Advanced HDX-MS and Native-MS Approaches for Improved Biologic Candidate Selection and De-Risked CMC and Clinical Development Programs

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Despite substantial efforts utilizing a diverse set of biologic evaluation and high-throughput techniques to select candidates with the best chance of success in preclinical development, the vast majority of biologic drug molecules fail in clinical trials. With most biologic drugs, including gene therapies, failures are realized in late-stage development, after substantial investment has been made in a candidate. Here, we discuss the potential for improving identification of potentially successful biologic drugs and de-risking CMC and clinical designs earlier through strategic application of advanced mass spectrometry, and specifically, how detailed assessment of target engagement using HDX-MS and Native-MS analyses may provide understanding to guide biologic development decisions.

Efficacy of Biologic Drugs

We are experiencing unprecedented innovation, growth and opportunity within the biopharmaceutical industry, with increasingly promising yet complex biologic therapeutic platforms in development, including complex monoclonal antibodies (mAbs), recombinant human proteins, enzyme-replacement therapies, fusion proteins, antibody-drug conjugates, bispecific drugs and gene and cell therapy approaches. However, despite significant advances in therapeutic platform development, almost 90% of biologic drug candidates fail from Phase I to Approval,1,2 and over 50% of biologics still fail in Phase III. This is due, in part, to a limited understanding and control of a candidate’s target site engagement, particularly at differing dose levels, and related mechanism of action (MOA), as well as poorly established dosing. Accordingly, obtaining a more accurate and complete assessment of a drug candidate’s target-site binding profile at an earlier stage, including defining the binding site interaction and related stoichiometry over the range of clinically relevant dose levels, should help improve candidate selection, better inform CMC program controls, support clinical programs and improve outcomes. Increasingly, advanced mass spectrometry-based approaches, specifically including both hydrogen-deuterium exchange mass spectrometry (HDX-MS) and Native mass spectrometry (Native-MS),
are being applied to generate such drug-target binding site profiles both early in candidate selection and throughout CMC and clinical program development. When detailed characterization provided by HDX-MS and Native-MS are combined with other well-established analytical techniques, there is further opportunity to utilize such detailed binding site profiling to more reliably select and optimize candidates, de-risk decisions and increase controls in CMC program development and enhance clinical development success rates overall.

Target engagement is known to be a major factor in eventual drug efficacy, and understanding and controlling how drug candidates interact with their expected targets can be enhanced using combined profiling approaches, specifically high-resolution analyses that provide details about the interaction. HDX-MS and Native-MS are powerful, information-rich approaches that increasingly are used for binding site profiling and analysis of stoichiometry. With interrogation and more accurate profiling of these properties, there is opportunity to de-risk and advance development of biologic drugs by assessing target engagement, particularly at different dose levels, and correspondingly, to leverage the understanding obtained to more reliably select and optimize candidates with greater probability of success.

High target affinity and MOA also are important criteria in candidate selection. Accordingly, analytical approaches that can generate insights into the molecular and chemical attributes of therapeutic molecules and enable correlation with biological function and/or clinical outcomes can improve selection and increase potential clinical success of candidates. While several analytical approaches have been used for this purpose, increasingly HDX-MS has been used to map interactions between antibodies and antigens and to understand the importance of specific epitopes with respect to efficacy. It also has been used to inform reengineering approaches, including identification of anti-drug antibody (ADA; e.g., neutralizing antibodies) binding epitopes on therapeutic proteins, conformational changes induced by binding and hot spots for aggregation. Further, recent advances in Native-MS have been applied to characterize stoichiometry of non-covalent complexes, and when combined with HDX-MS, can provide useful details about the nature of target engagement to inform candidate selection and optimization. For example, these approaches have been used to confirm co-engagement of targets with bispecific antibodies. Together, there is significant opportunity for using combined HDX-MS and Native-MS approaches to potentially integrate low-resolution data from high throughput methods and further profile such interactions and MOA even more effectively to improve development from candidate selection through manufacturing approaches.

Analytical Approaches for Analyzing Binding Interactions

High-throughput, Low-resolution Methods

Several tools have been in common use for detecting binding between molecules and establishing binding affinities. High-throughput, low-resolution techniques include surface plasmon resonance (SPR; BIACore) and biolayer interferometry (BLI; Octet), while analytical ultracentrifugation (AUC), light scattering techniques, isothermal titration calorimetry (ITC) and size exclusion chromatography (SEC) are used for further assessment of binding and examination of complex size. While these data can be obtained quickly, interpreting the results and utilizing the information to make strategic decisions about drug candidate selection is limited. Each method has different considerations that confer advantages and disadvantages, but a common problem with low-resolution approaches is that fundamental assumptions must be made when interpreting the data. For example, AUC and SEC measure hydrodynamic properties and interpretation (calculation of size) depends on assuming a shape of the molecule/complex. If it is unknown that the shape is irregular or elongated,
the calculated value will be inaccurate and may lead to a lack of clarity as to the stoichiometry of a complex. For example, if the calculated size is midway between theoretical values, it may not be possible to determine whether the data reflect an extended arrangement of fewer protomers or a greater number of subunits in a compact assembly. A globular arrangement of the same molecular weight complex, for example a hexamer, will sediment differently than if the hexamer is arranged as a plate or a hollow barrel. In such cases, knowledge of shape or subunit interactions from higher resolution structure studies can be leveraged to guide interpretation. Analysis also can be complicated by multi-component systems when components are of similar molecular weight or multiples of one component are similar in size to another component. Therefore, data from low-resolution approaches is much more accurately interpreted in the context of high-resolution data, and in each of these scenarios, the use of Native-MS can provide better understanding of structural organization of multimeric complexes and aid in assessing related implications for target engagement by biologic drugs.

**Detailed Molecular Characterization Methods**

High-resolution structure characterization tools provide much greater detail about the interaction between a biotherapeutic and its target and such detailed data can enable understanding about how complexation may influence MOA and clinical outcomes. High-resolution methods require significantly greater commitment of resources and time compared to low-resolution analyses; however, they are able to identify residues/regions directly involved in and allosterically effected by binding. X-ray crystallography (XC) is often considered to be the best tool for characterizing a complex at high-resolution, as it shows binding interactions in atomic detail, and in theory, is not size limited. The primary challenge is the substantial difficulty of obtaining high-quality crystals of complexes, which are often disadvantaged by being large, non-globular/asymmetric, conformationally labile and in dynamic equilibrium. Often data from XC provide very useful details about binding interactions to guide drug development, however, even if a crystal structure is generated, it may not reflect a functionally relevant form because crystallization typically selects for a single, static conformational state, which can be influenced by crystallization conditions. Like XC, NMR provides atomic-level detail, but it has the advantage of being conducted in solution under more physiologically or pharmaceutically relevant conditions. It requires significant effort to assign data, which must be done in order to interpret subsequent studies of interactions, dynamics or conformational changes. The approach is limited by size such that assignment is easier with smaller species (<40 kDa; e.g., scFV, Fab fragments) and becomes more challenging at mAb size, making analysis of mAb-Ag complexes at least a significant undertaking. Nonetheless, once assignments are made an array of different NMR experiments can quickly identify binding interactions and conformational changes. Many biologics are glycosylated or have other post-translational modifications, which can confound study by XC and NMR. Cryo-electron microscopy (EM) also may be used to examine the shape and structures of large, heterogeneous complexes. The technique now can determine complex interactions at resolution down to 3Å as well as reveal orientation between associated proteins in complexes and aggregates, and it can be further informative when coupled with MS-based analyses to control for artifacts that can be induced by dehydration, freezing and sectioning. MS characterization of binding interactions may be reliably applied to examine highly diverse biologic species having a broad range of properties (e.g., size, glycosylation/modifications, structures, shapes) and the analysis is relatively robust and fast compared to other high-resolution techniques. It may also be used to effectively examine heterogeneous species and specific components in complex mixtures. MS typically is utilized to provide peptide-level resolution but may be adapted for higher resolution by invoking additional MS dimensions (e.g., MS/MS, MSn) in
the acquisition scheme to fragment and further interrogate specific features of individual peptides. Adding dimensions increases the time required to analyze the data but may be valuable for gaining more detailed knowledge of complex systems, particularly those not amenable to X-ray or NMR. A broad range of experimental approaches are possible with MS, and HDX-MS and Native-MS techniques are particularly useful for analysis of binding interactions (Table 1).

**Mass Spectrometry Approaches—Advances in HDX-MS and Native-MS Analytics**

HDX-MS analysis is used to map binding site interactions, including epitopes on antigens, paratopes on antibodies, protein-protein/ligand interfaces and self-association, as well as to identify conformational changes induced by binding.\(^{10-12}\) The H/D exchange data reflect three-dimensional structure and conformational dynamics of a biologic in solution and report on relative solvent accessibility of regions within the structured protein. Studies can be conducted to identify changes to the biologic under different conditions, such as comparison of an antigen (alone and when bound by antibody) or an enzyme with and without an inhibitor to better understand the interaction and associated implications of binding. The technical approach involves exposing protonated sample to deuterated solvent (D\(_2\)O) for various lengths of time followed by quenching at low pH and low temperature (to slow the rate of back exchange), rapid proteolysis to generate peptide fragments and automated data collection with LC-MS (Figure 1). The amount of deuterium (D) taken up by individual peptide fragments at each time point is determined by mass difference and the data plotted to compare D uptake as a function of time (Figure 2). (For a technical review of modern HDX-MS, see Oganesyan *et al.*\(^ {24}\))

In an epitope mapping experiment, the free antigen is analyzed and compared to the antibody-bound state. Decreased D uptake in the bound state indicates that the peptide region is less solvent exposed than when unbound, suggesting that the region directly participates in the binding interaction (Figure 2, Panels A and B). Peptides where faster D uptake is observed in the bound state typically indicate regions that undergo a conformational change to become more exposed.

**Table 1. MS Approaches for Characterizing Binding**

<table>
<thead>
<tr>
<th>MS Approach</th>
<th>Purpose</th>
<th>Information Provided</th>
<th>Outcomes</th>
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<tbody>
<tr>
<td>HDX</td>
<td>Epitope mapping</td>
<td>• Reliably applied to diverse proteins and complexes with</td>
<td>• Identify sites of target engagement by therapeutic</td>
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<td></td>
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<td>• Large size, non-globular shape, flexible</td>
<td>• Assess changes to target induced by binding</td>
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<td></td>
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<td>• Heavy glycosylation (N-/O-glycans)</td>
<td>• Assess ADA epitopes for personalized treatment</td>
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<td>• Identify regions involved in binding interactions</td>
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<td>• 3D conformational (non-linear) epitopes</td>
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<td></td>
<td>• Identify regions that undergo conformational change upon binding</td>
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<td>Native</td>
<td>Stoichiometry of complexes</td>
<td>• Exact mass unambiguously identifies number and types of subunits in large complexes</td>
<td>• Determine whether formed complexes support intended MOA</td>
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<td></td>
<td></td>
<td>• Analysis of solution state (compare conditions)</td>
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<td></td>
<td></td>
<td>• Assess binding of competing species</td>
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<td></td>
<td></td>
<td>• Distinguish homo vs heterodimeric bispecific mAbs</td>
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<tr>
<td>Combined</td>
<td>Improve decision-making</td>
<td>• Potential for informing MOA and efficacy</td>
<td>• Improve efficacy</td>
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<td></td>
<td></td>
<td>• Co-engagement</td>
<td>• Optimize dosing</td>
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<td></td>
<td>• Valency</td>
<td>• Understand bioavailability and activity</td>
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<td></td>
<td>• Oligomerization</td>
<td>• Reduce counterproductive clearance</td>
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<td></td>
<td>• Identify potential synergies and competition</td>
<td>• Improve stability, reduce aggregation</td>
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<td></td>
<td>• Combine therapeutics</td>
<td>• Reduce potential immune reactions</td>
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<td></td>
<td>• Assess displacement by native ligands</td>
<td>• Enable re-engineering for further optimization and biobetters</td>
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<td></td>
<td></td>
<td>• Assess IC structures for potential immune reactivity</td>
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as a result of the binding interaction (Figure 2, Panel C). Often conformational changes are observed distal to the binding site and these allosteric changes may imply potential impact, for example, on effector function or stability in a mAb. Differences in the oligomeric state of a biologic can depend on solution conditions, and these differences can be detected by comparing samples with different formulations or stress conditions to predict potential impacts on clinically relevant parameters, such as was done with insulin to evaluate onset and duration of action with respect to oligomeric state.25, 26

Native-MS is a gentle technique used to examine proteins in their folded states and can be applied to determine the size of macromolecules, complexes and aggregates.4 It has been used effectively to analyze masses up to ~9 MDa6 and has been applied to characterize multi-protein assemblies,19, 27 viral capsids,28 mAb-antigen complexes,29 protein-small molecule complexes5, 30 and self-association.31 Native-MS data can reveal the stoichiometry of subunits in heterogeneous complexes (Figure 3) and further provide verification of individual components involved in detected complexes through additional MSn analyses4, 19. For example, Native-MS has been used to determine the relative levels of correctly paired heteromeric bispecific chains and incorrectly paired homomeric species in the production of a bispecific antibody candidate.32 It may be used further for determining the level of homotypic anti-CD3 pairings in a bispecific product, which is important for assessing potential risk of immune reactions due to CD3 cross-linking.33

Evaluation of Target Engagement to Assess Potential Efficacy Impact

Competition for Target Binding

Effective target engagement is central to efficacy and several facets of this present tractable areas for analytics to enable improvements in assessing pipeline viability. Binding can be optimized by identifying candidates with very high affinity and specificity for their target, and approaches such as affinity maturation have led to improved generation of such molecules. However, binding affinity is not the sole determinant and may not
even be the most important factor in conferring efficacy. The manner in which a therapeutic engages its target also makes a major contribution to the success of clinical candidates. Different antibodies with similar high binding affinities to the same target can perform very differently in vivo. From one perspective, this is not surprising because many validated targets for mAbs are large, multi-domain receptors, for example HER2, which has multiple therapeutic antibodies against it, and these different mAbs engage distinct epitopes and exert their effects through different mechanisms of action. Competition for target binding, however, impacts efficacy, even for extraordinarily high affinity compounds. Interestingly, target binding experiments have shown that higher affinity mAbs can be displaced by lower affinity binding partners that have partially overlapping binding sites and that this effect is driven by differences in kinetics, such as association rate \(k_a\), wherein lower affinity is offset by favorable \(k_a\) in a competition experiment. In addition, how well a therapeutic competes for binding a target depends on its relative concentration. If a lower affinity ligand is constitutively present at relatively high levels in circulation, the duration of effective action of a very high affinity drug may be much shorter than anticipated in vivo, as the actual and relative concentration decreases due to clearance while the native ligand persists. As such, identifying the epitope where a biotherapeutic engages its target can be a key step to determining potential efficacy of candidates, for example in the context of developing mAbs where native, competing ligands are present in serum. Moreover, being able to accurately analyze the drug candidate in the relevant context is important for assessing potential competing effects that diminish efficacy, which can be accomplished using Native-MS approaches.

**Linear versus Conformational Epitopes**

Epitope mapping is now more commonly applied to target molecules to understand how a therapeutic candidate interacts with it. There are different approaches to epitope identification. Several high throughput screening (HTS) approaches based on binding to peptide fragment libraries are used. Alternatively, high-resolution HDX-MS is used to identify binding regions, which are more protected from solvent exchange in the presence of a binding partner. HTS assays are fast and epitope identification relies on binding to peptide fragments, rather than intact proteins. The assay may utilize an ELISA format or cellular display approach and the peptides presented in these assays are relatively short amino acid sequences that represent linear epitopes. False negatives commonly occur with peptide-based
assays, because short linear sequences may not adopt the correct conformation and/or may only comprise part of the binding site, such that specific antibodies do not bind with sufficiently high affinity to be identified as hits in the screening process. Binding of the identified peptides is then cross-confirmed by mutagenesis of the antigen, resulting in reduced binding affinity, often using a SPR or BLI assay. The HDX-MS approach to epitope mapping is advantageous over these methods because the vast majority of epitopes (estimated at ~90%\(^{38}\)) are thought to be conformational epitopes comprised of noncontiguous sequence elements; such epitopes would not be effectively represented in a peptide-based screening analysis. While HDX-MS requires specialized expertise and more time to complete, the approach has much greater certainty of identifying the engagement site accurately and completely and providing more specific information about the drug-target interaction.

**Utilizing Understanding of Binding Interactions to Improve Efficacy**

Another advantage of HDX-MS mapping is that precise data facilitates understanding of how multiple target-drug interactions pertain to function/MOA; such data provides opportunity to select candidates with potential for greater efficacy. When this information is combined with knowledge of stoichiometry of drug-target complexes, rational design of therapeutic approaches and assessment of efficacy can be enabled. A straightforward application of this approach is to assess valency of a therapeutic mAb. Native-MS can be used to quickly characterize valency for a mAb and determine binding at one versus two arms. Bispecific molecules target two different entities and are a growing class of therapeutic candidates because employing more than one mode of action has potential to provide significantly enhanced specificity and potency in a single molecule.\(^{39}\) As such, over 100 formats have been presented as potential solutions to achieve the purported synergistic clinical improvement.\(^{39}\) Studies have shown that there are many aspects of a bispecific candidate’s construction that influence potential efficacy through target engagement.\(^{30-44}\) Most importantly, confirmation of productive co-engagement is critical for the assessment of therapeutic efficacy. Multi-specific and multi-valent therapeutics are also being developed, and inclusion of additional engagement sites adds complexity to evaluating potential efficacy and selectivity.\(^{42}\) A recent study reported the interdependent relationship of affinity at individual arms to avidity in mono and bivalent bispecific formats with respect to selectivity for cells containing one versus both targets.\(^{42}\) The data for the monovalent bispecific showed dual selectivity was negated when the affinity of a single arm for its target is too high, resulting in complete loss of the key benefit of a bispecific approach. Separately, the work also showed that addition of a duplicate binding module for a target to the candidate molecule increases avidity such that again selectivity for the second target was overcome. From the data, it is clear understanding co-engagement and valency is important for assessing and optimizing potency/efficacy of these complex therapeutic agents.

Many approved mAbs and candidates in the pipeline target soluble proteins as a means of inhibiting aberrant functions, for example suppressing autoimmune symptoms by inhibiting TNF (\(e.g.,\) adalimumab, rituximab, infliximab, etanercept, certolizumab pegol, golimumab) or mitigating angiogenesis to slow cancer or macular degeneration through binding up VEGF (\(e.g.,\) bevacizumab, ranibizumab). For soluble targets, binding at one versus both arms of a mAb may affect dosing. If the soluble factor circulates as an oligomer (\(e.g.,\) TNF alpha trimer), it can also be determined if binding to a biologic drug candidate alters oligomeric state, which has implications for dosing. A comparison of anti-TNF therapeutics showed that binding ratios to TNF with different approved products vary and the ratio influences pharmacological properties.\(^{19}\) Etanercept bound 1:1 with the TNF trimer, whereas adalimumab and
infliximab formed a range of complexes with the predominant ratio being 3 mAbs to 1 or 2 TNF trimers. This study demonstrates the importance of investigating target binding ratios to obtain a more comprehensive understanding of potential therapeutic behavior in vivo.

In addition to safety concerns, it is now well known that the immune system can contribute to sub-optimal efficacy of a therapeutic in patients. Interactions of biologic drugs with the immune system that thwart efficacy can involve soluble proteins and/or receptors on cells. Anti-drug antibodies, immune complex formation and binding to C1q or FcγRs, can hinder the ability of the biologic to productively engage the therapeutic target, reducing the therapeutic effect. Most biologic drugs are shown to have some level of ADA, but the clinical impacts of ADA are widely varied. ADA may be preexisting in patients due to prior exposure, such as with gene therapies that utilize AAV, or cross-reactivity with similar epitopes, and/or they may be induced by exposure or repeated exposure to therapeutic, such as with many anti-TNF drugs. In many cases, there appears to be no therapeutic consequence of ADA, while in others, the ADA can lead to inactivation and/or faster clearance of the biologic drug, thereby diminishing efficacy. Although rare, in some cases, ADA can promote severe or even fatal immune reactions, including hypersensitivity, anaphylaxis and cross-reactivity or breaking of tolerance to the native protein. Some data have been presented that suggest the size and structure of ADA-drug complexes influences these different outcomes. As such, being able to characterize attributes of ADA-drug complexes and to use that information to predict unwanted reactions has the potential for advancing biologic drug development. To improve selection of biologic candidates with greater certainty of clinical success requires integrating understanding these facets of human biology and molecular attributes of biologic drugs to better determine how their interactions impact MOA, immunological responses and effector functions. Combined HDX-MS and Native-MS analyses can provide strategic information to identify potential clinically relevant success factors in biologic drug candidates.

**Using Native-MS & HDX-MS Data for Candidate Selection and Development Decisions**

Understanding epitope targeting has proven to be important for achieving improved clinical outcomes, for example by combining two therapeutics. The validated oncology target HER2 is a large, membrane protein receptor with multiple extracellular domains found on the surface of certain cancer cells. Several approved mAbs target HER2 and many additional candidates are in development. In one study, the receptor was analyzed with several individual mAbs and the binding sites mapped to specific regions of the receptor. This type of detailed information has been used to determine the potential for...
combination therapies whereby the receptor is targeted at two unique locations for an enhanced effect, for example by invoking two MOA or by further disrupting dimerization and preventing growth signaling. By co-administering two such therapeutic mAbs (trastuzumab and pertuzumab), substantially greater progression-free survival was achieved for HER2-positive metastatic breast cancer patients. Clearly, more comprehensive understanding of target engagement can support improved selection and optimization of biologic drug candidates for greater efficacy, and utilizing a combination of HDX-MS and Native-MS is enabling these assessments.

Sevy et al. provided an excellent example of how HDX-MS epitope mapping can enable personalized treatment options for hemophilic patients. Based on patient-specific data, clinicians can use understanding about epitopes targeted by the patient’s anti-drug antibodies to make treatment decisions about administration of Factor VIII and VIIIa therapeutics. Up to that point, ADA titer was used as a guide, but these researchers demonstrated that epitope specificity and inhibitor kinetics are more important to predicting clinical response to Factor VIII treatment. In this study, HDX-MS was used to effectively distinguish patient ADA that target classic versus non-classic inhibitor epitopes as well as different types of classic inhibitors. The presented assay offers a robust approach to improving treatment efficacy based on reliable detection of key epitope-indicating peptides, showing how detailed molecular characterization is enabling greater efficacy through personalized medicine.

These elegantly designed studies clearly highlight the great power and potential HDX-MS and Native-MS based analytics have for advancing biologic drug development and elucidating the underlying biology that influences efficacy of these drugs for improving the ability to predict clinical success.

**Conclusion**

Findings from increasing applications in the space, including the example studies presented herein, demonstrate use of advanced HDX and Native mass spectrometry approaches can support improved identification of potentially successful biologic drugs, as well as early in development, enable de-risking of CMC and clinical designs. Specifically, these applications show how HDX-MS and Native-MS analyses may be used to obtain detailed assessments of target engagement by biologic drug candidates and provide improved understanding of attributes that influence efficacy to support development decisions for complex biologics.

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