OPINION ARTICLE

Seven perspectives on GPCR H/D-exchange proteomics methods [version 1; referees: 2 approved, 1 approved with reservations]

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Abstract

Recent research shows surging interest to visualize human G protein-coupled receptor (GPCR) dynamic structures using the bottom-up H/D-exchange (HDX) proteomics technology. This opinion article clarifies critical technical nuances and logical thinking behind the GPCR HDX proteomics method, to help scientists overcome cross-discipline pitfalls, and understand and reproduce the protocol at high quality. The 2010 89% HDX structural coverage of GPCR was achieved with both structural and analytical rigor. This article emphasizes systematically considering membrane protein structure stability and compatibility with chromatography and mass spectrometry (MS) throughout the pipeline, including the effects of metal ions, zero-detergent shock, and freeze-thaws on HDX result rigor. This article proposes to view bottom-up HDX as two steps to guide choices of detergent buffers and chromatography settings: (I) protein HDX labeling in native buffers, and (II) peptide-centric analysis of HDX labels, which applies (a) bottom-up MS/MS to construct peptide matrix and (b) HDX MS to locate and quantify H/D labels. The detergent-low-TCEP digestion method demystified the challenge of HDX-grade GPCR digestion. GPCR HDX proteomics is a structural approach, thus its choice of experimental conditions should let structure lead and digestion follow, not the opposite.

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Abbreviations

GPCR, G protein-coupled receptor; **HDX**, H/D-exchange; **TM**, transmembrane; **DDM**, n-dodecyl-β-D-maltopyranoside; **TCEP**, Tris-2-carboxyethylphosphine; **DLT**, DDM-low-TCEP; **CHS**, cholesteryl hemisuccinate; **β_2AR**, β_2 adrenergic receptor; **C*c*O**, cytochrome *c* oxidase; **TSPO**, translocator protein; **UPLC**, ultra-performance LC; **EM**, electron microscopy; **LCP**, lipidic cubic phase; **PC**, phosphatidylcholine; **DPC**, dodecyl phosphatidylcholine (12:0); **DMPC**, dimyristoyl phosphatidylcholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0); **PO**, 16:0/18:1, 1-palmitoyl-2-oleoyl; **DO**, 18:1/18:1, 1,2-dioleoyl; **PE**, phosphatidylethanolamine; **PG**, phosphatidylglycerol; **PS**, phosphatidylserine; **PA**, phosphatidic acid; **PI**, phosphatidylinositol; **PIPn**, PI phosphate.

Introduction

This opinion article is a response to the recent call to "strive for reproducible science"[1]. January 2010 saw the publication of the first fully automated membrane protein bottom-up H/D-exchange (HDX) proteomics method, which can map human G protein-coupled receptor (GPCR) dynamic conformations in solution at repeated HDX coverage of 89%, out of ~90% MS/MS coverage[2]. This method broke the years-long sub-25% coverage impasse, provided the first useful HDX proteomics protocol to obtain meaningful structural information of seven-transmembrane (TM) GPCR for drug discovery, and established HDX proteomics as a powerful mainstage tool for GPCR structure-function investigation. These peptides were robustly reproduced in over two hundred independent HDX runs, using several ligand-states of prototypic human GPCR β_2 adrenergic receptor (β_2AR) from numerous batches of purifications (2 and unpublished study by Xi Zhang and Patrick R. Griffin, *et al.*). Enabled by a DDM-low-TCEP (DLT) digestion method (DDM, n-dodecyl-β-D-maltopyranoside; TCEP, Tris-2-carboxyethylphosphine), this protocol integrates autosampler control programs to coordinate continuous full sets of HDX incubation, online digestion and data acquisition of high-performance liquid chromatography mass spectrometry (HPLC MS), and is flexible for users to choose 0-to-3600-second or longer-hour incubation modules, and regular or longer HPLC for MS/MS sequencing. Subsequently, this protocol has been applied in large-scale GPCR efforts and attracted broad interests from the GPCR community[3–9].

However, mis-representations have also emerged[3,6], reflecting misunderstanding of the GPCR HDX proteomics approach at multiple levels. Outstanding problems include: confusing the HPLC MS/MS and HPLC MS steps; confusing the various roles of detergents; incorrectly claiming the 2010 study analyzed HDX-labeled peptides with 120-min HPLC MS experiment; and calling the 89% HDX coverage invalid[3]. Questionable procedures include: neglecting the HPLC MS/MS part of HDX and the critical optimization of pepsin column digestion[3]; destabilizing membrane proteins by dilution with zero-detergent buffers and introducing Na/K interference to the GPCR protein system, such as using a quench/digestion buffer composed of 20 mM TCEP and 0.1 M KH_2PO_4 pH 2.01 to dilute GPCR DDM/NaCl solution[3]; disturbing proteins or labels with extra freeze-thaws[3]; neglecting the effects of the bicelle detergents, lipids and adducts on MS, data-dependent MS/MS acquisition and peptide identification[3]; and switching to manual mode for membrane protein HDX. Backed by these problematic procedures, the JASMS May 2015 paper repeatedly claimed CHAPSO/DMPC (dimyristoyl phosphatidylcholine, 14:0/14:0) bicelle specifically as a "better solubilization method than DDM for HDX-MS analysis of GPCRs"[3], but minimized discussing the structural concerns of CHAPSO/DMPC on proteins. The peptide MS spectra of CHAPSO/DMPC appeared unusually noisy compared with DDM[3], raising questions about potential effects on spectrum quality and HPLC column health over long-term practice. Meanwhile, another study submitted in May 2015 reported that human β_2AR purified from Sf9 did not predominantly sequester PC nor 14:0/14:0 chains from membrane, but enriched for cholesterol by 17.7 fold, and 18:1/18:1 chains by 80 fold[10], raising structural concerns against CHAPSO/DMPC.

This opinion article reasons that these problems reflect a common lack of systematic thinking and confusions of fundamentals in the GPCR bottom-up HDX proteomics approach, such as the MS *versus* MS/MS step, the protein structure HDX labeling *versus* label analysis step, and the roles of detergent/lipid additive for structure *versus* for digestion. Given the recent surging interest to study GPCRs using bottom-up HDX[3–9,11,12], this viewpoint clarifies critical technical nuances and logical thinking, and emphasizes systematically considering membrane protein structure stability and HPLC MS compatibility, throughout the pipeline. This article explains bottom-up HDX as two flexibly coupled modules to guide choices of detergent buffers and HPLC settings, and highlights the effects of metal ions, zero-detergent shock and freeze-thaws on membrane protein structure, stability and HDX result rigor. Rather than a comprehensive overview of HDX or membrane protein methods[8,9,12,13], or a refutation of particular publications, this article aims to provide a systematic practical guide to help scientists overcome cross-discipline pitfalls, and understand and reproduce the GPCR HDX methods at high quality. The ignorance of these nuances, rather than the lack of care or diligence, likely caused the previous impasse and emerging problems and endangers future success. Strengthened by important non-HDX biophysics studies published after 2010, these seven first-hand insights are critical to clear emerging misconceptions, but are not discussed in the original 2010 report.

1. Deep-sequencing-based bottom-up HDX MS: a two-stage analysis

Although HDX descriptions usually list multiple steps and elaborate on the well-established logistics of bottom-up proteomics[3,6], what has critically enabled the membrane protein HDX breakthroughs[2,14] is to think in terms of two distinct yet flexibly-coupled modules beyond the routines (Figure 1). The overall method workflow of bottom-up HDX structural proteomics of membrane protein GPCR can be viewed as two steps: (I) label via H/D-exchange, and (II) analyze—identify and quantify—H/D labels using bottom-up proteomics. H/D label analysis is peptide-centric and also has two stages: (1) identify and construct peptide matrix (a set of reproducibly identified peptides) using HPLC MS/MS deep sequencing, and (2) quantify H/D-labels in MS for each identified peptide, using MS peak area summed from the peptide's isotopic envelope (Figure 1). These two stages share the same protease digestion method and as similar as possible temperature and HPLC-MS instrument, but can differ in some other HPLC and MS/MS or MS conditions to best fulfill distinct purposes.

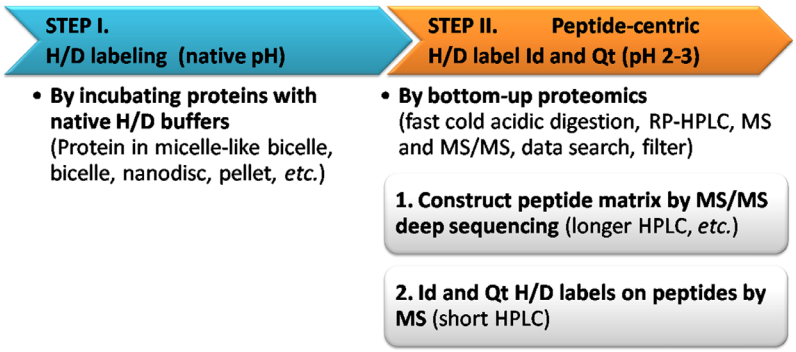

Figure 1. Two-step experiments of the deep-sequencing-based bottom-up differential HDX proteomics method for GPCR. Id, identification; Qt, quantitation.

The MS/MS deep sequencing stage aims to identify as many robust peptides as possible for target proteins. TM sequences often fall short of ionization efficiency (overdigestion is explained below), thus a longer HPLC gradient is desired to simplify elution population and allow these peptides a better chance to get picked for MS/MS scan in the ion-abundance-ranked data-dependent acquisition. Because these peptides do not carry H/D-labels, longer HPLC MS/MS analysis time causes no harm here. To obtain a robust MS/MS peptide matrix, the 2010 protocol then matched and iteratively filtered these MS/MS spectra using a multilayer method: (1) each peptide should score above 20 in MASCOT search against the target sequence, but spectra stay unmatched in decoy search against the reversed sequence (most decoy matches scored way below 10); (2) peptide sequences should comply with the pepsin preference sites reported by Hamuro *et al.* in 2008[15]; (3) fragment ions in MS/MS spectra appear reasonable in manual inspection; and (4) precursor ions should be repeatedly confirmed in high-resolution MS using the HDX MS experiment's HPLC gradient. This MS/MS stage provides an initial peptide matrix, which is further refined in subsequent HDX MS.

However, restricted by the minutes' time window of HDX pipeline to minimize H/D label back-exchange, the HDX MS stage uses shorter HPLC gradient and just MS scans. Peptide identification in HDX MS data is based on: (1) accurate peptide mass matching to those in the pre-constructed MS/MS peptide matrix; (2) retention time reproducibility over all HDX runs and correlation with the longer gradient; and (3) iterative confirmations via checking consistency across redundant peptide ladders, multiple charge states, and overall HDX profile trend throughout the H/D-incubation time points. Targeted MS/MS may further confirm ambiguous peptide ions. Ideally peptide MS/MS identification should be performed at the same HPLC gradient as used in HDX MS quantitation, but it challenges the capacity and scan speed of current popular HPLC and mass spectrometer instruments, and proved often unnecessary for simpler purified protein samples on high-resolution orbitrap analyzers (2,16 and unpublished study by Xi Zhang and Patrick R. Griffin *et al.*). Nonetheless, the rapidly growing data-independent acquisition MS/MS, which uses wide precursor isolation window for simultaneous fragmentation, may reconcile this gap[17–20].

Therefore, constructing the MS/MS peptide matrix favors longer HPLC gradient for exhaustive identification (no H/D labels), but the MS-based H/D label quantitation can apply short HPLC to minimize H/D label loss, as this step is based on MS peptide mass matching. The 2010 protocol achieved the 89% HDX coverage by devising a total 9.5-min HPLC method for HDX MS[2], not the 120 min claimed by *Duc et al.*[3]. This short HPLC for HDX MS was repeated in subsequent large-scale GPCR HDX studies. As a part of the 2010 strategy, changing from the regular 60-min to the 120-min HPLC method for MS/MS sequencing successfully recovered multiple TM peptides, and they were robustly identified throughout HDX MS mapping.

2. DDM as a tool for making structural-grade protein versus a tool for digestion

DDM/cholesteryl hemisuccinate (DDM/CHS) bicelle-like micelle served as a tool to prepare upstream structural-grade membrane protein solution samples, and to mark these conformations with matching D_2O buffer. As a tool for downstream digestion, DDM-low-TCEP alone suffices to support protease activity and to solubilize and stabilize substrates against aggregation throughout digestion. Importantly, the combination of these two modules—protein preparation-labeling and digestion—is flexible (Figure 1).

Upstream protein states can vary vastly with sample preparation methods, which should thus be screened with rigorous function assays (multi-facet, including activity, ligand binding and stability) and matched by the H/D-labeling buffer. However, this does not void the broad utility of DLT method for downstream digestion. Not only can DLT digestion be applied to various upstream protein preparations, including myriad detergent/lipid bicelles, lipid bilayer nanodiscs, DDM/CHS bicelle-like micelle, membrane pellets and intact organelles[21], but also DLT HDX-proteomics provides a tool to visualize their different effects on protein in-solution conformations. Remarkably, the DLT digestion method proved highly compatible with soluble protein projects to share the same regular reversed-phase (RP)-HPLC ESI MS and MS/MS instrument platform. Across large-scale applications, no deterioration was observed in peptide MS spectra (smooth not noisy), column health or sample carryover, similar to non-DLT soluble proteins

(2 and unpublished data by Xi Zhang and Patrick R. Griffin, *et al.*) (Supplementary Figure 1 and Supplementary Figure 2B). Therefore, the HDX-grade digestion of GPCRs is technically solved and is no longer hampered by solubilization during the digestion.

By contrast, CHAPSO/DMPC is less suitable as a tool for digestion in broader proteomic applications. Both CHAPSO and DMPC form net strong fixed positive charges under acidic pH, combined with high concentration (CHAPSO critical micelle concentration cmc is 8 mM, 5x cmc is 40 mM), they are long observed to dominate ionization, likely interfere with peptide RP-HPLC data-dependent MS/MS, and may harm long-term RP-HPLC column health, despite possible chromatograph improvement in ultra-performance LC (UPLC). Even anionic cholate entailed UPLC[22], and anionic deoxycholate (cmc 6 mM, no fixed positive charges) proved to require removal by ethyl acetate extraction before HPLC injection[23]. Samples that contain CHAPS, similar to CHAPSO (same charged groups, same 8 mM cmc, one less hydroxyl) find routine rejections at proteomics facilities: "Non acceptable buffers include NP40, CHAPS, Triton X, and PEG" (https://mass-spec.stanford.edu/sample-preparation; Sample Preparation, Stanford University Vincent Coates Foundation Mass Spectrometry Laboratory; Sept 28, 2015 access).

3. CHAPSO/DMPC bicelle versus DDM/CHS bicelle-like micelle: not unique to the HDX approach

How the presentation methods influence membrane proteins' native structures is the premier concern common to all solution-based biophysical approaches that aim to measure their functional/native states[14,24]. For solution-based structural technologies, the freedom from high-resolution crystallogenesis—itself a quality control of how comfortable (though not always native) membrane proteins are in these conditions—presents both advantages and pitfalls, and calls for extra rigor and caution in protein handling, data interpretation, and cross-examination with other function and structure measurements. To avoid masking the effects of intended perturbations, such as ligand stimulation, protein buffers often aim to approach native-like and function-neutral: stabilize the protein and minimize distortion (deactivation or over-activation).

The 2010 study prepared human GPCR protein in DDM/CHS solution[2], because mammalian GPCR natural habitats include 20–25% cholesterol, and membrane proteins are increasingly resolved to contain conserved binding sites for cholesterol, CHS and other derivatives[25–29]. The natural 20–25% cholesterol habitat proved possible to be re-established by using DDM/CHS that forms wide bicelle-like micelles around membrane proteins, and greatly enhances GPCR activity and stability from just DDM[25,30]. Although open to improvement, DDM/CHS emerges as a viable method to unify solution-phase means—crystallography, electron microscopy, structural proteomics and nuclear magnetic resonance (NMR)—to spearhead charting the solution-phase structures of GPCRs and complexes. Such actionable atomic clarity is in urgent need and provides the pivotal foundation to further understand interactions with molecules, such as certain lipids. Although CHAPSO/DMPC bicelle has produced membrane protein crystals and NMR results[31,32], the 2010 protocol is cautious and chooses not to present proteins in CHAPSO/DMPC for multiple reasons, as specified below and in Supplementary File 1, and increasing evidence since 2010 supports these cautions. NMR appears to favor zwitterionic CHAPSO/DMPC for technical convenience[33–35], but mass spectrometry-based structural proteomics approaches are free from such technical constraints.

The chemical structures of lipids matter. Indeed CHAPSO/DMPC presents a lipid-rich environment, but by no means resembles human GPCRs' native lipid bilayer habitats. In-depth consideration of lipids is essential (Figure 2) and is detailed in Supplementary File 1 and briefly summarized here. First, as a tool to present human GPCRs and complexes in near-native states (Step I, Figure 1), bilayer reconstitution is not restrained to 14:0/14:0 DMPC. CHAPSO/DMPC differs vastly from GPCR native lipid bilayer habitats in chemistry, and shall not dictate the choice of lipids to recreate bilayers. Neither does CHAPSO/DMPC bring much technical advantage to Step I for HDX-proteomics and most other solution-phase biophysical methods. To the contrary, the broad adaptability of downstream proteomics readout allows Step I to maximally prioritize protein structures, such as using various micelle, bicelle, bilayer, pellet, nanodisc or cell organelles (Figure 1). Second, as a tool to solubilize GPCR for HDX-grade digestion and peptide-centric label analysis (Step II, Figure 1), the compatibility of high-dose CHAPSO/DMPC with large-scale direct HPLC MS and MS/MS runs appears controversial. By contrast, DDM alone with optional low TCEP proves effective and well suited for RP-HPLC MS instruments when applied rationally (further cross-discipline pitfalls discussed below), thus

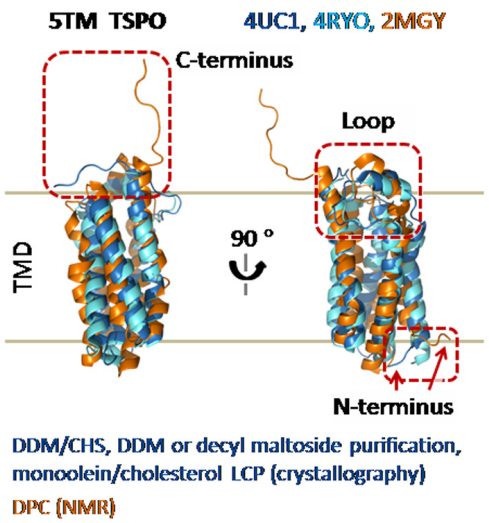

Figure 2. The 100% quaternary ammonium head groups of CHAPSO/DMPC call for cautions: structure alignment shows 100% DPC imposed variations to 5TM TSPO conformation. Blue or cyan, structures from two independent crystallizations in monoolein/cholesterol LCP (4UC1 or 4RYO); orange, structure from DPC-micelle NMR (2MGY). Distortions in all three domains of 5TM TSPO were seen in DPC-produced NMR structure (2MGY), contrasting the well-aligned independently acquired crystal structures from LCP (4UC1 and 4RYO) or DDM micelle and EM structure (not shown)[62,63]. TSPO structures were directly aligned in PyMOL.

HDX-grade GPCR digestion (Step II) is no longer limited by solubilization. As a structural approach, HDX-proteomics choice of experimental conditions should let structure lead (Step I) and digestion follow (Step II). Touting CHAPSO/DMPC specifically for the HDX-proteomics approach—by arguing CHAPSO/DMPC is a better solubilization method than DDM for GPCR digestion based on questionable practices, yet minimizing structural considerations on proteins—is misleading.

Therefore, lipid choice in bilayer reconstitution is unrestricted to just CHAPSO/DMPC, NMR-favored zwitterionic head groups, or 14:0/14:0 chains, but should and could prioritize protein conformation, activity and stability. Indeed proteins may differ, and extensive method optimization is necessary. Membrane proteins' responses to bilayer environment can be highly dynamic, diverse and sensitive; thus, multifaceted structure-activity measurements are essential to data interpretation. Recent rigorous bilayer reconstitutions for activity measurement typically examined various phospholipid head groups, chain lengths and cholesterol additive[36–38], and increasingly chose POPS[38], POPE[27], POPE/POPG[39–41], POPC/POPE/POPG[27] or DOPE/POPC/POPS[40] mixtures, with 16:0/18:1 or 18:1/18:1 fatty acid chains[37,40], rather than 100% 14:0/14:0 DMPC.

4. Optimization of pepsin column reaction is a key for coverage

In the chosen digestion buffer, HDX proteolysis is completed within seconds of column residence time: the highly reactive pepsin column is obviously the most sensitive component of the platform to affect coverage and reproducibility. Pepsin column length, diameter, manufacturing of beads and column, temperature and flow rate may all change digestion products' peptide length and reproducibility. Particularly, the pepsin-beads coupling reaction conditions affect pepsin surface density, activity and extent of autolysis—thus the effective enzyme surface concentration of final columns—and may vary between operators and manufacturers.

Because the 2010 HDX method is a completely automated protocol that integrates all experimental conditions, such as HDX incubation time, pepsin column flow rate, HPLC gradients and MS methods, manual operation only involves placing samples in designated sample trays, and selecting whether to use or not use the additional long-hour incubation module. However, the typical shelf life of each batch of pepsin beads and columns for peptide reproducibility is only about 10 months at 4°C. Therefore, rigorous practice means at least checking the optimal digestion flow rate and temperature based on the batch of beads and columns in use. These parameter updates are allowed in the 2010 protocol by simply typing the numbers, without changing the programming for sample handling and data acquisition.

Instead of under-digestion, low TM coverage is often caused by pepsin over-digestion, and may be rescued by optimizing pepsin column flow rate and temperature, and by applying longer HPLC for MS/MS[2]. Alternatively, longer TM peptides may be generated by reversible partial deactivation of the pepsin column, and by blocking TM substrate access with bulkier, tighter or more facial amphiphiles and lipids.

During initial digestion method development, the 30-min incubation with one column volume of pepsin bead slurry is commonly used to predict whether the pepsin column can reach digestion completion at seconds scale. But, the bead slurry format falls behind in peptide reproducibility, so all actual HDX data acquisitions in this protocol use pepsin-column digestions at precisely controlled flow rates.

Besides these four major points, this article further emphasizes systematic considerations of the subtle yet critical effects on membrane protein stability as follows.

5. Na^+ or K^+ matters

To the structure-function of membrane proteins, especially GPCRs, Na^+ and K^+ are not always inter-exchangeable: thus the measurement itself shall not introduce Na/K interference. To proteomics, mixing Na^+ and K^+ may cause adduct ion formation of both Na^+ and K^+ with peptides and other components, complicating peptide MS spectra. Upon 2009, development of the DLT GPCR HDX protocol started with asking whether to use Na^+ or K^+ buffers, and chose Na^+ for all buffers (protein dilution, H/D-incubation and quench/digestion buffers) for multiple reasons. First, Na^+ and K^+ may differ in structural and functional effects on TM proteins. Previous projects used K^+-based buffers to purify physiological-state high-activity C*c*O because both mitochondria and *Rs* bacteria have high internal K^+, whereas Na^+ was empirically screened as a tool to aid crystallogenesis[29,42–44]. Na^+ and K^+ affect C*c*O Ca^{2+} binding differently[45,46], though the exact actions remain obscure. For GPCRs, unique among all common cations, Na^+ was long-observed to act as a fast allosteric mediator itself and control agonist/antagonist-distinct GPCR activities, and the structural bases started to be resolved by crystallography[26,36,47–56]. Physiological levels of Na^+ (and Li^+ to less extent) favored opiate receptor binding with antagonists against agonists[48–50]; thus the predominant use of NaCl buffers in GPCR purification may partly account for the larger difficulty to obtain stable GPCR-agonist complex upfront, than GPCR-antagonist complex. Consistently, crystallography revealed that Na^+ also modulates the ion flux activity of TM G protein-gated K^+ channel (GIRK2) by binding at specific sites in its intracellular domain immediate to the TM domain interface[57,58]. Consistently using NaH_2PO_4 rather than KH_2PO_4 for the digestion buffer avoided fast side effects that may occur even within the short pre-digestion time window.

Second, avoiding Na^+/K^+ mixing also prevented forming both Na^+ and K^+-adduct ions, such ions may exponentially complicate MS spectra and increase the risk of interfering with useful peptide peaks for both MS/MS isolation and HDX MS. The recent open data search method revealed extensive formation of peptide-Na^+ adduct ions[59]. Third, continuing with Na^+ buffers provides a common ground for structure-function and dynamic-static structure correlations, as most other function and structure characterizations of GPCRs were performed in Na^+ buffers. Lastly, abrupt changes of chemicals may destabilize membrane proteins; thus the method design sought to achieve effects with minimal disturbance of upstream conditions. Likewise, when membrane protein solution uses KCl[60], the H/D labeling and digestion buffers should ideally switch to K^+ versions accordingly.

6. 0.1% + 0 does not equal 0.05% + 0.05%: the buffering effect on membrane protein stability

Abrupt changes in buffer concentration, particularly dilution with zero (or sub-cmc)-detergent or zero-electrolyte solutions, tend to immediately impact membrane proteins and cause destabilization and aggregation. To provide such buffering protection, ~3× cmc DDM was included in quench solution and proved to increase digestion coverage better than zero-detergent quench[2]. Similarly, destabilization and aggregation were seen in soluble proteins, such as human peptidyl arginine deiminase, upon zero-electrolyte dilution (unusually high occurrence of bimodal peptide HDX isotope envelopes despite pre-HDX removal of visible aggregates), and were solved also by buffering (unpublished results by Xi Zhang and Patrick R. Griffin). Often GPCR-CHAPSO/DMPC bicelles were prepared by adding CHAPSO/DMPC to, not replacing, the original DDM protein solution; thus the GPCR-CHAPS/DMPC bicelle sample contained double doses of micelle/bicelle, and may present an unequal ground for shielding/buffering effects. High occurrence of bimodal peptide HDX profile could also be artifacts from non-optimized HPLC or MS settings[61].

7. Full automation facilitates both structural and analytical rigor of membrane protein HDX

The DLT digestion method enabled membrane proteins to be analyzed on a fully automated HDX platform that orchestrates continuous sample handling and analysis. Rather than a dispensable convenience, the DLT-enabled automated protocol presents special advantages to maximize the structural/analytical rigor and sensitivity for membrane proteins. First, it eliminated detrimental post-labeling freeze-thaws. Post-H/D-labeling freeze-thaws of H/D-bearing membrane proteins or peptides may not only distort their H/D-label profiles, but also destabilize/aggregate membrane proteins, which worsens digestion peptide reproducibility and structural coverage. Second, it precisely controlled the time and temperature during and after GPCR H/D labeling and digestion, presenting a robust level ground that is vital for large-scale sensitive rigorous comparison to precisely locate stimuli-caused conformation changes. Membrane proteins' non-TM domains are often highly sensitive to ligand and protein interactions, their amide HDX dynamics can vary on a split-second scale, though HDX data recording often starts with seconds. Third, its random acquisition order and insertion of one or more blank buffer runs between every two protein samples minimized carryover, facilitating analytical rigor. Indeed the technical error bars of %D from quadruplicates were tiny throughout the GPCR HDX examination, peptides of the ~89% HDX coverage were well reproduced proving analytical robustness and presented structural validity (2 and unpublished study by Xi Zhang and Patrick R. Griffin, *et al.*). Such rigor risks being compromised when operators have to frequently see and manually freeze, thaw and transfer protein and peptide samples. This fully automated enclosed membrane protein protocol also offers facile rigor to measure the effects of various light and electromagnetic stimuli. The longer-hour HDX incubation could be useful to directly profile the membrane protein complex stability.

Previous reviewer(s) since Nov 2015 repeatedly claimed the necessity of including high-pressure digestion and ion-mobility peptide separation for membrane protein HDX, and insisted that GPCR solubilization in HDX is not solved and that automation belongs to the future. However, most GPCR samples that entered the HDX pipeline have indeed been solubilized, many already studied by crystallography, which requires not only GPCR solubilization but also mono-dispersion. Automation was decided to be critical for the membrane protein HDX rigor and was included in the GPCR HDX protocol since its invention in late 2009; thus automation has been a reality since then. This protocol solved the high-coverage GPCR HDX challenge without needing high pressure for digestion or ion mobility MS for peptide separation. These perspectives shall help operators to achieve high-quality applications of this protocol.

Conclusions

These perspectives, from the original method development, clarify critical technical nuances and logical thinking for the GPCR bottom-up HDX proteomics approach. The DDM-low-TCEP method resolved the technical barrier of HDX-grade GPCR digestion, and showed 7TM GPCR structures can be robustly approachable with bottom-up HDX proteomics; thus GPCR HDX is no longer hampered by solubilization during the digestion step. For effective application, it helps to view the GPCR HDX experiments as two modules that allow different flexibility in choosing detergent tools and HPLC MS settings (Figure 1). Systematically considering membrane protein conformation and stability throughout the pipeline is vital, because Na^+/K^+ mixing, zero-detergent shock, freeze-thaws and imprecise sample handling may all affect the structural and/or analytical rigor of GPCR HDX results. The 2010 89% HDX coverage was obtained with both structural and analytical rigor. HDX proteomics is a structural technology, its choice of experimental conditions should—and now could—let structure lead and digestion follow, not vice versa.

Author contributions

X.Z. conceived and wrote the article.

Competing interests

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Supplementary material

Supplementary Figure 1: Contrast of hGPCR β_2AR coverage using 2010 DLT versus urea digestion method, showing that appropriate application of DDM method did not cause solubilization problem.

Click here to access the data.

Supplementary Figure 2: Contrast of 285 kDa hGABA$_A$R coverage using 2015 FDD DDM-based digestion method versus common brutal force, further confirming that DDM is effective in protein solubilization during digestion.

Click here to access the data.

Supplementary File 1: CHAPSO/DMPC bicelle versus DDM/CHS bicelle-like micelle is not unique to HDX approach; lipid chemical structures matter.

Click here to access the data.

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Current Referee Status: ? ✓ ✓

Version 1

Referee Report 08 May 2017

doi:10.5256/f1000research.11496.r22463

✓ **Jun Qin**[1], **Chenxi Jia**[2]

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[2] State Key Laboratory of Proteomics, Beijing Proteomics Research Center, Beijing Institute of Radiation Medicine, National Center for Protein Sciences (PHOENIX Center), Beijing, China

This manuscript presented seven perspectives for investigation of GPCR structure using H/D-exchange with mass spectrometry. The manuscript was well-written and clarified many misconceptions in using H/D exchange in getting GPCR rough structure information. It is nice that the author put emphasis in the description of how to maintain the solution structure of GPCR with its relevant lipid environments during the H/D exchange reaction. It can be accepted after further addressing the following questions.

1). HDX can be used to illustrate the effect of post-translational modifications[1] on protein dynamics, ligand binding, and substrate specificity. It is an important part of application of this intriguing technology and should be included in this manuscript.

2). Crosslinking (XL) mass spectrometry of GPCR in its native state (with proper lipids and solubilizing detergents) may be a rival to HDX in the future. XL may be used with endogenous GPCRs. This should be discussed.

3).The manuscript need to be further proof-read. There are many typos and grammar errors.

One example:
(Page 4, Line 25) “How the presentation methods” should be corrected as “how the present methods”.

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Is the topic of the opinion article discussed accurately in the context of the current literature?
Yes

Are all factual statements correct and adequately supported by citations?
Yes

Are arguments sufficiently supported by evidence from the published literature?
Yes

Are the conclusions drawn balanced and justified on the basis of the presented arguments?
Yes

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 20 April 2017

doi:10.5256/f1000research.11496.r21854

Dapeng Chen
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The coupling of amide hydrogen/deuterium exchange (HDX) with mass spectrometry has been successfully used for the determination of protein dynamics. Recent studies[1-2] have shown that this method can reach high protein sequence coverage (>80%) in the study of GPCRs. However, frustrations exist, such as the confusion of the HPLC method and the inconsistency of the use of detergents. In the opinion article, Zhang discussed three critical technical aspects that should be carefully examined including 1) bottom-up MS analysis, 2) the use of detergents, and 3) the proteolysis strategy. Particularly, the author expanded the discussion on the problematic use of CHAPSO/DMPC for the membrane protein solution. The author claimed that this solution could form positively charged ions and interfere with MS analysis. In addition, the author raised their concerns of the presence of Na^+ and K^+ in the buffer, which could encourage the production of ion-added peptides and hinder MS data interpretation.

It is an undeniable fact that the DIA MS/MS strategy has been highly successful in bottom-up proteomics analysis. However, the application of this method could be very challenging due to the limitation of the development of bioinformatics tools. Therefore, the author's statement that DIA could improve MS/MS analysis independently is open to argument. Nevertheless, the combination of DDA and DIA is preferable, which would further improve sequence coverage in bottom-up proteomics analysis.
Recently, there have been advancements in top-down proteomics analysis. GPCRs are low-mass proteins (~40 kDa) and it is feasible to obtain high resolution of MS/MS data when using the ETD fragmentation method. When coupled with bioinformatics tools, top-down proteomics analysis can be used to determine protein topology[3]. The author may consider the potential use of top-down proteomics analysis on GPCRs.

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Is the topic of the opinion article discussed accurately in the context of the current literature?
Yes

Are all factual statements correct and adequately supported by citations?
Yes

Are arguments sufficiently supported by evidence from the published literature?
Yes

Are the conclusions drawn balanced and justified on the basis of the presented arguments?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Protein mass spectrometry

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 21 February 2017

doi:10.5256/f1000research.11496.r20226

? **John J Bergeron**
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The author has provided a description of the methodological issues that were resolved by the author for a 2010 paper using hydrogen deuterium exchange and mass spectrometry to characterize the beta adrenergic GPCR. The article may be a response to another article published by another group in 2015 also on the use of hydrogen deuterium exchange and mass spectrometry to characterize the beta adrenergic GPCR. A recently published review [1] attempts to summarize how hydrogen deuterium exchange and mass spectrometry has transformed not only GPCR characterizations (the author of the submitted opinion article here, Dr. Zhang is credited for his work in the review[1]) but also the study of peripheral membrane proteins. In the Opinion article submitted by the author, Dr. Zhang, the two-step approach for the characterization of the beta-adrenergic receptor is indicated in section 1 and Fig1. Detergent considerations are indicated in sections 2,3 as well as Fig2. Proteinase digestion is considered in section 4, monovalent cation considerations in section 5, buffer concentrations in section 6 and automation in section 7. Three supplementary figures are used to compare sequence coverage using different protocols and an extensive 7 page supplementary section on detergent (solubilisation) choices.

It is difficult for this reviewer to see the conceptual advance in this submitted Opinion piece and that of the recent review by the same author (Dr. Zhang) in a 2015 review in Molecular and Cellular Proteomics (indeed some of the figures are similar for GPCR coverage and credited as such in this submitted Opinion

piece).

One innovation that may be an extension of hydrogen deuterium exchange mass spectrometry may be the application of “Native” mass spectrometry[2] to integral membrane proteins such as the beta-adrenergic receptor.
Perhaps the author could consider the important biological discoveries made through hydrogen deuterium exchange mass spectrometry for integral membrane proteins using, as an example, the beta adrenergic receptor and the hope that this can be extended though “Native” mass spectrometry, especially for resolving the dynamics of the interactions with the intracellular subunits of the hetero trimeric signaling complex.

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Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.