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Mass Spectrometry Methods for Measuring Protein Stability

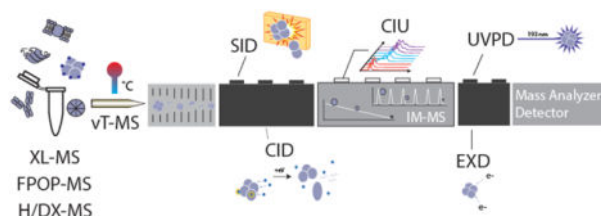
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Abstract

Mass spectrometry is a central technology in the life sciences, providing our most comprehensive account of the molecular inventory of the cell. In parallel with developments in mass spectrometry technologies targeting such assessments of cellular composition, mass spectrometry tools have emerged as versatile probes of biomolecular stability. In this review, we cover recent advancements in this branch of mass spectrometry that target proteins, a centrally important class of macromolecules that accounts for most biochemical functions and drug targets. Our efforts cover tools such as hydrogen-deuterium exchange, chemical cross-linking, ion mobility, collision induced unfolding, and other techniques capable of stability assessments on a proteomic scale. In addition, we focus on a range of application areas where mass spectrometry-driven protein stability measurements have made notable impacts, including studies of membrane proteins, heat shock proteins, amyloidogenic proteins, and biotherapeutics. We conclude by briefly discussing the future of this vibrant and fast-moving area of research.

Graphical Abstract



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1. Introduction

Stability-shift measurements have been a key component of biochemical research since the 1920s. For example, early work by Eggerth and colleagues in the 1920s monitored the changes in *Escherichia coli* (*E. coli*) flocculation as a function of added colloidal proteins in suspension.¹ A follow up study further probed the stability of *E. coli* as a function of strain variation, suspension media, as well as storage conditions.² As such cellular stability shift assays gained importance, measurements targeting purified proteins soon surfaced. An example of such work is included in a series of publications by Northrop and colleagues wherein they explored the stability of trypsin by measuring its activity as a function of temperature.^{3,4} Similar studies measuring protein activity as a function of pH, temperature, and aging were performed for a variety of enzymes such as pepsinogen,⁵ and ribonuclease,⁶ wherein a loss of enzymatic activity can be interpreted as a measurement of protein stability. Together, these types of studies helped build the foundations of modern biochemistry.

Later, spectrophotometry-based measurements emerged to enable in-depth investigations of protein stability. Early examples include absorbance spectrophotometry experiments, as demonstrated in early studies of rhodopsins,^{7,8} and polarimetry measurements, which were used extensively to characterize the stabilities of collagen in response to pH,⁹ temperature,¹⁰ organic solvent,¹¹ or changes in primary sequence.¹² These spectrophotometric approaches typically yield global protein stability information. Although these techniques were deployed extensively in the characterization of protein targets such as myoglobin^{13–16} and hemoglobin,¹⁷ novel techniques were beginning to emerge which promised insights into intermediate conformers populated during protein unfolding.

Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) quickly became the primary approaches for studying protein stability. DSC measures the heat capacity (C_p), and transition/melting temperature (T_m) at which 50% of protein population is denatured, with higher T_m values are indicative of higher stability, which is employed in a comparative manner to quantitate the stabilizing/destabilizing effects of buffer composition,^{18,19} protein-protein interactions,²⁰ ligand binding,^{21–23} or mutation.²⁴ ITC measures changes in the thermodynamic properties of a protein sample upon the controlled addition of known ligands or binding partners. Changes in these properties, discussed in more detail in section 2 below, can be interpreted as changes in sample stability.^{25–30} DSC and ITC remain standard techniques for protein stability measurements today.³¹

In the last three decades, techniques commonly used for small molecule structure determination such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) have developed into tools capable of targeting larger analytes such as intact proteins. Advancements in NMR spectroscopy enabled for the first time, higher resolution

measurements of protein secondary structure stability based on changes in chemical shifts of residue sidechains.^{32–37} Similarly, the development of electrospray ionization (ESI)³⁸ proved crucial for the growth of MS as a probe of protein stability.^{39,40} Since these early studies, researchers have sought to expand the MS toolbox to encompass a greater number of biophysical descriptors associated with protein conformation and folding (Figure 1).

Tandem MS (MS-MS or MS²) approaches were utilized to determine primary sequence,⁴¹ while charge state distributions (CSD)⁴⁰ were used to monitor protein structures incubated under different solvent conditions,^{42–44} or temperatures.^{45–51} These studies led to the realization that, under the appropriate conditions, ESI-MS was capable of transmitting non-covalent protein complexes in a manner closely mirroring their native states^{52–56}. In the three decades since these initial discoveries, innovations in protein ionization^{57,58} and instrumentation⁵⁹ have led to the emergence of native mass spectrometry, a technique capable of directly analyzing a wide array of multimeric protein machines,^{55,60–62} including chaperones,^{63,64,73–79,65–72} ribosomes,^{80–82} and intact viral particles.^{83–85}

MS-based techniques soon emerged capable of measuring protein stabilities. For example, covalent labeling techniques such as hydrogen-deuterium exchange MS (HDX-MS),⁸⁶ fast photochemical oxidation of proteins (FPOP),⁸⁷ and chemical cross-linking MS (CXL-MS)⁸⁸ are three such techniques which are capable of yielding protein structure and dynamics information at the level of individual amino acid residues within the targeted sequences. Briefly, these techniques covalently label proteins in solution, and the results of this labeling are then typically analyzed by bottom-up liquid chromatography MS (LC-MS) workflows. Such approaches can be used to directly assess the impacts of stress, ligand binding, or post-translational modification upon protein structure and stability within complex mixtures.

Another such approach, ion mobility-mass spectrometry (IM-MS), is capable of separating a variety of gas-phase ions based on their interactions with gaseous neutrals. IM-MS has been used to separate the electronic states of transition metals,^{89–91} as well as the shapes of varying carbon clusters,^{92–94} and PEG polymers.⁹⁵ Pioneering instrument development efforts combined ESI with IM-MS enabling the initial separations of intact protein ions,⁹⁶ and helped to establish a research area now referred to as gas-phase structural biology.⁹⁷ IM-MS technology provides the foundation for collision-induced unfolding (CIU) assays, wherein native-like protein ions are incrementally activated in the gas phase prior to IM separation. This results in gas phase unfolding of the ions, which is detected by IM as an increase in CCS.^{98,99} CIU has been applied to a variety of biological targets⁹⁸ including biotherapeutics,^{100–102} membrane proteins,^{103–107} chaperone complexes,¹⁰⁸ and kinases.¹⁰⁹ This review will expand on the various gas phase stability measurement techniques summarized here, with focused sections covering applications targeting biotherapeutics, membrane proteins, intrinsically disordered proteins, and heat shock proteins.

2. Survey of MS-based Protein Stability Measurement Techniques

2.1 Reference Techniques for measuring protein Stability

Thermal stability is a key determinant of protein structure and function.¹¹⁰ As such, stability measurements are extensively used throughout biochemistry, and especially in context

of pharmaceuticals, where protein stabilities are used to support the quality, safety and efficacy of biotherapeutics, or protein-based therapies, throughout their development.^{111,112} Technologies such as circular dichroism (CD),¹¹³ DSC,^{111,114} ITC,³¹ and differential scanning fluorimetry (DSF)¹¹⁵ are widely used for the analysis of protein stabilities.¹¹⁶ In particular DSC, ITC, and DSF function as reference protein stability probes due to their ability to directly track changes in thermodynamic stability^{117–121}.

Despite offering robust assessments of protein stability, the spectrometric and calorimetric methods briefly surveyed above often require large quantities of purified protein and lengthy acquisition times. In contrast, MS-based protein stability measurement techniques require substantially less sample and have the potential to be orders of magnitude faster when compared to their solution counterparts¹²². As indicated above, MS techniques also excel in extracting stability measurements from protein mixtures. Furthermore, when MS is combined with structurally sensitive labeling and separation techniques, such approaches can provide granular structural information that makes it possible to link specific regions of the protein sequence to changes in both protein structure and stability.

2.2 Mass Spectrometry-based Probes of Protein Stability

2.2.1 Native Mass Spectrometry

2.2.1.1 Introduction: For nearly three decades, a large number of MS based observations have indicated that protein complexes can be directly transferred into the gas phase in a manner that retains their native oligomeric states and ligand-binding properties.^{123,124} From the initial reports of nMS measurements, questions have emerged regarding the structural and functional states of the proteins captured in flight. While many of these questions persist today, a number of reports have greatly informed our current understanding of gas-phase protein structures and their potential fidelity to native conformations. For example, it has been shown that gas-phase peptide and protein ions retain much of their structural integrity following soft-landing on a surface housed within MS equipment.¹²⁵ Specifically, apoferritin, a protein known for its iron-storage capabilities and cage-like structure was shown to retain its native configuration after transport in the gas-phase and soft-landing.¹²⁶ Also, ESI-MS has been employed to measure binding constants of protein ligand interactions.¹²⁷ Additionally, nIM-MS enables comparisons between measured CCS values and those estimated from high-resolution structure determination experiments (such as x-ray crystallography and NMR), revealing both strong correlations between kinetically-trapped gas-phase protein structures and those associated with their native conformational states, as well as specific areas of structural compaction.¹²⁸ For more details on how CCS measurements are obtained from nIM-MS and their application for structural refinement please refer to targeted reviews covering this topic.^{129–131} Recently, the optimization of sample preparation, ionization, and ion transport conditions have advanced dramatically our knowledge of gas-phase biomolecular structure, and this area is covered by a number of excellent recent reviews.^{128,132–138}

ESI has proven to be a key enabling technology for MS-based protein stability measurements.¹³⁹ The technique involves application of high voltage to a conductive emitter, resulting in the creation of charged populations of aerosolized droplets. Subsequent

droplet fission and evaporation events give rise to analyte ions that can be analyzed by MS with both positive and negative polarities. Typical ESI-MS experiments employ high source temperatures ($> 100\text{ }^{\circ}\text{C}$), drying gas, and organic co-solvents in order to facilitate ion desolvation. Since such conditions can be detrimental to the preservation of native-like protein structures, nMS experiments utilize nanoESI (nESI), aqueous solutions and lower source temperatures ($20 - 30\text{ }^{\circ}\text{C}$).⁵⁷ In nESI, smaller droplets are produced which allow for more complete protein ion desolvation, resulting in significantly improved mass resolution and dramatic reductions in artifact protein complex signals. The fundamental principles of ESI, along with recent advancements in its theory of operation and implementation, have been discussed in detail previously.^{57,140–142}

2.2.1.2 Charge State Distributions as a Probe of Protein Stability: The native mass spectra of protein ions produced by nESI-MS typically reveals a series of ion signals corresponding to a narrow range of charge states, the magnitude of which correlate strongly with the available surface areas of the structures adopted by the analyte proteins in solution. As such, the charge state distribution (CSD) recorded for protein ions can be treated as a direct probe of protein solution structures, and measured over a wide array of preionization sample conditions.¹⁴³ By extension, such CSDs have been used as a measure of protein stability.^{42,144} For example, Chowdhury *et al.* showed that at least three CSDs were observed in ESI-MS data recorded for bovine cytochrome c (cyt c) when incubated at pH values ranging from 2.6–5.2. Critically, the existence of at least three cyt c conformational states had been observed in both NMR and CD datasets.⁴² Using a similar approach, Loo *et al.* demonstrated that ubiquitin CSDs could be varied based on the amount of organic solvent used, serving to denature the protein and alter its structure prior to ESI-MS.¹⁴⁴ In 1997, Konnerman & Douglas studied the CSDs of cyt c in detail and correlated their findings with both CD and DSF data.¹⁴⁵ Together, the studies discussed above laid the foundation for future developments in the application of CSD measurements to protein stability and structure.

Since their first use as a measure of protein structure and stability, CSD data have engendered questions surrounding the potential projection of solution-phase biophysical information into the gas-phase. Early IM-MS results for of cyt c showed that the +7 and +8 of the protein ions observed possessed more than one CCS value when compared to higher cyt c charge states.¹⁴⁶ Intrinsically disordered proteins (IDPs), which are often implicated in protein misfolding diseases, typically produce significantly wider CSDs and a greater plurality of CCS values for individual charge states than globular proteins studied by nIM-MS.^{147,148} Storage time and conditions in the gas-phase can further influence the range of structures accessed by protein ions in a charge state dependent manner.¹⁴⁹ Taken together, the studies above strongly suggest that protein ion structure is a product of the conditions surrounding their preparation, creation, storage, and transport. All of the previous factors must be considered alongside properties such as charge state, stability, sequence, and native structure when evaluating relationships between solvated and gas-phase biophysical data.¹⁵⁰ For example, ubiquitin ions having identical charge states, but prepared through different routes, can display distinct stability differences.¹⁵¹ As such, while CSD data has a

clear relationship to protein structure and stability in solution, the evaluation of associated gas-phase protein structure can prove more challenging.

2.2.2 Variable Temperature Mass Spectrometry—Variable temperature electrospray ionization (vT-ESI) is an analog of standard solution phase temperature annealing techniques, utilizing MS detection to monitor changes in protein structures (Figure 2A).⁴² The experimental setup includes a heat-conductive material that encapsulates and houses the ESI emitter.¹⁵² Throughout the experiment, higher currents are drawn through this housing, generating resistive heating in a controllable manner for the direct assessment of protein CSD,¹⁵³ and the oligomeric status of protein complexes.^{154,155} The largest advantages of vT-ESI devices remains their ability to probe protein melting point (T_m) values that are comparable to reference technologies (see Section 2.1), without need of large amounts of purified sample (Figure 2A ii).^{156–158} Recently, efforts in this space have been made to expand the structural information that can be obtained by vT-ESI-MS,¹⁵⁹ by incorporating IM-MS and detailed forms of data analysis in order to more deeply probe the connections between protein stability and gas-phase protein ion structure.^{160,161}

2.2.3 Overview of MS-based Footprinting Methods—MS-based footprinting encompasses techniques that probe the solvent accessible surface area (SASA) of proteins using a wide range chemical modifications that act to shift the measured mass of an analyte in a manner dependent on its structure and stability (Figure 2B).^{162,163} MS-enabled footprinting techniques offer high-throughput, amino-acid level resolution information, low limits of detection, and the ability to access protein stability values from within complex mixtures. In addition to the specific technologies covered in the sections below, a wide range of reagents, including carbene and diethylpyrocarbonate chemistries, that seek to comprehensively label solvent exposed residues within folded protein sequences.^{162,163} Our coverage of MS-based footprinting tools will focus on specific labeling technologies and their applications to protein stability measurements, and will include examples from Hydrogen-Deuterium Exchange (HDX), chemical cross linking (CXL), and fast photo-oxidation of proteins (FPOP) data.

2.2.3.1 Hydrogen-Deuterium Exchange: HDX-MS enables the acquisition of protein structure and stability information through the exchange of labile hydrogens with deuterium in solution at the level of individual amino-acid residues.^{164,165} The concept of HDX goes back to the late 1960s when it was first used in conjunction with NMR spectroscopy.¹⁶⁶ HDX exchange rates vary widely, and are based on the local environment of the backbone amide in question. Specifically, solvent accessibility and intramolecular hydrogen bonding can influence greatly the “protection factor” of an amino-acid residue in the context of HDX. In general, there are two procedures for measuring HDX by MS: Continuous-labeling and pulse-labeling. In continuous-labeling experiments, proteins are incubated with D₂O and then analyzed at fixed timepoints.¹⁶⁷ Continuous-labeling is primarily used to monitor slow (min – hours) structural transitions in intact proteins and is often performed using standard MS hardware.¹⁶⁶ In contrast, pulse-labeling HDX is capable of measuring faster transitions (seconds - hours), and functions by exposing samples briefly to a deuterium source following a structural perturbation.¹⁶⁸

The MS equipment and techniques used to measure HDX are continually evolving. Key challenges include limiting the back-exchange of absorbed deuterium,^{86,166} as well as improving both the throughput and coverage of the protein digestion step.^{169,170} Many excellent reviews cover the current applications and practice of HDX-MS.^{86,162,163,165,168,171–174} As such, the discussion below focuses mainly on the application of HDX-MS for the assessment of protein stability. The earliest examples of such experiments focus on model proteins, such as ubiquitin and lysozyme, with the latter protein probed both under conditions promoting intact and reduced disulfide bonds.¹⁷⁵ From these initial data, HDX-MS has grown significantly to encompass stability data targeting large proteins and their functional assemblies.

Specific examples of HDX-MS protein stability measurements in action span work associated with protein-ligand complexes, biotherapeutic antibodies, and protein-surface adsorption. In the latter area, HDX-MS has quantified the destabilization of proteins during surface adsorption,^{176,177} and have extended to free energy assessments for myoglobin-silica adsorption events.¹⁷⁷ HDX-MS played a central role in the evaluation of the National Institute of Standards and Technology monoclonal antibody (NISTmAb) standard.¹⁷⁸ Specifically, HDX-MS revealed the role of net protein charge on the stability and aggregation of the NISTmAb, an insight verified using a battery of reference protein stability measurement tools. HDX-MS also led the way in uncovering the role of glycosylation in stabilizing mAb structure in general, linking the presence of high-mannose or complex glycans to stability increases in model immunoglobulin G1 (IgG1) and IgG2 antibodies.¹⁷⁹ The influence of ligand binding on protein stability has also been examined in detail using HDX-MS.^{180,181} For example, a recent study utilized HDX-MS to determine two hotspots adjacent to the core binding interface of the SARS-CoV-2 Spike Receptor Binding Domain and the human Angiotensin-converting enzyme 2 (hACE2) protein. These hotspots could represent potential targets for therapeutics that act to destabilize the spike protein-hACE2 interactions.¹⁸⁰

Tandem MS (MS/MS) technologies are central to most high-resolution HDX-MS workflows. The migration of deuterium tags from their original labeling sites remains a challenge in evaluating residue-level HDX information. Such H/D scrambling is prevalent in datasets utilizing collision induced dissociation (CID) for ion activation,¹⁸² but is dramatically lessened (or eliminated) for workflows using electron capture dissociation (ECD) or electron transfer dissociation (ETD).^{164,183–185} Scrambling is caused by the increased vibrational energy and larger timescales of the CID process compared to ECD/ETD.¹⁸⁶ For example, an intact protein HDX-MS workflow incorporating ECD fragmentation has localized deuterium incorporation to residue pairs within the target sequence.^{164,187} Clearly, avoiding protein digestion steps allows for both the acquisition of global exchange data while simultaneously limiting back-exchange due to the large decrease in sample preparation time and likelihood of back-exchange.¹⁸³ It is clear that the advantages associated with the direct sequencing of intact proteins will spur further advancements in HDX-MS technology.

The peak capacities of LC-MS methods associated with HDX-MS have a clear impact on the acquisition of protein stability information that can be accessed from larger protein systems.

As such, IM separation has been broadly deployed in HDX-MS workflows in order to expand the total number of resolved features accessed by HDX-MS technology.^{188,189} The deconvolution of isotopic patterns for co-eluting peptides allows for accurate determination of exchange rates. In addition to HDX in solution, proteins can undergo HDX reactions with reagent gases and background neutrals while trapped in the gas phase. Such gas-phase HDX-MS experiments can be challenging to interpret, but a number of reports demonstrate how such HDX data, when combined with IM-MS, can provide synergistic datasets from which to resolve and identify the plurality of structural microstates that evolve during protein unfolding reactions.^{190,191} Future work will undoubtedly reveal further complimentary aspects of IM-MS and HDX data for the evaluation of protein stability in even greater detail.

Lastly, HDX has been utilized to track shifts in protein stability associated with ligand-binding events, often as a function of ligand concentration or ligand to protein ratio. For example, using Stability of Unpurified Proteins from Rates of H/D Exchange (SUPREX) analyses, thermodynamic parameters have been evaluated for the binding of anions to ferric-binding protein.¹⁹² Unlike, SUPREX, the Protein–ligand interactions by mass spectrometry, titration, and H/D exchange (PLIMSTEX) method does not need denaturants to obtain binding constant values for protein-ligand interactions using HDX-MS data.¹⁹³ For more information on HDX-MS studies of protein-small molecule binding and HDX-MS and other non-MS tools as a quantitative approaches for assessing protein stability, see the recent reviews by Williams¹⁹⁴ and Fordyce¹⁹⁵, respectively.

2.2.3.2 Chemical Crosslinking: Over the last 20 years, chemical crosslinking (CXL) combined with MS has proved to be an invaluable tool for evaluating the structures of protein complexes.⁸⁸ CXL-MS is most often accomplished by exposing proteins to compounds that possess two reactive sites spaced by a known distance. If both sites within the CXL reagent undergo a successful linking reaction with residues on the surface of the protein, then a covalent link between disparate regions of the protein can be established that, if located through MS/MS experiments, can reveal those sequence elements located nearby in the native protein structure. CXL-MS is also a potent tool for examining the stabilities stoichiometries, flexibilities, and binding interfaces associated with protein complexes.¹⁹⁶ Such CXL-MS datasets are most often used to create distance maps, a network of interactions between crosslinked proteins, that can be used as constraints for generating low-resolution models of protein assemblies.¹⁹⁷ Integration with nIM-MS data enables CXL-MS maps to directly account for protein complex stoichiometry, orientation, and size and thus improve the resolution of the models generated.^{198,199} The gas-phase stabilities of cross-linked proteins and complexes reveal expected increases in stability, with greater increases provided by charged reagents.^{198,199} Future developments in CXL chemistries and associated MS methods will clearly promote more detailed assessments of the stabilities of proteins and their associated complexes²⁰⁰.

2.2.3.3 Fast Photochemical Oxidation of Proteins: Fast photochemical oxidation of proteins (FPOP) methods generate hydroxyl radicals that irreversibly label solvent-exposed amino-acid side chains within proteins for MS and MS/MS evaluation.^{201,202} FPOP

reactions typically occur on the sub-millisecond timescale,^{203,204} enabling wide ranging studies of protein stability.^{201,205} Additionally, like other chemical footprinting techniques combined with MS detection, FPOP provides a unique set of restraints that can be used to generate, or improve, low-resolution protein structure models.²⁰⁶ FPOP experiments often require laser irradiation within flow injection manifolds to control the oxidation reactions used for protein labeling.²⁰⁷ More recent versions of FPOP operate without the need of lasers,²⁰⁸ offering the potential to measure protein stabilities directly through the direct observation of protein oxidation rates,²⁰⁹ and are moving towards *in vivo* proteome wide assessments of protein stability values. Future advancements in FPOP will likely continue along this trajectory, further enabling the measurement of protein and protein complex stabilities on a proteome scale.^{210,211}

2.2.4 Overview of protein ion activation methods—The activation of gas-phase ions is central to a number of methods associated with assessing the stabilities of proteins.²¹² Several approaches are available for increasing the internal energies of protein ions,²¹³ and if activated sufficiently, each method can provide unique patterns of fragment ions that, in many cases, can provide protein stability information. For example, collision induced dissociation (CID) often utilizes multiple low energy collisions with neutral gas, resulting in ions that undergo a relatively slow (microsecond timescale) accumulation of rotational/energy (Fig. 2C).²¹⁴ In contrast, surface induced dissociation (SID) utilizes fast (sub-microsecond) ion-surface collisions to impart rotational/vibrational energy to protein ions in a single step.²¹⁵ The different ion activation timescales accessed by CID and SID methods can lead to dramatically different fragmentation patterns in large protein complexes, with the former favoring the ejection of unfolded subunits and the latter producing multi-protein sub-assemblies as product ions.²¹⁵ ECD and ETD methods utilize electron capture/transfer and can produce fragment ions on shorter timescales than those accessed by CID and SID, leading to the rapid excitation of protein electronic states and the formation of product ions that can reveal unstructured regions within protein ions, adding further granularity to gas-phase protein stability assessment efforts.²¹⁶ Lastly, photo-activation of gas-phase ions provides a flexible platform for assessing protein stability values, with black body infrared dissociation (BIRD) and infra-red multi-photon dissociation (IRMPD) providing direct access to ion internal temperature information through a slow (microsecond) heating process, and thus, information regarding protein ion dissociation energetics. Ultra-violet photo-dissociation (UVPD), in contrast, produces a fast (sub-nanosecond) activation step primarily used to efficiently fragment the protein backbone. For each of the ion activation techniques listed above, the energy deposition timescale accessed dictates much of the eventual product ion population, as well as the utility of such data for protein stability measurements.^{217,218}

2.2.4.1 The energetics of protein ion unfolding and dissociation in the gas phase: Since the inception of Rice–Ramsperger–Kassel–Marcus (RRKM) theory,²¹⁹ which provides access to micro-canonical rate constants and barrier heights associated with primarily small molecule unimolecular decay reactions, a detailed description of the energetics underlying molecular decomposition events observed by MS has been developed. A similarly detailed description has been sought for gas-phase protein ions, with most efforts focusing on

activation methods that utilize relatively slow energy addition steps.²¹⁷ For example, BIRD has been used to evaluate the energetic details associated with the gas-phase dissociation of both small and large molecules.²²⁰ Specifically, BIRD measurements revealed exceptionally large pre-exponential factors associated with the temperature dependent rate constants determined for the dissociation of Shiga toxin oligomers, indicating the formation of highly disordered intermediates.²²¹ More recently, a framework for quantifying energy deposition during collisional activation has been described and used to evaluate the internal temperatures of protein complex ions undergoing SID.²²² In addition, this framework has been extended to evaluate the energetics associated with CID and CIU processes in protein ions.²²³ Future efforts will seek to deploy such detailed knowledge of ion temperature to further evaluate the information content of gas-phase protein stability measurements.

2.2.5 Activation and Dissociation Techniques for Protein Stability Assessment

2.2.5.1 Collision Induced Dissociation: Early observations of protein complex CID^{60,224,225,226} revealed product ion distributions dominated by highly-charged monomers and remaining oligomers stripped of both monomers and their associated charge. Such asymmetric charge partitioning between monomers and stripped oligomers can be rationalized based on the mobile proton model^{227,228} coupled with the unfolding ejected monomers. Initial observations of asymmetrically charged CID product ion populations for protein complexes were rapidly broadened to include a wide range of protein assemblies, establishing the asymmetric charge partitioning model as the standard mechanism underlying protein complex CID.^{150,221,229} Subsequent measurements indicated that this mechanism can be readily shifted to include folded monomers and sub-complexes, as well as peptide fragmentation channels, upon charge manipulation of the precursor complex.²³⁰ A more detailed understanding of protein complex dissociation and unfolding was obtained by deploying IM-MS to monitor the sizes of collisionally-activated protein complexes.¹³³ Early IM-MS experiments focused on the tetrameric transthyretin (TTR), directly observing increases in IM drift time upon collisional activation that allowed protein unfolding to be confidently invoked within protein complex CID mechanisms for the first time.²³¹ In addition, these observations gave rise to current generation CIU technology (*see* Section 2.3.2), a method capable of directly assessing gas-phase protein stabilities.

Quantitative assessments of the dissociation pathways produced upon the collisional activation of protein complexes can be used to investigate the stabilities of target complexes, alongside subunit composition, topology and protein-protein interaction strengths.^{232–234} Such an analysis was conducted for a series of small heat shock protein oligomers, and revealed the presence of different protein-protein interaction strengths and subunit stability values could be detected and quantified in such systems.²³⁴ In a separate study, CID pathway analysis was used to assess the stabilities of TTR and one of its amylogenic variants, ultimately determining that the wild type (WT) protein complex to be the more stable variant.²³⁵ For further detail concerning the theory and practice of protein complex CID, a number of excellent review papers are available.^{213,214,227,228,236–238}

The extent to which the CID mechanism can be altered through precursor charge manipulation remains an active area of research, and progress in this area may open new avenues for gas-phase protein stability measurements.^{234,236–240} For example, a gas-phase corona discharge probe can be used to on-line charge reduction, and this approach can prepare ions that eject compact protein product ions upon CID over a wide range of oligomeric states and masses (12 – 233 kDa).²⁴⁰ Furthermore, in cases where no subunit was ejected, peptide fragmentation can be captured from such charge-reduced precursors.²³⁰ Clearly, precise control over both the charge states and internal energies of proteins is necessary to maximally utilize CID for protein stability measurements.

2.2.5.2 Surface Induced Dissociation: In the context of protein stability measurements, SID is often utilized to evaluate the binding strength associated with protein-protein interfaces within larger assemblies.²¹⁵ Previous work has indicated that charge reduced complexes provide a dramatically improved ability to access to sub-complex product ions and a greater fidelity to the relative sizes of protein-protein interfaces.^{241,242} SID has been deployed to evaluate the structures of tetrameric protein complexes in this fashion, finding strong correlations to SID product ion populations and the relative stabilities of the interfaces between dimers and monomers within such structures.^{243–245} SID has also been used to detect differences in the stabilities of ligand-bound tetrameric protein complexes in a manner dependent upon ligand binding strength and location.²⁴⁵ The stabilities and structures of large protein complexes, including the GroEL tetradecamer, have also been reported.²⁴² A broader evaluation of SID applications and instrumentation is available in recent reviews.^{215,246}

2.2.5.3 Electron-Capture and Transfer Dissociation: ECD utilizes low energy electrons (1eV), (Figure 2C), whereas ETD uses anions to transfer electrons to precursors, both of which typically act to produce a similar population of product ions associated with protein backbone cleavage.^{247,248} A large number of excellent reviews cover the theory and application of ECD and ETD technology, much of which is focused on protein sequencing.^{216,249–251} Many reports also focus on evaluating ECD/ETD fragmentation from the point of view of evaluating protein structure, as data strongly indicates that fragment ion production can be linked to unstructured regions within protein sequence or surface-accessible reaction sites.^{216,252,253} The content below will focus primarily on the use of these tools for assessing protein stabilities.

ECD has been combined with collisional activation in order to survey the stabilities of multiple protein systems.^{254,255} For example, ECD has been used to evaluate the stability of the kinase inducible domain (KIX), revealing that its three helices unfold similarly in both the gas phase and in solution.²⁵⁴ In a separate study, ECD data was able to differentiate horse and tuna heart cytc on the basis of stability.²⁵⁵ A large number of reports have focused on the ability of ECD to evaluate monomeric protein structures, and in this area is the subject of multiple excellent reviews.^{216,252,253} Initial applications of ECD to multi-protein complexes focused on alcohol dehydrogenase (ADH) tetramer ions, which revealed the presence of an N-terminal fragmentation pattern correlating with those regions of its X-ray structure possessing high b-factors,²⁵⁶ Atomic displacement parameter,

temperature factor or b-factor indicate regions of disorder in a crystal structure.²⁵⁷ ECD fragmentation being produced from regions with high b-factors is an observation that was subsequently extended to other protein assemblies, indicating the utility of ECD to characterized disordered regions.^{258–260} When combined with IM-MS, ECD acquired for protein complexes can provide a deeper level of structural insight associated with detected differences in protein stability.^{151,261–265} For instance, the gas-phase unfolding of Hb tetramer ions was characterized by both IM and ECD, establishing that protein unfolding occurs first at the termini of both the α and β subunits of the complex, and that these unfolded regions are correlated with elevated b-factor regions within X-ray data.²⁶⁶ As a further example, the structural differences between WT and mutants of the metamorphic protein lymphotactin have been quantified based on a combination of IM-MS and ECD data.²⁶² In another study, ECD fragmentation and IM-MS data streams were used to jointly constrain a coarse-grained model of variant apolipoprotein E oligomers, which together suggested the presence of a tetramer possessing C4 symmetry.²⁶⁵ Finally, IM-MS combined with ECD was able to identified a compacted bound state alongside binding site information for a molecular tweezer-type compound attached to tau protein monomers.²⁶⁴

More recently, ETD fragmentation has been used to investigate protein stability and structure.²⁶¹ In an early set of experiments in this area, ETD data was collected on ADH tetramers.²⁵⁶ In contrast to prior ECD work, ETD fragmentation was not correlated to regions of the sequence possessing enhanced b-factors, but instead was correlated with the solvent accessible surface area (SASA) of the complex. ETD methods have subsequently been extended to monitor the unfolding of both Concanavalin (ConA) and Hemoglobin (Hb) tetramer ions.²⁶³ When combined with IM-MS, ECD acquired for protein complexes can provide a deeper level of structural insight associated with detected differences in protein stability.^{151,260–264,267} For instance, the gas-phase unfolding of Hb tetramer ions was characterized by both IM and ECD, establishing that protein unfolding occurs first at the termini of both the α and β subunits of the complex, and that these unfolded regions are correlated with elevated b-factor regions within X-ray data.²⁶⁰ As a further example, the structural differences between WT and mutants of the metamorphic protein lymphotactin have been quantified based on a combination of IM-MS and ECD data.²⁶² In another study, ECD fragmentation and IM-MS data streams were used to jointly constrain a coarse-grained model of variant apolipoprotein E oligomers, which together suggested the presence of a tetramer possessing C4 symmetry.²⁶⁷ Finally, IM-MS combined with ECD was able to reveal a compacted bound state alongside binding site information, for a molecular tweezer-type compound attached to where in the structure the tweezer had bound to tau protein monomers.²⁶⁴ Taken together, the studies above project a bright future for ECD/ETD methods as a vital technology for annotating the structural details associated with shifts in protein stability.

2.2.5.4 Ultraviolet Photodissociation: Through the absorption of monochromatic UV light, proteins and their assemblies can quickly cross the energy barriers associated with covalent bond dissociation, making UV photodissociation (UVPD) a useful technology for top-down protein sequencing applications.²⁶⁸ When applied to intact protein complexes, UVPD at lower laser powers engenders the formation of highly-charged unfolded protein

product ions and stripped complexes similar to those observed in CID datasets, but when laser fluence is increased, symmetric charge portioning among product ions is typically observed.²⁶⁹ Recent work points to the potential of UVPD for characterizing of protein stability. For example, 193 nm UVPD has been used to assess the stabilities of a range of model protein tetramers.²⁷⁰ Additionally, UVPD has been used to localize and characterize the conformational changes that occur upon ligand-binding.^{266,271,272} Further, UVPD has detected the effects mutations within the proto-oncogene K-Ras and its kinase binding partner Raf.^{273,274} Lastly, UVPD fragmentation has been observed to reflect IM-based measurements tracking protein unfolding in either solution or the gas phase.^{275,276} Recent reviews are available covering advancements in UVPD technology.²⁶⁸

2.3 Ion Mobility Spectrometry

2.3.1 Fundamental principles and general Applications—IM is a technique that separates gas-phase protein structures based on their size and charge and has emerged as a useful technique for the characterization of protein structures and stabilities.²⁷⁷ In a simple IM experiment, ions are collected in a pre-IM region, and released in concentrated ion packets into a drift tube filled with inert gas (typically N₂ or He). In the drift tube, ions are separated based on their mobility under the influence of a weak electric field. The amount of time an ion takes to traverse the drift tube, defined as drift time (DT), can be converted to a collision cross section (CCS), a parameter directly related to the size and shape of an ion.²⁷⁸ Early native IM-MS (nIM-MS) data confirmed that gas phase ions retain compact native-like structures in the absence of bulk solvent.^{231,239,279–281} Many IM analyzers are available and offer a range of capabilities for the detailed analysis of gas-phase protein structure and stability, primarily through CCS values, which can often be directly correlated to molecular models in order to assess protein structures.^{132,282} When coupled to MS, CCS values can be correlated with ion compositions that reveal the influences of sequence changes^{283,284}, ligand binding²⁸⁵, or post-translational modifications^{286,287} on protein structure and stability.

2.3.2 Collision Induced Unfolding—Recent advancements in IM-MS technology have enabled the acquisition of both protein structure and stability information simultaneously through CIU experiments. CIU is accomplished by increasing the internal temperature of protein ions in a stepwise fashion prior to IM separation through exposure to activating collisions with a background gas (Figure 2E). Typically, large proteins will undergo several CIU transitions resulting in increased IM drift times. Plots of collision energy against IM drift time (or CCS) can be generated in order to capture and quantify the transitions detected. The resulting CIU ‘fingerprints’ have the ability to capture stability shifts associated with changes in protein domain structure^{283,284,288–292}, anion and cation adduction^{293–295}, disulfide bonding patterns¹⁰⁰, glycosylation²⁹⁶, as well as ligand and cofactor binding^{109,297–300}. Currently, efforts are being made to hyphenate CIU with online separation methods^{301–303}, expand the number of commercially available IM-MS instrument platforms capable of CIU^{108,304–306}, develop the technique for middle-level protein structure analysis³⁰⁷, and to further illuminate the fundamental principles of CIU²²². There have also been considerable efforts to integrate CIU with other structurally-sensitive MS-based probes,

producing datasets of high dimensionality and enabling a deeper level of protein structure analysis than was previously possible (Figure 3).

2.4 Proteome-wide Stability Measurements

An array of LC-MS technologies based on the bottom-up assessment of protein mixtures are currently providing exciting insights into protein stabilities across entire proteomes. Tools based on HDX and oxidative labeling technology are now used to regularly probe the impact of bioactive compounds on protein stabilities on a proteomic scale.^{163,209,308} Other methods capable of comprehensively surveying protein stability seek to quantify the soluble proteins that remain following a thermal shock.^{110,309} The remaining soluble, folded proteins are then quantified by LC-MS based proteomics. Using this method, protein stabilities have been captured across the cell cycle, revealing that protein stability is correlated with enzyme activity, DNA-binding, and protein complex formation. Similar thermal shock based proteome assays have been applied across 13 species covering all domains of life.¹¹⁰ These experiments have produced the most comprehensive assessment of protein stability currently available, and the resulting ‘meltome’ atlas highlighted stability differences between protein classes, with those involved in cellular respiration granted particularly large stabilities. LC-MS analysis following the limited digestion of protein mixtures under native conditions with a non-specific protease prior to LC-MS interrogation is another widely used method to probe protein stabilities on a proteome-wide scale.³¹⁰ Stable proteins remain more protease resistant than those that are less stable, and thus methods associated with quantitative proteomics can readily quantify protein stabilities under such conditions. Finally, FPOP experiments have recently been carried out within cells, paving the way for a new generation of proteome-wide stability data. The technology relies on a Platform Incubator with movable XY stage (PIXY), which allows for both the growth of cultures and FPOP experiments to be carried out within the same optical bench.³¹¹ Irradiation of one sample well takes 20s of analysis, which allows the study of protein folding and signaling in a time-dependent manner. For more details regarding current MS-based workflows for proteome-wide structural studies, a recent review is available.³¹²

3. Biotherapeutics

Monoclonal antibodies, or mAbs, currently represent the largest and most successful class of biotherapeutics available.^{313,314} Details surrounding the structures and immunological action of IgGs, the most common antibody isotype used for biotherapeutic development, can be found in previous reports.³¹⁵ Unlike small molecule therapeutics, IgGs are composed of over 12,000 atoms, thus dramatically increasing their relative complexities, and projecting myriad challenges for current pharmaceutical measurement science.^{316–318} Specifically, the relative dynamism of mAbs (relating to their structures’ hinge region which provides flexibility allowing the structure to dynamically encompass a large conformational range of positions) creates difficulties in rapidly assessing higher order structure (HOS), a key factor associated with biotherapeutic safety and efficacy.^{319–322} Structurally-sensitive MS methods have evolved into critical tools in the rapid analysis of mAb HOS characterization.¹²² MS is compatible with a wide range of LC methods, and as such, LC-MS based methods are used throughout the pharmaceutical industry within multi-attribute monitoring (MAM)

workflows.^{323–325} Furthermore, recent advancements in nMS and nIM-MS techniques have demonstrated potential for MAM characterization of mAb HOS, aggregation, degradation, and stability. Figure 4 illustrates the information content that can be achieved with thermal stability, MS, and IM-based methods.

3.1. Stability Analysis of Biotherapeutic Antibodies

3.1.1 Trends in MS-based mAb Stability data—MS-enabled stability assessments can be carried out in solution and in the gas-phase, depending upon the needs of the application in question. Variable temperature experiments are often used to monitor the shift from native mAb solution structures to unfolded structures and quantify melting point temperatures, T_m , while gas-phase technologies (e.g. IM-MS) can be deployed to detect changes in gas-phase mAb structures in a manner correlated with temperature changes in solution, or in a CIU mode (Fig 4A). Importantly, different mAb compositions and mass-resolved therapeutic modalities can be individually interrogated in MS-enabled stability assessments in manner that is challenging to replicate with other technology platforms. Biosimilar mAb products by design aim to recapitulate the T_m of their mAb reference. Shifts in variable temperature data can indicate either destabilized or stabilized structures, respectively (Fig 4B). Shifts in variable temperature MS data acquired for fusion protein samples will depend strongly on the type of fusion protein being measured (e.g. Fc vs Fab, smaller vs larger protein, etc.), but such assays can reveal both shifts in stabilities as well as new T_m features when compared with parent molecule data (Figure 4C). For variable temperature experiments targeting antibody-drug conjugates (ADCs), shifts in mAb stability can be tracked as more drug molecules and linkers are conjugated to the protein, which is often progressively destabilized as the drug-to-antibody (DAR) ratio is increased (Figure 4D). For bispecific mAbs, variable temperature MS data is expected to reveal an intermediate stability relative to the T_m values recorded for parent mAb structures used in its generation (Figure 4E).

3.1.2. Standard and Variable Temperature MS—Clearly, the assessment of mAb primary structure remains a key MS-related task in biotherapeutic discovery and development, as such changes can induce altered therapeutic stability and efficacy profiles.^{122,326,327,328,329,330} In concert with these standard MS measurements, variable temperature ESI-MS data is growing in utility in this area.¹⁶¹ For example, such methods have been used to characterize changes in IgG stability through the detection of both degradation products and non-native disulfide bonds within mAb samples heated within the ESI source.³³¹ In general, MS-based methods have a central and growing role in assessing mAb HOS and stability.

3.1.3 Footprinting MS—Footprinting techniques such as covalent labelling, HDX, and FPOP provide a direct and granular assessment of antibody stabilities.³³² Covalent labelling measurements can reveal subtle changes to HOS,³³³ structural integrity,³³⁴ and antigen interactions.¹⁶² HDX exchange methodologies are well established for the assessment of mAb stability,³³⁵ and are increasingly used to determine elements of biotherapeutic HOS.^{336,337} FPOP methods have also demonstrated their general utility in

the characterization mAb HOS,^{338,339} and have specifically excelled in the area of epitope mapping.^{340,341}

3.1.4 IM-MS and CIU—IM-MS and its associated methods have been shown to be able to differentiate between mAb subclasses based on disulfide bonding patterns,^{100,284,342,343} differences between innovators and biosimilars,^{323,344–347} glycosylation patterns,^{100,296,348} HOS structure associated with domain exchange,²⁸³ and ADC drug loadings.¹⁰¹ Variable temperature IM-MS, achieved altering the temperature of the IM drift gas, can also be used to detect changes to antibody HOS.³⁴⁹ In general, IM data has been particularly useful as a probe for subtle changes in HOS that may go undetected by MS alone³⁵⁰ despite the fact that gas-phase mAbs typically undergo significant collapse in the gas-phase.³⁵¹

In parallel with the above approaches, the capabilities of CIU to capture stability shifts associated with protein domain structure,^{283,284,288–292} anion and cation adduction,^{293–295} as well as ligand and cofactor binding in mAbs has been well documented.^{109,297–300} Additionally, CIU has been shown to be able to track differences in mAb disulfide bonding¹⁰⁰ (Figure 5A), glycosylation patterns,²⁹⁶ ADC drug loading¹⁰¹ (Figure 5D), domain exchange²⁸³ (Figure 5A), HDX-uptake,³⁴⁴ light chain variants,³⁰⁴ and bispecific stoichiometries^{284,307} (Figure 5C), in addition to probing the subtle differences between innovator and biosimilar mAbs^{323,346,347,352} (Figure 5B). IM-MS and CIU technologies are currently well positioned to provide information-rich, rapid assessments of mAb HOS across a wide range of therapeutic modalities.

4. Membrane Proteins

Membrane proteins (MPs) are important therapeutic targets which play vital roles in cellular function^{353,354}, they represent over 60% of therapeutic drug targets and nearly 80% of drugs approved by the FDA act on membrane proteins.^{354–357} As the structure of a protein is closely linked to its function, the characterization of membrane proteins is vital to elucidating their involvement in disease and potential druggability.³⁵⁸ Despite this, membrane proteins are underrepresented in structural databases due to challenges associated with their hydrophobicity and difficulty in obtaining high purity samples. Recent reviews form an excellent resource that outline both the current challenges and promise surrounding membrane protein structural biology.^{359–365} Native MS has emerged as a method capable of handling the polydispersity of membrane proteins samples to yield details of their structure and function,³⁶⁶ this section will discuss how MS has elucidated new information regarding membrane protein stability.

To conserve the native structure of membrane proteins in the absence of a cell membrane, multiple solubilization techniques have been developed. Historically, detergent micelles have been the most popular technique for solubilizing purified membrane proteins and they remain widely used today.^{367,368} Detergents vary in structures and charges, but all possess the ability to form a micelle that can encapsulate membrane protein targets. The hydrophobic membrane protein residues interact with the hydrophobic tails of the detergent and the polar head groups of the detergent allow the complex to be solubilized^{365,368,369}. Among the many newer approaches to create a lipid bilayer structure in contrast to the single

lipid layer created by micelles, bicelles and nanodiscs are most commonly used^{370–372}. In bicelles, detergents are used to surround the edges of the bilayer to create a fluid, yet discrete, bilayer structure^{373,374}. Other bilayer-based solubilization techniques include Styrene maleic acid lipid particles (SMALPs)^{375,376}, amphipols³⁷⁷, and lipid vesicles or liposomes.³⁷⁸ The details surrounding membrane mimetics are well covered in a number of recent review articles.^{370,379–381}

4.1 Stability Analysis of Membrane Proteins

4.1.1. Mass Spectrometry—Mass spectrometry has recently emerged as a method especially suited for the analysis of native MPs, due in part to its ability to handle complex mixtures and lower sample concentrations.^{366,382–390} For the purposes of this work we will focus on ESI (and nESI) methodologies, which dominate the analysis of native protein samples. MP ions generated through ESI or nESI for nMS analysis are most often still encapsulated in one of solubilization agents mentioned above, and therefore collisional activation must be applied to remove bound detergents or lipids from the target membrane protein ion³⁹¹. Additionally, the independent solubilization agents themselves, *e.g.* detergents, also ionize and can thus result in an abundance of noise signals in the resulting native mass spectra. While time-of-flight (TOF) mass analyzers have shown great success in this field, higher resolution technologies, such as Orbitrap mass analyzers³⁹², can be helpful for resolving the intended membrane protein signal from noise. It is important to note that not all solubilization agents are equally effective in this endeavor, and screening detergents, solution conditions, and optimizing instrument parameters is a necessity for striking the delicate balance between the removal of solubilization agents and optimizing the stability of native MP structure³⁹³. However, with successful optimization, nMS has been used to study discrete lipid^{393–397} and ligand binding³⁹⁸ (Figure 6 A & F) events, as well as quantifying the thermodynamics associated with lipid binding^{399–402} (Figure 6B), and specific protein-protein interactions associated with a wide range of MPs^{403–405}, and their functional assemblies^{374,378,406–409} (Figure 6C).

4.1.2. Footprinting MS—Deeper structural insights can be gained from MP by deploying LC-MS techniques in combination with chemical labeling, where the solvent accessible sites of native MPs are labeled permanently or reversibly prior to digestion. HDX,^{410–413} CXL,^{414–416} and FPOP,^{201,417–419} have all been used to probe MP tertiary structures, as well as the interactions between MPs and both protein binding partners and solubilization agents (Figure 6G). Much of the mechanics of MS-based footprinting tools targeting MPs remain similar to those directed towards water soluble protein systems, and typically provide a valuable readout capable of monitoring the conformational responses of MPs upon stimulation, both temporally and spatially. Among the labeling techniques surveyed here, FPOP has most often been applied to assess MP structure and stability. Favorable attributes of FPOP for MP-associated applications include its fast labeling times, the irreversible nature of the chemical modifications generated, the neutrality of pH maintained during the measurement, and its ability to access non-polar residues for labeling.^{206,417}

4.1.3. IM-MS and CIU—The addition of IM separation coupled to MS is beneficial for nMS of MPs. Additionally, IM-MS platforms include supplemental trapping regions that can provide opportunities to perform collisional activation aimed at both the liberation of membrane proteins from their solubilization agents as well as the dissociation of detergent or lipid clusters, which can greatly increase signal quality. The energy experienced by ions in these trapping regions is a function of an accelerating potential (collision voltage, CV), and, ideally, optimized solubilization systems can be removed at relatively low CVs. At CVs higher than the threshold for removal of the solubilization agents, the membrane protein can experience CIU, and this unfolding can be tracked through the resulting IM arrival time distribution⁹⁸. These CIU experiments are valuable for assessing the relative gas phase stability of membrane protein complexes^{397,420} and have been used in the past to classify soluble protein systems¹⁰⁹. The addition of IM to nMS allows for the elucidation on protein stability as a function of lipid and or ligand binding^{103,400,420,421} (Figure 6F), amino acid sequence in relationship to disease pathologies^{104,409} (Figure 6D), and lipid and or ligand binding locations¹⁰³ (Figure 6E).

The study of membrane proteins represents an exciting, high-risk, high-reward area of research with the potential for groundbreaking medical discoveries. Methods to study MP stability in native-like environments are essential to understanding how they perform their cellular functions and, as many MPs are implicated in human disease, how pharmaceuticals may be developed to correct their dysfunction. Due to their insolubility in aqueous solutions and the complex environments in which they exist natively, MPs are challenging analytes, and MS has emerged as frontier tool for determining their stabilities.

5. Amyloidogenic Proteins

Amyloidogenic proteins are soluble proteins that can undergo conformational changes that result in the formation of amyloid fibrils which are typically characterized as highly organized states, rich in beta-sheet secondary structure.⁴²² Amyloids and amyloidogenic proteins are commonly associated with protein misfolding and a wide range of human diseases. The most well-known examples of amyloidogenic proteins include amyloid beta (A β) and α -synuclein (α -syn), key proteins associated with Alzheimer's disease (AD) and Parkinson's disease (PD) respectively. AD and PD together account for over 60% cases of dementia and created healthcare cost of over 400 billion USD in 2021 in the U.S.^{423,424} However, not all amyloidogenic proteins are disease-related, as some produce functional amyloids that have well-defined physiological roles in many organisms, including in humans, bacteria and an array of animal species.⁴²⁵ Interestingly, amyloid formation appears to be a general property of proteins. With the sufficient application of heat, mechanical, or pH-based stress, stable proteins, such as lysozymes, can misfold and form amyloid fibers.⁴²⁶ The stability of amyloidogenic proteins related to human diseases is of great importance, as this characteristic is likely linked to their disaggregation and clearance *in vivo*. In this section, we will focus on how the stabilities of amyloidogenic proteins are studied through MS related techniques.

5.1. MS-related Stability Analysis of Amyloidogenic Proteins

Proteins prone to misfolding and amyloidogenesis present unique challenges in their stability measurements, as such systems aggregate to produce a wide range of oligomers, some of which are insoluble under the conditions designed to promote native protein structures. While most MS tools target only soluble oligomers for analysis, charge detection MS has been used to study the mass distribution and polymorphism of insoluble amyloid fibers.⁴²⁷ Conventional mass spec measures m/z alone, with high molecular weight ions, the charge must be deducted from the charge state envelope, this resolution is often lacking for very large ions. CDMS allows for the simultaneous detection of m/z and the charge. This allows the centroid mass of individual ions to be calculated regardless of the complexity of the spectral features contained within the data analyzed. In addition, since CDMS allows for direct mass determinations, the technology enables larger ions to be measured than is typically achievable using standard MS technologies. CDMS is especially useful in interrogating the heterogeneous aggregates generated by amyloid proteins during fibril formation. For example, fibrils of A β 1–42, tau and α -synuclein have been individually assessed by CDMS by Pansieri et al.⁴²⁸ In combination of TEM data, CDMS mass distribution data revealed two bundles of A β 1–42 with a low mass of 20 MDa and a high mass of 55 MDa. The authors also reported on the diversity of fibril mass and morphology of tau and α -synuclein.⁴²⁸ A detailed review on CDMS and its various applications including amyloid proteins has been recently covered by Keifer et al.⁴²⁹

In general, native MS can transmit soluble protein oligomers in order to make targeted stability measurements linked to specific variants or ligand-bound oligomers.^{430,431} (Figure 7A) Amyloidogenic proteins that natively occupy an assembly state can also be targeted by nMS. For example, amyloid inhibitors targeting the misfolding-prone L55P mutant of TTR have been screened using nMS, where the intensity of the intact tetramer was tracked, alongside subunit exchange dynamics, to assess potential compound efficacy.^{432,433}

Identifying and quantifying the oligomeric species present in amyloidogenic protein samples is critically important for assessing the mechanisms surrounding amyloid-associated disease.^{434,435} When probed by nMS, amyloidogenic proteins typically produce multimodal CSDs with the lower charge states correlating to compact, native-like conformers, and higher charge states corresponding to partially unfolded forms of the protein. The addition of IM separation prior to nMS has served to help quantify the conformational flexibility of amyloidogenic proteins.⁴³⁶ (Figure 7B) Interestingly, IM-MS has supported the classification of many amyloidogenic proteins as intrinsically disordered proteins (IDP), due to their polydisperse distributions of ground state structures.^{437–439} IM-based CCS information has been used to probe the stability of β -2 microglobulin (β -2m) through a series of titration experiments.⁴⁴⁰ Barran *et al.* has reported stability changes associated with both WT and mutated p27 IDR, the disordered region of the cell cycle inhibitory protein p27kip1.⁴⁴¹ The use of IM-MS also enables CIU analysis (Figure 7C).⁴⁴² For example, Dong *et al.* employed CIU to track metallothioneins, a group of intrinsically disordered proteins, and their interactions with a series of metals, finding that stability changes can be used to separate the metal associated complexes that are otherwise difficult to distinguish based solely on their IM profiles.⁴⁴³ Finally, Sanders *et al.* observed that binding of the

chaperon protein β -casein can stabilize α -lactalbumin from forming amyloids using CIU.⁴⁴⁴ Overall, nMS, IM-MS, and CIU have provided key stability information for a wide range of amyloidogenic proteins and complexes.

If more localized stability information is desired, HDX-MS allows the exploration of the site-specific conformational status of amyloidogenic proteins (Figure 7D). An increasing number of reports have focused on HDX-based stability values, demonstrating the wide applicability of this technique. For instance, HDX-MS has been used to examine the stability of protofibrils associated with many amyloid-related proteins, including $A\beta^{445}$ and β -2m.⁴⁴⁶ Moreover, pulsed HDX can capture $A\beta$ stability data on the millisecond timescale, providing unique snapshots of the protein aggregation process. Pulsed HDX-MS is especially useful in studying proteins that undergo rapid aggregation, like $A\beta_{(1-42)}$, where continuous HDX-MS labeling methods have proven challenging to use in the same manner.⁴⁴⁷ Lastly, both FPOP and covalent crosslinking can be readily coupled to MS for online evaluation of protein aggregation, and have been covered in depth by Johnson *et al.*²⁰¹ and O'Reilly *et al.*⁴⁴⁸ respectively.

6. Heat Shock Proteins

Heat shock proteins (HSPs) are a class of molecular chaperones which particularly benefited from the emergence of nMS, and gas-phase methods for assessing protein stability. HSPs are biologically expressed in response to a variety of cellular stressors, and play critical roles in cell survival, preserving cellular homeostasis.⁴⁴⁹ A class of smaller HSPs (sHSP) with monomeric molecular masses ranging from 12–42 kDa are largely ATP-independent chaperones which sequester early unfolded intermediates, or misfolded proteins in the cell to prevent aggregation.⁴⁵⁰ All sHSPs share a conserved α -crystallin domain (ACD), and these domains interact to first form a dimer which then can be a part of a range of large heterogenous complexes often exceeding 1 MDa.⁴⁵⁰ The inherent complexity of these mixtures has precluded their characterization by traditional biophysical techniques, however nMS enables the separation of these complexes via their molecular masses, enabling a detailed dissection of the sHSP assemblies which often coexist in solution. Historically, MS-enabled work in this space has been dominated by three main approaches: Tandem nMS experiments using CID, HDX-MS, and subunit exchange experiments evaluated by MS detection. In recent years, nIM-MS has also emerged for the assessment of sHSP 3D structure, given the heterogeneous nature of the complexes typically encountered in such samples. Most of the studies discussed in the section below integrate multiple MS-based data streams in order to comprehensively probe the stability of HSP complexes.

6.1. Stability Measurements Based on native and HDX-MS

The sHSP α -crystallin (α C) has been investigated extensively by MS leading to a wealth of structure and stability information for this otherwise refractory system. Found primarily in the eye lens, α C is a hetero-oligomer of α B-crystallin (α BC), and α A-crystallin (α AC). Large α C complexes act as ATP-independent chaperones to prevent the non-specific aggregation of β - and γ -crystallins, the main protein components of the eye lens.⁴⁵¹ Initial MS analysis of α C complexes primarily employed nMS and CID to dissect the

extreme complexity of α C samples. Gas phase activation by CID allowed the authors to deconvolute this protein mixture, demonstrating that α C hetero-oligomers ranging from 24-mers to 33-mers (up to ~ 1 MDa), co-exist in such samples.⁴⁵² In the years since, nMS in combination with CID⁴⁵³ has been used to measure changes in the stabilities of α C complexes in response to post-translational modifications^{454,455} or changes in primary sequence.^{453,456,457} HDX-MS studies have also played a key role in characterizing the stability of α C complexes in response to heat shock, showing that despite the inherent thermos-stability encoded in sHSPs, α C undergoes a structural transition that weakens protein-protein interfaces when incubated at elevated temperatures.⁴⁵⁸ Lastly, using nMS to track subunit exchange within large complexes, a 5 residue C-terminal truncation of one of the two α C subunits was revealed to retard complex formation substantially while also leading to a marked shift in the equilibrium of complex stoichiometry in order to significantly favor even numbered complexes.⁴⁵³ Further subunit exchange MS experiments explored the varying stabilities of the individual α AC and α BC subunits,⁴⁵⁹ as well as their stabilities in heterocomplexes in response to changes in temperature,⁴⁶⁰ and pH.⁴⁶¹ Together tandem nMS, HDX, and subunit exchange are responsible for much that is currently understood surrounding α C oligomer structure and stability.

6.2. Other MS-Enabled Stability Measurements

As nMS-based approaches have gained recognition for their ability to characterize α Cs, such tools were quickly adopted to characterize sHSP homologues from other organisms. Many of these studies utilize tandem nMS and subunit exchange to evaluate the stabilities sHSPs that contain α -Crystallin-like domains, and further established the mechanism by which these domains dictate sHSP complex formation and stability (Figure 8).^{51,234,462–469} Furthermore, variable temperature ESI sources in conjunction with aforementioned approaches have been used to explore the impact of solution-phase temperature increases on the stability of sHSPs.^{51,458,462,465} MS was also increasingly applied to the analysis of larger HSPs such as HSP70,^{470,471} and HSP90,^{472–475} which also oligomerize in a largely mono-disperse format to carry out their biological functions. Recent work has featured IM-MS to characterize the stabilities of sHSPs,^{73,76,476–478} as well as larger HSPs,⁴⁷⁹ often leveraging CIU^{73,76,476,478} to study the gas phase stabilities of such assemblies. Methods such as tandem nMS and subunit exchange have been deployed on IM-MS instruments, providing increased peak capacity and the ability to record CCS values for the ions detected, thus aiding future efforts to model large oligomeric HSP assemblies. The recent release of a cyclic IM-MS (cIM) instrumentation capable of IMⁿ offers unique opportunities to deconvolution the intricately complex mixtures such as those observed for α C and other sHSP complexes. Overall, MS-based approaches have contributed substantially to our knowledge of HSPs, as well as other chaperones,^{79,289,480–482} and these techniques are poised to continue playing a major role in the characterization of such complexes.

7. Conclusions and Future Outlook

Clearly, MS technologies are illuminating many previously hidden facets of protein stability. The range of technologies available, combined with the capabilities of MS to evaluate individual components within complex mixtures on the cellular scale, opens up new avenues

of investigation that will continue to be explored in the coming decades. As MS technology advances, armed with an ever-increasing appreciation for the correlations that exist between native proteins and their desolvated analogues, we can look forward to deeper, more granular assessments of protein stability shifts linked to a detailed view of protein structure. Ultimately, we can expect that MS-driven biophysical probes will give rise to comprehensive catalogs of protein stabilities across the universe of human proteoforms and structural states adopted under a wide range of conditions, ultimately leading to breakthroughs in biochemistry and the treatment of human disease.

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ABBREVIATIONS

Aβ	Amyloid Beta
ACD	α -Crystallin Domain
AD	Alzheimer's disease
ADCs	Antibody drug conjugates
ADH	Alcohol Dehydrogenase
BIRD	blackbody infrared radiative dissociation
CCS	Collision Cross Section
CD	Circular Dichroism
CID	Collision Induced Dissociation
CIU	Collision Induced Unfolding
CSD	Charge State Distribution
ConA	Concanavalin A
CV	Collision Voltage
DSC	Differential Scanning Calorimetry
DSF	Differential Scanning Fluorimetry
DT	Drift Time

ECD	Electron-Capture Dissociation
ESI	Electrospray Ionization
ETD	Electron-Transfer Dissociation
FDA	Food and Drug Administration
FPOP	Fast Photochemical Oxidation of Proteins
hACE2	human Angiotensin-converting Enzyme 2
Hb	Hemoglobin
HDX	Hydrogen Deuterium Exchange
HOS	High Order Structure
HSPs	Heat-Shock Proteins
ITC	Isothermal Titration Calorimetry
IM – MS	Ion Mobility - Mass Spectrometry
IR	Infrared
IRMPD	Infrared Multiphoton Dissociation
LC	Liquid Chromatography
mAb	Monoclonal Antibody
MAM	Multiple Attribute Monitoring
MP	Membrane Protein
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectrometry
NISTmAb	National Institute of Standards and Technology Monoclonal Antibody
NMR	Nuclear Magnetic Resonance
nESI	Nano-electrospray Ionization
nIM-MS	Native Ion Mobility – Mass Spectrometry
nMS	Native Mass Spectrometry
PD	Parkinson's Disease
PIXY	Platform Incubator with movable XY stage
RRKM	Rice–Ramsperger–Kassel–Marcus

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SASA	Solvent Accessible Surface Area
sHSP	Smaller Heat-Shock Proteins
SID	Surface-Induced Dissociation
SMALPs	Styrene Maleic Acid Lipid Particles
SUPREX	Stability of Unpurified Proteins from Rates of H/D Exchange
PLIMSTEX	Protein–ligand interactions by mass spectrometry, titration, and H/D exchange
TOF	Time-of-Flight
T_m	Melting Temperature
TTR	Transthyretin
vT	Variable Temperature
UV	Ultraviolet
UVPD	Ultraviolet Photodissociation
CXL	Chemical Cross-linking
αAC	αA-crystallin
αBC	αB-crystallin
αC	α-Crystallin
α-syn	α-synuclein

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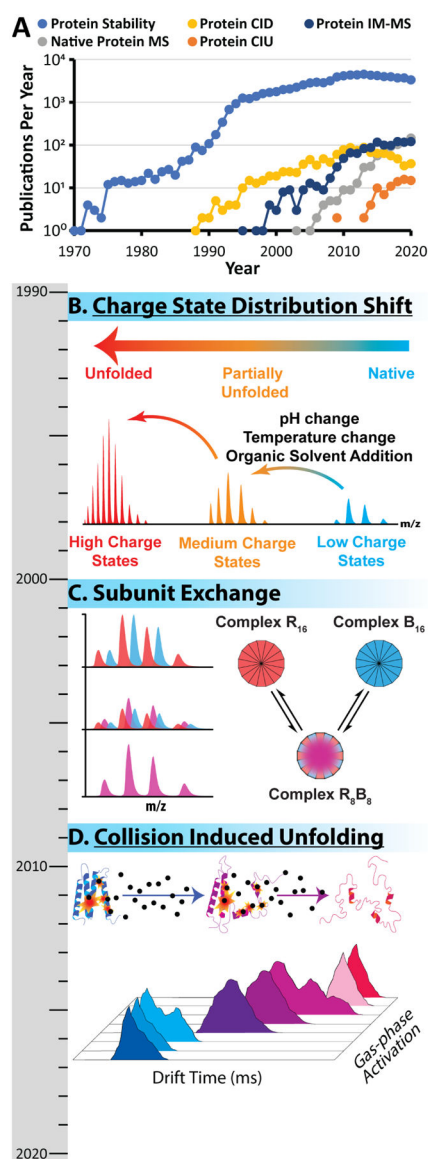
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**Figure 1.**

A) The total number of publications in the [pubmed.gov](https://pubmed.ncbi.nlm.nih.gov/) database for the search terms indicated in legend. **B)** The majority of early protein stability measurements conducted by MS (pre-2000) relied on the observation of CSD shifts. The blue CSD represents a “native” protein possessing lower charge states. Upon denaturation, the protein unfolds presenting a CSD centered at higher charge (lower m/z), occupying partially unfolded structures (orange CSD) enroute to a fully unfolded population (red CSD). **C)** Native MS emerged in the early 2000s, and one of the initial biological systems studied by this approach were α -crystallin proteins. MS is able to track the exchange between co-incubated homo-oligomers, which eventually leads to the formation of hetero-oligomers. **D)** Building upon these initial measurements, CIU employs gas phase activation in conjunction with IM, to observe the gas phase change in structure due to collisional heating. This approach is growing rapidly and is being applied to a broad variety of biological systems.

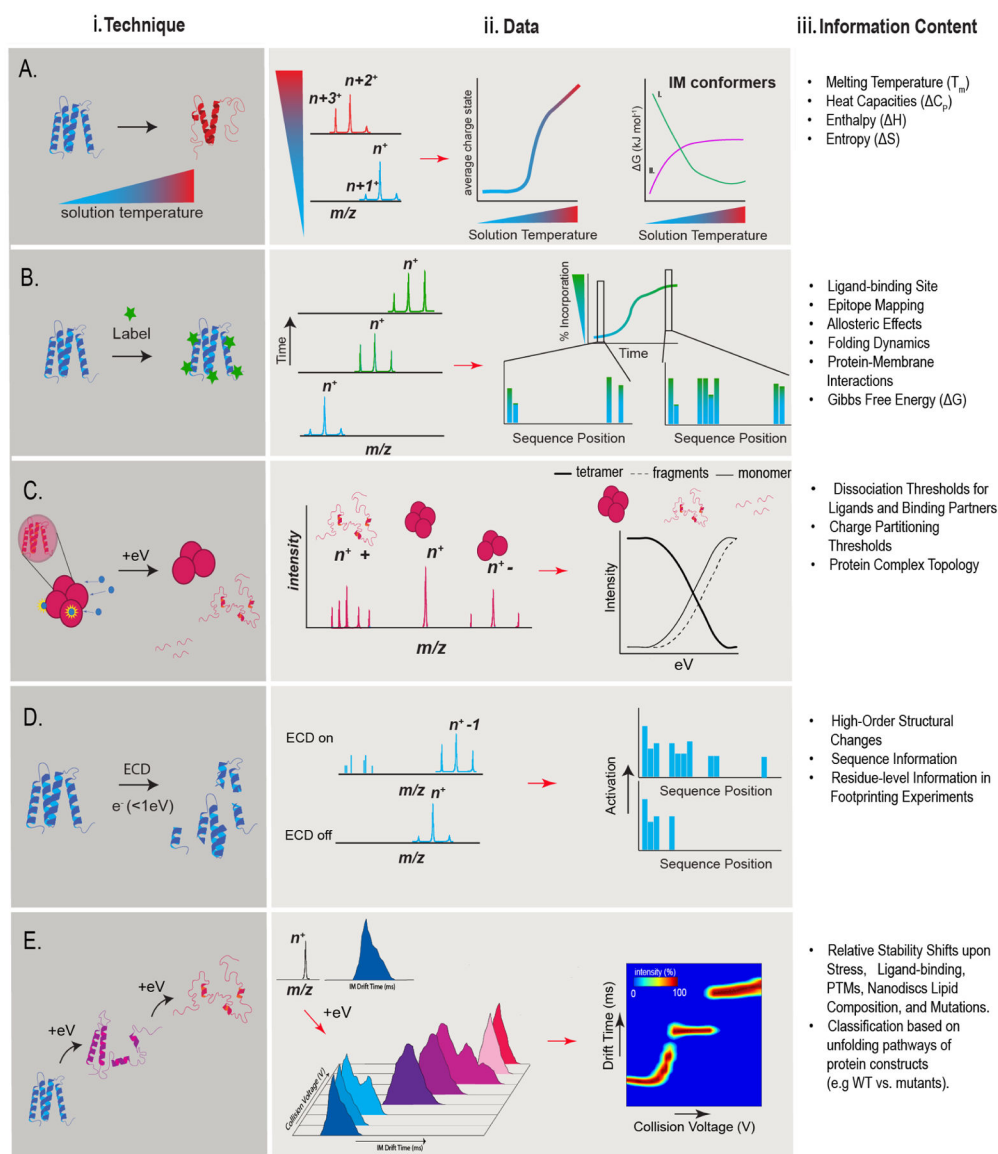


Figure 2. Mass-Spectrometry Techniques for Protein Conformational Stability Measurements. For each technique described, there is a corresponding summary figure, **ii)** data and **iii)** information content column. **A) Variable Temperature.** A protein experiences a temperature gradient as it is introduced into the mass spectrometer. A shift in CSD indicates conformational changes. By monitoring IM conformers, insights into thermodynamic values can be made. **B) Footprinting** uses selective or non-selective reagents (green stars) to monitor changes to the solvent-accessible regions of a protein. **C) Electron Capture Dissociation** is a fragmentation technique where proteins are exposed to low energy electrons, which are captured and produce backbone fragmentation. ECD fragmentation patterns can be changed based on the precursor conformation and charge state. **D) Collision Induce Dissociation** allows the evaluation of protein complex subunit stoichiometry and composition. By monitoring protein ejection from protein complex precursors, dissociation thresholds can be determined and related to the stabilities of subunits and interfaces. **E)**

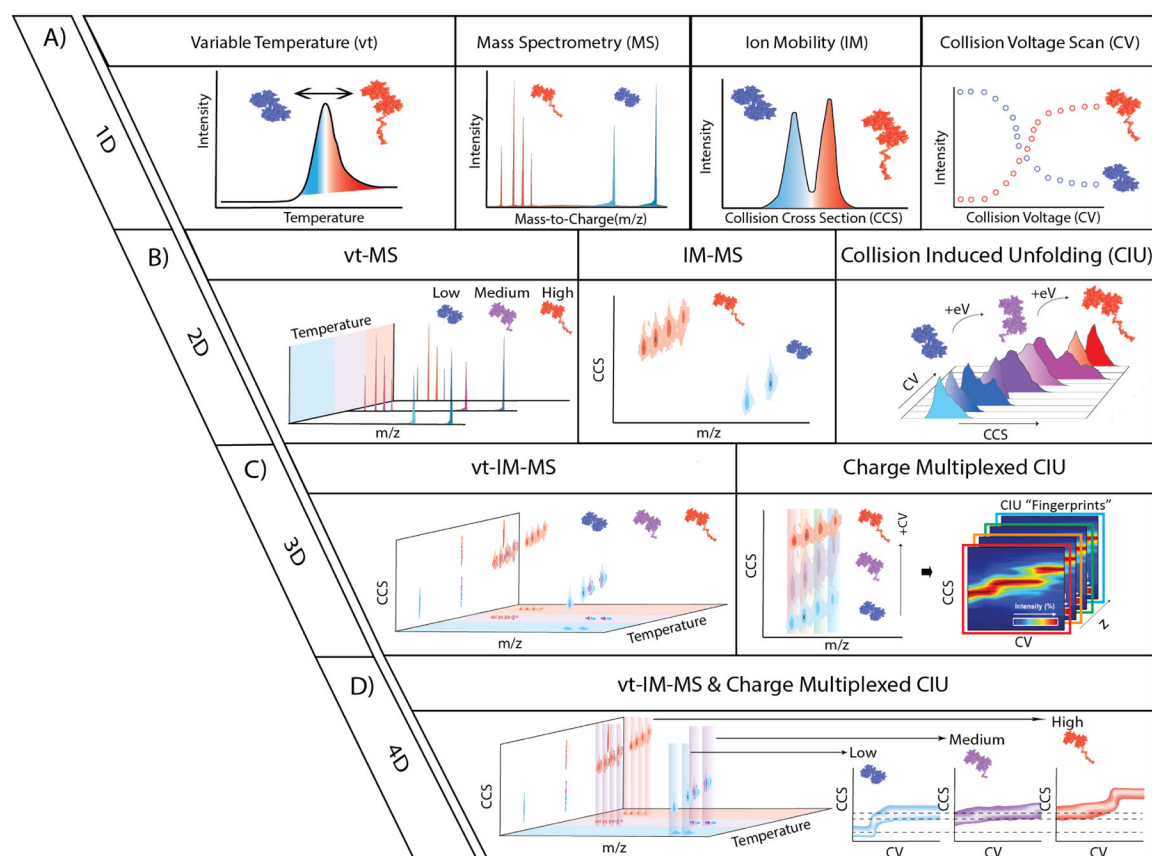
Collision Induced Unfolding is an IM-MS technique where a protein conformation is monitored as its internal energy is increased using collisional activation. A shift in voltage required for eliciting the unfolding transitions observed is indicative of stability shifts when comparing between fingerprints of different states (e.g. apo vs holo).

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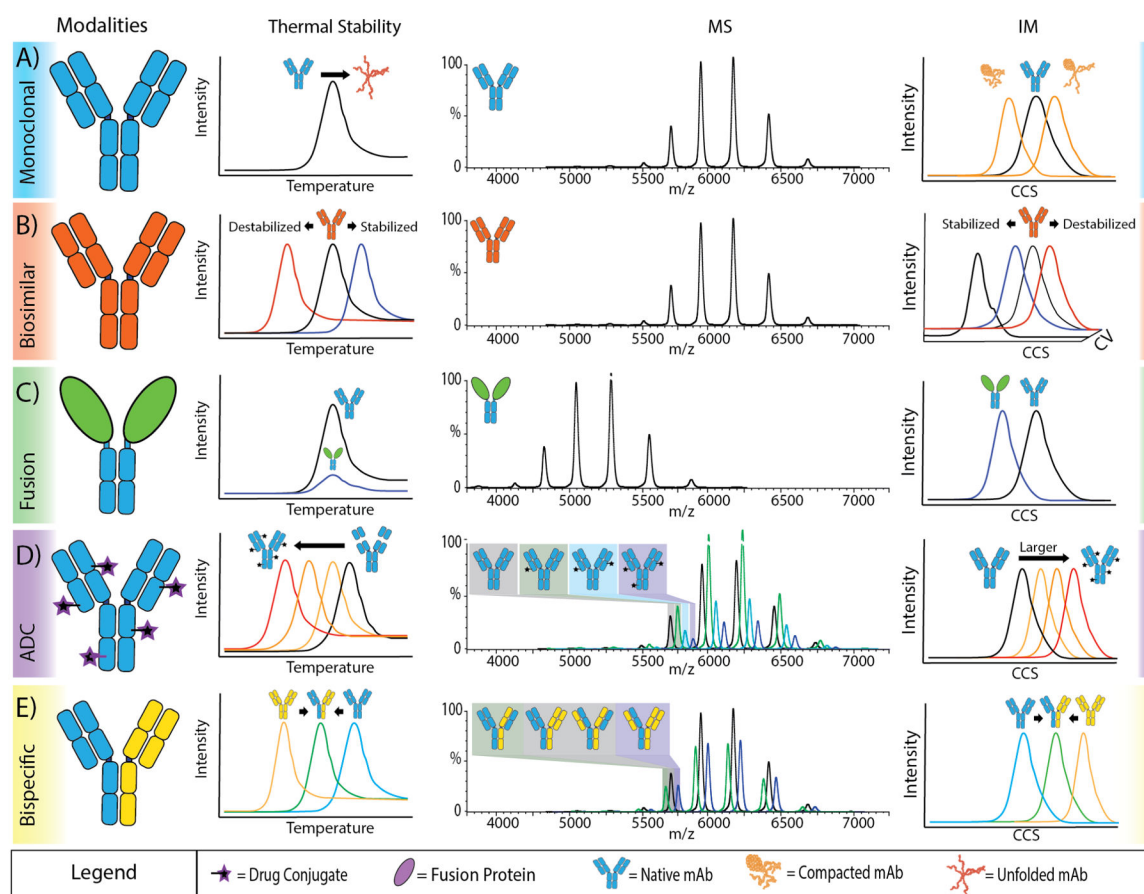
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**Figure 3:**

Scaling the dimensionality of MS-based protein stability measurements. A) One-dimensional assays of protein stability can include variable temperature, mass spectrometry, ion mobility, and collision voltage scanning. B) Hyphenation of the 1-D techniques in A provides access to powerful 2-D techniques such as vt-MS, IM-MS, and CIU that can separate and generate a wide range of unfolded protein structures. C) Further enhancements can be achieved with 3-D hyphenated techniques based on those shown in A and B above. D) A logical extension of the techniques shown in C leads to a "4-D" assay of protein stability governed by MS methods, capable of measuring changes in protein stability across multiple phases/conditions simultaneously.

**Figure 4.**

Data and information content that can be expected from variable temperature, MS and IM datasets for the following biotherapeutic modalities: **A)** mAbs, **B)** biosimilars, **C)** fusion proteins, **D)** antibody-drug conjugates, and **E)** bispecific antibodies. Generally, for variable temperature experiments shifts to lower T_m values indicate a decrease in stability and higher values indicate an increase in stability. Changes in mass spectrometry generally indicate different structures or stoichiometries. For IM shifts to lower CCS values indicate more compact structures while larger values indicate larger, often unfolded structures. By applying activation energy and monitoring unfolding, ex. Biosimilar IM, shifts in stability can be monitored by shifts in the IM peak relative to the activation energy.

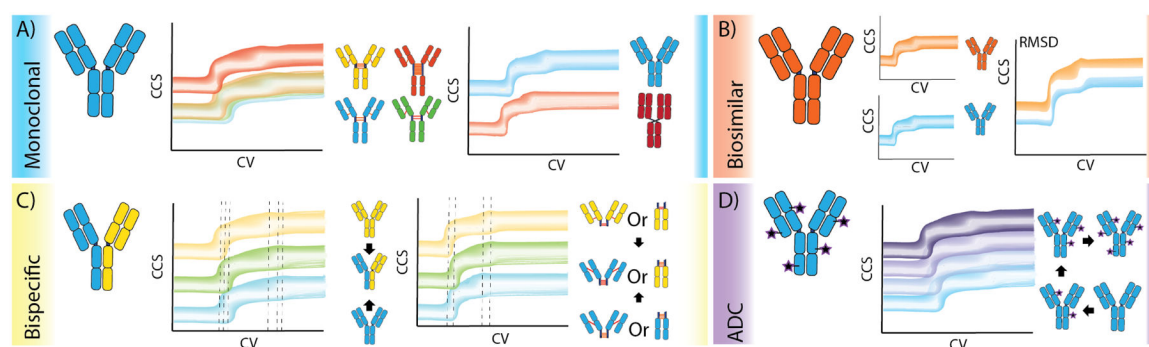


Figure 5. CIU applications for biotherapeutic mAbs.

A) Differentiation of monoclonal IgG subclasses by disulfide bonding patterns and difference in CIU unfolding due to domain exchange. **B)** Biosimilar antibodies have qualitatively similar fingerprints, but contemporary CIU analyses are able to quantitate subtle differences in stability. **C)** Bispecific antibodies present CIU characteristics centered between the precursor structures. **D)** Shifts in CCS and stability can be quantified as a function of increasing drug load in ADC biotherapeutics.

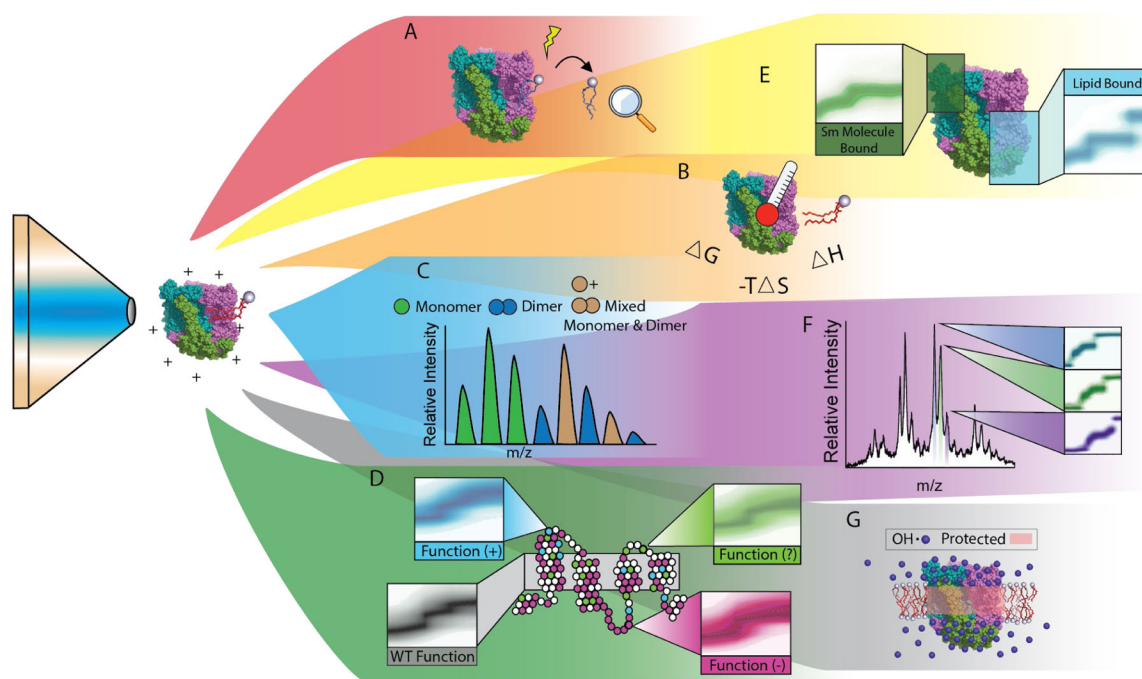


Figure 6. Summary of various MS-related measurements associated with MP stability assessments.

A) identification of endogenous lipid binding **B)** thermodynamics of lipid binding to membrane protein **C)** oligomeric state assignment **D)** evaluation of disease state mutations in amino acid sequence **E)** site-selective ligand binding events **F)** resolving multiple simultaneous ligand bound states **G)** oxidative labeling.

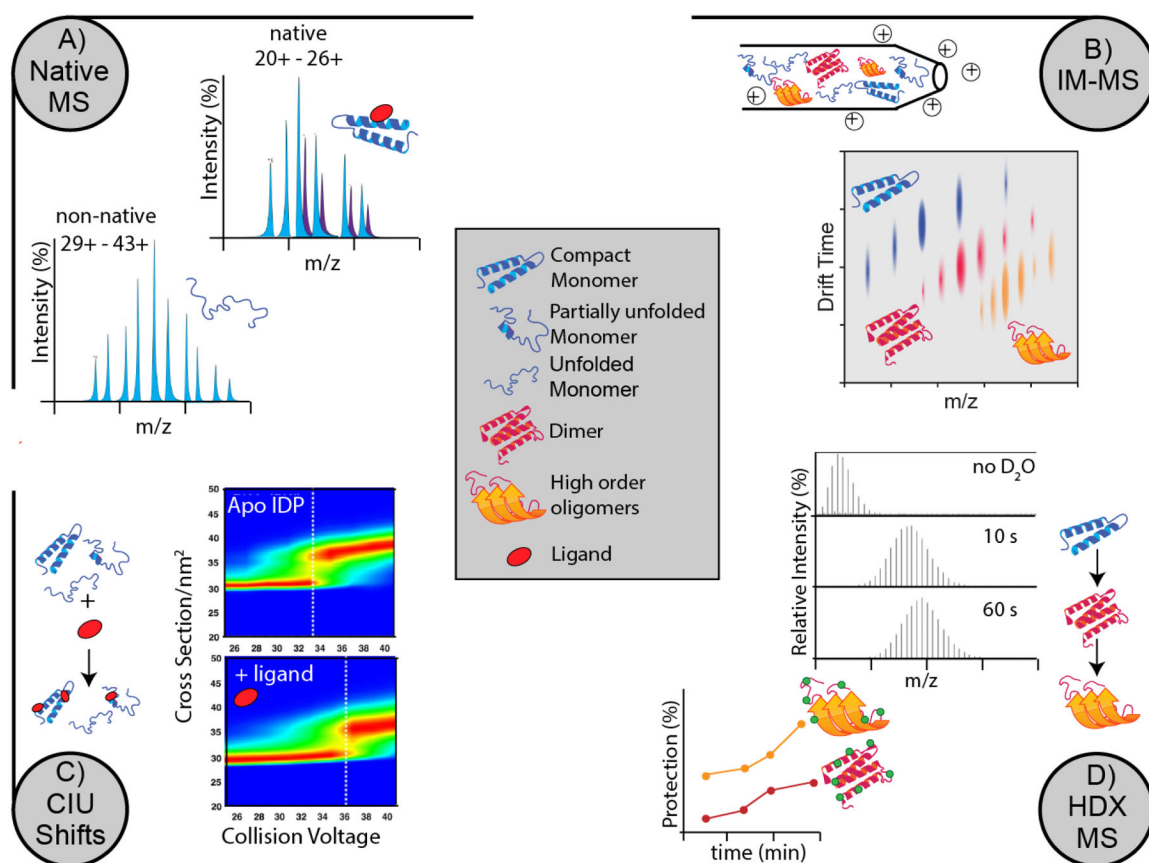


Figure 7.

Overview of select MS techniques that allow direct stability measurements with challenging amyloidogenic proteins. A) Native MS usually produces a narrow range of charge states compared to non-native MS. Native MS can retain the native structures of amyloidogenic proteins and even the non-covalent complexes formed through ligand or protein binding through gentle ionization parameters. B) nESI needle is filled with a mixture of different oligomers of an amyloidogenic protein. IM-MS is able to separate the complex population of oligomers in drift time space based on their size, charge and shape. C) CIU shifts otherwise known as CIU50 values can be obtained through a series of IM-MS experiments at increasing collision energy. If a ligand binding event caused an amyloid protein to increase in stability, CIU50 values will reflect this increase. D) HDX-MS can capture localized information, in as little as few milliseconds or as long as days, allowing us to take snapshots of an amyloid aggregation process.

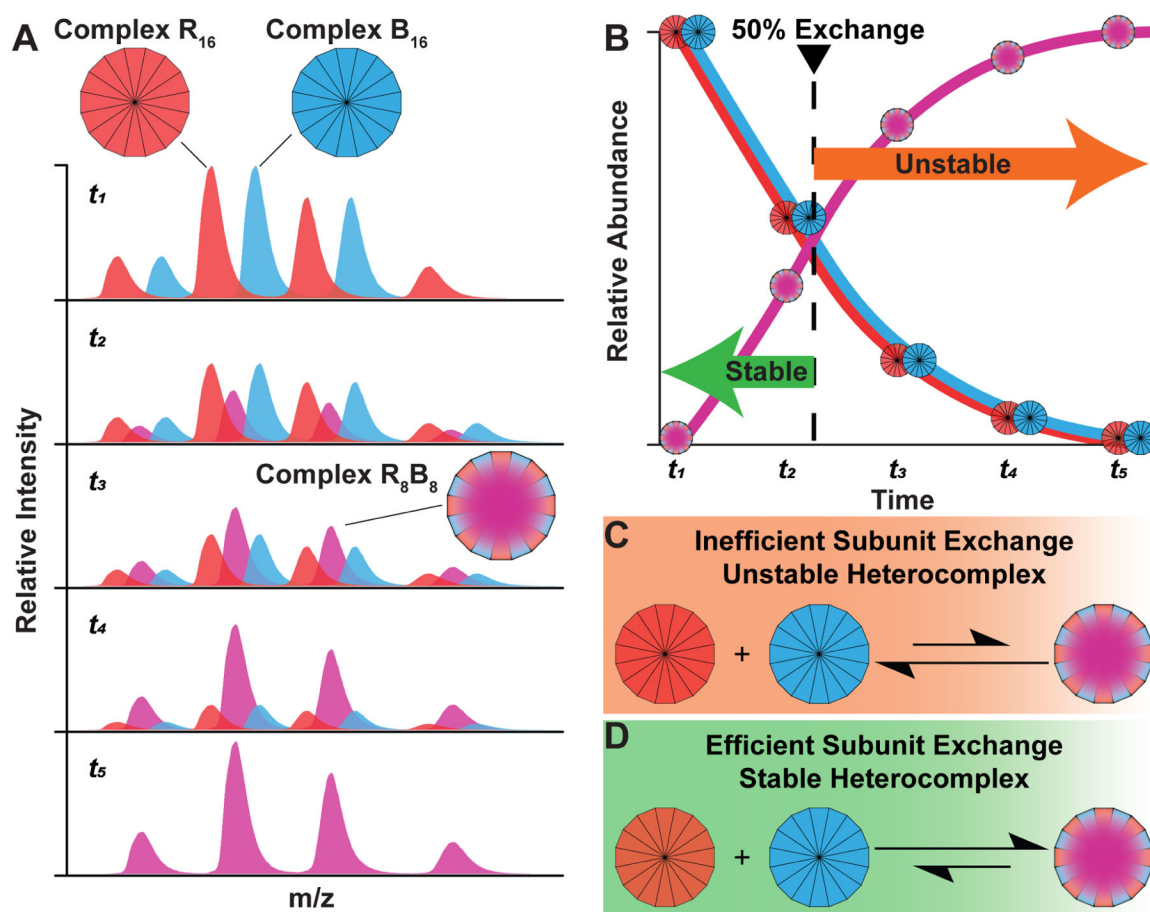


Figure 8. Subunit Exchange between sHSP Oligomers Illuminated through MS.

A) Two sHSP homo-oligomers (R and B, numbers indicated the number of subunits within the example oligomers) with suspected hetero-oligomeric interactions are co-incubated in solution, and MS data is collected at various time points (t_1 , t_2 , t_3 etc.). **B)** The MS data is quantified versus time to measure the decay of homo-oligomers and the growth in abundance of hetero-oligomeric sHSP species. The rate of this conversion from homo-oligomers to hetero-oligomers can be measured as a function of time, and can serve as an indication or stability. **C)** When hetero-oligomeric sHSP complexes are stable their subunit exchange is expected to occur quickly. **D)** Conversely, when hetero-oligomeric complexes are unstable, the equilibrium shifts in the direction of homo-oligomers.