backbone amides to deuterium. This can be described by the following equation, where F is folded, U is unfolded, H is hydrogenated, D is deuterated, K_1/K_{-1} are (un)folding kinetics, and K_{ch} is the chemical exchange rate:

$$F_H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U_H \underset{D_2O}{\overset{k_{ch}}{\rightarrow}} U_D \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} F_D \quad (2)$$

The HDX of backbone amide hydrogens in folded proteins can occur in two different ways. Most will exchange via EX2 kinetics, where $k_{-1} > K_{ch}$, meaning the intrinsic rate of HDX is much slower than the folding/unfolding rates, and therefore multiple opening/closing events must occur before the amide hydrogen can exchange (James et al., 2022; Weis, 2016). Alternatively, few protein regions may exhibit EX1 kinetics, where $K_{ch} > k_{-1}$, meaning that during an unfolding event, exchange will occur rapidly. It is rare for stable, folded proteins to exhibit EX1 kinetics, but often important regulatory regions such as hinges can exchange via this pathway. EX1/2 kinetics present with characteristic experimental readouts: EX1 presents as a bimodal isotopic distribution in the spectra whereas EX2 exhibits a binomial isotopic distribution (an example of EX2 is shown in Fig. 1) (Ozohanics & Ambrus, 2020). For a more in-depth review of HDX rates and kinetics, we direct the reader to these excellent reviews (Hamuro, 2024; James et al., 2022; Wales & Engen, 2006).

HDX-MS reports on protein dynamics, secondary structure features and protein-ligand interactions (Deng et al., 2016; Konermann & Scrosati, 2024).

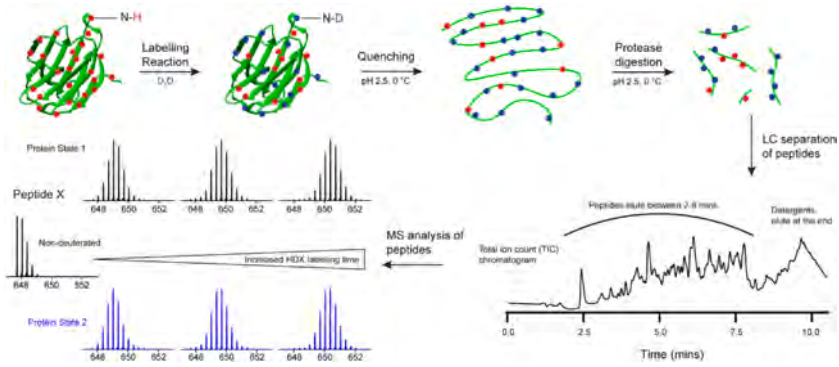

Fig. 1 Bottom-up hydrogen/deuterium exchange mass spectrometry (HDX-MS) workflow.

It is widely applied across structural biology; for example, HDX-MS can provide conformational dynamics information to the static structures provided by cryo-electron microscopy (EM), creating a valuable combination (Engen & Komives, 2020). HDX-MS integrated with molecular dynamics (MD) simulations provides a comprehensive understanding of the dynamics of a system (Anderson et al., 2022; Jia et al., 2023) and provides a higher resolution, atomistic view. Previously, we have shown that HDX-MS and MD simulations match very well for efflux pump systems, as we have deployed these techniques to study the resistance nodulation and cell division (RND) proteins AcrB and AcrA (Reading et al., 2020a; Russell Lewis et al., 2023, 2025).

Typically, membrane protein samples contain detergents or lipids, both of which can be problematic for MS, and their inherent hydrophobicity can lead to low sequence coverage. However, in recent years, advances in HDX-MS methodology (Section 2) mean they are increasingly applicable to the study of membrane proteins (Martens & Politis, 2020; Trabjerg et al., 2018), and therefore bacterial efflux proteins, which can consist of both integral and peripheral membrane proteins (Kumawat et al., 2023). Efflux proteins exist across almost all bacterial species and can act independently or in partnership, including the tripartite protein 'nanomachines' that span both membranes of Gram-negative bacteria (Russell Lewis, Lawrence, Hammerschmid, & Reading, 2023; Sun, Deng, & Yan, 2014). They are responsible for mediating the internal cellular environment of the host bacterium and ensuring cellular integrity by exporting toxic substances to the external environment (Piddock, 2006; Saier et al., 1998). Some efflux proteins are highly specific to single substrates, whilst others are highly poly-specific and can effuse a wide range of chemically diverse compounds, thus are significantly implicated in the rapid rise of multidrug antimicrobial resistance (AMR) (Levy & Bonnie, 2004).

We have previously deployed HDX-MS, supported by molecular dynamics (MD) simulations, biophysics and cell-based assays to reveal the structural dynamics underpinning efflux behavior. This yielded success in deciphering the dynamical behavior that underpins AcrB inhibition and a clinically relevant efflux-profile modifying mutant (Reading et al., 2020), inhibition against the periplasmic adaptor protein (PAP) AcrA (Russell Lewis et al., 2023), and the role of Mg^{2+} as a structural cofactor of AcrA during conditions of shifting pH (Russell Lewis et al., 2025). HDX-MS has been used by many others to reveal the conformational movements of other efflux pumps, for example investigating BmrA, an ATP binding cassette (ABC)

efflux protein from *Bacillus subtilis,* during ATP hydrolysis (Javed et al., 2022; Mehmood et al., 2012). HDX has also yielded success for other bacterial transporters such as LeuT, LacY, XylE and GlpT (Adhikary et al., 2017; Martens et al., 2018). These examples exemplify the versatility of HDX-MS when applied to membrane protein transporters, as it can reveal information on transport dynamics, cation cofactors, pH-induced conformational switches, inhibitor binding and impact of mutations.

In this chapter, we seek to highlight the experimental considerations for designing HDX-MS investigations on efflux proteins. Whilst HDX-MS is becoming increasingly popular, it can be technically demanding to optimize a protocol and troubleshoot potential challenges. This chapter will serve as a detailed guide for HDX-MS on efflux proteins, with a focus on an original case study of AcrB in membrane-scaffold protein (MSP) lipid nanodiscs in the presence of an efflux pump inhibitor (EPI). The approach can be applied to other membrane proteins and serve as an important resource for designing HDX-MS experiments.

2. Unlocking HDX-MS for membrane proteins

In this section, we detail the overall workflow of an HDX-MS experiment and the practical considerations required for investigations on membrane proteins. Membrane proteins naturally exist in lipid bilayers and need a membrane mimetic to remain soluble in vitro. This can either be detergent micelles, or lipid environments such as nanodiscs or liposomes (Denisov & Sligar, 2016). In recent years, there has been a drive to consider the lipid environment when studying membrane proteins, as detergent micelles strip the proteins of its native lipids, which can be destabilizing and affect structure, function and folding (Klenotic et al., 2021; Saliba et al., 2015). Here, we will also detail recent advances in HDX-MS methodologies for membrane proteins in lipid environments.

2.1 The HDX-MS workflow

Continuous labeling, bottom-up HDX-MS, is currently the most widely used approach in the field. This workflow consists of D_2O labeling, quench, proteolytic digestion, and liquid chromatography (LC)-MS analysis at peptide level (Fig. 1) and has been integrated into fully automated setups (James et al., 2022; Wales et al., 2008). MS can also be coupled to ion mobility (IM), and more recently cyclic IM (cIMS), which adds an

additional dimension for separation. The IM-MS coupling increases the dynamic range of HDX-MS, enabling the analysis of complex samples with greater speed and accuracy (Engen et al., 2021; Griffiths et al., 2024). Automation facilitates the laborious sample handling involved and addresses the methods' inherent time constraint imposed by the nature of hydrogen exchange (Englander et al., 1972). The exchange reaction is minimized at pH 2.3–2.5 and 0 °C (typical quench conditions), yet it cannot be brought to a standstill (Englander & Kallenbach, 1983). Hence, post-labeling sample processing, e.g. protein digestion and LC-MS analysis, requires timely integration under a tight pH and temperature regime.

HDX-MS relies on obtaining sufficient sequence coverage and redundancy to maximize the output of structural information on the protein of interest. This requires effective protein digestion, which depends on both target protein unfolding and protease performance. The significant decrease in pH during the quench reaction can already be an efficient measure to denature the protein, however denaturing agents such as urea, thiourea or guanidine hydrochloride (GnHCl) have become common quench buffer additives to enhance unfolding. To reliably investigate protein dynamics with HDX-MS, the interplay between protein denaturation and digestion is therefore a crucial aspect (refer to Section 3.3 for a detailed discussion).

Seamless workflow integration has played a key role in the success of HDX-MS, initiating its growing adoption beyond academia. These advancements however were realized on soluble proteins, making it less applicable for membrane proteins, as they require a hydrophobic environment for solubilization, adding an additional layer of complexity to the setup, which can impact protein unfolding, digestion, and chromatographic separation. Hence, workflow adaptations were in demand for HDX-MS to meet the increasing interest in membrane protein research (Overington et al., 2006; Rask-Andersen et al., 2011; Thakur et al., 2021).

2.2 Expanding the HDX-MS workflow for membrane proteins

Detergents have played a pivotal role for membrane protein characterization for decades. They can also be integrated into the existing bottom-up HDX-MS approach, assuming that they are compatible with MS. Non-ionic, sugar-based detergents such as *n*-Dodecyl-β-D-maltopyranoside (DDM) and Lauryl Maltose Neopentyl Glycol (LMNG)/cholesteryl hemisuccinate (CHS) are commonly applied for membrane protein investigations in HDX-MS (Josephs et al., 2021; Zhang, 2017). However, detergents strip

membrane proteins of their native lipid environment, which may perturb the conformation and functional state of the solubilized protein. Consequently, sustaining the native lipid environment is becoming increasingly popular to preserve protein-lipid interactions, that are often crucial for the structural and functional integrity of the protein (Bolla et al., 2019; Nji et al., 2018; Pyle et al., 2018).

A variety of different lipid systems have become available for membrane protein solubilization ranging from simpler forms such as bicelles (Sanders & Prosser, 1998) and liposomes (Rigaud & Lévy, 2003) to more sophisticated structures such as nanodiscs (Bayburt et al., 2002; Denisov et al., 2004), styrene maleic acid lipid particles (SMALPs) (Knowles et al., 2009), and even native membranes (Zeev-Ben-Mordehai et al., 2014). The higher sample complexity and lipid-content complicates workflow integration in HDX-MS. The lipid system counters the rapid protein unfolding that is needed for protein digestion. Moreover, lipids can impair proteolytic digestion, lower chromatographic performance, suppress analyte ionization, and complicate spectral analysis (Lin et al., 2025). One approach can be to simply inject the entire sample (peptides and lipids) over the chromatography system, and monitor peptide separation/identification, column pressure and ion suppression, and to extensively clean the system after a practitioner defined maximum of lipid-containing samples (Vadas, Jenkins, Dornan, & Burke, 2017). An alternative preventative approach is to disrupt lipid-membrane protein system and then remove the lipids prior to injection into the MS system, preferably via an automated setup, which can be beneficial to the user due to reduced manual intervention and a possible increase in MS durability (Anderson et al., 2018; Hammerschmid et al., 2023). However, including an extra lipid removal preparation step prior to injection carries a risk of protein loss or increased signal to noise due to interactions with lipid-extraction phases. Therefore, HDX-MS experiments on membrane protein-lipid environments often require a three-step process for successful workflow integration: (i) the disruption of the lipid-membrane system, (ii) the managed fouling of the system by phospholipids, and (iii) optimization of sample loss prevention (Lin et al., 2025).

Detergents are effective for lipid environment disassembly for two reasons. Firstly, they have readily been applied in the context of bottom-up HDX-MS applications (Martens & Politis, 2020; Möller et al., 2020; Reading et al., 2020), and secondly, they are extremely efficient in membrane disruption whilst keeping the protein soluble. Quench buffers

often contain detergent additives to unlock the protein from the membrane system and prevent aggregation during unfolding. Other membrane-active agents such as the bile acid cholate, the peptide mellitin (Ae et al., 2007), or the antibiotic polymyxin B (Moubareck, 2020) also provide alternatives or may be used in combination with detergents to enhance the disruption of the membrane. More challenging however is the removal of phospholipids preceding the LC-MS, to avert detrimental effects on downstream protein digestion and LC-MS peptide analysis, particularly when considering the underlying time constraint of the method.

Various strategies have been developed for sample delipidation and/or management of column fouling, including washing out and absorption methods. Donnarumma et al. developed a protocol based on acetone wash for lipid removal to study the outer membrane protein F (OmpF) in outer membrane vesicles (OMVs) (Donnarumma et al., 2018). This approach relies on trichloroacetic acid (TCA) precipitation of the protein directly after labeling. Subsequently, the pellet is washed with ice cold acetone (−20 °C) to extract the lipids, and after centrifugation, the protein pellet is resuspended for injection to the LC-MS system with online digestion. Another method applies di- or trichloromethane to wash lipids and/or detergents directly from the trap column after protein digestion and peptide trapping (Rey et al., 2010; Rey, Forest, & Pelosi, 2012). This approach is an attractive alternative as it allows for circumventing laborious sample handling (Rey, Mrázek, et al., 2010). To remove lipids via absorption, zirconium dioxide (ZrO_2) beads are highly effective. ZrO_2 acts as a Lewis acid that binds phosphates from the head groups of phospholipids. This technology was first exploited by Hebling et al., who established a protocol where lipids are manually removed using ZrO_2 beads after cholate-based nanodisc disassembling and in-solution protein digestion (Hebling et al., 2010). Later, this workflow was refined allowing for lipid removal before online protein digestion (Martens et al., 2018, 2019), which was also adopted for SMALPs (Reading et al., 2017) and for liposomes (Lin et al., 2025). Alternatively, titanium oxide (TiO_2) beads can be used for sample delipidation, as they provide the same chemistry for phosphate group binding, albeit with lower efficiency compared to ZrO_2 (Hammerschmid et al., 2023) – in this same work it was also identified that 'bead-blocking', i.e. using BSA (bovine serum albumin) protein, glycine buffer, or similar alternatives, helps to prevent sample loss during these procedures. These methods are now widely adopted for membrane protein research in HDX-MS,

particularly when with samples in nanodiscs, liposomes and SMALPs. Yet, they require laborious skills at the cost of time and therefore being at odds with the methods' time constraint and can present complicated hurdles to overcome during HDX-MS optimization. This problem has been tackled by various automation endeavors in recent years.

Anderson et al. developed a system that allows sample filtration after phospholipids bind to ZrO_2 beads at the bottom of an X-press module. This was integrated into the LEAP HDX platform where the robot applies the necessary downward pressure before aspirating and injecting the sample into the LC-MS setup with online digestion (Anderson et al., 2018). Another automated delipidation system was developed using a phospholipid trap column based on ZrO_2 or TiO_2 beads. The implementation of an additional valve and chromatographic pump enables trap column integration both up- or downstream the proteolytic column. This system not only allows for online sample delipidation but also cleaning and regeneration of the phospholipid trap column, circumventing the need for bead disposal (Hammerschmid et al., 2023). A further column-based approach was developed by Calvaresi et al., exploiting the principle of size exclusion chromatography (SEC) to separate the labeled protein from lipids. Here, a SEC column including a Rheodyne valve was positioned before the protease column enabling unwanted sample components to be sent to waste by manual switching (Calvaresi, Redsted, Norais, & Rand, 2021). This system benefits from the size exclusion principle which also applies to other compounds that may interfere with the LC-MS system, such as tris(2-carboxyethyl) phosphine (TCEP) or high concentrations of denaturing agents.

With membrane protein characterization by means of HDX-MS gaining momentum over the last decade (Yang et al., 2023), promising protocols have been established to overcome some of the major challenges. Excitingly, first endeavors have been undertaken for studying membrane proteins directly in their cellular context (Lin et al., 2022). For a more in-depth discussion of current scenarios and future perspectives for HDX-MS of membrane proteins in native-like environments, the interested reader is directed to Section 5 as well as the recent review article by Javed et al. (Javed et al., 2023). Overall, these developments present encouraging news for research on multidrug efflux pumps, as HDX-MS positions as viable player for future discoveries in this field.

3. Establishing an HDX-MS protocol to study efflux pump proteins

The design of an HDX-MS experiment to study efflux proteins and other membrane proteins can be summarized in a decision tree, as shown in Fig. 2. We describe below in detail the main points of the decision tree.

3.1 Sample optimization

In establishing a protocol for HDX-MS investigations, it is important to consider the sample under investigation. When dealing with efflux proteins, most samples will be membrane proteins, and thus require a membrane mimetic, but it is possible that soluble constructs may be used. PAPs are a component of tripartite efflux pumps in Gram-negative bacteria, that exist in the periplasm and link the inner membrane efflux proteins to the outer membrane factors (OMF) (Zgurskaya et al., 2015). These proteins are often anchored to the inner membrane through an N-terminal transmembrane domain (e.g. EmrA, *Aquifex aeolicus;* MacA, *E. coli*) or lipid moiety (e.g. AcrA, *E. coli*; MexA, *Pseudomonas aeruginosa*) (Hinchliffe et al., 2014). Therefore, PAPs lacking lipid modification are soluble constructions that do not necessarily need a lipid environment for HDX-MS. Conversely, PAPs that have a lipid modification can be mutated to lack the lipid attachment, as it can cause oligomerization and a subsequently heterogenous sample (Russell Lewis et al., 2023, 2025; Tikhonova et al., 2011). If a soluble construct is being used, the reader can move onto the experimental design section (Section 3.2).

When investigating membrane proteins, the first consideration is whether to have lipid membrane in your sample. This may be to directly observe the impact of lipids on your target protein, or to ensure a more physiologically relevant environment when performing experiments. If lipids are not required, the protein sample should be purified in a detergent that ensures a stable sample and is amenable to HDX-MS; a detergent screen can be useful to find the best option, but DDM and LMNG/CHS is often the detergent system of choice (Möller et al., 2020). If lipids are required, the protein sample should be purified or reconstituted into a suitable lipid environment, the choice of environment being important (discussed below). When detergent is used as the membrane mimetic then detergent should be present in the sample, labeling and quench buffers at a concentration 2x higher than the critical micelle concentration (CMC), along with required buffering components and salts, to protect from non-

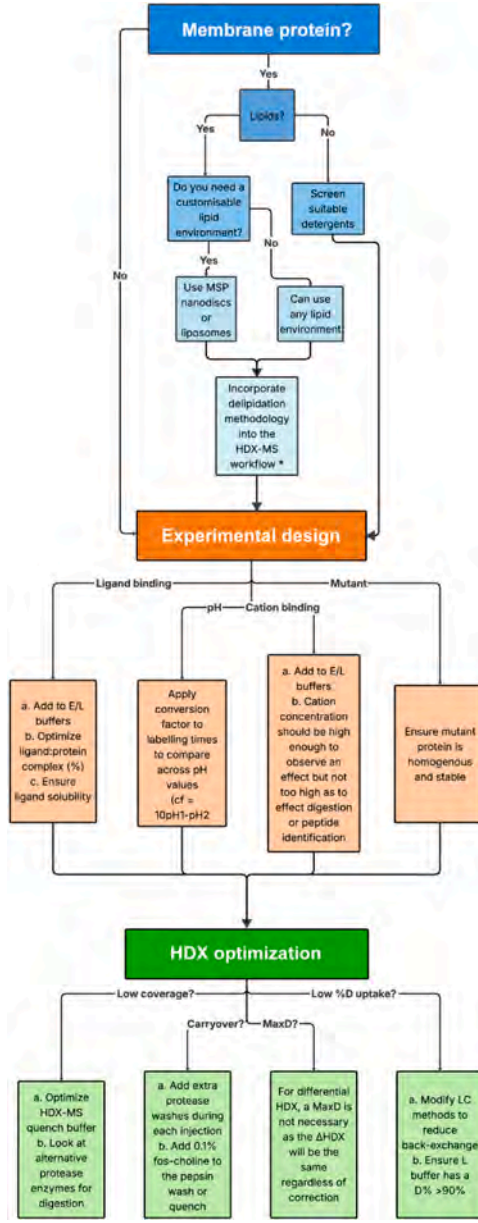

Fig. 2 Experimental decision tree to design an HDX-MS experiment to study efflux proteins, or membrane proteins in general.

specific aggregation in these conditions caused by exposure of their hydrophobic regions. If a lipid-membrane protein system is used, then the sample and labeling buffers will be devoid of detergent but then detergent will need to be introduced in the quench step to facilitate lipid dissociation from the membrane protein and maintain membrane protein solubility. Careful consideration of what detergent to use, and its concentration, in the quench is required, as this solution, containing the protein sample, will be injected into the LC-MS system.

For customizable lipid environments (e.g. to investigate the impact of different lipid compositions), liposomes or MSP nanodiscs present good options (Denisov & Sligar, 2016; Hebling et al., 2010; Javed et al., 2023; Martens et al., 2018; Redhair et al., 2019). Liposomes are self-closed phospholipid bilayers that proteins can be reconstituted into, yet the protein orientation cannot be controlled, and reconstitution efficiency can be low, both of which can hamper HDX-MS interpretation (Seddon et al., 2004). Another option is MSP nanodiscs; this involves reconstituting a detergent solubilized protein into lipid nanodiscs, which are in turn bound by MSP (Bayburt et al., 2002). They confer advantages over liposomes, such as stability and the ability to modify the lipid:protein ratio; however, the presence of MSP peptides can impede the LC-MS performance and identification of target protein peptides.

If a customizable lipid environment is not required, liposomes and MSP nanodiscs can still be used, though native nanodiscs offer an alternative approach; polymers can directly solubilize proteins from their native membranes to form nanodiscs with the native lipid environment (Lee et al., 2016). This confers the advantage of avoiding detergent solubilization at any stage of purification, which can affect sample function and stability. Traditionally, SMA has been used to form SMALP nanodiscs, and we have previously described an HDX-MS protocol for membrane proteins in SMALPs that involves the removal of lipids and polymer before injection into the LC-MS (Reading et al., 2017). Unfortunately, SMA and other first-generation polymers are not soluble under quench conditions and are therefore not suitable to automated delipidation methods as the polymer would precipitate throughout the MS system (Scheidelaar et al., 2016). Therefore, an offline filtration step was necessary before LC-MS analysis as well as optimization of workflow conditions (e.g. spin speed, temperature, filter type and condition compatibility, detergent and denaturant quench concentration, etc) to minimize sample loss during filtration (Reading et al., 2017). However, next-generation polymers such as styrene

maleimide (SMI) remain soluble under quench conditions and are, in theory, amenable to online HDX-MS methodology (Hall et al., 2018). Regardless of the lipid environment chosen, a suitable modification to the HDX-MS method is required to deal with the presence of the lipids (Section 2.2).

3.2 Experimental considerations

It is important to characterize the purified protein sample to ensure HDX-MS is performed on functional protein. Usually, this will be an SDS-PAGE or western blot to confirm the protein of interest has been purified and survived in an intact form (i.e. lacking truncated versions); SEC to assess sample homogeneity; dynamic light scattering (DLS) to assess nanodisc/liposome integrity; and a functional and/or protein stability assay to confirm protein activity. After the protein sample is optimized and the HDX-MS workflow chosen, the next consideration is the aim of the experiment. This will govern what extra controls, checks or optimizations are necessary. For most applications, differential HDX (ΔHDX) is used, as it is adept at analyzing the differences in HDX between two states (e.g. apo versus holo, wildtype versus mutant), allowing for the characterization and localization of the effect of a condition on protein dynamics (Masson et al., 2019). When performing differential HDX-MS, all conditions should be identical between the states except for the component under investigation. A simple experimental example is when testing the effects of mutations on protein secondary structure and dynamics; we have previously used HDX-MS to investigate the clinically relevant AcrB G288D mutant (Reading et al., 2020) where all buffers and experimental conditions were identical, only the protein sequence is different (i.e. wildtype versus mutant). Importantly, extra experiments should be performed to ensure homogeneity, stability, and function of the mutant sample to achieve the most reliable data. Below we detail considerations for HDX-MS experiments when environmental components of the protein system are explored.

3.2.1 Ligand/drug binding

For efflux pump proteins, HDX-MS can be used in several ways to reveal different structural information. One key area is monitoring ligand binding; this could be investigating antibiotic (substrate) binding, or inhibition by EPIs, or both. To ensure HDX-MS can provide biologically relevant information, it is helpful to have data related to binding affinities and stoichiometries of relevant ligands. Binding dissociation constant (K_D) and other

parameters to evaluate the binding property of the protein:ligand complex can be measued by techniques such as isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR). Once known, the percentage of protein:ligand complex under the specific conditions of the planned HDX experiment can be calculated using Eq. (3) (O'brien et al., 2020; Yang et al., 2014) where L_T is the total ligand concentration; P_T is the protein concentration; K_D is the equilibrium dissociation constant. When studying ligand-bound states, the aim should be to achieve the highest percentage of protein:ligand complex possible (>90 %), but sometimes this is not possible for weak binders, and compromises can be considered. For more information on optimizing HDX-MS investigations with weak binders, we direct the reader to this review (Hamuro & Coales, 2022).

$$\text{fraction of bound protein} = \frac{(L_T + P_T + K_D) - \sqrt{(L_T + P_T + K_D)^2 - 4L_T P_T}}{2P_T} \tag{3}$$

Due to the polydispersity of many efflux pumps to transport many chemically diverse compounds, we often encounter weak binders. To ensure the protein:ligand complex remains high, we spike in the ligand at the desired concentration to the protein stock and incubate for 30 min before the labeling experiment. Furthermore, the ligand is also added to the labeling buffer at the desired concentration, to mitigate the impact of dilution that occurs during the labeling step (Hamuro & Coales, 2018).

It is important to check the solubility of the ligand in the labeling buffer, and whether it needs solvents such as dimethyl sulfoxide (DMSO) to remain in solution. If so, the labeling buffers (both ± ligand) should contain the necessary concentration of DMSO (<5 %; with a minimal amount of DMSO as possible favoured) and this should be kept consistent across both protein states. It is also important to check the ligand remains in solution for the duration of the HDX time course; this can be checked by adding the ligand to labeling buffer and leaving it on the bench for several hours to see if it precipitates (Masson et al., 2019). In case of precipitation, extra steps such as sonication or extra solvent may be required to ensure ligand solubility.

3.2.2 Monitoring changes across different pH situations

HDX-MS experiments can be performed away from physiological pH, and ΔHDX can be used to track changes in protein dynamics between different pHs. This is becoming increasingly relevant for PAPs, which exist in the periplasm of Gram-negative bacteria which is often more

acidic than the cytosol (Miller & Salama, 2018). Furthermore, we have previously shown that several PAPs in the RND superfamily have a conserved histidine residue that has been shown to act as a pH-induced conformational switch (Russell Lewis et al., 2025). Since the rate of HDX is affected by pH, labeling experiments performed at conditions away from physiological pH will exchange faster or slower within the same 'labeling time point'. Therefore, to accurately compare protein dynamics between different pH values, a time window expansion can be applied to the labeling time points (Eq. (4)). For example, the intrinsic HDX rate decreases $10^{7.4-6}$-fold when the pH decreases from 7.4 to 6.0, and thus a 10 s labeling timepoint at pH 7.4 can be treated as being relatively equivalent to a 251 s incubation at pH 6.0.

$$\frac{k_{ch1}}{k_{ch2}} = \frac{[OH^-]_1}{[OH^-]_2} = \frac{k_w/[H^+]_1}{k_w/[H^+]_2} = \frac{10^{-pH_2}}{10^{-pH_1}} = 10^{pH_1-pH_2} \quad (4)$$

3.2.3 Considering cation influence on dynamics

HDX-MS may be applied to study the effect of cation binding to efflux pump proteins. Multidrug and toxic compound extrusion (MATE) transporters efflux cationic drugs in exchange for H^+ or Na^+ (Omote et al., 2006), and in recent years, several PAPs have been shown to bind cations such as AcrA and Mg^{2+}, ZneB and Zn^{2+}, and CusB and Cu^+/Ag^+ (Bagai et al., 2007; De Angelis et al., 2010; Russell Lewis et al., 2025). To investigate the effect of cation binding on protein dynamics, ΔHDX can be performed with or without cation presence in the labeling buffer. Similarly to ligand binding, K_D values can be calculated for protein:cation complexes, and this can guide the concentration used throughout the experiments. However, sometimes the affinity can be very weak (mM affinity), and therefore some optimizations need to be performed. This involves non-deuterated reference samples, which are diluted with an equilibration buffer instead of labeling buffer, before being 'quenched' and injected into the LC-MS. The resultant peptides are analyzed to achieve a peptide map. When investigating cation binding, several injections should be run using a range of different cation concentrations. A balance needs to be found that allows for a high proportion of protein:cation complex, but low enough to still achieve high peptide coverage. If cation concentration is too high, this can affect the number of peptides identified, through various mechanisms such as ion suppression, which involves less volatile solutes such as divalent cations changing the efficiency of droplet formation or desolvation in

electrospray ionization (ESI) so less ions reach the detector (Annesley, 2003). High cation concentration may also affect protease activity in an unpredictable way and disrupt the desalting ability of the trap column, leading to an abundance of $[M+Mg]^{2+}$ peptides, for example, that cannot be accurately identified due to the increased mass, consequently reducing the ion population of the corresponding $[M+H]^{+}$ peptide.

3.3 HDX-MS optimization

3.3.1 Sequence coverage

Once the sample has been characterized and necessary stability experiments have been performed, the HDX-MS workflow can then be optimized and tested. Firstly, the general workflow and instrumentation is tested using a protein standard (non-deuterated), typically well-studied and behaved proteins like phosphorylase B (PhosB), and close to 100 % coverage should be achieved with hundreds of peptides identified. Then, the workflow can be tested using the sample of interest, and several parameters help assess data quality. This can involve the acquisition of coverage maps, using non-deuterated reference samples, and preliminary labeling data across a range of timepoints.

The first step when working on a new sample is to achieve a set of peptides, which in turn provides a peptide database (leveraging peptide fragmentation and tandem MS) with a sufficient sequence coverage map and redundancy score. Sequence coverage (%) is the number of residues covered by identified peptides divided by the total number of residues in the protein multiplied by 100, whereas peptide redundancy is the sum of the peptide lengths divided by the total number of residues covered by peptides (Ball et al., 2022). A coverage map is achieved by diluting the protein sample in equilibration buffer; this is identical to the labeling buffer except it is made with H_2O instead of D_2O. The sample still undergoes quench, digestion, trapping and LC-MS, akin to a deuterated sample. In the MS, tandem MS is utilized (MS^2 domain) to fragment peptides, and peptides are identified from the fragmentation data using computer software and relevant algorithms (Zhang & Smith, 1993). Fragmentation data can be collected in data-dependent acquisition (DDA) or data-independent acquisition (DIA) modes; in DDA, precursor ions are selected for fragmentation based on their abundance and charge, but the reproducibility or precursor selection and long instrument cycle times makes this less applicable for HDX-MS (Plumb et al., 2006). Thus, DIA methods are more commonly used, where all ions are fragmented within a small

isolation window regardless of abundance or charge. MS^E is a common DIA methodology where high and low collision energy scans are acquired one after another, providing information on precursor ions and fragments in a single run. MS^E can be further enhanced by optimizing the collision energy profiles associated with IM data (Rincon Pabon et al., 2024). Furthermore, it is often useful to include a list of common contaminants to help minimize the risk of false positives during peptide identification (James et al., 2022). The retention time information (provided by the LC) in this peptide database is then used to confirm the identity of a peptide, supported by the accurate mass of a deuterium-shifted signal. Proper optimization of (a) quench and (b) digestion conditions are critical to achieving high coverage of protein samples.

3.3.1.1 Quench buffer optimization

To aid protein denaturation and solubilization, the quench buffer usually contains chaotropic agents such as urea, thiourea, andGnHCl, although full denaturation of some proteins can be achieved simply by lowering the pH. Chaotropic agents not only drive protein denaturation but also prevent the protein from precipitating while undergoing unfolding (Hamuro & Coales, 2018; Lim, Rg Rö Sgen, & Englander, 2009; Möller et al., 2019). For membrane proteins, urea is preferred as high concentration of GnHCl can trigger protein precipitation due to its high ionic strength (Forest Eric & Man & Mus-Veteau, 2016). Also, proteases are less sensitive to urea than GnHCl – e.g. pepsin remain active up to 4 M urea while almost inactive at 3 M GnHCl (Yang et al., 2015). Moreover, urea forms H-bonds with exposed amide hydrogens or deuteriums during protein unfolding which may, theoretically, help to reduce both back- and forward-exchange in deuterated samples (Lim, Rg Rö Sgen, & Englander, 2009). Among other denaturants are organic solvents and detergents; organic solvents usually induce protein unfolding (Guo et al., 2020), while detergents, especially in low concentration, may denature protein but also avoid protein aggregation by shielding hydrophobic residues from contact with polar solvent (Hamuro & Coales, 2018). Furthermore, detergents are effective at disrupting lipid nanodiscs and addition to the quench buffer aids downstream digestion and delipidation (Martens et al., 2019). Different detergent types and concentrations may need to be screened in order to achieve optimal membrane protein extraction, solubility and extent of system interference; the most common detergents used in the quench condition are DDM, Fos-Choline 12 (FC-12), OG, and C12E8, with the avoidance of any

PEG-derived detergent (e.g. Tween 20, Triton X-100, etc) being strongly encouraged as, even with the highest purity specifications, they can still have trace PEG which causes significant contamination of the LC-MS chromatogram (Hamuro & Coales, 2018; Lin et al., 2025).

Other quench buffer additives include TCEP which is a reducing agent for disulfide bonds (Zhang et al., 2010). Membrane proteins often exhibit rigid structures, particularly in their transmembrane segments, often stabilized by disulfide bridges. Using urea in combination with TCEP is an efficient measure to drive protein unfolding within the restricted timeframe of the HDX workflow. One caveat to this is that high concentrations of chaotropes/TCEP may impact protease activity, however this is less of a problem when working with immobilized protease columns, which have become standard in HDX-MS workflows (Rey et al., 2009; Wang et al., 2002; Woods & Hamuro, 2001).

3.3.1.2 Digestion optimization

In the case of low sequence coverage, even after testing several different quench conditions, optimizing the protease enzyme may be helpful. After quenching, the sample is either directly incubated with a soluble acid functional protease (offline) or passed through a column with immobilized protease (online). Immobilized protease columns offer considerable advantages over offline methods, as they immediately precede a reverse-phase trapping column which reduces autolysis peptide fragments from the protease itself (Vadas, Jenkins, Dornan, & Burke, 2017), enhances protease stability/reusability, increasing experimental reproducibility (Möller et al., 2019), and digestion can occur under high back pressure which significantly increases efficiency (Ahn et al., 2012). The most common protease in HDX-MS is pepsin; it provides non-specific cleavage at the C-terminus of all residues except for histidine, lysine, arginine and proline, with a preference for cleavage adjacent to hydrophobic residues (Smith et al., 1997). To increase the number of peptides obtained during HDX-MS, and to subsequently increase sequence coverage, alternative proteases with different cleavage preferences have been implemented into workflows. Recently, several acid proteases have emerged with enhanced stability, reduced sensitivity to chaotropic agents, and provide non-specific digestion with broader residue selectivity; all of which make them exciting options for HDX-MS experiments (James et al., 2022).

Commonly used alternatives are the fungal proteases Aspergillopepsin (protease type XIII) and Rhizopuspepsin (protease type XVIII) (Rey et al., 2009;

Zhang et al., 2008), which have different cleavage preferences compared to pepsin and have been applied to HDX-MS workflows. Other options are proteases from the *Nepenthes* species of carnivorous plants, Nepenthesin I or II (Kadek et al., 2014; Yang et al., 2015). These proteases can cleave C-terminally of pepsin-inaccessible residues, providing a broader specificity. This makes these enzymes particularly useful for integral membrane proteins, benefiting from superior digestion efficiency (Rey et al., 2013). These enzymes also exhibit higher stability, lower auto-digestion activity and resistance against denaturing conditions. Nepenthesin enzymes are becoming more commonplace in HDX-MS investigations on membrane proteins and have yielded success with G-protein coupled receptors (Otun et al., 2024).

Möller and co-workers demonstrated how optimizing different proteases and subsequent additives in the quench buffer is necessary to carefully optimize digestion conditions (Möller et al., 2019). They have also reported that a combination of proteases within the same column can be successful in increasing the number of peptides obtained. Furthermore, they observed that urea in quench buffer rather than GnHCl may surpass pepsin digestion efficiency for some proteins, but this was not universal. This highlights that digestion conditions are protein-specific and need to be optimized for each system individually.

3.3.2 Peptide carryover

After an optimized peptide list has been obtained, with sufficient sequence coverage and redundancy, then preliminary labeling data can be collected to assess for peptide carryover (Fang et al., 2011; Majumdar et al., 2012). This occurs when peptides remain in the MS system from a previous run, often due to intrinsic chemical properties such as hydrophobicity. Peptides can remain in the system in various places, such as the injection syringe barrel, dead volumes in the system, tubing connections and trap/analytical columns. The trap column is a particular 'hot spot' for carryover as strong interactions occur between peptides and the stationary phase (often C18 octadecyl alkyl chains), and in some cases an increasing organic gradient is not strong enough to elute all the peptides. Furthermore, carryover can be particularly high risk for membrane protein samples such as efflux proteins, as they contain many transmembrane helices which create substantially hydrophobic peptides after digestion.

Peptides exhibiting carryover present a problem for HDX-MS analysis. As they remain in the system, the peptides undergo extensive back-exchange and are eluted in a subsequent run. Carryover can be detected when

analysing the spectra of a dataset, as they appear as isotopic doublets consisting of a higher m/z feature caused by the deuterated peptide, and a lower m/z feature caused by a partially or fully undeuterated peptide that has remained in the system and undergone back exchange. Often this feature can be mistaken as bimodal EX1 kinetics, which would suggest conformational heterogeneity. To determine whether bimodal spectra is carryover or EX1 kinetics can be difficult, but the strongest indication of peptide carryover is a consistent intensity ratio of both envelopes throughout the data set. We direct the reader to this review for an in-depth discussion on the different spectral features and how to accurately assess them (James et al., 2022). Injecting a blank run after a sample measurement is an effective measure to estimate the extent of peptide carryover. Regardless, carryover needs to be minimized and should ideally be <5–10 %.

Several methods can be implemented to reduce carryover. Performing SEC on the protein sample before HDX-MS experiments can help to remove aggregates from the sample, consequently reducing carryover. Furthermore, 0.1 % FC-12, OG, or C12E8 can be added to quench and/or pepsin wash solution, to solubilize hydrophobic peptides that are prone to carryover (Hamuro & Coales, 2018; Lin et al., 2025; Masson et al., 2019; Russell Lewis et al., 2023). Importantly, FC-12 does not interfere with the analysis of peptides as it elutes later than the peptides in the LC and its inclusion in wash buffers is usually sufficient to prevent most carryover (Hamuro & Coales, 2018). It is advised to run a clean blank between samples, which is an injection of a pepsin wash solution throughout the MS system and the trap/LC columns are run under a sawtooth gradient of organic solvent. This helps flush any remaining peptides out of the system before the next run. The number of protease washes can be increased between runs as well until sufficient carryover is repressed. Lastly, it could be worth considering trap and LC columns with C8 stationary phase as the weaker stationary phase will lead to less peptides becoming trapped within the MS system, or use of a buffer injection chase (Lin et al., 2025), especially when dealing with membrane protein samples and lipids which introduce components with high hydrophobic 'stickiness'.

It is important to note that protein aggregation can also occur during an HDX-MS experiment. Similarly to peptide carryover, this presents as non-EX2 (bi- or multi-modal) behavior and/or tailing can be observed at the ends of the spectral envelope. Protein aggregation often also presents with a consistent intensity drop throughout the data. Therefore, before HDX-MS experiments, it is best to leave the sample on the benchtop at room

temperature for the entire duration of the HDX time window, to test whether the sample is stable or whether aggregation occurs (Masson et al., 2019). This is an advantage that lipid nanodiscs systems confer to membrane proteins, as they are often more stable being surrounded by a lipid environment (Denisov & Sligar, 2016).

3.3.3 Maximum deuteration (MaxD) control

One consideration when performing HDX-MS experiments is whether a MaxD is required (Peterle et al., 2022). As the sequence of a polypeptide will govern the extent of back-exchange under quench conditions, each peptide will exhibit a different extent of back-exchange (Bai et al., 1993). Therefore, to accurately measure the absolute deuterium incorporation of each individual peptide within a dataset, a full deuterated protein sample should be measured, and each peptide can be back-exchange corrected using the corresponding maximally deuterated control (i.e. MaxD). A MaxD is required when quantifying protein folding/dynamics or characterizing intrinsic disorder (Fowler et al., 2016; Stofella et al., 2025). It is not necessary to collect a MaxD when performing ΔHDX, as the generated peptides should experience the same back exchange in both states as long as experimental conditions remain constant, thus the ΔHDX will be unaffected. Although it can be useful to classify the degree of back exchange within a ΔHDX dataset, for example when considering differences between isoforms containing high-degrees of mutational difference (Calvaresi et al., 2023; Wales et al., 2016).

If a MaxD is desired, there are several methods available, and often the right method depends on the individual protein system, requiring a degree of trial and error. To collect a maximally labelled sample, all backbone amides are required to be in an exchange competent state. One method is to incubate the protein in 100 % deuterated buffer at high temperatures; this can be effective for some proteins but may cause some membrane proteins to aggregate (Zhang & Smith, 1993). One modification to this is to heat the protein to 5 °C below the melting point and label it for 10 min (Mayne, 2016). This is more suitable for membrane protein samples as it reduces the likelihood of aggregation, yet it does require knowledge of the melting temperature and maximum deuteration may not be achieved. Another approach is to increase the exposure of protein to deuteration (> 24 h), without using any artificial deuteration-enhancers (Singh et al., 2013). However, despite its simplicity the approach may not account for very stable regions that may never achieve maximal deuteration. Proteins

can be acid denatured to achieve MaxD values, but this also requires long labeling times and proteins may aggregate (Sowole & Konermann, 2014). Chemical chaotropes can also be used to denature the protein before labeling; urea is the preferable choice as GnHCl has a higher propensity to cause membrane protein aggregation (Yan & Maier, 2009). Collecting MaxD values often involves testing several methods, and combining multiple approaches can be effective (Peterle et al., 2022).

4. Case study: MBX efflux pump inhibitor class action on AcrB

In this section, we detail an HDX-MS workflow to investigate the inhibition of AcrB, the prototypical RND inner membrane efflux protein from *Escherichia coli* (Kobylka et al., 2020). EPIs have the potential to be an important adjunctive therapy, to 'revive' the activities of antibiotics during a multidrug resistant infection. The pyranopyridines (MBX) series of EPIs show potent activity against RND efflux pumps of Enterobacteriaceae species (Alenazy, 2022; Opperman et al., 2014). MBX-2319 was the first MBX EPI to be discovered and was shown to bind tightly to the lower section of the AcrB distal binding pocket, blocking the access of substrates via steric hinderance. It also interacts with the hydrophobic trap, potentially preventing AcrB protomers transitioning between conformational states and thus halting its efflux mechanism. Since MBX-2319, many derivatives have been designed to enhance pharmacological properties and activity (Sjuts et al., 2016; Wang et al., 2017). One example is MBX-3756, which is a *trans*-isomer of the previously reported MBX-3132; however, it lacks experimental data on its mechanism of action. Therefore, the aim was to use HDX-MS to reveal how MBX-3756 affects AcrB structural dynamics.

We utilize our recently described automated delipidation workflow (Hammerschmid et al., 2023) (Section 2), enabling us to investigate AcrB within a lipid environment. This is important, as detergents can often destabilize membrane proteins and compromise function (Denisov & Sligar, 2016; Gulamhussein et al., 2019); having a lipid environment provides a more physiologically relevant sample. Therefore, we perform ΔHDX of AcrB in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) MSP nanodiscs, ± MBX-3756. As ΔHDX monitors the change between two states (e.g. drug-free and drug-bound), data is not corrected for back-exchange.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2024) partner repository with the dataset identifier PXD066556.

4.1 Equipment and reagents

All reagents purchased from ThermoFischer Scientific or Sigma Aldrich/Merck unless otherwise stated.

General.

- Microcentrifuge tubes
- Mini cooling centrifuge
- Nano-drop UV-Vis Spectrophotometer
- 4-point pH probe
- Ice bucket
- Orbital shaker
- Vortex
- Tris(hydroxymethyl)aminomethane
- Sodium chloride
- Ethylenediaminetetraacetic acid (EDTA)
- Sodium phosphate
- Glycerol
- Hydrochloric acid (HCl)

Preparation of nanodisc sample.

- AcrB in detergent (Reading et al., 2020)
- MSP1E3D1 (Merck)
- 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) lipids (Avanti)
- SM2 Bio-beads (Bio-Rad)
- Sodium cholate
- Superdex 200 10/300 increase GL column

HDX-MS experiments.

- Xevo G2-XS mass spectrometer (Waters)
- ACQUITY UPLC M-Class System with extended three-valve configuration and HDX technology (Waters)
- PAL3 RTC HDX robot (Trajan Scientific, Morrisville)
- Vanguard column (BEH C18, 130 Å, 1.7 μm, 2.1 mm × 5 mm; Waters)
- Acquity UPLC column (BEH C18, 130 Å, 1.7 μm, 1.0 mm × 100 mm; Waters)
- Microbore guard column (2.0 mm ID x 2 cm C130-B, IDEX)

- Acetonitrile, Optima grade
- Water, Optima grade
- Formic acid, Optima grade
- DCl, 99.96 % D
- NaOD, 99.5 % D
- D_2O, 99.96 % D
- Tris-glycine
- Guanidinium hydrochloride (GnHCl)
- Zirconium oxide (5425-U, Supelco)
- Ammonium hydroxide
- Methanol

HDX-MS analysis software.
- MassLynx 4.2 (Waters)
- ProteinLynx Global Server (PLGS) 3.0.3 (Waters)
- DynamX 3.0 (Waters™)
- Deuteros 2.0 (Lau et al., 2021)

These were the software used for this work, but many software packages are available to use (Stofella et al., 2024).

4.2 Preparing nanodisc samples for HDX-MS

AcrB nanodiscs are prepared according to previous protocols (Daury et al., 2016; Denisov et al., 2004). Briefly, dried POPC lipids are resuspended in nanodisc buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 0.5 mM EDTA) supplemented with 200 mM sodium cholate, vortexed, heated and sonicated until clear. MSP1E3D1 is added to the solubilized lipids and the mixture incubated for 15 min at the transition temperature of the lipid (4 °C). Detergent solubilized AcrB is added to the reconstitution mixture, creating a final molar ratio of 40:1:0.5 of lipid:MSP:AcrB. The sample is incubated at 4 °C for up to 2 h, and 0.8 g per mL of sample of damp SM2 Bio-beads is added. The mixture is incubated overnight for 16 h on an orbital rotator at 4 °C. AcrB nanodisc samples are filtered the next morning and injected onto a Superdex 200 10/300 increase GL column in Protein Buffer (50 mM $NaHPO_4$, 150 mM NaCl, 10 % Glycerol). Peak fractions are pooled, flash frozen in liquid nitrogen, and stored at −80 °C until ready for HDX-MS experiments. It is important to characterize the nanodisc sample before HDX-MS experiments to assess sample quality and homogeneity; usually SDS-PAGE and DLS should be run to complement the SEC (Fig. 3).

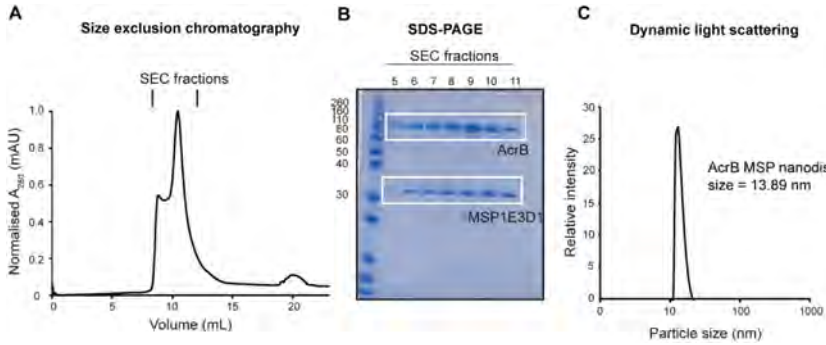

Fig. 3 Sample characterization of AcrB prepared in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) MSP nanodiscs using (A) size exclusion chromatography, (B) SDS-PAGE, and (C) dynamic light scattering.

4.3 Preparing buffers and samples for HDX-MS

For ΔHDX, we need two versions of the labeling buffers, differing only by the presence/absence of the EPI, MBX-3756.

1. The equilibration buffer should contain the necessary components to keep the sample in a stable condition. For AcrB nanodiscs, the equilibration buffers are: 50 mM $NaHPO_4$, pH 7.4, 150 mM NaCl, 5 % DMSO. The undeuterated samples act as reference samples and provide a retention time for identified peptides that can be used to search for the same deuterated peptides. Note: DMSO is commonly required to keep small molecule drugs and ligands soluble and, to keep conditions identical for differential HDX, the amount added must be matched in both ligand-absent and -present conditions.
2. The labeling buffers should have the same composition as the equilibration buffers; except they are made with D_2O instead of H_2O. The concentration of the inhibitor that is used should be carefully optimized. It needs to ensure a high percentage of protein:ligand complex but also remain soluble in solution throughout the HDX time course. Usually, knowing the binding constant for the inhibitor allows estimation of protein:ligand complex concentration. In this instance, binding parameters are unknown, therefore we use at least >10 molar excess of ligand (100 μM final), guided by previous cryo-EM work with MBX inhibitors (Wang et al., 2017). It was ensured that 100 μM ligand remained soluble in the buffer for the several hours of the HDX experiment.

The labeling buffers are: 50 mM $NaHPO_4$, pD 7.4, 150 mM NaCl, 5 % DMSO, ±100 μM MBX-3756. It is important to ensure the pD of the labeling buffer is accurate, by applying the right correction:

$$pD_{corrected} = pH_{read} + 0.4 \quad (5)$$

3. Lastly, the quench buffer should be prepared. It is required to bring the pH down to ~2.5 to minimize the exchange reaction at the end of each timepoint. Furthermore, quench buffers often contain additives such as urea, thiourea or GnHCl to unfold proteins to aid digestion. Our workflow requires a glycine-HCl based quench buffer for optimal delipidation efficiency. The recommended quench buffer is composed of 500 mM glycine-HCl, pH 2.35, 1.6 M GnHCl.

   It is imperative that the quench buffer is tested with the equilibration buffer, to ensure it can reduce the pH to 2.5. It should be tested by adding 1:1 with equilibration buffer on ice, and ensuring the pH drops to 2.3-2.5, and the final pH recorded.
4. Although the degree of sensitivity will depend on each LC-MS system and setup we recommend that 10-100 pmol of protein (after dilutions from labeling and quenching) is injected per run. Protein MSP nanodiscs complicate sample concentration calculations, as MSP are also proteins thus absorb UV at 280 nm. An estimated total protein concentration is used by combining the molecular weights and extinction coefficients of AcrB and MSP (5.6 μM). If a flash-freeze step was chosen for storage of purified protein before an HDX experiment then it is important to assess sample condition after freeze-thaw cycles or long-term storage to ensure integrity; this can be carried out by SDS-PAGE, SEC, circular dichroism (CD), or using other biophysical techniques.

4.4 Procedure

1. The first task is to ensure the MS system is set up with the correct solvents. Solvent A: H_2O (Optima) + 0.23 % formic acid (pH 2.5), Solvent B: Acetonitrile + 0.23 % formic acid, Solvent B2: Methanol + 3 % NH_4OH. Our automated delipidation workflow has a unique set up compared to standard HDX-MS workflow (Fig. 4). Usually, the auxiliary solvent manager (ASM) uses solvent A to flow over the digestion column and trap column, and the binary solvent manager (BSM) mixes solvent A and B to create an organic gradient over the trap and analytical columns. In our set up, an additional delipidation valve

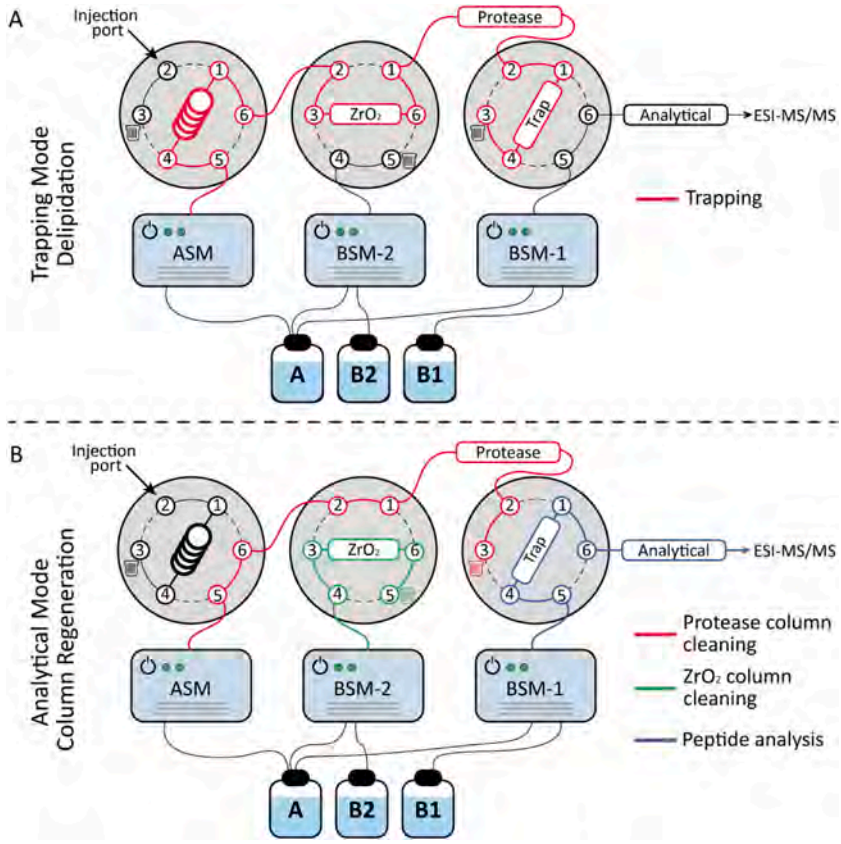

Fig. 4 Schematic illustration of the automated phospholipid trapping workflow in (A) trapping/delipidation and (B) analytical/column regeneration modes.

flanks the injection and trapping valves and is operated by a second BSM. Therefore, the sample is injected and passes through the phospholipid trap column and further to the protease/trap columns, in solvent A from the ASM. Then, in analytical mode, the sample is eluted into the MS using a gradient created by BSM-1, whilst the phospholipid trap column is washed in solvent B2 and re-equilibrated in solvent A, controlled by BSM-2.

2. Next, optimized peptide identification is needed to ensure optimal protein coverage before proceeding to ΔHDX experiments. 5 μL of protein sample is diluted in 45 μL equilibration (or labelling) buffer. This 1:10 dilution would provide 90 % D_2O.

3. 50 μL of ice-cold quench buffer is added to the sample and 100 μL of quenched sample are injected into a 50 μL sample loop within the MS, and the MS method is run. The sample is passed over the self-packed ZrO_2 phospholipid trap column, pre-blocked with 3 % BSA solution in solvent A (Hammerschmid et al., 2023) and onto an online Enzymate™ pepsin digestion column (Waters) in solvent A (kept at 10 °C, 200 μL/min flow rate). The delipidated, peptic fragments are trapped onto an ACQUITY BEH C18 1.7 μM VANGUARD pre-column (Waters) for 3 min and then eluted using an 8-40 % gradient of solvent B (40 μL/min flow rate) into a chilled ACQUITY UPLC BEH C18 1.7 μM 1.0 x 100 mm column (Waters). Both trap and analytical columns are kept at 0 °C. The eluted peptides are ionized by ESI into the Xevo G2-XS Q-Tof mass spectrometer. MS^E data are acquired with a 20–30 V trap collision energy ramp for high-energy acquisition of product ions. Argon is used as the trap collision gas at a flow rate of 2 mL/min. Leucine enkephalin is used for lock mass accuracy correction and the mass spectrometer is calibrated with sodium iodide before the run.
4. During the analytical run, the pepsin column is manually washed twice with pepsin wash solution (1.5 M Gu-HCl, 4 % (v/v) MeOH, 0.8 % (v/v) formic acid, 0.1 % (w/v) FC-12).
5. The total ion chromatogram (TIC) can be used to assess the run. Peaks should be well resolved and appear in the 9-minute gradient window, there should not be significant amounts of undigested protein at the end of the LC run and the intensity should be $\sim e^6$-e^7 for this system. If this standard is not met, sample may need concentrated further, the quench buffer modified, or the digestion column changed, cleaned or conditions optimized further.
6. These steps can be repeated to obtain HDX reference samples in minimum triplicate. MS^E data can be used to identify recovered peptides, using PLGS.
7. If sufficient sequence coverage is obtained, steps 2-4 can be repeated for both protein states (±MBX-3756) in labeling buffer. Labeling times are determined to be 10 s, 100 s and 1000s, and each time point is performed in minimum triplicate for ± MBX-3756. Ensure a cleanblank run is performed between every labelled sample – a cleanblank run is an injection of pepsin wash solution using a saw-tooth gradient of solvent B, to clean the LC system and limit peptide carryover (Majumdar et al., 2012).

8. After collecting the data, the identified peptides are filtered further using DynamX (v. 3.0) using recommended parameters (Sørensen & Salbo, 2018), and the labelled data uploaded and processed. All the spectra are visually examined and only those with a suitable signal to noise ratio are used for analysis. The amount of relative deuterium uptake for each peptide was determined using DynamX (v. 3.0).

4.5 Statistics

When performing ΔHDX experiments, statistics are useful to accurately identify peptides with significant differential HDX uptake. The guidelines generally support a hybrid significance filter (Weis, 2019). The first filter is the calculation of a confidence interval (CI). This uses the pooled standard deviation (SD) of deuterated peptides for the time points performed in triplicate. The pooled SD is used to calculate the CI at the 95 % significance level, considering a two-tailed distribution with four degrees of freedom. For this work, it gives a CI of ±0.25 Da. The second filter is passing a Welch's *t*-test with a *P*-value of ≤0.05. Only peptides that satisfy both the CI and the Welch's *t*-test are deemed significant. Post-analysis software such as Deuteros 2.0 perform these statistics calculations when processing the analyzed data (Lau et al., 2021). For a more extensive review of HDX software and statistics, we direct the reader to here (Stofella et al., 2024).

4.6 Data visualization

Deuteros 2.0 takes statistically significant peptides and plots them in various ways to enable data interpretation. One main plot is a Woods plot (Fig. 5B); this depicts each peptide as a horizontal bar, with the length representing the peptide length. The x-axis is the peptide number, and the y-axis is the ΔHDX (Da). This is a particularly effective way to present data, as individual woods plots can be stacked to show the changes across all time points, or the uptake data can be compressed by summing differences across timepoints. For this case study, we do the former. As well as ΔHDX data, uptake plots also show peptide coverage and redundancy all in one plot. Furthermore, domain maps can be added to the plot to highlight structural features of the protein of interest. Aside from Woods plots, peptide uptake plots are often shown for regions of interest, highlighting the quality of data, and uptake data can be visualized on the protein structure to show the proximity of significant peptides within the folded protein. This is effectively useful when investigating ligand binding and can help interpret ligand-protein interactions and allosteric effects.

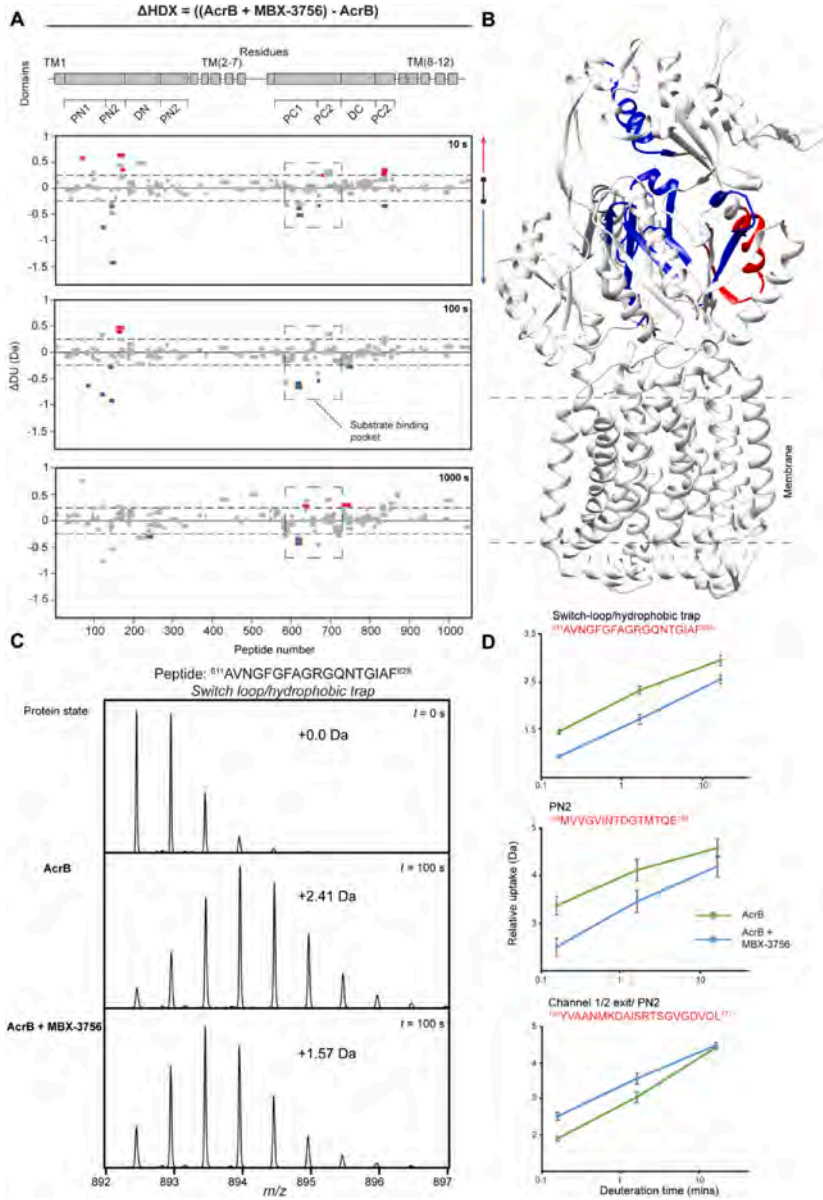

Fig. 5 Effect of EPI MBX-3756 on AcrB structural dynamics and function within a POPC lipid nanodisc. (A) Differential HDX (ΔHDX) plots (ΔHDX = (AcrB+MBX-3756)-AcrB) for all time points collected. Red signifies statistically significant peptides with increased HDX between states and blue represents those with decreased HDX (see Section 4.5). All measurements were performed at least in triplicate. (B) ΔHDX extent is coloured onto the L-state monomer of AcrB (PDB:2HRT). (C) A representative *m/z* spectrum

For our case study on AcrB and MBX-3756, we observe 177 high-quality peptides, with a coverage of 77.8 %. In the presence of MBX-3756, residues 610–630 exhibit reduced ΔHDX across the entire time course (Fig. 5) – this is highlighted by peptide ^{611}AVNGFGFAGRGQNTG-IAF^{628} (Fig. 5D). This area of AcrB is located within the substrate binding pocket and contains several residues found in the hydrophobic trap (F610, V612 F615, I626, F628) (Reading et al., 2020). Region 116–149 also contains several protected peptides in the first two timepoints, which also contain several residues found in the distal binding pocket (S128, E130, S132, S134, F136, V139). This is consistent with previous findings on other MBX derivatives; the cryo-EM structure of MBX-3132, of which MBX-3756 is a *trans*-isomer, observed its interactions with multiple residues in the hydrophobic trap, including V612 and F615 which are also in the significant peptides highlighted by HDX (Wang et al., 2017). However, areas of increased HDX upon EPI binding were also found within some regions of AcrB's PN2 subdomain in the first two time points suggesting structural dynamics could be increased there. Our HDX-MS data supports that MBX-3756 interacts with the hydrophobic trap and inhibits AcrB by preventing the proper movement of substrates through the pump by perturbing its proper backbone motions as well as steric blocking of substrate binding. Analogous perturbed dynamical behavior was also found by HDX-MS for other hydrophobic trap targeting inhibitors, such as PAβN, supporting a general mechanism of inhibitory action (Reading et al., 2020). This HDX-MS experiment provides a useful insight into MBX class EPI action (Vargiu et al., 2014).

5. Limitations of HDX and future outlooks

There are inherent limitations to bottom-up equilibrium HDX-MS measurements which are important to be aware of when investigating bacterial efflux systems. The reliance of peptide-level information generated

(peptide 611–628) under non-deuterating conditions and deuterating conditions with and without MBX-3756. The mass change of the deuterated samples is written in Daltons. (D) Representative HDX uptake plot data are presented with the average and standard deviation from repeated measurements (n = 3). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2024) partner repository with the dataset identifier PXD066556.

by acidic protease digestion affords only medium resolution information on protein dynamics. Efforts are continually being made in computational analytics and top-down fragmentation (e.g. electron capture dissociation) to improve resolution towards single residue detail (Seetaloo et al., 2022; Stofella et al., 2024); however, these strategies have their own merits and drawbacks associated, and usually require careful control experiments, all which should be understood before pursuing. Most often, as performed here in the case study, the inherent medium resolution is simply accepted as it has consistently been shown to be highly informative on locating areas of dynamic behavior and change.

Increasing sequence coverage, redundancy and deuteration time-window aid in improving resolution and confidence on identifying dynamic changes between states. However, achieving up to 100 % sequence coverage and high redundancy values is never guaranteed and more often is not reached for a protein of interest. This is especially true for membrane proteins, where digestion of its transmembrane regions is inefficient, often due to their high degree of hydrophobicity, and their protection within a membrane mimetic vehicle, required for maintaining solubility throughout digestion. Relatedly, the very nature of the transmembrane portions being maintained within a hydrophobic environment protects them from solvent, which can significantly reduce their deuteration levels, even if detected, which can limit the ability to differentiate perturbed dynamics between states. Moreover, membrane proteins are often more intractable and unstable over time compared to soluble proteins, becoming more prone to aggregation and 'crashing out' of solution. This can limit deuteration time window breadth and make practical MaxD information more difficult to obtain (Peterle et al., 2022). Ultimately, the practitioner will need to make decisions on what sequence coverage, redundancy and deuteration time window is satisfactory after optimization. Achieving *high-quality* membrane protein sequence coverage – across an entire, processed deuterated data set - at >60 % has been shown to provide biologically relevant information for important systems such as GPCRs and efflux pumps (Krishna Kumar et al., 2023).

It is also useful to reiterate that HDX-MS data delivers sequence-level reporting only on backbone dynamics. This has some important realizations when evaluating what the data means, and we recommend reading this accessible perspective by Hamuro to understand HDX-MS interpretation in more detail (Hamuro, 2024). Some important aspects to highlight for efflux systems are: (i) Not seeing a HDX response does not necessarily mean that dynamic changes are not present. Events that take place with only miniscule

H-bonding alterations will be almost "HDX-silent" (Scrosati et al., 2021); (ii) HDX-MS identifies allosteric effects. For example, HDX-MS may identify a change in dynamics upon binding or mutation that are in addition to the direct effects of mutation or binding. This could be a disadvantage to identify the direct binding site but could be an advantage to understand the energy flow of the binding event; (iii) Homo-oligomers measured by HDX-MS provide an average for all its identical protein subunits and are unable to distinguish between homomers. From high-resolution structural studies we know that the ground states of efflux pumps can be asymmetric in support of dynamic, interconverting states that enable substrate translocation. Therefore – even if bimodal HDX spectra is present suggesting slowly interconverting states and/or mixed conformational species – then interpretation of asymmetry is difficult with HDX data alone. Taken together, we encourage that HDX is used in partnership with other biophysical, computational, and structural tools and datasets to comprehensively define protein functional dynamics, to provide consistent but distinct information on the same protein folding well.

HDX-MS is powerful, and the future looks exciting for the technique. There has always been the promise for HDX-MS to offer a simple 'just add (heavy) water' approach to sensitively assay the structural malleability and movements that underly protein folding and function. Although we do not comprehensively cover all past advancements here (these are best obtained from a complete review by the Guttman group (James et al., 2022)) we wanted to highlight important ones that demonstrate the ambition of the HDX-MS field in the coming years.

First, it is important to highlight the seminal organization of HDX-MS understanding and the establishment of general data reporting in the 2019 white paper by Masson, Burke, Schriemer and Rand et al., which provides recommended community-guided ways to make HDX-MS data more FAIR (Findable, Accessible, Interoperable, and Reusable) (Masson et al., 2019). This is essential for HDX-MS to be used in a more holistic way with other structural biology and proteomics techniques to achieve a more integrated view of biology (Beck et al., 2024). It also ensures that HDX-MS data can be best utilized in the AI-revolution with the first preliminary investigations into the use of HDX-MS data to predict dynamics and interactions already happening recently (Yu et al., 2023). Linked to data acquisition is the reality that comprehensive data analysis has been the rate-limiting step for bottom-up HDX for a long-time. However, a recent development of a DIA method to automated HDX analysis, AutoHX

(Filandr et al., 2024), could significantly improve accessibility, throughput and – dare we say – enjoyment for HDX-MS, through reducing the pain of a traditionally laborious analysis regime. It currently relies on the requirement for narrow DIA window data obtainable only in partnership with certain vendor machines but with future expansion into the breadth of instrument compatibility then we predict AutoHX (and its progeny) to significantly improve and enhance HDX-MS use.

The field has made significant progress recently on analytical workflows to meet the challenge of making HDX-MS both more accessible and informative in new contexts. A way to miniaturize the HDX on to a 'HDX-chip' procedure has recently been developed which could potentially reduce the costs and throughput of performing HDX-MS by a general practitioner (Hansen et al., 2025). Subzero chromatography has the potential to make back-exchange negligible during chromatography which would enable longer gradients and better separation of peptides during analysis, offering the ability to deal with increased sample complexity. In recent years millisecond HDX has been achieved with flow and droplet systems which offer ways to measure these rapid dynamic events and expand the time window down to the theoretical limits of HDX reactivity (Anacleto et al., 2023; Kish et al., 2023). This is important when considering intrinsically disordered proteins, as proteins containing intrinsic disorder deuterate completely so fast that information of their dynamics was lost with the previous 'bottom-limits' of experimental HDX time windows (1–10 s). We pose that recent achievement of a robust droplet microfluidic HDX paves the way to discretely control analyte (Hammerschmid et al., 2025), HDX and quench processes which could open the possibility to higher throughput towards single-droplet (Kempa et al., 2020) and single-cell capabilities (Gebreyesus et al., 2022). Successful workflows have been demonstrated to achieve in situ insight on proteins within the complexity of living cells and cell lysate systems (Kaldmäe et al., 2020; Lin et al., 2022; Moroco et al., 2025; Quanico et al., 2016); although, no generic method yet exists that can provide a protein-targeted investigation (instead relying on analyzing only the most abundant proteins in a system) we anticipate the community to add this capability soon. Overall, these developments will, in partnership, help to create an HDX-MS technique capable of contributing enriched molecular details on dynamic changes difficult to measure with any other technique. Doing so in ever more interoperable and quantitative detail, as well as expansion into more directly biologically relevant contexts, will

facilitate simulations of molecular processes to predict novel and experimentally testable molecular biology and contribute to structural biology efforts to better understand the cell (Beck et al., 2024).

Acknowledgements

The authors thank Timothy J Opperman, Attiliio V. Vargiu and Microbiotix for provision of MBX-3756. DH and ER were supported by a UKRI Future Leaders Fellowship to ER (MR/X009580/1). JS and ER were supported by Eli Lilly and Company through the Lilly Research Program. BRL were supported by a King's College London PhD studentship and a BBSRC Pioneer Award (BB/Y512849/1). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2024) partner repository with the dataset identifier PXD066556.

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