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Recent Developments in the Field of Intrinsically Disordered Proteins: Intrinsic Disorder–Based Emergence in Cellular Biology in Light of the Physiological and Pathological Liquid–Liquid Phase Transitions

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intrinsically disordered protein, intrinsically disordered protein region, liquid–liquid phase transition, liquid–liquid phase separation, proteinaceous membrane-less organelle

Abstract

This review deals with two important concepts—protein intrinsic disorder and proteinaceous membrane-less organelles (PMLOs). The past 20 years have seen an upsurge of scientific interest in these phenomena. However, neither are new discoveries made in this century, but instead are timely reincarnations of old ideas that were mostly ignored by the scientific community for a long time. Merging these concepts in the form of the intrinsic disorder–based biological liquid–liquid phase separation provides a basis for understanding the molecular mechanisms of PMLO biogenesis.

Contents

INTRODUCTION: SOME OLD BUT CONCEPTUALLY RELEVANT NEWS ABOUT INTRINSICALLY DISORDERED PROTEINS

Intrinsically disordered proteins (IDPs; i.e., functional proteins without unique 3D structures), or hybrid proteins with ordered domains and functional IDP regions (IDPRs), play important roles in various emergent events taking place in a living cell (28, 122, 137). In fact, some act as major facilitators of biological liquid–liquid phase separation (LLPS) [or liquid–liquid phase transitions (LLPTs), in which two solutions, due to their immiscibility, separate into two phases] that serves as an important molecular mechanism of the biogenesis of various proteinaceous membrane-less organelles (PMLOs). Because of the importance of disorder-based LLPS for PMLO biogenesis, I introduce some of the major features of IDPs and IDPRs, despite the coverage of the various aspects of intrinsic disorder in multiple dedicated reviews. Therefore, in the sections below, some of the

most important disorder-related concepts are briefly reintroduced, starting with the discovery of the common existence of these proteins, followed by a consideration of their (multi)functionality, interactability, multilevel spatiotemporal heterogeneity, and engagement in the pathogenesis of numerous human maladies.

They Do Exist

Despite early protein researchers' focus on the biological catalysts and enzymes that confirmed a long-standing belief that the unique 3D structure of a protein determines its specific functionality, the concept of protein intrinsic disorder is currently widely accepted. The fact that the corresponding branch of protein science is currently blooming is reflected in the results of a simple bibliometric analysis, where the Web of Science tools of Clarivate Analytics were used to look at the papers dealing with disorder-related topics, such as (intrinsically disordered protein) OR (natively unfolded) OR (intrinsically unstructured) OR (natively unstructured). This analysis revealed that, as of June 16, 2020, there were 7,274 such papers published in 1,133 journals by 19,485 authors from 2,856 organizations in 90 countries or territories (see **Figure 1*a***). Furthermore, for the past three years, more than two papers on these subjects have been published daily. **Figure 1*a*** also shows that the turn of the century represents a point of no return that marks the beginning of an exponential growth in the number of intrinsic disorder–related publications. Furthermore, while **Figure 1*a*** shows the most recent state of the field, scientists described biologically active proteins without unique structures as early as the 1930s (e.g., 73). What, then, made 2000 a point of no return? The answer to this question can be found in the need to overcome a famous lock-and-key model-based sequence-structure-function paradigm dominating in structural biology, which basically stated that for a protein to be functional, a unique 3D structure is needed. In light of this model, the likelihood of a structure-less protein having a defined biological function was considered, if not impossible, then at the very least highly improbable. Although such proteins were periodically described in scientific literature, for a very long time, each of them was considered to be a rare exception from the general rule that protein function relies on a rigid 3D structure. It seems that, by 2000, a critical mass of exceptions was reached, and as a result, three research groups independently and almost simultaneously developed an important hypothesis that the functional structure-less proteins (which are currently known as IDPs) are not unique and rare exceptions but, in fact, constitute a new realm within the protein universe (35, 138, 151). The time was right, grains fell into fertile soil, and the ideas that IDPs and hybrid proteins containing ordered domains and IDPRs do exist and should not be ignored while describing physiological functions and pathological dysfunctions of proteins rapidly became accepted by the scientific community and eventually revolutionized the protein science field.

They Are Common

One of the important factors that contributed to the acceptance of the protein intrinsic disorder phenomenon was a compelling demonstration of the natural abundance of IDPs and IDPRs. In fact, it is now accepted that IDPs and IDPRs are not mere exceptions, but instead are universally present in all living organisms. Although different computational studies produced a rather broad range of estimates of the prevalence of intrinsic disorder in various proteomes, it is clear that the abundance and penetrance of disorder typically increase with an increase in organism complexity (36, 97, 146, 154). In fact, 25–30% of eukaryotic proteins are predicted to be mostly disordered, more than half of eukaryotic proteins have long regions of disorder (36), and more than 70% of signaling proteins have long disordered regions (67).

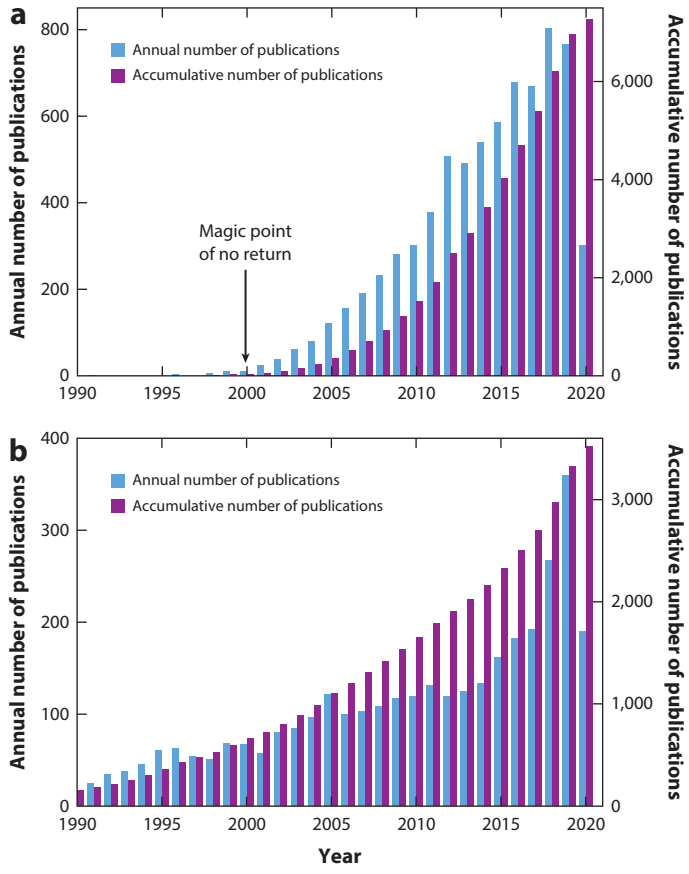

Figure 1

Time course of the development of the interest of researchers in IDP-, IDPR-, LLPS-, and PMLO-related subjects. (*a*) Web of Science data related to publications dedicated to IDPs and IDPRs. (*b*) Web of Science data related to publications dedicated to LLPS and PMLOs. In both plots, blue and purple bars show the annual and accumulative numbers of publications, respectively. Abbreviations: IDP, intrinsically disordered protein; IDPR, intrinsically disordered protein region; LLPS, liquid–liquid phase separation; PMLO proteinaceous membrane-less organelle.

They Have Specific Functions

The increased amount of disorder in eukaryotes is attributed to the presence of specific disorder-dependent functions. What, then, can IDPs and IDPRs do that cannot be done by ordered proteins and domains? Ordered proteins, with their unique 3D structures, are most frequently involved in catalysis and transport, acting within the frames of the lock-and-key or induced fit models, since catalysis and specific ligand binding are facilitated by the precise locations of certain amino acid side chains within the active or binding sites. In contrast, IDPs and IDPRs, being often multifunctional promiscuous binders, are commonly involved in regulation and control of various signaling processes (33–35, 55, 127, 138, 143). Functionally, IDPs and IDPRs are grouped into several broad classes, such as molecular recognition, molecular assembly, protein modification, entropic chain activities, and RNA and protein chaperones (34, 116). Besides providing multiple functional advantages over the ordered proteins, the structural floppiness of IDPs and IDPRs also defines the

multitude of multilevel means of their functional regulation and control (55, 125, 129, 143), e.g., various post-translational modifications (PTMs) (68, 96).

Interaction Specialists: They Do Just What They Like

The majority of disorder-related functions pertain to the ability of IDPs and IDPRs to engage in interactions with other proteins, lipids, membranes, nucleic acids, polysaccharides, and small molecules of organic and inorganic origin and to form a multitude of static complexes, which are typically characterized by very unusual and complex topologies ranging from the interaction-induced local folding generating structural elements bound to the surface of a partner to folding of a whole IDP, and from wrapping around the binding partner to penetrating deep inside the binding partner; IDPs and IDPRs also form semistatic or dynamic complexes (125). Often, IDPs and IDPRs either form fuzzy complexes, with the flanking fuzziness in the bound state disordered regions flanking the interaction interface but not the interface itself, or remain disordered or characterized by the random fuzziness attributed to the ability to resist folding at interaction and preserve highly dynamic structure in the bound state (50–52, 54, 86, 106, 117). Some IDPs and IDPRs are able to bind to multiple partners and to gain very different structures in the bound state, and this adjustable promiscuity represents an important method to increase the complexity of the disorder-based interactomes (6, 92). Disorder allows formation of the most and least stable protein complexes. Importantly, although specific disorder-based interactions often engage binding-induced folding, formation of stable complexes does not always require folding, and IDPs and IDPRs were shown to form tight complexes (with the affinity approaching picomolar levels) without gaining any ordered structure and retaining long-range flexibility and highly dynamic character (15). Finally, IDPs and IDPRs are capable of weak multivalent interactions, which are crucial for biological phase separation and formation of membrane-less organelles (see below) (130, 132, 133).

Spatiotemporal Heterogeneity of Intrinsically Disordered Proteins and the Structure–Function Continuum

The inability of IDPs and IDPRs to form unique 3D structures under physiological conditions is determined by the peculiarities of their amino acid sequences, such as compositional biases (depletion in order-promoting residues Trp, Tyr, Phe, Ile, Leu, Val, Cys, and Asn and enrichment in disorder-promoting residues Ala, Arg, Gly, Gln, Ser, Glu, Lys, and Pro), high net charge, low overall hydrophobicity, low sequence complexity, and presence of repeats (148). Importantly, such sequence peculiarities are unequally distributed within the protein molecule. This determines the multilevel spatiotemporal heterogeneity of such proteins and defines their mosaic structure, where different parts of a protein can be (dis)ordered to different degrees. As a result, the entire IDP or IDPR can be described as a combination of foldons (independent foldable units of a protein), inducible foldons (disordered regions that can fold at least in part due to the interaction with binding partners), nonfoldons (nonfoldable protein regions), semifoldons (regions that are always in a semifolded form), and unfoldons (ordered regions that have to undergo an order-to-disorder transition to render a protein functional) (128, 129). Importantly, these differently (dis)ordered pieces of the protein structural mosaic might have well-defined and specific functions (130). Therefore, such a complex structural mosaic serves as a basis of the heterogeneous and complex anatomy of proteins that defines the complex molecular physiology reflected in their multifunctionality and ability to become involved in interaction with multiple structurally unrelated partners. In other words, IDPs and IDPRs are structurally and functionally heterogeneous

complex systems that form the core of the protein structure–function continuum (130, 131). Disorder is also an important contributor to the proteoform concept, according to which a single gene can efficiently encode for a set of distinct protein molecules (109). Such structural and functional diversification is reached by several means that affect the chemical structure of proteinaceous product(s) of a given gene, such as allelic variations (i.e., single or multiple point mutations, indels, single-nucleotide polymorphisms) at the DNA level, alternative splicing, and other pretranslational mechanisms affecting messenger RNA (mRNA), complemented by a wide spectrum of PTMs of a polypeptide chain, as well as by the presence of intrinsic disorder and structural alterations induced by functioning (131). Therefore, the existence of various proteoforms contributes to the protein structure–function continuum, which indicates that the actual gene–protein relationship is better described by a model in which one gene corresponds to many functional proteins or many functions, rather than the classical (but heavily oversimplified) paradigm in which one gene corresponds to one protein (130, 131).

They Are at the Edge

One might ask what defines the ability of these structure-less and highly dynamic protein clouds to serve as multifunctional, tunable, and extrasensitive sensors, controllers, and regulators. Since IDPs and IDPRs operate in a region between order and complete randomness or chaos, they were classified as complex biological systems positioned at the edge of chaos, where the complexity is maximal (129). In fact, all the features ascribed to complex or edge-of-chaos systems (13) can be identified in IDPs and IDPRs (129). For example, these proteins are characterized by an amusing spatiotemporal heterogeneity and nested structural organization spanning several scales. Structural elements of IDPs are themselves interdependent complex systems that interact nonlinearly and can feel and respond to various neighboring elements. The behavior of a given IDP is complex and represents an interplay between chaos (disorder) and order. It cannot be expressed as a sum of the behaviors of its parts, and small perturbations may cause a large effect, a proportional effect, or no effect at all. These proteins might contain both positive (amplifying) and negative (damping) feedbacks, indicating that there is an interplay between cooperation and competition. Because of their dynamic nature, IDPs and IDPRs change over time, and their prior states may have an influence on present states, indicating the existence of some kind of memory. Finally, IDPs and IDPRs are capable of emergent behavior and self-organization resulting in the unanticipated appearance of novel structures, patterns, and properties (129). Furthermore, the functional properties of IDPs can be additionally tuned by alternative splicing, interaction with numerous binding partners of different physicochemical nature, mutations, and different PTMs.

Protein Intrinsic Disorder in Human Diseases and Drugability of Protein Clouds

Because IDPs and IDPRs are intimately involved in the regulation of numerous biological processes, their misbehavior and dysfunction are interlinked with the pathogenesis of various human diseases. The prevalence of intrinsic disorder in proteins associated with a variety of maladies ranging from amyloidosis to cancer, cardiovascular disease, diabetes, and neurodegeneration gave rise to the D^2 (disorder in disorders) model (135, 140) and associated disease-related unfoldome and unfoldomics concepts (141). Disorder is also common in many viruses, which are characterized by the widest spread of disorder in comparison with the proteomes of other kingdoms of life (97, 154). Systematic computational studies revealed that functionally important IDPRs are abundantly present in various viruses that utilize disorder to highjack, regulate, control, and exploit

various host pathways and to evade the host immune system (153). In its turn, the host utilizes intrinsic disorder in proteins associated with innate antiviral immunity (155).

The broad penetrance of intrinsic disorder in proteins associated with human diseases defines the attractiveness of IDPs and IDPRs as potential drug targets. There are several approaches that use intrinsic disorder in drug discovery (22, 37, 70, 84, 121, 126, 136). One of these approaches utilizes the ability of the drug molecules to mimic a critical foldable region of the disordered partner that undergoes a binding-induced disorder-to-order transition and compete with this foldable IDPR for its binding site on the structured partner (22, 71). Another approach uses IDPRs as targets of small molecules capable of inducing local misfolding in the IDPR via stabilizing an ordered structure incapable of protein–protein interaction (37, 84, 121). Still another approach utilizes the capability of small molecules to stabilize different members of the functionally misfolded ensemble [i.e., a conformational ensemble, where preformed potential binding elements are involved in a set of non-native intramolecular interactions, thereby becoming sequestered inside the noninteractive or less interactive cage, which prevents such potential elements from engaging in unnecessary and unwanted interactions with non-native binding partners (124)], and therefore prevents the targeted protein from establishing biological interactions (126). Finally, small molecules may target and stabilize some specific members of the disordered conformational ensemble, and such small molecules can be found using an in silico structure-based computational docking screen (in this case, metastable structures are first extracted from the conformational ensemble of a target IDP and then used in a virtual screening to identify potential ligands) (120). However, despite the fact that IDPs and IDPRs are involved in pathogenesis of numerous human diseases and are highly abundant in eukaryotes, and despite the existence of several drug discovery strategies to target disorder-based interactions, a comprehensive computational analysis of the current druggable human proteome revealed that there exists a significant bias toward ordered proteins, leading to a low abundance of drugs targeting IDPs (64). **Figure 2** provides an illustration of some of the difficulties associated with utilization of IDPs and IDPRs as potential drug targets, showing the results of a simulation of interactions between the typical IDP, c-Myc$_{370–409}$, and its 10074-A4

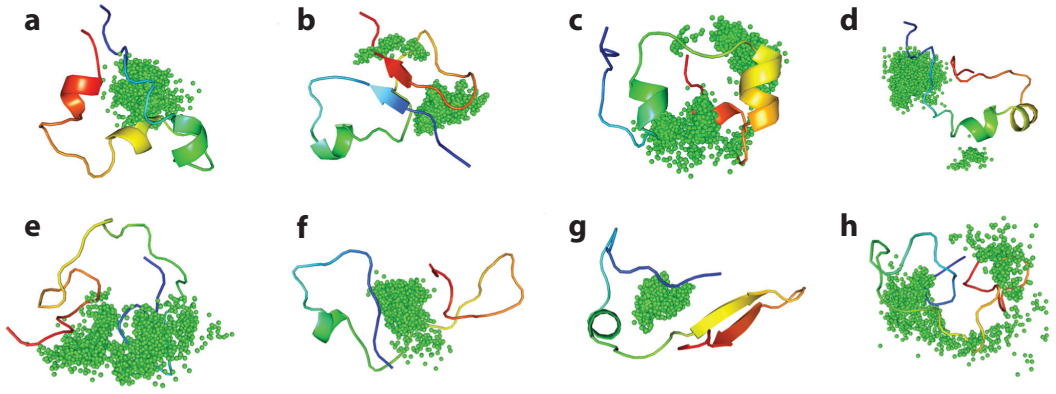

Figure 2

Illustration of the ligand cloud concept. Holoconformations of a c-Myc$_{370–409}$ fragment from the explicit solvent simulations were clustered using the backbone-RMSD clustering with a cutoff of 2.0 Å, and the representative c-Myc$_{370–409}$ structures (from *blue* at the N terminal to *red* at the C terminal) for the first eight clustering groups were displayed in the schematic, whereas the structures of 10074-A4 ligand from each group were depicted as green dots at the centers of mass. The fractional cluster populations are (*a*) 14.3%, (*b*) 13.9%, (*c*) 13.7%, (*d*) 10.4%, (*e*) 7.5%, (*f*) 6.9%, (*g*) 5.4%, and (*h*) 5.2%. Figure reproduced with permission from Reference 69. Abbreviation: RMSD, root mean square deviation.

ligand (69). The structure of c-Myc represents a conformational ensemble or protein cloud that can be clustered in several structurally different groups. Importantly, the diverse members of this ensemble are all capable of binding the ligand, and for a given c-Myc structure, the binding of ligand occurs at a broad set of sites (see **Figure 2**). Therefore, this IDP–ligand binding scenario can be described as a ligand cloud around the protein cloud (69). Such a binding mode is principally different from the traditional binding of small molecules to ordered proteins, where a dominant binding structure is formed (69). Obviously, dealing with such cloud-around-cloud or cloud-in-cloud binding scenarios is a challenging task, which might explain the rarity of drugs targeting IDPs and IDPRs.

INTRINSIC DISORDER–BASED EMERGENCE IN CELLULAR BIOLOGY: PHYSIOLOGICAL AND PATHOLOGICAL LIQUID–LIQUID PHASE TRANSITIONS

Intrinsic Disorder and Emergence in Biological Systems

As mentioned above, IDPs and IDPRs represent edge-of-chaos systems and show emergent behavior, which relies on complex self-organization processes and results in the formation of unforeseen novel structures, patterns, and properties (28, 122, 129). Among the illustrative examples of disorder-based emergent behavior are the spatiotemporal oscillations of the Min protein system (MinD, MinC, and MinE), which determines the division plane of the rod-shaped *Escherichia coli* cells via spatial regulation of the positioning of the cytokinetic Z ring (80, 81) by oscillating from pole to pole with an intrinsic wavelength comparable to the size of the *E. coli* cell (100). Such oscillating behavior of the Min system can be reproduced on the supported bacterial and artificial membranes in vitro (63, 72, 83), where mesoscale patterns of traveling protein surface waves can be found (78, 79). Functionally important oscillatory behavior related to the spatial regulation of the development, cell division, and segregation of chromosomes and plasmids was also described for several members of the WAKA protein family (Walker A cytomotive ATPase; also knows as ParA) (10, 38, 39, 119). Some other examples of disorder-based emergent behavior are given by the LLPS-driven formation of various PMLOs, formation of large protein clusters on the membrane surface, and physiological and pathological liquid–gel phase transitions (see below). PMLOs, which are known to play several important roles in the organization of various intracellular processes, are very diverse and commonly found in the cytoplasm, nucleus, and mitochondria of various eukaryotic cells; in the chloroplasts of plant cells; and in bacterial cells (2–5, 11, 23, 26, 47, 53, 60, 66, 105, 108, 118, 132, 133). The diversity and multiplicity of PMLOs are illustrated by **Figure 3*a***; there are at least 40 different PMLOs in eukaryotic and bacterial cells (159). Recently, a manually curated database of phase separation associated proteins, PhaSepDB (**http://db.phasep.pro/**), was created that currently includes 2,914 nonredundant proteins found in different organelles and represents a useful centralized resource of the LLPS-associated proteins (156).

Some Properties of Proteinaceous Membrane-Less Organelles

PMLOs are highly dynamic protein-based assemblages (118) that can be considered as constituting a special form of disorder-based protein complexes (91, 132, 139) and serve as an illustration of the disorder-based emergent behavior of IDPs and IDPRs (28, 122, 129, 134). **Figure 3*b*** shows that the proteomes of eukaryotic PMLOs are enriched in intrinsic disorder (27). Although they vary in size, PMLOs are typically rather large, possessing macroscopic dimensions and being detectable by light microscope. In fact, the dimensions of at least some PMLOs are

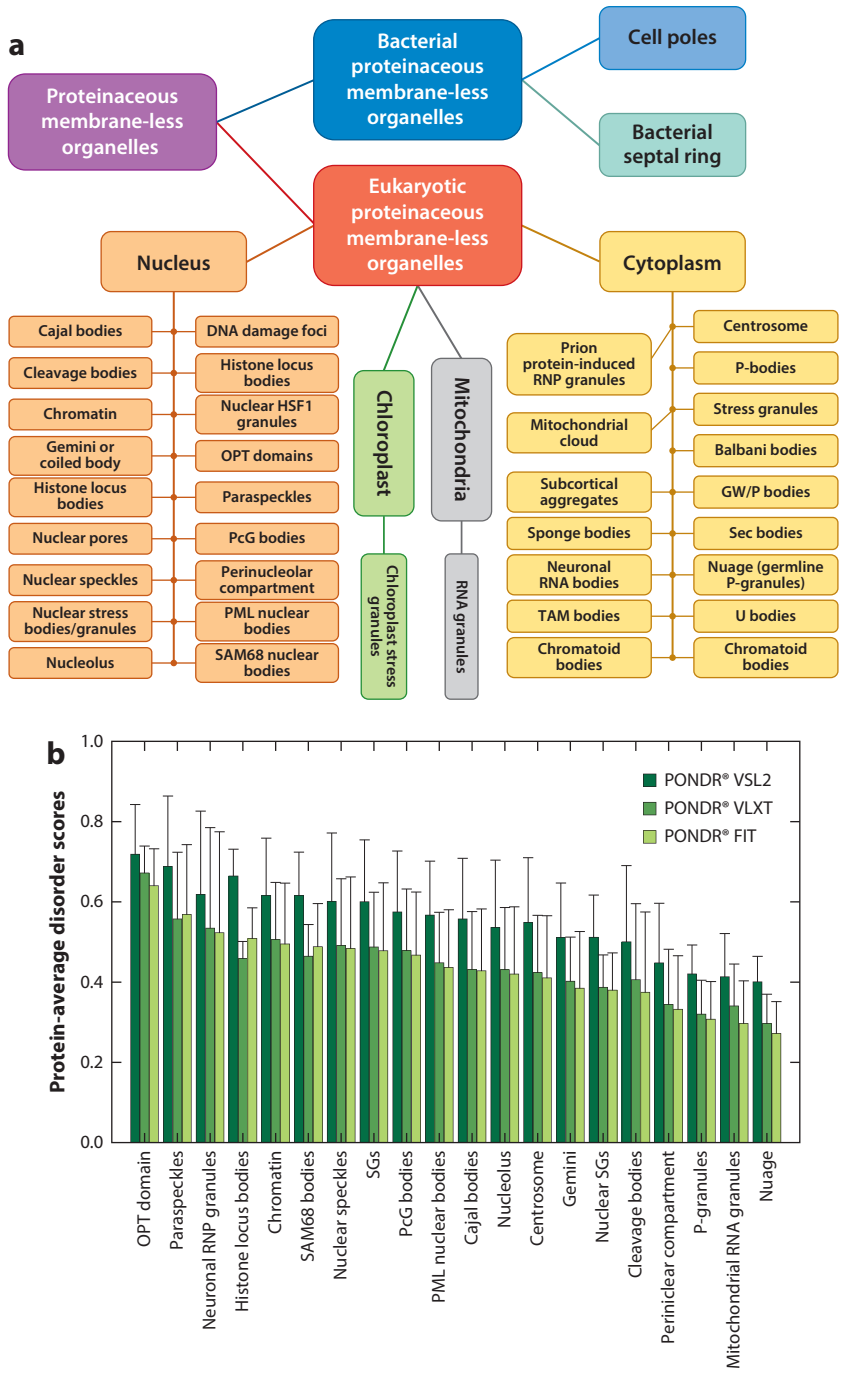

Figure 3

Some characteristics of PMLOs. (*a*) Multitude of cytoplasmic, nuclear, mitochondrial, and chloroplast PMLOs in eukaryotes and bacterial PMLOs. Panel adapted with permission from Reference 159. (*b*) Intrinsic disorder status of PMLO-related proteins. Shown is an evaluation of the overall disorder levels in human proteins associated with PMLOs. Spread of the protein-average disorder scores in individual PMLOs, evaluated by PONDR® VSL2 (*dark green bars*), PONDR® VLXT (*green bars*), and PONDR® FIT (*light green bars*), is shown. Bars represent mean protein-average disorder scores in corresponding PMLOs, whereas error bars reflect the corresponding standard deviations. Panel generated using data presented in Reference 27. Abbreviation: PMLO, proteinaceous membrane-less organelle.

dependent on cell size (16). Because of the lack of an enclosing membrane, the components of PMLOs are involved in direct exchange and contact with the exterior environment (95, 98), and the structural integrity and biogenesis of these intracellular bodies is exclusively dependent on protein–protein and/or protein–nucleic acid interactions (32, 82). PMLOs demonstrate liquid-like behavior, such as dripping, fusion, and wetting (17, 18, 43, 149). PMLOs represent liquid-droplet phases of the nucleoplasm or cytoplasm (1, 17, 18, 43, 75, 149), which, despite possessing high concentrations of constituents, are not very different from the surrounding cytoplasm or nucleoplasm, being characterized by rather low intrinsic density and viscosity (56, 123). The fluidity of PMLOs is determined by the weak multivalent interactions between IDPs or proteins containing IDPRs and their partners. These light touching interactions are not accompanied by essential structural changes in proteins undergoing LLPTs (29, 132, 133), as evidenced by nuclear magnetic resonance (NMR) analysis of several PMLOs and liquid droplets (7, 8, 88, 102, 103).

Importantly, many PMLOs are nonhomogeneous entities. For example, the structure of stress granules (SGs) was shown to have a clear heterogeneous (at least biphasic) nature. These abundant PMLOs possess a more densely packed core and a more diffused shell, with these different phases being formed at different SG biogenesis stages; for example, the dense core is assembled at an early event of granule assembly (147).

Liquid–Liquid Phase Separation as a Root for the Biogenesis of Membrane-Less Organelles

The ability of supersaturated protein solutions to undergo LLPS is a well-known phenomenon in the field of protein crystallography. However, this mechanism was mostly ignored by the scientific community until quite recently, when it was recognized that LLPS can drive cellular compartmentalization and biogenesis of various membrane-less organelles (MLOs, also known as biomolecular condensates, foci, liquid droplets, puncta, speckles, nonmembranous cytoplasmic or nucleoplasmic granules, cellular or nuclear microdomains, intracellular or intranuclear bodies, etc.). **Figure 1*b*** shows an exponential increase in the number of publications dealing with LLPS and PMLOs over the past 30 years, reflecting a dramatic increase in the interest of researchers working in various fields of protein science, cellular biology, biotechnology, and biomedicine in these intriguing phenomena. This recent upsurge of LLPS- and MLO-related publications is peculiar, since the scientific community knew about the existence of some of the cellular membrane-less compartments for a long time [e.g., the nucleolus was described as early as the 1830s (142, 145), and SGs were found in the 1980s (24, 25)]. The fact that PMLOs were mostly overlooked for a long time has its roots in the scientific reductionist approach, which is based on the presumption that the functionality of a complex system can be understood by the isolation and analysis of the functions of its individual parts, and which was successfully utilized for the analysis of the functionality of traditional membrane-encapsulated organelles, but which obviously failed for PMLOs due to the impossibility of their isolation from the cell. This impossibility of isolation in an unperturbed form, combined with their transient existence, has placed PMLOs into the category of potential artifacts. This perspective changed recently, when it was recognized that PMLOs might represent a natural method of compartmentalization of various biological processes in different regions of the cell (47) and may also be related to the pathogenesis of various diseases, most notably cancers and neurodegenerative diseases. These intracellular bodies act as important controllers of the different aspects of cellular life, since they are able to respond to different extra- and intracellular stimuli and can also facilitate, mediate, and regulate numerous biological functions (108).

Biogenesis of PMLOs is driven by intracellular LLPS processes, which are also known as liquid–liquid demixing phase separation (16, 139) and which are highly controllable, fast, and

reversible. Since LLPS is a concentration-dependent process, the local colocalization of the high concentrations of participating molecules within a small cellular microdomain might trigger PMLO formation (95, 98). In addition to the fluctuations in the concentrations of proteins undergoing LLPT, formation of PMLOs can be initiated by alterations of the environmental conditions affecting protein–protein or protein–nucleic acid interactions; by variations in the concentrations of some small molecules or salts; or by changes in solvent osmolarity, pH, and temperature, as well as by alternative splicing and various PTMs of the phase-forming proteins or their binding to some specific partners (16, 19, 62, 139, 161).

The Mechanisms of Phase Separation

The exact molecular mechanisms defining the ability of highly dynamic PMLOs to form and to keep their specific structures in the absence of membranes remain mostly unknown. In fact, although LLPS in nonpolar polymer solution systems can be adequately described by a classical Flory-Huggins theory (FHT) (45, 65), which was developed for nonpolar polymer systems (46), which centers on the expression for free energy of mixing derived from a lattice model, and which is successfully used to understand the thermodynamics and phase behavior of homopolymer mixtures (157), the applicability of this theory for aqueous mixtures of polar biomolecules is questionable (159). As early as 1956, it was also emphasized that the model should not be used to analyze mixtures of polar components, where the energy of intermolecular interactions may depend on the mutual orientations of the molecules (115). This is further supported by a very important observation that, due to the heteropolymeric nature of biomolecules, their LLPTs in aqueous solution are sequence dependent (77), whereas FHT was originally developed for chemically synthesized homopolymers with identical monomers (46) and therefore does not address sequence-dependent biological LLPS. Detailed analysis of this theory and other theoretical models is beyond the scope of this review. Interested readers are referred to a recent comprehensive review in which Lin et al. (77) provide a detailed overview of several current theories explaining LLPTs in biological systems that lead to the formation of PMLOs.

Importantly, some general patterns characteristic of the proteins capable of undergoing LLPTs have been noted. Since many of the PMLO-resident proteins are IDPs or contain IDPRs, and since the formation of all of the PMLOs analyzed to date relies on IDPs and IDPRs, it is clear that intrinsic disorder is important for PMLO biogenesis (139). Furthermore, in addition to invariably containing high levels of intrinsic disorder, proteins capable of biological phase separation are characterized by structural or sequence modularity and are able to participate in weak multivalent interactions, which are often determined by the presence of intrinsic disorder in these proteins (130, 132, 133). These weak multivalent interactions needed for the polyvalent binding can represent the heterologous electrostatic attraction between the oppositely charged biological polymers, such as oppositely charged proteins or positively charged proteins and nucleic acids, or homologous interactions of the same protein molecules containing repetitive donor and acceptor domains or regions (e.g., multiple stretches or alternating blocks of positively and negatively charged residues). Alternatively, they can represent heterologous and/or homologous interactions between repetitive units of various physicochemical nature [e.g., homorepeats of the SH3 domain (Src homology 3 domain) and its PRM (proline-rich motif) partners, such as novel neural Wiskott-Aldrich syndrome protein (N-WASP); multiple copies of the VPGXG pentapeptide in elastin-like peptides (ELPs); polyQ tract in the Whi3 protein; arginine-rich linear motifs (R-motifs) in nucleophosmin (NPM1); RNA recognition motifs (RRMs) in the TIA-1 protein; or short helical leucine-rich motifs (HLMs) in Dcp2 (132, 133)]. Therefore, these examples show that some modular multidomain proteins containing structured domains connected by flexible

linkers can be engaged in LLPS as well (42). In addition, LLPT might be controlled by cation–π interactions, as evidenced, for example, by the ability of several proteins containing YG or FG/GF motifs clustered within the positively charged blocks in close proximity to the arginine residues to efficiently form phase-separated droplets (40, 91). Furthermore, in addition to specific environmental cues, PMLO biogenesis is regulated via various PTMs (61) and alternative splicing of the phase-forming proteins (139). As some of the outlined features of proteins capable of LLPS are sequence based and recognizable, several predictors of biological protein phase separation have been developed (144). A recent comprehensive review (144) described and compared some such first-generation phase-separation predictors that were developed using different perceptions and physical bases and utilized diverse sequence features and computational designs. It also emphasized the fact that, because of the multitude of features associated with phase separation, novel and more comprehensive second-generation predictors are needed to generate an unbiased description of LLPS predispositions (144).

Recently, a comparative analysis of the composition of various PMLOs indicated that, despite the fact that there are probably thousands of proteins potentially capable of phase separation, numerous common components are shared between many different PMLOs (20). This important observation indicated that, inside the cells, both timing and specificity of LLPTs are tightly controlled and regulated, and this complex regulation serves as a safeguard that not only orchestrates the parallel functioning of multiple PMLOs and preserves their identity and physical separation, but also ensures that the right PMLO is formed in the right place and at the right time (20).

Techniques to Observe Liquid–Liquid Phase Separation and Look into the Proteinaceous Membrane-Less Organelles

A wide range of techniques can be used for the visualization and analysis of the morphological, structural, and dynamical properties of liquid droplets and PMLOs. Some of these techniques are briefly outlined below. Techniques for the visualization of liquid droplets and PMLOs include different modes of light microscopy, such as wide-field and confocal fluorescence microscopy, 4D fluorescence imaging (time-resolved 3D image reconstruction), phase contrast and differential interference contrast (DIC) microscopy (which are contrast-based imaging methods), and electron microscopy, as well as novel imaging methods that exceed the visible light diffraction limit on spatial resolution, e.g., super-resolution microscopy (SRM) (e.g., stochastic optical reconstruction microscopy, which achieves spatial resolution down to tens of nanometers, or lattice light sheet microscopy) (87).

Since PMLOs and phase-separated droplets have finite size and can scatter visible light, they can be detected by optical density measurements or direct light scattering (4). Information on particle size and shape over a large range of molecular weights and hydrodynamic radii can be obtained by several light scattering techniques, including dynamic light scattering, static light scattering, and multi-angle light scattering (87). Small-angle scattering techniques, such as small-angle X-ray scattering and small-angle neutron scattering, provide low-resolution (1–2 nm) information on the molecular size and shape (87). Light scattering techniques can also be used for kinetic analysis of the LLPS processes (4, 87).

Quantification of the macromolecules inside PMLOs or phase-separated droplets can be achieved via evaluation of the amount of the fluorescently labeled macromolecule within a microscopically imaged region of interest (ROI) (87). In addition, single-molecule fluorescence spectroscopy and fluorescence correlation spectroscopy provide important means for the direct observation of diffusional and conformational dynamics of macromolecules during the LLPS process and within the phase-separated droplets (87).

Macromolecular dynamics and the viscoelastic properties of phase-separated droplets can be analyzed using time-resolved microscopy images (87). Quantitative information on the dynamics of fluorescently labeled components within the phase-separated droplets and PMLOs can be retrieved using the fluorescence recovery after photobleaching and fluorescence loss in photobleaching approaches, where controlled photobleaching of the labeled molecules within an ROI is used to evaluate the kinetics of fluorescence intensity re-equilibration after photobleaching (4, 87). There are also various microrheology techniques for the analysis of the material properties of membrane-less organelles (e.g., viscosity, surface tension, and molecular network mesh size) (4, 87).

Although cryo-electron microscopy and cryo-electron tomography can provide some structural information on the features of PMLOs and phase-separated droplets, NMR spectroscopy continues to serve as the gold standard tool for the procurement of site-specific information on the structural and dynamic properties of IDPs and IDRs in both dilute and phase-separated environments, representing also a unique tool for finding connections between the amino acid sequence features and protein phase-separation propensity (4, 87, 150).

Finally, the recently developed optoDroplet system offers selective temporal and spatial control of PMLO formation inside the cell (107). This optogenetic platform is based on the IDP-Cry2 fusion protein, which includes an LLPS-prone multivalent IDP fused to the light-sensitive protein Cry2 from *Arabidopsis thaliana*, which is known to self-associate upon blue light exposure (107). The resulting system allows dynamical modulation of intracellular protein interactions, enabling the spatiotemporal control of LLPS within living cells, both globally and at specific subcellular locations (107). Obviously, this platform represents a unique tool for understanding light-induced and spatiotemporally controllable phase transitions within living cells and thereby provides an important means for exploring the intracellular phase space (107).

A detailed description of various biophysical techniques that can be used for analysis of LLPTs and PMLOs is outside the scope of this review, and the interested reader is advised to look for the corresponding focused studies (e.g., 4, 87).

This Goes In, That Comes Out: Proteinaceous Membrane-Less Organelles and Partitioning

It has been pointed out that PMLOs resemble polymer-based aqueous two-phase systems (ATPSs) (158). In fact, both entities are formed as a result of LLPS, both contain high concentrations of constituents (polymers in ATPSs or biopolymers in PMLOs), and their separated phases are characterized by noticeable changes in the solvent properties of water (158). Such changed solvent properties drive partitioning of various compounds (proteins, nucleic acids, and polysaccharides, as well as organic low-molecular-weight molecules, metal ions, etc.) between the phases of ATPSs or in and out of the PMLOs (158). As a result, similar to phases of ATPSs, PMLOs can be specifically enriched or depleted in some particular constituents. Characteristic examples of this phenomenon are the enrichment of PMLOs found within the *Xenopus* oocyte nucleus in some macromolecules present in nucleoplasm (56) and the recruitment and concentration of specific proteins in Negri bodies (NBs), where viral RNAs are synthesized (90). Furthermore, since some components (e.g., nucleic acids and proteins) can accumulate inside of the PMLOs to high concentrations, these increased concentrations can accelerate various cytoplasmic or nucleoplasmic reactions, thereby allowing PMLOs to serve as specific liquid-phase microreactors (16, 110, 112). Similarly, due to the increased concentrations of specific sets of mRNAs and regulatory proteins, some nuclear PMLOs can act as dynamic sensors of localized signals and regulators of the associated mRNA translation (48). In addition to the aforementioned partitioning, composition of PMLOs is controlled by a

set of specific interactions, which includes interactions between modular binding domains; weaker interactions between IDPRs or nucleic acid base pairing; and nonspecific interactions, such as electrostatic and hydrophobic interactions (30).

They Are Coming in 3D, 2D, and. . . 1D?

Although the vast majority of PMLOs are described as liquid droplets (i.e., they represent phase-separated three-dimensional entities), it is possible to find two-dimensional protein clusters on the membrane surface formed via the dynamic interactions between the multivalent cytoplasmic tails of transmembrane proteins and their multivalent binding partners (12, 101). Such LLPS-driven 2D clustering on the membrane surface has been described for several systems, such as the phosphorylated cytoplasmic domain of Nephrin and its intracellular targets, Nck and N-WASP (12); 2D functional clusters formed on the model membranes by the 12-component signaling system that were enriched in kinases, were depleted in phosphatases, and enhanced actin filament assembly by recruiting and organizing actin regulators (113); and SNARE proteins forming cell membrane microdomains, which are almost circular spots smaller than 100 nm (29). Furthermore, clustering at the endocytic sites was described for the nucleation promoting factors, such as WASP and WIP, promoting actin-related protein 2/3 (Arp2/3) complex activation, and thereby initiating the Arp2/3-mediated actin filament assembly that drives endocytic membrane invagination and vesicle scission (114). Some additional examples of functions of 2D PMLOs include formation of the presynaptic active-zone-like condensates driven by RIM and RIM-BP multivalent interactions and containing voltage-gated Ca^{2+} channels (VGCCs) (152), environmental cue–driven LLPS of various cell surface transmembrane receptors leading to the formation of the nanometer- to micrometer-scale clusters that initiate transduction of the diverse signals (21), formation of lipid rafts triggered by phase separation of membrane-anchored proteins (74), phase separation and clustering of the ABC transporters within the *Mycobacterium* membrane (57), and phase separation of zona occludens (ZO) proteins into condensed membrane-bound compartments that drive tight junction formation (14). All of these observations suggest that the multivalent protein interactions leading to 2D phase separation and generation of spatially organized micron-scale protein clusters on the membrane surface can be responsible for the regulation and control of various signaling pathways.

Phase separation plays an important role in chromatin organization and formation of chromatin-associated subcompartments (41, 49, 59, 93). Since DNA is a long string that, when completely extended, ranges in human chromosomes from 2 to 8 cm (99), it can be potentially considered as a 1D entity. In line with this idea, some proteins interacting with DNA search for their binding site using 1D diffusion (85). Therefore, although not exactly correct, it is tempting to coin the term 1D PMLO to describe at least some of the chromatin subcompartments.

Aberrant Liquid–Liquid Phase Separation, Anomalous Proteinaceous Membrane-Less Organelles, and Disease: The Center of the Storm?

There are several ways in which LLPS and PMLOs can be associated with the pathogenesis of human diseases, with some alterations in PMLO formation, composition, and material properties contributing to several disease states (28, 104, 111). First, many PMLOs have a specific window of safe existence. This window includes specific times, locations, and conditions that define the physiological biogenesis of functional PMLOs. Outside this window is the danger zone, where the pathological conversion from liquid to solid form within the highly concentrated milieu of PMLOs might happen due to the misfolding and pathological aggregation of PMLO-residing

proteins. Such pathological conversions can be triggered by extended time of PMLO existence (or pathological aging of PMLOs), as well as by elevated levels of proteins undergoing LLPTs, their aberrant PTMs, pathological mutations, or chromosomal translocation (94). In other words, deregulated biogenesis of PMLOs and/or the loss of their dynamics can initiate some pathological transformations, and in turn, the biogenesis and dynamics of PMLOs can be altered by inclusion of some pathology-related proteins (9). In fact, although many PMLOs (e.g., SGs) are liquid-like in the norm, they are able to mature or age into a much less dynamic state, the appearance of which typically coincides with the formation of amyloid-like fibrils (76). Such maturation or aging leads to characteristic alterations of the mechanical and physical properties of cellular bodies (76), with time-dependent changes in the dense core of these PMLOs serving as a potential source of insoluble protein aggregates (31).

Second, aberrant PTMs can affect protein disorder–based LLPTs and alter the biogenesis of PMLOs (28). The complexity of this mechanism is illustrated by one of the IDPs associated with the pathogenesis of Alzheimer's disease, microtubule-associated protein tau, which undergoes LLPS (7), the efficiency of which is altered by mutations and various PTMs, such as hyperacetylation, hyperphosphorylation, and truncation, (7, 44). The LLPS behavior of tau is differently modulated by different factors, with hyperacetylation disfavoring LLPS and inhibiting the heparin-induced aggregation of this protein (44) but with truncation, mutations, and hyperphosphorylation enhancing LLPS and tau aggregation (7).

Third, pathological mutations in proteins either undergoing LLPTs or involved in LLPT regulation might have noticeable effects on PMLO biogenesis. Similarly, chromosomal translocations might generate fusion oncogenes capable of LLPS, leading to the formation some pathological PMLOs associated with cancer pathogenesis. There are many illustrations of both of these phenomena, and interested readers are encouraged to look for the corresponding reviews (e.g., 28, 104, 111).

Fourth, viral infection can be associated with the formation of specific PMLOs. In fact, for many viruses, replication and assembly take place in the so-called cytoplasmic viral factories, which are specialized intracellular compartments formed during viral infection. An illustration example of such viral factory is given by the NBs, where mRNAs and genomic and antigenomic RNAs of rabies virus are synthesized (90). NBs were shown to possess all of the characteristic properties of PMLOs, being fluid, spherical, able to fuse together, and able to reversibly deform when encountering a physical barrier (90). Formation of NBs is driven by interactions among rabies virus, an RNA-binding nucleoprotein N, and intrinsically disordered phosphoprotein P (90). Since similar bodies with properties of liquid droplets are also formed by other viruses, such as measles virus (160) and vesicular stomatitis virus (58), it is clear that formation of such PMLOs, which concentrate replicase proteins, virus genomes, and host proteins required for replication, is crucial for efficient viral replication (89).

Drugs Affecting Liquid–Liquid Phase Transitions and Targeting Proteinaceous Membrane-Less Organelles: Myth or Reality?

Given the various contributions of PMLOs to pathogenesis of many diseases and viral infections, it is clear that drugs that can affect LLPS and modulate PMLO biogenesis are needed. As pointed out above, despite the attractiveness and exceptional potential of the ability to regulate intrinsic disorder–based interactions, targeting IDPs and IDPRs by drugs represents a challenging task. Fortunately, this task is challenging but not impossible, and several successful approaches have been developed to find small molecules capable of modulating such interactions. In light of these difficulties, is it possible to have a drug that could modulate LLPTs and thereby affect the

biogenesis of PMLOs? At the moment, this idea definitely sounds like a dream, since the field is too premature, and it is rather difficult to imagine a mechanism by which a small molecule would be able to directly act on LLPT. However, it was recently hypothesized that one of the possible solutions for this seemingly impossible problem can be found in the targeted modulation of PTMs that can alter phase separation and modulate PMLO formation (104).

CONCLUDING REMARKS: PROTEIN INTRINSIC DISORDER AS A UNIVERSAL TOOL FOR SEEING THE INVISIBLE AND SOLVING THE UNSOLVABLE

In light of the lock-and-key model, which dominated scientific minds for more than a century, the idea that a structure-less protein could be functional was considered to be nonsense. However, recognition of the wide penetrance of IDPs and IDPRs in proteomes is changing protein science. The concept of protein intrinsic disorder provides solutions for many scientific problems that cannot be easily comprehended and explained based on the classic structure–function paradigm. Some of the corresponding examples include, but are not limited to, protein multifunctionality, binding promiscuity, the ability to connect different structures in complexes with different partners, the capacity to avoid the conformational catastrophe caused by alternative splicing, the ability to transmit signal via low-affinity–high-specificity interactions with binding partners, the potential to serve as extremely sensitive and highly responsive sensors, potential regulation by a multitude of PTMs, the ability to be involved in the formation of the most stable as well as the weakest complexes, the propensity to be engaged in multivalent weak interactions, and the ability to demonstrate emergent behavior. Recent studies also added the crucial dependence of biological LLPS and biogenesis of PMLOs on the intrinsic disorder–based multivalent weak interactions that define the fluidity of phase-separated droplets to this list.

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