Emergent functions of proteins in non-stoichiometric supramolecular assemblies

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Abstract

Proteins are the basic functional units of the cell, carrying out myriads of functions essential for life. There are countless reports in molecular cell biology addressing the functioning of proteins under physiological and pathological conditions, aiming to understand life at the atomistic-molecular level and thereby being able to develop remedies against diseases. The central theme in most of these studies is that the functional unit under study is the protein itself. Recent rapid progress has radically challenged and extended this protein-function paradigm, by demonstrating that novel function(s) may emerge when proteins form dynamic and non-stoichiometric supramolecular assemblies. There is an increasing number of cases for such collective functions, such as targeting, localization, protection/shielding and filtering effects, as exemplified by signaling complexes and prions, biominerals and mucus, amphibian adhesions and bacterial biofilms, and a broad range of membraneless organelles (bio-condensates) formed by liquid-liquid phase separation in the cell. In this short review, we show that such non-stoichiometric organization may derive from the heterogeneity of the system, a mismatch in valency and/or geometry of the partners, and/or intrinsic structural disorder and multivalency of the component proteins. Either way, the resulting functional features cannot be simply described by, or predicted from, the properties of the isolated single protein(s), as they belong to the collection of proteins.

Keywords

emergent function, dynamic assembly, biomolecular condensate, contextual function, intrinsically disordered protein, phase separation, membraneless organelle

Introduction

Proteins are protagonists of life. They carry out a broad diversity of functions, from catalyzing chemical reactions and transporting key metabolites to recognizing signaling molecules and regulating gene expression. What they do is described by their "function", a term that has been used and abused excessively in the literature. Actually, the function of a protein can be approached at different levels, such as describing the chemical reaction, e.g. catalysis or binding, it carries out (molecular function, MF), or the cellular process it takes part in (biological process, BP) [1]. The first is usually approached by characterizing the protein in the test tube, whereas the second can be described by manipulating the protein in cellular studies [2]. Ingenious high-throughput "omics" approaches are also at our disposal for these various endeavors [3, 4].

Our general notion in approaching the function of an isolated protein is the structure-function paradigm, which describes the function of a protein at the atomistic level by its structure. It is underlined by more than 100k structures in the Protein Data Bank [5], also supported by high-throughput omics (structural genomics) programs [6]. Its descriptive power is also attested by the success of rational structure-based approaches of drug design [7]. The rather recent discovery of intrinsically disordered proteins/regions (IDPs/IDRs) does not contradict, only extends, this paradigm, suggesting that function can also emanate from a highly dynamic conformational ensemble, devoid of a well-defined, dominant structure [8, 9].

The functional insight extracted from structures is also important in appreciating the cellular (BP) function of a protein, which can be considered as a contextual (cellular or organismal) readout of the MF it carries out. In this sense, the BP function of a protein is an "emergent" property, which is only realized in the complex cellular environment. It follows from this caveat that the "function" of exactly the same protein may actually be different in different cells or cell states.

The broad assumption in all these considerations is that a well-defined function can be ascribed to each protein. This is, however, a definite oversimplification, as many proteins have more than one functions, at both levels [10, 11]. A simple functional variant may result from switching the function of the protein by post-translational modification or alternative splicing, but true multifunctionality (e.g. gene sharing, moonlighting) is also witnessed when the protein has the capacity to do completely different things in the cell. In retrospect, this is of no great surprise as many proteins have complex domain organization, and/or are capable of interacting with hundreds of interaction partners [4], i.e., it would appear rather naïve to assume that they always do the same in all different molecular settings. It is of note, though, that diversity of functions can also be the sign of the lack of absolute specificity, i.e. promiscuity [12].

Either way, whether well-defined or promiscuous, we traditionally ascribe function(s) to individual proteins, i.e., consider them as the functional units of the cell. This view, however is somewhat challenged by functional complexes, which form a unit, the function of which cannot be ascribed to any of its constituent subunits. To name just one example, the anaphase promoting complex (APC) has 13 subunits in human cells and functions as an E3 ubiquitin ligase that marks proteins for degradation in conjunction with the progression of cell cycle [13]. Its emergent function cannot be simply derived from that of its subunits, it only arises when the complex is fully assembled, i.e., is a collective property of the proteins arranged in a particular way in time and space. While our knowledge may be insufficient to predict it from those of its building blocks, we can at least rationalize their assembly and collective functioning on structural means, by assuming their sequential assembly from structurally complementary building blocks [14]. Thus, according to the

traditional view, the functional unit of life is the protein, either in itself or as part of a multi-subunit complex with a well-defined stoichiometry and structure.

In this review we survey a variety of recent results showing that physical attraction can also drive the assembly of multidomain and ID proteins into higher-order non-stoichiometric assemblies [15, 16]. As defined through common features of the diverse examples shown, non-stoichiometric assemblies are dense entities made of hundreds or thousands of protein (and other components) confined in space by multivalent interactions, lacking internal symmetry and strict number and/or stoichiometry of components. In these, the collective action of molecules convey a broad range of emergent functions [17, 18]. These assemblies are usually very dynamic and can have different structural states ranging from highly ordered structures (amyloids, prions), through open and extendable complexes (signaling complexes, signalosomes) to liquids/hydrogels and biominerals (assemblages, bio-condensates, liquid droplets, membraneless organelles (MOs), adhesive secretomes). By describing these various systems, we illustrate this emerging and probably general principle, and also point out that similar physicochemical phenomena have already been observed and described many times in other areas, such as surface chemistry [19], soft-matter physics [20], colloid chemistry [21] and even in membrane biology [22] and cell biology [23]. In these analogous systems, it is also generally accepted that the formation of such assemblies entails the appearance of emergent features, not apparent at the level of isolated components. Through all these examples and functional considerations, we purport that the collective functioning of dynamic protein assemblies represents a novel, general paradigm in molecular cell biology.

Amyloids, prions and signalosomes

Perhaps the simplest structural manifestation of this principle is the appearance of amyloids and prions, which may be considered as supramolecular signaling complexes [24]. Whereas traditionally thought of as pathological self-organizing protein assemblies, we now have many examples of "physiological" prions, which derive their altered functional state from their extended orderly assembled state termed amyloid (Figure 1Aa). Its structure is most often a β-sheet extended indefinitely in one dimension, composed of perpendicular β-strands [25]. In the amyloid core, there is a characteristic tight packing with self-complementing "steric zipper" interactions (Figure 1Ab), from which the solvent is excluded and is supported by an extended H-bond network between side chains [26].

Amyloids, pathological and physiological prions

Amyloids/prions are traditionally thought of as pathological entities, as exemplified by the human prion protein, or amyloidogenic proteins associated with neurodegeneration, such as alpha-synuclein in Parkinson's disease or tau protein in Alzheimer's disease [24, 27]. The self-stimulated transition to an altered structural and functional state, and the emergent functional properties of the amyloid are also harnessed for functional purposes in cells [28]. This phenomenon is most extensively characterized in yeast, as exemplified by Ure2, which can promote the uptake of poor nitrogen sources [29] and Sup35, which in the prion state confers a new phenotype on cells by suppressing stop-codons and facilitating translation readthrough [30]. In higher organisms, a novel function emerging from the amyloid state explains the functioning of the neuron-specific cytoplasmic polyadenylation-element binding protein (neuronal CPEB) in the marine snail *A. californica*, involved in memory formation [31]. In human, the Pmel17 protein forms fibrous striations inside melanosomes to support melanin granule formation [32]. Also, the receptor-interacting serine/threonine-protein kinases 1 and 3 (RIP1 and RIP3) form a heterodimeric, fibrillar functional amyloid signaling complex mediating tumor necrosis factor-induced programmed cell necrosis. The amyloid state of the RIP1/3 homotypic interaction motifs is a prerequisite for function, as mutations affecting their amyloid core compromise kinase activation and subsequent necrosis [33].

In all these cases, the function of prions/amyloids differs from that of their components, and may appear due to the suppression of the function of associated functional domains, or form the emergent physicochemical properties of the amyloid itself. Furthermore, in case of amyloids the degree and extent of supramolecular organization can also influence the functional readout, since while the conversion of a peptide/protein into amyloid state creates a novel pathological or functional entity, further assembly of the fibrils or precursor oligomers into large assemblies often neutralizes their toxicity, leading to an additional functional state [34-36].

Signalosomes and signaling complexes

Complex multidomain proteins feature in the formation of other kinds of functional, non-stoichiometric supramolecular condensates, which appear in signal transduction. These signaling complexes are very large, open-ended assemblies of lose structural organization and composition [37, 38]. As probably follows from their ill-defined stoichiometries, these signalosomes are very dynamic, they quickly respond to signaling cues by extension, contraction and morphological rearrangements and shedding or incorporating novel components, often linked with signaling post-translational modifications [37].

A prime example of such higher-order signaling assemblies is exemplified by the head-to-tail polymerization of signaling proteins (Figure 1B) containing a Phox and Bem1 (PB1), dishevelled (Dvl), homologous (DIX), and sterile alpha motif (SAM) domains [39]. These are encountered in distinct signaling processes, such as autophagy, ephrin signaling and the Wnt signaling pathway. The primary functional outcome of their dynamic and extendable, often competing domain-domain associations results from high local concentrations and the ensuing rapid signal propagation, also effectively showcasing enzyme-substrate targeting, specificity and substrate selection and signal amplification [37, 38].

Even more complex and heterogeneous organization appears in postsynaptic densities (PSD) in neurons [40, 41]. Excitatory (glutamatergic) synapses in the mammalian brain are usually localized on dendritic spines, where a very high concentration of receptors, scaffolds, signaling proteins and cytoskeletal elements assemble into an organized electron-dense structure, PSD (Figure 1C, 1D). The major organizational proteins of PSD are large multidomain scaffold proteins, such as PSD95, which engage in multivalent domain-domain and domain-motif interactions with many other proteins. Anchored to this scaffold are receptors and channel proteins (e.g. glutamate-activated NMDA receptor, AMPA receptor, K^+-channel), signaling enzymes (e.g., Ca^{2+}-CaM dependent kinase II (CaMKII), Fyn Src kinase, neuronal nitric oxide synthase…), their anchoring proteins (e.g. AKAP, GKAP, Shank, etc…), cytoskeletal proteins (e.g. actin, cortactin), and other signaling proteins (e.g. synGAP, SPAR, IRSp53, etc…) (cf. Figure 1C).

Due to its complexity, PSD has multiple emergent properties, such as large ion fluxes, effective channel regulation by localized modifying enzymes, and localized processing of signaling information [41]. Co-localization of enzymes with their substrates (such as receptors and channels) provides specificity of reactions and modifications (cf. next section and Figure 2). A further emergent function of physical origin, metaplasticity, has also been proposed for PSD [42]. In short, plasticity of synaptic communication means the general ability of the nervous system to change synaptic strength, primarily by PTM-altered activity of synaptic receptor ion channels, such as the NMDA channel [43]. It is much less appreciated that plasticity itself is also plastic, i.e. the prior history of the synapse changes its ability to undergo plastic changes. As a highly emergent function of the PSD, it was suggested that metaplasticity results from the extremely high local concentration of autophosphorylated CaMKII molecules, which generates a local electrostatic field high enough to affect membrane potential and alter the direction of synaptic plasticity [42].

Liquid-liquid phase separation

Organelles are mesoscale compartments within cells that fulfil a specific function and are separately enclosed either within their own lipid bilayer (membranous organelles) or by phase boundaries (membraneless organelles) [44]. Membraneless organelles (MOs) are non-stoichiometric supramolecular condensates that largely influence the normal operation [45, 46] as well as stress tolerance of living cells [15]. Although several of them have been known to exist for a very long time (e.g. the nucleolus, nuclear speckles or the Balbiani body), it has been only very recently recognized that they form without membranes through liquid-liquid phase separation (LLPS), a process driven by multivalent weak interactions between IDPs/IDRs [47-49]. As described by classical polymer theories [50], polymers above a critical concentration may separate from solution to form a polymer-rich and solvent-rich phase by LLPS. On an atomic scale, protein LLPS can be achieved through distinct, and most often co-occurring mechanisms [16, 51]: simple coacervation of hydrophobic residues, cation-pi interactions between G/SYG/S, RG-GR and FG-GF motifs, electrostatic attraction and repulsion between regions of alternating charge patterns, or multivalent domain-motif interactions. Such supramolecular condensates confer a wide range of functional advantages on cells [52] due to their unique material properties and responsiveness [44]: they are highly diverse in physical properties, shape, size, viscosity, molecular composition, subcellular location and functions [15, 52]. They may contain hundreds of different proteins and thousands of mRNAs, as shown in omics analyses of the composition of stress granules and P-bodies [53, 54]. Their assembly can be dictated by dedicated regions of one or a few constitutive components, the drivers of LLPS (scaffolds) [55]. For example, PML is the only protein essential for PML nuclear body formation [56], and spindle-defective protein 5 (SPD-5) is sufficient for the formation of centrosomes in *C. elegans* [57]. Often, scaffolds can drive LLPS on their own, and recapitulate many features of the given condensate. Critical for their emergent biological function, however, is the recruitment of associated proteins (clients), which cannot phase separate on their own, but critically contribute to the functional output of the condensate. As outlined next, these emergent functions can fall into five broad types, which often appear in combination in the functioning of a particular organelle.

1) Bioreactors: increasing reaction kinetics through proximity effects

Formation of a phase boundary through LLPS reflects a special way of concentrating molecules (e.g. enzymes and their substrates) in one place in a cell, leading to optimal concentrations, stoichiometry and orientation for reactions/interactions to occur (Figure 2).

Most MOs hitherto described function as such reactors. Examples include nucleoli that condense the players of ribosome biogenesis [58] and P-bodies that condense the factors required for mRNA degradation [53]. PSDs [59] and signalosomes/membrane clusters [60] described before harness the same operational principle. A less well-known example is miRISC, a multi-protein assembly for microRNA-mediated mRNA repression. The condensation of the two core components of human miRISC, Argonaute2 (Ago2) and TNRC6B, into phase-separated droplets facilitates accelerated deadenylation of target RNAs bound to Ago2 [61]. Also, in *C. elegans*, efficient RNA silencing requires small-RNA amplification mediated by RNA-dependent RNA polymerases. This is facilitated by the perinuclear germline Mutator foci that assemble through LLPS of the C-terminal IDR of the Mutator complex protein MUT-16. MUT-16 functions as a scaffold, bringing together many other proteins required for small-RNA biogenesis and amplification [62].

Another example is the coacervation of ZNF207/BuGZ, which induces microtubule bundling and concentrates tubulin, promoting microtubule polymerization and assembly of spindle and spindle matrix by concentrating its building blocks [63]. Within animal cells, microtubule arrays are organized by the centrosome that comprises two centrioles surrounded by an amorphous protein mass called pericentriolar material (PCM). In *C. elegans*, PCM assembly requires SPD-5, a coiled-coil protein that forms micrometer-sized porous networks *in vitro*. Only this assembled network, but not the unassembled SPD-5 protein, functions as a scaffold for PCM client proteins, including microtubule-associated proteins that recruit tubulin and form microtubule asters [57].

2) Biomolecular filters: selective recruitment of macromolecules

Macromolecular constituents of MOs are never randomly selected from the cell. Different MOs selectively recruit certain macromolecules, while exclude others, i.e., they act as biomolecular filters (Figure 2).

This function is best showcased by the hydrogel-like mesh filling the central transport channel of the nuclear pore complex (NPC). The mesh is formed by ID phenylalanine-glycine-rich nucleoporins (FG-Nups) and acts as a permeability barrier, ensuring critical selective control for nucleocytoplasmic transport. While small molecules (<40 kDa) can cross the pore by passive diffusion, the passage of larger molecules needs to be facilitated by nuclear transport receptors (NTRs), which are selectively recognized by FG-Nups and can guide cargos across the barrier [64].

Another example is provided by DDX4 liquid droplets that were demonstrated to exclude chromatin and double-stranded DNA and RNA, while selectively partitioning single-stranded DNA and RNA with a preference for structured RNAs (hairpins and regulatory RNAs) [65]. P-bodies and stress

granules also recruit transcripts selectively, since only a well-defined subset of the transcriptome could be identified within them [53, 54].

3) Regulators of spatial patterns: maintenance of large concentration gradients in the cytoplasm

By stabilizing concentration gradients of selected macromolecules in particular regions of cells (Figure 2), LLPS processes are also well-suited for facilitating the formation and maintenance of unique spatial patterns [66]. For example, the adhesion receptor Nephrin undergoes LLPS through multivalent interactions with its cytoplasmic partners, Nck and N-WASP [67], which leads to the grouping of the receptors into micrometer-sized clusters on the membrane surface. In the presence of the Arp2/3 complex, these receptor clusters promote local actin assembly at membranes [68], thus also functioning as reactors.

Probably the most penetrating example of this behavior is the development of cell polarity in asymmetric cell divisions. In *D. melanogaster* neuroblasts, the cell-fate determinant Numb undergoes LLPS through multivalent domain-motif interactions with Pon, to form a basal crescent that then segregates into the basal daughter cell to shape its differentiation [69]. In *C. elegans*, RNA- and protein-rich P-granules show increased condensation at the posterior end of the one-cell embryo that will form the first germ cell after cell division [70]. This spatial patterning of P-granules is achieved by opposing concentration gradients of two polarity proteins: MEG-3, which forms the granules through RNA-induced phase separation, and MEX-5, a competitor of MEG-3 for binding RNAs [71]. These observations suggest that such phase transitions could represent a general mechanism for creating and maintaining spatial patterns within cells [66], like receptor clusters [68] and cell polarity [70].

4) Biomolecular shields: shielding viral macromolecules from the immune response

Phase-separated condensates are also capable of hiding recruited macromolecules so that those could not be recognized by the ones excluded (Figure 2).

RNA viruses replicate in the hostile environment of the host cytoplasm, as they are exposed to the immune system and also to RNAses ready to degrade viral RNA. That is, the virus has to juggle the subsequent steps of unpacking, replicating and re-packing its RNA genome without providing access to host enzymes, which they achieve by concentrating and segregating their replication machinery within specialized compartments. The liquid-like replication compartments of vesicular stomatitis virus form by phase separation of 3 viral proteins required for replication [72]. For example, the

replication and assembly of Mononegavirales occurs in specialized intracellular compartments known as viral factories, viral inclusions or viroplasms. For rabies virus, these viral inclusions are called Negri bodies that also show characteristics similar to those of liquid organelles [73]. These condensates not only ensure favorable local concentrations of the components for enzymatic reactions, they also shield viral RNA from detection by cytosolic pattern-recognition receptors mediating cellular antiviral response [72, 73].

5) Reservoirs: temporary storage of macromolecules

Phase-separated MOs are also frequently employed for transiently storing select macromolecules in an inactive, dormant state; dissolution of such reservoirs upon a particular cellular signal enables then the rapid restoration of the halted functions (Figure 2).

For example, to survive stressful conditions, cells have evolved stress response pathways that arrest the cell cycle, re-adjust cell metabolism and upregulate stress-protective factors. Stressed eukaryotic cells form stress granules (SGs) that concentrate stalled translation preinitiation complexes of selected transcripts and mRNAs released from polysomes [54]. Budding yeast and bacteria employ a related stress-adaptive strategy that involves changes in the physical state of the cytoplasm, from a fluid to a protective, solid-like state, on energy-depletion. In budding yeast, the RNA-binding SG protein Pub1 forms condensates upon starvation or heat stress that is associated with cell-cycle arrest. While starvation-induced Pub1 condensates form by LLPS and subsequently convert into reversible gel-like particles, heat-induced condensates are more solid-like and require chaperones for disaggregation [74]. In Drosophila S2 cells amino-acid starvation leads to the inhibition of protein transport through the secretory pathway, and to the formation of reversible stress assemblies, so called Sec bodies, which incorporate components of the ER exit sites. They require Sec23 and Sec24AB Sec for their formation, they have liquid droplet-like properties, and act as a protective reservoir for ERES components to rebuild a functional secretory pathway after re-addition of amino-acids [75].

Heterochromatin is a compacted state of chromatin, wherein unused genes are kept in a repressed state. Heterochromatin protein 1 alpha (HP1α) binds to chemical modifications on histones that mark transcriptionally silent regions of chromatin and packs such regions together into liquid droplets through LLPS. The dynamic properties and selectivity of this liquid-like state could explain how heterochromatin stays accessible for DNA repair [76].

Protective reservoirs are also useful to store cell-cycle components when they are not needed. For instance, unphosphorylated CPEB4 phase separates into inactive, liquid-like droplets through its N-

terminal IDR. In contrast, phosphorylation by ERK2- and Cdk1 on several residues additively activate CPEB4 by pushing the equilibrium towards its monomeric state in M-phase, required for cytoplasmic polyadenylation and meiotic progression [77].

Extracellular condensation of secreted proteins: biominerals, mucus, adhesive secretions and bacterial biofilms

Secretion and subsequent large-scale assembly of proteins in the extracellular space is a prevalent strategy for the formation of a protective physical layer used in all domains of life, as demonstrated through a few illustrative examples.

Biomineralization: hard tissues of inorganic minerals for a variety of purposes

Biomineralization is a generic process of making an orderly crystalline structure of proteins and inorganic minerals in tissues as diverse as bones and teeth in vertebrates, egg shell in aves and reptiles, and hard tissues in mollusks. Their common feature is that a few specific proteins serve as nucleators and regulate the growth of crystal nanostructures in a specific manner. The resulting tissues generally contain around 95% crystallized inorganic mineral and a small proportion of water and protein. A wide variety of structured and disordered proteins participate with each having their distinctive function in crystal formation and growth. While ordered domains such as tyrosinases, carbonic anhydrases, chitin-binding domains and Von Willebrand factor A are well-known coordinators of biocalcification, the also abundant IDRs are important for both the binding of inorganic ions or to serve as phosphorylation donors [78].

A convenient model for the study of biomineralization is the formation of sea urchin embryo skeletal element (spicule), and several recent observations highlight the importance of the self-assembling capacity of the participating proteins in this process (Figure 3).

The *S. purpuratus* spicule matrix protein SpSM30B/C was shown to form hydrogel-like structures that mediate amorphous calcium carbonate (ACC) crystal formation [79]. It was proposed that the hydrogel structure promotes the organization and growth of elongated single crystal calcite nanoparticles, while hydrogels are capable to form on the surface of already existing crystals, mediating textural changes that affect the properties of the mineral phase. The observed glycosylation pattern of the protein was suggested to affect the kinetics of phase transition.

Another sea urchin model protein, SpSM50, also forms hydrogels in a calcium-dependent manner, facilitating the formation of both vaterite and calcite nanoparticles [80]. It was suggested that the C-

terminal ordered CTLL domain serves as a mediator of protein-protein or protein-mineral interactions, while the repetitive, disordered N-terminal part of the protein destabilizes its internal structure and improves its overall self-association propensity [80]. The same behavior was observed for other nacre and spicule matrix hydrogels [81, 82], underlining the general importance of hydrogel formation as a regulatory step in biomineralization. Whereas self-assembly is generally accompanied by an elevation of secondary structure propensities, as shown in the case of nacre framework protein n16 [83], in other cases, like PFMG1 [82], a significant amount of disorder is retained in the assembled state. Amelogenin, the major protein component of mammalian tooth enamel also adopts a mostly disordered structure in vitro but shows a prominent alpha-helical tendency on the addition of TFE, which also induces self-assembly of the protein [84].

The currently accepted model for bionucleation is the polymer-induced liquid precursor (PILP) phase system [85], which describes nucleation process as a liquid-liquid phase separation mediated by disordered polymers binding to different solutes, resulting in a metastable precursor (Figure 3). These amorphous precursors were indeed shown to occur in vivo [86, 87] and calcium containing nanospheres were directly observed during the development of sea urchin embryos [88], indicating the applicability of this model to living systems.

Mucus: a protective endothelial layer

Another example of extracellular protein associations is mucus, a viscous material found in many different metazoan species from corals and gastropods to humans. Their functions span a broad range, including filtering nutrients from the environment, aiding movement of gastropods, and providing protection against predators.

In vertebrates, where mucus covers all epithelial tissues in direct contact with the environment, the most important function of this hydrogel is to hydrate and lubricate the mucosal surfaces [89]. The main protein components of mucus hydrogels in humans are gel-forming mucins (GFMs). e.g. MUC2, MUC5A, and MUC5B, all of which contain D-domains on their N terminus and a cystine knot (CK) domain at their C terminus. These globular domains are capable of forming stable protein-protein interactions, thus facilitating the formation of higher order structures. MUC2, MUC5A and MUC5B also contain several repeats of Cys domains (named after their high cysteine content) in their central region. All GFMs are characterized by a long central region that is enriched in serine, threonine and proline residues (S/T/P region) and is heavily glycosylated [89], except for the Cys domains. Since Cys domains contain mostly hydrophobic residues, the alternating pattern of negatively charged glycosylated regions and Cys domains contribute to the reversible forces

between mucin proteins in the hydrogel [90]. The S/T/P region is shown to adopt an extended, disordered conformation that is indispensable for the elasticity of the mucus hydrogel [91]. The main driving force behind phase separation of GFMs is concentration [92], but other factors such as salt concentration, pH and the presence of other protein factors also play crucial roles in regulating structure and rheological properties of the mucus blanket [89]. In cystic fibrosis, mutation(s) in the cystic fibrosis transmembrane conductance regulator (CFTR) channel leads to electrolytic imbalance and the formation of the highly viscoelastic mucus, causing the disease [89].

Adhesive exudates: sticky gels for protection and predation

Assembled secreted protein(s) (termed exudates) also play interesting and important roles in invertebrates: they either use the adhesive, gluey material for protecting themselves against predators or attaching themselves to surfaces. For example, the Australian fossorial frog *N. bennetti* secretes from its dorsal skin a protein-rich liquid exudate when attacked by a predator, i.e. a snake [93]. Once outside, the protein solution rapidly forms a tacky elastic gel, causing discomfort to the predator. This behavior has also been observed in several other frogs [94]. Interestingly, larvae of the frog secrete a similar material that forms a cement, which is used by the embryos and early larvae to adhere to stationary supports [95]. Other amphibia, such as some salamander species, also rely on proteinaceous adhesive exudates to protect themselves [96].

The application of adhesive secretions may be an even broader, general strategy of invertebrates to attach themselves, their nests or their eggs to biological or mineral surfaces [97]. For example, the moth *O. eucalypti* uses a protein-based hydrogel to secure its egg [98]. At variance, some velvet worms eject a proteinaceous solution turning instantaneously gluey, to immobilize and capture their prey [99].

Bacterial biofilms: protection and colonization

Bacteria often form colonies stuck to each other and to biological and environmental surfaces. Their cells are embedded in a self-produced protein matrix of extracellular polymeric substance (EPS), often also termed biofilm [100]. The biofilm matrix can contain soluble, gel-forming polysaccharides, proteins and DNA, and also insoluble components such as functional amyloids or cellulose, which all impart their individual physical properties on the heterogenous and highly dynamic matrix. Interestingly, not only components of the biofilm give rise to the emergent properties of an artificial habitat, bacteria in biofilms also exhibit emergent properties that differ substantially from those of the free-living bacterial cells.

Specific and emergent structural and functional properties of the biofilm specify the characteristic features (emergent properties) of the colony within the biofilm, such as social cooperation, resource capture and enhanced resistance or tolerance to antibiotics and environmental stress. For example *E. coli* biofilms have desiccation tolerance, a group of proteins called hydrophobins secreted by *B. subtilis* form highly hydrophobic biofilms that float at the air–liquid interface, and in *B. subtilis* biofilms DNA from lysed cells is a source of phosphorus, carbon and energy [100].

Conclusions: principles and applications of bio-condensates

Through many distinct examples, we have shown that assemblies of proteins form in many biological settings and convey a very broad range of emergent properties. Importantly, the different possible functional (Figure 2) and structural (Figure 1) categories probably combine in many distinct concrete assemblies. Certain generalizations regarding the structural and atomistic background of the formation of such assemblies, nevertheless, can be made. Protein-protein interactions can be mediated by domains, motifs and residues, which can all lead to the formation of stoichiometric complexes but also to non-stoichiometric ones (Figure 4), if: i) multiple interacting partners cause heterogeneity of the system (Figure 4A), ii) there is a mismatch in the binding valency of the partners (Figure 4B), and iii) some partners have a high level of structural disorder (Figure 4C). The underlying physical interactions then lead to the formation of a non-stoichiometric and extendable assembly with completely novel, emergent properties. Whereas the noted features can be derived from indirect observations and considerations, basic challenges of this emerging field is to unravel the underlying structural determinants promoting the assembly process and to provide quantitative description of thermodynamics, kinetics and dynamics of the various functional states that arise. Although we have a battery of techniques to look into these details [101, 102], heterogeneity and diversity of the systems already reported will represent an enduring challenge for many years to come.

Understanding all the details of supramolecular assemblies is also of special interest because they display the potential of special applications in medical and material sciences. Hydrogels are excellent candidates for targeted drug delivery and tissue engineering, because they are biocompatible, highly responsive to their environment and their physical properties such as stiffness, mesh size and biodegradability can be fine-tuned through varying their components and applying cross-linking [103]. Hydrogels can encapsulate therapeutic agents, such as drugs [104], peptides, proteins [105] or nucleic acids [106], capable of releasing them in a controlled and gradual manner. This property was utilized for the delivery of taxol to human hepatocarcinoma tumor in mice [107], resulting in a stable

release and reduced toxicity. The same effect was observed with doxorubicin, where the hydrogel-encapsulated drug showed similar anti-tumor activity with less side-effects compared to the conventional application [108]. Hydrogels can also be applied to accelerate wound healing [109] and cardiac regeneration [110], whereas artificial mimicking of biomineralization is a promising field in regenerating tooth enamel [111] and bones [112]. One strategy is to encapsulate regenerative agents into hydrogels, like bone marrow-derived mesenchymal stem cells, and utilize them as 3D scaffolds for bone regeneration [113]. With the ever-growing insight into the molecular organization of the supramolecular structures participating in tissue regeneration and the formation of "smart interfaces" [114], artificial materials that are capable of communicating with and responding to their cellular environment seems soon to be within our reach.

First and foremost, however, the basic challenge lies with our ability to harness this novel paradigm of organization for a better understanding of cell biology. As primordial coacervation may have been instrumental in the emergence of life [115], and extant organelles can have as basic functions as decelerated aging of oocytes [116] or assembly of ribosomes [117], the importance of these cellular compartments can hardly be overstated. As studies aimed at their detailed description keep unrolling, we are entering one of the most exciting phase of our quest for the molecular understanding of life.

Legends of figures

Figure 1 *Amyloids, prions and signalosomes as non-stoichiometric signaling complexes*

There are various ways by which multidomain proteins can assemble into non-stoichiometric signaling complexes. (A) Prions form by the orderly assembly of amyloid-type structures, as shown schematically for the human prion protein (a) and by the high-resolution X-ray structure of the heptapeptide GNNQQNY of the physiological yeast prion Sup35 (b, based on PDB 1yjp, cf. [26]). (B) Head-to-tail organization of p62 autophagy scaffolding protein forms a signalosome, via its N-terminal PB1 domain forming a filamentous structure (adapted from ref. [38]). (C) Schematic diagram of the network of proteins in the PSD, with various groups of proteins color-coded. For simplicity, only a few key proteins are named and shown (adapted from ref. [41]). (D) Electron microscopic image of an excitatory glutamatergic synapse, displaying a prominent electron-dense zone, PSD, juxtaposed to the postsynaptic membrane (from ref. [40], Copyright (2008) National Academy of Sciences).

Figure 2 *LLPS results in different types of emergent functions*

Phase separation brings a large number of macromolecules (protein, RNA) in close proximity and/or physical contact, endowing five broad types of emergent functional features on them. Here we show them separately starting from the upper left corner and proceeding clockwise: 1) bioreactors (affecting catalytic efficiency and specificity by enzyme-substrate proximity and targeting), biomolecular filters (selecting molecules incorporated by size and chemical differences), regulators of spatial patterns (generating concentration gradients in cells), biomolecular shields (protection of content from outside effects, such as immune system of degrading enzymes), and reservoirs (temporarily storing components for later re-use), but in most of the cases they appear in combination in the functioning of a particular organelle.

Figure 3 *Crystal nucleation through phase separation as a mechanism of biomineralization*

Biomineralization, such as the formation of bone and tooth, egg shell and hard tissues of mollusks results from the orderly deposition of inorganic minerals by large, repetitive, mostly disordered proteins. Increasing evidence attests to a nucleation model of biomineralization (polymer-induced liquid precursor (PILP) phase), which describes the nucleation process as LLPS mediated by ID polymers binding to different solutes.

Figure 4 *Various assembly logics of non-stoichiometric assemblies*

All different processes leading to the formation of non-stoichiometric assemblies rely on proteins that can engage in multivalent interactions with each other. Various combinations of domain-domain, domain-motif and residue-residue interactions may lead to assemblies of distinct internal symmetry and dynamics, preventing the formation of stoichiometric complexes, due to heterogeneity multiple interacting partners, A), a mismatch in the binding valency of the partners (B), and/or a high level of structural disorder that remains even in the mutually bound state (C).

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Emergent functions of proteins in non-stoichiometric supramolecular assemblies

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Abstract

Proteins are the basic functional units of the cell, carrying out myriads of functions essential for life. There are countless reports in molecular cell biology addressing the functioning of proteins under physiological and pathological conditions, aiming to understand life at the atomistic-molecular level and thereby being able to develop remedies against diseases. The central theme in most of these studies is that the functional unit under study is the protein itself. Recent rapid progress has radically challenged and extended this protein-function paradigm, by demonstrating that novel function(s) may emerge when proteins form dynamic and non-stoichiometric supramolecular assemblies. There is an increasing number of cases for such collective functions, such as targeting, localization, protection/shielding and filtering effects, as exemplified by signaling complexes and prions, biominerals and mucus, amphibian adhesions and bacterial biofilms, and a broad range of membraneless organelles (bio-condensates) formed by liquid-liquid phase separation in the cell. In this short review, we show that such non-stoichiometric organization may derive from the heterogeneity of the system, a mismatch in valency and/or geometry of the partners, and/or intrinsic structural disorder and multivalency of the component proteins. Either way, the resulting functional features cannot be simply described by, or predicted from, the properties of the isolated single protein(s), as they belong to the collection of proteins.

Keywords

emergent function, dynamic assembly, biomolecular condensate, contextual function, intrinsically disordered protein, phase separation, membraneless organelle

Introduction

Proteins are protagonists of life. They carry out a broad diversity of functions, from catalyzing chemical reactions and transporting key metabolites to recognizing signaling molecules and regulating gene expression. What they do is described by their "function", a term that has been used and abused excessively in the literature. Actually, the function of a protein can be approached at different levels, such as describing the chemical reaction, e.g. catalysis or binding, it carries out (molecular function, MF), or the cellular process it takes part in (biological process, BP) [1]. The first is usually approached by characterizing the protein in the test tube, whereas the second can be described by manipulating the protein in cellular studies [2]. Ingenious high-throughput "omics" approaches are also at our disposal for these various endeavors [3, 4].

Our general notion in approaching the function of an isolated protein is the structure-function paradigm, which describes the function of a protein at the atomistic level by its structure. It is underlined by more than 100k structures in the Protein Data Bank [5], also supported by high-throughput omics (structural genomics) programs [6]. Its descriptive power is also attested by the success of rational structure-based approaches of drug design [7]. The rather recent discovery of intrinsically disordered proteins/regions (IDPs/IDRs) does not contradict, only extends, this paradigm, suggesting that function can also emanate from a highly dynamic conformational ensemble, devoid of a well-defined, dominant structure [8, 9].

The functional insight extracted from structures is also important in appreciating the cellular (BP) function of a protein, which can be considered as a contextual (cellular or organismal) readout of the MF it carries out. In this sense, the BP function of a protein is an "emergent" property, which is only realized in the complex cellular environment. It follows from this caveat that the "function" of exactly the same protein may actually be different in different cells or cell states.

The broad assumption in all these considerations is that a well-defined function can be ascribed to each protein. This is, however, a definite oversimplification, as many proteins have more than one functions, at both levels [10, 11]. A simple functional variant may result from switching the function of the protein by post-translational modification or alternative splicing, but true multifunctionality (e.g. gene sharing, moonlighting) is also witnessed when the protein has the capacity to do completely different things in the cell. In retrospect, this is of no great surprise as many proteins have complex domain organization, and/or are capable of interacting with hundreds of interaction partners [4], i.e., it would appear rather naïve to assume that they always do the same in all different molecular settings. It is of note, though, that diversity of functions can also be the sign of the lack of absolute specificity, i.e. promiscuity [12].

Either way, whether well-defined or promiscuous, we traditionally ascribe function(s) to individual proteins, i.e., consider them as the functional units of the cell. This view, however is somewhat challenged by functional complexes, which form a unit, the function of which cannot be ascribed to any of its constituent subunits. To name just one example, the anaphase promoting complex (APC) has 13 subunits in human cells and functions as an E3 ubiquitin ligase that marks proteins for degradation in conjunction with the progression of cell cycle [13]. Its emergent function cannot be simply derived from that of its subunits, it only arises when the complex is fully assembled, i.e., is a collective property of the proteins arranged in a particular way in time and space. While our knowledge may be insufficient to predict it from those of its building blocks, we can at least rationalize their assembly and collective functioning on structural means, by assuming their sequential assembly from structurally complementary building blocks [14]. Thus, according to the

traditional view, the functional unit of life is the protein, either in itself or as part of a multi-subunit complex with a well-defined stoichiometry and structure.

In this review we survey a variety of recent results showing that physical attraction can also drive the assembly of multidomain and ID proteins into higher-order non-stoichiometric assemblies [15, 16]. As defined through common features of the diverse examples shown, non-stoichiometric assemblies are dense entities made of hundreds or thousands of protein (and other components) confined in space by multivalent interactions, lacking internal symmetry and strict number and/or stoichiometry of components. In these, the collective action of molecules convey a broad range of emergent functions [17, 18]. These assemblies are usually very dynamic and can have different structural states ranging from highly ordered structures (amyloids, prions), through open and extendable complexes (signaling complexes, signalosomes) to liquids/hydrogels and biominerals (assemblages, bio-condensates, liquid droplets, membraneless organelles (MOs), adhesive secretomes). By describing these various systems, we illustrate this emerging and probably general principle, and also point out that similar physicochemical phenomena have already been observed and described many times in other areas, such as surface chemistry [19], soft-matter physics [20], colloid chemistry [21] and even in membrane biology [22] and cell biology [23]. In these analogous systems, it is also generally accepted that the formation of such assemblies entails the appearance of emergent features, not apparent at the level of isolated components. Through all these examples and functional considerations, we purport that the collective functioning of dynamic protein assemblies represents a novel, general paradigm in molecular cell biology.

Amyloids, prions and signalosomes

Perhaps the simplest structural manifestation of this principle is the appearance of amyloids and prions, which may be considered as supramolecular signaling complexes [24]. Whereas traditionally thought of as pathological self-organizing protein assemblies, we now have many examples of "physiological" prions, which derive their altered functional state from their extended orderly assembled state termed amyloid (Figure 1Aa). Its structure is most often a β-sheet extended indefinitely in one dimension, composed of perpendicular β-strands [25]. In the amyloid core, there is a characteristic tight packing with self-complementing "steric zipper" interactions (Figure 1Ab), from which the solvent is excluded and is supported by an extended H-bond network between side chains [26].

Amyloids, pathological and physiological prions

Amyloids/prions are traditionally thought of as pathological entities, as exemplified by the human prion protein, or amyloidogenic proteins associated with neurodegeneration, such as alpha-synuclein in Parkinson's disease or tau protein in Alzheimer's disease [24, 27]. The self-stimulated transition to an altered structural and functional state, and the emergent functional properties of the amyloid are also harnessed for functional purposes in cells [28]. This phenomenon is most extensively characterized in yeast, as exemplified by Ure2, which can promote the uptake of poor nitrogen sources [29] and Sup35, which in the prion state confers a new phenotype on cells by suppressing stop-codons and facilitating translation readthrough [30]. In higher organisms, a novel function emerging from the amyloid state explains the functioning of the neuron-specific cytoplasmic polyadenylation-element binding protein (neuronal CPEB) in the marine snail *A. californica*, involved in memory formation [31]. In human, the Pmel17 protein forms fibrous striations inside melanosomes to support melanin granule formation [32]. Also, the receptor-interacting serine/threonine-protein kinases 1 and 3 (RIP1 and RIP3) form a heterodimeric, fibrillar functional amyloid signaling complex mediating tumor necrosis factor-induced programmed cell necrosis. The amyloid state of the RIP1/3 homotypic interaction motifs is a prerequisite for function, as mutations affecting their amyloid core compromise kinase activation and subsequent necrosis [33].

In all these cases, the function of prions/amyloids differs from that of their components, and may appear due to the suppression of the function of associated functional domains, or form the emergent physicochemical properties of the amyloid itself. Furthermore, in case of amyloids the degree and extent of supramolecular organization can also influence the functional readout, since while the conversion of a peptide/protein into amyloid state creates a novel pathological or functional entity, further assembly of the fibrils or precursor oligomers into large assemblies often neutralizes their toxicity, leading to an additional functional state [34-36].

Signalosomes and signaling complexes

Complex multidomain proteins feature in the formation of other kinds of functional, non-stoichiometric supramolecular condensates, which appear in signal transduction. These signaling complexes are very large, open-ended assemblies of lose structural organization and composition [37, 38]. As probably follows from their ill-defined stoichiometries, these signalosomes are very dynamic, they quickly respond to signaling cues by extension, contraction and morphological rearrangements and shedding or incorporating novel components, often linked with signaling post-translational modifications [37].

A prime example of such higher-order signaling assemblies is exemplified by the head-to-tail polymerization of signaling proteins (Figure 1B) containing a Phox and Bem1 (PB1), dishevelled (Dvl), homologous (DIX), and sterile alpha motif (SAM) domains [39]. These are encountered in distinct signaling processes, such as autophagy, ephrin signaling and the Wnt signaling pathway. The primary functional outcome of their dynamic and extendable, often competing domain-domain associations results from high local concentrations and the ensuing rapid signal propagation, also effectively showcasing enzyme-substrate targeting, specificity and substrate selection and signal amplification [37, 38].

Even more complex and heterogeneous organization appears in postsynaptic densities (PSD) in neurons [40, 41]. Excitatory (glutamatergic) synapses in the mammalian brain are usually localized on dendritic spines, where a very high concentration of receptors, scaffolds, signaling proteins and cytoskeletal elements assemble into an organized electron-dense structure, PSD (Figure 1C, 1D). The major organizational proteins of PSD are large multidomain scaffold proteins, such as PSD95, which engage in multivalent domain-domain and domain-motif interactions with many other proteins. Anchored to this scaffold are receptors and channel proteins (e.g. glutamate-activated NMDA receptor, AMPA receptor, K^+-channel), signaling enzymes (e.g., Ca^{2+}-CaM dependent kinase II (CaMKII), Fyn Src kinase, neuronal nitric oxide synthase…), their anchoring proteins (e.g. AKAP, GKAP, Shank, etc…), cytoskeletal proteins (e.g. actin, cortactin), and other signaling proteins (e.g. synGAP, SPAR, IRSp53, etc…) (cf. Figure 1C).

Due to its complexity, PSD has multiple emergent properties, such as large ion fluxes, effective channel regulation by localized modifying enzymes, and localized processing of signaling information [41]. Co-localization of enzymes with their substrates (such as receptors and channels) provides specificity of reactions and modifications (cf. next section and Figure 2). A further emergent function of physical origin, metaplasticity, has also been proposed for PSD [42]. In short, plasticity of synaptic communication means the general ability of the nervous system to change synaptic strength, primarily by PTM-altered activity of synaptic receptor ion channels, such as the NMDA channel [43]. It is much less appreciated that plasticity itself is also plastic, i.e. the prior history of the synapse changes its ability to undergo plastic changes. As a highly emergent function of the PSD, it was suggested that metaplasticity results from the extremely high local concentration of autophosphorylated CaMKII molecules, which generates a local electrostatic field high enough to affect membrane potential and alter the direction of synaptic plasticity [42].

Liquid-liquid phase separation

Organelles are mesoscale compartments within cells that fulfil a specific function and are separately enclosed either within their own lipid bilayer (membranous organelles) or by phase boundaries (membraneless organelles) [44]. Membraneless organelles (MOs) are non-stoichiometric supramolecular condensates that largely influence the normal operation [45, 46] as well as stress tolerance of living cells [15]. Although several of them have been known to exist for a very long time (e.g. the nucleolus, nuclear speckles or the Balbiani body), it has been only very recently recognized that they form without membranes through liquid-liquid phase separation (LLPS), a process driven by multivalent weak interactions between IDPs/IDRs [47-49]. As described by classical polymer theories [50], polymers above a critical concentration may separate from solution to form a polymer-rich and solvent-rich phase by LLPS. On an atomic scale, protein LLPS can be achieved through distinct, and most often co-occurring mechanisms [16, 51]: simple coacervation of hydrophobic residues, cation-pi interactions between G/SYG/S, RG-GR and FG-GF motifs, electrostatic attraction and repulsion between regions of alternating charge patterns, or multivalent domain-motif interactions. Such supramolecular condensates confer a wide range of functional advantages on cells [52] due to their unique material properties and responsiveness [44]: they are highly diverse in physical properties, shape, size, viscosity, molecular composition, subcellular location and functions [15, 52]. They may contain hundreds of different proteins and thousands of mRNAs, as shown in omics analyses of the composition of stress granules and P-bodies [53, 54]. Their assembly can be dictated by dedicated regions of one or a few constitutive components, the drivers of LLPS (scaffolds) [55]. For example, PML is the only protein essential for PML nuclear body formation [56], and spindle-defective protein 5 (SPD-5) is sufficient for the formation of centrosomes in *C. elegans* [57]. Often, scaffolds can drive LLPS on their own, and recapitulate many features of the given condensate. Critical for their emergent biological function, however, is the recruitment of associated proteins (clients), which cannot phase separate on their own, but critically contribute to the functional output of the condensate. As outlined next, these emergent functions can fall into five broad types, which often appear in combination in the functioning of a particular organelle.

1) Bioreactors: increasing reaction kinetics through proximity effects

Formation of a phase boundary through LLPS reflects a special way of concentrating molecules (e.g. enzymes and their substrates) in one place in a cell, leading to optimal concentrations, stoichiometry and orientation for reactions/interactions to occur (Figure 2).

Most MOs hitherto described function as such reactors. Examples include nucleoli that condense the players of ribosome biogenesis [58] and P-bodies that condense the factors required for mRNA degradation [53]. PSDs [59] and signalosomes/membrane clusters [60] described before harness the same operational principle. A less well-known example is miRISC, a multi-protein assembly for microRNA-mediated mRNA repression. The condensation of the two core components of human miRISC, Argonaute2 (Ago2) and TNRC6B, into phase-separated droplets facilitates accelerated deadenylation of target RNAs bound to Ago2 [61]. Also, in *C. elegans*, efficient RNA silencing requires small-RNA amplification mediated by RNA-dependent RNA polymerases. This is facilitated by the perinuclear germline Mutator foci that assemble through LLPS of the C-terminal IDR of the Mutator complex protein MUT-16. MUT-16 functions as a scaffold, bringing together many other proteins required for small-RNA biogenesis and amplification [62].

Another example is the coacervation of ZNF207/BuGZ, which induces microtubule bundling and concentrates tubulin, promoting microtubule polymerization and assembly of spindle and spindle matrix by concentrating its building blocks [63]. Within animal cells, microtubule arrays are organized by the centrosome that comprises two centrioles surrounded by an amorphous protein mass called pericentriolar material (PCM). In *C. elegans*, PCM assembly requires SPD-5, a coiled-coil protein that forms micrometer-sized porous networks *in vitro*. Only this assembled network, but not the unassembled SPD-5 protein, functions as a scaffold for PCM client proteins, including microtubule-associated proteins that recruit tubulin and form microtubule asters [57].

2) Biomolecular filters: selective recruitment of macromolecules

Macromolecular constituents of MOs are never randomly selected from the cell. Different MOs selectively recruit certain macromolecules, while exclude others, i.e., they act as biomolecular filters (Figure 2).

This function is best showcased by the hydrogel-like mesh filling the central transport channel of the nuclear pore complex (NPC). The mesh is formed by ID phenylalanine-glycine-rich nucleoporins (FG-Nups) and acts as a permeability barrier, ensuring critical selective control for nucleocytoplasmic transport. While small molecules (<40 kDa) can cross the pore by passive diffusion, the passage of larger molecules needs to be facilitated by nuclear transport receptors (NTRs), which are selectively recognized by FG-Nups and can guide cargos across the barrier [64].

Another example is provided by DDX4 liquid droplets that were demonstrated to exclude chromatin and double-stranded DNA and RNA, while selectively partitioning single-stranded DNA and RNA with a preference for structured RNAs (hairpins and regulatory RNAs) [65]. P-bodies and stress

granules also recruit transcripts selectively, since only a well-defined subset of the transcriptome could be identified within them [53, 54].

3) Regulators of spatial patterns: maintenance of large concentration gradients in the cytoplasm

By stabilizing concentration gradients of selected macromolecules in particular regions of cells (Figure 2), LLPS processes are also well-suited for facilitating the formation and maintenance of unique spatial patterns [66]. For example, the adhesion receptor Nephrin undergoes LLPS through multivalent interactions with its cytoplasmic partners, Nck and N-WASP [67], which leads to the grouping of the receptors into micrometer-sized clusters on the membrane surface. In the presence of the Arp2/3 complex, these receptor clusters promote local actin assembly at membranes [68], thus also functioning as reactors.

Probably the most penetrating example of this behavior is the development of cell polarity in asymmetric cell divisions. In *D. melanogaster* neuroblasts, the cell-fate determinant Numb undergoes LLPS through multivalent domain-motif interactions with Pon, to form a basal crescent that then segregates into the basal daughter cell to shape its differentiation [69]. In *C. elegans*, RNA- and protein-rich P-granules show increased condensation at the posterior end of the one-cell embryo that will form the first germ cell after cell division [70]. This spatial patterning of P-granules is achieved by opposing concentration gradients of two polarity proteins: MEG-3, which forms the granules through RNA-induced phase separation, and MEX-5, a competitor of MEG-3 for binding RNAs [71]. These observations suggest that such phase transitions could represent a general mechanism for creating and maintaining spatial patterns within cells [66], like receptor clusters [68] and cell polarity [70].

4) Biomolecular shields: shielding viral macromolecules from the immune response

Phase-separated condensates are also capable of hiding recruited macromolecules so that those could not be recognized by the ones excluded (Figure 2).

RNA viruses replicate in the hostile environment of the host cytoplasm, as they are exposed to the immune system and also to RNAses ready to degrade viral RNA. That is, the virus has to juggle the subsequent steps of unpacking, replicating and re-packing its RNA genome without providing access to host enzymes, which they achieve by concentrating and segregating their replication machinery within specialized compartments. The liquid-like replication compartments of vesicular stomatitis virus form by phase separation of 3 viral proteins required for replication [72]. For example, the

replication and assembly of Mononegavirales occurs in specialized intracellular compartments known as viral factories, viral inclusions or viroplasms. For rabies virus, these viral inclusions are called Negri bodies that also show characteristics similar to those of liquid organelles [73]. These condensates not only ensure favorable local concentrations of the components for enzymatic reactions, they also shield viral RNA from detection by cytosolic pattern-recognition receptors mediating cellular antiviral response [72, 73].

5) Reservoirs: temporary storage of macromolecules

Phase-separated MOs are also frequently employed for transiently storing select macromolecules in an inactive, dormant state; dissolution of such reservoirs upon a particular cellular signal enables then the rapid restoration of the halted functions (Figure 2).

For example, to survive stressful conditions, cells have evolved stress response pathways that arrest the cell cycle, re-adjust cell metabolism and upregulate stress-protective factors. Stressed eukaryotic cells form stress granules (SGs) that concentrate stalled translation preinitiation complexes of selected transcripts and mRNAs released from polysomes [54]. Budding yeast and bacteria employ a related stress-adaptive strategy that involves changes in the physical state of the cytoplasm, from a fluid to a protective, solid-like state, on energy-depletion. In budding yeast, the RNA-binding SG protein Pub1 forms condensates upon starvation or heat stress that is associated with cell-cycle arrest. While starvation-induced Pub1 condensates form by LLPS and subsequently convert into reversible gel-like particles, heat-induced condensates are more solid-like and require chaperones for disaggregation [74]. In Drosophila S2 cells amino-acid starvation leads to the inhibition of protein transport through the secretory pathway, and to the formation of reversible stress assemblies, so called Sec bodies, which incorporate components of the ER exit sites. They require Sec23 and Sec24AB Sec for their formation, they have liquid droplet-like properties, and act as a protective reservoir for ERES components to rebuild a functional secretory pathway after re-addition of amino-acids [75].

Heterochromatin is a compacted state of chromatin, wherein unused genes are kept in a repressed state. Heterochromatin protein 1 alpha (HP1α) binds to chemical modifications on histones that mark transcriptionally silent regions of chromatin and packs such regions together into liquid droplets through LLPS. The dynamic properties and selectivity of this liquid-like state could explain how heterochromatin stays accessible for DNA repair [76].

Protective reservoirs are also useful to store cell-cycle components when they are not needed. For instance, unphosphorylated CPEB4 phase separates into inactive, liquid-like droplets through its N-

terminal IDR. In contrast, phosphorylation by ERK2- and Cdk1 on several residues additively activate CPEB4 by pushing the equilibrium towards its monomeric state in M-phase, required for cytoplasmic polyadenylation and meiotic progression [77].

Extracellular condensation of secreted proteins: biominerals, mucus, adhesive secretions and bacterial biofilms

Secretion and subsequent large-scale assembly of proteins in the extracellular space is a prevalent strategy for the formation of a protective physical layer used in all domains of life, as demonstrated through a few illustrative examples.

Biomineralization: hard tissues of inorganic minerals for a variety of purposes

Biomineralization is a generic process of making an orderly crystalline structure of proteins and inorganic minerals in tissues as diverse as bones and teeth in vertebrates, egg shell in aves and reptiles, and hard tissues in mollusks. Their common feature is that a few specific proteins serve as nucleators and regulate the growth of crystal nanostructures in a specific manner. The resulting tissues generally contain around 95% crystallized inorganic mineral and a small proportion of water and protein. A wide variety of structured and disordered proteins participate with each having their distinctive function in crystal formation and growth. While ordered domains such as tyrosinases, carbonic anhydrases, chitin-binding domains and Von Willebrand factor A are well-known coordinators of biocalcification, the also abundant IDRs are important for both the binding of inorganic ions or to serve as phosphorylation donors [78].

A convenient model for the study of biomineralization is the formation of sea urchin embryo skeletal element (spicule), and several recent observations highlight the importance of the self-assembling capacity of the participating proteins in this process (Figure 3).

The *S. purpuratus* spicule matrix protein SpSM30B/C was shown to form hydrogel-like structures that mediate amorphous calcium carbonate (ACC) crystal formation [79]. It was proposed that the hydrogel structure promotes the organization and growth of elongated single crystal calcite nanoparticles, while hydrogels are capable to form on the surface of already existing crystals, mediating textural changes that affect the properties of the mineral phase. The observed glycosylation pattern of the protein was suggested to affect the kinetics of phase transition.

Another sea urchin model protein, SpSM50, also forms hydrogels in a calcium-dependent manner, facilitating the formation of both vaterite and calcite nanoparticles [80]. It was suggested that the C-

terminal ordered CTLL domain serves as a mediator of protein-protein or protein-mineral interactions, while the repetitive, disordered N-terminal part of the protein destabilizes its internal structure and improves its overall self-association propensity [80]. The same behavior was observed for other nacre and spicule matrix hydrogels [81, 82], underlining the general importance of hydrogel formation as a regulatory step in biomineralization. Whereas self-assembly is generally accompanied by an elevation of secondary structure propensities, as shown in the case of nacre framework protein n16 [83], in other cases, like PFMG1 [82], a significant amount of disorder is retained in the assembled state. Amelogenin, the major protein component of mammalian tooth enamel also adopts a mostly disordered structure in vitro but shows a prominent alpha-helical tendency on the addition of TFE, which also induces self-assembly of the protein [84].

The currently accepted model for bionucleation is the polymer-induced liquid precursor (PILP) phase system [85], which describes nucleation process as a liquid-liquid phase separation mediated by disordered polymers binding to different solutes, resulting in a metastable precursor (Figure 3). These amorphous precursors were indeed shown to occur in vivo [86, 87] and calcium containing nanospheres were directly observed during the development of sea urchin embryos [88], indicating the applicability of this model to living systems.

Mucus: a protective endothelial layer

Another example of extracellular protein associations is mucus, a viscous material found in many different metazoan species from corals and gastropods to humans. Their functions span a broad range, including filtering nutrients from the environment, aiding movement of gastropods, and providing protection against predators.

In vertebrates, where mucus covers all epithelial tissues in direct contact with the environment, the most important function of this hydrogel is to hydrate and lubricate the mucosal surfaces [89]. The main protein components of mucus hydrogels in humans are gel-forming mucins (GFMs). e.g. MUC2, MUC5A, and MUC5B, all of which contain D-domains on their N terminus and a cystine knot (CK) domain at their C terminus. These globular domains are capable of forming stable protein-protein interactions, thus facilitating the formation of higher order structures. MUC2, MUC5A and MUC5B also contain several repeats of Cys domains (named after their high cysteine content) in their central region. All GFMs are characterized by a long central region that is enriched in serine, threonine and proline residues (S/T/P region) and is heavily glycosylated [89], except for the Cys domains. Since Cys domains contain mostly hydrophobic residues, the alternating pattern of negatively charged glycosylated regions and Cys domains contribute to the reversible forces

between mucin proteins in the hydrogel [90]. The S/T/P region is shown to adopt an extended, disordered conformation that is indispensable for the elasticity of the mucus hydrogel [91]. The main driving force behind phase separation of GFMs is concentration [92], but other factors such as salt concentration, pH and the presence of other protein factors also play crucial roles in regulating structure and rheological properties of the mucus blanket [89]. In cystic fibrosis, mutation(s) in the cystic fibrosis transmembrane conductance regulator (CFTR) channel leads to electrolytic imbalance and the formation of the highly viscoelastic mucus, causing the disease [89].

Adhesive exudates: sticky gels for protection and predation

Assembled secreted protein(s) (termed exudates) also play interesting and important roles in invertebrates: they either use the adhesive, gluey material for protecting themselves against predators or attaching themselves to surfaces. For example, the Australian fossorial frog *N. bennetti* secretes from its dorsal skin a protein-rich liquid exudate when attacked by a predator, i.e. a snake [93]. Once outside, the protein solution rapidly forms a tacky elastic gel, causing discomfort to the predator. This behavior has also been observed in several other frogs [94]. Interestingly, larvae of the frog secrete a similar material that forms a cement, which is used by the embryos and early larvae to adhere to stationary supports [95]. Other amphibia, such as some salamander species, also rely on proteinaceous adhesive exudates to protect themselves [96].

The application of adhesive secretions may be an even broader, general strategy of invertebrates to attach themselves, their nests or their eggs to biological or mineral surfaces [97]. For example, the moth *O. eucalypti* uses a protein-based hydrogel to secure its egg [98]. At variance, some velvet worms eject a proteinaceous solution turning instantaneously gluey, to immobilize and capture their prey [99].

Bacterial biofilms: protection and colonization

Bacteria often form colonies stuck to each other and to biological and environmental surfaces. Their cells are embedded in a self-produced protein matrix of extracellular polymeric substance (EPS), often also termed biofilm [100]. The biofilm matrix can contain soluble, gel-forming polysaccharides, proteins and DNA, and also insoluble components such as functional amyloids or cellulose, which all impart their individual physical properties on the heterogenous and highly dynamic matrix. Interestingly, not only components of the biofilm give rise to the emergent properties of an artificial habitat, bacteria in biofilms also exhibit emergent properties that differ substantially from those of the free-living bacterial cells.

Specific and emergent structural and functional properties of the biofilm specify the characteristic features (emergent properties) of the colony within the biofilm, such as social cooperation, resource capture and enhanced resistance or tolerance to antibiotics and environmental stress. For example *E. coli* biofilms have desiccation tolerance, a group of proteins called hydrophobins secreted by *B. subtilis* form highly hydrophobic biofilms that float at the air–liquid interface, and in *B. subtilis* biofilms DNA from lysed cells is a source of phosphorus, carbon and energy [100].

Conclusions: principles and applications of bio-condensates

Through many distinct examples, we have shown that assemblies of proteins form in many biological settings and convey a very broad range of emergent properties. Importantly, the different possible functional (Figure 2) and structural (Figure 1) categories probably combine in many distinct concrete assemblies. Certain generalizations regarding the structural and atomistic background of the formation of such assemblies, nevertheless, can be made. Protein-protein interactions can be mediated by domains, motifs and residues, which can all lead to the formation of stoichiometric complexes but also to non-stoichiometric ones (Figure 4), if: i) multiple interacting partners cause heterogeneity of the system (Figure 4A), ii) there is a mismatch in the binding valency of the partners (Figure 4B), and iii) some partners have a high level of structural disorder (Figure 4C). The underlying physical interactions then lead to the formation of a non-stoichiometric and extendable assembly with completely novel, emergent properties. Whereas the noted features can be derived from indirect observations and considerations, basic challenges of this emerging field is to unravel the underlying structural determinants promoting the assembly process and to provide quantitative description of thermodynamics, kinetics and dynamics of the various functional states that arise. Although we have a battery of techniques to look into these details [101, 102], heterogeneity and diversity of the systems already reported will represent an enduring challenge for many years to come.

Understanding all the details of supramolecular assemblies is also of special interest because they display the potential of special applications in medical and material sciences. Hydrogels are excellent candidates for targeted drug delivery and tissue engineering, because they are biocompatible, highly responsive to their environment and their physical properties such as stiffness, mesh size and biodegradability can be fine-tuned through varying their components and applying cross-linking [103]. Hydrogels can encapsulate therapeutic agents, such as drugs [104], peptides, proteins [105] or nucleic acids [106], capable of releasing them in a controlled and gradual manner. This property was utilized for the delivery of taxol to human hepatocarcinoma tumor in mice [107], resulting in a stable

release and reduced toxicity. The same effect was observed with doxorubicin, where the hydrogel-encapsulated drug showed similar anti-tumor activity with less side-effects compared to the conventional application [108]. Hydrogels can also be applied to accelerate wound healing [109] and cardiac regeneration [110], whereas artificial mimicking of biomineralization is a promising field in regenerating tooth enamel [111] and bones [112]. One strategy is to encapsulate regenerative agents into hydrogels, like bone marrow-derived mesenchymal stem cells, and utilize them as 3D scaffolds for bone regeneration [113]. With the ever-growing insight into the molecular organization of the supramolecular structures participating in tissue regeneration and the formation of "smart interfaces" [114], artificial materials that are capable of communicating with and responding to their cellular environment seems soon to be within our reach.

First and foremost, however, the basic challenge lies with our ability to harness this novel paradigm of organization for a better understanding of cell biology. As primordial coacervation may have been instrumental in the emergence of life [115], and extant organelles can have as basic functions as decelerated aging of oocytes [116] or assembly of ribosomes [117], the importance of these cellular compartments can hardly be overstated. As studies aimed at their detailed description keep unrolling, we are entering one of the most exciting phase of our quest for the molecular understanding of life.

Legends of figures

Figure 1 *Amyloids, prions and signalosomes as non-stoichiometric signaling complexes*

There are various ways by which multidomain proteins can assemble into non-stoichiometric signaling complexes. (A) Prions form by the orderly assembly of amyloid-type structures, as shown schematically for the human prion protein (a) and by the high-resolution X-ray structure of the heptapeptide GNNQQNY of the physiological yeast prion Sup35 (b, based on PDB 1yjp, cf. [26]). (B) Head-to-tail organization of p62 autophagy scaffolding protein forms a signalosome, via its N-terminal PB1 domain forming a filamentous structure (adapted from ref. [38]). (C) Schematic diagram of the network of proteins in the PSD, with various groups of proteins color-coded. For simplicity, only a few key proteins are named and shown (adapted from ref. [41]). (D) Electron microscopic image of an excitatory glutamatergic synapse, displaying a prominent electron-dense zone, PSD, juxtaposed to the postsynaptic membrane (from ref. [40], Copyright (2008) National Academy of Sciences).

Figure 2 *LLPS results in different types of emergent functions*

Phase separation brings a large number of macromolecules (protein, RNA) in close proximity and/or physical contact, endowing five broad types of emergent functional features on them. Here we show them separately starting from the upper left corner and proceeding clockwise: 1) bioreactors (affecting catalytic efficiency and specificity by enzyme-substrate proximity and targeting), biomolecular filters (selecting molecules incorporated by size and chemical differences), regulators of spatial patterns (generating concentration gradients in cells), biomolecular shields (protection of content from outside effects, such as immune system of degrading enzymes), and reservoirs (temporarily storing components for later re-use), but in most of the cases they appear in combination in the functioning of a particular organelle.

Figure 3 *Crystal nucleation through phase separation as a mechanism of biomineralization*

Biomineralization, such as the formation of bone and tooth, egg shell and hard tissues of mollusks results from the orderly deposition of inorganic minerals by large, repetitive, mostly disordered proteins. Increasing evidence attests to a nucleation model of biomineralization (polymer-induced liquid precursor (PILP) phase), which describes the nucleation process as LLPS mediated by ID polymers binding to different solutes.

Figure 4 *Various assembly logics of non-stoichiometric assemblies*

All different processes leading to the formation of non-stoichiometric assemblies rely on proteins that can engage in multivalent interactions with each other. Various combinations of domain-domain, domain-motif and residue-residue interactions may lead to assemblies of distinct internal symmetry and dynamics, preventing the formation of stoichiometric complexes, due to heterogeneity multiple interacting partners, A), a mismatch in the binding valency of the partners (B), and/or a high level of structural disorder that remains even in the mutually bound state (C).

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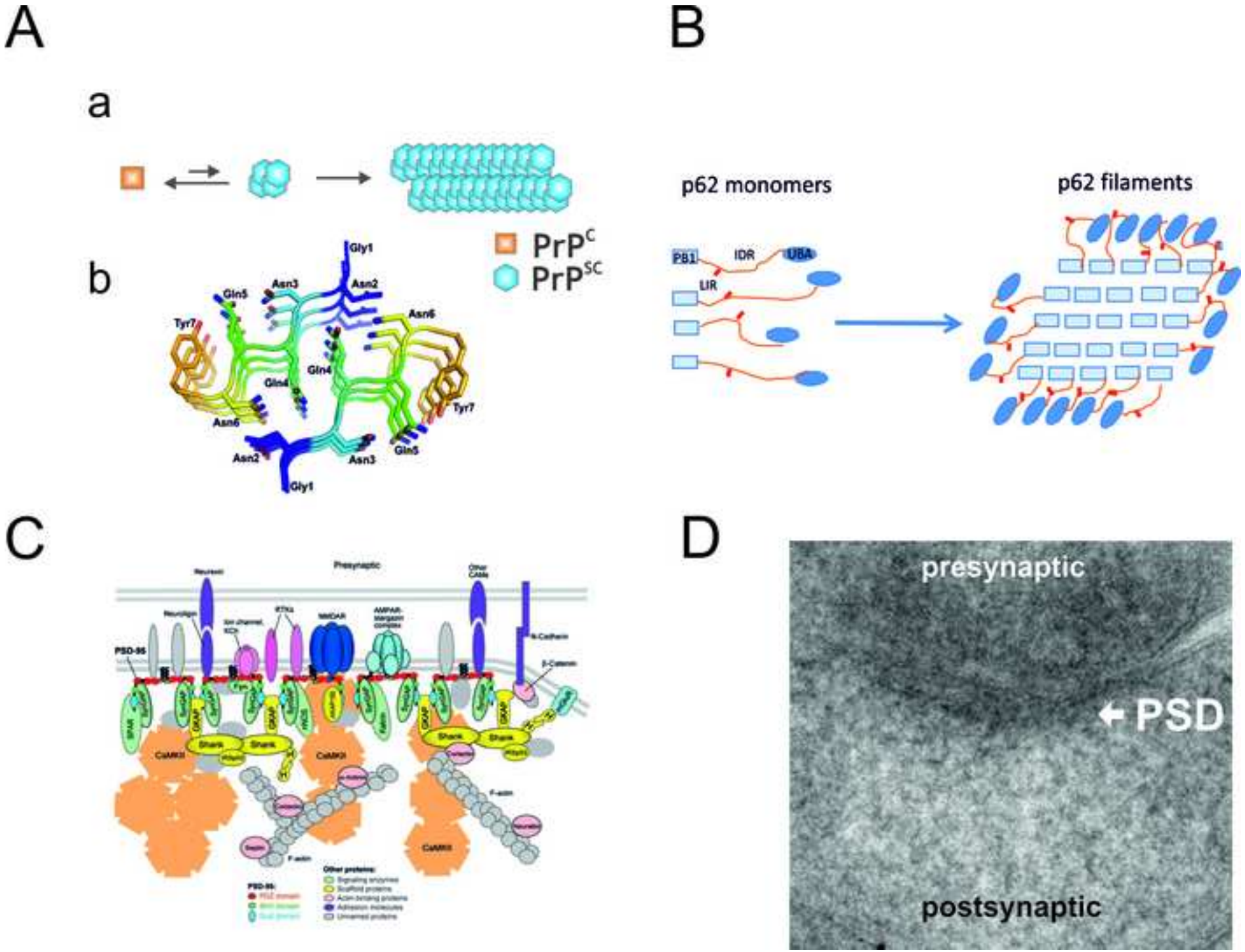

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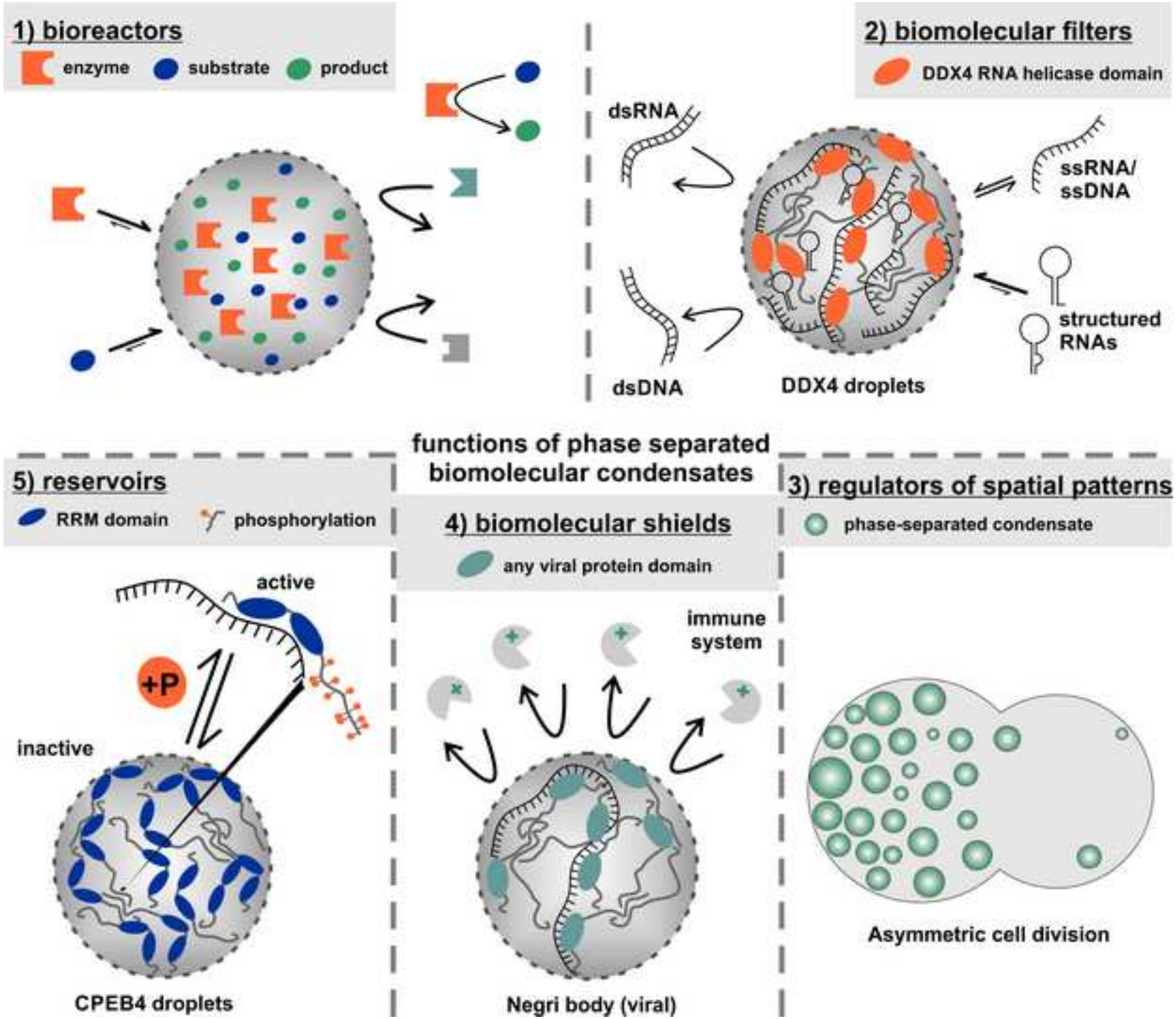

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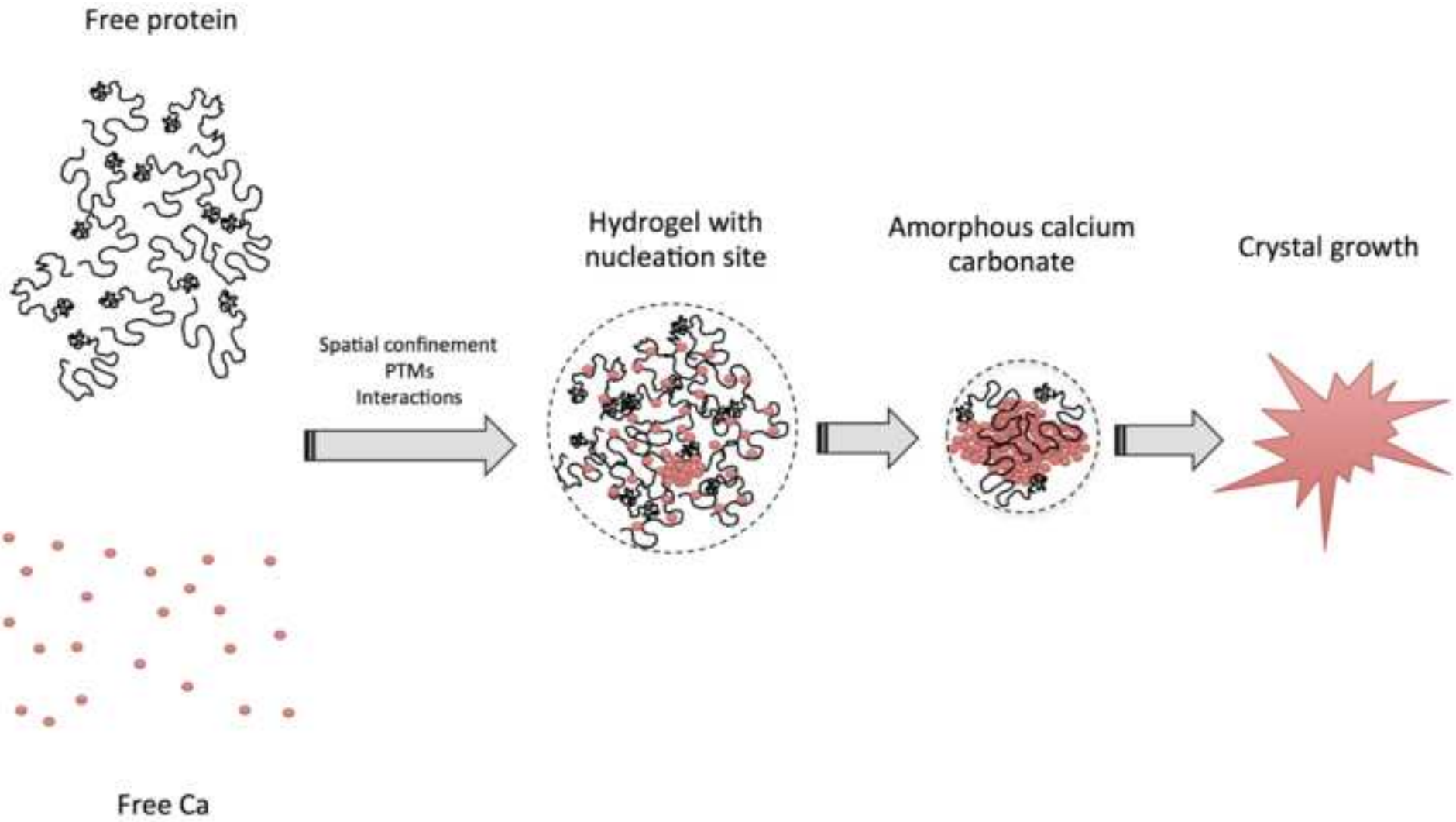

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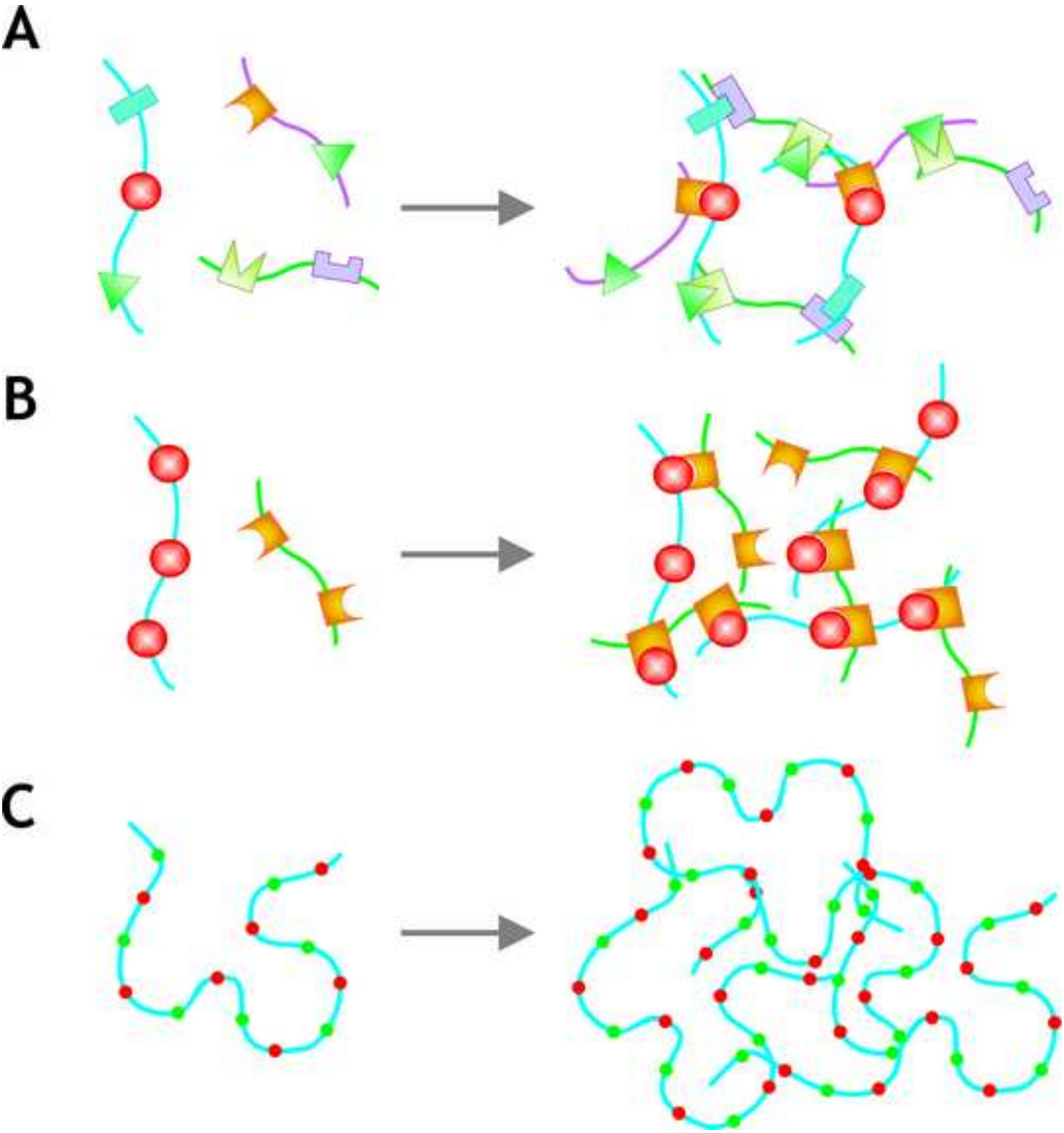

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