Table 6: Structural MS - New Developments in Limited Digestion, HDX, FPOP and Ion Mobility

Facilitator: Chris Chumsae, *Bristol-Myers Squibb Company, Devens, MA USA*
Scribe: Alyssa Stiving, *Merck & Co., Inc., West Point, PA USA*

Scope:
In recent years, mass spectrometry has become a powerful tool for various detailed structural studies on proteins and protein therapeutics. Pioneering work in limited proteolysis allowed scientists to understand relaxed regions of protein structure through their susceptibility towards proteases. More recently, labelling techniques such of hydrogen-deuterium exchange (HDX) and fast photochemical oxidation of proteins (FPOP) have enabled residue level labelling of solvent exposed backbone amides and solvent exposed residue sidechains, respectively. These technologies have contributed to our understanding of structure as well as protein interactions. Additionally, advances in ion mobility have allowed for the spatial separation of proteins and proteo-forms based on the molecule's shape and collisional cross-section. This roundtable will provide an open forum to discuss the most recent advances in the use of mass spectrometry for structural studies of proteins. Advances in workflows, instrumentation and technologies will be discussed.

Discussion Notes:

Overall summary of discussion:

- HDX is a pretty mature technique that is well accepted, and a "go-to" technique, but there are other methods like covalent labeling that are easy to do using traditional peptide mapping approaches and give good complementary information on side chain interactions plus don't have the limitations of back exchange.
- Crosslinking is still an important technique and can provide some spatial information that isn't going to be obtainable by other techniques.
- Being able to take all of the generated structural information and graft or evaluate that using models or structures brings a lot of value.
- IM definitely has some real power for looking at complexes, shapes, and also being able to be hyphenated with techniques that give us chromatographic separation.
- FPOP is making some progress and there seems to be some sort of commercial application and that can be another approach. FPOP is specific for sidechains as well.
- Limited proteolysis is something that you would only do if you don't have access to these other approaches where you could get this information.

Discussion points:

One of the biggest perceived challenges of HDX is needing to make an investment for a dedicated system. For those doing labeling techniques, do you have dedicated instruments for that work?

- One lab uses a custom apparatus that allows for reversible labeling and uses a C8 desalting column to capture the labeled protein. These lines can be switched out for pepsin, C13, etc., enabling switching between HDX, covalent labeling, and denaturing MS.
- Another lab uses the Leap robot but they are not using anything else because of its footprint. They want to protect the instrument for the robot. Unfortunately, that means that instrument is off-limits for anything else.

Comparing HDX, FPOP, and covalent labeling methods:

- There is a higher bar to get into HDX in addition to training when comparing with many other structural MS techniques.
- With HDX, one of the big challenges is back exchange whereas with FPOP and covalent labeling, they are more permanent.
- Biologically-focused collaborators think HDX is more established and it is viewed as more legitimate as reviewers.
- Even if FPOP data is more convincing, there tends to be a priority for having HDX data.

Is the current trend driving more towards HDX or are covalent labeling and FPOP gaining more momentum?

- In the past, HDX was definitely the go-to method because of familiarity and it is relatively easy to do compared to FPOP.
- For FPOP there is a learning curve for collaborators in addition to the SME themselves.
- On the other hand, all of these techniques are powerful and focus on different aspects of the structure so none is better than the rest and they have to work together.
- Most of the time, it is really about what you have in your hand and what you can do with your existing/current experimental setup, so from that perspective, FPOP has a pretty large barrier.
- Covalent labeling is relatively easy, after incubation with your protein mixture it is really just a peptide mapping experiment.
- All of these techniques have pros and cons, it really just depends on what information you need.
- FPOP is the furthest from a commercial solution.

NHS ester chemical crosslinking vs. HDX:

- HDX results interpretation is more user friendly, but crosslinking is unique because it can give you distance constraints.
- It has been demonstrated that combining crosslinking with labeling gives a better understanding of your binding system.
- From a broader perspective, crosslinking is often used nowadays to study the interactome, the interaction from one protein to other proteins, stoichiometries of their interactions, etc.

HDX data and structure modeling:

- Is modeling the data on the protein structure used as part of the general workflow nowadays or not as much as it potentially could be? We usually see it get mapped on the structure, usually kinetics plots or some sort of global view of kinetic plots across the entire sequence taken one step further and put on the structure. One challenge is that sometimes there isn't a structure available. This is mostly done for visualization purposes.
- From the actual uptake, trying to generate a predicted structure or even based on homology modeling, this isn't very easy. There are some efforts in predicting structures on the basis of MS data, but this could the future of the field.
- One of the benefits of the mature state of HDX is that there is an established understanding of the kinetics. You can get an idea of higher-order structure based on exchange. However, back exchange can impact the efficacy of determining higher-order structure from HDX alone.

HDX data analysis: with respect to deuterium uptake, is there a particular software that is used?

- HDX workbench and HDX examiner were both discussed as popular choices.
- There is also a recent release of Protein Metrics/PMI that supports HDX.

The use of liquid handlers in HDX:

- Liquid handlers are a good option since they relieve a lot of manual labor, but there are also cases where some prefer to do it manually.
- For manual incubation time, you can go down to ~10 seconds but for liquid handler, the shortest is really 30 seconds so there is definitely a gap there. It all depends on whether the system is highly dynamic or not.
- The liquid handler provides an opportunity where there are small changes or a lot of different states and changing the titration of the ligand that you are adding.
- Some hope to start using liquid handlers as a way to screen mapping conditions. You could screen several quench reagents, test different quench incubation times to see if there are difficult disulfide bonds, you can set up the peptide mix to do the exact same mapping experiment and measure back-exchange of several peptides. It really increases the amount of testing you can do beforehand.

HDX and IM:

- It is helpful for cross-section calculations to elucidate if we have one or many conformations present in solution.
- Historically the two techniques haven't been combined very much but this is starting to change.

Native MS:

- One of the most exciting things is the ability to integrate with chromatographies that were not typically amenable to MS. Now, we use charge-based separation with MS-friendly mobile phases, getting MS information on different subpopulations or isoforms that are chromatographically resolved.
- Mostly SEC is being used in native MS, more than direct infusion and IEX (according to those attending the roundtable).
- There are challenges with fewer charge states and lower sensitivity. One example of this is the CASSS MS talk where they utilized the post-column addition to do some unfolding and boost the signal.
- The Newomics source allows you to get nano-like performance at micro flow rates, giving the best of both worlds

Do you typically do tandem with native MS? Or other techniques where we get more protein-protein interaction information?

- One group uses SEC before IMS separation when interested in D-isomer impurities of pharmaceuticals. It is difficult to characterize with MS alone because of having the same mass, but these other techniques allow an additional dimension of separation. Can separate some with HPLC but they still experience a lot of D-isomer impurities, so they try to combine the separation along with ion mobility.

Complementary/integrated approaches:

- It is important to study your protein from both top-down and bottom-up approaches to get the whole picture.
- It is also important to look to other methods like native MS to provide that complementary information.
- Many are always looking at what is "new" in native MS since it is sometimes challenging to get that data. For example, Dr. Ruotolo's keynote talk about using CIU to probe protein interactions and conformations is allowing us to move outside the space of labeling techniques to get this information or using this for complementary information.
- Even if collaborators show binding, that doesn't necessarily give any high-resolution information, which is where MS can come into play.

- It saves time to do quality control work with denaturing and native MS before getting started.

Ion mobility:

- One of the biggest challenges with IM is the resolving power.
- While there are exciting new technologies like Waters Select Series Cyclic and MOBILion MOBIE where you can try to optimize these separations, it really depends on the question you are asking/what information you need whether IM can help to answer that.
- The architecture of the Agilent 6560 isn't amenable to having a passthrough mode, you need to have IM be part of your experiments.
- With limited funds, you often have to choose the most flexible MS for all applications.

Limited proteolysis as a way to probe structure:

- Can see additional proteolysis occurring on a more relaxed structure. This is something that was done a lot more previously.
- Limited proteolysis gives some low resolution information on the domain level.
- As you increase the chaotrope, you start to see decreased efficiency and integrity of the protease itself, which is one of the biggest challenges with limited proteolysis and often why it isn't the best choice.
- Overall, it was agreed upon that limited proteolysis is an outdated technique with the options we have available now.