Trends in Biochemical Sciences

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Review

Stochasticity of Biological Soft Matter: Emerging Concepts in Intrinsically Disordered Proteins and Biological Phase Separation

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At the turn of this century, cardinal changes took place in the perceptions of the structure and function of proteins, as well as in the organizational principles of membrane-less organelles. As a result, the model of the organization of living matter is changing to one described by highly dynamic biological soft matter positioned at the edge of chaos. Intrinsically disordered proteins (IDPs) and membrane-less organelles are key examples of this new outlook and may represent a critical foundation of life, defining its complexity and the evolution of living things.

Highlights

The perceptions of protein structures and functions are changing due to the abundance of IDPs and IDP regions.

It is now recognized that many cellular processes occur within proteinaceous membrane-less organelles (PLMOs) rather than only in membrane-encapsulated chambers.

IDPs and PLMOs can be considered as biological soft matter.

By considering IDPs and PLMOs as edge of chaos systems, a better understanding of their mechanism can be gained.

Success in studying these systems depends on interdisciplinary collaborations that bridge biological studies with physical approaches.

Biological Soft Matter

Over the past 20 years or so, the emergence, acceptance, and penetrance of the **intrinsically disordered proteins** (**IDPs**; see Glossary) has caused molecular biology, biochemistry, molecular biophysics, structural biology, and other protein-related branches of science to undergo a paradigm shift, a so-called 'softening'. In fact, for decades, the prevailing consideration of proteins, protein–protein interactions, and other protein functions was considered to be rigid, where, for a given protein, a unique 3D structure defined a unique biological activity. It is now realized that many protein functions rely on the lack of specific structure [1–7]. This recognition has changed the classical consideration of a functioning protein from a quasi-rigid entity with a unique 3D structure resembling an aperiodic crystal into a softened conformational ensemble representation, with **intrinsic disorder** affecting different parts of a protein to different degrees [8]. This generates a highly heterogeneous, mosaic-like structure of a protein molecule comprising differently folded regions, each with a specific spectrum of biological functions [9]. Although some IDPs (soft proteins) can, at least partially, rigidify upon their binding to specific interaction partners, this folding-at-binding mechanism represents just one of many potential means by which IDPs or **IDP regions** (IDPRs) can bind to their partners [10]. However, many IDP/IDPR-based complexes retain high levels of intrinsic disorder [11] and, therefore, continue to be mostly soft. Box 1 provides examples of the major structural and functional features of IDPs.

In this review, we represent the intriguing concept of **biological soft matter** (matter that has unique abilities to self-assemble into supramolecular structures under stress) by considering some peculiar features of IDPs/IDPRs and their complexes with preserved intrinsic disorder. A special case of biological soft matter is given by **proteinaceous membrane-less organelles (PMLOs)**, which are commonly found in eukaryotic cells. PLMOs are specific cellular bodies [often ribonucleoprotein (RNP) particles] that originate as a result of **liquid–liquid phase transitions** (LLPTs) and provide many important biological functions [12]. We focus mainly on the consequences of the stochasticity of IDP/IDPR structure on protein functions. We introduce the **edge of chaos** representation of IDPs/IDPRs, and then show how viewing these proteins as

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Box 1. Major Structural and Functional Features of IDPRs are common in nature. They and IDPRs

(i) IDPs/IDPRs are common in nature. They are found in all proteomes, and their abundance at the proteome levels typically increases with the increase of the complexity of organisms [107].
(ii) Amino acid sequences of IDPs/IDPRs are biased. They are depleted in hydrophobic (order-promoting) residues and enriched in polar, charged, and structure-breaking (disorder-promoting) residues. They typically contain multiple repeats and are less complex than sequences of ordered domains. However, the disorder-encoding sequence space is vast and noticeably exceeds the sequence space of ordered proteins [2,3,9].
(iii) IDPs are characterized by high spatiotemporal heterogeneity. Their structures are represented as highly dynamic and heterogeneous conformational ensembles [9,14].
(iv) Structures and functions of IDPs/IDPRs are regulated and modulated by various means, such as post-translational modifications, alternative splicing, and interactions with binding partners. Therefore, they represent an important illustration of the proteoform concept, where, due to all these aforementioned factors, one gene encodes several structurally and functionally different proteoforms that have an important role in defining the protein structure–function continuum [42].
(v) IDPs/IDPRs are interaction masters. Many proteins serving as hubs in protein–protein interaction networks rely on intrinsic disorder. Interaction modes attainable by IDPs/IDPRs are diverse. On interacting with their binding partners, IDPs/IDPRs can noticeably fold or retain significant levels of disorder, or even remain completely disordered. Some IDPs/IDPRs can fold differently on interacting with different binding partners. Some IDPs/IDPRs can be engaged in specific but weak interactions. The weakest and strongest protein-centric complexes depend on intrinsic disorder [10].
(vi) Intrinsic disorder is not compatible with protein catalytic activities, but is vital for functions associated with control, signaling, recognition, and regulation. As a result, the functional repertoire of IDPs/IDPRs is complementary to the catalytic and transport functions of ordered proteins and domains [3].
(vii) Misbehavior of IDPs/IDPRs is at the root of various human diseases, such as amyloidoses, cancer, cardiovascular disease, diabetes, and neurodegenerative diseases [108].

edge of chaos systems (or **complex systems**) can be used to gain interesting insights into their functions. We also demonstrate how these concepts can be related to PMLO biogenesis.

IDPs/IDPRs as Edge of Chaos Systems

It is well known that natural polypeptides, even IDPs, are never **random coils** and always contain noticeable levels of residual structure. This is even the case for proteins in solutions containing high concentrations of strong denaturants, such as urea or guanidinium hydrochloride [13], and is definitely applicable to proteins in physiological aqueous media [8,9,14]. Therefore, an IDP cannot serve as an example of a completely chaotic system and, instead, should be considered as an edge of chaos system. The term 'edge of chaos' is used to define a transition region between order and complete randomness or chaos, which is characterized by the bounded instability (i.e., a constant dynamic interplay between order and chaos) [15]. Mitchell Waldrop pointed out [16] that the components of the edge of chaos system 'never quite lock into place, yet never dissolve into turbulence, either. These are the systems that are both stable enough to store information, and yet evanescent enough to transmit it. These are systems that can be organized to perform complex computations, to react to the world, to be spontaneous, adaptive, and alive.' Edge of chaos systems are systems with the highest complexity and adaptability and are believed to serve as a main source of evolution [16]. The positioning of such systems between randomness and order allows them to use both order and disorder to evolve to become more complex. Complex or edge of chaos systems are characterized by a set of specific features [17] (Box 2).

Many of the specific features ascribed to edge of chaos systems can be found in IDPs/IDPRs [9]. Box 2 lists major properties of these systems, and considerations discussed later show how these general properties of the edge of chaos systems are applicable to IDPs/IDPRs. Accumulated experimental data leave no doubts that IDPs/IDPRs are structurally heterogeneous at different times and in different locations [8,9,14]. Their spatiotemporal heterogeneity penetrates to multiple levels, where IDPs/IDPRs are differently disordered not only at a global level (as a

Glossary

Biological soft matter: many different types of matter of biological origin that combine viscous and elastic elements and originate from self-assembled supramolecular structures typically stabilized by noncovalent interactions. These types of matter include colloids, elastomers, foams, gels, liquid crystals, and polymers. They are united by showing large responses to modest stresses.
Butterfly effect: an expression used in chaos theory to emphasize the critical dependence of the behavior of a complex system on initial conditions, where small changes in the initial conditions can dramatically and nonlinearly affect the results, resulting in strikingly disproportional consequences, such as a hurricane in China caused by a butterfly flapping its wings in New Mexico.
Chaos theory: a field of modern mathematics for predicting the behavior of seemingly unpredictable complex (or edge of chaos) systems.
Complex systems: systems comprising many different components that interact with each other nonlinearly or nonadditively, where the overall interactions between the components are not equal to the sum of the two-body and many-body interactions.
Edge of chaos: a transition region between order and complete randomness or chaos characterized by a constant dynamic interplay between order and chaos.
Edge of chaos systems: systems positioned at the transition region between order and chaos and that use both order and disorder to evolve to become more complex. They are characterized by the highest complexity and adaptability and serve as a main source of the evolution. Minimal changes in the environment of the edge of chaos system can generate large and diversified changes.
Emergent behavior: behavior of a complex system that cannot be predicted or extrapolated from the examination of the behavior of its individual parts. It originates from the interactions between parts of a system and their relationships to one another. Emergent behavior is also related to the appearance of complex systems and patterns out of a multiplicity of relatively simple interactions between individual parts.

Box 2. Examples of the Characteristic Features of Complex or Edge of Chaos Systems

(i) Complex or edge of chaos systems are characterized by the presence of heterogeneous, nonlinearly interacting, and interdependent components. This means that the behavior of a system cannot be described as a sum of the behaviors of its parts, and the response of such a system to a small perturbation is not easily predictable [17].
(ii) Components of a complex system may be edge of chaos systems themselves, generating a nested structure spanning several scales [17].
(iii) A complex system can self-organize to form novel patterns and structures with properties that cannot be directly derived from the properties of its constituents. This unanticipated behavior shown by an edge of chaos system is known as emergence [17].
(iv) An intricate interplay between disorder and order determines the complexity of a system.
(v) Components of an edge of chaos system can compete or cooperate, defining the presence of negative (damping) or positive (amplifying) feedbacks [17].
(vi) In a complex system, the history may be important, since prior states may affect present states, thereby generating the memory of a system [17].
(vii) Properties of a complex system are characterized by the roughness or insensitivity of the qualitative features of a system to small modifications [17].
(viii) The behavior of a complex system is characterized by integrity reflected in the spatiotemporal coordination of the behavior of its constituents [17].

whole, they can be compact or extended to different degrees), but also at the domain and subdomain levels (see later).

At the domain and subdomain levels, IDPs are also differently disordered, with a highly heterogeneous structural organization, components of which can be classified as **foldons**, **inducible foldons, nonfoldons**, **semifoldons**, and **unfoldons** [9]. Some of these structural constituents are independent, others are interdependent, and some are able to interact nonlinearly (i.e., nonadditively, where the overall interactions between constituents in a system are not equal to the sum of the two-body and many-body interactions), thereby defining the irreducibility of the system to the simple sum of its constituent parts. One of the examples of this phenomenon is given by a flanking binding model of some IDPs/IDPRs [18]. Here, a short recognition motif that folds at binding to a specific partner is flanked by disordered regions that, despite being mostly disordered in the bound form, increase binding affinity, since their deletion reduces stability of the complex [18]. This is illustrated by the phosphorylation-induced binding of the disordered kinase-inducible domain (KID) of the cAMP response element-binding protein (CREB) to the KIX domain of CREB-binding protein (CBP) [19].

The behavior of disordered proteins and regions and their exquisite ability to be controlled and regulated are defined by their positioning at the transition region between order and chaos. In fact, minimal alterations in the environment of an edge of chaos system (e.g., small environmental perturbations or different post-translational modifications in proteins) are expected to generate large and diversified responses. This is illustrated by the family of G-protein-coupled receptors (GPCRs), which are considered as a cellular 'control panel' that is 'able to detect the presence of a strikingly diverse array of molecules outside the cell and to initiate a variety of intracellular signaling cascades in response' [20]. In humans, the GPCR family includes over 800 members that can interact with more than 1000 ligands of different nature, such as amines, lipids, nucleotides, odorants, peptides, photons, and proteins, initiating a variety of intracellular signaling cascades [21,22]. Here, activated GPCR interacts with a member of one of four major families of guanine nucleotide-binding proteins (Gα proteins) encoded by 16 human genes [22–24], leading to the modulation of various downstream effector proteins (such as adenylate cyclase and phospholipase C) and key secondary messengers (e.g., cAMP, Ca^{2+}, and IP3) [22,25,26]. The interaction of activated GPCRs with Gα proteins is characterized by complex coupling selectivity, where several different GPCRs can couple to the same Gα protein and one GPCR can couple to more than

Foldon: independent foldable unit of a protein, which is not equivalent to the domain, since single-domain proteins might have multiple foldons.
Functional memory: ability of a protein molecule to encode, store, and retrieve information when needed for function. Functional memory of an IDP manifests, for example, by the presence of functional prestructured motifs.
Inducible foldon: disordered region of a protein that can fold due ,at least in part, to its interaction with binding partners.
Intrinsic disorder: lack of a unique structure in a functional protein.
Intrinsically disordered protein/region (IDP/IDPR): biologically active protein and/or region without a unique structure.
Liquid–liquid phase transition: physical process leading to the separation (demixing) of a mixture of two or more liquids into nonmiscible phases. This phase separation depends on the thresholds of critical concentrations of the components and can also be modulated by physicochemical alterations of the system.
Molecular recognition feature (MoRF): a disorder-based protein–protein interaction site, located within a longer IDPR, that is disordered in the unbound state, but can specifically fold on interacting with a specific partner.
Morphing MoRF: MoRF that can differently fold on interacting with different binding partners; disorder-based protein–protein interaction site that morphs between different structures at binding to different partners.
Nonfoldon: nonfoldable protein region (i.e., a region that is always disordered).
Partitioning: behavior of solutes in a two-phase (or multiphase) liquid system, where solutes have unequal distribution between the phases, being preferentially included into (or excluded from) one liquid phase.
Polyelectrostatic model: model describing interactions between oppositely charged disordered polymers, where all charges contribute to binding either via more specific, spatially short-range contacts between specific charged groups or via the less specific, spatially long-range polyelectrostatic interactions between binding partners.
Proteinaceous membrane-less organelle (PMLO): a cellular compartment not embedded into a membrane.

one Gα protein. As a result, GPCRs mediate most cellular responses to hormones, neurotransmitters, ions, photons, and other environmental stimuli, and are responsible for vision, olfaction, and taste [20].

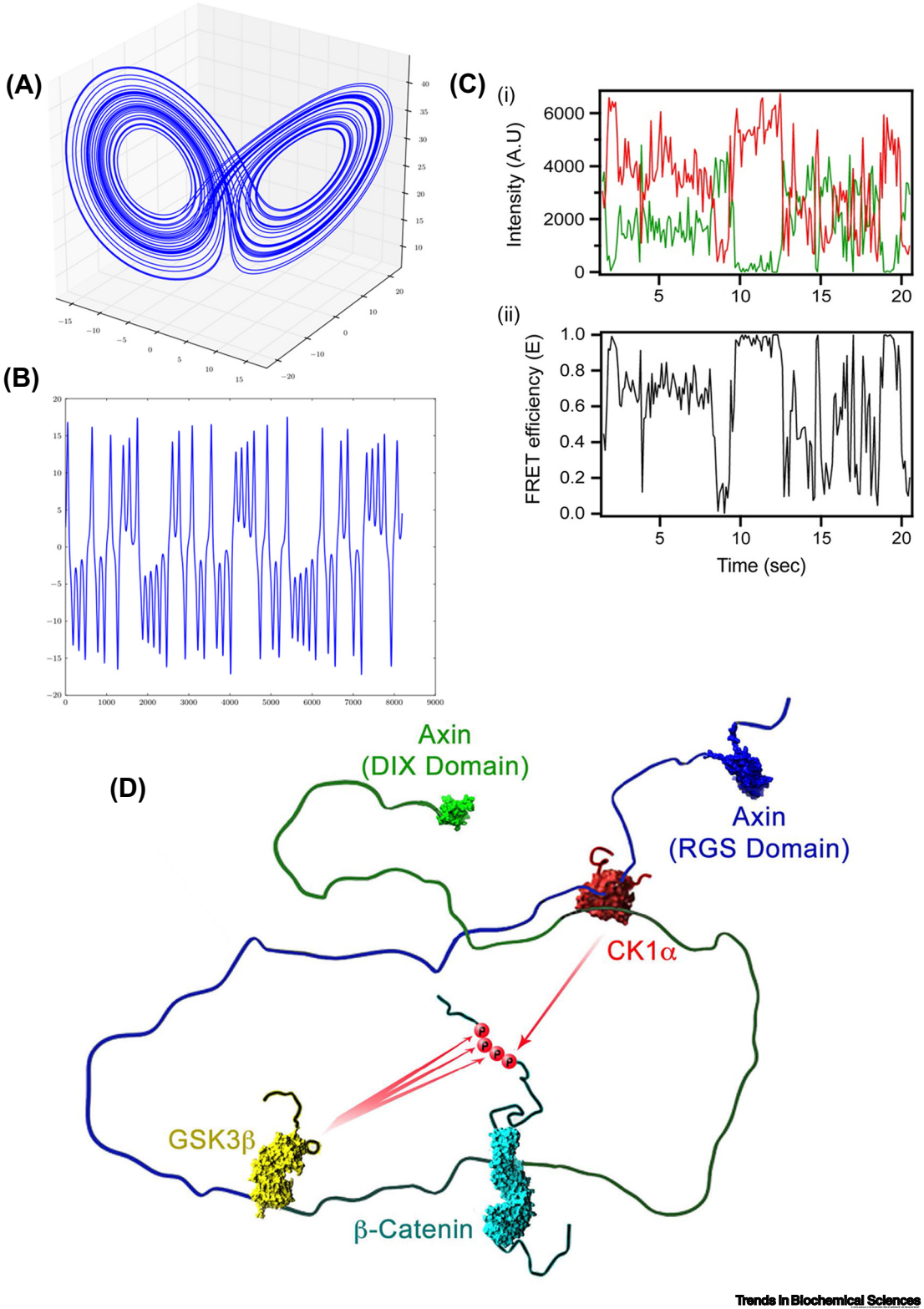
Furthermore, the structural and functional behavior of IDPs is complex and does not represent a simple sum of the behaviors of their parts. This is because many structural components of IDPs/IDPRs are edge of chaos systems themselves, with some of them being engaged in permanent transitioning between order and disorder [8,9,14]. As a result, different parts of a protein can differently respond to changes in the environment, thereby generating a new means of functional regulation. This can be illustrated by conditionally and transiently disordered proteins {i.e., ordered proteins, the functions of which require local or even global unfolding that can be promoted by a variety of factors of passive and/or environmental (changes in pH, temperature, redox potential, mechanical force, or light exposure) and active (interaction with partners, post-translational modifications, release of autoinhibition, etc.) nature [27]}.

By contrast, IDPs/IDPRs are generally less sensitive to mutations than ordered proteins and domains, thereby illustrating the roughness of their properties. This is reflected in the faster evolution rates of IDPRs compared with ordered domains in several families of hybrid protein containing both ordered and disordered regions [28]. Generally, but not always, IDPs/IDPRs evolve faster and exhibit higher rates of insertions and deletions compared with ordered proteins and domains, and their patterns of accepted point mutations are noticeably different from those of ordered proteins [28]. An illustration is given by the p53 family members, IDPRs of which are highly diversified in evolution, whereas the ordered DNA-binding domain is evolutionary conserved [29].

Stimuli-induced disorder-to-order transitions represent a clear indication of the ability of IDPs/IDPRs to show **emergent behavior** leading to self-organization, and there are also other illustrations of such emergent behavior. For example, emergent behavior is seen in the Min proteins in *Escherichia coli*, where spatiotemporal oscillations due to repetitive cycles of binding and detaching of the Min proteins to and from the membrane enable self-organization into specific patterns within a cell. *In vivo*, this results in spatial regulation of the positioning of the cytokinetic Z ring before cell division [30,31]. Visualization of the repetitive cycles of Min proteins *in vitro* depicts surface-traveling protein waves on support lipid bilayers [32,33]. The pattern-forming ability of the proteins from Min system reflects the potential applicability of **chaos theory** for the description of IDPs [9]. Another example is observed in PMLO biogenesis and is discussed further later.

Finally, at the subdomain level, the presence of structural and **functional memory** in IDPs/IDPRs is given by the existence of various disorder-based binding sites (DBBSs), such as **molecular recognition features** (MoRFs) [34–36], short linear motifs (SLiMs) [37], eukaryotic linear motifs (ELMs) [38], and prestructured motifs (PreSMos) [39]. Often, these DBBSs manifest in the nonbound state as transiently populated semiordered motifs that can, at least partially, rigidify at the IDP binding to its partners. Importantly, some DBBSs can fold differently at interaction with different binding partners. This is illustrated by the p53 tumor suppressor protein, which controls the expression of genes involved in the regulation of apoptosis, cell cycle, DNA repair, response to cellular stress, and so on [40], and dysfunction of which is associated with cancerous transformation [41]. All domains of this protein are engaged in interaction with multiple partners, with the unstructured C-terminal tetramerization and regulatory domain interacting with more than 45 different proteins [40,42,43]. The most C-terminal part (residues 376–393) of p53 represents an intrinsically

Random coil: a polypeptide conformation with randomly oriented side chains, the distribution of which can be described by the Gaussian probability function. Random coils have no specific structure except that inherent in the local interactions. They represent a highly dynamic ensemble of random conformers that contains both extended and compact states.
Semifoldon: protein region that is always in a semifolded state.
Stochastic machine: protein complex that operates via random movements of its parts and not via coordinated conformational changes.
Unfoldon: ordered protein region that has to undergo an order-to-disorder transition to become functional.



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disordered negative regulatory domain that, among many other partners, binds to cyclin A, sirtuin, CBP, and the $S100B_{\beta\beta}$ dimer and folds differently at binding to these four different partners, becoming an α-helix, a β-strand, or two coils with different configurations respectively [43]. Being an example of the one-to-many disorder-based binding mode (where one IDPR binds to many different partners) [44], this negative regulatory subdomain of p53 also illustrates the **morphing MoRF** concept.

On the Chaotic Side of IDPs/IDPRs

Chaos is not equivalent to disorder, but explores the transitions between order and disorder. Therefore, positioning between order and disorder implies that the edge of chaos systems might integrate features of both ordered and ordered systems. In agreement with this hypothesis, some of the structural behaviors of an IDP can be described in terms of a strange attractor (e.g., Lorenz attractor [45–47]), where chaotic systems will neither converge to a steady state (IDPs do not form fully ordered state) nor diverge to infinity (IDPs do not behave as random coils), and will stay in a bounded but chaotically defined region, and where small changes in initial conditions may produce large changes in the long-term outcome (the so-called '**butterfly effect**') [9]. Furthermore, although the 3D evolution of a trajectory describing the behavior of the Lorenz's system also resembles a butterfly (Figure 1A), the time-course of one of the variables in such a system shows nonconverging oscillations between two states (Figure 1B). Importantly, illustrations of such butterfly-like dynamics can be found in the IDP world. For example, using single-molecule fluorescence to characterize conformational dynamics of several IDPs, an intriguing 'hopping' behavior was described for neuroligin and the NMDAR-2B glutamate receptor, which, despite being highly disordered, were characterized by stochastic conformational switching between two states with different degrees of compaction (Figure 1C) [48].

Another demonstration of the chaotic functional behavior of IDPs is given by so-called **stochastic machines** [49]. An illustrative example of such a stochastic machine is the β-catenin destruction complex between Axin, β-catenin, casein kinase Iα (CKI-α), and glycogen synthetase kinase 3β (GSK3β) [49]. Here, the two kinases and the β-catenin bind to long IDPRs of Axin and form a highly dynamic structure, where ordered subunits and/or domains are connected by long flexible linkers [49]. In such a stochastic machine, productive kinase-substrate collisions leading to phosphorylation are enabled by uncoordinated random movements of the linkers and their bound proteins and not by cooperative conformational changes in catalytic subunits (Figure 1D) [49].

Furthermore, many biological processes represent nonlinear and unpredictable phenomena that can be described, analyzed, and understood utilizing apparatus of the chaos theory, which is a field of modern mathematics that can be used for predicting the behavior of inherently unpredictable systems. The corresponding examples include interactions between IDPs/IDPRs and their partners [9], the emergent behavior of complex systems that can give rise to the self-organization and formation of specific patterns [50], as well as various outputs of the nonlinear reaction and/or diffusion dynamics that take place inside living cells, such as the abilities to

Figure 1. Chaos, Intrinsically Disordered Proteins (IDPs), and Complex Systems. (A) The phase-space representation of the behavior of the variable in the Lorenz attractor [115] defined by a set of three nonlinear interdependent equations [46,47]. Here, the variable is plotted against its rate of change, generating the characteristic butterfly-like loops indicating that the trajectories of the chaotic system converge on a complex shape, known as strange attractor [46,47]. (B) Stochastic time course of one of the three variables in the Lorenz attractor [115]. This variable changes stochastically, and its stochastic changes resemble the time dependence of the fluorescence resonance energy transfer (FRET) efficiency describing the conformational dynamics of the neuroligin cytoplasmic domain. (C) Single-molecule FRET analysis of the conformational dynamics of the neuroligin cytoplasmic domain [48]. In (i), emissions of donor and acceptor are shown by green and red colors, respectively. (ii) The time course of FRET efficiency. The protein clearly shows hopping behavior, with stochastic transitions between different FRET efficiency values [48]. (D) Illustration of the stochastic machine showing a possible configuration of the Axin, β-catenin, casein kinase Iα (CKI-α), and glycogen synthetase kinase 3β (GSK3β) complex. Axin is shown with color variations to make its pathway easier to follow. Random motions of flexible regions of Axin can readily bring about the substrate–enzyme collisions needed for function of this complex. Abbreviation: A/U, arbitrary unit. Reproduced, with permission, from [49].

coordinate cell division [51,52] and cell motility [53,54], to organize information flow [55,56] and encode the positional information within the cell [55,57–59], or to ensure division of a rod-like bacteria into two equally-sized daughter cells by the Min-driven positioning of the division septum at the center of the bacterial cell [60], or the formation of various PMLOs (see later).

PMLOs as Edge of Chaos Systems

PMLOs serve as another important illustration of stochastic, but highly organized, biological soft matter. PMLOs are many [61–68] and their key characteristics are listed in Box 3. They can be found at the cell poles of rod-shaped bacteria [69] and within their septal rings [70]. They are also in the nucleus, cytoplasm, mitochondria, chloroplasts, and liquid-like receptor clustering compartments of eukaryotic cells [71]. Although a detailed description of PMLOs is outside the scope of this article and can be found elsewhere [71], examples of the specific characteristics of PMLOs are outlined in Box 3. Figure 2 illustrates the variability of PMLOs found in eukaryotes and bacteria.

As suggested by their name, PMLOs are not encapsulated in a membrane; instead, their interior and constituents are in direct contact with the environment [72,73], and the integrity of PMLOs is determined by a multitude of dynamic protein–RNA, protein–protein, and protein–DNA interactions [74], which have undergone LLPTs [63,75–77]. As such, PMLOs exhibit liquid-like behavior and are able to coalesce, drip, fuse, relax to spherical structures upon fusion, and wet [78–81]. Biophysically, PMLOs are similar to the intracellular fluids in which they are located in [82,83]. Therefore, PMLOs are considered as a different liquid state of cytoplasm, nucleoplasm, mitochondrial matrix, or chloroplast stroma [84].

Although PLMOs share these characteristics, these subnuclear organelles are diverse; they have different functions, rather different morphologies, divergent cellular distribution, and typically highly dissimilar compositions. Therefore, the biogenesis, structural dynamics, and morphology of PMLOs are all critically dependent on proteins, which thereby serve as a common denominator. Furthermore, proteomes of many PMLOs contain significant levels of intrinsic disorder [71,85–88]. The importance of intrinsic disorder for PMLO biogenesis was considered in several studies [84–93], and examples of the related observations are summarized in Box 4.

Data shown in Boxes 2–4 indicate that PMLOs can be described in terms of the edge of chaos systems. In fact, being the result of the emergent behavior of interacting IDPs and

Box 3. Specific Characteristics of PMLOs

(i) PMLOs contain from a few to >1500 proteins. Not all proteins found in PMLOs are directly related to their assembly, and at least some are present in PMLOs because of their preferential partitioning into these subcellular bodies [89].
(ii) In relation to their roles in PMLO biogenesis, proteins can be grouped into two major categories: drivers that are directly involved in the PMLO formation, and passengers that are attracted to PMLOs by specific solvent properties of aqueous media inside these liquid droplets [109].
(iii) Proteins within PMLOs are 10–300-fold more concentrated than corresponding proteins in the dilute phase [92,95,110], potentially affecting the solvent properties of water inside PMLOs. Changed solvent properties in PMLOs promote the partition of various molecules in and out of PMLOs [109].
(iv) The biogenesis of PMLOs is controlled by the concentrations of their constituents and changes in the local environment. It is also dependent on the capability of 'driver' proteins to undergo post-translational modifications [110,111].
(v) Some PMLOs can undergo re-LLPTization, where more stable PMLOs can cause the dissociation of less stable droplets and then use the resulting freed constituents for their own growth [75,111].
(vi) The fluid nature and low-density structure of PMLOs define the easiness of the access of their interior to various environmental factors. Together with the increased concentrations of related protein and RNA components, the high accessibility and fluidity define the ability of some PMLOs to act as microreactors accelerating cytoplasmic reactions [12].

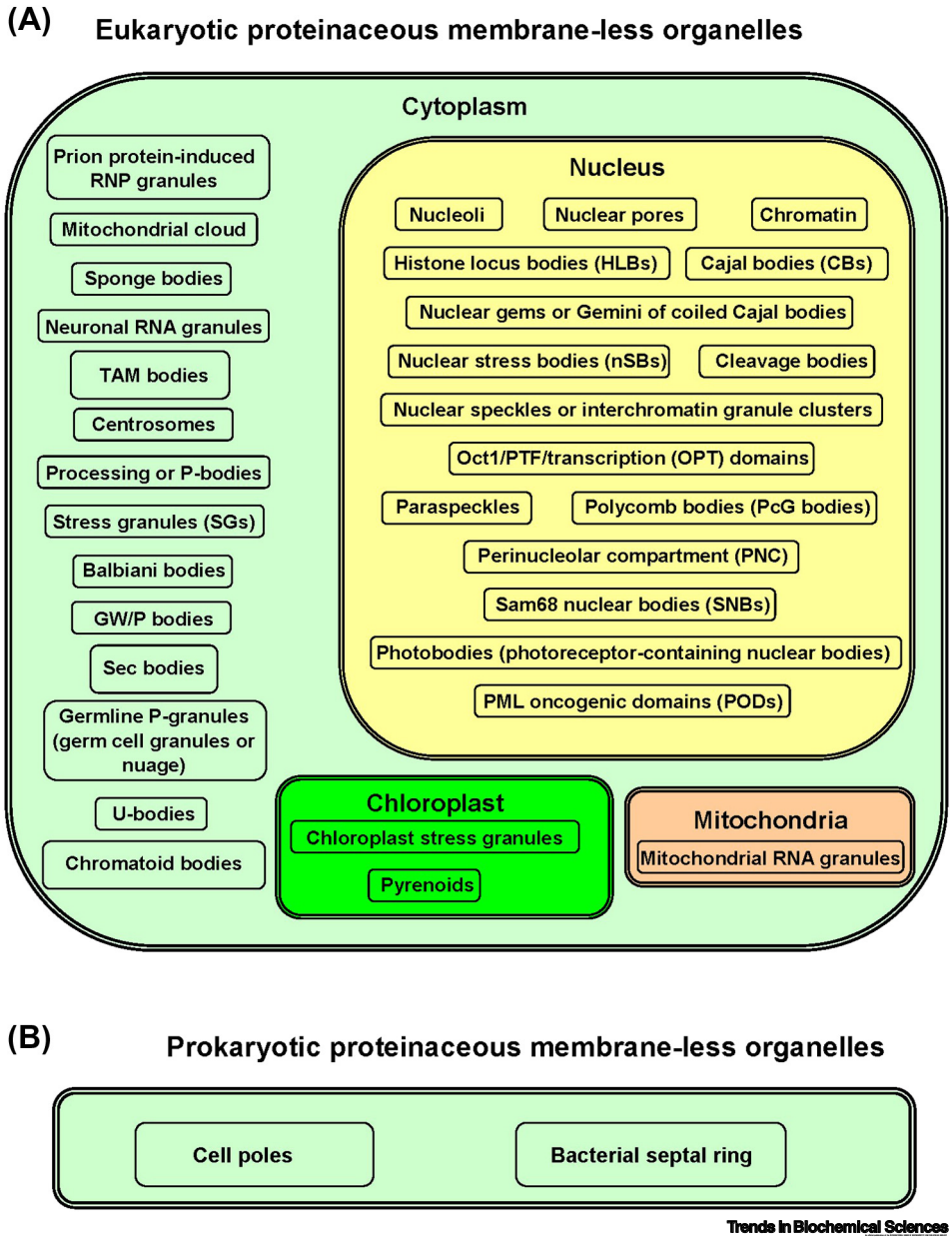

Figure 2. The Multitude of Cytoplasmic, Nuclear, Mitochondrial, and Chloroplast Proteinaceous Membrane-Less Organelles (PMLOs) in (A) Eukaryotes and (B) Prokaryotic (Bacterial) PMLOs. The cytoplasm of eukaryotic cells contains prion protein-induced ribonucleoprotein (RNP) granules, mitochondrial cloud, sponge bodies, neuronal RNA granules, TAM bodies, centrosomes, processing or P-bodies, stress granules (SGs), Balbiani bodies, GW/P bodies, Sec bodies, germline P-granules (germ cell granules or nuage), U-bodies, and chromatoid bodies. In the nucleosome, one can find nucleoli, nuclear pores, chromatin, histone locus bodies (HLBs), Cajal bodies (CBs), nuclear gems or Gemini of coiled of Cajal bodies, nuclear stress bodies (nSBs), cleavage bodies, nuclear speckles or interchromatin granule clusters, Oct1/PTF/transcription (OPT) domains, paraspeckles, polycomb bodies (PcG bodies), perinucleolar compartments (PNCs), Sam68 nuclear bodies (SNBs), and PML oncogenic domains (PODs). In chloroplasts and mitochondria, there are chloroplast SGs (cpSGs) and mitochondrial RNA granules, respectively. Furthermore, the nucleus of plant cells and chloroplasts of many algae and hornworts contain photobodies (photoreceptor-containing nuclear bodies) and pyrenoids (specific organelles associated with the operation of a carbon-concentrating mechanism), respectively. Abbreviations: TAM bodies, temporal asymmetric mitochondrial RNA processing bodies; GW/P bodies, glycine- and tryptophan-rich cytoplasmic bodies also known as processing (P) bodies.

Box 4. Importance of Intrinsic Disorder for PMLO Biogenesis

(i) All proteins shown so far to undergo LLPTs are either IDPs or hybrid proteins containing ordered domains and long IDPRs [87].
(ii) Many PMLO-related proteins are highly disordered, suggesting that the presence of intrinsic disorder represents an important property that defines the ability of a protein to undergo LLPT and to be related to the PMLO biogenesis [89].
(iii) IDPs capable of LLPT and thereby related to PMLO biogenesis are characterized by high conformational flexibility, sequence simplicity connected with the enrichment in specific residues, multivalency based on the abundant presence of repetitive units in the form of alternating oppositely charged blocks, or in the form of repetitive donor–acceptor (or ligand–receptor) units connected by flexible linkers, and accessibility to various post-translational modifications [85,112,113].
(iv) IDPs/IDPRs can be engaged in polyelectrostatic interactions, where, instead of presenting discrete charges, rapidly interconverting and diverse conformers found within the conformational ensembles of IDPs/IDPRs create mean electrostatic fields that are used for polyelectrostatic attraction [114].
(v) The lack of a stable structure of IDPs involved in LLPTs defines the stability and resilience of the phase-separated droplets [87].

RNAs, these liquid droplets are edge of chaos systems, constituents of which are interdependent edge of chaos systems characterized by high spatiotemporal heterogeneity [63,94]. Biogenesis of PMLOs relies on the nonlinear interactions of their components and can be easily distorted by small environmental perturbations (e.g., the efficiency of PMLO formation can be modulated by various post-translational modifications [95]). LLPTs are not accompanied by significant structural changes in the assembling proteins, which remain mostly disordered [92]. In fact, PMLOs are held together by a multitude of weak nonspecific interactions [71], which can be exemplified by a **polyelectrostatic model** [96] where multiple charged groups of one protein stochastically and dynamically palpate the oppositely charged groups of another protein (or RNA) via the spatially distributed long-range polyelectrostatic interactions . Such polyelectrostatic interactions between mostly disordered and highly solvated entities generate enormous interfaces, which are dynamic and mostly wet, since polyelectrostatic attractions via mean electrostatic fields do not expel much water. This generates in PMLOs an overcrowded environment [87] with unique solvent properties of water [97] that may have an additional role in PMLO biogenesis and contribute to the specific **partitioning** of various solutes to and from PMLOs. For example, as an extreme case, interfaces might include entire molecules, such as when nucleophosmin (NPM1) undergoes LLPS via homotypic interactions between its polyampholytic IDPRs or when it utilizes its multivalent arginine-rich linear motifs (R-motifs) for heterotypic interaction with RNAs when they phase separate to form granular component regions within the nucleolus [98].

The edge of chaos status of PMLOs can also define their overall resilience and insensitivity to small changes in their composition [71]. Here, the lack of fixed structure in IDPs involved in the formation of phase-separated droplets might be related to the stability of these droplets, since the resilience of fluid complexes made of flexible constituents is different from the stability of rigid complexes formed by rigid blocks via specific and high-affinity block–block interactions. This can be visualized by comparing the resilience of a bowl of noodles (PMLO) and a brick wall: ‘taking a few or even just one brick can lead to the collapse of the wall, whereas a bowl of noodles remains a bowl of noodles even after many noodles are eaten’ [71].

Finally, some PMLOs even have a role in the cellular memory. For example, in budding yeast, in response to the unproductive exposure to mating pheromone (i.e., when no successful mating takes place in a reasonable amount of time), a pheromone-refractory state is initiated, allowing cells to resume vegetative proliferation [99]. In this pheromone-refractory state, the G1/S inhibitor RNA-binding protein Whi3 is inactivated due to the formation of superassemblies, allowing cells to escape from the pheromone-induced cell cycle arrest and resume proliferation. Although such a Whi3 superassembly-driven pheromone-refractory state is stable for many cell cycles, it

is present in mother cells only and is not inherited by daughter cells [99]. Another example of cellular memory-related activities of PMLOs is given by P-bodies. In fact, although the major function of P-bodies is to serve as a specific compartment where most of the mRNA metabolism (e.g., decapping and degradation of translationally repressed mRNAs and miRNA-associated gene silencing) takes place [100], these PMLOs can also serve as storage depots for the translationally silent mRNAs that are capable of re-entering the translation machinery in response to changes in cellular conditions [101].

Moving Means Alive, Not Moving Means Dead

One should keep in mind that physical, dynamic, and mechanical properties of PMLOs vary broadly. Many PMLOs are highly dynamic, liquid-like droplets [61–68], whereas others are less dynamic, almost nondynamic, biomolecular condensates or bioreactive gels (e.g., Balbiani bodies, centrosomes, nuclear pores, and amyloid bodies), showing material states that span from viscous liquids to gels and even to the solid-like state seen in functional amyloids [102]. Importantly, the biogenesis of nondynamic biomolecular condensates or bioreactive gels starts with the formation of dynamic, liquid-like droplets that quickly mature into less dynamic structures [102]. Furthermore, it appears that the normally liquid-like PMLOs (e.g., stress granules) are able to mature or age into a less dynamic state typically coinciding with the formation of fibrous structures [103].

Although such maturation can be related to the biological need of the cell to produce structures with varying physical properties [103], aberrant PMLO biogenesis and abnormal aging of liquid-like RNP droplets can be accompanied by the misfolding and pathological aggregation of PMLO-residing IDPs/IDPRs, related to the pathogenesis of various human diseases, such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, and cancer [91,104,105]. Therefore, for a typical liquid PMLO, there is a specific time and condition window of safe existence that defines the biogenesis of functional RNP droplets; outside of this window, the pathological conversion from a liquid to a solid form might happen. This conversion can be triggered by spending more time in the condensed phase than required for the safe existence of the PMLO, or by an increase in the protein concentration, or by a pathological mutation [105]. In other words, in terms of liquid PMLOs, one can clearly see the validity of the 'moving means alive, not moving means dead' concept. Here, the presence of highly dynamic structure defines functional (alive) PMLOs, whereas the transition of supposed-to-be-fluid PMLOs to solid, nondynamic forms defines their functional death and even can be associated with triggering the development of cell death and disease.

Concluding Remarks and Future Perspectives

There is a remarkable similarity in the historical perception of IDPs and PMLOs by the scientific community. Both phenomena were first ignored and considered as artifacts because of their contradiction of the established paradigms (wherein protein functionality is defined by the presence of a unique structure and organelles are membrane-encapsulated cellular compartments that can be isolated and characterized), but then became a commonly accepted reality. This acceptance of the existence of intrinsic disorder and the associated ability of a protein to undergo liquid–liquid phase transitions clearly brought about a new level of complexity. At first, the complexity of IDPs and PMLOs appeared overwhelming. In fact, the modes of their action appeared to be in conflict with general logic. However, consideration of IDPs/IDPRs and PMLOs as edge of chaos systems helps to recognize the roots of their complexity and, therefore, provides a means to better understand their mechanics.

Synergetics, a science aimed at elucidating the universal mechanisms of the organization and functionality of systems of various nature, predicts that, to be realizable, complex systems must

Outstanding Questions

How can the better integration of modern biology and physics to study biological systems be achieved?

How can the biological soft matter concept be utilized in the future development of protein science?

What can one learn from considering proteins as edge of chaos systems?

What structural, functional, and dynamic properties of proteins can be explained by the edge of chaos nature of IDPs/IDPRs?

Besides the biogenesis of known PMLOs, what other cellular processes and structures can be attributed to the emergent behavior of IDPs/IDPRs?

How many different PMLOs are there?

What are the actual biological roles of PMLOs and the actual functional advantages of LLPTs leading to the formation of various PMLOs?

Can activities ascribed to PMLOs be conducted by their components in the disassembled state?

What are the actual solvent properties of aqueous media inside PMLOs and how can these properties be analyzed?

How can the partitioning of various molecules in and out of PMLOs be studied?

What are the roles of driver proteins beside their direct involvement in PMLO biogenesis?

Are there specific protein disassemblers that can initiate the disintegration of PMLOs?

Can specific small molecules be designed to control LLPT and PMLO biogenesis?

What are the differences between physiological and pathological LLPTs and normal and harmful PMLOs?

Can pathological PMLOs be converted back to physiological cellular bodies, and is there a specific time frame for such a conversion?

be simply organized. Universality and organizational simplicity of the complex systems define their cognizance [106]. In relation to the living matter, these considerations indicate that the organizational principles of PMLOs, the existence of some of which was already known two centuries ago, and the organization of intracellular space may become understandable by incorporating knowledge from proteins and polymer physics. All PMLOs are formed by disordered biopolymers, such as IDPs/IDPRs and, often, RNAs. The variety of PMLOs and their functions are defined by the variety of PMLO-forming IDPs. The formation of all PMLOs is based on a general principle rooted in the liquid–liquid phase separation of the polymers under crowded conditions. Therefore, the current status of the life sciences clearly indicates that the success of modern biological science relies on interdisciplinary research focused on the synthesis of knowledge from various scientific fields and on the incorporation and spread of natural science, or physical, approaches to life sciences. Obviously, biological soft matter concept is in its infancy, and further research is needed to answer multiple important questions (see Outstanding Questions) to bring it to maturation.

Can LLPTs and PMLOs be used as potential drug targets?

Is it safe to target LLPTs and PMLOs?

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