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A blurry view of fuzzy objects: on the roles of low-resolution structural techniques in discovery and early characterization of intrinsically disordered proteins

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The discovery of intrinsically disordered proteins (IDPs) (and, therefore, the establishment of the field of protein intrinsic disorder) was initially driven by low-resolution techniques, which overturned the established "lock-and-key" paradigm of structural biology by showing that some proteins exist as a dynamic conformational ensemble rather than a single fixed structure. Though unable to provide atomic-level detail offered by X-ray crystallography or NMR, these methods were the first to reveal that many functional proteins exist as a dynamic ensemble of conformations rather than a single fixed structure. Furthermore, these techniques highlighted a limitation of high-resolution methods such as X-ray crystallography, which often could not resolve disordered regions. Curiously, despite the fact that X-ray crystallography requires rigid, crystalized samples and portrays the proteins as aperiodic crystals, this technique provided some early hints of intrinsic disorder that came from the "missing residues" in X-ray structures. Ultimately, by identifying proteins that lacked stable structures, these initial experiments utilizing low-resolution techniques drove the development of advanced approaches, such as specialized NMR techniques, to better characterize the dynamics of these proteins. The goal of this review is to emphasize the roles of low-resolution structural techniques in establishing the IDP field by showing some illustrative examples of IDPs they helped to discover in the years preceding the formal acceptance of the protein intrinsic disorder concept.

KEYWORDS

intrinsically disordered protein, multiparametric approach, low-resolution techniques, intrinsically disordered region, integrative structural biology

1 Introduction

1.1 The intrinsic disorder phenomenon in a few words

Intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) do not have unique rigid structures that typically observed in globular proteins/domains. Instead, they exist as dynamic ensembles of conformations, varying at both secondary and tertiary levels (Dunker et al., 1998; Wright and Dyson, 1999; Uversky et al., 2000a; Dunker et al., 2001; Tompa, 2002; Daughdrill et al., 2005; Uversky and Dunker, 2013). Based on their global structural properties at the whole protein/region level, IDPs/IDRs can be broadly categorized as containing

collapsed disorder (forming molten globule-like structures) or extended disorder (resembling random coils or pre-molten globules) (Dunker et al., 2001; Uversky, 2003; Daughdrill et al., 2005). Proteins and regions can also exhibit semi-disorder with an approximately equal likelihood of being ordered or disordered (Zhang et al., 2013). These semi-disordered regions are crucial for various physiological and pathological processes, such as protein aggregation and protein-protein interactions, often folding upon binding to other molecules (Zhang et al., 2013).

However, one should keep in mind that with a very few exceptions, a real protein (not necessary an IDP) has a very sophisticated structural organization characterized by an intricate spatiotemporal heterogeneity, akin to a mosaic or a kaleidoscope, encompassing different functional units with various levels of disorderedness: foldons (independently foldable units), inducible foldons (IDRs that fold upon binding partners), inducible morphing foldons (IDRs that fold differently upon binding to different partners), non-foldons (non-folded regions essential for function), semi-foldons (always partially folded), and unfoldons (ordered regions that must unfold to become active) (Uversky, 2013c;a;b;Jakob et al., 2014). This intricate structural diversity, where differently (dis)ordered structural elements might have well-defined and specific functions, enables IDPs/IDRs to perform multiple functions and interact with, regulate, and be regulated by numerous diverse partners (Uversky, 2015b).

Being complex systems with sophisticated structurally and functionally heterogeneous organization, IDPs/IDRs are central to the structure-function continuum concept, demonstrating a shift from the simplistic "one gene-one protein-one function" model to a more complex "one gene-many proteins-many functions" model (Uversky, 2015b; 2016). This is crucial for understanding the proteoform concept (Smith et al., 2013) highlighting the idea that the complexity of biological systems is not solely dictated by their genome size, but rather by the vast and functionally diverse proteome, consisting of a multitude of unique proteoforms derived from a relatively small number of genes (Schluter et al., 2009). This diverse array of proteoforms is generated through several key mechanisms, including genetic variations, alternative splicing that produces different mRNA transcripts, and various post-translational modifications (PTMs) that chemically alter proteins (Uhlen et al., 2005; Farrah et al., 2013; Farrah et al., 2014; Kim et al., 2014; Reddy et al., 2015). Furthermore, IDPs/IDRs are critical contributors to this proteoform diversity, as they further amplify this protein variability through their unique disordered nature (Uversky, 2016).

A brief summary of some key facts about IDPs/IDRs is offered below in a form of short bullets.

- The biological activities of IDPs and IDRs are not dependent on a unique, fixed structure (Dunker et al., 1998; Wright and Dyson, 1999; Uversky et al., 2000a; Dunker et al., 2001; Tompa, 2002; Daughdrill et al., 2005; Uversky and Dunker, 2010).
- IDPs/IDRs are ubiquitous across all known proteomes (Dunker et al., 2000; Dunker et al., 2001; Ward et al., 2004; Uversky, 2010; Uversky and Dunker, 2010).
- The amino acid makeup of IDPs/IDRs is fundamentally different from that of ordered proteins, defining their inability to form stable structure (Dunker et al., 1998; Garner et al., 1998; Uversky et al., 2000a; Dunker et al., 2001; Romero et al., 2001; Williams et al., 2001; Uversky, 2002b; Radivojac et al., 2007; Vacic et al., 2007; Uversky and Dunker, 2010).
- Differences in the amino acid compositions between ordered proteins/domains and IDPs/IDRs allow for the prediction of intrinsic disorder based solely on the primary sequence (He et al., 2009).
- Because of the lack of stable 3D-structures, IDPs can sustain exposure to extremely harsh environmental conditions that would typically denature or unfold ordered proteins and render them dysfunctional (Uversky, 2017b).
- Extended IDPs are characterized by a "turned out" response to heat and changes in pH, where high temperatures or extreme pH values induce their partial folding (Uversky, 2009).
- IDPs and IDRs are structurally highly variable. They exhibit significant structural diversity, varying in compactness, the amount of flexible secondary structure they possess, and the number of tertiary contacts they form (Dunker and Obradovic, 2001; Uversky, 2002b; Daughdrill et al., 2005; Uversky and Dunker, 2010).
- IDPs and IDRs are highly flexible, constantly shifting their conformations (Dunker and Uversky, 2010). However, despite this dynamic nature, their structures can be adequately characterized by a limited collection of relatively stable, low-energy conformations that effectively describe their overall structural preferences (Choy and Forman-Kay, 2001; Huang and Stultz, 2008).
- IDPs and IDRs perform functions that synergize with the roles of ordered proteins and domains (Iakoucheva et al., 2002; Dunker et al., 2005; Uversky et al., 2005).
- IDPs/IDRs serve diverse functions, which can be broadly classified into various overarching groups, such as assemblers, chaperones, display sites, effectors, entropic chains, and scavengers (Tompa, 2002).
- The functions of some IDPs/IDRs, categorized as entropic chain activities, are based solely on the continuous movement of their flexible, random-coil-like polypeptide chains (Dunker et al., 2001).
- IDPs/IDRs rarely exhibit enzymatic catalysis, with a few exceptions where collapsed IDPs show catalytic activity (Uversky et al., 1996; Pervushin et al., 2007; Vamvaca et al., 2008; Woycechowsky et al., 2008).
- The flexible nature of IDPs and IDRs makes them particularly susceptible to a wide range of PTMs (Dunker et al., 2002; Iakoucheva et al., 2004).
- IDPs/IDRs are versatile binders, interacting with a diverse range of targets such as nucleic acids, metal ions, heme groups, other small molecules, proteins, polysaccharides, and membrane bilayers (Uversky et al., 2000a; Dunker et al., 2002).
- The remarkable adaptability of some IDPs and IDRs allows them to specifically interact with multiple partners, even if those partners are structurally dissimilar (Oldfield et al., 2008).
- Many IDPs/IDRs exhibit both one-to-many and many-to-one binding capabilities (Dunker et al., 2005; Uversky et al., 2005).
- IDPs frequently play a central role in protein interaction networks, acting as hubs that connect to many other proteins (Dunker et al., 2005; Uversky et al., 2005).

- Binding to specific partners can induce a disordered-to-ordered transition in IDPs/IDRs (Dyson and Wright, 2002; Oldfield et al., 2005).
- Some IDPs/IDRs can assume different structures upon interaction with different partners (Oldfield et al., 2008).
- IDPs/IDRs exhibit a wide variety of ways of interacting with other molecules, resulting in many unconventional complexes (Uversky, 2011).
- Some IDPs/IDRs bind to other molecules without undergoing a complete or partial folding process, resulting in the formation of dynamic, disordered, or fuzzy complexes (Nash et al., 2001; Mittag et al., 2008; Mittag et al., 2010; Uversky, 2011).
- IDPs/IDRs, with their multifunctionality, binding promiscuity, and binding plasticity, are crucial players in the rapid and well-organized adaptation to changing conditions and in the efficient orchestration of complex cellular responses vital for survival and function (Bondos et al., 2022; Chakrabarti and Chakravarty, 2022; Hsiao, 2024).
- Due to their ability to interact with multiple partners, IDPs/IDRs can act scaffolds that orchestrate activities of partners (Cortese et al., 2008; Buday and Tompa, 2010; Barbar and Nyarko, 2015; Clark et al., 2015).
- Many human diseases are characterized by the involvement of IDPs/IDRs in their pathogenesis (Uversky et al., 2008; Uversky, 2014a;b; Uversky et al., 2014; Uversky, 2022).
- IDPs/IDRs, being complex 'edge of the chaos' systems, exhibit emergent behavior through intricate self-organization, meaning they develop unexpected new structures, patterns, and properties (Uversky, 2013c).
- IDPs/IDRs play crucial roles in cellular liquid-liquid phase separation (LLPS) and in the biogenesis of various membraneless organelles (MLOs) and biomolecular condensates (BMCs) (Uversky, 2015b; 2017a;c).
- Being complex systems, IDPs/IDRs exhibit a basic form of "intelligence", being capable of processing information, adapting to their environment, changing their environment, and showing memory-like behavior (Tripathi et al., 2025).

All these facts clearly emphasize that IDPs/IDRs are blurry or fuzzy subjects by default, and this blurriness/fuzziness is manifested in both structure (manifested by highly dynamic and heterogeneous spatiotemporal organization) and function (displayed by multifunctionality and binding promiscuity). In other words, IDPs/IDRs, with their fuzzy structures and blurry functions, contrast with classic globular proteins with crisp structures and specific functions, thereby challenging the traditional "structure-function paradigm". Furthermore, due to their intrinsically dynamic nature, IDPs and IDRs do not exhibit a single, well-defined conformation under experimental conditions. This inherent conformational heterogeneity reflected in a "cloud-like" representation of their conformational ensembles poses a significant obstacle to conventional structural determination techniques, as it prevents the resolution of a unique, high-resolution structure. This idea is demonstrated by Figure 1 which compares the NMR solution structures of several IDPs serving as illustrations of the "blurry view" of IDP conformational ensembles from low-resolution methods with the "crisp view" of ordered proteins generated by high-resolution methods, such as X-ay crystallography and NMR. Consequently, a diverse array of advanced experimental methods is required to obtain constraints and gain insights into the ensemble of states sampled by these disordered polypeptide chains (Uversky and Dunker, 2012c; Uversky, 2015a).

1.2 A multiparametric approach in structural biology

The ultimate goal of structural biology is to obtain information on the structure of a biological macromolecule with the highest possible resolution. Ultra-high resolution structures can provide an unparalleled level of detail, showing individual atoms and their arrangement within the protein molecule, capturing the dynamic nature of proteins and showing their regions with multiple conformations, as well as allowing evaluation of hydrogen bonding and solvent networks. Even for a well-folded protein, achieving this goal is challenging and requires special techniques, such as X-ray crystallography and cryo-electron microscopy. A recently reported 0.70 Å, room-temperature crystal structure of a small globular protein crambin (4.7 kDa) that was refined to an *R* factor of 0.0591 represents the highest-resolution ambient-temperature structure of a protein achieved to date (Chen et al., 2024). The resolution can be further increased by using crystallography at low temperature. For example, for the same protein crambin, diffraction data were measured to ultra-high resolution (0.54 Å) at low temperature with short-wavelength synchrotron radiation, which is close to a diffraction resolution limit $d_{min} \approx 0.5$ Å (Jelsch et al., 2000). The highest resolution achieved in cryo-EM for protein structure determination is currently 1.25 Å (as reported for apoferritin, which is composed of 24 subunits arranged in a symmetrical structure, with a total molecular mass of 440–480 kDa) (Yip et al., 2020).

Recent breakthroughs in instrumentation and analytical techniques are rapidly pushing both X-ray crystallography and cryo-EM closer to their theoretical diffraction limits. However, in addition to the advanced instrumentation and analytical techniques, the quality and behavior of the biological samples remain crucial for successful structure determination using both methods, with flexibility and conformational dynamics being the usual reasons for decreasing resolution. In X-ray crystallography, protein flexibility can hinder the formation of high-quality crystals with well-ordered structures necessary for high-resolution diffraction. Even if crystals form, internal motions within the protein can lead to averaged electron density maps, blurring out the finer details and limiting the resolution of the determined structure (Hekstra, 2023). In cryo-EM, flexibility and conformational heterogeneity present challenges during image processing. Averaging thousands of particle images with differing conformations can blur the structural details and decrease resolution. This is particularly problematic for symmetric complexes, where flexibility within individual subunits can be difficult to resolve (Suder and Gonen, 2024). Therefore, in both techniques, getting a crisp image of protein structure with high resolution typically requires reduction of protein mobility on multiple time and spatial scales.

It is clear that neither X-ray crystallography nor cryo-EM can obtain a high resolution structure for intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) because of a very simple reason: unlike ordered, well-folded proteins and domains, IDPs/IDRs do not adopt a single, stable three-dimensional structure.

Protein structural characterization methods all have limitations. As mentioned above, the traditional structural biology techniques X-ray crystallography and cryo-EM are not suitable for the analysis of highly flexible macromolecules existing as rapidly interconverting conformational ensembles.

The highly varied structures and complex, multi-timescale dynamic fluctuations of IDPs make it impossible to characterize their full range of properties with a single experimental or computational technique. A comprehensive understanding requires a multi-pronged approach utilizing various methods (Uversky and Dunker, 2012c; Uversky, 2015a). Just as the compound eyes of insects provide a much wider field of view and excel at detecting rapid movement compared to simpler eyes (Völkel et al., 2003), a multiparametric analysis provides a more comprehensive and dynamic understanding of IDPs. This implies that examining multiple characteristics simultaneously allows researchers to gain insights that would not be possible with a single approach (Uversky and Dunker, 2012c; Uversky, 2015a). A range of biophysical tools, forming a multiparametric approach, were developed to analyze various aspects of a protein's structure, including its overall shape, folding patterns (secondary structure), temporary interactions (long-range contacts), stability, and how flexible different regions are. As no single technique can reveal the complete picture, to fully understand a protein, it is essential to use multiple techniques that are sensitive to these different structural details simultaneously.

2 Low-resolution techniques in the discovery of IDPs

Although the role of NMR in understanding the dynamic nature of IDPs/IDRs and characterization of their conformational ensembles is undeniable and it "illuminates intrinsic disorder" (Dyson and Wright, 2021), very important information, especially in the early days of the IDP field, was derived using a wide spectrum of low-resolution techniques. Furthermore, these low-resolution techniques were used long before the scientific community fully accepted the concept of biologically active proteins lacking a fixed 3D structure, and were instrumental in laying the groundwork for this vital discovery, played a crucial role in the discovery of the protein intrinsic disorder phenomenon and, later, offered tremendous help with establishing the IDP/IDR field.

Various biophysical techniques suitable for the analysis of the dynamic structures of IDPs/IDRs have been thoroughly explained in comprehensive reviews and books (Daughdrill et al., 2005; Receveur-Brechot et al., 2006; Eliezer, 2009; Jensen et al., 2010; Longhi and Uversky, 2010; Uversky and Dunker, 2012a; Uversky and Dunker, 2012b; Uversky and Dunker, 2012c; Gibbs and Showalter, 2015; Uversky, 2015a; Schramm et al., 2019; Evans et al., 2023; Maiti et al., 2024), and the focus of the present review is on the roles of different low-resolution techniques in the discovery of the protein intrinsic disorder phenomenon. To this end, Figure 2 introduces a timeline of key discoveries linking specific techniques to the acceptance of the IDP concept, whereas Table 1 summarizes information on low-resolution techniques discussed in this review and briefly discusses their physical principles, specific parameters they measure, as well as their advantages and limitations in the context of the IDP analysis.

2.1 X-ray crystallography on intrinsic disorder: the lowest possible limit of low-resolution data

One should keep in mind that none of the structure-centered experimental approaches of structural biology was specifically designed for the analysis of disorder. In fact, because of the then-dominating "lock-and-key" model of protein action and the associated central paradigm of structural biology stating that a unique 3D structure of a protein determines its function, all techniques for protein analysis were specifically designed for the analysis of the structural properties, conformational behavior, and thermodynamic stability of ordered proteins. Therefore, when using most of these techniques, the information on the presence of intrinsic disorder in a protein was derived from the absence of a signal characteristic for an ordered protein.

The best illustration of this fact is given by X-ray crystallography, which serves as the primary tool for determining the 3D atomic structure of proteins. This technique is rooted in the classical experiments of Walther Otto Ernst Friedrich (1883–1958), Paul Knipping (1883–1935), and Max von Laue (1879–1960), who, in 1912, showed that the X-rays passing through a crystal produced a diffraction pattern on a photographic plate (Friedrich et al., 1912). Interpretation of the X-ray scattering patterns connecting the X-ray wavelength and diffraction angles to the atomic spacing within a crystal and design of the first X-ray spectrometer by William Henry Bragg (1862–1942) and William Lawrence Bragg (1890–1971) have opened a way for determination of crystal structures. These important developments happened long before the X-ray crystallography became a go-to tool for protein structure analysis. In fact, protein crystallography was started by the pioneering work of John Desmond Bernal (1901–1971) and Dorothy Crowfoot Hodgkin (1910–1994), who reported the first X-ray photographs of crystalline pepsin in 1934 (Bernal and Crowfoot, 1934). This study, while not leading to the determination of protein structure, provided crucial evidence that proteins possess a definite, ordered atomic arrangement that could theoretically be decoded. The very first protein crystal structure (myoglobin) was solved in 1958 by John Cowdery Kendrew (1917–1997) and colleagues (Kendrew et al., 1958).

The resolution of the diffraction pattern depends on the degree of order in the crystal, and where the disordered regions are "invisible", since the flexibility of their atoms results in the non-coherent X-ray scattering, making them regions with missing electron density. Often, these regions with missing electron density correspond to loops, tails, hinges, and linkers of a protein. When protein crystallographers first observed "missing electron density" is not a single, documented event with a specific date because it is inherent to the process of X-ray

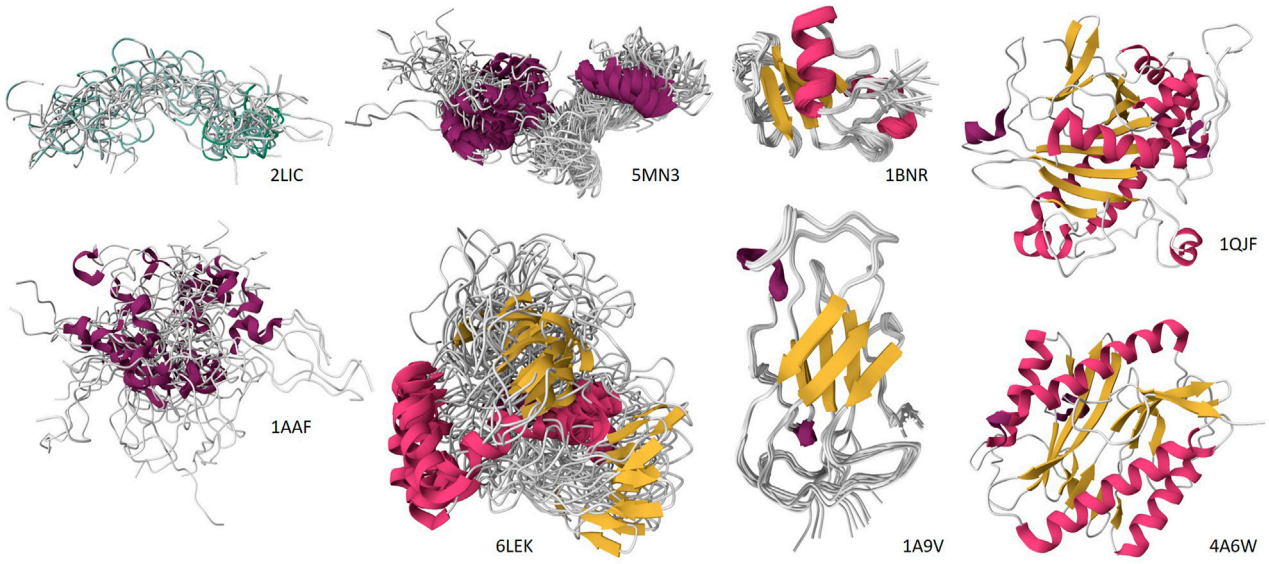

FIGURE 1
Comparison of the "blurry view" of IDP conformational ensembles from low-resolution methods with the "crisp view" of ordered proteins generated by high-resolution methods, such as X-ay crystallography and NMR. Top row: NMR structure of the polyserine tract of *Apis mellifera* vitellogenin, residues 358–392 (PDB ID: 2LIC) (Havukainen et al., 2012), NMR structure of the *Littorina littorea* metallothionein (PDB ID: 5MN3) (Baumann et al., 2017), NMR structure of barnase from *Bacillus amyloliquefaciens* (PDB ID: 1BNR) (Bycroft et al., 1991), and X-ray crystal structure of isopenicillin N synthase (IPNS) from *Emericella nidulans (strain FGSC A4/ATCC 38163/CBS 112.46/NRRL 194/M139)* (PDB ID: 1QJF) (Burzlaff et al., 1999). Bottom row: NMR solution structure of the nucleocapsid protein from human immunodeficiency virus type 1 group M subtype B (isolate MN) (PDB ID: 1AAF) (Summers et al., 1992), NMR structure of *Megabalanus rosa* Cement Protein 20 (PDB ID: 6LEK) (Mohanram et al., 2019), NMR structure of the major house dust mite allergen Der p 2 (PDB ID: 1A9V) (Mueller et al., 1998), and X-ray crystal structure of *E. coli* methionine aminopeptidase (PDB ID: 4A6W) (Huguet et al., 2012). The corresponding structures are shown exclusively for the illustrative purposes and ar not discussed in the text.

crystallography, especially when working with biological macromolecules. However, in 1968, missing electron density was reported in the C-terminal residues of hemoglobin, suggesting the presence of flexibility or disorder in this region (Perutz et al., 1968). Already in 1971 [i.e., 13 years after the first crystal structure of a protein, myoglobin, was solved in 1958 (Kendrew et al., 1958), and when only seven protein crystal structures were known (Minor et al., 2016)], a study on the X-ray crystal structure of *staphylococcus* nuclease first used the term "disordered" to describe two regions of missing electron density (Arnone et al., 1971). Therefore, the initial awareness of disorder in protein crystallography can be traced back to the early 1970s, and it was pointed out early on that many proteins in the Protein Data Bank (PDB) contain regions with missing electron density (Bloomer et al., 1978; Bode et al., 1978).

More recent analyses confirmed the prevalence of regions with missing electron density in protein structures deposited in the PDB (Le Gall et al., 2007; Djinovic-Carugo and Carugo, 2015; DeForte and Uversky, 2016). In one of these studies, it was reported that "more than 80% of the structures refined at a resolution lower than 1.75 Å contain at least one missing string and about 20% of the structures refined at a resolution better than 0.75 Å contain at least one missing string" (Djinovic-Carugo and Carugo, 2015). Clearly, the fact of missing electron density in a region of a protein molecule does not provide any structural information besides making a claim that this region is too flexible to be resolved by X-ray crystallography. Perhaps this represents one of the lowest possible limits of the low-resolution information about disorder. However, one should not take this lack of structural information lightly, as while missing electron density might seem like a simple absence of information, it in fact does offer important insights into the dynamics and function of a protein.

Curiously, in 1984 it was recognized that the actual reasons for the electron density missing from a protein structure can be very different (Bennett and Huber, 1984). In some cases, missing electron density arises from perpetual motion at the backbone level, characterizing dynamic disorder. In other cases, missing electron density corresponds to regions with static disorder that have multiple conformations within the crystal (Bennett and Huber, 1984). Still other regions with missing electron density correspond to domain wobble (a concept introduced by Garner et al., in 1998 (Garner et al., 1998)), i.e., the wholesale movement of a structured domain facilitated by a flexible hinge.

2.2 NMR spectroscopy

Atomic-resolution studies of ordered protein structures have been possible through the use of nuclear magnetic resonance (NMR) since 1957, when the first proton resonance spectrum was reported for a small globular protein, bovine pancreatic ribonuclease (Saunders et al., 1957). Historically, NMR spectroscopy primarily targeted the structure determination of globular proteins with regular secondary structure motifs, following the path laid by X-ray crystallography. And similar to X-crystallography, protein NMR studies prioritized the 3D structure determination, mostly neglecting flexible regions, such as linkers, loops, and sequence termini (Marion, 2013). As a result, the entire protein NMR toolbox has been developed over about 3 decades for applications on folded

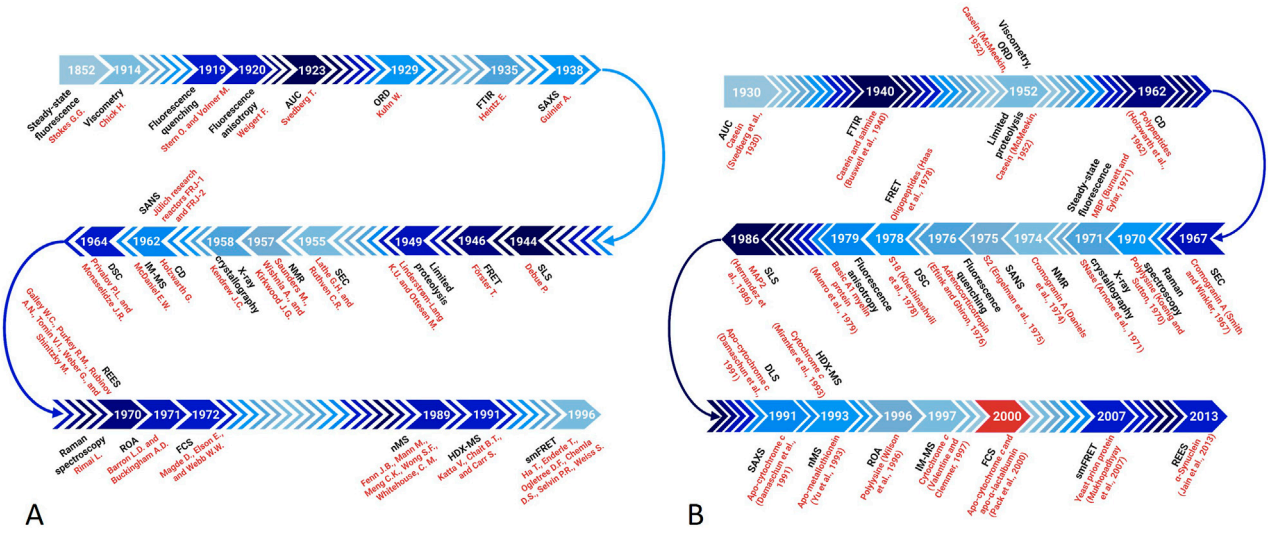

FIGURE 2
A timeline of key discoveries linking specific techniques to the acceptance of the IDP concept. **(A)** Timing of the introduction of biophysical techniques. **(B)** Timing of the first use of these techniques in the IDP analysis.

proteins (Bolik-Coulon et al., 2019). For example, a crucial move toward solving high resolution protein structures by NMR was made in 1976, when the two-dimensional (2D) NMR was introduced by Aue et al. (Aue et al., 1976) followed by the transfer of the 2D NMR analysis to the field of biomolecules by the group of Kurt Wüthrich, who, in 1982, outlined the framework for using NMR to determine protein structures (Wuthrich et al., 1982) followed by actually solving the first protein structure (proteinase inhibitor IIA from bull seminal plasma) by ^{1}H-NMR in 1985 (Williamson et al., 1985; Wüthrich, 1990).

Since the information on molecular conformations in NMR experiments is retrieved from the analysis of the unique chemical shift signals generated by the local environment of each atomic nucleus, and since no stable local environment for chemical groups is present in IDPs/IDRs, NMR spectra of IDPs are different from those of ordered proteins and contain some peculiar characteristics, such as the limited dispersion in proton NMR (Bolik-Coulon et al., 2019). Although it is impossible to pinpoint when an IDP was analyzed by NMR for the first time, it is likely that the recognition of the distinct spectral properties of IDPs/IDRs coincided with the emergence of awareness and research interest in IDPs themselves. In his 1962 survey of the proton magnetic resonance spectra of a number of globular proteins (ribonuclease, bovine serum albumin, insulin, aldolase, myoglobin, chymotrypsin, pepsinogen, and cytochrome *c*) under various conditions, Arthur Kowalsky reported that although the peaks in the proton NMR spectra of native proteins are broad, they became sharper under various denaturing conditions (Kowalsky, 1962). These observations were further elaborated by McDonald and Phillips, who reported in 1969 that the experimental NMR spectra of many denatured proteins (lysozyme, ribonuclease, pepsin, trypsin, apoferredoxin, apo-flavodoxin, oxytocin, and cytochrome *c*) were closely matched by the spectra computed by summing the spectra of the equivalent free amino acids, indicating the random coil-like structure of unfolded forms of globular proteins (McDonald and Phillips, 1969; Smith, 1999).

In 1974, the team of R. J. P. Williams reported the results of the 1D ^{1}H-NMR analysis of the intact chromaffin granule of the adrenal gland and isolated from it a mixture of chromogranin proteins (largely chromogranin A) and noted that "the n.m.r. spectrum of these chromogranin proteins is in fact similar to that of the sum of its component amino acids. Its profile is readily recognizable as that of a random coil protein" (Daniels et al., 1974). The fact that the authors recognized the NMR spectrum of a random coil protein indicates that the characteristic features of the random coil spectra were already well-known by that time.

Several reviews were dedicated to the description of how the application of NMR was involved in the discovery and characterization of intrinsic disorder (Mittag and Forman-Kay, 2007; Marsh et al., 2012; Kosol et al., 2013; Felli and Pierattelli, 2014; Konrat, 2014; Novacek et al., 2014; Brutscher et al., 2015; Dunker and Oldfield, 2015; Kurzbach et al., 2015; Bolik-Coulon et al., 2019; Dyson and Wright, 2019; 2021; Camacho-Zarco et al., 2022; Shahrajabian and Sun, 2024). While NMR spectroscopy excels at providing high-resolution structural details of IDPs in solution (Daughdrill et al., 2005; Eliezer, 2009; Jensen et al., 2010), it is not without its own drawbacks. Some of these limitations, challenges, and shortcomings include (Uversky and Dunker, 2012c):

- Size constraints: as protein size increases, molecular tumbling becomes slower, leading to shorter spin-spin relaxation times and more complex spectra, making analysis challenging.
- Challenges with sequence redundancy: tandem repeats in IDPs/IDRs can create redundancy in NMR spectra, making it harder to assign specific signals to individual amino acids.
- Limited spectral dispersion: due to the relatively uniform environments experienced by residues within IDPs/IDRs, their NMR spectra often exhibit poor dispersion, hindering the ability to resolve individual signals.
- Dynamics and line broadening: rapid conformational fluctuations within IDPs/IDRs, particularly on the

TABLE 1 Characteristics of low-resolution biophysical techniques contributed to the discovery and initial characterization of IDPs.

Technique	Physical principles	Parameters measured	Advantages	Disadvantages
X-ray crystallography	Relies on the interaction of X-rays with the ordered, periodic arrangement of atoms in a crystal lattice. The technique uses X-ray diffraction and the resulting interference patterns to determine the 3D-structure of molecules, such as proteins, at an atomic level	Measures the angles and intensities of X-rays diffracted by a crystal to ultimately determine the 3D arrangement of atoms within it. From this primary data, numerous structural parameters can be derived, such as unit cell dimensions, atomic positions, bond lengths, and bond angles	Shows the presence of IDRs as regions with missing electron density Was crucial for raising the awareness of the common presence of disorder in proteins	Does not provide any structural information besides making a claim that this region is too flexible to be resolved by X-ray crystallography
NMR spectroscopy	Founded on the principles of quantum mechanics, utilizing the intrinsic angular momentum, or spin, of specific atomic nuclei. Because these nuclei act as microscopic magnets, they interact predictably with an external magnetic field	Measures chemical shift, spin-spin coupling, relaxation times, and signal intensity (integration) to provide information on molecular structure, dynamics, and interactions	Provides atomic-resolution dynamics over multiple timescales Enables detection of transient and residual structures Allows studies under near-physiological conditions Allows characterization of the biomolecular interactions Provides rich information despite spectral crowding	Poor chemical shift dispersion, signal loss from fast exchange, low sensitivity, challenges with larger proteins, challenges with repeat-containing proteins, need for complex experimental setups, need for isotopic labeling, difficulties with ensemble interpretation, distinguishing transient structure from random coil
Optical rotatory dispersion (ORD)	Rooted in circular birefringence, where a chiral material causes left and right circularly polarized light to travel at different speeds due to different refractive indices. This velocity difference creates a phase shift between the two light components, causing the plane of polarization of a combined beam to rotate. This rotation varies with wavelength, and an optical rotatory dispersion (ORD) curve, a plot of rotation versus wavelength, can be used to study the molecule's chiral and electronic properties	Analyzes the relationship between an optical rotation of chiral compound and the wavelength of light. The characteristic ORD curve produced from this data can be used for estimation of key parameters related to the protein secondary structure and conformational changes	Ability to measure outside of absorption bands which makes it insensitive to protein concentration and suitable for analysis of highly concentrated samples Sensitivity to overall molecular conformation Complementary structural information to CD.	Complexity of spectral interpretation, limited information for dynamic ensembles, insensitivity to subtle conformational changes, poor signal resolution in the far-UV region, mostly obsolete
Circular dichroism (CD)	Based on the differential absorption of left- and right-circularly polarized light by a chiral, or "optically active", molecule. This difference in absorbance leads to the light emerging from the sample as elliptically polarized, rather than linearly polarized. Ellipticity, a measure of the shape of this ellipse, is directly proportional to this difference in absorbance and provides information about the conformation and structure of a molecule under study	Measure several key parameters of proteins. The far-ultraviolet CD spectrum of a protein (190–250 nm) is characteristic of protein secondary structure (e.g., α-helices, β-sheets, irregular structure). The near-UV CD spectrum of a protein (250–350 nm) characterizes the uniqueness of the local environment of aromatic residues and therefore gives information about 3D-strucutre. This allows CD spectroscopy to be used for rapid conformational analysis and for monitoring folding and unfolding transitions	Solution-based analysis, confirms disordered nature, monitors induced folding, low sample requirements, speed of analysis, monitors effects of environment, enables kinetic studies, non-destructive, versatile, cost-effective	Low-resolution information, not residue-specific, susceptibility to conditions and contaminants, poorly represented reference data leading to inaccuracy of deconvolution, averaging over multiple domains, difficulty detecting small changes, spectral overlap
Fourier-transform infrared (FTIR) spectroscopy	Works on the principle of molecular vibration and infrared light absorption. Molecules absorb specific frequencies of infrared light, causing their bonds to vibrate at that same frequency. The two types of vibration are stretching (a change in the distance between two atoms along the bond axis) and bending (change in the angle between two chemical bonds). This absorption is unique to each molecule, creating a "fingerprint" spectrum, when the data is converted from an interferogram to a spectrum using a Fourier transform	Provides information about secondary structural motifs and can be used in conformational studies, but offers very limited insight into the overall 3D structure of a protein. Can provide quantitative information on glycosylation and phosphorylation, as well as hydration and solvent effects. Can be used for analysis of protein conformational stability, misfolding and aggregation	Provides direct insight into secondary structure (position of the amide I band is highly sensitive to the protein secondary structure, such as α-helices, β-sheets, and unordered conformations), no need for isotopic labeling or extensive sample preparation, monitors induced folding and interactions, deal for studying protein aggregation (not affected by scattering from large particles), can be used in hydrogen-deuterium (H/D) exchange studies	Water interference, buffer and solvent interference, poor sensitivity to secondary structural elements, difficulty with complex mixtures, limited structural details, overlapping spectral signals, poor distinction between disordered states, need for deconvolution, no spatial information, potential for artifacts and inaccurate results,
Raman spectroscopy	Raman spectroscopy is based on the inelastic scattering of light, where interaction of molecules with monochromatic light causes them to scatter at a different frequency (energy) than the incident light. This inelastic scattering occurs when a molecule absorbs a photon, is excited, and then emits a scattered photon. The energy difference between the incident and scattered photons corresponds to the vibrational energy of the molecule, which provides a unique "fingerprint" for material identification	Raman spectroscopy can measure a variety of parameters related to a protein structure, dynamics, conformational stability, interactions, and environment. Detailed information on the protein backbone and its secondary structure components (e.g., α-helices, β-sheets, irregular structure) is provided from the analysis of amide I (~1630-1700 cm^{-1}) and amide III bands (~1230-1340 cm^{-1}), as well as backbone skeletal stretches (~870-1150 cm^{-1}). Tertiary structure and microenvironment are evaluated by observing the vibrational modes of specific amino acid side chains, such as tyrosine doublet (~850/830 cm^{-1}), tryptophan band (~1550 cm^{-1}), and disulfide bond (~500-545 cm^{-1})	Label-free and non-destructive analysis in native aqueous environments, sensitivity to secondary and tertiary structure, high spatial resolution and single-molecule capability, characterization of protein interactions and aggregation, applicability at dilute concentrations, quantitative analysis	Weak Raman effects, strong fluorescence interference, conformational diversity and related spectral overlap, as well several technical limitations, such as difficulties with low concentrations, potential laser-induced damage, difficulties with quantitative analysis
	Resonance Raman spectroscopy enhances Raman scattering by tuning the laser to an electronic absorption band of the molecule, leading to a significant increase in intensity for vibrational modes coupled to that electronic transition. This technique selectively probes chromophores, minimizing interference from other parts of the sample	Resonance Raman spectroscopy is used to study protein structure, dynamics, and function by measuring vibrations in specific parts of the protein. This technique can provide information on proteins secondary structure (using bands like amide III), amino acid side chain environment (sensitive markers for aromatic residues), heme group properties (core size, spin state, and oxidation state), and metal-ligand interactions	Real-time, non-destructive monitoring, enhanced sensitivity for low concentrations, selective probing of local environments, reduced spectral complexity, compatibility with aqueous solutions, detection of transient secondary structure elements, capability to distinguish between native and aggregated states	Potential for fluorescence interference, risk of sample damage, dependence on chromophors, provides indirect information and cannot directly determine the protein structure, technical complexity, misleading structural interpretations, limited insight into conformational ensembles, signal convolution in complex environments, broad overlapping signals due to dynamic and heterogeneous structures
	Raman optical activity (ROA) is based on the observation that chiral molecules interact differently with left and right circularly polarized light, leading to a difference in the scattering intensity of each. This phenomenon is an interference effect between the scattering from a molecule polarizability tensor, which causes normal Raman scattering, and its optical activity tensor. The optical activity tensor is only present in chiral molecules	ROA provides highly detailed structural information about proteins, including their conformation and secondary structure elements such as α-helices, β-sheets. It is also sensitive to changes in a protein's tertiary structure, which result from overall folding. Furthermore, ROA can detect subtle changes caused by a protein's microenvironment, such as interactions with solvent molecules or the presence of specific side chains like aromatic residues and stabilizing disulfide bonds	This non-destructive and label-free technique probes conformational ensembles in solution, highly sensitive to backbone conformation and secondary structure, investigates chirality and stereochemistry, works in aqueous solutions, provides insights into dynamics and disorder and can be used for analysis of misfolding and aggregation, can distinguish conformers, contains information about the conformation of both the polypeptide backbone and the amino acid side-chains	Weak signal and low sensitivity (low signal-to-noise ratio), requires high sample concentrations, spectral overlap, complexity of spectral assignments, technique limitations, interference from fluorescence, accuracy of data interpretations
	In size exclusion chromatography (SEC), molecules are separated according to their size as they flow through a column filled with porous beads; large	Measures hydrodynamic radius (R_H), or Stokes radius (R_S), which is the radius of a sphere that diffuses identically to the protein. The protein size in	Separation of proteins by their hydrodynamic radius under native, non-denaturing conditions. Deviation from expected elution behavior of globular	Limited resolution and sensitivity, low loading capacity, poor resolution of conformers and related limited insight into conformational changes, lack of

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TABLE 1 (*Continued*) Characteristics of low-resolution biophysical techniques contributed to the discovery and initial characterization of IDPs.

Technique	Physical principles	Parameters measured	Advantages	Disadvantages
Size-exclusion chromatography (SEC)	molecules, unable to fit into the pores, move quickly through the column and elute first, while smaller molecules can penetrate the pores, taking a longer and more winding path and eluting later	solution is affected by both its molecular weight and its shape. These data can be used for estimation of molecular mass using calibration of a column with globular proteins with known molecular weights. Can also provide information about quaternary structure and quantify population of different components. Can be used for the analysis of conformational stability and conformational changes	proteins of given molecular mass is a strong indicator of intrinsic disorder. Can physically separate different IDP conformers or distinct association states based on differences in their hydrodynamic dimensions. Can be used for analysis of interactions and complex formation. Can be used under a variety of conditions. This technique is non-destructive, versatile, and cost-effective	appropriate size markers for column calibration, challenges with mass and size correlation (misleading molecular weight estimations if using globular proteins for column calibration), interaction with column matrix
Viscometry	Fluid viscosity is measured using internal resistance of fluid to flow and drag. Different methods can be used for this purpose and viscometers measure time it takes for a fixed volume of fluid to flow through a capillary under gravity (in capillary viscometers), or time required for a sphere of known size and density to fall through a fluid (in falling sphere viscometers), or the torque needed to rotate a spindle (rotor) at a constant speed while submerged in the fluid (in rotational viscometers), or the damping effect of a fluid on an oscillating element (in vibrational viscometers)	Measure several parameters of protein solutions, providing insights into size, shape, conformation, and molecular interactions of proteins. Intrinsic viscosity is defined as the limit of the reduced viscosity as the solute concentration approaches zero. It reflects the contribution of an individual molecule to the viscosity of a solution under study. Being a key hydrodynamic parameter, intrinsic viscosity can be used to evaluate hydrodynamic radius, molecular size and weight, structure and shape, and conformation. For example, globular proteins, which are not perfect spheres, are characterized by the intrinsic viscosity values ranging from 3 to 4 dL/g, whereas for a rigid sphere, the theoretical intrinsic viscosity is 2.5 dL/g. Intrinsic viscosity also can be used for evaluation of protein-protein interactions (PPI), as the viscosity of concentrated protein solutions is highly sensitive to PPI, which can be attractive or repulsive	Viscometry is a sensitive and quantitative method provided insights into size, shape, and conformational changes of IDPs. This non-destructive and low-volume analysis is sensitive to conformational changes and provides insights into protein-protein and solvent interactions. Viscometry is a robust, well-established, accessible and reliable measurement technique	Provides only an ensemble-averaged measure of size and shape of IDPs, masking their specific dynamics, conformational heterogeneity, and structural transitions that define their biological function Lacks the resolution to differentiate between multiple, coexisting conformations and cannot distinguish between the contribution of the folded domains and the flexible disordered regions Cannot detect transient interactions Shows strong concentration dependence Cannot differentiate between a global conformational expansion and the formation of larger oligomers or aggregates
Small-angle X-ray scattering (SAXS)	Based on elastic scattering of X-rays by variations in electron density of a material. Measures intensity of these scattered X-rays at very small angles, typically less than 10°, to reveal information about nanoscale structure of the analyzed sample	Is highly efficient at measuring the overall size and dimensions of an IDP by calculating its radius of gyration (R_g) Generates pair distribution function providing information on the overall shape, anisotropy, and maximum dimensions (D_{max}) of a protein Kratky plot can quickly differentiate between a globular folded protein that has the bell-shaped curve and a disordered protein leveling off into a plateau at high scattering angles	Uniquely suited to study flexible, heterogeneous, dynamic molecules in their native solution state Provides global, ensemble-averaged information Can handle dynamic and flexible structures Non-destructive technique requiring minimal sample preparation and can be used in *in-situ* studies	Has an inherent low-resolution nature (typical resolution ranges from 1 to 20 nm) and tendency to average structural signals across a highly flexible and heterogeneous ensemble of conformations Multiple, structurally different models can produce scattering curves that fit the experimental SAXS data equally well Might have difficulty in ensemble modeling and issues with the reliability of R_g measurements for highly extended IDPs
Small-angle neutron scattering (SANS)	Relies on the wave-particle duality of neutrons, their interaction with atomic nuclei, and the use of elastic scattering at small angles to probe structures on a mesoscopic (nanoscale) scale	Measures the overall low-resolution structure of proteins and protein complexes Provides information on a nanometer scale (typically 1–100 nm) about shape, size, and conformation of proteins and complexes, and shows how different components within a complex are arranged Can be used for the analysis hydration shell and in the investigation of conformational changes	Since neutrons have no charge and interact directly with the atomic nucleus via the strong nuclear force, they can penetrate deeply into a sample and provide unique structural information. Adjusting the proportion of normal water (H_2O) to heavy water (D_2O) in a solvent allows using a "contrast matching" technique in neutron scattering. This method makes one component of a complex system invisible to neutrons, revealing the structure of the other components	SANS measurements are ensemble-averaged, and provide information about the entire population of IDP conformations, rather than a single, high-resolution structure Cannot capture dynamic details and is characterized by limited resolution Getting detailed insight requires computational modeling Require high concentrations, large sample volume, and cautious sample preparation
Static light scattering (SLS)	Based on the physical principles that when light interacts with particles, the particles re-radiate a portion of that light in all directions, and that the intensity of light scattered by a particle depends on its size and molecular weight	Measures the weight-average molecular weight (M_w) of a protein and the average root mean square (RMS) radius or radius of gyration (R_g), and quantifies protein-protein interactions by calculating the second virial coefficient (A_2), which is determined from the concentration dependence of the scattering data and serves as a key parameter for predicting protein solubility, interactability, crystallization, and the stability	Provides accurate measure of M_w without any assumptions and can be used for the R_g determination Detects and quantify protein aggregation Characterizes intermolecular interactions by providing information about the second virial coefficient (A_2) that is important for understanding propensity of IDPs for self-association or liquid-liquid phase separation (LLPS)	IDPs, with their high flexibility and lack of stable, defined structure, violate key assumptions of traditional SLS models and complicate the interpretation of scattering data SLS is highly sensitive to contaminants
Dynamic light scattering (DLS)	DLS is rooted on the physical principles of Brownian motion of a particle in a solvent (speed of such motion is size-dependent), the interference of scattered light, and the mathematical autocorrelation apparatus. By observing the time-dependent fluctuations in scattered light, the size distribution of nanoparticles and macromolecules can be accurately determined	By observing the time-dependent fluctuations in scattered light, the hydrodynamic size, size distribution of proteins, and polydispersity index can be accurately determined. DLS provide information on the translational diffusion coefficient (D), which is used for calculation of the hydrodynamic radius, R_H, or Stokes radius (R_S), using the Stokes-Einstein equation. Tool can be used for evaluation of the oligomerization state, protein-protein interactions, and conformational stability	DLS is versatile, fast, label-free, and non-destructive technique that can study a wide range of sizes and concentrations using low sample volumes Can be used for characterization of dynamic and flexible structures, monitor conformational changes, analyze conformational stability, assess sample homogeneity, and detect aggregation	DLS is an ensemble average technique that measures the average properties of a population of molecules in solution and provides a composite picture of the conformational ensemble It has a low resolution and, being strongly dependent on particle size, is dominated larger aggregates that dominate the signal and can obscure information about the smaller, monomeric IDPs Requires clean samples, specific concentration range, and assumes a spherical shape of a scattering particle
Analytical ultracentrifugation (AUC)	AUC involves subjecting a sample in solution to a high centrifugal force. This process causes particles to sediment at a rate that is a function of their size, shape, and density, providing important information that helps determine their hydrodynamic and thermodynamic properties. The overall behavior of the particles is determined by the interplay between four fundamental forces: Centrifugal, buoyant (based on the Archimedes' law), frictional, and diffusive	Provides an accurate and model-free method for determining the molecular mass of a protein and can distinguish between different oligomeric states Provides information on purity and heterogeneity Can be used for the analysis of conformational changes and provides information on the hydrodynamic shape of a protein by measuring its frictional ratio, which can distinguish between compact globular proteins and more elongated or flexible conformations SV-AUC provides a distribution of sedimentation coefficients (s), which measures how fast a molecule sediments in the centrifugal field, representing its size and shape	AUC is a first principle technique that does not require calibration standards, any labeling, chemical modifications or immobilization of a protein under study It does not rely on a stable, rigid structure and can characterize the size, shape, and interactions of highly flexible proteins Probes protein in a biologically relevant environment Is sensitive to overall shape and conformation Quantifies protein-protein and protein-ligand interactions Can assess heterogeneity and oligomerization Requires very small sample volumes	Due to the inherent flexibility and lack of stable 3D structure in IDPs, their sedimentation profiles are complex and difficult to deconvolute AUC cannot easily distinguish species with very similar sedimentation profiles Often requires combination with other biophysical methods and molecular dynamics simulations Interpretation of frictional ratio for IDPs could be challenging Requires high sample purity Significant expertise is required for performing experiments and properly analyzing the resulting data

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TABLE 1 (*Continued*) Characteristics of low-resolution biophysical techniques contributed to the discovery and initial characterization of IDPs.

Technique	Physical principles	Parameters measured	Advantages	Disadvantages
		SE-AUC measures the apparent molecular weight, oligomerization state, and binding stoichiometry and affinity of proteins in solution at thermodynamic equilibrium	Can provide thermodynamic and hydrodynamic information being used in SE-AUC and SV-AUC modes, respectively Can be combined with other methods and used as an orthogonal, gold standard approach to validate date generated by other techniques	Provides information about the overall size and shape distribution of the IDP ensemble, but cannot provide structural details at high resolution
Limited proteolysis	Limited proteolysis is based on the selective cleavage of polypeptide chains by an enzyme, which is determined by the three-dimensional structure and inherent dynamics of the substrate protein. Structured proteins are generally resistant to degradation, as a protease can only cleave peptide bonds within flexible, exposed, and unstructured regions of a protein, since these areas have the high conformational flexibility needed to fit into the active site	Limited proteolysis assesses the accessibility and flexibility of protein regions to a protease. It provides information on surface accessibility and structural flexibility, domain organization, conformational changes associated with binding of interaction partners, environmental stress, and posttranslational modifications, as well as folding and conformational stability	Limited proteolysis can identify and map disordered regions; screens for conformational changes; detects binding-induced folding; analyze dynamic conformational changes; reveals structural features, such as presence of stable domain; can be used for assessing molecular interactions; can be performed using small samples	High susceptibility of the entire polypeptide chain of an IDP to proteolytic cleavage can lead to excessive cleavage, irreproducible results and loss of information about the locations of transient folded regions. The method is characterized by limited resolution. The peptide abundance data for IDPs can be difficult to interpret in limited proteolysis coupled with mass spectrometry
Fluorescence	Steady-state fluorescence is best described by the Jabłoński diagram showing how molecules absorb light, transition to an excited state, and then return to the ground state via both radiative (light-emitting) and non-radiative pathways. Depending on the nature of the fluorophore, fluorescence ban be intrinsic or extrinsic	Major parameters of steady-state fluorescence in proteins are the maximum emission wavelength (λ_{max}), fluorescence intensity, quantum yield, and anisotropy. This technique allows investigation of protein folding and unfolding, protein conformational stability, protein interactions, as well as probing local environment by providing information on the polarity of the fluorophore environment	Provides a range of ensemble-averaged information on IDP conformation, dynamics, and interactions with other molecules using relatively simple, non-destructive, and highly sensitive measurements. Can be used for probing changes in the microenvironment of a fluorophore and tracking protein behavior. As applicable for analysis of interactions and binding, conformational ensembles	Since the long-term average properties of the entire population of molecules is measured at once, dynamic information and information about individual conformations is lost. Provides a single, averaged snapshot of the conformational ensemble and is characterized by limited resolution of conformational flexibility. Insensitive to fast dynamics and cannot detect short-lived states. Changes in fluorescence can be cause by various factors, making interpretation of the results difficult. Fluorophore can be permanently destroyed by prolonged exposure to light. High background noise can be produced by fluorescence of other molecules in the sample
	Fluorescence quenching decreases fluorescence intensity of a molecule through physical interactions with a quencher, where either quencher collides with an excited fluorophore deactivating it (dynamic or collisional quenching) or a non-fluorescent complex is formed between the quencher and fluorophore in the ground state (static quenching)	Fluorescence quenching measures changes in protein fluorescence parameters, such as intensity, quantum yield, and lifetime, caused by a quencher molecule that reduces fluorescence through collisions or by forming a complex. Being sensitive molecular properties, such as size, charge, and polarity, as well as environmental factors, such as temperature, pH, oxygen, and the presence of specific ions or chemicals, fluorescence quenching can report on molecular interactions and conformational changes	This highly sensitive and technically simple approach can be used to probe fast, local conformational dynamics and investigate and resolve distinct conformational ensembles. Can be used for the analysis of binding events and investigation of interaction mechanisms. Can report on collapse and folding transitions. Being sensitive to local environmental changes can report how the local environment of a specific residue is affected by binding or conformational transition	Data interpretation for highly flexible and heterogeneous structures is hindered by the limited, ensemble-averaged information provided by this method. Cannot be used for the analysis of transient interactions, provides limited spatial resolution, and can generate misleading ensemble average
	Red edge excitation shift (REES) originates when the fluorescence lifetime (τ_F) is comparable to or longer than the solvent relaxation time (τ_S) and the environment of a fluorophore is characterized by slow solvent relaxation (e.g., the interior of a protein globule). Ander such conditions, the system becomes a collection of fluorophores in different microenvironments, each with a distinct energy level. For the sub-population of fluorophores excited at the red-edge (lowest energy), the slow-moving solvent may not have enough time to relax before the photon is emitted. As a result, fluorophores in more polar environments are selectively exited, which results in a shift in the fluorescence emission spectrum to longer wavelengths (a red shift)	The magnitude of the REES provides information about the physical properties of the local environment of fluorophore, where more restricted or rigid environment with particularly slow solvent relaxation is characterized by larger REES. Therefore, REES, which is sensitive to the polarity and dynamics of the immediate surroundings of a fluorophore, can be used to analyze protein dynamics, flexibility, conformational stability, protein interactions, and folding and unfolding processes, as well as it can reveal the equilibrium between different conformational states within a protein population	REES is a sensitive and relatively inexpensive technique that provides unique insights into conformational dynamics of IDPs and interactions with other molecules. It probes dynamics of the local the local environment and the rate of solvent relaxation around a fluorophore and can detect subtle changes in the local structure and dynamics of IDPs and distinguish between different conformational states. It also can be used to monitor functionally relevant conformational changes (e.g., "disorder-to-order" transition upon binding to a target molecule) and to reveal heterogeneity within IDP ensembles	REES is not observed in highly disordered proteins, where environment relaxes too quickly. It depends on the presence of an environmentally-sensitive fluorophore (such as tryptophan). Experiments require careful control to avoid ambiguity if data interpretations
	Fluorescence anisotropy measures how much a fluorescent molecule rotates by analyzing the polarization of the light it emits after being excited by polarized light. Molecules that rotate faster due to their smaller size, lower viscosity, or fewer interactions will have their emitted light depolarized, resulting in lower anisotropy. Conversely, larger molecules rotate more slowly, are less likely to tumble, and therefore emit light with higher anisotropy	Fluorescence anisotropy measures the rotational motion and tumbling of a fluorophore attached to a protein, changes in which can reveal information about the size, shape and flexibility of a protein. It can be used to study various biological phenomena in real-time within a solution, such as protein-protein, protein-ligand, and protein-nucleic acid interactions, protein folding and unfolding, oligomerization and aggregation, enzymatic assays. It also represents a robust method for analyzing protein-membrane interactions revealing crucial details about a size, shape, and rotational motion of a protein upon membrane binding, as well as characterizing the orientation, order, and dynamic properties of proteins and lipids within the membrane, and is useful for studying complex processes, such as receptor assembly	It can characterize flexible and dynamic behavior of IDPs in real-time and under near-physiological conditions, provide information on different motional dynamics (local, segmental, or global), and can be used in analysis of disorder-to-order transitions and studying local environment and structure. It is suitable for the real-time monitoring of binding and can be used to generate titration curves for accurate determination of binding constants and kinetics. Has a potential to be used in single-molecule and live-cell analysis, providing means for observing function *in vivo*	Limitations in the use of fluorescence anisotropy for analysis of IDPs are rooted in their high flexibility and large range of motion that decouple the movement of the fluorophore from the overall dynamics of the protein (probe-protein decoupling). This also defines limitations of this technique for characterization of protein size and binding, as reflected in small binding-induced changes in anisotropy
	Fluorescence (Förster) resonance energy transfer (FRET) involves the non-radiative transfer of energy between two fluorescent molecules, a donor and an acceptor. This non-radiative, long-range event is mediated by dipole-dipole coupling. For an excited donor molecule to transfer its energy to a ground-state acceptor, three key conditions must be met: The donor and acceptor must be separated by a short, critical distance (around 1–10 nm); there must be a strong spectral overlap between the donor's emission profile and the acceptor's absorption profile; and the transition dipole moments of the two molecules must have a favorable relative orientation	Since FRET measures distance between donor and acceptor, acting as a "spectroscopic ruler", it can be used for evaluation of the distance between two fluorescently labeled molecules, as well as analysis of protein-protein interactions, protein conformation and folding, and protein complex dynamics	Ensemble FRET is a high-throughput and cost-effecting method that allows efficient characterization of conformational states, provides reliable ensemble-averaged distance between two labeled points on the protein, monitors conformational changes, and provides insights into the environmental effects	Ensemble FRET masks structural and dynamic heterogeneity of IDPs. It provides a single, average FRET efficiency representing the population average of the heterogeneous and continuously interconverting ensemble of conformations. It conceals rare sub-populations cannot resolve kinetic details, and complicates distance quantifications

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TABLE 1 (*Continued*) Characteristics of low-resolution biophysical techniques contributed to the discovery and initial characterization of IDPs.

Technique	Physical principles	Parameters measured	Advantages	Disadvantages
	Single molecule FRET (smFRET) is a biophysical technique that uses two fluorescent molecules—a donor and an acceptor—to measure distances within a single molecule. The method relies on the principle of non-radiative energy transfer, where the efficiency of energy transfer is highly dependent on the distance (inverse sixth-power distance dependence, R^{-6}) between the fluorophores. This sensitivity allows smFRET to function as a "molecular ruler" to measure conformational changes and distances in the 1–10 nm range with high precision	By attaching fluorescent labels to specific sites, smFRET is used to observe the complex internal motions of individual proteins, including their folding and conformational shifts. It measures nanometer-scale distances within individual protein molecules in real-time. This technique is sensitive enough to track how individual proteins explore different structural states and how quickly they transition between them. The data can reveal conformational stability of a protein, characterize its intermediate states, and study the effect of binding partners or genetic variations on protein structure and function	Unlike traditional ensemble measurements that mask dynamic behaviors, smFRET provides a clear view of the conformational diversity and dynamic fluctuations of IDPs. This technique is particularly well-suited for IDPs because it is sensitive to the large-scale distance changes characteristic of these proteins and can resolve distinct structural forms and transient events by observing individual molecules. It can even provide detailed insights into how these proteins behave in complex cellular environments. This approach is characterized by high specificity and sensitivity, can analyze molecules at very low concentrations and can investigate rare events	The application of smFRET to IDP faces several major obstacles, including potential perturbation of protein dynamics by the labeling process, low signal-to-noise ratio requiring advanced equipment and leading to high measurement uncertainty, and difficulties in data interpretation due to the inherent conformational heterogeneity of IDPs. Furthermore, standard smFRET provides only a single-distance measurement, which is insufficient for fully capturing the complex, multi-dimensional dynamics of IDPs
	Fluorescence correlation spectroscopy (FCS) uses a focused laser to excite fluorescent molecules within a small detection area. As these molecules randomly diffuse, the total fluorescence intensity fluctuates over time. FCS uses statistical analysis (autocorrelation) to measure the "flicker rate" of this light. Faster fluctuations indicate smaller, quicker molecules, while slower fluctuations suggest larger, slower-moving ones. This data are used to calculate the number of molecules and their diffusion rates, which in turn reveals concentration, molecular size, and other properties	Analyzing the natural fluctuations in fluorescence intensity and by fitting the autocorrelation curve to a theoretical models, FCS provides quantitative information on the behavior of fluorescently labeled proteins, such as concentration, diffusion coefficient and mobility, hydrodynamic radius, molecular interactions, oligomerization state, conformational changes an chemical kinetics	FCS is a highly sensitive technique uniquely suited for characterization of IDPs and their dynamic properties in real time. FCS provides access to single molecule dynamics, can measure diffusion and size, characterize interactions, quantify conformational changes, does not require immobilization, and can be used for the analysis of LLPs. Has single-molecule sensitivity, and can be used to study biomolecules at low (picomolar to nanomolar) concentrations	Inherent flexibility and conformational heterogeneity of IDPs present challenges in FCS data interpretation and complicate the ability to extract meaningful information. Some of these limitations are difficulty modeling conformational ensembles, broadening of correlation curves, challenges with deconvolution and interpretation of FCS curves representing complex superposition of signals from multiple species, potential labeling-induced alterations in protein dynamics, and photobleaching
Mass spectrometry	Hydrogen-deuterium exchange mass spectrometry (HDX-MS) measures changes in the mass of a protein resulting from the exchange of its backbone amide hydrogens with deuterium from a heavy water (D_2O) solvents. As the mass of deuterium is about twice as large as that of hydrogen, each exchange event is measurable. The rate of hydrogen-deuterium exchange depends on local protein structure, with solvent-exposed, flexible or unstructured regions undergoing rapid exchange, and with ordered regions, where hydrogens are protected from the solvent, exchanging slowly	HDX-MS measures changes in conformation, dynamics, and interactions of proteins by monitoring the rate of hydrogen-deuterium exchange. Can analyze allosteric effects and protein aggregation. Is uniquely suited to analysis of IDPs, as high deuterium uptake at very short time period indicates dynamic and disordered regions	HDX-MS probes transient, dynamic structures; allows for millisecond-scale analysis; can identify IDRs and characterize residual structure, as well as analyze folding and study binding transitions by monitoring stabilization upon interaction and mapping interaction sites	The primary disadvantage of HDX-MS for analysis of IDPs is their rapid exchange kinetics (low protection), making subtle changes in dynamics difficult to capture and interpret. Other issues are limited special resolution, incomplete sequence coverage, signal overlap, complex HDX-MS spectra that difficult to deconvolute
	Ion mobility-mass spectrometry (IM-MS) is rooted in separating ions by their mobility through a gas-filled cell under an electric field. The basis for separation lies in the fact that size, shape, and charge of an ion govern how often it collides with neutral gas molecules. These collisions slow the ion down, controlling its velocity and drift time	IM-MS measures shape, size, charge, and conformational landscape of a protein It evaluates a collision cross-section (CCS), which is a quantification of protein shape in the gas phase Can capture the variety of shapes adopted by a protein assembly and be used in conformational analysis	Can characterize dynamics and heterogeneous nature of IDPs in gas phase. It reveals conformational distributions, characterizes protein-partner interactions, separates isomers and conformers, and can be used for native state analysis by using soft ionization techniques, such as electrospray ionization (ESI)	IDPs are characterized by complex, broad, or overlapping arrival time distributions (ATDs) deconvolution of which could be challenging Different conformations can have different ionization efficiencies leading to inaccuracies in relative quantification IM-MS has a suboptimal resolving power, and prone to artifacts related to structural perturbations in IDPs during ionization, insensitivity to conformational changes during the experiments, and interference from solvents unwanted adducts
	Native mass spectrometry (nMS) is based on the fundamental principle of mass spectrometry, which is to ionize molecules, separate them by their mass-to-charge ratio (m/z), and detect them. It uses soft ionization techniques, such as electrospray ionization (ESI) that applies a high voltage to a liquid sample, creating a spray of charged droplets. Droplets shrink due to solvent evaporation until the electric field at their surface is strong enough to desorb solvated ions, expelling them into the gas phase	nMS measures mass-to-charge ratio (m/z) of intact proteins and their associated complexes and deconvolutes the resulting spectrum to give a precise molecular mass Can reveal oligomeric state, number of subunits, and stoichiometry Provides information about overall shape and folded state Can detect conformational changes Can resolve micro-heterogeneity within a protein sample (e.g., PTMs)	Enables the study of IDPs in their native state by gently moving them from a liquid to a gas phase (likely) without disrupting their structure Allows revealing different co-populated conformations present in a highly heterogeneous structural ensemble of an IDP. Can investigate dynamic interactions	Because of the natural flexibility, heterogeneity, and distinct ionization behaviors, nMS analysis IDPs can be problematic at various stages, starting with ionization and transfer into the gas phase challenges, to conformational distortion in gas phase, to charge-state artifacts, and to limitations in data analysis and interpretation. Furthermore, instead of capturing the full, dynamic range of conformations and interactions of an IDP, nMS provides a snapshot of the conformational ensemble at a specific point
Differential scanning calorimetry (DSC)	DSC measures the difference in heat flow between a sample and a reference as they are heated or cooled at a controlled rate. A measurable difference in temperature or heat flow between the sample and the reference reflects either absorption or release energy associated with global physico-chemical changes in a sample, such as melting or crystallization. Corresponding differences are recorded as a thermogram that is used for determination of the thermodynamic properties of the system	Key parameters generated by DSC for proteins are Melting temperature (T_m) at which half of the protein population is denatured and half is still in its folded state Enthalpy (ΔH), which is calculated from the area under the thermogram peak and corresponds to the total heat energy absorbed during the thermal unfolding process Change in heat capacity (ΔC_p), which is the heat capacity difference between the folded and denatured states Cooperativity of the denaturation process, with a sharp, narrow peak reflecting a highly cooperative, two-state melting process, and with a broader peak corresponding to a less cooperative process, possibly accompanied by the population of intermediate states, or may indicate an oligomeric/multidomain protein	Can distinguish between ordered protein and IDP. Can be used for evaluation of hydration capacity Can assess changes in conformational stability induced by environmental factors Can be used for comparison of variants and analysis of interactions (e.g., ligand binding) that affect thermal properties of a protein Can provide complete thermodynamic profile Represents a label-free and direct measure of the heat absorbed or released during thermal transitions Is insensitive to optical artifacts	Since IDPs do not have rigid structure, they do not undergo clear, cooperative unfolding transitions and the transition detected by DSC represents a shift in the population of conformations within the ensemble, leading to inability to determine a single T_m ΔH measured by DSC for IDPs does not correspond to the breaking of bonds in a single, stable folded structure but reflects the total energy absorbed during the heating-induced redistribution of conformational states within the ensemble Thermal "transitions" in IDPs are weak and broad Obviously, melting of IDPs cannot be described within a two-state unfolding model
High-speed atomic force microscopy (HS-AFM)	HS-AFP is based on the same principle forming foundation of a conventional ATM, namely, using a sharp, nanometer-sized tip to sense the surface of a sample and map its topography. The technological refinements causing the dramatic acceleration of the scanning process enable rapid	HS-AFM is a label-free imaging technique for visualizing the real-time structural dynamics, functional activities, and molecular interactions of single proteins. The generated data allows for the creation of high-resolution	A single-molecule label-free technique that enables direct visualization of individual IDP molecules in real-time and under near-physiological conditions, providing insights into their structural dynamics and functional behavior	Has strong technical demands for sample preparation Is prone for tip-induced artifacts Cannot be used for capturing biological processes that occur on sub-100 ms timescales

(Continued on following page)

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TABLE 1 (*Continued*) Characteristics of low-resolution biophysical techniques contributed to the discovery and initial characterization of IDPs.

Technique	Physical principles	Parameters measured	Advantages	Disadvantages
	imaging that can directly visualize dynamic processes, such as protein in action	videos capturing movements, conformational changes, and binding events of a query protein under near-physiological conditions	Provides high spatiotemporal resolution Allows for quantitative and detailed structural characterization of IDP structural properties at the single-molecule level	Has a limited field of view and cannot be used for analysis of internal structure High sensitivity to external vibrations and acoustic noise Highly expensive and requires specialized expertise Potential disagreement with smFRET due to the differences in how each method observes and influences behavior of a query molecule

millisecond to microsecond timescale, can lead to significant line broadening in NMR spectra, sometimes preventing direct studies of partially folded intermediates like molten globules.
- Limited global information: NMR primarily focuses on nuclei and their local environments, providing less information about the overall size and shape of IDPs, though some estimations of size can be gleaned from diffusion data derived from line broadening.

Furthermore, other structural biology techniques suggested the existence of IDPs and IDRs before they were actually described by NMR and X-ray crystallography. For example, in their paper reporting the random coil-like appearance of the 1D ^{1}H-NMR spectrum of chromogranin A, Daniels et al. (1974) indicated that physical properties of this protein evaluated in 1967 by a set of biophysical techniques, such as gel-filtration chromatography, viscometry, and optical rotatory dispersion (ORD) have led to the conclusion that chromogranin A has a conformation approaching that of a random-coil polypeptide (Smith and Winkler, 1967).

2.3 Optical rotatory dispersion (ORD) spectroscopy

Optical Rotatory Dispersion (ORD) is one of the unique spectroscopic techniques that can be used for the analysis of molecule stereochemistry and explore the world of chiral molecules and their unique properties (Djerassi, 1960). ORD measures the wavelength dependence of the optical rotation (i.e., the rotation of the plane of polarization of light when it passes through a chiral substance), which, in relation to proteins, can be used for evaluation of protein secondary structure (Jirgensons, 1964). This technique has long pre-history, being observed for the first time in 1811 by François Arago (1786–1853) who noticed that quartz crystals produced colors in sunlight that had passed through two polarizing filters (Arago, 1811). In 1812, Jean-Baptiste Biot (1774–1862) described rotation of the plane of polarized light by a substance and made first observations of ORD; i.e., that optical rotation varied depending on the wavelength (color) of the light (Biot, 1812). Although Werner Kuhn (1899–1963) demonstrated the possibilities of studying molecular structure by ORD in 1929 (Kuhn, 1929), the actual exploration of these possibilities started much later.

In 1952, Thomas LeRoy McMeekin (1900–1979) conducted a systematic ORD-based analysis of milk proteins and established that that the milk protein casein exists in an "unfolded configuration" (McMeekin, 1952). He also noted that "As compared with other proteins, casein is remarkably stable. In solution it may be heated, or treated with organic solvents, specific denaturing agents such as urea and guanidine hydrochloride, and small amounts of acid or alkali without apparent change in properties. *In vitro*, however, casein is digested with the greatest ease by proteolytic enzymes . . . Since casein cannot be denatured, it is frequently considered to be already denatured or to have an unfolded structure. Studies of physical properties of casein solutions, such as viscosity and streaming birefringence are consistent with the idea that casein is a long molecule resembling denatured proteins (McMeekin, 1952).

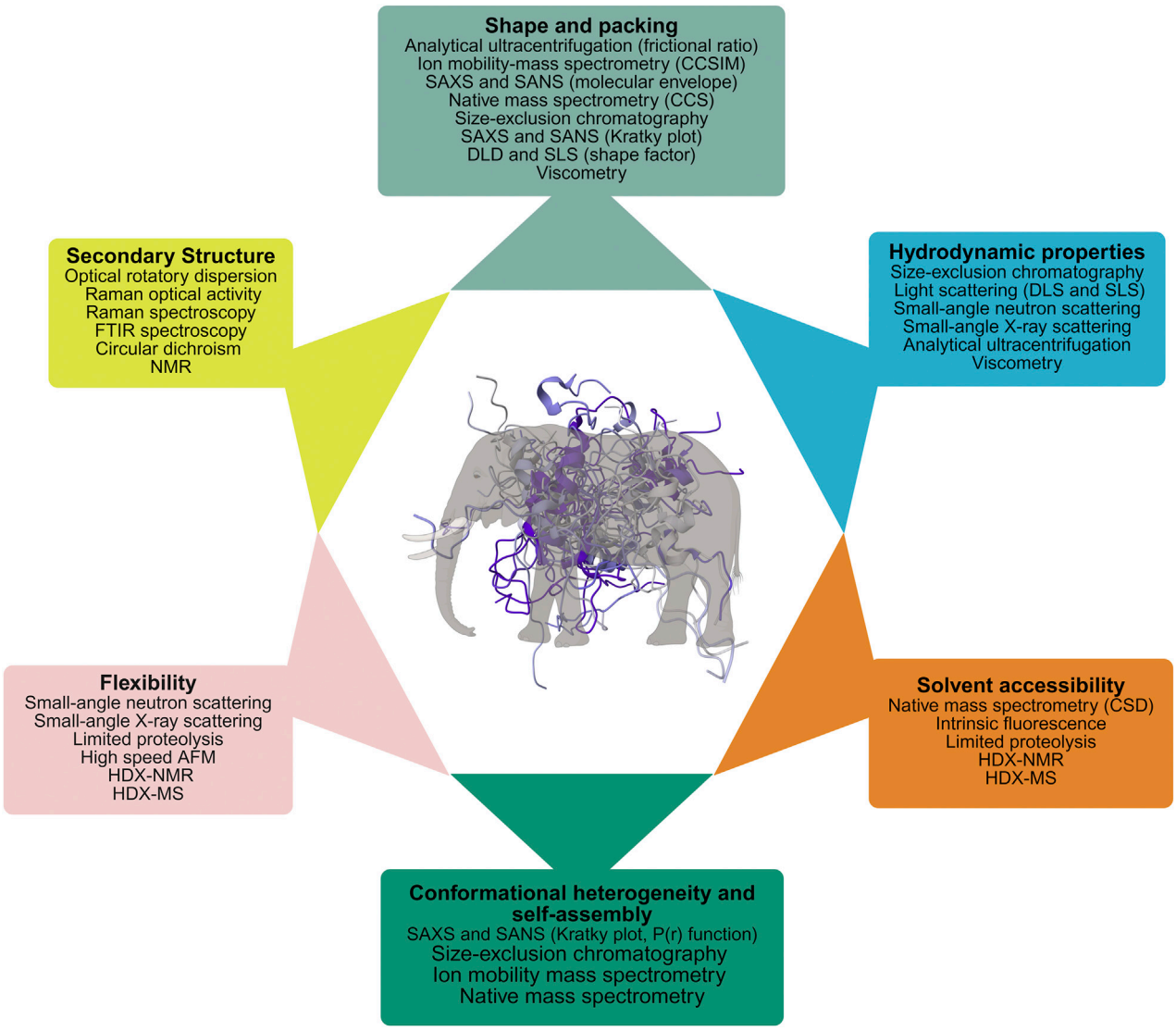

FIGURE 3
Multiparametric approach as a tool to see the "whole elephant". A fuzzy structure of an IDP (shown in the middle) can be evaluated by different biophysical techniques sensitive to different structural features of the protein.

Bruno Jirgensons (1904–1982) was a very noticeable figure in the field of protein ORD, making significant contributions to the development and application of ORD as a technique for investigating the structure of proteins and other macromolecules (Jirgensons, 1964; Jirgensons, 2012). In relation to the subject of this review, it seems that Prof. Jirgensons was one of the first researchers who conducted the biophysical characterization of an intrinsically disordered protein. In fact, in 1958, he reported the results of the analysis of a highly phosphorylated protein from egg yolk, phosvitin (curiously, with more than 90% of its serine amino acids being phosphorylated and with 10% phosphorus content, phosvitin is considered as the most phosphorylated protein in nature (Yilmaz and Agagunduz, 2020)), by viscometry and ORD, and suggested that this protein is characterized by a flexible, disordered structure, which is similar to that of disordered polyglutamate (Jirgensons, 1958).

Poly-L-lysine (polylysine) and poly-L-glutamic acid (polyglutamic acid) have long been used as models for the spectroscopic identification of secondary structure sequences in proteins since, being disordered at neutral pH, they adopt well-defined conformations under certain conditions of temperature and pH (Adler et al., 1973; Yu and Krimm, 1977). In fact, polylysine at alkaline pH and polyglutamic acid at acid pH form α-helical conformations, since under these conditions they have neutral side chains, whereas at high pH and high temperature, polylysine adopts an extended, flat, multistranded β-sheet conformation, and polyglutamic acid forms β-sheet structure at low pH.

In 1965, Bruno Jirgensons and Lubomir S. Hnilica reported that in aqueous solutions, the unfractionated calf-thymus histone and its four main fractions (F1, F2a, F2b and F3) were in the form of disordered chains (Jirgensons and Hnilica, 1965). In 1966 (the manuscript was actually received by the journal on 10 April 1965), Bruno Jirgensons reported a comprehensive analysis of multiple proteins by optical rotatory dispersion (ORD) to classify them according to the conformation of their polypeptide chains

(Jirgensons, 1966). Based on the result of this analysis, a database was created which grouped proteins based on their secondary structure and which contained a "disordered" category for some of the proteins (Jirgensons, 1966). Furthermore, some proteins were described as containing ordered secondary structure and some disorder (Jirgensons, 1966). Illustrative examples of the fully disordered proteins in that database are phosvitin and histones in water (Jirgensons, 1966). Jirgensons also indicated that the disordered nature of phosvitin and histones in water is supported by their anomalous behavior during viscosity measurements. One of the conclusions in the 1966 study by Jirgensons reads: "There is no doubt that large portions of the polypeptide chains of all of the proteins are disordered, and that in all cases of proteins which have compact macromolecules (yielding solutions of low viscosity) the disorder is more or less fixed" (Jirgensons, 1966).

2.4 Circular dichroism (CD) spectroscopy

Similar to ORD, circular dichroism (CD) is rooted in the analysis of optical activity, with CD measuring the difference in absorption of left and right circularly polarized light by a chiral molecule. Due to this difference, an elliptically polarized light wave results when a linearly polarized light wave passes through an optically active chiral compound (Rogers et al., 2019). The phenomenon was first observed and named by Aimé Cotton (1869–1951) who, in 1895, reported that certain materials, known as chiral molecules, absorb left- and right-circularly polarized light at different rates (Cotton, 1895). However, the technique and instrumentation for modern CD spectroscopy were developed later (see below).

Although ORD studies of polypeptides and proteins were conducted prior to CD, the dominance of ORD lasted only a few years, as in 1960, a French group presented an innovative approach to measure CD based on the use of a Pockel's cell as an electro-optic retardation modulator (Grosjean and Legrand, 1960), which in combination with the adoption of the photoelastic modulator in 1966 (Billardon and Badoz, 1966), led to the development of accurate CD instruments. This instrumental breakthrough resulted in the widespread application of CD spectroscopy, making this method one of the major tools in protein structure investigation. Because of the relatively low cost compared to NMR instrumentation and ability to work on a wide range of proteins and under diverse conditions providing a wealth of structural information (such as the secondary and tertiary structures of proteins in solution, as well as details about protein folding, interactions, and conformational changes induced by environmental factors or ligand binding) by using relatively small amounts of protein sample, spectropolrimetry became a very popular technique, and spectropolarimeters are commonly found in many structural biology laboratories.

An advantage of CD in the analysis of protein structure is determined by the ability of this technique to provide complementary structural information from different spectral regions (Kelly et al., 2005). In fact, since the peptide bond acts as the chromophore in the far-UV region (also known as "peptide region"), CD spectra measured in this spectral range are used to characterize protein secondary structure. On the other hand, CD spectra in the near-UV region (also known as "aromatic region") report on the asymmetry of the environment of aromatic residues and therefore provide information about protein tertiary structure (Kelly et al., 2005; Rogers et al., 2019). Similar to ORD, the shape of the CD spectrum in the peptide region (240 nm and below) is determined by the contributions of different secondary structure elements (with α-helices, β-sheets, β-turns, and unordered/random coil being the most frequently considered structural motifs) and can therefore be used for the estimation of the secondary structure composition of a protein (Kelly et al., 2005; Greenfield, 2006; Whitmore and Wallace, 2008; Rogers et al., 2019). Qualitative analysis of the far-UV CD spectra is based on their decomposition into contributions of various secondary structure elements. The procedure requires CD reference spectra for different types of secondary structure and depends on the crystallographically determined fraction of each secondary structural element present in globular proteins. In their seminal paper published in 1969, Norma J. Greenfield and Gerald D. Fasman (1925–2003), demonstrated how CD spectra could be computationally analyzed to estimate the secondary structural composition of proteins (Greenfield and Fasman, 1969).

Highly disordered proteins can be readily recognized by CD, since they lack any significant organized secondary structure and therefore have a peculiar far-UV CD spectrum resembling that of an unordered polypeptide, with a strong negative band near 200 nm and either a weak negative shoulder or a weak positive maximum near 220 nm (Woody, 2010). The very first descriptions of the far-UV CD spectra of "randomly disordered polypeptides" (which can be served as an oversimplified IDP) can be found in a 1962 paper by George Holzwarth (1937–2024), Walter Bruno Gratzer (1932–2021), and Paul Mead Doty (1920–2011) (Holzwarth et al., 1962), and in follow-up 1965 papers by Holzwarth and Doty (Holzwarth and Doty, 1965) and Léon Velluz (1904–1981) and Michel Legrand (Velluz and Legrand, 1965), who reported corresponding data for the poly-L-glutamic acid and poly-L-lysine at neutral pH.

Phosvitin, with its unique amino acid composition (this protein contains only 15% nonpolar amino acids but has 66% anionic and 17% cationic residues), was expected to behave similarly to highly charged polypeptides, such as polyglutamic acid. In line with this hypothesis, the far-UV CD analysis of this protein reported in 1967 by Serge N. Timasheff (1926–2019), Robert Townend, and Gertrude E. Perlmann (1912–1974) was qualitatively similar to spectra observed with polypeptides in unordered conformation, indicating a mostly disordered nature (Timasheff et al., 1967). In 1972, Earle Stellwagen, Richard Rysavy, and George Babul used far-UV CD spectroscopy as one of the techniques to characterize the structural properties of apo-cytochrome *c* (Stellwagen et al., 1972). Based on the results of their analysis, the authors concluded that this protein is in a randomly coiled conformation and that the apoprotein retains none of the conformational features of the native holo-protein (Stellwagen et al., 1972). In 1976, Colin Crane-Robinson et al. revealed that in aqueous media, chicken erythrocyte histone H5 is mostly disordered (it is essentially in the form of a flexible random coil) but the N-terminal part of this protein gains globular helical structure in the presence of salts (Crane-Robinson et al., 1976). Importantly, the C-terminal region of this protein (residues 59–197) remains mostly disordered even in the presence of 1 M kF (Crane-Robinson et al., 1976).

In a 1978 study, R. Wade Warrant and Sung-Hou Kim revealed that the protamine molecule is characterized by a random coil conformation in the unbound form, but folds to a structure containing α helices on binding to tRNA (Warrant and Kim, 1978). A monograph published in 1981 by Venyaminov et al. assembled far-UV CD spectra of all the ribosomal proteins from *Escherichia coli*, many of which showed characteristic features of mostly disordered proteins (Venyaminov et al., 1981). CD was used by Bonora et al. to investigate how various factors, including pH, salt concentration, helix-promoting solvents, and temperature, affect the structure of a protamine, salmine AI (Bonora et al., 1981). This small, basic protein was found to be largely unstructured in water. However, introducing certain counter-ions, particularly perchlorate and the solvent 2-chloroethanol, led to varying degrees of α-helical structure formation (Bonora et al., 1981). Similar behavior was also described for the fowl protamine, galline (Nakano et al., 1989).

This list can be expanded, indicating that the overall importance of far-UV CD for establishing and advancing the IDP field cannot be overstated. Especially in early days of this filed, most of the experimentally validated IDPs were those found using CD. This is reflected, for example, by the fact that in 2002, CD data for more than 100 IDPs were compiled and analyzed, leading to the conclusion that highly disordered proteins can be classified into two groups: coil-like and pre-molten globule-like (Uversky, 2002a). CD continues to be a popular tool for IDP analysis, as evidenced by more than 650 papers published since 1999, where the use of CD in IDP discovery and characterization is discussed. Furthermore, several amendments were introduced to make this approach more accurate while estimating the secondary structure of IDPs (Miles et al., 2023; Nagy et al., 2024).

The first systematic study on IDPs that triggered the interest of the scientific community to these proteins by claiming their abundance was published in 2000 (Uversky et al., 2000a). This study assembled a set of 91 "natively unfolded" proteins (i.e., IDPs with extended disorder, such as native coils and native pre-molten globules), which at physiological conditions have been reported to have the NMR chemical shifts of a random-coil, and/or lack significant ordered secondary structure (as determined by CD or FTIR), and/or show hydrodynamic dimensions close to those typical of an unfolded polypeptide chain (Uversky et al., 2000a). The disorder status of most of the protein in this dataset (72 of 91) was established or confirmed by far-UV CD (Uversky et al., 2000a), indicating crucial importance of this low-resolution technique in the establishing the IDP field.

2.5 Fourier-transform infrared (FTIR) spectroscopy

Infrared (IR) spectroscopy is a method for studying molecules by observing how they absorb infrared light. Since each molecule has a distinct set of vibrating bonds, the absorption pattern, or "fingerprint", can be used to identify unknown compounds and determine their structure. IR is a powerful tool for analysis of secondary structure and dynamics of proteins. This is primarily done by analyzing the Amide I band (around 1600-1700 cm^{-1} originating from the C=O stretching and N-H in-phase bending vibration of the amide group), which is sensitive to protein secondary structure due to the variations in hydrogen bonding and dipole-dipole interactions within α-helices, β-sheets, and disordered regions (Sutherland, 1952; Schwaighofer and Lendl, 2023). As a result, different elements of protein secondary structure are characterized by different frequencies of C=O vibrations and absorb in specific sections of the amide I band, which is reflected in the characteristic band maxima and shapes (Sutherland, 1952; Schwaighofer and Lendl, 2023).

The use of IR in protein/polypeptide structure analysis has a long history, with the first spectra of proteins being reported as early as 1935 (Heintz, 1935; Stair and Coblentz, 1935), and in 1940, Buswell et al. analyzed the IR spectra of 16 proteins, including three IDPs, acid casein, rennet casein, and salmine, and pointed out that presence of characteristic bands in the spectra of all the proteins studied suggest the important role that the hydrogen bond plays in protein structure (Buswell et al., 1940). In 1947, S.E. Darmon and Gordon Brims Black McIvor Sutherland (1907–1980) reported the IR spectra of high molecular weight synthetic polypeptides [protein analogs synthesized by Robert Burns Woodward (1917–1979) and Charles H. Schramm (Woodward and Schramm, 1947)] and noted that they showed a noteworthy similarity to a film of denatured keratin (Darmon and Sutherland, 1947). This was a remarkable conclusion, since the actual elements of protein secondary structure were introduced in 1951 by Linus Carl Pauling (1901–1994) and Robert Corey (1897–1971), who predicted the α-helix and β-sheet based on the hydrogen bonding of the protein backbone (Pauling and Corey, 1951), whereas the concept of "secondary structure" itself was formalized by Kaj Ulrik Linderstrøm-Lang (1896–1959) in 1952 (Linderstrøm-Lang, 1952). Structural transitions in a synthetic polypeptide from α-structure to β-structure were shown to be accompanied by the noticeable changes in the C=O frequency (Elliott and Ambrose, 1950), and dramatic changes in the IR spectra were shown to accompany insulin denaturation (Elliott et al., 1950).

Since those early days, the use of infrared spectroscopy to explore the conformation of proteins and polypeptides is well-documented (Sutherland, 1952; Elliott, 1954; Susi, 1972; Cooper and Knutson, 1995; Jackson and Mantsch, 1995; Barth, 2007; Glassford et al., 2013; Lorenz-Fonfria, 2020). The popularity of this approach increased dramatically after introduction of the Fourier-transform infrared spectrometers in 1950s that combined mathematical theory with advancements in optical engineering, followed by further instrumental improvements that made FTIR systems more compact and user-friendly. In the field of protein science alone, this popularity is reflected in almost 36,500 papers on infrared spectroscopy of proteins published between 1949 and 2025.

IR spectroscopy played an important role in establishing the IDR field by providing information on synthetic polypeptides and natural proteins lacking regular structure. Some of the first reports have already been mentioned above. Additional examples of IDPs characterized by IR in years preceding publications of the first systematic study on natively unfolded protein in 2000 (Uversky et al., 2000a) include coil-like structure reported for:

- soluble form of elastin by Mammi et al. (1968),
- the high-mobility-group chromosomal protein HMG 17 by Abercrombie et al. (1978),

- myelin basic protein by Cynthia S. Randall and Zand (1985),
- the chromosomal protein MC1 from the archaebacterium *Methanosarcina barkeri* by Imbert et al. (1990),
- the heat-stable inhibitor of the cAMP-dependent protein kinase by Thomas et al. (1991),
- Alzheimer's disease-related tau protein by Schweers et al. (1994),
- non-Aβ component of Alzheimer's disease amyloid plaque precursor (NACP), now known as α-synuclein by Weinreb et al. (1996),
- the photosystem II manganese-stabilizing protein of the thermophilic cyanobacterium *Synechococcus elongates* by Sonoyama et al. (1996),
- and α_s-casein by Byler and Susi (1986), Bhattacharyya and Das (1999).

This list is likely far from being complete. Although IDPs characterized by IR represented a small fraction of the experimentally characterized "natively unfolded proteins" included in the original study (6 of 91; 6.6%) (Uversky et al., 2000a), the role of this technique in establishing the IDP field is indisputable.

2.6 Raman spectroscopy

Raman spectroscopy is based on the Raman effect discovered in 1928 by Chandrasekara Venkataraman (known as C.V. Raman, 1888–1970) and Kariamanikkam Srinivasa Krishnan (1898–1961) (Raman and Krishnan, 1928) and independently by Grigory V. Landsberg (1890–1957) and Leonid I. Mandelstam (1879–1944), who described this effect also in 1928 as an inelastic combinational scattering of light (Landsberg and Mandelstam, 1928). Since the phenomenon of inelastic light scattering was predicted by Adolf Gustav Stephan Smekal (1895–1959) in 1923 (Smekal, 1923), it has been referred to as the Smekal-Raman-Effekt in older German-language literature (Kohlrausch, 1931). By detecting the frequency shifts from the frequency of the incident light caused by the inelastic scattering of laser light, Raman spectroscopy can produce a unique vibrational "fingerprint" of a protein molecule, which reveals its specific structure and dynamics. This method enables the characterization of individual amino acids, the observation of secondary and tertiary structural elements, the investigation of protein folding and unfolding pathways, and the detection of conformational transitions driven by external influences such as temperature or other molecular binding events. Furthermore, being complementary to CD, Raman spectroscopy is immune to the effects of light scattering, and can therefore be used for the analysis of proteins in micelles and other scattering media (Chi et al., 1998). Although Raman spectroscopy has been used for the analysis of proteins since the 1960s following the invention of the laser [in 1970, Rimai et al. reported the visible resonance Raman spectra of the rhodopsin from frozen bovine retinae (Rimai et al., 1970)], the use of the technique in early days was restricted by the need to use concentrated protein samples of 20–50 mg/mL because of low instrument sensitivity (Tu, 1982; Wen, 2007; Carey, 2012).

The sensitivity and selectivity of this approach are dramatically enhanced by utilizing resonance Raman spectroscopy that uses an excitation wavelength within an analyte absorption band. As a result of selective enhancement of vibrations coupled to the resonant electronic transition, resonance Raman spectroscopy (RR spectroscopy) can achieve up to 10^8-fold signal enhancement relative to the non-resonance Raman spectroscopy (Jakubek et al., 2018). Another modification of the method is Raman optical activity (ROA) that detects chiral molecules by measuring a small difference in their vibrational Raman scattering intensity when illuminated with right- and left-circularly polarized light (incident circular polarization, ICP) (Zhu et al., 2005). Alternatively, it can measure the small circularly polarized component within the scattered light itself, using a fixed-polarization incident light source (scattered circular polarization, SCP). Being discovered in 1971 by Laurence D. Barron and A. David Buckingham (1930–2021) (Barron and Buckingham, 1971) and discussed in detail in 1973 (Barron et al., 1973), ROA became a powerful tool for the structural analysis of proteins and other biomolecules (Barron et al., 2000; Barron et al., 2004; Zhu et al., 2005).

Since the Raman spectra are dominated by the amide bands of the peptide backbone, similar to ORD and CD, it can be used for the analysis of protein secondary structure by finding correlations between the positions of the amide I and amide III vibrations and fraction of each secondary structural element present in globular proteins determined from X-ray crystal structures (Williams, 1986; Berjot et al., 1987; Miura and Thomas Jr, 1995; Sane et al., 1999; Rygula et al., 2013). In 1970, Jack L. Koenig and Preston Sutton analyzed Raman spectra of poly-L-lysine in both aqueous solution and solid state, and showed that although this homopolymer behaves as a random coil in aqueous solutions, it folds into α-helical structure in solid state (Koenig and Sutton, 1970). In 1971, Ramachandran et al. demonstrated that the polypeptide having the repealing sequence $(Tyr\text{-}Ala\text{-}Glu)_n$ (where n ~175) was in a helical conformation at low pH and a random coil conformation at high pH (Ramachandran et al., 1971).

Although this tool was successfully used for the analysis of secondary structure in globular proteins, it was pointed out that "extrapolating empirical Raman parameters from globular proteins to natively unfolded proteins will presumably lead to significant errors, as some of the features that necessarily typify globular proteins are not present in natively unfolded proteins" (Maiti et al., 2004). Human α-synuclein, a disordered protein linked to the pathogenesis of Parkinson's disease and several other neurodegenerative diseases collectively known as synucleinopathies, was likely one of the first IDPs analyzed by Raman spectroscopy (Maiti et al., 2004). Since disordered α-synuclein can be induced to adopt both extensive α-helical and β-sheet conformations, this single protein can be used to characterize features in the Raman spectra that are associated with these secondary structures (Maiti et al., 2004). These observations showed that Raman spectroscopy can be used for the analysis of conformational changes induced in IDPs by environmental cues, as well as for the identification of the conformational constituents of IDPs in their conformational ensembles (Maiti et al., 2004). These authors also analyzed Raman spectra of three other IDPs, phosvitin, α-casein, and β-casein, as well as ionized polyglutamate and polylysine, and showed that a three-component band fitting can characterize their Raman amide I band (Maiti et al., 2004).

ROA is also used in the characterization of secondary structure of proteins and polymers, as illustrated by early studies on α-helical and unordered polylysine (Wilson et al., 1996), polyglutamic acid in α-helical and disordered conformations, bovine α-lactalbumin in native and acid molten globule states, and human immunoglobulin (Hecht et al., 1999). A comprehensive review on the use of ROA in the analysis of solution structure and dynamics of different biomolecules was given by Barron et al. (2000), where it was indicated that ROA can provide important information on the structural organization of IDPs, such as hen phosvitin, rat metallothionein, soybean Bowman–Birk inhibitor, bovine α-casein, and yeast invertase (Barron et al., 2000). An important feature of ROA is that this technique is well-suited to measure the extent of polyproline II (PPII) conformation (Smyth et al., 2001), which could be dominant in unfolded peptides and proteins (Shi et al., 2002).

2.7 Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC), also known as gel-filtration or gel-permeation chromatography, is a hydrodynamic technique that separates proteins based on their size and shape. The basic physical principle behind the separation power of this tool is the use of the porous beads with a well-defined range of pore sizes as the stationary phase. When a solution containing molecules (mobile phase) is passed through a column packed with those porous beads (stationary phase), solutes are separated based on their size. Here, molecules that are too large to fit inside any pore of stationary phase only have access to the mobile phase between the beads. Because of their size, these molecules are excluded from the stationary phase and will follow the shortest path through the column. On the other hand, small molecules with hydrodynamic dimensions smaller than the lower limit of the pore size can fit inside all the pores in the beads. They will be drawn in pores by the force of diffusion, where they will stay for a short time and then move out. All these molecules are included into the stationary phase as they have the complete access to all the mobile phase inside and between the beads. As a result, these molecules will have the largest retention on the column, and will therefore elute last during the gel filtration separation. All other molecules with sizes between these two extremes are partially included, as they can fit inside some but not all of the pores in the beads, and therefore possess an intermediate retention on the column and elute between the large ("excluded") and small ("totally included") molecules. This range of the pore sizes in the beads defines the fractionation range of a column, and within this range, molecules are eluted in order of decreasing size.

This technique was invented in 1955 by Grant H. Lathe and Colin R. Ruthven who used starch gel as the column matrix to separate substances "on the basis of their molecular weights" (Lathe and Ruthven, 1955; Lathe and Ruthven, 1956). This makes SEC a relatively novel approach in comparison to classical hydrodynamic methods such as viscometry (early work on the viscosity of globulin protein solutions was conducted by Harriette Chick (1875–1977) in 1914 (Chick, 1914; Chick and Lubrzynska, 1914)) and sedimentation [Théodor Svedberg (1884–1971) developed the first analytical ultracentrifuge in 1923 (Svedberg and Nichols, 1923; Svedberg and Rinde, 1924)]. As early as 1959, it was recognized that the SEC-based fractionation of macromolecules is determined by their molecular sizes (Porath and Flodin, 1959). This brought the molecular sieve hypothesis of the gel-forming polymer action to existence. SEC is now considered as a general separation technique, where the size and shape of molecules are the prime separation parameters (Porath, 1968). Therefore, the elution behavior of proteins on SEC column is determined by their Stokes radii rather than by molecular masses (Andrews, 1965; Ackers, 1967; Ackers, 1970; Fish et al., 1970; Corbett and Roche, 1984; Le Maire et al., 1986; Le Maire et al., 1987; Potschka, 1987; Uversky, 1993; Uversky, 1994). One should keep in mind, however, that since the hydrodynamic radii of similarly shaped and chemically similar molecules are proportional to the molecular mass, one can consider SEC as a mass-based separation technique, even though this is not strictly true. Based on these premises, estimation of molecular mass of a globular protein by a column calibrated using a set of globular proteins with known molecular masses is among the most frequent uses of SEC in the protein field.

A protein's hydrodynamic volume is a key structural feature that changes significantly when the globular protein denatures and unfolds (Tanford, 1968; Uversky, 1993; Uversky, 1994; Uversky, 2003). Therefore, assessing the hydrodynamic dimensions of a protein—whether it is compact, extended, or partially expanded—is essential for accurate classification of its conformation. Therefore, it is not surprising that SEC is broadly used for estimating a molecule's dimensions and monitoring how these dimensions change under different conditions. For example, it can be used to track the unfolding of globular proteins by observing changes in their retention time, which corresponds to changes in their Stokes radius (Corbett and Roche, 1983; Endo et al., 1983; Corbett and Roche, 1984; Lau et al., 1984; Brems et al., 1985; Uversky, 1993; Uversky, 1994) or by following the appearance of a new elution peak corresponding to some intermediate forms (Corbett and Roche, 1983; Endo et al., 1983; Gupta, 1983; Corbett and Roche, 1984; Withka et al., 1987; Uversky et al., 1992; Uversky, 1993; Uversky, 1994; Uversky and Ptitsyn, 1994; Uversky and Ptitsyn, 1996).

Hydrodynamic dimensions of IDPs can be studied by SEC as well. In fact, due to their lack of compact structure and extended conformations, IDPs are known to migrate faster on an SEC column than globular proteins of similar molecular mass (Uversky et al., 1999; Uversky et al., 2001; Uversky et al., 2002a; Uversky et al., 2002b; Receveur-Brechot et al., 2006). For example, in their 1967 study describing purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla (chromogranin A), Albert David Smith and Hans Winkler reported that although this protein was shown to have a molecular weight of 77 kDa based on the sedimentation, diffusion, and approach-to-equilibrium measurements, results of chromatography on a Sephadex G-200 column calibrated with globular proteins gave a molecular weight 7 times that given above. Based on the careful analysis of the outputs of several biophysical tools, these authors have made a set of very important conclusions: "...good agreement between the ultracentrifuge and Sephadex experiments was obtained on the assumption that Sephadex chromatography depends on the effective hydrodynamic radii of proteins and not on their

molecular weights. The hydrodynamic properties of the protein differed from those of a typical globular protein. Thus the protein had a high intrinsic viscosity, a high frictional ratio and a large effective hydrodynamic volume. The hydrodynamic properties of the protein, but not its molecular weight, were dependent on the ionic strength of the solvent. Increasing the ionic strength caused an increase in the sedimentation and diffusion coefficients, but a decrease in the intrinsic viscosity and in the frictional ratio of the protein. . . These results are compatible with the protein's having a conformation approaching that of a random-coil polypeptide, the volume occupied by the molecule being determined by electrostatic repulsion between the excess of negative charges" (Smith and Winkler, 1967).

In 1978, Gilllian A. Nimmo and Philip Cohen reported purification of protein phosphatase inhibitor-1 (PPI-1) from rabbit skeletal muscle utilizing an approach that includes heat treatment at 90 °C as one of the purification steps (Nimmo and Cohen, 1978). Based on the gel-filtration analysis, PPI-1 was shown to have an apparent molecular weight of 60 kDa, which is more than 3 time larger that the molecular weight measured by sedimentation equilibrium centrifugation (19.2 kDa). Based on the observed experimental features (gel-filtration behavior, stability to heating at 100 °C, and amino acid) the authors concluded that PPI-1 possesses little ordered structure (Nimmo and Cohen, 1978). The G-substrate isolated in 1981 by Dana W. Aswad and Paul Greengard from cytosol of rabbit cerebellum was shown to be characterized by an unfolded, nonglobular structure based on its hydrodynamic properties (it has a Stokes radius of 31 Å that corresponds to an apparent molecular mass of 54 kDa, whereas sedimentation analysis showed that the actual molecular weight of this protein is 21.7 kDa), heat stability, and acid solubility (Aswad and Greengard, 1981). In 1996, based on the analysis of the circularly permuted variants of dihydrofolate reductase from *E. coli* by SEC (among many other biophysical techniques) it was concluded that permuteins, being intrinsically disordered, are characterized by high compactness degree, as evidenced by their Stokes radii exceeding those of globular proteins with the same molecular weight by a factor ~1.2 (Uversky et al., 1996), which is a characteristic feature of the molten globule-like folding intermediate of globular proteins (Uversky, 1993; Uversky, 1994; Ptitsyn, 1995).

The 2000 survey of the experimentally verified IDPs included 16 proteins, whose extended conformation was evidenced by the abnormal mobility on gel-filtration column (Uversky et al., 2000a), supporting the idea that SEC played an important role in establishing the commonness of IDPs. These proteins are:

- horse heart apocytochrome *c* (Stellwagen et al., 1972),
- protein phosphatase inhibitor-1 from rabbit skeletal muscle (19.2 kDa by sedimentation and 60.0 kDa by gel-filtration) (Nimmo and Cohen, 1978),
- a dopamine-and adenosine 3':5′-monophosphate-regulated phosphoprotein from bovine caudate nucleus, DARPP-32 (27.6 kDa by sedimentation and 59 kDa by gel-filtration) (Hemmings et al., 1984),
- bovine cardiac troponin C in the absence of calcium (McCubbin and Kay, 1985), heat-stable microtubule-associated protein MAP2 (Hernandez et al., 1986),
- smooth muscle caldesmon (Lynch et al., 1987),
- brain-specific 14-kDa protein (14 kDa by Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 57 kDa by gel filtration) (Nakajo et al., 1990),
- carboxyl-terminal transactivation domain of Vmw65 from herpes simplex virus type 1 (11.0 kDa from sedimentation and 55.0 kDa from gel-filtration) (Donaldson and Capone, 1992),
- prothymosin α (Stokes radius is 1.77 larger than those expected for a compactly folded protein consisting of 109 amino acid residues) (Gast et al., 1995),
- chromogranin B (~100 kDa by SDS-PAGE and 220 kDa by gel-filtration) (Yoo, 1995),
- human topoisomerase I (~66 kDa by sedimentation and ~300 kDa by gel-filtration) (Stewart et al., 1996),
- N-terminal domain of human topoisomerase I (198 residues, ~66 kDa by sedimentation and ~96 kDa by gel filtration) (Stewart et al., 1996),
- oncoprotein 18 (Op18)/stathmin from fetal calf thymus (17.22 kDa by mass-spectrometry and ~64 kDa by gel-filtration) (Schubart et al., 1987; Belmont and Mitchison, 1996),
- human cardiac troponin I (Ferrieres et al., 1998), and
- N-terminal domain of the Pf1 gene 5 protein (theoretical mass is 31 kDa vs 53 kDa by gel-filtration) (Bogdarina et al., 1998).

2.8 Viscometry

Viscometry is a technique to measure the viscosity of fluids, which is a resistance of fluid to flow or the internal friction within a fluid that opposes its motion when subjected to stress, such as pouring or stirring. The concept of viscosity has a long history, dating back to the 17th century, when Isaak Newton (1643–1727) provided a qualitative description of viscosity in his 1687 publication *Philosophiæ Naturalis Principia Mathematica* as "the lack of slipperiness of the parts of the liquid" reflecting internal friction of fluids and developed a formal description of the relation between the shear stress of fluid and its flow rate (Newton, 1687). A formal description of the laminar flow rate of an incompressible, viscous fluid through a cylindrical tube, which is known as the Hagen-Poiseuille equation was independently proposed by Jean Léonard Marie Poiseuille (1797–1869) in 1838 (Poiseuille, 1838) and Gotthilf Heinrich Ludwig Hagen (1797–1884) in 1839 (Hagen, 1839; Sutera and Skalak, 1993). This long historical excursion is concluded by a work of George Gabriel Stokes (1819–1903), who, in 1851, provided a formal description of the force of viscosity on a small sphere moving through a viscous fluid at a low speed in a form now known as Stokes' law (Stokes, 1851).

The concept of intrinsic viscosity as a measure of the contribution a polymer or macromolecule to the viscosity of a solution, independent of its concentration was introduced in the second half of the 1930s based on works by Elmer O. Kraemer (1898–1943) and William D. Lansing, who used the symbol [η] for limiting the value of $-\frac{\eta_r - 1}{c}$ as concentration c approaches zero but did not define this parameter (Kraemer and Lansing, 1935), and Toivo A. Kauppi and Shailer L. Bass (1906–1988) who coined the term in 1937 to describe the [η] parameter introduced by Kraemer and Lansing (Kauppi and Bass, 1937). The concept represented by [η]

became a fundamental parameter in characterizing polymers and proteins, since intrinsic viscosity is highly dependent on the shape and volume of a molecule.

As already indicated, Harriette Chick (1875–1977) was one of the first researchers who started using viscometry for protein analysis. In 1914, she published two papers where viscometry was used to analyze several proteins, including horse serum albumin, ovalbumin (egg-albumin), pseudoglobulin, and euglobulin from horse serum (Chick, 1914; Chick and Lubrzynska, 1914). These studies formed grounds for using the viscosity measurement of proteins as a biochemical tool. In 1916, Emil Hatschek (1869–1944) used viscometry to evaluate how the physical properties of colloids including proteins (albumin and gelatin), such as their hydration and particle volume, affect their viscosity (Hatschek, 1916). In the following year, Svante August Arrhenius (1859–1927) published a paper on the viscosity of colloidal solutions (including proteins), where he used physical chemistry principles to explain how the concentration and denaturation of proteins affect the viscosity of biological fluids (Arrhenius, 1917). In 1926, Moses Kuntz (1887–1978) proposed an empirical formula correlating viscosity of solution and volume of solute (Kunitz, 1926). Wilfrid James Loughlin published two papers in 1932, in which the effect of denaturation on the viscosity of protein solutions was analyzed and demonstrated that the unfolding of proteins increases the viscosity of their solutions, resulting in an increase in protein volume (Loughlin, 1932; Loughlin and Lewis, 1932).

These and many other early experiments that used viscometry to study protein denaturation and unfolding laid the groundwork for understanding intrinsically disordered states by showing that a rigid, compact, globular protein has a small effect on viscosity because it occupies a small hydrodynamic volume (they typically have a low intrinsic viscosity, in the range of 3–4 mL/g, which is independent of protein molecular mass), whereas an unfolded, flexible, or disordered protein adopts a random coil-like conformation, creating a much larger hydrodynamic volume that increases the solution's viscosity (intrinsic viscosity of unfolded proteins increases with increase in molecular mass). In 1966, based on the viscosity measurements for a set of 12 proteins unfolded in 5–6 M GdmCl, Charles Tanford (1921–2009), Kazuo Kawahara, and Savo Lapanje (1925–1997) concluded that unfolded proteins demonstrate random coil behavior (Tanford et al., 1966).

Since intrinsic viscosity serves as a specific characteristic of the overall size and shape of a protein, and since this parameter can be accurately measured in a relatively straightforward and cost-effective way, viscometry became a rather broadly used method in protein studies, leading to the early observation that some proteins lacked the defined, globular structures in their native states. For example, in 1950, Eugene L. Hess and Aspascia Cobure, while measuring the intrinsic viscosity of mixed proteins, including blood plasma and serum, observed that pathological samples had a higher intrinsic viscosity than normal ones, reflecting the increase in large, flexible proteins such as fibrinogen, α_2-globulins, and γ-globulins in the disease states (Hess and Cobure, 1950). In 1952, Thomas LeRoy McMeekin reported that casein is characterized by high viscosity and therefore resembles denatured proteins (McMeekin, 1952). In 1958, based on the viscometric analysis of aforementioned phosvitin, Bruno Jirgensons concluded that this protein is similar to that of disordered polyglutamate, being characterized by a flexible, disordered structure (Jirgensons, 1958). In 1966, Jirgensons pointed out that the anomalous behavior of phosvitin and histones during viscosity measurements reflects their disordered nature in water (Jirgensons, 1966). Based on the viscometry results (among the outputs of several other techniques), Albert David Smith and Hans Winkler concluded that conformation of chromogranin A approaches that of a random-coil polypeptide (Smith and Winkler, 1967). As per results reported by Earle Stellwagen, Richard Rysavy, and George Babul in 1972, apo-cytochrome *c* intrinsic viscosity of 15.5 mL/g and sedimentation behavior consistent with monomeric protein (Stellwagen et al., 1972).

2.9 Scattering

2.9.1 Small-angle X-ray scattering (SAXS)

Small-angle X-ray scattering (SAXS) was introduced in 1938 by André Guinier (1911–2000), who showed the ability of this technique to produce structural information from the metallic alloys (Guinier, 1938) and developed, together with Peter Joseph William Debye (1884–1966), Otto Kratky (1902–1995), and other scientists, the foundational theory and techniques for small-angle scattering, transforming SAXS into an essential tool for materials science and crystallography. SAXS and its complementary tool small-angle neutron scattering (SANS) are small-angle scattering (SAS) techniques. SAS involves the analysis of scattering patterns obtained at small angles, typically a few degrees. This method provides structural information within a resolution range of 1–25 nm, and it can reveal repeat distances in partially ordered systems up to 150 nm in size.

The first SAXS experiments on proteins were performed in 1950s. These utilized the standard X-ray tubes with low X-ray flux, required long exposure times, and were originally limited to the evaluation of the radius of gyration (R_g) for easily purified proteins such as hemoglobin and ovalbumin (Guinier et al., 1955). A systematic application of this tool to study the biological macromolecules in solution began in the 1960s, as SAXS provided low-resolution structural data on molecular shape and internal features without requiring crystallization (Svergun and Koch, 2003). Currently, SAXS is often used to analyze the structure of a variety of biological objects, such as solutions of biological macromolecules, nanocomposites, alloys, and synthetic polymers (Doniach, 2001; Svergun and Koch, 2003). The development of synchrotron radiation in the 1970s significantly advanced SAXS, transforming it into a powerful and accessible tool in structural biology. Synchrotron light sources provide X-rays with exceptional properties that dramatically improve the speed, quality, and versatility of SAXS experiments compared to traditional laboratory X-ray tubes (Svergun and Koch, 2003).

SAXS measures the scattering of X-rays as they pass through a solution of the protein, delivering low-resolution information about the overall size, shape, and flexibility of the molecule. It can be used for evaluation of the radius of gyration (R_g), maximum dimension (D_{max}), distance distribution functions ($p(r)$), hydrated volume, 3D molecular envelope, and molecular mass (M_r) (Glatter and Kratky, 1982; Svergun and Koch, 2003). The R_g indicates the average root-

mean-square distance of the electrons from the center of mass of a protein and thereby provides a measure of overall compactness, with higher R_g values for a protein with a given molecular mass indicating a more elongated or unfolded shape. The D_{max}, which is the maximum distance between any two points within the protein, can be obtained by analyzing the pair-wise distance distribution function, $p(r)$ that represents the probability of finding two atoms within a molecule separated by a specific distance and can be derived from the scattering data.

Hydrated volume is a key measurement for estimating a protein's molecular mass because it accounts for the total volume the protein occupies in a solution, which includes the protein itself and the surrounding layer of associated water molecules. The 3D molecular envelope represents a computationally-generated low-resolution 3D model of the shape of a protein, which is consistent with the scattering data. The M_r of a protein in solution can be accurately determined by SAXS, and conducting SAXS analysis at a range of protein concentrations can be used for evaluation of the self-association equilibria (Glatter and Kratky, 1982; Svergun and Koch, 2003). Furthermore, SAXS offers a unique opportunity to evaluate the globularity of a protein. To this end, the scattering intensity (I(q)) is presented in a form of a Kratky plot ($q^2I(q)$, against the scattering vector q) (Heine et al., 1962), which contains characteristic peaks or plateaus indicating the protein conformation (where compact and globular proteins show a bell-shaped peak, whereas extended, random coil peptides (or homopolypeptides) produce a curve that increases at higher scattering angles) (Glatter and Kratky, 1982; Bernado and Svergun, 2012; Kikhney and Svergun, 2015).

Since a SAXS pattern is sensitive to the size, shape, and internal electron density distribution of a scattering molecule, this technique provides important means to measure the change in the overall dimensions of a protein molecule (Glatter and Kratky, 1982). As a result, for decades, SAXS was successfully used for the analysis of intramolecular protein folding (Stamatoff, 1979; Phillips et al., 1988; Tsuruta et al., 1989; Tsuruta et al., 1990; Eliezer et al., 1993; Kataoka et al., 1993; Koide et al., 1999; Segel et al., 1999; Kojima et al., 2000), characterization of various conformational states of globular proteins (Damaschun et al., 1991a; Damaschun et al., 1991b; Eliezer et al., 1995; Konno et al., 1995; Semisotnov et al., 1996; Zhou et al., 1997; Uversky et al., 1998; Gualfetti et al., 1999; Koide et al., 1999), and even analysis of the enzyme behavior, including subtle domain movements (Byer et al., 2023) and structural characterization of chaperones and their complexes (Shi et al., 1996).

SAXS is a rather widely used technique for characterizing IDPs. It is particularly powerful for studying the global dimensions and conformational heterogeneity of IDPs that lack a single, stable 3D structure. The technique can reveal the presence of disordered regions and the flexibility of multidomain proteins by observing characteristics, such as large R_g, expanded distance distribution functions (p(r)), and, for highly disordered proteins, the lack of a characteristic bell-shaped peak in Kratky plot (Bernado and Svergun, 2012).

Among the first SAXS-based studies of disordered proteins was structural characterization of the acid-denatured form of apo-cytochrome *c*, which was shown to behave as a random coil, being characterized by high R_g (evaluated by SAXS) and Stokes' radius R_s values (determined by dynamic light scattering), the R_g/R_s ratio of 1.55 typical for a random-coil polymer, and the lack of the peak in the Kratky plot (Damaschun et al., 1991a). Furthermore, it was shown that the R_g of apo-cytochrome *c* at pH 2.3 strongly depends on the quality of the solvent (Damaschun et al., 1991b).

In a comprehensive SAXS-based study, Semisotnov et al. (1996) compared globular (native and "molten globular") and non-globular states (unfolded protein and randomly coiled, partially α-helical, and partially β-structural synthetic polypeptides) of several proteins and model polypeptides using Guinier (used for the R_g evaluation) and Kratky plots, as well as analyzed the globularization process in protein folding using the integrated SAXS intensity (Semisotnov et al., 1996). This analysis revealed that the unfolded polypeptides (proteins or synthetic polypeptides) have high R_g values and are invariantly characterized by the lack of a peak in Kratky plot. Furthermore, in synthetic polypeptides, the formation of the intramolecular α-helical elements joined by flexible coils without the formation of a hydrophobic core does not affect the shape and intensity of the SAXS pattern including the shape of the Kratky plot (Semisotnov et al., 1996). It was pointed out that this behavior was in line with the previously reported data for poly (D-glutamic acid) in random coil, helical, and a mixture of helix and coil forms (Muroga et al., 1988). By analyzing different partially folded conformations induced by various anions in the acid-unfolded staphylococcal nuclease using a set of biophysical techniques including SAXS, it was shown that the acid-unfolded and pre-molten globule states of this protein are not globular (their Kratky plots devoid a characteristic peak) (Uversky et al., 1998).

Prothymosin α was likely the first actual IDP characterized by SAXS (Gast et al., 1995; Uversky et al., 1999; Uversky et al., 2000b). It was shown that this protein, which is characterized by a global charge of −54 at neutral pH, is completely unfolded at neutral pH but can gain some helical structure at acidic pH (Gast et al., 1995; Uversky et al., 1999) or in the presence of Zn^{2+} ions (Uversky et al., 2000b). Acid pH- or Zn^{2+}-induced forms of this protein were classified as pre-molten globules characterized by a dramatic decrease in R_g (from 37.8 ± 0.9 Å at neutral pH to 27.6 ± 0.9 Å at acidic pH or 28.1 ± 0.8 Å at neutral pH in the presence of Zn^{2+}) and lack of globular structure (based on the absence of a bell-shaped peak in the corresponding Kratky plots) (Uversky et al., 1999; Uversky et al., 2000b). Among other IDPs subjected to SAXS analysis in the early 2000s were the members of the human synuclein family, α-synuclein (Uversky et al., 2001) and its mutants associated with the familiar form of Parkinson's disease (Li J. et al., 2001), as well as β- and γ-synucleins (Uversky et al., 2002a).

Interestingly, introduction and eventual acceptance of IDPs (which do not have unique structure and exist as highly dynamic conformational ensembles) played an important role in the subsequent SAXS developments reflected in the elaboration of approaches for characterization of protein mobility and flexibility by generating realistic ensemble models of unstructured states (Bernado and Svergun, 2012; Kikhney and Svergun, 2015). Among these SAXS-based ensemble-generating approaches are the Ensemble Optimization Method (EOM) (Bernado et al., 2007; Tria et al., 2015), the Minimal Ensemble Search (MES) (Pelikan et al., 2009), Basis-Set Supported SAXS (BSS-SAXS) (Yang et al., 2010), and the Ensemble Refinement of SAXS (EROS) (Rozycki et al., 2011). The logics behind these ensemble-generating methods is based on the assumption that the experimental SAXS profile

originates from a number of coexisting conformational states. In these approaches, at the first step, a large set of static random models of various possible conformations of a given protein is computationally generated, from which a sub-ensemble of conformations that collectively describe the experimental profile is selected (Bernado and Svergun, 2012; Kikhney and Svergun, 2015).

Therefore, combined with ensemble modeling, SAXS can help define the range of conformations consistent with experimental data, offering insights into the degree of disorder and compaction within the protein. Furthermore, SAXS is currently efficiently combined with other techniques, such as NMR, to provide a more comprehensive structural picture of flexible systems, such as a tumor suppressor p53 (Wells et al., 2008). For example, one of the commonly used methods, ENSEMBLE, generates ensembles of IDPs that collectively describe SAXS curves in addition of several NMR experimental constrains (Marsh et al., 2007; Marsh and Forman-Kay, 2009).

2.9.2 Small-angle neutron scattering (SANS)

Small-angle neutron scattering (SANS) is another small-angle scattering (SAS) technique that uses neutrons to probe the structure of materials on a mesoscopic scale, typically from 0.5 to 500 nm. The technique was developed in 1960s, with the first specialized SANS diffractometers being developed at the Jülich research center in Germany in the early 1970s (Jacrot, 1976). However, the first experiments on biological samples were conducted in 1969 using less-specialized neutron scattering instruments at early research reactor, with the membrane myelin (Parsons and Akers, 1969), haemoglobin solutions (Schneider et al., 1969), and a myoglobin single crystal (Schoenborn, 1969) being the first such subjects. A key benefit of small-angle neutron scattering (SANS) is its ability to use selective deuteration and contrast variation. By leveraging the significant difference in how hydrogen and deuterium scatter neutrons, researchers can precisely alter the scattering contrast of specific components within a system. This makes it possible to highlight one molecule, such as a protein, against a surrounding solvent or a binding partner.

Not surprisingly, among the first applications of SANS in biology was analysis of the structure of larger complexes, such the nucleosomes (Hjelm et al., 1977; Uberbacher et al., 1983; Hammermann et al., 2000; Sugiyama et al., 2015) or ribosomes (Engelman et al., 1975; Crichton et al., 1977; Laughrea et al., 1978; Osterberg et al., 1980; Malhotra and Harvey, 1994; Svergun et al., 1994; Svergun et al., 1996). For example, based on the analysis of the SANS curves of nucleosomes in various H_2O/D_2O solvents, the overall organization of this fundamental subunit of chromatin was described as "a spherically averaged structure with most of the histones closely packed into a core of radius 3.2 nm surrounded by a loosely packed DNA-rich shell of 2.0 nm thickness resulting in a particle of 5.2 nm average radius" (Hjelm et al., 1977).

Furthermore, using contrast variation, it was shown that the nucleosome unfolding induced by a decrease in ionic strength represents a complex process, where DNA became partially unfolded below the transition point, whereas only small conformational changes occur in the protein core (Uberbacher et al., 1983). Curiously, in 1975, the analysis of the 30S ribosomal subunit of *E. coli*, where a specific pair of ribosomal proteins were deuterated (S2-S5, S5-S8, and S3-S7) not only provided information on the distances between the centers of gravity of three protein pairs, but also showed that S5 and S8 were compact and S2 was extended (Engelman et al., 1975).

Although the capability of SANS to selectively analyze a query protein using contrast variation and specific deuterium-labeling seems to be particularly powerful for investigating IDPs within complex, biologically relevant environments that mimic cellular conditions, this method was not broadly applied in IDP analysis until quite recently (Gabel, 2012) and therefore did not contribute much to the establishing of the IDP field. However, since this tool is ideally suited for the analysis of the properties of a protein of interest in the presence of very high concentrations of other macromolecules, especially proteins, SANS is efficiently used for the analysis of the effects of molecular crowding on IDPs (Johansen et al., 2011; Goldenberg and Argyle, 2014; Balu et al., 2016; Moreno et al., 2016; Fagerberg et al., 2020). In the 21st century, hybrid approaches unlocked the full potential of SANS for studying IDPs (Gabel, 2012). This involved integrating SANS data with complementary techniques such as NMR and computational modeling to map an entire structural ensemble of an IDP, as well as utilization of contrast variation, which employs deuteration to selectively visualize specific components within IDP-containing complexes or residue-specific deuterium labeling with the remaining hydrogenated portion contrast matched out (Chen et al., 2023).

2.9.3 Static light scattering (SLS)

Static light scattering (SLS) is a physical chemistry technique for molecular characterization that measures the average intensity of scattered light to determine a weight-average (or absolute) molecular weight M_w and size (radius of gyration, R_g) of a macromolecule, such as a polymer or a protein in solution. The theoretical foundation for SLS was laid by a classic paper published in 1908 by Gustav Mie (1868–1957), where the first mathematically rigorous and complete solution for the scattering of an electromagnetic plane wave by a homogeneous sphere was presented (Mie, 1908). This formalism successfully explained the optical properties of colloidal metal solutions, particularly the different colors seen in suspensions of gold nanoparticles of various sizes (Mie, 1908; Horvath, 2009). In 1944, Peter Debye (1884–1966) published a paper detailing the first measurements of M_w of small polymers using single-angle SLS (Debye, 1944). This work formed the theoretical foundation for applying light scattering to macromolecules. Finally, Bruno Hasbrouck Zimm (1920–2005) further advanced the SLS technology by developing the theory of the angular dependence of light scattering. In 1948, he reported in a single paper both the theoretical basis for a technique that measures the angular dependence of light scattered by large molecules in solution and the design of a new photometer that improved the accuracy of light-scattering measurements at different angles (Zimm, 1948). The method described in that paper is now known as the Zimm plot, which allows determination of M_w, R_g, and second virial coefficient (A_2) of a macromolecule in a solution by measuring light scattering across various angles and concentrations.

The very first paper describing the determination of molecular weights of proteins (ovalbumin, amandin, exelsin, and hemocyanin)

was published in 1935 by P. Putzeys and Jeanne Brosteaux (Putzeys and Brosteaux, 1935), and in 1941 these authors reported the M_w values for more than 20 proteins (Putzeys and Brosteaux, 1941). These and many other early SLS studies of proteins, including the application of this technique to determine the molecular weights of proteins, were comprehensively summarized in a 1951 paper by Paul Mead Doty (1920–2011) and John Tileston Edsall (1902–2002) (Doty and Edsall, 1951).

The development of goniometers and photometers in the 1950s and the invention of the laser in the 1960s, which provided a monochromatic, well-collimated light source, significantly improved the accuracy and ease of light scattering measurements. Subsequent technological advances included the introduction of online light scattering detection, development of low-angle laser light scattering, and multi-angle laser light scattering (MALS) combined with size-exclusion chromatography (SEC-MALS). All this improved the utility of SLS which became a standard method for the analysis of molecular properties of proteins and protein aggregation (Wyatt, 1993). In 1986, Hernandez et al. conducted a comprehensive multiparametric analysis of microtubule-associated protein 2 (MAP2), with SLS being one of the techniques to show that it was not a compact globular protein, being instead a monomeric, highly elongated, and flexible protein (Hernandez et al., 1986). A clear example of using light scattering to define a protein as disordered prior to the modern understanding of IDPs is given by 1995 study of prothymosin α by Gast et al. (Gast et al., 1995). In 2016, it was emphasized that hundreds of publications have appeared in which SEC-MALS was "employed as a routine tool for quality control during the process of purification or production of a biological macromolecule, i.e., to ensure the homogeneity and lack of aggregation of the source material" (Minton, 2016).

2.9.4 Dynamic light scattering (DLS)

Dynamic light scattering (DLS) (also known as quasi-elastic light scattering, QELS) is another physical chemistry technique for molecular characterization based on the analysis of light scattering. DLS measures the fluctuations in scattered light intensity over time to assess the speed at which particles move due to Brownian motion. This information is then used for evaluation of the translational diffusion coefficient (D_T) used via the Stokes-Einstein equation for calculation of the hydrodynamic radius (R_h or R_S) of particles in a liquid (which is the effective size of a particle as it moves through a solution, including any associated solvent molecules), finding a distribution of particle sizes within a sample that allows for the calculation of the size distribution/polydispersity index (PDI), as well as providing estimates for the relative amount of mass or number of particles in a given size range.

Conceptually, this method is rooted in the quantitative explanation of Brownian (i.e., the random movement of particles in a fluid) independently provided in 1905, by Albert Einstein (1879–1955) (Einstein, 1905) and in 1906, by Marian von Smoluchowski (1872–1917) (Von Smoluchowski, 1906), who presented what is known now as the Smoluchowski equation, which is a cornerstone of stochastic process theory, that details the time-dependent behavior of the probability density function for a particle experiencing Brownian motion under the influence of both external forces and diffusion. However, despite the theory, measuring Brownian motion to determine particle size remained impractical for several decades due to technological limitations. In fact, it took more than 50 years to transform these ground-breaking theoretical considerations into a practical tool, DLS, which measures the speed of this motion to determine particle size. In 1964, Robert Pecora developed the formalism showing that the diffusion of macromolecules in solution could cause a broadening of the frequency spectrum of scattered light, thereby laying a framework for interpreting experiments on Doppler shifts in light scattering from pure liquids and polymer solutions and indicating that "Doppler shift measurements should yield information about the shape of the polymer molecule, and its translational and rotational diffusion coefficients" and pointing out that "the recently developed optical maser has made such experiments practical" (Pecora, 1964). The validity of this formalism and assumption was experimentally demonstrated by Cummins et al., who were the first to employ the use of a laser (a continuous wave (CW) optical maser) in the study of polymer solutions (Cummins et al., 1964).

The practicality of DLS requires the use of a high-intensity, monochromatic light source creating a reliable scattering signal and is therefore rooted in using lasers, which were invented in late 1960s. Another key component for the efficient analysis of the intensity fluctuations in scattered light was a digital autocorrelator (a means for data analysis), the foundation for which was laid by Eric Jakeman and Edward Roy Pike (1929–2025), who, in 1969, reported a method to analyze the temporal correlation of photon events in light scattering experiments (Jakeman and Pike, 1969). In 1970, the successful application of this method for determination of the diffusion coefficients of haemocyanin at low concentration was reported by Foord et al. (1970), becoming the first experimental study on using intensity fluctuation spectroscopy of scattered laser light in the study of protein solutions. A 2019 review by Anthony Wishard and Bruce C. Gibb provides a more detailed description of a historical background of DLS development, summarizes the strengths and limitations of the technique, introduces data interpretation, and outlines some important features of DLS in the analysis of supramolecular structures (Wishard and Gibb, 2019).

It was also suggested that DLS can be successfully applied to monitor changes in the molecular size of proteins upon their unfolding and refolding (Nicoli and Benedek, 1976; Gast et al., 1986; Damaschun et al., 1991a). Furthermore, DLS was shown to provide useful information on the time-dependent conformational changes taking place during the unfolding-refolding reactions of proteins that proceed with time constants in the range of seconds or minutes (Gast et al., 1992). Combining a stopped-flow system with a dynamic light scattering apparatus allowed for reduction of the time-resolution of the method to the order of 1 s (Gast et al., 1997).

Since SLS and DLS provide complementary information on protein size, shape, and aggregation state, these techniques are commonly used together. In fact, the ratio of the R_g (measured by SLS) to R_h (evaluated by DLS) is known as a shape factor, as it reveals the shape or conformation of a macromolecule. Here, an R_g/R_h value of ~0.775 is characteristic of a compact, globular protein (sphere), whereas the larger values indicate a less compact or elongated structure, approaching 1.55 for a random-coil polymers (Tanford, 1966; Damaschun et al., 1991a). Changes in R_g and R_h can reflect aggregation or conformational changes, and evaluating the shape factor under different conditions can be used for the analysis of protein stability and folding status. Furthermore, the combined

use of SLS and DLS generates a more complete picture than either method alone, especially for complex systems, such as self-associating proteins or proteins forming aggregates. This is illustrated, for example, by Parupudi et al. who demonstrated the power of fitting DLS and SLS data jointly to determine protein-protein interactions, as a global fit of both DLS and SLS data is necessary to obtain meaningful interaction parameters, since DLS alone can lead to different interpretations (Parupudi et al., 2021).

In 2016, Allen P. Minton provided a comprehensive review on recent applications of light scattering measurement in the biological and biopharmaceutical sciences and indicated that "measurements of both SLS and DLS have been used extensively to monitor unfolding and aggregation of proteins in solution under a variety of conditions" (Minton, 2016). This review also contains numerous recent examples of the use of these two techniques (often in combination with other methods) for characterization of mass and size of individual macromolecular solutes, protein conformational and colloidal stability, nonspecific protein-protein interactions in slightly concentrated solutions, reversible specific associations in dilute solutions, and macromolecular interactions in concentrated protein solutions, as well as analysis of supermolecular assemblies (Minton, 2016).

Reported in 1991 by Damaschun et al., structural characterization of the acid unfolded apocytochrome *c* by DLS and SAXS (in combination with other biophysical approaches) likely represent one of the first applications of this approach for analysis of IDPs (Damaschun et al., 1991a). In 1995, the same group reported structural characterization of a prototypical IDP, prothymosin α, using SLS and DLS in combination with SAXS, circular dichroism, and mass spectrometry, indicating that the protein is a biologically active protein with random coil conformation characterized by the shape factor of 1.55 and complete lack of ordered secondary structure and concluding: "The finding that a biologically active protein molecule with 109 amino acid residues adopts a random coil conformation under physiological conditions raises the question whether this is a rare or a hitherto-overlooked but widespread phenomenon in the field of macromolecular polypeptides" (Gast et al., 1995).

2.10 Analytical ultracentrifugation (AUC)

Analytical ultracentrifugation (AUC) was invented in the 1920s by Theodor Svedberg (1884–1971), who developed the first analytical ultracentrifuge to determine the molecular weight and analyze the sedimentation behavior of disperse systems and macromolecules, such as proteins and colloids (Svedberg and Nichols, 1923; Svedberg and Rinde, 1924). The power of this approach was demonstrated by the first paper published in 1926, where Svedberg described the sedimentation equilibrium study of hemoglobin and determined the molecular weight of this protein (Svedberg and Fåhraeus, 1926). Since AUC sedimentation velocity (SV-AUC, where the rotor is spun at sufficiently high speed to allow measurement of the rate at which macromolecules move through a solution under high centrifugal force) and sedimentation equilibrium (SE-AUC, where the rotor speed is lower permitting to reach a state of equilibrium, where the centrifugal force driving sedimentation is balanced by diffusion) provide crucial information about the macromolecular properties, such as molecular weight, sedimentation, diffusion, frictional coefficients, shape, and formation of protein complexes, this technique rapidly became a major tool for the analysis of polymers, colloids, and proteins (Cölfen, 2023). The use of AUC in protein studies has undergone two major phases of popularity: a period of initial growth between the 1950s and 1970s (caused by the appearance of commercially available ultracentrifuges), and a resurgence period in the 1990s fueled by significant technological advances including computerized data acquisition, rotor speed, and temperature regulation.

AUC can be used for the analysis of unfolded proteins and IDPs because of their specific hydrodynamic properties, such as larger hydrodynamic radius and slower sedimentation velocity than compact proteins of the comparable molecular mass (Salvay et al., 2012; Scott and Winzor, 2015). A brief description of the parameters measured by AUC in application to IDPs is provided below.

SV-AUC is the most common and versatile AUC method for protein analysis, which provides rich hydrodynamic information, such as sedimentation coefficient (*s*), diffusion coefficient (*D*), size, shape, and oligomeric state. Being a measure of the sedimentation rate per unit of centrifugal field (i.e., the rate of boundary movement per unit centrifugal force), sedimentation coefficient, *s*, is the migration parameter that characterizes size and density of a protein molecule, where larger and more compact molecules sediment faster and have higher *s* value. Since IDPs/unfolded proteins have larger size and lower packing density than the folded proteins of the same molecular mass, they are characterized by lower sedimentation coefficients.

As the diffusion coefficient (*D*) characterizing the random motion (diffusion) of the particles is size-dependent, extended IDPs have smaller *D* compared to their compact, well-folded counterparts. Furthermore, the diffusion coefficient can be used to calculate the hydrodynamic (Stokes) radius R_h (or R_S, which is the radius of the equivalent hydrodynamic sphere) of a protein using the Stokes-Einstein equation relating the diffusion of a spherical particle in a fluid to its size and the properties of the fluid. Furthermore, since the derived R_h value can be used for the evaluation of the frictional coefficient, *f*, and the theoretical minimum frictional coefficient f_0 can be calculated for a perfect, unhydrated/unsolvated sphere with a radius of R_u and a volume equivalent to that of the macromolecule being studied, one can obtain information about the frictional ratio, $f/f_0 = R_S/R_u$, which is independent of molecular mass and provides information on the shape of a studied protein, being equal to unity for a spherical unsolvated protein. A high f/f_0 is characteristic of an extended, flexible, and unfolded protein. Finally, since SV-AUC separate species based on their size, this technique can distinguish between monomers, oligomers, and aggregates in a sample. For IDPs, this can confirm whether a protein exists as a monomer or is undergoing self-association (Scott and Winzor, 2015).

SE-AUC is used to direct measure of the buoyant molecular mass of a particle/protein from the concentration gradient at equilibrium. Furthermore, for proteins that reversibly associate, SE-AUC can be used to determine the equilibrium constants (K_a or K_d) and stoichiometry of the interacting species (Schuck, 2013).

In its early days, AUC was intensively used by Svedberg's team for evaluation of the molecular mass of proteins, such as hemoglobin (Svedberg and Fåhraeus, 1926), egg albumin (Svedberg and Nichols,

1926), phycoerythrin and phycocyan (Svedberg and Lewis, 1928), serum albumin and of serum globulin (Svedberg and Sjögren, 1928), hemocyanin (Svedberg and Chirnoaga, 1928; Svedberg and Heyroth, 1929), Bence-Jones protein (Svedberg and Sjögren, 1929), edestin (Svedberg and Stamm, 1929), casein (Svedberg et al., 1930a; Svedberg et al., 1930b), amandin and excelsin (Svedberg and Sjögren, 1930), legumin (Sjögren and Svedberg, 1930), insulin (SVEDBERG, 1931), and the blood pigments of the invertebrates (Svedberg and Eriksson, 1932; Svedberg and Hedenius, 1933). Since that time, AUC was widely used to characterize proteins in their native state, providing information on their molecular weight, homogeneity, and interactions.

Although casein was one of the first proteins subjected to the AUC analysis as early as 1930 (Svedberg et al., 1930a; Svedberg et al., 1930b), originally, no conclusion was made about its disordered nature. However, Svedberg and his colleagues reported that instead of being a single, homogeneous protein, casein samples contained components of different sizes, the prevalence of which was strongly dependent on the method used for preparation of protein samples, and that calcium caseinate formed a very polydisperse suspension with colloidal properties (Svedberg et al., 1930a; Svedberg et al., 1930b). Later studies showed that the colloidal particles in milk known as casein micelles represent a complex aggregate of individual casein proteins, stabilized by calcium phosphate (Svedberg and Pedersen, 1940). Furthermore, the analysis of the frictional ratio for casein provided early insights into the non-globular and flexible nature of the casein molecule (Svedberg and Pedersen, 1940).

In 1968, a comprehensive analysis of the lysine-rich fraction of calf thymus histones by sedimentation equilibrium and other physicochemical techniques indicated that this protein had a high frictional ratio and an extended shape, whereas the ORD measurements indicated that it had a low helical content (Haydon and Peacocke, 1968). Later, AUC was used to measure the size and salt-dependent assembly state of histones and to identify and characterize the core histone octamer, a complex of eight histone proteins (two each of H2A, H2B, H3, and H4), with derived results being essential for developing the nucleosome model of chromatin organization (Kornberg, 1974; Elgin and Weintraub, 1975; Thomas and Kornberg, 1975; Weintraub et al., 1975).

In 1981, Pretorius et al. showed that human clathrin extracted from the coated pits of the plasma membrane was characterized by a very large frictional ratio of 3.06 ± 0.18 (Pretorius et al., 1981). Since this protein was shown to contain high levels of ordered secondary structure (49% α-helical structure, 17% β-structure, and 34% random coil structure) it was pointed out that clathrin, being characterized by a very large frictional ratio, cannot represent a random coil polypeptide chain, and it was therefore hypothesized that this protein consisted of several globular (likely α-helical) domains connected by flexible polypeptide sequences to form a common locus (Pretorius et al., 1981). In 1988, Haritos et al. used AUC to characterize the solution state of prothymosin α, paratymosin α, thymosin α_1, and thymosin β_4, resolving early contradictory gel-filtration findings, according to which these proteins appeared as oligomers which were 4–5 times larger than those calculated from their amino acid sequences (Haritos et al., 1989). It was also pointed out that thymosin α_1 was characterized by a frictional ratio of 1.3 indicating its extended, non-globular shape, which differs significantly from an ordered protein (Haritos et al., 1989). Several more recent studies reported large f/f_0 values for several IDPs, such as nucleoporin (Denning et al., 2003), securin (Sánchez-Puig et al., 2005), plasmid partition protein KorB (Rajasekar et al., 2010), and neuroligin (Paz et al., 2008), for which the corresponding f/f_0 values were 2.8, 2.1, 1.9, and 1.6, respectively.

2.11 Limited proteolysis

Limited proteolysis involves controlled digestion of a protein with a protease under specific conditions to see which parts of a protein are flexible or exposed and therefore more accessible to protease, and which are protected and rigid. The resulting fragments provide insight into the overall structure of a protein, its domain organization, and conformational dynamics. Limited proteolysis, which results in the accumulation of specific fragments corresponding to the more rigid parts of a protein, is different from the total proteolysis (also through the action of proteases) leading to the complete or near-complete degradation of a protein into its constituent amino acids, which is useful for determination of amino acid sequence. Because of its ease from the viewpoint of both the theory (flexible parts are digested, rigid parts are not) and practice (mix a query protein with a limited quantity of a protease, wait for a limited time, and separate the components of the resulting mixture) and usefulness of the generated information, this approach has a long history of useful utilization in protein research.

In the aforementioned study describing the analysis of milk proteins by ORD, Thomas LeRoy McMeekin also mentioned: "*In vitro*, casein is digested with the greatest ease by proteolytic enzymes. It is well-known that the ease of digestion of some proteins is greatly increased by denaturation or cooking, which appears to make a more accessible molecular structure by unfolding" (McMeekin, 1952). In this quote, McMeekin cited a study by Hans Lineweaver (1907–2009) and Sam R. Hoover published in 1941, were the action of pepsin on native and urea-denatured proteins were compared (Lineweaver and Hoover, 1941). However, these authors themselves pointed out that a number of investigators have shown earlier that the denatured proteins are digested by enzymes more rapidly than native ones. Among the rather long list of their predecessors, the authors mentioned Mortimer Louis Anson (1901–1968) and Alfred Ezra Mirsky (1900–1974), who, in 1934, reported that denatured hemoglobin can be digested by trypsin, which does not attack the native protein (Anson and Mirsky, 1934).

However, the actual "father" of limited proteolysis as a research tool was Kaj Ulrik Linderstrøm-Lang, who, together with Martin Ottesen, coined the term in 1949, to differentiate the restricted specificity of certain enzymes under certain conditions from the random proteolysis accompanying total protein degradation (Linderstrøm-Lang and Ottesen, 1949). The fact that ordered parts of a protein molecule are insensitive to proteolysis gives raise to the sequence/structure paradigm of limited proteolysis, where "higher order structure and not primary sequence is the main determinant of the site of initial hydrolysis" (Hubbard, 1998). Hans Neurath (1909–2002) emphasized that in addition to being a

useful tool for protein structure analysis, limited proteolysis (in a form of proteolytic processing) represents a very important biological process leading to the activation of many proteins (including many proteases themselves), which are synthesized as precursors (Neurath and Walsh, 1976; Neurath, 1979; Neurath, 1986; Neurath, 1999).

It was also pointed out that limited proteolysis represents "a convenient and accurate method for determining the "ratio of denaturation" – quantitative determination of denatured and native states–of partially denatured proteins" (Okunuki et al., 1956). This opens a possibility to use limited proteolysis as a tool for conformational analysis of proteins, where a very significant role was played by the laboratory of Angelo Fontana, who emphasized that the proteolytic probes can pinpoint the sites of local unfolding in a protein chain (Fontana et al., 1986; Fontana et al., 1993), as well as to analyze the partially folded states of globular proteins (such as molten globules) induced in several proteins by specific conditions (Fontana et al., 1997a; Fontana et al., 1997b; Fontana et al., 1999; Polverino de Laureto et al., 2002; Fontana et al., 2004).

Finally, in relation to the subject of this review, observation of faster proteolysis rates in certain proteins, compared to structured counterparts, provided an early indicator of intrinsic disorder. For example, high proteolytic sensitivity of casein was reported by McMeekin in 1952 (McMeekin, 1952). In 1970, James Bartley and Roger Chalkley showed that histones of calf thymus nucleohistone are protease resistant when complexed with DNA, whereas when freed from DNA, they rapidly degraded, indicating the presence of DNA-binding-induced structure (Bartley and Chalkley, 1970). In 1973, Russell Doolittle (1931–2019) indicated that a large disordered region of fibrinogen enabled its cleavage and activation, indicating functional importance of IDR (Doolittle, 1973). In 1978, T. S. K. Chang and Barry R. Zirkin showed that protamine released during the rabbit sperm nuclei decondensation is rapidly degraded, with degradation reaching maximum extent before the decondensation was completed (Chang and Zirkin, 1978).

Ignacio V. Sandoval and Klaus Weber reported in 1978 that large microtubule-associated proteins MAP1 and MAP2 (that are crucial for tubulin assembly) are rapidly degraded in crude brain extracts by a calcium-dependent protease, which does not affect tubulin, and this event results in the permanent loss of tubulin polymerization (Sandoval and Weber, 1978). This study supported the earlier observation of Richard B. Vallee and Gary G. Borisy that MAP1 and MAP2, which form the projections from cytoplasmic microtubules, are rapidly degraded by trypsin, with tubulin being little affected under the conditions employed (Vallee and Borisy, 1977). In 1980, Allan et al. used trypsin digestion to show that only a central domain of a lysine-rich histone H1 from calf thymus (residues 37–120) is in a folded conformation, whereas N- and C-terminal regions (residues 1–36 and 121–213, respectively) are very accessible in chromatin and therefore likely not in a compact, folded conformation (Allan et al., 1980).

Grzelczak et al. reported in 1982 that the most abundant cytosolic wheat embryo protein, early-methionine-labelled (E_m) polypeptide, characterized a distinctive amino acid composition, with Gly and Glx accounting for almost 40% of the total amino acids, is rapidly and completely degraded soon after the first contact of the embryo with water (Grzelczak et al., 1982). In 1986, Sohar et al. demonstrated that although the calcium-binding proteins troponin C and calmodulin are protected from digestion by the chymotrypsin-like serine proteinase in the presence of the elevated calcium levels, both proteins are rapidly degraded by the same protease in the absence of calcium (Sohar et al., 1986), indicating that the structures of these proteins are calcium-dependent. In line with these observations, Mamoru Ohnishi and Reinhart A. F. Reithmeier showed in 1987 that the structure of another calcium-binding protein, calsequestrin (which binds 40-50 calcium ions and is characterized by high levels of acidic residues), is calcium-sensitive as well, as this protein was rapidly degraded by trypsin in the absence of calcium, but showed remarkable stability in calcium-loaded form (Ohnishi and Reithmeier, 1987).

In 1988, Kashima et al. demonstrated that a very significant part of high molecular weight (300 kDa) histidine-rich protein from rat epidermis is rapidly degraded by the epidermal proteinases, leading to the accumulation of a 56 kDa fragment (Kashima et al., 1988). In 1993, Leontiev et al. showed that while the dihydrofolate reductase from *E. coli* variants with the Thr35 → Asp and Thr35 → Asp/Asn37 → Ser/Arg57 → His mutations in the active site were compact and have pronounced secondary structure, they did not possess rigid 3D structure and were ~10 times more susceptible to limited trypsinolysis in comparison with the wild type protein, emphasizing low proteolytic stability of these intrinsically disordered molten globular mutants (Leontiev et al., 1993).

Cohen et al. showed that in the absence of DNA, a member of the basic/helix-loop-helix/zipper family of DNA-binding proteins, transcription factor Max, is rapidly digested by several endoproteases, indicating an open and flexible structure of the protein in the unbound form (Cohen et al., 1995). On the other hand, dimerization and DNA binding were shown to facilitate α-helix formation in this protein, making it less susceptible to proteolytic degradation (Horiuchi et al., 1997). The lack of defined three-dimensional structure in the native state of dehydrin-like desiccation stress protein from the resurrection plant *Craterostigma plantagineum* was supported by fast proteolytic degradation (Lisse et al., 1996). Based on the comprehensive structural analysis using proteolytic mapping, circular dichroism spectropolarimetry, and nuclear magnetic resonance spectroscopy, Kriwacki et al. revealed that the cyclin-dependent kinase (Cdk) inhibitor $p21^{Waf1/Cip1/Sdi1}$ lack stable secondary or tertiary structure in the free solution state, but its N-terminal region adopts an ordered stable conformation when bound to Cdk2, thereby providing a mechanical explanation to the remarkable ability of $p21^{Waf1/Cip1/Sdi1}$ to bind and inhibit a diverse family of cyclin-Cdk complexes, including cyclin A-Cdk2, cyclin E-Cdk2, and cyclin D-Cdk4 (Kriwacki et al., 1996). Interaction between the eukaryotic translation initiation factor eIF4G and the cap-binding protein eIF4E was shown to be accompanied by the unfolded to folded transition of the 98-amino acid domain of *Saccharomyces cerevisiae* eIF4G1, as evidenced by multiple biophysical techniques, including limited proteolysis (Hershey et al., 1999).

Admittedly, the presented list of the proteins characterized by limited proteolysis as rapidly digestible polypeptides with limited structure is far from complete. Also, all the cases described here cover a "pre-IDP" period; i.e., a time-frame before the publication of our survey in 2000 (Uversky et al., 2000a). Among the

experimentally characterized "natively unfolded proteins" included in that study (Uversky et al., 2000a), there were 7 proteins (7.8%), whose disordered status was confirmed by limited proteolysis, indicating the important role of this technique in establishing the IDP field.

2.12 Fluorescence

2.12.1 A brief introduction to fluorescence and its major characteristics

Fluorescence is the instant emission of absorbed light at a longer wavelength. This phenomenon occurs when a substance absorbs a high-energy photon, prompting its electrons to move to a higher energy level. As the electrons return to their ground state, they emit the energy as a photon of light with a longer wavelength. Historically, work on fluorescence is rooted in studies of "epipolic dispersion" by John Herschel (1792–1871), who described a blue glow emanating from a thin, superficial layer ("epipolic stratum" from Greek ἐπίπόλη, a surface) of a colorless quinine solution near the surface where the light enters, with such "epipolized light" been changed in some way, as it was incapable of being epipolized again, indicating that epipolic dispersion is different from scattering (Herschel, 1845a; Herschel, 1845b).

However, other researchers of the 19th century also observed the luminescence phenomena in various materials. For example, in 1819, Edward Daniel Clarke (1769–1822) reported that certain varieties of fluorite crystals had different color depending on the direction of illumination; i.e., when viewed by reflected light versus transmitted light (Clarke, 1819). Similar observations on some varieties of fluorites were reported by René Just Haüy (1743–1822) in 1822, but the effect was incorrectly interpreted as a form of light scattering, similar to opalescence (Haüy, 1822). While describing a similar effect in chlorophyll, David Brewster (1781–1868) also considered it as a form of opalescence (Brewster, 1834). Finally, in 1843, Alexandre-Edmond Becquerel (1820–1891) described the emission of light by calcium sulfide deposited on paper when exposed to solar light beyond the violet part of the spectrum, being, therefore, the first to state that the emitted light is of longer wavelength than the incident light (Becquerel, 1843). In 1852, George Gabriel Stokes (1819–1903) summarized the accumulated information about these optical effects and provided the first detailed description of the phenomenon, which he named "fluorescence", where certain materials, such as fluorspar and uranium glass, convert invisible ultraviolet light into visible light of a longer wavelength (Stokes, 1852; 1853). The observed phenomenon, where the wavelength of the fluorescent (emitted) light is always longer than the wavelength of the exciting (absorbed) light, represents a fundamental principle is now known as the Stokes shift.

The physics of fluorescence is best illustrated by Jabłoński diagram (Jablonski, 1933; Jabłoński, 1935) showing electronic and vibrational energy states of a molecule, as well as the radiative and non-radiative transitions after light absorption. This diagram serves as an effective visual tool demonstrating the energy transitions and relaxation processes that occur in molecules after the absorption of light and showing corresponding processes, such as absorption, fluorescence and delayed fluorescence (from singlet states), phosphorescence (from triplet states after intersystem crossing), and non-radiative transitions. Although being commonly known as Jabłoński diagram, the more correct name of this model is the Perrin-Jabłoński diagram (after Jean Baptiste Perrin (1870–1942), Francis Perrin (1901–1992), and Aleksander Jabłoński (1898–1980)) modified by Alexander N. Terenin (1896–1967), Gilbert N. Lewis (1875–1946), and Michael Kasha (1920–2013) to emphasize the contribution of other researchers to the development of this concept.

Fluorescence is characterized by several parameters, such as the fluorescence quantum yield, fluorescence intensity, extinction coefficient, peak excitation wavelength, peak emission wavelength, Stokes shift, fluorescence lifetime, fluorescence anisotropy, and fluorescence resonance energy transfer (FRET). *Fluorescence quantum yield* quantifies the efficiency of the fluorescence process, being the ratio of the number of photons emitted to the number of photons absorbed. The idea is rooted in a series of papers by Emil Gabriel Warburg (1846–1931) reporting quantitative experiments on photochemical reactions [e.g. (Warburg, 1920)], which were crucial for subsequent establishment of the method for the quantum yield determination as described by Sergey I. Vavilov (1891–1951) (Wawilow, 1924). *Fluorescence lifetime* provides a measure of the average time spent by a fluorophore in an excited state before emitting a photon and returning to its ground state. For the first time, luminescence decay times characterizing the duration of phosphorescence of uranyl salts were measured by Edmond Becquerel in 1859 (Becquerel, 1859). In 1926, Ramón Enrique Gaviola (1900–1989) developed a first instrument for measuring fluorescence decay in the nanosecond range (Gaviola, 1926), and in the same year, Francis Perrin derived a mathematical relationship between fluorescence lifetime and molecular rotation, thereby contributing to the theoretical understanding of fluorescence lifetime (Perrin, 1926).

Fluorescence anisotropy measures the rotational motion of a fluorophore-labeled molecule by analyzing the polarization of its emitted light. Although the modern definition and use of fluorescence anisotropy were introduced by Aleksander Jabłoński (Jablonski, 1960) and independently by Gregorio Weber (1916–1997) (Weber, 1952; 1967), this work was built upon the earlier studies of Fritz Weigert (1876–1947) (Weigert, 1920) and Francis Perrin (Perrin, 1926). For a flexible or non-rigid protein, the intrinsic fluorophores (tryptophan residues) have freedom to rotate independently of the rest of the molecule. This faster, local rotational motion causes a significant and measurable decrease in fluorescence anisotropy. By contrast, in a rigid protein, the tryptophan is held in place, and its rotation is constrained, leading to higher anisotropy.

Fluorescence resonance energy transfer (FRET, also known as Förster resonance energy transfer) is the radiationless transfer of energy from an excited donor fluorophore to a suitable acceptor fluorophore, a physical process that depends on spectral overlap and proper dipole alignment of the two fluorophores. Although it was observed for the first time in 1922, in the experiments on fluorescence behavior of a vapor composed of mercury and thallium atoms conducted by Günther Cario (1897–1984) and James Franck (1882–1964) (Cario and Franck, 1922) and the finalized theory was introduced in 1940s by Theodor Förster (1910–1974) (Forster, 1946) following the earlier work by Francis Perrin (Perrin, 1932), FRET became a go-to technique in the 1960s,

when it was demonstrated that FRET measurements could be carried out with fluorescently labeled peptides, and thereby the ruler for molecular interactions was discovered (Stryer and Haugland, 1967; Szabo et al., 2022).

2.12.2 Fluorescence and protein flexibility

Despite all these developments, the actual use of fluorescence in life sciences did not start before the 1950s, when the first spectrophotofluorometer was invented by Robert L. Bowman (1916–1995) (Bowman et al., 1955) and when it was established that some naturally occurring amino acids, such as tryptophan, tyrosine and phenylalanine, were responsible for the ultraviolet fluorescence observed in proteins (Weber, 1953; Duggan and Udenfriend, 1956; Shore and Pardee, 1956; Konev, 1957; Teale and Weber, 1957). A book "Fluorescence and Phosphorescence of Proteins and Nucleic Acids" published in 1967 by Sergei V. Konev (1931–2005), being the first book written specifically about luminescence of biopolymers, contains a comprehensive overview of the then-major achievements in the field of protein fluorescence and describes the use of fluorescence as a means to analyze the structure and conformation of macromolecules in intact cells (Konev, 1967).

As per PubMed, the current literature on protein fluorescence includes more than 475,000 studies. The power of fluorescence spectroscopy in application to proteins is reflected in the ability of this technique to provide characterization of protein flexibility across a wide range of timescales, from picoseconds to seconds and even minutes, with the specific timescale observed being dependent on the type of motion being measured and the fluorescence method used. As a result, fluorescence methods allow for detection and analysis of rapid, local motions, such as fluctuations of individual amino acid side chains and the local relaxation of the protein matrix (10^{-12} to 10^{-9} s), capturing structural changes, such as domain motions or transitions between different protein states (10^{-6} to 10^{-3} s), as well as analysis of slower, large-scale motions and conformational changes, ranging from folding-unfolding processes to binding or dissociation of other molecules (10^{-3} s to minutes).

Fluorescence spectroscopy was crucial for discovery of protein conformational breathing, which was the foundation of the shift of the traditional view of proteins as rigid, static structures to a more accurate model of flexible, fluctuating molecules essential for biological function. For example, in 1973, Joseph R. Lakowicz and Gregorio Weber reported that despite their common location in the interior of the protein matrix and the apparent inaccessibility to solvent, no tryptophan residues were excluded from quenching by oxygen (which is an "ideal" quencher of the fluorescence of singlet excited states, as every collision with an excited fluorophore results in quenching), indicating that "proteins, in general, undergo rapid structural fluctuations on the nanosecond time scale which permit diffusion of oxygen" (Lakowicz and Weber, 1973). Based on these observations, Gregorio Weber pointed out in 1975: "From these results it can be definitely concluded that the time-average structure observed by X-ray diffraction is something of an abstraction, since it is not itself widely–or even sparsely–represented at any given time in the population of molecules . . . Indeed the protein molecule model resulting from the X-ray crystallographic observations is a "platonic" protein, well removed in its perfection from the kicking and screaming "stochastic" molecule that we infer must exist in solution. The great importance of the former lies in that it has permitted us to see the origin of the "bulk properties" of the protein, which result from averaging over the whole population, However, when it comes to the conversion of one structure into another, and to render account of any dynamic function of the protein, consideration of the "stochastic" species is indispensable" (Weber, 1975). Fluorescence-based analysis also revealed the conformational inhomogeneity of protein structure, as evidenced, for example, by the multiexponential fluorescence decay in single-tryptophan proteins (Grinvald and Steinberg, 1976).

2.12.3 Intrinsic fluorescence

Depending on their origin, fluorophores are classified as intrinsic (occurring naturally in the system under study) and extrinsic (fluorophores that do not occur in nature), which are further subdivided to fluorescent dyes (fluorophores that can interact with biomolecules but are not covalently attached to them) and fluorescent labels/probes/tags (fluorophores that are covalently attached to the biomolecules). One should keep in mind that obtaining fluorescent characteristics of a protein is typically not sufficient for its structural classification, and fluorescence analysis is therefore commonly complemented by other techniques, such as CD or FTIR, to obtain at least information about protein secondary structure.

Long before IDPs were widely studied, intrinsic fluorescence was used to analyze the unfolding of globular proteins, which helped establish a foundation for understanding large-scale protein conformational changes. In fact, the most common application of intrinsic protein fluorescence is to detect and monitor conformational changes in a query protein. This is because the parameters of intrinsic fluorescence (particularly those of tryptophan (Trp) fluorescence), such as intensity, lifetime, anisotropy, and peak emission wavelength, are highly sensitive to the local environment and provides valuable, label-free insights into protein structure, dynamics, and conformational changes. This sensitivity arises from the indole ring of Trp, which is characterized by a relatively large dipole moment both in the ground and excited states it extensively interacts with polar and charged groups in its environment, and whose photophysical properties are affected by surrounding residues, solvent exposure, and molecular motion (Lakowicz, 1983; Permyakov, 2018). As a result, proteins can be classified based on their tryptophan fluorescence parameters, such as shape and position of their intrinsic fluorescence spectra, which indicate location and environment of a tryptophan residue within a protein (Eftink, 1991; Chen and Barkley, 1998; Engelborghs, 2003).

The more hydrophobic and rigid the environment, the shorter the wavelength of the fluorescence maximum, the phenomenon known as a blue-shift (Vivian and Callis, 2001). One of the most extreme blue-shifts was reported for azurin, whose tryptophan residue is in an unusual rigid environment within the β-barrel structure lacking hydrogen bonding or other polar interactions. The resulting fluorescence spectrum was notable for its distinct vibrational structure and a maximum emission (λ_{max}) at an unusually short wavelength of 308 nm (Burstein et al., 1977). Conversely, as the environment becomes more polar and flexible, the maximum shifts to a longer wavelength, known as a red-shift, and unfolded proteins are typically characterized by fluorescence

spectra with λ_{max} at 350–353 nm (Eftink, 1991). It was also indicated that the fluorescence properties of Trp residues in the proteins can be described based on the existence of three discrete spectral classes, a buried in nonpolar regions of the protein (λ_{max} = 330–332 nm, spectral band width $\Delta\lambda$ = 48–49 nm, quantum yield q = 0.11, and lifetime τ = 2.1 ns), a completely exposed to water (λ_{max} = 350–353 nm, $\Delta\lambda$ = 59–61 nm, q = 0.2, τ = 5.4 ns); and in limited contact with water (λ_{max} = 340–342 nm, $\Delta\lambda$ = 53–55 nm, q = 0.3, τ = 4.4 ns) (Burstein et al., 1973).

2.12.4 Quenching of fluorescence

A very promising spectroscopic technique for evaluation of solvent accessibility of aromatic residues in a protein involves the quenching of their intrinsic fluorescence by the addition of various low molecular weight agents (Lehrer, 1971; Eftink and Ghiron, 1976; Lehrer and Leavis, 1978), whose efficiency can be evaluated using Stern-Volmer plot (named after Otto Stern (1888–1969) and Max Volmer (1885–1965) who proposed this approach in 1919) correlating changes in fluorescence intensity with the concentration of quencher, and whose slope gives the Stern-Volmer constant, K_{SV}, a quantitative measure of fluorescence quenching efficiency (Stern and Volmer, 1919). The quenching reaction involves physical contact between the quencher and an excited indole ring, and can be kinetically described in terms of a collisional and a static component (Eftink and Ghiron, 1976). In 1976, Maurice R. Eftink and Camillo A. Ghiron published a comprehensive study utilizing acrylamide as quencher and showed that this approach is very discriminating in sensing the degree of exposure of tryptophan residue in proteins, including a randomly coiled peptide adrenocorticotropin (Eftink and Ghiron, 1976). The power of fluorescence at the single molecule level was demonstrated in 2000 by Zhuang et al., who showed that fluorescence self-quenching (i.e., fluorescence quenching between identical fluorophores) is sensitive to the collapse of intrinsically disordered protein titin (Zhuang et al., 2000).

2.12.5 Red edge excitation shift

Combining Vavilov's law stating that the fluorescence quantum yield is constant regardless of the excitation wavelength, and as a result, emission energy is independent of the excitation energy within the absorption band (Wawilow, 1924; Wawilow, 1927) with Kasha's rule emphasizing that "the emitting level of a given multiplicity is the lowest excited level of that multiplicity" (Kasha, 1950) gives rise to an important principle that the shape of the fluorescence spectrum is independent of the excitation wavelength. However, in 1970, it was found that the spectroscopic properties of the fluorescence spectra of aromatic fluorophores embedded into rigid and highly viscous media do not conform to classical rules and can depend on excitation wavelength and the excited-state energy transfer between identical molecules can be undetectable on excitation at the red edge of the absorption spectrum (Galley and Purkey, 1970; Rubinov and Tomin, 1970; Weber and Shinitzky, 1970). These and subsequent observations were described as "red-edge effects" (REE), and the analysis of these phenomena, especially red edge excitation shift (REES) provided a useful approach for the investigation of protein structure and dynamics by analyzing how its intrinsic fluorescence spectrum shifts when excited by different wavelengths of light (Demchenko, 2002).

The appearance of REES is linked to the reorientation time of the polar solvent molecules (water) surrounding an excited polar fluorophore (tryptophan) with changed electronic state (and, thus, changed dipole moment) to reach a new, lower-energy equilibrium with the excited fluorophore. The timescale of such solvent relaxation depends on the environment, being very fast in non-viscous solvent (happening well within the excited-state lifetime of the fluorophore) but being much slower in motionally restricted environments, such as viscous solvents or the compact, tightly packed interior of a folded protein. Under such conditions, a shift of the excitation wavelength towards the "red edge" (lower energy) of the absorption spectrum causes excitation of only a sub-population of fluorophores that are already in a lower-energy ground state. At the excitation of such red-edge molecules, their surrounding solvent cannot fully relax before fluorescence emission occurs, leading to the red-shift of the emission spectrum compared to the emission observed when exciting at the absorption maximum (Demchenko, 2002). This shift provides information about the local environment's polarity, dynamics, and rigidity, making it useful for monitoring protein stability, conformational changes, and ligand binding.

A comprehensive analysis of REE in single-Trp proteins revealed that the effects can be found in proteins possessing fluorescence emission spectra with λ_{max} ranging from 320 to 340 nm, whereas in proteins and peptides with fluorescence spectra above 340 nm, REE are not observable because of the high flexibility of these proteins and the access of tryptophan residues to the rapidly relaxing solvent. In proteins with λ_{max} below 320 nm REE is not observed as well, likely due to the highly hydrophobic Trp environment where dielectric effects are not significant (Demchenko, 1988; Demchenko, 2002; Demchenko, 2013). A recent comprehensive review by Rupasree Brahma and H. Raghuraman provides a detailed description of using REES for linking solvent relaxation dynamic and protein conformation and emphasizes that this technique can be used as a sensitive tool to monitor the structural plasticity of IDPs and distinguish subtle but important structural alterations in these proteins (Brahma and Raghuraman, 2021). However, most REES-based studies of IDPs were conducted very recently and therefore did not contribute to the establishing of the IDP field [for example, REES was used for analysis of α-synuclein and amyloidogenic k-casein in 2013 and 2014, respectively (Jain et al., 2013; Arya and Mukhopadhyay, 2014)].

2.12.6 Analysis of IDPs by steady-state fluorescence

Because of their known amino acid biases, IDPs are commonly depleted in aromatic residues. Therefore, intrinsic fluorescence-based studies are typically restricted to a limited number of aromatic residue-containing IDPs, which are usually characterized by surface location of Trp residues characterized by the red-shifted intrinsic fluorescence spectra with λ_{max} at 340 – 353 nm (Permyakov and Uversky, 2010). Often, the fluorescence spectrum of an IDP coincides with that of free Trp in water. However, interactions of such IDPs with partners can promote transfer of Trp residues to a more hydrophobic or more rigid environment, leading to a blue shift of fluorescence maximum position. For example, the intrinsically disordered C-terminal domain of chicken gizzard caldesmon (CD136) has a λ_{max} of

350.2 nm, but interaction with its binding partner, calmodulin, results in a pronounced (17 nm) blue shift of the CaD136 fluorescence spectrum (Permyakov et al., 2003). Analysis of the intrinsically disordered N-terminal fragment of human glucocorticoid receptor fragments revealed that it is characterized by the λ_{max} of 340 nm, suggesting an external location of Trp residues, whereas trimethylamine N-oxide-induced folding of this fragment resulted in a blue shift of fluorescence maximum by more than 10 nm (Baskakov et al., 1999).

In 1971, Paul R. Rurnett and I. H. Eplar showed that the myelin basic protein (MBP) is characterized by red-shifted intrinsic fluorescence (Burnett and Eylar, 1971). In 1975, Jones and Rumsby reported that the tyrosine and tryptophan residues of this protein are largely exposed to the solvent and that the addition of 8 M urea had little effect on the fluorescence properties of the protein (Jones and Rumsby, 1975). Furthermore, the resonance energy transfer from tyrosine to tryptophan was very inefficient (Jones and Rumsby, 1975), suggesting that MBP is characterized by extended conformation. Later, Monferran et al. showed that the intrinsic fluorescence of MBP is characterized by the λ_{max} of 346 nm and this maximum remains practically unchanged in chemically modified (acetylated and succinylated) MBP (Monferran et al., 1986). These authors also showed that only non-modified protein was able to efficiently interact with membranes, and this interaction resulted in a dramatic blue-shift of intrinsic fluorescence below 335 nm (Monferran et al., 1986). In 1991, Cavatorta et al. used intrinsic fluorescence to characterize interaction of MBP with divalent cations and showed that Zn^{2+} can bind to MBP (Cavatorta et al., 1991).

In 1975, Kevin N. Pearce showed that the intrinsic fluorescence of bovine β-casein A^1 at low temperature (where a protein is known to be a monomer with random coil conformation) is characterized by λ_{max} of 340 nm, but the spectrum became noticeably blue-shifted as a result of temperature-induced micelle formation (Pearce, 1975). Conformational analysis of the extracellular domain of the low affinity NGF receptor (LNGFR or gp75) revealed that this protein is mostly disordered, as evidenced by its low content of regular secondary structure and red-shifted intrinsic fluorescence with λ_{max} of 345 nm (Timm et al., 1992). However, this protein was shown to undergo a temperature-induced transition to a more disordered state with the emission peak shifted to ~353 nm (Timm et al., 1992).

Based on the steady-state and time-resolved fluorescence analysis, Neyroz et al. concluded that a potent inhibitor of protein phosphatase 1, DARPP-32 (dopamine- and CAMP-regulated phosphoprotein with an apparent molecular mass, of 32,000 on SDS- polyacrylamide gel electrophoresis), although this protein is characterized by mostly random coil conformation, has a λ_{max} of 352 nm, low fluorescence anisotropy values indicative of the independent segmental motions of the peptide chain surrounding fluorophore, and is characterized by accessibility to quencher comparable to that of proteins containing exposed fluorophores, it can be further unfolded by 8 M urea, as evidenced by further decrease of fluorescence anisotropy (Neyroz et al., 1993). Josefsson et al. revealed that the B-repeat segment of a cell surface protein of *Staphylococcus aureus* SdrD is capable of binding of 14 Ca^{2+} ions, and that, based on the circular dichroism and Trp fluorescence analysis, concluded that although the metal-free form is strongly disorganized, the Ca^{2+}-bound form is well folded (Josefsson et al., 1998).

2.12.7 Fluorescence anisotropy in analysis of IDPs

Fluorescence anisotropy was used to characterize the flexibility and conformational changes of IDPs in several early studies [see, e.g., aforementioned DARPP-32 analysis (Neyroz et al., 1993)]. In 1973, Munro et al. used time-resolved tryptophan fluorescence polarization spectroscopy to analyze the dynamics of several proteins in the subnanosecond and nanosecond time range (Munro et al., 1979). This analysis revealed that the tryptophan residues of the analyzed proteins exhibit different degrees of rotational freedom, ranging from almost no mobility to nearly complete freedom in the subnanosecond time range, as evidenced by an intrinsically disordered bovine basic A1 myelin protein (Munro et al., 1979). Y. Gloria Chu and Charles R. Cantor used static and nanosecond fluorescence polarization techniques to analyze the ribosomal protein S1 fluorescently labeled by iodoacetylethylenediamine (1,5)-napthol sulfonate (IAEDANS) and revealed that this protein is characterized by segmental flexibility, suggesting that it contains at least two domains that can rotate independently (Chu and Cantor, 1979). In 1981, Lubert Stryer (1938–2024) emphasized that protein molecules are not inflexible crystal-like entities, but are characterized by very rapid motions of selected regions, since nanosecond fluorescence polarization analysis revealed that segmental flexibility, where domains can rotate over an appreciable angular range of times of nanoseconds, is a characteristic feature of multidomain proteins, whereas time-resolved fluorescence polarization studies have demonstrated that internal tryptophan residues can be flexible in the subnanosecond time range (Stryer, 1981).

Based on the steady-state and lifetime-resolved fluorescence anisotropy measurements of intrinsic protein fluorescence, Lakowicz et al. showed that tryptophan residues of carbonic anhydrase, carboxypeptidase A, alpha-chymotrypsin, trypsin, pepsin, and bovine and human serum albumins are characterized by the remarkable freedom of motion within the protein matrix, which implies that these matrices are highly flexible on the nanosecond time scale (Lakowicz et al., 1980). Fluorescence polarization studies of dansylcadaverine-labeled fibronectin indicated that this protein has a significant degree of chain flexibility under physiologic conditions and may assume an unfolded conformation upon binding to collagen in the tissue matrix (Williams et al., 1982).

Hauer et al. used time-resolved fluorescence anisotropy to analyze the 8 kDa protein kinase inhibitor (PKI alpha) of cAMP-dependent protein kinase (cAPK) free in solution and bound to cAPK (Hauer et al., 1999). To this end, three single mutants of PKI alpha were created (V3C, S28C, and S59C) and labeled with iodoacetamide. Analysis revealed that in the unbound state, all three variants were characterized by high flexibility, whereas binding to the catalytic (C) subunit of cAPK caused local folding of the N-terminal region of PKI alpha, whereas the segment around Ser59 remained highly flexible when bound to the C-subunit (Hauer et al., 1999), suggesting the "fuzzy" nature of the resulting PKI alpha-cAPK complex. Over the years, fluorescence anisotropy was successfully used for characterization of protein flexibility.

However, the unique power of this technique was revealed in a 2010 study of Mattheyses et al., who used fluorescence polarization microscopy to analyze fluorescence anisotropy of fluorescently tagged specific domains of individual (Mattheyses et al., 2010). The analysis resolved order and disorder of individual protein domains nucleoporins within the nuclear pore complex (NPC) and showed that "the tips of the FG domains are disordered, whereas the NPC-anchored domains are ordered" (Mattheyses et al., 2010).

2.12.8 Fluorescence (Förster) resonance energy transfer (FRET)

Fluorescence (Förster) resonance energy transfer (FRET) analysis is used to measure protein flexibility by tracking changes in distance between two fluorescent molecules (fluorophores) attached to a protein. In early studies, the proximity of fluorescently labeled sites on biological macromolecules was analyzed by the ensemble FRET, which provided averaged information about the distances between labeled parts of proteins. A comprehensive review published by Lubert Stryer in 1978 entitled "Fluorescence energy transfer as a spectroscopic ruler" provided a description of the Förster's theory and of the experimental tests of its validity, systemized experimental strategy for the measurements of transfer efficiency, and summarized data on using energy transfer for elucidation of the proximity relationships in a wide variety of biological macromolecules and assemblies, and thereby represented FRET as a powerful tool in the analysis of protein structure and interactions (Stryer, 1978).

Although most of the FRET applications in the early days were related to the estimation of distances between interacting partners, in 1978, Elisa Haas, Ephraim Katchalski-Katzir, and Izchak Z. Steinberg analyzed the translational dynamics of the ends of oligopeptides by measuring energy transfer between chromophores attached to the N- and C-termini (Haas et al., 1978). The logics of this approach, where FRET is enhanced due to the translational diffusion as a donor-acceptor pair that is too far apart for efficient transfer at the instant of excitation may diffuse toward each other during the excited-state lifetime so that the distance between them becomes short enough for efficient transfer (Elkana et al., 1968; Steinberg and Katchalski, 1968), is a founding principle of the single molecule FRET (smFRET) that become one of the most efficient tools for evaluation of flexibility and conformational dynamics of proteins. Though smFRET was demonstrated for the first time by Ha et al., in 1996 by obtaining simultaneous dual color images and emission spectra from donor and acceptor fluorophores linked by a short DNA molecule (Ha et al., 1996), the first actual application of this tool in analysis of IDPs was reported in 2007 by Mukhopadhyay et al., who demonstrated that the natively unfolded yeast prion protein adopts an ensemble of collapsed and rapidly fluctuating structures (Mukhopadhyay et al., 2007). Although it did not contribute to the establishment of the protein intrinsic disorder concept, this powerful technology rapidly became a popular tool in the IDP field (Ferreon et al., 2010; Lee et al., 2015; Schuler et al., 2016; Gomes and Gradinaru, 2017; LeBlanc et al., 2018; Nasir et al., 2019; Metskas and Rhoades, 2020).

2.12.9 Fluorescence correlation spectroscopy (FCS)

An important technique for the analysis of molecular dynamics is fluorescence correlation spectroscopy (FCS) that measures spontaneous fluorescence intensity fluctuations in a microscopic detection volume. Being, in essence, the fluorescent counterpart to the dynamic light scattering, FCS provides information about diffusion coefficients and hydrodynamic radii (Rigler et al., 1979; Sorscher et al., 1980; Kask et al., 1987) of even a single fluorescent molecule (Krichevsky and Bonnet, 2002). FCS was introduced in 1972 by Douglas Magde, Elliot Elson, and Watt W. Webb who analyzed the binding of ethidium bromide to DNA (Magde et al., 1972).

Currently, FCS is used for the analysis of the photodynamic properties of fluorescent dyes, study of translational and rotational diffusion, in various binding assays, as well as investigation of conformational fluctuations of biomolecules, and even analysis of living cells (e.g., counting fluorescence particle *in vivo*, probing cellular organelles, and analysis of nuclear structure) (Maiti et al., 1997; Krichevsky and Bonnet, 2002). Most frequently, FCS, which, in addition to the translational diffusion time of the molecules through the small detection volume provides information on the average number of fluorescent molecules in the detection volume, is utilized in detection and identification of single molecules in solution (Eigen and Rigler, 1994; Kinjo, 1998) and in the analysis of molecular interactions (Sterrer and Henco, 1997; Widengren and Rigler, 1998; Hink et al., 2002; Berland, 2004; Levin and Carson, 2004), providing a means for molecular recognition at the single molecule level (Van Craenenbroeck and Engelborghs, 2000), even in living cells (Lippincott-Schwartz et al., 2001; Hink et al., 2002; Pramanik, 2004).

An illustrative example of using FCS in interactions of IDPs is given by a study published in 2000 by Pack et al., where the peculiarities of binding of disordered substrates (apo-cytochrome *c* and disulfide-reduced apo-α-lactalbumin) to GroEL was analyzed (Pack et al., 2000). One of the first applications of FCS in structural IDP analysis was reported in 2006, when Crick et al. quantified the hydrodynamic sizes of monomeric polyglutamine (which acts as a model IDP) as a function of chain length and showed that this homopolypeptide exists as a heterogeneous conformational ensemble of collapsed structures (Crick et al., 2006). In the aforementioned 2007 study, Mukhopadhyay et al. used FCS in combination with smFRET to analyze the conformational behavior of yeast prion protein (Mukhopadhyay et al., 2007).

2.13 Mass spectrometry

2.13.1 Hydrogen-deuterium exchange mass spectrometry (HDX-MS)

Time-resolved spontaneous hydrogen-deuterium exchange (HDX) of covalently bonded hydrogens of a protein with deuterium in solution provides information about protein conformation, flexibility, and solvent accessibility (Englander et al., 2016). In fact, since protected regions exchange more slowly than exposed, flexible regions, protein secondary and tertiary structure can be deduced from the rate at which backbone amide protons exchange with solvent deuterium. Application of HDX for analysis of proteins was pioneered by Kaj Ulrik Linderstrøm-Lang (1896–1959), who, in addition to the aforementioned development of the protein secondary structure concept and elaboration of the limited proteolysis as a research tool, proposed the foundational theory for the use of this technique in

studying protein structure and dynamics (Hvidt and Linderstrom-Lang, 1954; Hvidt and Linderstrom-Lang, 1955; Linderstrom-Lang, 1955; Berger and Linderstrøm-Lang, 1957; Benson and Linderstrøm-Lang, 1959; Englander et al., 1997).

Although in those early studies, HDX was analyzed for insulin, β-lactoglobulin, myoglobin, and poly-DL-alanine, because of the use of densitometry as analytical technique, the application of this method was limited by poor spatial resolution (early densitometric analysis measured average deuterium incorporation over large segments of a protein, which made it impossible to pinpoint specific sites of exchange). The situation changed dramatically when it was proposed to use NMR as a method for HDX detection (Dempsey, 2001), and NMR-based HDX detection was the leading technique in the field until the early 2000s, when mass spectrometry (MS) emerged as an extremely powerful alternative (Deng et al., 2016; Masson et al., 2019; James et al., 2022; Stofella et al., 2022), although the first report demonstrating the use of MS to monitor hydrogen-deuterium exchange in intact proteins was published by Viswanatham Katta, Brian T. Chait, and Steven Carr in 1991 who also mentioned that this technique can efficiently detect conformational changes of proteins in solutions (Katta et al., 1991).

In HDX analysis, NMR relies on the different magnetic properties of hydrogen and deuterium and provides residue-specific information, whereas MS utilizes change in mass due to deuteration and offers information that is specific to peptides obtained by proteolytic digestion. While HDX-MS is characterized by somewhat lower spatial resolution than HDX-NMR [although there are several computational strategies to extract single-residue protection factors from peptide-level HDX-MS data (Zhang et al., 2012; Kan et al., 2013; Gessner et al., 2017; Saltzberg et al., 2017; Babic et al., 2019; Skinner et al., 2019; Zhang, 2020; Salmas and Borysik, 2021; Smit et al., 2021)], mass spectrometry has several important advantages, such as no sample size limitations, no labeling required, low protein concentration, low costs, and highly automated processing (Stofella et al., 2022).

There are more than 540 studies describing the application of HDX-NMR for characterization of structure, dynamics, and interactions of proteins, investigation of protein folding, analysis of hydrogen bond formation within the intermediate on protein folding pathways, and evaluation of the global and local folding free energies in proteins. Similarly, more than 2200 studies represent the outputs of the HDX-MS utilization for similar purposes. Although the use of HDX-NMR dates back to the 1970s and 1980s, the corresponding studies were primarily focused on the analysis of ordered proteins and their folding and interactions. Based on the timing of its invention, HDX-MS, which began to replace HDX-NMR around the early 2000s, did not contribute much to the early studies of IDPs. However, the ability of this technique to follow the structural evolution of a protein from an unfolded to a folded state, providing insight into the flexibility of folding intermediates, was demonstrated in 1993 by Miranker et al. using cytochrome *c* folding as an example (Miranker et al., 1993). In 1999, Eyles et al. showed that unfolding of cellular retinoic acid-binding protein I (CRABP I) is characterized by the formation of transiently populated intermediate states (Eyles et al., 1999).

Furthermore, several recent studies show power of this technique in application to IDPs. For example, in 2006, Hamuro et al. reported the use of HDX-MS in analysis of a ligand-dependent transcription factor involved in glucose homeostasis and adipocyte differentiation, a nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) (Hamuro et al., 2006). These authors showed that a ligand-free ligand binding domain (LBD) of PPARγ is significantly more flexible than the rest of the LBD, indicating that this binding pocket is intrinsically disordered in order to accommodate different ligands (Hamuro et al., 2006).

MacDonald et al. generated HDX-MS and NMR-based model for the tropomyosin-binding domain of the calponin homology-associated smooth muscle (CHASM) protein, where the CHASM intrinsically disordered N-terminal region interacts with the globular C-terminal calponin homology (CH) domain and the cooperativeness between these regions is required for CHASM binding to smooth muscle tropomyosin (MacDonald et al., 2012). Kumar et al. used HDX-MS to demonstrate that the structurally dynamic N-terminal domain of progesterone receptor is regulated through folding coupled to binding to the TATA-binding protein (TBP) (Kumar et al., 2013). In 2013, Diana Resetca and Derek J. Wilson provided a comprehensive review outlining the use of time-resolved electrospray ionization mass spectrometry (TRESI-MS) and HDX in analysis of rapid, activity-linked conformational transitions in proteins (Resetca and Wilson, 2013). In the same year, Deepa Balasubramaniam and Elizabeth A. Komives presented a comprehensive review discussing the utility of HDX-MS in discovery of IDRs in proteins, monitoring coupled folding and binding, and characterization of protein aggregates and oligomers (Balasubramaniam and Komives, 2013).

2.13.2 Ion mobility-mass spectrometry (IM-MS)

Ion mobility-mass spectrometry (IM-MS) is an analytical technique that separates ions based on their collision cross-section (CCS, which is a measure of the size and shape of ion that defines its collision rate) in the gas phase. In application to proteins, IM-MS provides information about the protein conformational landscape, and can be used for characterizing its size, shape, and dynamics, analyzing different conformational states of a protein, and tracking how it folds, interacts with other molecules, or responds to changes in its environment, such as salt concentration. Although biomolecule-focused IM-MS is one of the relatively recently commercialized MS-based analytical tools (Causon, 2021), this method represents a combination of two well-established technologies, ion mobility spectrometry (IMS) and MS, both with long individual histories of application.

IMS, which studies the movement of ions in gases under the influence of an electric field, is rooted in the seminal work of Joseph J. Thomson (1856–1940) and Ernest Rutherford (1871–1937), who investigated the relationship between electrical conductivity and gaseous media in 1896 (Thomson and Rutherford, 1896). However, movement of ions through a gas under the influence of an electric field was described for the first time by John Zeleny (1872–1951) in 1898, who indicated that "the positive and negative ions, which take part in the conduction of gases exposed to Röntgen rays, move with different velocities when in the same electric field" (Zeleny, 1898). MS was invented in 1913 by Joseph J. Thomson (1856–1940) as a method to discover non-radioactive isotopes by separating ions based on their mass-to-charge ratio (Thompson and Thomson, 1913), with the first fully functional mass spectrograph being

built in 1919 by Francis William Aston (1877–1946) (Aston, 1919). For some time, these techniques developed in parallel. For example, IMS, which is characterized by high sensitivity and fast screening capabilities, became a major technology behind detection of explosives and chemical warfare agents in military operations, sporting events, and airports (Makinen et al., 2010; Dodds and Baker, 2019).

In its early years (until the 1940s), MS played crucial role in the field of physics being used to resolve questions about the fundamental nature of the atom, to discover and analyze isotopes and atomic weights, as well as to separate uranium-235 from the more abundant uranium-238 using "calutrons", which were large, industrial-scale mass spectrometers (Griffiths, 2008; Smoluch and Silberring, 2019). At that time, MS technology was also utilized by industrial chemists, who knew the identities and structures of most of the molecules in their mixtures, and used MS for measuring the abundances of small hydrocarbons in process streams (to ensure the correct ratios of components for high-quality fuels and synthetic rubber production), improving quality control, and process monitoring (Griffiths, 2008; Smoluch and Silberring, 2019).

Powered by advances in commercial technology, mass spectrometry grew from a physics/industrial chemistry tool into a major method for organic chemistry by the mid-1950s. Its applications initially focused on hydrocarbon analysis, and later progressed to the study of complex organic molecule fragments (Griffiths, 2008). Application of MS to large polar organic molecules, such as proteins and other biomolecules, was originally limited by the fact that the ionization techniques available at the time were too violent (Clarke, 2019). The situation changed in the late 1980s with the advent of the so-called "soft" ionization techniques of electrospray ionization (ESI) (Fenn et al., 1989) and matrix-assisted laser desorption/ionization (MALDI) (Clarke, 2019). This eventually made MS the cornerstone technology in the field of proteomics (Aebersold and Mann, 2003; Malmstrom et al., 2006; Prakash et al., 2006; Prakash et al., 2007; Griffiths, 2008; Gstaiger and Aebersold, 2009; Bodenmiller and Aebersold, 2010; Pan et al., 2011; Bensimon et al., 2012; Sabido et al., 2012; Guo and Aebersold, 2023).

Combination of IMS with MS gave rise to the ion mobility mass spectrometry (IM-MS), which, being an electrophoretic gas-phase technique, provides a means for distinguishing and separation of analytes based on their charge (z), mass (m), and mobility (K) in a given buffer gas (Harvey et al., 2011). The invention of IM-MS is ascribed to Earl Wadsworth McDaniel (1926–1997), who, in the early 1960s, coupled a low-field ion mobility drift cell to a sector mass spectrometer (McDaniel et al., 1962). Some of the first studies discussing application of IM-MS include identification and mobility of ions in a Townsend discharge by time-resolved mass spectrometry (McAfee Jr and Edelson, 1963), analysis of the ionic polymerization of ethylene at near atmospheric pressures (Kebarle and Hogg, 1965), and analysis of ammonium ions produced by the alpha radiolysis of ammonia and their solvation in the gas phase by ammonia and water molecules (Hogg and Kebarle, 1965). Importantly, different forms of MS can be coupled with IMS (e.g., Fourier transform ion cyclotron resonance (ICR) (Solouki et al., 1994a; Solouki et al., 1994b;Bluhm et al., 2000), linear quadrupoles (Lawrence et al., 1991), magnetic sector spectrometers (Koizumi et al., 1980; Kemper and Bowers, 1990), time-of-flight (TOF) (Hoaglund et al., 1998; Henderson et al., 1999), and trapping devices (Valentine and Clemmer, 1997)), which dramatically increases the potential for analysis of complex samples (Harvey et al., 2011).

In application to proteins, IM-MS provides information about protein shape by measuring how fast protein ions move through a gas-filled chamber. This determines a rotationally averaged collision cross-section (CCSIM), which reflects the size of a protein and its 3D shape, thereby providing a structural snapshot. The technique is useful for distinguishing between different protein shapes, studying protein dynamics, and providing experimental data on shape and size that can enhance computational protein prediction (Turzo et al., 2022; Christofi and Barran, 2023). Furthermore, it was shown that IM-MS can be used for the analysis of protein conformation dynamics (Yang et al., 2023).

One of the first IM-MS-based analyses of protein structure was investigation of the conformations of cytochrome *c* ions (+8 through +18) in the gas phase by simultaneous ion-mobility and hydrogen–deuterium exchange measurements (Valentine and Clemmer, 1997). In 1998, IM-TOFMS analysis of bradykinin and ubiquitin ions formed by ESI revealed that it is possible to simultaneously record mass-resolved mobilities for all ions present in ESI distributions, thereby providing a means for direct measurement of conformer- and mass-to-charge (m/z)-resolved abundances for the total distribution of ions and generating 3D spectra that contain collision cross section, mass-to-charge, and ion abundance information (Hoaglund et al., 1998). In the late 1990s, research conducted in groups of David E. Clemmer (Clemmer et al., 1995; Clemmer and Jarrold, 1997; Hoaglund et al., 1998; Hoaglund-Hyzer et al., 1999), Michael T. Bowers (von Helden et al., 1995; Wyttenbach et al., 1996), Martin F. Jarrold (Hudgins et al., 1999; Mao et al., 1999; Jarrold, 2000), and David H. Russell (Gillig et al., 2000) demonstrated that IM-MS can be used to analyze the structures of peptides and proteins, as well as to study changes in the collision cross-section (CCS) of a protein (which is related to its size and shape) associated with the changes in its charge state.

Since IDPs have a range of flexible, unfolded conformations, this ability to measure different protein shapes was critical for their subsequent characterization by this technique. Because of the timing of the IM-MS development, this technique did not contribute to the discovery of the protein intrinsic disorder phenomenon and was not used in early studies on IDPs. However, it does represent a powerful tool for the structural characterization of IDPs and for the evaluation of the extent of disorder in proteins (Jurneczko et al., 2012; Beveridge et al., 2013; Beveridge et al., 2014; Beveridge et al., 2015; Stuchfield and Barran, 2018). It was also indicated that the applicability of IM-MS, which provides rotationally averaged CCSs of molecular ions that can be correlated with conformation, to the study of IDPs is further enhanced by the ability of this technique to examine absolute conformation(s), populations of conformation, and conformational changes (Jurneczko et al., 2012).

Several early studies of IDPs by IM-MS are outlined below. In 2004, Bernstein et al. conducted conformational analysis of human α-synuclein by IM-MS and nano-electrospray ionization-mass spectrometry (N-ESI-MS) and revealed the presence of stable compact and extended monomeric structures (Bernstein et al., 2004). IM-MS-based analysis of high mobility group A (HMGA) chromatic architectural factors serves as an important illustration of the power of this approach, which, in combination with limited

proteolysis and electrospray ionization-mass spectrometry (ESI-MS), revealed that this protein can assume a compact form with the degree of compactness being dependent on the presence of the acidic tail and its constitutive phosphorylation (Maurizio et al., 2011). In 2011, Brocca et al. used IM-MS to investigate the conformational properties of an intrinsically disordered cell-cycle regulator from *S. cerevisiae*, Sic1, and its kinase-inhibitor domain, and showed that the isolated Sic1 KID populates collapsed states of different compactness, including a metastable, highly compact species (Brocca et al., 2011). Canon et al. investigated the folding of an intrinsically disordered human salivary proline-rich protein IB5 induced by binding of the epigallocatechin gallate (EgCG) tannin (Canon et al., 2011).

2.13.3 Native mass spectrometry (nMS)

Native mass spectrometry (nMS, which is a form of electrospray ionization mass spectrometry (ESI-MS)), being a relatively recent addition to the analytical toolbox of mass spectrometry, can be used to measure the size, stoichiometry, and interactions of macromolecules (such as proteins) in their native, folded state, thereby directly exploring the different conformational components in terms of geometry and structural compactness (Konijnenberg et al., 2015; Leney and Heck, 2017; Li et al., 2017; Natalello et al., 2017; van Dyck et al., 2017; Keener et al., 2021; Webb, 2022). Development of nMS is rooted in the development of the electrospray ionization (ESI), a soft ionization method permitting the transfer of intact, fragile macromolecules, such as proteins, from a solution into the gas phase for MS analysis. ESI was introduced in 1989 by John B. Fenn as "a powerful technique for producing intact ions *in vacuo* from large and complex species in solution", thereby acting as a crucial means for MS-based analysis of large biomolecules (Fenn et al., 1989). In that study, it was also indicated that this technology allows obtaining mass spectra "for biopolymers including oligonucleotides and proteins, the latter having molecular weights up to 130,000, with as yet no evidence of an upper limit" (Fenn et al., 1989).

In 1990, a group of Brian T. Chait reported ESI-MS results for thirteen proteins with molecular masses ranging from 5000 to 77,000 Da (Chowdhury et al., 1990a), and in 1991, this group demonstrated that ESI-MS represents an effective method for probing conformational changes of proteins in solutions, since the charge state distribution (CSD) observed in an ESI mass spectrum provides insights into the protein's solution-phase conformation (Katta and Chait, 1991). In the same year, the group of Joseph A. Loo also reported that the solvent-induced conformational changes of polypeptides can be probed by electrospray-ionization mass spectrometry (Loo et al., 1991). Following these initial observations, several groups expanded nMS (note that the term "native mass spectrometry" was coined in 2004 by Albert J. R. Heck and coauthors (Van den Heuvel and Heck, 2004)) into a powerful analytical technique for structural biology.

By preserving non-covalent interactions and analyzing intact complexes, nMS is an important analytical technique that provides details on protein folding, protein-protein interactions, protein-ligand binding, post-translational modifications, proteoform characterization, as well as complex heterogeneity, stability, and assembly, composition and identity of subunits, and assembly and dissociation pathways (Heck, 2008; Struwe and Robinson, 2019; Chen et al., 2021; Tao et al., 2021; Tamara et al., 2022). nMS can also be used for evaluation of the solvent accessible surface area (SASA) of a protein in solution by analyzing its charge state distribution (CSD) in the gas phase (Yang et al., 2025). In fact, a correlation between SASA and the average charge state (z_{av}) of a protein derived from its CSD was independently established by Igor Kaltashov (Kaltashov and Mohimen, 2005; Abzalimov et al., 2007), Rita Grandori (Testa et al., 2011), and Carol V. Robinson (Hall et al., 2012). The foundation of CSD analysis is that the spatial arrangement of a protein in solution determines its ionization pattern during the electrospray process and subsequent transfer to the gas phase (Chowdhury et al., 1990b; Kaltashov et al., 2006; Benesch et al., 2007). Deconvolution of MS spectra by Gaussian fitting can identify main conformers present in sample (Dobo and Kaltashov, 2001; Borysik et al., 2004; Frimpong et al., 2010), and the average charge state and the relative abundance can then be retrieved for each conformer (Santambrogio et al., 2022), making possible SASA estimation (Kaltashov and Mohimen, 2005; Abzalimov et al., 2007; Testa et al., 2011; Hall et al., 2012).

The structural studies of proteins by nMS via CSD analysis started in 1990 (Chowdhury et al., 1990b; Katta and Chait, 1991; Loo et al., 1991), and the 1993 description of a high charge state of the intrinsically disordered metal-free apo-metallothionein and lower charge states of the folded metallothionein-metal complexes (Yu et al., 1993) likely represents the first report on application of this technique to the analysis of IDPs. Although similar to IM-MS, because of the timing of its invention and development, nMS was not broadly used in early IDP studies; nonetheless, this technique represents a powerful addition to the modern arsenal of IDP analytic tools. Several recent reviews outlined various applications of nMS in the IDP field (Testa et al., 2013; Natalello et al., 2017; Mitra, 2019; Santambrogio et al., 2019; Santambrogio et al., 2022; Reid et al., 2023; Osterholz et al., 2025).

2.14 Differential scanning calorimetry (DSC)

Being a tool that provides a thermodynamic profile of protein unfolding/denaturation process by measuring the heat absorbed or released by a protein sample as its temperature is changed, differential scanning calorimetry (DSC) is different from the biophysical technique discussed so far, all of which provide information on some structural features of a protein, such as its secondary structure, flexibility, hydrodynamic dimensions, or solvent/protease accessibility. Instead of looking at some peculiar structural features, DSC gives information on the stability of the overall structure or structural elements. In other words, scanning microcalorimetry is a technique that is capable of revealing folded polypeptide chains without showing preference for special structural features, thereby serving as a macroscopic indicator of folding (Privalov, 1979).

The idea of DSC was invented by Emmett S. Watson and Michael J. O'Neill in 1962 to measure the heat flow of solid materials and determine phase transitions, melting, crystallization, and heat capacity (Watson and O'Neill, 1962), whereas in 1964, Peter L. Privalov (1932–2020) and Jamlet (Damlet) R. Monaselidze created the first adiabatic scanning

microcalorimeter to study biopolymers (Privalov et al., 1964). Since globally disordered proteins lack rigid tertiary structure, they do not reveal cooperative endothermic thermal denaturation, which is reflected as a positive peak characteristic for folded proteins (Privalov, 2009). However, IDPs exhibit characteristic values of specific heat capacity C_p, which are elevated at all temperatures due to greater solvent-exposed surface area, and which exceed the C_p values of folded proteins, and in the case of extensively disordered proteins, reaching the values characteristic for fully unfolded protein (Privalov and Makhatadze, 1990). Furthermore, due to the lack of a cooperative transition from a single, stable conformation, IDPs are characterized by the lack of a large unfolding enthalpy ΔH. These features, lack of heat absorption heat, lack of large ΔH, and high specific heat capacity values are considered as the DSC hallmarks of globally disordered proteins (Permyakov, 2012).

Based on the calorimetric analysis of several ribosomal proteins from *E. coli* (S4, S7, S8, S16, S18, and L11) conducted in 1978 by Khechinashvili et al., it was concluded that although S4, S7, S8, S16, and L11 possessed a cooperative tertiary structure in solution as evidenced by their cooperative melting, S18 did not show conformational transitions in the range of 10–100 °C, suggesting that this protein was disordered (Khechinashvili et al., 1978).

Colin Crane-Robinson and Peter L. Privalov revealed in 1983 that intrinsically disordered histones H1 and H5 (defined by these authors as "denatured random coil proteins") can partially fold in the presence of salt, with folding taking place within their N-terminal 80-residue-long fragments that can be derived from both proteins by trypsin digestion in high salt (Crane-Robinson and Privalov, 1983). Analysis of these proteins by DSC revealed that the folded structures are located entirely within these peptides, whereas a thermorgram of the C-terminal domain of H1 contains no peak and shows high C_p values, thereby indicating that this domain is not folded (Crane-Robinson and Privalov, 1983). In 1990, Marie Paulsson and Petr Dejmek analyzed the calorimetric behavior of several whey proteins and pointed out that, contrarily to other whey proteins whose melting produced a distinct thermal peak, pure caseins showed no endotherms within the analyzed temperature range (25–140 °C), suggesting that thermal behavior of caseins is fundamentally different from the unfolding of globular whey proteins (Paulsson and Dejmek, 1990).

In 1996, Uversky et al. conducted the multiparametric analysis of circularly permuted dihydrofolate reductase variants from *E. coli* and revealed that these proteins behave as native molten globules, being compact disordered molecules with high degree of secondary structure but without rigid 3D-structure as evidenced by the lack of heat absorption peak in their DSC thermograms and the high C_p values. However, after addition of ligands, these permutants gained specific activity and the native-like structural properties, including a large change in their melting behavior, such as lower C_p values at low temperatures and the presence of a distinct heat absorption peak (Uversky et al., 1996). Based on these observations it was concluded that folding of permuted dihydrofolate reductase variants was terminated at the stage of the molten globule formation, whereas their interaction with ligands promoted structural adjustments and formation of active protein molecules, indicating ligand-induced transition from the molten globule to the native state (Uversky et al., 1996). In 1997, our comparative analysis of ligand-containing and ligand-free forms of human α-fetoproteins by several techniques including DSC revealed the release of ligands converted this protein into the molten globular form, which was evidenced by the absence of any visible heat absorption in the DSC thermograms and high C_p values (Uversky et al., 1997).

In 2003, Soulages et al. reported that a group 2 late embryogenesis abundant (LEA) protein from soybean does not display a cooperative unfolding transition upon heating in DSC experiments (Soulages et al., 2003). DSC-based analysis of the C-terminal domain of chicken gizzard caldesmon CaD, (CaD_{136}, residues 636–771) and series of its single tryptophan mutants (W674A, W707A, and W737A) and a double tryptophan mutant (W674A/W707A) revealed that all these proteins are characterized by high specific heat capacity values (ranging from ~2–3 J/(g K)) and the absence of distinct heat absorption peaks within the temperature region from 10 to 100 °C suggested that their structure is predominantly unfolded (Permyakov et al., 2015).

2.15 High-speed atomic force microscopy (AFM)

Atomic force microscopy (AFM) is a very-high-resolution imaging technique (in some instances, it can have a resolution on the order of fractions of a nanometer) based on using a mechanical probe consisting of a cantilever and a sharp tip, which can be modified in many ways to investigate surface properties, to gather information by "sensing", "feeling", or "touching" the surface of a sample. AFM was invented in 1985 by IBM scientists Gerd Binnig, Calvin Forrest Quate (1923–2019), and Christoph Gerber, who reported a development of a new type of microscope, which, being a combination of the principles of the scanning tunneling microscope [STM that was invented in 1981 by Gerd Binnig, Heinrich Rohrer (1933–2013), Christoph Gerber, and Edi Weibel (Binnig et al., 1982)] and the stylus profilometer, achieved a lateral resolution of 30 Å and a vertical resolution less than 1 Å (Binnig et al., 1986). The major difference between STM and AFM is in the basic principles of their operation, where STM measures a tunneling current between a conductive tip and sample (thus being limited to the analysis of conductive and semiconducting samples), whereas AFM measures forces, such as van der Waals forces, between a tip and the surface and can be used for analysis of any material, conductive or not. Since AFM can visualize and manipulate individual molecules, study their properties, and measure their interactions, it is considered as a single-molecule technique.

Biomedical applications of AFM, which can operate in ambient air, high vacuum, and liquid environments, are centered at topographic imaging [e.g., it can resolve DNA double helix (Mou et al., 1995) or probe structure and dynamics of nucleosomes (Stumme-Diers et al., 2019), or visualize and provide topological information about amyloid fibrils (Adamcik and Mezzenga, 2018; Aubrey et al., 2020; Lutter et al., 2020; Lutter et al., 2022), or show the surface structure of purple membranes (Worcester et al., 1988) and the extracellular surface of the gap junction (Hoh et al., 1991; Hoh et al., 1993), or generate molecular-resolution images of individual proteins (Hansma et al., 1991; Arakawa et al., 1992)], force measurement [e.g., it can detect interaction between histones and nucleosomes (Stormberg et al., 2021; Rafa et al., 2024)], and manipulation [e.g., can be used to orient protein aggregates (Lea

et al., 1992)] (Xia and Youcef-Toumi, 2022). Introduction of the amplitude modulation (AM) mode (also known as tapping mode) in 1993, where the cantilever is oscillated in the Z-direction at (or near) resonant frequency, and this oscillation amplitude is measured to detect the tip-sample interaction, facilitated the observation of biomolecular processes allowing analysis of molecules weakly attached to a substrate surface (Zhong et al., 1993).

Current literature on the utilization of AFM in protein-related studies is vast (with almost 18,000 publications in PubMed mentioning atomic force microscopy and protein) and coverage of even a small fraction of this set of papers is outside the scope of this review. Although almost from the time of its inception, AFM was successfully used in structural analysis of ordered proteins, utilization of this tool for structural and dynamics characterization of IDPs was originally limited due to the fact that IDPs do not have stable 3D structure but dynamically sample a multitude of conformational states. Since it takes at least 30 s to capture an image using the AM mode, the AFM performance of was obviously too slow to capture dynamic biomolecular processes or provide reliable images of moving objects.

The situation changed due to the invention of small cantilevers with high resonant frequencies and small spring constants, as well as an optical beam deflection (OBD) detector for small cantilevers independently by the groups of (Viani et al., 1999) and Toshio Ando (Ando et al., 2001). The resulting AFM "can capture a 100×100 pixel^2 image within 80 ms and can therefore generate a movie consisting of many successive images (80-ms intervals) of a sample in aqueous solution", a dramatic acceleration in comparison with the original AFM (Ando et al., 2001). It took a few more years and several additional inventions to establish high-speed AFM (HS-AFM) suitable for nano-visualization of dynamic biomolecular processes (Ando et al., 2008). Modern HS-AFM can image protein molecules at 10–20 frames per second. Among impressive illustrations of the capability of HS-AFM "to visualize the dynamic behavior of structured proteins during their functional activity at 2–3 nm lateral and ~0.1 nm vertical spatial resolution and at ~100 ms temporal resolution under near-physiological conditions" (Ando, 2022) are a video of myosin V walking along actin filaments (Kodera et al., 2010), representation of the rotary catalysis of a rotorless adenosine triphosphate (ATP)–driven motor F_1 (Uchihashi et al., 2011), and visualization of the dynamic structural states of ClpB involved in its disaggregation function (Uchihashi et al., 2018). More examples of HS-AFM imaging studies on proteins and cells can be found in a comprehensive review by Toshio Ando, Takayuki Uchihashi, and Simon Scheuring (Ando et al., 2014).

Although HS-AFM was invented after the IDP field was established, this technique provides unique structural and dynamic information at the single molecule level, generating actual movies showing how IDP moves and directly observing individual protein molecules in action at high spatiotemporal resolution (Ando et al., 2013). Some of the illustrative examples of information generated by HS-AFM for IDPs are outlined below. HS-AFM captured images of heterodimeric FACT (facilitates chromatin transcription) protein at rates of 5–17 frames per second and clearly revealed two distinct wiggling tail-like segments that protrude from the main body of FACT, have different contour lengths (17.5 and 27.5 nm on average), fluctuate in position, and have similar macroscopic flexibility, being more structurally relaxed than random coils (Miyagi et al., 2008).

A subsequent study revealed that a small globule temporally appears but quickly vanishes within each mobile tail-like image of FACT, corresponding to the IDR containing high-mobility-group domain, with the lifespan of the globule increasing upon phosphorylation (Hashimoto et al., 2013). The intrinsically disordered interdomain region between the helicase and nuclease domains of an archaeal Hef protein from *Thermococcus kodakarensis* was shown to be involved in interactions with multiple proteins including PCNA1 and a RecJ-like protein, suggesting that Hef acts as a scaffold and uses this IDR for sequential interaction with several proteins to control the repair pathway at the stalled fork (Ishino et al., 2014).

The flagellar hook-length control protein FliK from *Salmonella enterica* serovar Typhimurium was shown by HS-AFM to adopt a shape of two balls linked by a flexible string (Kodera et al., 2015). In 2016, Sakiyama et al. analyzed by HS-AFM structural dynamics of intrinsically disordered, barrier-forming phenylalanine-glycine nucleoporins (FG Nups) within the nuclear pore complexes (NPCs) from the *Xenopus laevis* oocyte and showed that FG Nups are highly flexible and dynamically fluctuating chains that rapidly elongate and retract, consistent with the diffusive motion of tethered polypeptide chains (Sakiyama et al., 2016). Finally, it was emphasized that "successive HS-AFM images of an IDP molecule can not only identify constantly folded and constantly disordered regions in the molecule, but can also document disorder-to-order transitions. Moreover, the number of amino acids contained in these disordered regions can be roughly estimated, enabling a semiquantitative, realistic description of the dynamic structure of IDPs" (Kodera et al., 2021)

Finally, an example of successful application of AFM for the characterization of IDP in the "pre-IDP" period is given by the 1997 report of H G. Brown and Jan H. Hoh, who showed that the neurofilament sidearms form an entropic brush required for maintaining the interfilament spacing (Brown and Hoh, 1997). In 2001, Li et al. conducted conformational analysis of the human cardiac titin PEVK region (186-residue-long) and showed that conformational ensemble populated by this protein is characterized by a wide range of elastic conformations with end-to-end distances ranging from 9 to 24 nm and persistence lengths from 0.4 to 2.5 nm (Li H. et al., 2001).

3 A multiparametric approach in structural biology: the power of integration

Due to their conformational flexibility and lack of stable 3D structures, IDPs/IDRs present a unique challenge for detailed structural analysis. They are characterized by extreme spatiotemporal heterogeneity, as instead of possessing a single unique structure, they exist as dynamic, interconverting ensembles of multiple conformations. This presents an interesting conundrum: individual ordered proteins possess unique, specific 3D-structures, yet their folding and final shapes are governed by a limited set of universal physicochemical principles, allowing for a seemingly infinite variety of protein structures to be classified and systematically studied. On the contrary, individual IDPs/IDRs are disordered differently, and their different degrees of disorder make

each IDP/IDR unique. In other words, one can rephrase the famous opening line of the "Anna Karenina" novel by Leo Tolstoy to read: "All ordered proteins are alike; each intrinsically disordered protein is disordered in its own way". In other words, this defines the "Anna Karenina" principle for ordered proteins, which must satisfy a uniform set of conditions to be "successful" and functional, whereas IDPs, being "disordered in their own way," use their unique dynamic ensembles for function.

Furthermore, structural analysis of IDPs/IDRs that rapidly fluctuate between a wide range of conformations in a dynamic ensemble, rather than settling into a single, rigid structure cannot be conducted by X-ray crystallography, which, for an ordered protein, produces a single time- and space-averaged model. This task requires a combination of specialized techniques developed for analysis of specific structural characteristics. In other words, integrative structural analysis, which combines multiple experimental and computational approaches, is the most powerful strategy for accurately characterizing the dynamic and fluctuating landscape of IDPs/IDRs. Since each method typically provides unique information about a specific properties of a protein over different timescales and length scales, the use of such multiparametric approach allows avoiding "five blind men and elephant" situation (this analogy is based on the ancient parable of the blind men and an elephant, where each person feels a different part of the animal and comes to a completely different conclusion about what an elephant is) (Uversky, 2015a).

Typically, a single technique provides specific and limited information about a particular "piece of the elephant" (e.g., one technique might describe the average overall shape, while another gives data on the local motion of a specific segment). Therefore, the use of a multiparametric approach, known as integrative structural biology, is essential for piecing together a more complete picture of an IDP. Figure 3 illustrates this idea by showing how a combination of various tools creates a possibility to see the "whole elephant" by integrating the different structural details of IDPs, such as shape, packing, secondary structure, flexibility, hydrodynamic properties, solvent accessibility, conformational heterogeneity, and self-assembly.

One should also keep in mind that the use of various tools for the analysis of each of the individual structural features of an IDP opens additional ways of getting more reliable information about this "fuzzy elephant". This is because different tools are not only sensitive to different structural characteristics of the protein but also have different averaging properties. For example, some techniques (which besides NMR and MD simulations include such powerful time-averaging techniques as HDX-MS, DLS, SLS, viscometry, SAXS, SANS, FRET, and FCS), capture structural information that is averaged over time. Other methods capture a snapshot of the entire population of molecules at a single point in time, revealing the distribution of different conformations. These ensemble-averaging techniques include NMR, CD, FTIR, Raman spectroscopy, ROA, AUC, smFRET, fluorescence anisotropy, REES, SAXS, SANS, HDX-MS, native-MS, and ion mobility-MS. Note that although HDX-MS is included among the time-averaging techniques, it can also be implemented by pulse labeling in a time-resolved fashion. Also, while HDX-MS, native-MS, and ion mobility-MS are listed as ensemble-averaging techniques, their actual strength is to sort ions and measure properties of each singly detected ion.

It is important to remember that the image generated for an IDP by integration of data generated by multiple complementary experimental techniques is admittedly fuzzy. This is not surprising taking into account the highly flexible nature of IDPs/IDRs, which are best described as a "protein clouds", and the fact that the utilized ensemble low-resolution techniques measure signals representing an average of all the states present in the conformational ensemble, and thereby conceal information about individual structures and their specific features. However, this fuzziness is not an experimental error but is instead a true reflection of the dynamic, ensemble-based structure of the protein. The information concealed by the averaging technique(s) are the specific snapshots of single transient conformations, which would exist for only a fraction of a second. Furthermore, there are far more possible conformations for an IDP than there are experimentally determined restraints, making the problem of building a complete ensemble mathematically "underdetermined".

4 Looking at the "fuzzy herd of fuzzy elephants": low-resolution techniques for analysis of membraneless organelles

The modern field of IDPs is dominated by the analysis of their roles in liquid-liquid phase separation (LLPS) and biogenesis of biomolecular condensates or membraneless organelles (MLOs) (Uversky et al., 2015; Mompean and Laurents, 2017; Uversky, 2017a; Uversky, 2017c; Cuevas-Velazquez and Dinneny, 2018; Darling et al., 2018; Drino and Schaefer, 2018; Owen and Shewmaker, 2019; Pancsa et al., 2019; Turoverov et al., 2019; Uversky, 2019;Martin and Holehouse, 2020; Murthy and Fawzi, 2020; Borcherds et al., 2021; Uversky, 2021; Antifeeva et al., 2022; Darling and Uversky, 2023). This obviously raises a question on the applicability of low-resolution techniques for the analysis of these phenomena. Since the study of LLPS and MLOs/BCs began after the IDP concept was generally accepted, a thorough consideration of this very important point is outside the scopes of this historical perspective. However, a very brief discussion of some of the low-resolution techniques used in LLPS/MLO analyses is provided below.

Being typically in the micrometer to sub-micrometer size range, MLOs are definitely microscopic objects. However, they are larger than a single protein by several orders of magnitude. MLOs/BCs are stabilized by weak, transient, multivalent, "touch-and-go" interactions (of palpation type) between IDPs and other biomolecules. In other words, instead of forming a single rigid complex, MLO formation involves multiple, individually weak interaction sites that are constantly associating and dissociating. This creates a highly adaptable and context-dependent molecular assembly, which is formed, dissolved, and reorganized through LLPS, is characterized by a fluid-like nature with specific solvent properties, and has the collective behavior, such as response to cellular stimuli. Therefore, MLOs represent a kind of enormous, "fuzzy herd of fuzzy elephants running within a colossal dust cloud". Such metaphorical view of MLOs clearly emphasizes the complexity of their analysis, where one needs to look at the shape and behavior of the whole herd and study its response to various stimuli, and on another side should have a possibility to identify individual elephants, follow their behavior, interactions, and responses, and also look at all this within the cloud of dust. Therefore, study such systems requires principally different

techniques to be able to observe both the "forest" (the entire MLO or herd of elephants) and the "trees" (the individual elephants/molecules) at the same time.

Clearly, not all the low-resolution techniques considered in this study are suitable for such analyses. However, some ensemble techniques, such as CD (Cinar et al., 2018; Chen and Huang, 2020; Amiri et al., 2025), FTIR (Cinar et al., 2018; Edun et al., 2020; Amiri et al., 2025), and Raman spectroscopy (Murthy et al., 2019; Agarwal et al., 2021; Avni et al., 2022), as well as analytical ultracentrifugation (AUC) (Mitrea et al., 2018), SAXS (Mitrea et al., 2018; Martin et al., 2021; Koning et al., 2025), SANS (Koning et al., 2025), and dynamic light scattering (DLS) (Hochmair et al., 2023; Amiri et al., 2025) were successfully used to study various structural features of IDPs undergoing LLPS.

Single-molecule fluorescence-based techniques are well suited for conducting such kind of studies (Nasir et al., 2019). For example, using single molecule fluorescence resonance energy transfer (smFRET) Joshi et al. dissected critical molecular events associated with phase separation of an intrinsically disordered prion-like low-complexity domain of Fused in Sarcoma (FUS), revealed the coexistence of two conformationally distinct subpopulations in the monomeric forms, and described conformational shapeshifting events associated with phase separation of this protein (Joshi et al., 2023). (Joshi et al., 2023) Wen et al. used smFRET in combination with molecular dynamics simulations to demonstrate that the microtubule-associated protein tau undergoes conformational transitions from compact to extended states during LLPS (Wen et al., 2025).

It was pointed out that FCS, being a single-molecule fluorescence technique, can be used in the analysis of physical (e.g., diffusional coefficient, hydrodynamic radius) and chemical (e.g., concentration, binding affinity) properties of fluorescent or fluorescently labeled molecules (Wang et al., 2022). Currently, FCS is increasingly adopted to study LLPS and MLOs. For example, in 2020, Peng et al. showed that dual-color fluorescence cross-correlation spectroscopy (dcFCCS) can capture formation of nanoscale condensates beyond the detection limit of conventional fluorescence microscopy (Peng et al., 2020), whereas Yamamoto et al. revealed that polarization-dependent fluorescence correlation spectroscopy (Pol-FCS) can be used for evaluation of macromolecular crowding and simultaneously measure the relaxation times of rotational and translational diffusion of fluorescent molecules at the same position, even in living cells (Yamamoto et al., 2021). Recently, Wang et al. provided a comprehensive review summarizing FCS applications in the LLPS studies in live cells and in aqueous solutions (Wang et al., 2022). These authors also emphasized that the FCS-based techniques, such as fluorescence auto-correlation spectroscopy (FACS) and fluorescence cross-correlation spectroscopy (FCCS), being combined, can be used in quantitative analysis of molar concentration, diffusion coefficient and hydrodynamic radius, homo- or hetero-interaction, dissociation constant, and fluorescence brightness (Wang et al., 2022).

In conclusion, it is important to emphasize that almost none of the aforementioned techniques can be used for studying IDPs in crowded, condensed phases without at least some adaptations. Furthermore, methods to study MLOs must be carefully selected and amended based on the stage of the MLO life. This highlights the need for specialized and integrative approaches in biophysical research to address the complexities of these dynamic biological structures. Obviously, all these and related subjects deserve more focused analysis and should be considered in a dedicated review.

5 Concluding remarks: a blurry view of fuzzy objects

To sum up, the observations presented here clearly show that low-resolution techniques were crucial for assembly of information leading to the establishment of the field of protein intrinsic disorder. In fact, these tools helped with the accumulation of the "critical mass" of data to convincingly show that structure-less biologically active proteins are rather common, emphasizing that they are not the mere flukes or mistakes of nature, but are crucial constituents of the protein universe. They also gave researchers a possibility to see these fuzzy objects. Note that the IDPs/IDRs are fuzzy by default, as they exist as highly dynamic conformational ensembles; i.e., highly dynamic sets of many rapidly interconverting, structurally distinct conformations, and this structural fuzziness (inherent structural heterogeneity), is often not a limitation but a fundamental aspect of their functions. Admittedly, the view created by these low-resolution techniques is blurry and does not come even close to the sharp static images generated for structures of ordered proteins by high-resolution techniques. However, even a blurry view of an IDP/IDR provides an undeniably real depiction of these important proteins/regions. This ability to visualize a physical entity, even imperfectly, is a significant advance over the previous "invisibility" of unresolved structures in electron density maps: a fuzzy image of an IDP/IDR is still more informative than having no structural data at all.

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