A robust "bottom up" proteomics pipeline is integral for assessing protein structure using hydroxyl radical protein footprinting mass spectrometry.

Rohit Jain[1,2,3], Nayeon Jeon[3], Janna Kiselar[2,3], Mark Chance[1,2,3]*

[1]Center for Synchrotron Biosciences, Case Western Reserve University, School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106, USA; [2]Center for Proteomics and Bioinformatics, Case Western Reserve University, School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106, USA; [3]Department of Nutrition, Case Western Reserve University, School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106, USA.

Abstract:

Hydroxyl Radical Protein Footprinting (HRPF) monitors macromolecular structure and dynamics by utilizing hydroxyl radicals to probe the solvent-accessible side chains of proteins. Hydroxyl radicals form irreversible covalent bonds with protein side chains based on their solvent accessibility and intrinsic reactivity. Following labeling, bottom-up proteomics which involves protease digestion and liquid chromatography (LC-MS/MS) coupled with mass spectrometry, is routinely employed to detect and quantify the modified protein side chains. The HRPF technique has been a breakthrough in the field of structural biology, enabling the assessment of structures and interrelationships between proteins, protein-drug complexes or such macromolecular mixtures. It is now being extended to complex applications such as *in-cell* and *in-vivo* studies. This perspective focuses on detailing aspects of peptide separations technology in HRPF, with a particular emphasis on chromatography. The discussion further encompasses the HRPF methodology, its current limitations, recent developments, and proposed ideas for future developments for selected research fields.

What is hydroxyl radical protein footprinting mass spectrometry (HRPF-MS)?

Structural biology spans both experimental and theoretical techniques that identify the molecular structure and dynamics of biological macromolecules to understand how these molecules function and interact. Recently, the artificial-intelligence (AI) network, AlphaFold2, developed by DeepMind, has revolutionized the field of structural biology. AlphaFold2 has predicted and released the high-accuracy models of more than 200 million proteins from some 1 million species, covering almost every known protein on the planet.[1, 2] The reliability of few AlphaFold predicted models has been validated against high-resolution experimental structures obtained from techniques such as X-ray crystallography or cryo-electron microscopy. AlphaFold2 and other AI

platforms, like RoseTTAFold, are now instrumental in solving three-dimensional structures that remained unsolved for years.[3] However, the current reliance of AI platforms on information about related protein sequences introduces inherent limitations in predicting the effects of mutations on protein structure, studying protein interactions with other interacting proteins, or molecules like ligands/drugs, and capturing protein dynamics in processes such as allostery and folding.[4, 5] In these cases, the full potential of AI prediction models can be realized through complementary structural biology approaches.

Biophysical techniques, such as Fluorescence Resonance Energy Transfer (FRET) and Small-Angle X-ray Scattering (SAXS), have provided structural biology information where high-resolution techniques like X-ray crystallography and NMR have limitations.[6-8] Structural Mass Spectrometry (MS) methods, including protein footprinting MS comprising hydrogen-deuterium exchange (HDX) and hydroxyl radical footprinting, native MS, ion-mobility MS ,and chemical cross-linking MS, yield valuable complementary structural information to validate three-dimensional protein structures and track their structure and dynamics in various experimental conditions beyond the immediate scope of AI predictions.[9, 10] Among these structural MS methods, Hydroxyl Radical Protein Footprinting (HRPF) offers several advantages for characterizing protein structure, folding, interactions and dynamics. HRPF technologies for have dramatically improved sensitivity and resolution, providing detailed sub-peptide and residue-level information to validate macromolecular structure and dynamics. HRPF encompasses different covalent labeling approaches where reactive hydroxyl radicals are generated by radiolysis of water using X-rays, gamma rays, electron beams, electric discharge, plasma source, decomposition of hydrogen peroxide using transition metal-based Fenton chemistry, and photolysis of hydrogen peroxide using lasers (FPOP) or a high pressure flash oxidation lamp.[11-14] In a HRPF experiment, protein side chains form irreversible covalent bonds with free radicals based on solvent accessibility and intrinsic reactivity of residues. HRPF offers a favorable target macromolecular size range (from small proteins in the kDa size range to MDa megacomplexes), permits high-throughput experimentation, and can be performed in physiological environment for purified samples in buffer or native-environment in cells.[15] Hydroxyl radicals have van der Waals surface areas and solvent properties similar to those of water molecules, making them effective probes of a similar surface.[16]

Labeling in HRPF experiments is independently performed at several reaction time points for pairwise comparisons between a protein and its interacting partners. The covalent modifications in labeled samples are primarily detected and quantified using classical “bottom-up” mass

spectrometry (Figure 1).[15] Labeled protein complexes are proteolyzed to the peptide level, followed by the separation of resulting peptides through ultra-high performance liquid chromatography (UHPLC). The identification of modified and unmodified peptides and residues is achieved using tandem mass spectrometry (MS/MS), where the integration of extracted ion chromatogram (EIC) peaks is used to quantify the amount of unmodified and modified species for each peptide. Each peptide's unique fragmentation pattern allows the acquired MS/MS scans to identify unmodified and modified peptides/residues within the samples. The fraction of unmodified peptide/residues is calculated for each reaction time and then plotted to generate the dose-response curves. These curves are then fitted to single-exponential functions to determine the first-order rate constants. The rate constants for peptides/residues are subsequently compared across experimental variables to monitor changes in solvent accessibility for protein side chains in different functional and structural states. HRPF data analysis is now semi-automated, and software suites are available to assist users with data analysis.[17-20]

HRPF has evolved into a "state-of-the-art" biophysical technique, enhancing our understanding of various macromolecular system. It has proven particularly useful for systems challenging to analyze using other techniques. Recent examples include studies on how G-protein coupled receptors (GPCRs) select and bind their target G proteins, the photoactivation of orange carotenoid proteins, epitope mapping of anti-drug antibodies, confirmation of Fab-Fc receptor interactions, and detailing structural differences between major human prion strains.[21-24] Structural details of intermediates during protein misfolding are crucial for understanding neurodegenerative diseases, and HRPF has analyzed amyloid fibrils and prefibrillar intermediates with residue-specific resolution for the amyloid-β peptide in Alzheimer's disease.[25, 26] HRPF has been beneficial for integrative structural modeling of multidomain proteins like Polo-like kinase 1, the human estrogen receptor and mapping the protein-protein interaction surface of the Hemoglobin-Haptoglobin complex.[27-29] HRPF serves as an impetus in drug discovery, identifying inhibitor binding sites for a type 2 diabetes target protein and allosteric conformational changes during the binding of a small drug molecule with the prostate cancer target protein phosphatase PP2A.[30, 31] In a recent example, HRPF calculated protein-ligand binding sites, binding stoichiometry, and site-specific binding constants on the holo-calmodulin:melittin model system.[32] In these HRPF applications, high sequence coverage of proteins at the peptide and residue levels has been critical. The required high spatial resolution in macromolecular systems has been made possible by combined developments in instrumentation, fragmentation methods, and sample handling strategies in "bottom-up" proteomics. The following section will showcase the challenges for "bottom-up" proteomics in obtaining a high-quality dataset for an HRPF experiment.

Challenges for “bottom-up” proteomics in HRPF-MS.

“Bottom-up” proteomics is an established method in proteomics used to identify proteins and characterize their amino acid sequences and post-translational modifications. This involves proteolytic digestion prior to analysis by liquid chromatography coupled with tandem mass spectrometry.[33] Hydroxyl labeling of protein side chains in HRPF leads to mass shifts at modified residues. “Bottom-up” proteomic workflows are routinely applied to identify and quantitate these peptides and residues across the protein sequence, providing details of the local structure.[34] HRPF experiments involve pairwise comparison of biomolecular states, and the labeled samples at different exposure times are analyzed using identical “bottom-up” proteomics workflows to reduce experimental variation. Oxidative modifications are covalent and therefore the downstream HRPF analysis is more flexible than HDX-MS (hydrogen deuterium exchange mass spectrometry).[15, 35] Back exchange in HDX-MS reduces signal and creates increased error and hence its controlled with low pH and cold temperature. Following labeling in HRPF, proteolytic digestion conditions are optimized for maximal coverage of the protein sequence. Strategies include desalting to remove salts for better MS ionization, using denaturants and reducing agents to improve proteolytic digestion, and employing a combination of proteases for multiple cleavage sites to provide overlapping fragments. Irreversible oxidative modifications in HRPF experiments allow the routine use of specific residue cleavage proteases like Trypsin (Arg, Lys), LysC (Lys), and rAspN (Asp, Glu), eliminating the need for acidic pH. Specific cleavage of proteins results in peptides with 5-20 residues range and eases their analysis with mass spectrometry. However, this strategy may have limitations as the region of interest could be missed based on the availability and location of cleavage sites in proteins. In these cases, acidic proteases like Pepsin could be useful, however, data analysis is laborious due to nonspecific Pepsin cleavage. Another alternative to map missing fragments is ProAlanase, which is a site specific and cleaves at proline and alanine residues. Downstream HRPF steps, such as sample cleanup using precipitation with organic solvents (acetone or methanol/chloroform) and buffer exchange methods followed by proteolytic digestion, are currently tedious.[36] The combination and automation of these procedures in HRPF would significantly increase processing speed, akin to past developments in HDX-MS.

Proteolytic digestion yields peptide mixtures containing both unmodified and modified peptides. Oxidative labeling on modified peptides can alter their hydrophobicity, and reverse-phase liquid chromatography is used to separate the peptide mixtures, followed by tandem mass spectrometry to identify and analyze these modifications. Modified species with oxygen adducts (+14, +16 Da)

usually elute prior to but close (~1-3 min.) to unmodified peptides because of higher polarity conferred by oxygen addition. While, modified species with Arginine (-43 Da) or Histidine (-10 Da, -22 Da, -23 Da) oxidation elute at various positions relative to unmodified peptides due to different labeling chemistries. Peptide modification extents and dose-response rates in HRPF are calculated using exact mass-based extracted ion currents (EIC) from LC-MS.

The separation of peptide mixtures is, therefore, a critical step in HRPF and requires special considerations in comparison to the general proteomics workflow. Firstly, the intensity and fraction of hydroxyl labeled peptides in HRPF, for most peptides, are much less (10-200 times) than unmodified peptides, as hydroxyl radical generation is controlled to prevent radiation damage (Figure 2a). Secondly, the hydroxyl radicals can modify 18 out of 20 amino acids, providing multiple probes, albeit with a large dynamic range in reactivity due to differences in the intrinsic reactivity of amino acid residues.[16] This large hydroxyl reactivity range may result in modifications of several residues in a peptide segment, generating multiple isoforms, complicating their separation and correct identification in contrast to specific covalent labeling approaches.[17] For specific labeling chemistries, like Glycl-ethel-ester, which can modify only solvent-accessible carboxyl groups on glutamic and aspartic acid residues, modified peptides (+57 Da and +85 Da mass shifts) generally elute later than unmodified peptides (Figure 2b). Last but not least, the multiplex labeling of protein side chains with trifluoromethyl chemistry (CF_3, +68 Da mass shift) in HRPF, generates additional peptide isoforms with different reactivity and chromatography profile compared to hydroxyl radicals (Figure 2c).[37] The large dynamic range for detecting modestly modified products in HRPF therefore necessitates a much higher signal-to-noise ratio during chromatography separation, achievable with thin and long nano-LC columns. Peptides elute based on their hydrophobicity, and the water:acetonitrile gradient on reverse-phase columns is optimized based on the size of protein and hydrophobicity of eluting peptides. The error in quantitation of modified peptides due to carryover can be reduced between different samples by including washing run with higher water:acetonitrile gradient. In addition, a quality control run with standard peptides to monitor the column separation could be added between samples.

The MS2 based examination of hydroxyl radical-modified residues is essential to provide residue-level resolution, and the scrambling of covalent labels in the gas phase is not a limitation for HRPF experiments. Collision-induced dissociation (CID) is routinely used to induce peptide fragmentation in the gas phase for HRPF, providing sensitive and specific detection of modified residues. The extent of modification for modified protein side chains varies reflecting intrinsic reactivity and solvent accessibility factors for each of them. The HRPF dataset is extraordinary

rich, as almost all side chains across the peptides can be reliably detected. While data-dependent acquisition (DDA) has been used for HRPF, this approach is limited in detecting low-abundance products, especially for residues with low hydroxyl reactivity.[38] This limitation can be overcome by using SRM-based fragmentation method, where knowledge about *m/z* values of low-abundance oxidation products, and their elution times from DDA analysis can be used for generating a "target inclusion list".[38] The individual quantification of all the modified species can also be performed by generating a "dynamic inclusion" target list, where the target *m/z* values change as a function of chromatography and expected retention time.[17] Alternative approaches for the localization of modified sites in HRPF include gas fragmentation with electron transfer dissociation (ETD). ETD method has been implemented for more accurate identification of oxidative modifications of cysteine, methionine, and histidine residues.[39, 40] ETD method is however, limited to larger peptides that can easily access higher charge states. Recently, the poor ETD fragmentation efficiency for doubly charged peptides has been overcome with supercharging agents like *m-nitrobenzyl alcohol*.[41] The ETD MS/MS quantification for HRPF experiments has been further improved by coupling size exclusion chromatography with ETD fragmentation, allowing coelution of isomeric modified peptides.[42]

Recent developments and future perspectives for selected HRPF research fields

HRPF experiments involve the modification of protein side chains by hydroxyl radicals, and knowing the administered hydroxyl radical dose is essential for several reasons. Firstly, the hydroxyl radical dose needs to be tailored for conducting HRPF experiment based on the "one-hit kinetics" principle, where protein molecules undergo only one modification event.[43] Secondly, experimental parameters like ligands, buffers, or excipients can influence the availability of hydroxyl radicals for protein modification; therefore, a quantifiable dosimeter is essential for tuning the hydroxyl radical dose. Last but not least, over-oxidation of proteins due to a higher hydroxyl radical dose can cause unfolding of proteins, further accentuating the need for a quantifiable dosimeter. As a result, different dosimetry strategies have been developed to tune the hydroxyl radical dose for various HRPF platforms. Examples include a fluorescent dye, Alexa Fluor 488, for X-ray synchrotron HRPF experiment, a nucleobase Adenine for HRPF experiments based on the photolysis of hydrogen peroxide (FPOP and Flash oxidation), and the use of a reporter peptide (leucine enkaphalin) as an internal dosimeter for FPOP data.[44-47] In HRPF experiments via FPOP platforms, minute changes in hydrogen peroxide concentration, laser fluence, and buffer composition can alter the effective hydroxyl radical concentration, necessitating real-time dosimetry.[48] A platform was designed for the real-time normalization of FPOP oxidation using

Adenine as a dosimeter, and this compensated HRPF method was used to study the effects of buffer and excipients in different Adalimumab biosimilar formulations.[48, 49] HRPF experiments are being conducted at high-flux X-ray synchrotrons, and the higher brightness of these synchrotrons could induce radiation damage during exposures. A high-throughput endstation device using a 96-well PCR plate form factor, supporting diagnostic instrumentation and a fluorescent Alexa Fluor 488 dosimeter for synchrotron HRPF experiments can carefully control the X-ray dose to the samples, while avoiding X-ray doses that can cause over-oxidation or radiation damage to the sample.[45] This high-throughput HRPF pipeline facilitated a comprehensive screening of the influence of sample chemistry on hydroxyl radical dose, with a study involving 26 organic compounds.[45] New dosimetry strategies for HRPF experiments could further prevent radiation damage from disrupting exposures quality. A microfluidic dosimetry cell has been developed for low penetrating soft X-ray Irradiation using sodium benzoate as a fluorescent dosimeter, representing a step towards online dosimetry for HRPF at synchrotron beamlines.[50] Liquid jets for sample delivery in HRPF experiments at an unfocused X-ray source demonstrated a tenfold increase in hydroxyl labeling compared to capillary flow.[51] Automated inline quantitative fluorescence dosage characterization and sample exposure have been demonstrated using liquid on a high flux density microfocused synchrotron beamline. This development may enable X-ray exposure time in the single-digit microseconds while retaining a high level of side-chain labeling and increasing ultrafast time-resolved in-situ HRPF experiments.[52]Recently, an intact mass spectrometry screening of hydroxyl labeled protein samples immediately following X-ray exposure, along with metrics to quantify the extent of observed labeling from the intact mass spectra, has been described. This direct approach to evaluating the extent of labeling can provide guidance on “safe” dose ranges, offering immediate feedback on experimental outcomes before embarking on detailed LC-MS analyses.[53]

Hydroxyl modifications on protein side chains are chemically complex, involving multiple mass changes and exhibiting at least a 1000-fold range of reactivity toward the 20 amino acid side chains.[16] Due to this wide dynamic range of reactivity, not all protein side chains are easily detected in a single experiment, resulting in low structural resolution. As a solution, chemical reagents and workflows capable of producing multiple labels in a “one-pot reaction” could advance HRPF toward a high-resolution structural biology technique.[37] Trifluoromethyl (TFM, CF_3) labeling reagents are being explored to provide an even labeling in HRPF experiments, offering an overall dynamic range of reactivity with protein side chains and enabling detection of “low reactivity” residues.[37, 54-56] TFM labeling of proteins like lactoglobulin, myoglobin, and vitamin K epoxide reductase was demonstrated on different HRPF platforms using Langlois’ reagent

(sodium triflinate) as a TFM reagent.[54, 56] In one example comparing apo/holo-myoglobins' footprints, TFM labeling was shown to be sensitive to changes in protein conformation and solvent accessibility, fulfilling the essential requirement for a suitable footprinting methodology.[54] A rigorous analysis of TFM labeling of free amino acids in biocompatible conditions has been performed using Langlois' reagent and X-ray synchrotron-mediated water radiolysis.[37] In this study, all 20 amino acids exhibited modifications with either CF_3 or hydroxyl radicals, emphasizing the utility of TFM chemistry in providing broader coverage for HRPF experiments, and the potential of multiplex labeling in a "one-pot reaction".[37] Further experimentation and improvements can fine-tune the reactivity between CF_3 and hydroxyl labeling channels, achievable by varying the concentration of TFM reagent and the hydroxyl radical dose.

HRPF is not limited to in-vitro based systems, and studies have been conducted on complex in-cell and in-vivo systems that better mimic the native cellular environment. For example, SILAC-SPROX experiments involving the denaturation of cellular lysates were utilized to differentiate three breast cancer cell lines and distinguish between subtypes of breast cancer.[57, 58] HRPF, employing a nanosecond laser photolysis method, was employed to investigate the EGF/EGFR interaction on the plasma membrane of intact mammalian cells.[59] An in-cell FPOP (IC-FPOP) flow system, designed to avoid cellular clumping, has been developed to conduct HRPF experiments in intact cells.[60] IC-FPOP served as a novel tool for proteome-wide structural biology, modifying over 1300 proteins across 27 different cellular compartments in Vero cells.[61] Subsequently, IC-FPOP was utilized to oxidatively modify proteins in five different cell lines, indicating that different cell lines may exhibit varying susceptibility to oxidative modification.[62]

The aforementioned examples showcase instances where HRPF has experienced exponential advancement since its initial usage for proteins, contributing to increased quantitative power and reproducibility of the method. Other significant developments in the HRPF field encompass integrative structural modeling, coupling HRPF with other structural methods, computational modeling of protein structure utilizing HRPF data as structural constraints, newer HRPF strategies for higher labeling of membrane proteins, and the availability of high-flux X-ray synchrotron beamlines with stopped-flow kinetics, providing a temporal window into high-resolution structures, as exemplified by the association of GPCR-G protein.[63-67] With these exponential advancements, HRPF has evolved into a powerful tool for studying the higher-order structure of proteins, proving useful for investigating a wide variety of proteins with a large favorable size range in their physiological environment. HRPF, with its current and anticipated capabilities outlined here, holds

strong promise for the next structural revolution, guided by artificial intelligence-based prediction models.

AUTHOR INFORMATION

Corresponding Authors

*Mark R. Chance - Center for Synchrotron Biosciences; Center for Proteomics and Bioinformatics; Department of Nutrition, Case Western Reserve University, School of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106, USA.

*Email: mrc16@case.edu

Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. M.R.C. is a Founder and Chief Scientific Officer of NeoProteomics, which provides access to footprinting technologies and services. J.K. is a consultant for NeoProteomics. M.R.C. owns shares and is a member of the scientific advisory board of GenNext® Technologies, Inc., makers of the benchtop flash oxidation system.

ACKNOWLEDGMENTS

Funding for development of the XFP beamline was provided by a Major Research Instrumentation award from the NSF (DBI-1228549) and Case Western Reserve University. This research used resources of the NSLS-II, a U.S. DOE Office of Science User Facility operated for the DOE Office of Science by Brookhaven National Laboratory under Contract No. DE-SC0012704. The DOE has established a portal for accessing X-ray footprinting beamlines at: https://berstructuralbioportal.org/x-ray-footprinting/

Figures:

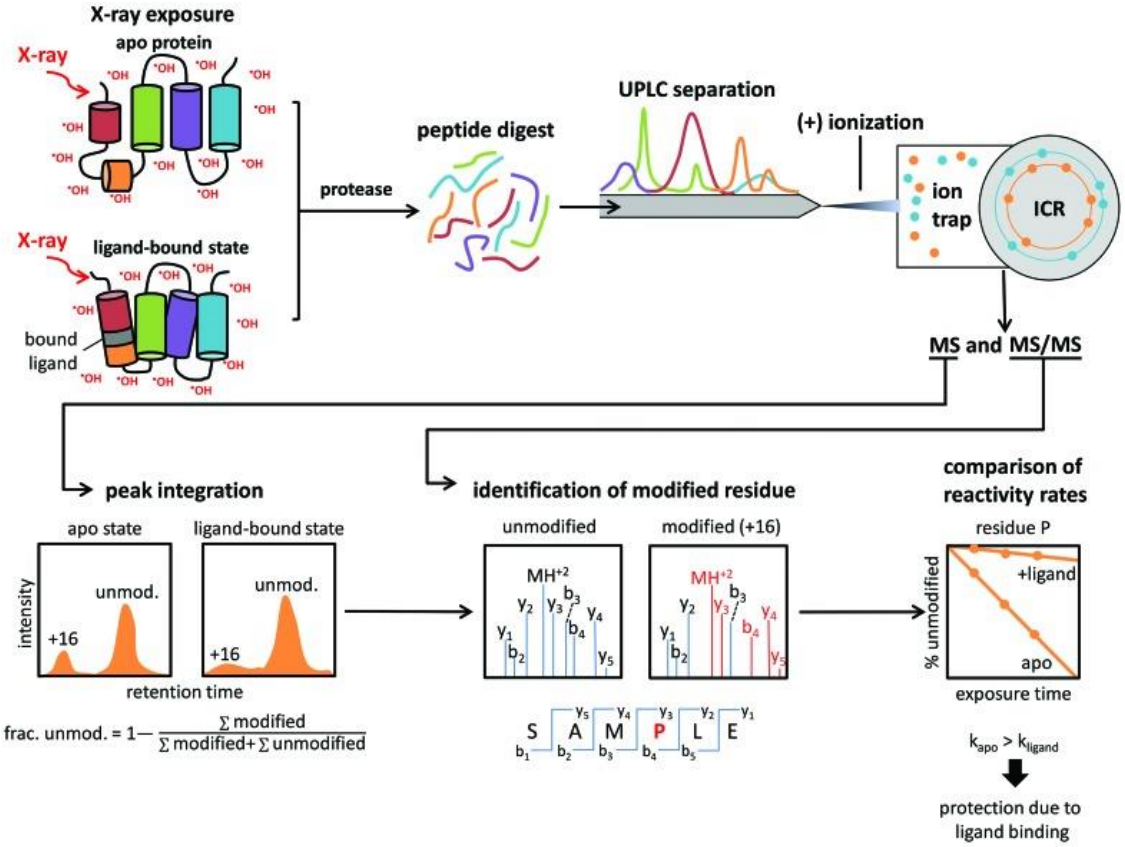

Figure 1: Schematic of HRPF experiments. The figure was taken with permission from Asuru *et al.* (2019). *J. Synchrotron Rad.* 26, 1388-1399.[15]

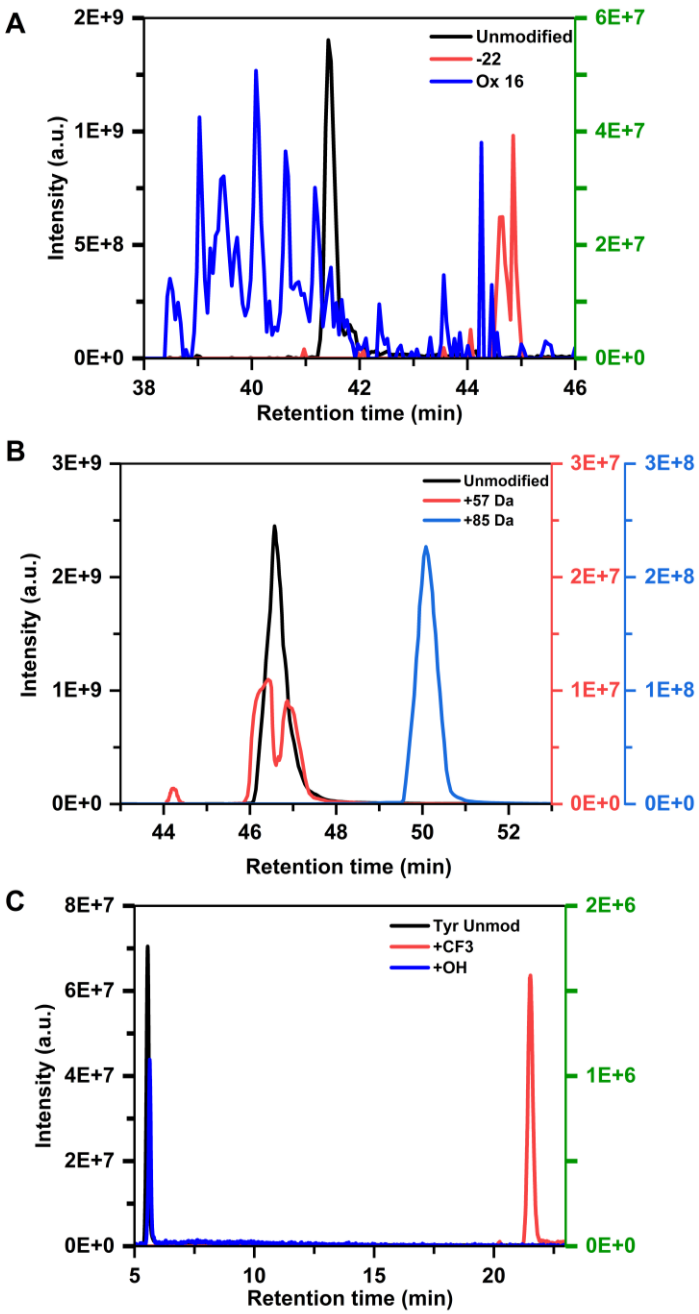

Figure 2: Extracted ion current (EIC) has been plotted against retention time for different protein footprinting techniques. (A) Hydroxyl labeling of a peptide segment from Lactate oxidase enzyme. (B) Multiplex labeling of tyrosine amino acid with trifluoromethyl chemistry. The figure was taken with permission from Jain *et al.*, (2022). *Anal. Chem.* 94, 27, 9819-9825.[37] (C) GEE labeling of a peptide segment from biotinylated Neuropeptide Y.

References

[1] Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A. W., Kavukcuoglu, K., Kohli, P., and Hassabis, D. (2021) Highly accurate protein structure prediction with AlphaFold, *Nature 596*, 583-589.

[2] Varadi, M., Anyango, S., Deshpande, M., Nair, S., Natassia, C., Yordanova, G., Yuan, D., Stroe, O., Wood, G., Laydon, A., Žídek, A., Green, T., Tunyasuvunakool, K., Petersen, S., Jumper, J., Clancy, E., Green, R., Vora, A., Lutfi, M., Figurnov, M., Cowie, A., Hobbs, N., Kohli, P., Kleywegt, G., Birney, E., Hassabis, D., and Velankar, S. (2021) AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models, *Nucleic Acids Research 50*, D439-D444.

[3] Baek, M., DiMaio, F., Anishchenko, I., Dauparas, J., Ovchinnikov, S., Lee, G. R., Wang, J., Cong, Q., Kinch, L. N., Schaeffer, R. D., Millán, C., Park, H., Adams, C., Glassman, C. R., DeGiovanni, A., Pereira, J. H., Rodrigues, A. V., van Dijk, A. A., Ebrecht, A. C., Opperman, D. J., Sagmeister, T., Buhlheller, C., Pavkov-Keller, T., Rathinaswamy, M. K., Dalwadi, U., Yip, C. K., Burke, J. E., Garcia, K. C., Grishin, N. V., Adams, P. D., Read, R. J., and Baker, D. (2021) Accurate prediction of protein structures and interactions using a three-track neural network, *Science (New York, N.Y.) 373*, 871-876.

[4] David, A., Islam, S., Tankhilevich, E., and Sternberg, M. J. E. (2022) The AlphaFold Database of Protein Structures: A Biologist's Guide, *Journal of molecular biology 434*, 167336.

[5] Nussinov, R., Zhang, M., Liu, Y., and Jang, H. (2022) AlphaFold, Artificial Intelligence (AI), and Allostery, *The Journal of Physical Chemistry B 126*, 6372-6383.

[6] Basak, S., and Chattopadhyay, K. (2014) Studies of protein folding and dynamics using single molecule fluorescence spectroscopy, *Physical chemistry chemical physics : PCCP 16*, 11139-11149.

[7] Jain, R., Khan, N., Menzel, A., Rajkovic, I., Konrad, M., and Techert, S. (2016) Insights into open/closed conformations of the catalytically active human guanylate kinase as investigated by small-angle X-ray scattering, *European Biophysics Journal 45*, 81-89.

[8] Jain, R., and Techert, S. (2016) Time-resolved and in-situ X-ray scattering methods beyond photoactivation: Utilizing high-flux X-ray sources for the study of ubiquitous non-photoactive proteins, *Protein and peptide letters 23*, 242-254.

[9] Sinz, A. (2018) Cross-Linking/Mass Spectrometry for Studying Protein Structures and Protein–Protein Interactions: Where Are We Now and Where Should We Go from Here?, *Angewandte Chemie International Edition 57*, 6390-6396.

[10] Wang, J.-H., Tang, Y.-L., Gong, Z., Jain, R., Xiao, F., Zhou, Y., Tan, D., Li, Q., Huang, N., Liu, S.-Q., Ye, K., Tang, C., Dong, M.-Q., and Lei, X. (2022) Characterization of protein unfolding by fast cross-linking mass spectrometry using di-ortho-phthalaldehyde cross-linkers, *Nature Communications 13*, 1468.

[11] Liu, X. R., Zhang, M. M., and Gross, M. L. (2020) Mass Spectrometry-Based Protein Footprinting for Higher-Order Structure Analysis: Fundamentals and Applications, *Chemical reviews 120*, 4355-4454.

[12] Minkoff, B. B., Blatz, J. M., Choudhury, F. A., Benjamin, D., Shohet, J. L., and Sussman, M. R. (2017) Plasma-Generated OH Radical Production for Analyzing Three-Dimensional Structure in Protein Therapeutics, *Scientific reports 7*, 12946.

[13] Sharp, J. S., Chea, E. E., Misra, S. K., Orlando, R., Popov, M., Egan, R. W., Holman, D., and Weinberger, S. R. (2021) Flash Oxidation (FOX) System: A Novel Laser-Free Fast Photochemical Oxidation Protein Footprinting Platform, *Journal of the American Society for Mass Spectrometry 32*, 1601-1609.
[14] Liu, X. R., Zhang, M. M., and Gross, M. L. (2020) Mass Spectrometry-Based Protein Footprinting for Higher-Order Structure Analysis: Fundamentals and Applications, *Chemical reviews 120*, 4355-4454.
[15] Asuru, A., Farquhar, E. R., Sullivan, M., Abel, D., Toomey, J., Chance, M. R., and Bohon, J. (2019) The XFP (17-BM) beamline for X-ray footprinting at NSLS-II, *Journal of synchrotron radiation 26*, 1388-1399.
[16] Xu, G., and Chance, M. R. (2007) Hydroxyl radical-mediated modification of proteins as probes for structural proteomics, *Chemical reviews 107*, 3514-3543.
[17] Kaur, P., Kiselar, J., Yang, S., and Chance, M. R. (2015) Quantitative protein topography analysis and high-resolution structure prediction using hydroxyl radical labeling and tandem-ion mass spectrometry (MS), *Molecular & cellular proteomics : MCP 14*, 1159-1168.
[18] Liu, X. R., Rempel, D. L., and Gross, M. L. (2020) Protein higher-order-structure determination by fast photochemical oxidation of proteins and mass spectrometry analysis, *Nature Protocols 15*, 3942-3970.
[19] Xu, H., and Freitas, M. A. (2009) MassMatrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data, *Proteomics 9*, 1548-1555.
[20] Chea, E. E., Prakash, A., and Jones, L. M. (2021) The utilization of the search engine, Bolt, to decrease search time and increase peptide identifications in hydroxyl radical protein footprinting-based workflows, *Proteomics 21*, e2000295.
[21] Ahn, D., Ham, D., and Chung, K. Y. (2021) The conformational transition during G protein-coupled receptor (GPCR) and G protein interaction, *Current opinion in structural biology 69*, 117-123.
[22] Schick, A. J., 3rd, Lundin, V., Low, J., Peng, K., Vandlen, R., and Wecksler, A. T. (2022) Epitope mapping of anti-drug antibodies to a clinical candidate bispecific antibody, *mAbs 14*, 2028337.
[23] Sun, Y., Izadi, S., Callahan, M., Deperalta, G., and Wecksler, A. T. (2021) Antibody-receptor interactions mediate antibody-dependent cellular cytotoxicity, *The Journal of biological chemistry 297*, 100826.
[24] Siddiqi, M. K., Kim, C., Haldiman, T., Kacirova, M., Wang, B., Bohon, J., Chance, M. R., Kiselar, J., and Safar, J. G. (2021) Structurally distinct external solvent-exposed domains drive replication of major human prions, *PLoS pathogens 17*, e1009642.
[25] Jain, R., Petri, M., Kirschbaum, S., Feindt, H., Steltenkamp, S., Sonnenkalb, S., Becker, S., Griesinger, C., Menzel, A., Burg, T. P., and Techert, S. (2013) X-ray scattering experiments with high-flux X-ray source coupled rapid mixing microchannel device and their potential for high-flux neutron scattering investigations, *The European physical journal. E, Soft matter 36*, 109.
[26] Klinger, A. L., Kiselar, J., Ilchenko, S., Komatsu, H., Chance, M. R., and Axelsen, P. H. (2014) A synchrotron-based hydroxyl radical footprinting analysis of amyloid fibrils and prefibrillar intermediates with residue-specific resolution, *Biochemistry 53*, 7724-7734.
[27] Loginov, D. S., Fiala, J., Brechlin, P., Kruppa, G., and Novak, P. (2022) Hydroxyl radical footprinting analysis of a human haptoglobin-hemoglobin complex, *Biochimica et biophysica acta. Proteins and proteomics 1870*, 140735.
[28] Ruan, H., Kiselar, J., Zhang, W., Li, S., Xiong, R., Liu, Y., Yang, S., and Lai, L. (2020) Integrative structural modeling of a multidomain polo-like kinase, *Physical chemistry chemical physics : PCCP 22*, 27581-27589.

[29] Huang, W., Peng, Y., Kiselar, J., Zhao, X., Albaqami, A., Mendez, D., Chen, Y., Chakravarthy, S., Gupta, S., Ralston, C., Kao, H. Y., Chance, M. R., and Yang, S. (2018) Multidomain architecture of estrogen receptor reveals interfacial cross-talk between its DNA-binding and ligand-binding domains, *Nat Commun 9*, 3520.
[30] McClinch, K., Avelar, R. A., Callejas, D., Izadmehr, S., Wiredja, D., Perl, A., Sangodkar, J., Kastrinsky, D. B., Schlatzer, D., Cooper, M., Kiselar, J., Stachnik, A., Yao, S., Hoon, D., McQuaid, D., Zaware, N., Gong, Y., Brautigan, D. L., Plymate, S. R., Sprenger, C. C. T., Oh, W. K., Levine, A. C., Kirschenbaum, A., Sfakianos, J. P., Sears, R., DiFeo, A., Ioannou, Y., Ohlmeyer, M., Narla, G., and Galsky, M. D. (2018) Small-Molecule Activators of Protein Phosphatase 2A for the Treatment of Castration-Resistant Prostate Cancer, *Cancer research 78*, 2065-2080.
[31] Stanford, S. M., Aleshin, A. E., Zhang, V., Ardecky, R. J., Hedrick, M. P., Zou, J., Ganji, S. R., Bliss, M. R., Yamamoto, F., Bobkov, A. A., Kiselar, J., Liu, Y., Cadwell, G. W., Khare, S., Yu, J., Barquilla, A., Chung, T. D. Y., Mustelin, T., Schenk, S., Bankston, L. A., Liddington, R. C., Pinkerton, A. B., and Bottini, N. (2017) Diabetes reversal by inhibition of the low-molecular-weight tyrosine phosphatase, *Nature chemical biology 13*, 624-632.
[32] Liu, X. R., Zhang, M. M., Rempel, D. L., and Gross, M. L. (2019) Protein-Ligand Interaction by Ligand Titration, Fast Photochemical Oxidation of Proteins and Mass Spectrometry: LITPOMS, *Journal of the American Society for Mass Spectrometry 30*, 213-217.
[33] Dupree, E. J., Jayathirtha, M., Yorkey, H., Mihasan, M., Petre, B. A., and Darie, C. C. (2020) A Critical Review of Bottom-Up Proteomics: The Good, the Bad, and the Future of this Field, *Proteomes 8*, 14.
[34] Wang, L., and Chance, M. R. (2017) Protein Footprinting Comes of Age: Mass Spectrometry for Biophysical Structure Assessment*, *Molecular & Cellular Proteomics 16*, 706-716.
[35] Jain, R., Muneeruddin, K., Anderson, J., Harms, M. J., Shaffer, S. A., and Matthews, C. R. (2021) A conserved folding nucleus sculpts the free energy landscape of bacterial and archaeal orthologs from a divergent TIM barrel family, *Proceedings of the National Academy of Sciences of the United States of America 118*.
[36] Rogers, J. C., and Bomgarden, R. D. (2016) Sample Preparation for Mass Spectrometry-Based Proteomics; from Proteomes to Peptides, *Advances in experimental medicine and biology 919*, 43-62.
[37] Jain, R., Dhillon, N. S., Farquhar, E. R., Wang, B., Li, X., Kiselar, J., and Chance, M. R. (2022) Multiplex Chemical Labeling of Amino Acids for Protein Footprinting Structure Assessment, *Analytical chemistry 94*, 9819-9825.
[38] Kiselar, J. G., and Chance, M. R. (2010) Future directions of structural mass spectrometry using hydroxyl radical footprinting, *Journal of mass spectrometry : JMS 45*, 1373-1382.
[39] Srikanth, R., Wilson, J., and Vachet, R. W. (2009) Correct identification of oxidized histidine residues using electron-transfer dissociation, *Journal of Mass Spectrometry 44*, 755-762.
[40] Li, X., Li, Z., Xie, B., and Sharp, J. S. (2013) Improved Identification and Relative Quantification of Sites of Peptide and Protein Oxidation for Hydroxyl Radical Footprinting, *Journal of the American Society for Mass Spectrometry 24*, 1767-1776.
[41] Li, X., Li, Z., Xie, B., and Sharp, J. S. (2015) Supercharging by m-NBA Improves ETD-Based Quantification of Hydroxyl Radical Protein Footprinting, *Journal of the American Society for Mass Spectrometry 26*, 1424-1427.
[42] Xie, B., and Sharp, J. S. (2016) Relative Quantification of Sites of Peptide and Protein Modification Using Size Exclusion Chromatography Coupled with Electron Transfer Dissociation, *Journal of the American Society for Mass Spectrometry 27*, 1322-1327.
[43] Galas, D. J., and Schmitz, A. (1978) DNAase footprinting a simple method for the detection of protein-DNA binding specificity, *Nucleic Acids Research 5*, 3157-3170.

[44] Gupta, S., Sullivan, M., Toomey, J., Kiselar, J., and Chance, M. R. (2007) The Beamline X28C of the Center for Synchrotron Biosciences: a national resource for biomolecular structure and dynamics experiments using synchrotron footprinting, *Journal of synchrotron radiation 14*, 233-243.
[45] Jain, R., Abel, D., Rakitin, M., Sullivan, M., Lodowski, D. T., Chance, M. R., and Farquhar, E. R. (2021) New high-throughput endstation to accelerate the experimental optimization pipeline for synchrotron X-ray footprinting, *Journal of synchrotron radiation 28*, 1321-1332.
[46] Niu, B., Mackness, B. C., Rempel, D. L., Zhang, H., Cui, W., Matthews, C. R., Zitzewitz, J. A., and Gross, M. L. (2017) Incorporation of a Reporter Peptide in FPOP Compensates for Adventitious Scavengers and Permits Time-Dependent Measurements, *Journal of the American Society for Mass Spectrometry 28*, 389-392.
[47] Xie, B., and Sharp, J. S. (2015) Hydroxyl Radical Dosimetry for High Flux Hydroxyl Radical Protein Footprinting Applications Using a Simple Optical Detection Method, *Analytical chemistry 87*, 10719-10723.
[48] Sharp, J. S., Misra, S. K., Persoff, J. J., Egan, R. W., and Weinberger, S. R. (2018) Real Time Normalization of Fast Photochemical Oxidation of Proteins Experiments by Inline Adenine Radical Dosimetry, *Analytical chemistry 90*, 12625-12630.
[49] Misra, S. K., Orlando, R., Weinberger, S. R., and Sharp, J. S. (2019) Compensated Hydroxyl Radical Protein Footprinting Measures Buffer and Excipient Effects on Conformation and Aggregation in an Adalimumab Biosimilar, *AAPS J 21*, 87.
[50] Huart, L., Nicolas, C., Hervé du Penhoat, M. A., Guigner, J. M., Gosse, C., Palaudoux, J., Lefrançois, S., Mercere, P., Dasilva, P., Renault, J. P., and Chevallard, C. (2021) A microfluidic dosimetry cell to irradiate solutions with poorly penetrating radiations: a step towards online dosimetry for synchrotron beamlines, *Journal of synchrotron radiation 28*, 778-789.
[51] Gupta, S., Chen, Y., Petzold, C. J., DePonte, D. P., and Ralston, C. Y. (2020) Development of Container Free Sample Exposure for Synchrotron X-ray Footprinting, *Analytical chemistry 92*, 1565-1573.
[52] Rosi, M., Russell, B., Kristensen, L. G., Farquhar, E. R., Jain, R., Abel, D., Sullivan, M., Costello, S. M., Dominguez-Martin, M. A., Chen, Y., Marqusee, S., Petzold, C. J., Kerfeld, C. A., DePonte, D. P., Farahmand, F., Gupta, S., and Ralston, C. Y. (2022) An automated liquid jet for fluorescence dosimetry and microsecond radiolytic labeling of proteins, *Communications biology 5*, 866.
[53] Farquhar, E. R., Vijayalakshmi, K., Jain, R., Wang, B., Kiselar, J., and Chance, M. R. (2023) Intact mass spectrometry screening to optimize hydroxyl radical dose for protein footprinting, *Biochemical and Biophysical Research Communications 671*, 343-349.
[54] Cheng, M., Asuru, A., Kiselar, J., Mathai, G., Chance, M. R., and Gross, M. L. (2020) Fast Protein Footprinting by X-ray Mediated Radical Trifluoromethylation, *Journal of the American Society for Mass Spectrometry 31*, 1019-1024.
[55] Cheng, M., Guo, C., and Gross, M. L. (2020) The Application of Fluorine-Containing Reagents in Structural Proteomics, *Angewandte Chemie (International ed. in English) 59*, 5880-5889.
[56] Cheng, M., Zhang, B., Cui, W., and Gross, M. L. (2017) Laser-Initiated Radical Trifluoromethylation of Peptides and Proteins: Application to Mass-Spectrometry-Based Protein Footprinting, *Angewandte Chemie International Edition 56*, 14007-14010.
[57] Adhikari, J., West, G. M., and Fitzgerald, M. C. (2015) Global Analysis of Protein Folding Thermodynamics for Disease State Characterization, *Journal of Proteome Research 14*, 2287-2297.

[58] Liu, F., Meng, H., and Fitzgerald, M. C. (2017) Large-Scale Analysis of Breast Cancer-Related Conformational Changes in Proteins Using SILAC-SPROX, *Journal of Proteome Research 16*, 3277-3286.
[59] Zhu, Y., Serra, A., Guo, T., Park, J. E., Zhong, Q., and Sze, S. K. (2017) Application of Nanosecond Laser Photolysis Protein Footprinting to Study EGFR Activation by EGF in Cells, *Journal of Proteome Research 16*, 2282-2293.
[60] Espino, J. A., Mali, V. S., and Jones, L. M. (2015) In Cell Footprinting Coupled with Mass Spectrometry for the Structural Analysis of Proteins in Live Cells, *Analytical chemistry 87*, 7971-7978.
[61] Rinas, A., Mali, V. S., Espino, J. A., and Jones, L. M. (2016) Development of a Microflow System for In-Cell Footprinting Coupled with Mass Spectrometry, *Analytical chemistry 88*, 10052-10058.
[62] Kaur, U., Johnson, D. T., and Jones, L. M. (2020) Validation of the Applicability of In-Cell Fast Photochemical Oxidation of Proteins across Multiple Eukaryotic Cell Lines, *Journal of the American Society for Mass Spectrometry 31*, 1372-1379.
[63] Sun, J., Li, W., and Gross, M. L. (2022) Advances in mass spectrometry-based footprinting of membrane proteins, *Proteomics 22*, 2100222.
[64] McKenzie-Coe, A., Montes, N. S., and Jones, L. M. (2022) Hydroxyl Radical Protein Footprinting: A Mass Spectrometry-Based Structural Method for Studying the Higher Order Structure of Proteins, *Chemical reviews 122*, 7532-7561.
[65] Chance, M. R., Farquhar, E. R., Yang, S., Lodowski, D. T., and Kiselar, J. (2020) Protein Footprinting: Auxiliary Engine to Power the Structural Biology Revolution, *Journal of molecular biology 432*, 2973-2984.
[66] Du, Y., Duc, N. M., Rasmussen, S. G. F., Hilger, D., Kubiak, X., Wang, L., Bohon, J., Kim, H. R., Wegrecki, M., Asuru, A., Jeong, K. M., Lee, J., Chance, M. R., Lodowski, D. T., Kobilka, B. K., and Chung, K. Y. (2019) Assembly of a GPCR-G Protein Complex, *Cell 177*, 1232-1242.e1211.
[67] Biehn, S. E., and Lindert, S. (2021) Accurate protein structure prediction with hydroxyl radical protein footprinting data, *Nat Commun 12*, 341.