Research Article

Simulations of Higher-Order Protein Organizations Using a Fuzzy Framework

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Spatiotemporal regulation of the biochemical information is often linked to supramolecular organizations proteins and nucleic acids, the driving forces of which have yet to be elucidated. Although the critical role of multivalency in phase transition has been recognized, the organization principles of higher-order structures need to be understood. Here, we present a fuzzy mathematical framework to handle the heterogeneity of interactions patterns and the resultant multiplicity of conformational states in protein assemblies. In this model, redundant binding motifs can establish simultaneous and partial interactions with multiple targets. We demonstrate that these multivalent, weak contacts facilitate polymer formation, while recapitulating the observed valency-dependence. In addition, the impact of linker dynamics and motif binding affinity, as well as the interplay between the two effects was studied. Our results support that fuzziness is a critical factor in driving higher-order protein organizations, and this could be used as a general framework to simulate different kinds of supramolecular assemblies.

1. Introduction

Proteins can form a wide variety of assemblies, in terms of composition, size, and dynamics. In addition to simple binary, ternary complexes, and middle-size oligomers, proteins may also assemble into higher-order organizations. These supramolecular assemblies are implicated in different biological processes ranging from normal physiology to disease [1, 2]. For example, to minimize signaling noise for low-affinity effectors, signaling complexes frequently increase the local concentration of binding sites via higher-order protein assembly [3]. Recent discoveries revealed that supramolecular organizations of proteins and nucleic acids can generate functional cellular compartments [4, 5], which lack a membrane boundary [6]. Such membraneless organelles appear at various points on the biological landscape, for example, can serve as biomolecular storages upon stress and bioreactors to accelerate chemical reactions as well as signaling devices, whose assembly/disassembly is regulated by a variety of pathways [7–9]. Seminal works by Brangwynne, Hyman, and Parker labs revealed that these organelles are created by a process of liquid-liquid demixing, once the component concentration exceeds the saturation limit [10, 11]. This process, which was termed as a phase transition, could be described by the Flory–Huggins theory [12].

Higher-order protein organizations exhibit a wide spectrum of states with distinct dynamics. Prions/amyloids are stabilized by β-zippers, resulting in static and solid-like inheritable entities [13]. Signalosomes, such as inflammasomes or necrosomes could resemble prion-like stable structures [14] or be dynamic, for example, the autophagosome [15]. Ribonucleoproteins (RNP) generate dynamic granules or liquid-like droplets [16]. Nuclear pore complexes (NPCs) are somewhat more stable and form hydrogels [17]. Intriguingly, the very same protein could be organized into different higher-order states with distinct dynamics. Pathological mutations may induce conversion of the material state, for example, liquid-like droplets to solid fibrils. In the case of the hnRNPA and Fus protein, familial mutations appear in Amyotrophic lateral sclerosis (ALS) [1, 2]. Interestingly, pathological mutations often affect intrinsically disordered linker regions [18] and not the interacting motifs themselves,
suggesting the importance of conformational dynamics in organizing higher-order structures [19].

These experimental observations are in agreement with the recently proposed framework for higher-order protein organizations [20]. This model suggests that the material state of higher-order structures could be decomposed into three factors. First, low-affinity elements/motifs such as cation-pi, pi-pi, aromatic hydrogens bonds [21, 22] mediate weak contacts with fast off-rates. Second, multivalency of binding elements increases the number of microstates in the bound state [23]. Third, conformational flexibility/dynamics or disorder must be retained to enable different topologies and reduce the entropic penalty of binding [16]. Along these lines, NMR data indicates similar conformational heterogeneity of Fus in its free and bound states [16]. Polymer physics approaches recapitulated the effect of multivalency of interacting motifs [23]. Monte Carlo simulations shed light on linker solvation [24, 25]. However, the problem of degenerate interaction patterns by weak affinity motifs [22] and how the resultant heterogeneous conformations impact higher-order protein organizations have not been addressed so far.

Our model considers the supramolecular assembly as a fuzzy complex [26–32]. These protein assemblies are characterized by structural multiplicity or dynamical disorder in their bound states, and the conformationally heterogeneous region is demonstrated to have a considerable impact on the biological function. This phenomenon is referred to as "fuzziness" in proteins, which concept has been supported by experimental evidence on a wide range of examples [33]. Fuzziness originates in transient and ambiguous interactions, which lead to redundant contact patterns in protein complexes [27, 34]. Although the view on degenerate alternative contacts in specific complexes contradicts to the traditional concept on specific molecular recognition, it is corroborated by a wide range of experimental data [35, 36] (and references therein). Furthermore, a variety of regulatory mechanisms are linked to protein fuzziness [29], which might also contribute to the organization of higher-order assemblies [20].

Fuzziness is known as a mathematical concept, where the membership in given sets is described by a function, varying between [0,1] instead of a single [0 or 1] value. Fuzziness has been derived from the seminal work of Zadeh [37] and has been implicated in the electronic control of ~3000 artificially intelligent devices [38]. We have developed a simulation method based on the mathematical concept of fuzziness to describe how weak degenerate motifs connected by flexible linkers can be organized into higher-order polymers. In this model, a single binding site can simultaneously interact with multiple binding motifs to different extents. We show that these partial and multiple contacts facilitate polymer formation as compared to the one-to-one binding models. Independently of the fuzzy description, the algorithm allows to study the influence of linker dynamics, motif affinity, and their interplay on molecular associations. Our results support the previous proposal [20] that fuzziness is a critical factor in driving higher-order protein organizations.

2. Methods

2.1. Model System. The simulations were run on 100 hypothetically identical proteins, which were placed into 64 periodic simulation boxes. All units are given in residues. The separate proteins can associate, the resultant polymers can dissociate, and both can diffuse with probabilities given below.

2.2. Protein. The model is a hypothetically biological chain, which is composed of N residues (depending on the valence, Figure 1). Each residue is characterized by two values: binding affinity and dynamics. Binding affinity characterizes the interaction preference, and this value could be derived from experimental studies or theoretical estimates of binding free energies. Here, dynamics refers to a conformational exchange in the bound state. That is, upon molecular interaction, to what extent flexibility or dynamical disorder can be retained in the assembly. If this value is high, the residue can interconvert between multiple states, generating multiple interaction patterns and heterogeneous conformations. If D = 1, linkers preserve their conformational heterogeneity similarly to their unbound state, while in case of D = 0, the linkers collapse and become rigid in the assembly. The value for dynamics could be derived from bioinformatics studies, as it can be predicted based on the protein sequence or from NMR measurements. In this work, the values for binding affinity and dynamics are hypothetical and not derived from observed or computed data on specific model systems.

Values for binding affinity and dynamics vary in a [0,1] range and were kept fixed during simulation. Binding affinity >0.3 designates residues involved in binding; fuzziness values >0.3 correspond to linker residues. Binding elements (α) are defined as a continuous stretch of at least 5 residues with binding affinity >0.3. Linkers (λ) are defined as all other segments connecting the binding elements. In case of the hypothetical protein, the length of each binding site was 7 and the length of each linker was 10 residues. The size of the molecule was varied between 34 and 102 residues.

2.3. Molecular Association/Polymer. If two molecules are linked by one or more nonzero fuzzy interactions, they are considered to form a complex. Interacting sites must be spatially close (i.e., their distance should be below a given threshold). Bound molecules move together in case of diffusion to the neighboring box. Parameters for binding (affinity, probability to associate/dissociate) are described below. Large polymers (higher-order assemblies) were defined as interconnected m > 25 molecules.

2.4. Simulation Box. 64 cubic simulation boxes were used, with lengths between 20 and 70 residues (units are given in residues). The molecules were placed randomly into the boxes, with no steric overlap.

2.5. Parameters. Binding preference of a binding element (α) is obtained as an average of the residue binding preferences.

\[ S_\alpha = \frac{\sum_{k=1}^{n_i} S_k}{n_i} \]
where $s_k$ is the binding preference of residue $k$ and the binding element contains $n_i$ residues.

The affinity between two binding elements is defined as the average of the binding preferences $[0,1]$.

$$K_{a_i,a_j} = \frac{s_{a_i} + s_{a_j}}{2},$$

where $s_{a_i}$ and $s_{a_j}$ are the binding preferences of the interacting $a_i$ and $a_j$ elements.

As the fuzzy framework allows one binding element to interact with multiple other elements, the number of possible binding elements available for interaction needs to be determined. All binding elements within the volume, which is defined by the neighboring linkers are considered (see below).

$$n_{\text{max int},a_i} = \sum_{j=1}^{n} \alpha_j,$$

where $\alpha_j \in V_i$, and $n_{\text{max int},a_i}$ is the maximum number of interaction sites around the binding element $a_i$. The available volume for $a_i$ interactions is defined by the length of the longer neighboring linker ($l_i$), which is measured from the center of the binding element. In the default case, $V_i$ is a spherical volume with a radius of $l_i$.

$$V_i = \frac{4\pi l_i^3}{3}.$$  

This volume could be rescaled according to linker dynamics, which is defined as the average of the residue dynamics.

$$D_i = \frac{\sum_{k=1}^{l_i} d_k}{l_i},$$

where $d_k$ is the dynamical value of residue $k$, and $l_i$ is the length of the linker.

If the linker dynamics ($D_i$) is 1, all the available binding sites are considered within the volume as defined in equation (4). If $D_i < 1$, the sphere radius is reduced proportionally to the linker dynamics.

$$l'_i = D_i \ast l_i,$$

where $D_i$ is the linker dynamics and $l_i$ is the length of the linker. $l'_i$ is used to obtain the volume by equation (4).

2.6. Computed Quantities. The association probability linearly depends on the binding affinity ($K_{a_i,a_j}$) and reciprocally on the available binding sites ($n_{\text{max int},a_i}$).

$$p_{\text{on}}^{\text{eff}} = \frac{p_{\text{on}}^{\text{int}} \ast (1 - p_{\text{off}}^{\text{int}}) \ast K_{a_i,a_j}}{n_{\text{max int},a_i} + n_{\text{max int},a_j}},$$

where the intrinsic association probability $p_{\text{on}}^{\text{int}} = 0.6$, the intrinsic dissociation probability $p_{\text{off}}^{\text{int}} = 0.1$, similarly to the reference [23]. The association probability is compared to a random number (rnd), and if $p_{\text{on}}^{\text{eff}} \geq \text{rand}$, the binding is realized.

Once interactions are formed in the first step, we define an occupancy value for each binding elements $[0,1]$. It is calculated with an algebraic sum, which is the fuzzy union ($s$-norm) operator.

$$\mu_{a_i} = s(K_{a_i,a_i}, K_{a_i,a_j}, \ldots, K_{a_i,a_n})$$

$$= s(s(K_{a_i,a_j}, K_{a_i,a_j}), \ldots, K_{a_i,a_n}) \ast (K_{a_i,a_i})$$

$$= K_{a_j,a_i} + K_{a_i,a_j} - K_{a_i,a_i} \ast K_{a_i,a_i},$$

where $n$ is the number of the binding elements in the polymer. The algebraic sum was chosen because of its super idempotent property $s(a,a) = a$. Equation (8) considers all binding sites within the polymer, but only the connected sites (where $K_{a_i,a_j} > 0$) are taken into account in the algebraic sum.

From the second step, the affinity of a given interaction between $a_i$ and $a_j$ also depends on the local concentration.
of the bound binding elements. The binding affinity between two elements must be weighted by the occupancies of the neighboring binding sites.

\[ K'_{\alpha_i, \alpha_j} = \frac{s_{\alpha_i} + s_{\alpha_j}}{2} \times \sqrt{1 + \sum_{m=1}^{n_{\text{max}, \alpha_j}} \mu_m + \sum_{m=1}^{n_{\text{max}, \alpha_j}} \mu_m}, \quad (9) \]

where \( \mu_n \) and \( \mu_m \) occupancies are summarized for all binding elements within the available volume for \( \alpha_i \) and \( \alpha_j \). If the local concentration is considered from the second step, the modified affinities \( (K'_{\alpha_i, \alpha_j}) \) are used to determine the occupancies in equation (8).

The association probability is also modified accordingly.

\[ p_{\text{on}}^{\text{eff}} = \frac{p_{\text{on}}^{\text{int}} \times (1 - p_{\text{off}}^{\text{int}}) \times K'_{\alpha_i, \alpha_j} \times 1}{n_{\text{max}, \alpha_j} + n_{\text{max}, \alpha_j} + 1 + n_{\text{int}, \alpha_j} + n_{\text{int}, \alpha_j}} \quad (10) \]

where \( K'_{\alpha_i, \alpha_j} \) is the modified binding affinity defined in equation (9), and \( n_{\text{int}, \alpha_j} \) and \( n_{\text{int}, \alpha_j} \) are the actual number of binding elements, which are bound to \( \alpha_i \) and \( \alpha_j \), respectively.

The dissociation probability has an inverse relationship to the binding affinity.

\[ p_{\text{off}}^{\text{eff}} = \frac{p_{\text{off}}^{\text{int}} \times (1 - p_{\text{on}}^{\text{int}})}{K'_{\alpha_i, \alpha_j}} \quad (11) \]

where the intrinsic association \( (p_{\text{on}}^{\text{int}}) \) and dissociation \( (p_{\text{off}}^{\text{int}}) \) probabilities are the same as in equation (7), and \( K'_{\alpha_i, \alpha_j} \) is the modified binding affinity defined in equation (9).

From the second step, the molecules could be present as individual chains, or chains organized into oligomers or larger polymers. Here, we need to define the interaction capacity (“freedom”) of the binding elements within the molecular assembly/polymer.

\[ F = \sum_{i=1}^{n} 1 - \mu_i \quad (12) \]

where \( (1 - \mu_i) \) is the available interaction capacity of a given \( \alpha_i \) binding element, which is summarized for all binding elements in the polymer.

Any molecule types in the system: individual molecules, oligomers, or larger polymers can diffuse to another box. Diffusion means repositioning into another box, in case of polymers molecules move together. The probability of the diffusion is defined as

\[ p_{\text{diff}} = \frac{1}{6} \times \frac{1}{\sqrt{\sum_{i=1}^{n} (1 - \mu_i)}} \quad (13) \]

where the square root of \( F \) (equation (12)) is used in the denominator.

2.7. Comparison of Fuzzy and Nonfuzzy Simulations. As in the fuzzy simulations, a partial binding is also considered to be a potential interaction (depending on the association probabilities as computed by equations (7) or (10)), we ensured that the probabilities of forming large polymers in the fuzzy and nonfuzzy simulations are comparable. First, the binding affinities in the fuzzy simulation are derived from equations (2) and (9), while in the nonfuzzy simulations they are always 1. Therefore, the association probabilities (equation (10)) of the nonfuzzy simulations are higher, unless the local concentration effect is considered. Fuzzy simulations only produce large polymers >4 valencies (binding elements) without considering the local concentration. Second, the association probability (equation (10)) is inversely proportional to the current number of binding interactions, so a potential to contact simultaneously via multiple sites does not necessarily increase the probability of polymer formation. Third, the dissociation probability (equation (11)) is inversely related to the binding affinity. Therefore, a weak (partial) binding in the fuzzy model is more likely to dissociate than a full binding (with \( K = 1 \)) in the nonfuzzy model. Overall, the fuzzy simulations without the local concentration effect are comparable to the nonfuzzy simulations. Please note that the nonfuzzy simulations in this work are not equivalent to those in the reference [23], as our simulations also account for spatial dimensions, linker dynamics, and motif affinity.

2.8. Computational Protocol. A periodic system was defined, which contained 64 boxes, with dimensions from 20 to 70 residue units (length was varied in separate simulations). The system was comprised of 100 molecules, each composed of 34 to 102 residues. The simulated molecules were placed randomly in the boxes. The simulation protocol was similar to the stochastic rule-based simulation in reference [23].

In the first simulation step, the molecules could associate according to the probabilities given in equation (7). From the second iteration step, the molecules had three options: associate (i), dissociate (ii), and diffuse to another randomly chosen neighboring cell (iii). Occupancies (equation (8)) and the interaction capacities (equation (12)) are determined in each step, and affinities were modified accordingly (equation (9)). Association probabilities (equation (10)) and dissociation probabilities (equation (11)) were also scaled by the modified affinities (equation (9)) to account for the local concentration and binding status of the available sites. Diffusion was inversely proportional to the interaction capacity, so larger polymers have less chance to move to the neighboring box.

3. Results

All previous simulations of higher-order assemblies assumed that interactions are well-defined and each site contacts only one partner at the same time (one-to-one) [23, 24]. In contrast, the fuzzy model allows multiple interactions for the same site to different extents. In principle, the potential to
form multiple partial contacts can increase the probability of polymer formation. To compare fuzzy and the one-to-one (nonfuzzy) binding models, however, we eliminated the artefact that increased binding potential which causes polymer formation (Methods). First, we studied the impact of valency on the generation of higher-order assemblies. The model system contained one molecule type, the size of which has been systematically varied between 2 to 6 binding elements and linkers, and the length of which were arbitrarily defined as 7 and 10 residues, respectively (Figure 1). Concentration was modulated by varying the size of the simulation box, while the number of molecules was kept fixed. The topologies and the parameters considered for interactions are shown in Figure 1 (for explanation see Methods). Three types of stochastic movements were performed, which are similar to reference [23]: (i) association, (ii) dissociation, and (iii) diffusion to a neighboring box. The results were averaged for 10 parallel simulations (10,000 steps) for each parameter combinations.

Multivalency is considered as the major driving force of phase transition [6, 23]. The impact of valency and concentration on the probability of formation of large polymers is shown in Figure 2. Concentration is varied by changing the size of the simulation box. Both one-to-one and fuzzy simulations show a strong dependence on the number of binding elements, recapitulating previous experimental observations [23].

Fuzzy interactions seem to facilitate the formation of higher-order molecular associations (Figure 2). The same number of molecules with the same valency generate polymers in an order of magnitude larger simulation box in the fuzzy simulations than in the nonfuzzy model. This suggests that weak partial interactions with multiple partners may enhance assembly at lower valency. This is consistent with recent experimental data on highly dynamical interacting partners [39]. Along these lines, in simulations, which were conducted using lower binding element affinities (0.35) and linker dynamics (0.35), no higher-order oligomers (m > 25) were observed in the one-to-one simulations, while polymers were formed in the case of >4 binding elements in the fuzzy model (Figure 3).

We also studied the impact of binding affinity on polymerization. Intuitively, higher affinity between the interacting elements increases the association probability (equation 10). In accord, in both fuzzy and nonfuzzy models, polymerization takes place at lower valency at higher binding affinity (Figure 4). Using the same simulation conditions, where no polymerization was observed in the nonfuzzy model (Figure 3), we have observed higher-order assembly at increased affinity. In the fuzzy model, polymerization occurs at lower affinity (Figure 4(b)) as compared to the one-to-one binding, illustrating that partial and heterogeneous contacts may compensate for weaker interactions [22]. Obviously, above a certain limit, increasing affinity and valency may induce the formation of aggregates or amyloid structures and not dynamical assemblies.

In the fuzzy model, we assume that the local concentration of the binding elements also influences affinity, which is in agreement with earlier theoretical [40] and experimental [41] results. This effect was taken into account via equation (9), and the modified affinities were incorporated into
the association (equation (10)) and dissociation probabilities (equation (11)). Considering the local concentration of the binding sites generates polymers at lower valency (Figure 5). This could be one of the reasons why fuzzy simulations produce higher-order states more frequently (see Figures 5(b) and 6(a)).
Intrinsically disordered regions play important roles in organizing higher-order structures [18, 42]. These proteins segments, while lacking a well-defined structure, exhibit enhanced plasticity that enables a large number of contact combinations [20, 24]. However, intrinsically disordered regions are not infinitively flexible; they exhibit a given range of dynamics, which certainly affects the available topologies.

In our fuzzy model, we take this effect into account via equation (6), which modifies the affinities and association/dissociation probabilities. Systematic increase of linker dynamics increases the probability of polymer formation (Figure 6). In agreement with experimental studies [16, 39], these results reflect that linker dynamics is a critical element of higher-order assembly, which is independent of motif.

**Figure 5:** Probability of large polymers as a function of valency and binding affinity in the fuzzy binding model with (a) and without (b) considering the local concentration effect. The local concentration is computed by equation (9). Linker dynamics = 0.35; box length = 20.

**Figure 6:** Probability of large polymers as a function of valency and linker dynamics in the one-to-one (a) fuzzy (b) binding models. Linker dynamics is computed as the average of the residue-based values (equation (5)). Binding element affinity = 0.35; box length = 20.
affinity. We might hypothesize that sticky chains, with low number of valencies, are also capable to generate higher-order structures [39].

In the nonfuzzy binding model, the impact of linker dynamics is comparable to that of increasing interaction affinity (Figure 4), illustrating that either stronger or more heterogeneous binding can promote assembly formation. Fuzzy simulations are more sensitive to linker dynamics (Figure 6), as it affects the number of possible interaction patterns, which could be generated at a given time point. This observation underscores that degenerated contacts and the resultant conformational heterogeneity are important driving forces of higher-order assembly.

In the fuzzy model, the interplay between the motif affinity and linker dynamics was studied systematically using models with different valencies (Figure 7). For any combinations of binding affinity and linker dynamics, the critical role of valency is observed.

In addition to the effect of valency, interaction affinity and linker dynamics act in synergy to promote polymerization. Lower affinity elements with more flexible linkers as well as high-affinity elements with less dynamical linkers can produce higher-order assembly. Obviously, these two scenarios are expected to result in distinct supramolecular assemblies along the dynamical continuum [20]. This is an important issue for pathological mutations, which studies are currently ongoing in our laboratory.

4. Discussion

Protein function is usually interpreted within the deterministic framework of the classical structure-function paradigm. This relationship establishes a connection between a well-defined three-dimensional organization of amino acid residues and the biological activity of the resultant conformer. The classical description also involves the assumption that the intra- or intermolecular interactions generate a well-defined pattern. Increasing experimental evidence contradicts this simple picture and demonstrates that biological function may require conformation and interaction heterogeneity [32, 33]. Sequences of proteins composing membraneless organelles, for example, are enriched in redundant/degenerate motifs [22], which appear to contact in multiple ways resulting in a heterogeneous assembly [16]. Indeed, structural and interaction heterogeneity is an intrinsic feature of higher-order protein assemblies, ranging from static to highly dynamical structures [20].

Developing computational approaches to describe heterogeneous systems is a challenge. Until now, a one-to-one binding model has been employed in both coarse-grained and lattice simulations [24], which could not account for the effect of heterogeneity, resulted by multiple alternative configurations. A fuzzy mathematical framework allows coexisting alternative structures or interaction patterns in the system, which are realized to different extents.
Within the fuzzy model, a binding element may interact with multiple partners simultaneously, and the contribution to alternative states are expressed via membership functions. The membership of a binding element varies in each configuration, and the system remains heterogeneous throughout the trajectory.

Here, we applied the fuzzy framework to a hypothetical polymer characterized by binding affinity and dynamics. The simulations recapitulate the observation that multivalency is a prerequisite for phase transition [23]. As compared to the one-to-one (nonfuzzy) binding model, the fuzzy simulations predict a lower phase boundary (Figures 2 and 3). This illustrates that weak partial interactions lead to degenerate patterns favor assembly. The partial contacts increase the probability of productive interactions via local concentration effects (Figure 5). We demonstrate that—in addition to motif affinity—linker dynamics is a critical factor in driving higher-order assembly. This effect is especially pronounced in the fuzzy model (Figure 6). Systematic studies on the interplay between binding affinity and linker dynamics outline two alternative ways to promote higher-order organization of proteins (Figure 7): higher affinity for the binding motifs or increasing dynamics in the bound system. This hypothesizes that weak interaction networks in fuzzy systems are capable to organize higher-order associates.

5. Conclusion

Understanding the driving forces of higher-order protein assembly is challenging, owing to the complexity of these systems. Here, we developed a fuzzy mathematical model to simulate associations of biological polymers. In this approach, the system is described by multiple coexisting states, capturing the inherent heterogeneity of higher-order assemblies. We propose that the fuzzy model provides a general framework to study higher-order systems along the structural and dynamical continuum.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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