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Crowding in Cellular Environments at an Atomistic Level from Computer Simulations

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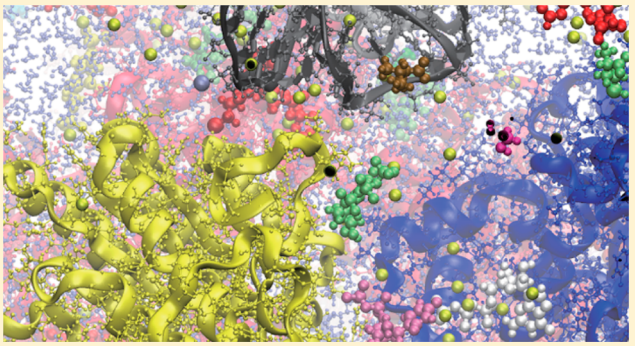
ABSTRACT: The effects of crowding in biological environments on biomolecular structure, dynamics, and function remain not well understood. Computer simulations of atomistic models of concentrated peptide and protein systems at different levels of complexity are beginning to provide new insights. Crowding, weak interactions with other macromolecules and metabolites, and altered solvent properties within cellular environments appear to remodel the energy landscape of peptides and proteins in significant ways including the possibility of native state destabilization. Crowding is also seen to affect dynamic properties, both conformational dynamics and diffusional properties of macromolecules. Recent simulations that address these questions are reviewed here and discussed in the context of relevant experiments.



INTRODUCTION

Realistic biological environments involve a high degree of complexity and crowding at the molecular level that is still only at the beginning of being fully understood.[1−5] Increasing evidence suggests that crowding can markedly affect biomolecular structure, dynamics, and thus possibly their function.[6] The most obvious aspect of cellular crowding, a greatly diminished free volume with mostly entropic consequences, has long been studied in various ways using experiments, theory, and simulation.[7−12] However, the degree to which artificial crowders approximate real cellular environments or even reproduce just excluded-volume effects without any other artifacts is unclear.[6,13−15] At the same time, increasing attention is now focused on the effects of interactions between the cellular components[16−24] and altered solvent properties,[25−30] often contributing enthalpically,[15,31,32] as well as the consequences of a substantial compositional heterogeneity present in biological cells.[33,34]

The concentration of biological macromolecules in cells is between 100 and 450 g/L, occupying 5−40% of the cytoplasmic volume.[35] In addition, structural elements such as the cytoskeleton, compartments, and membrane surfaces are present, whereas genomic DNA fills out eukaryotic nuclei and a good fraction of prokaryotic cells. A variety of metabolites, small molecules that are intermediates of metabolic reactions such as phosphates, carbohydrates, amino acids, alcohols, vitamins, and other cofactors, as well as ions at significant concentrations further contribute to the rich physicochemical environment inside living cells. Experiments that are carried out under *in vivo* conditions or in the presence of cell extracts face substantial technical challenges.[1,2,25,36] Often, it is difficult to achieve sufficient resolution to provide detailed insights, although recent progress especially with in-cell nuclear magnetic resonance (NMR)[24,37−55] and in-cell fluorescence spectroscopy[56−58] techniques is encouraging. An alternative is computational modeling, which can describe the structure and dynamics of biomolecules with high resolution in both space and time within model and resource limitations.[59] In particular, molecular dynamics (MD) simulations using atomistic force fields are a popular avenue for studying biological systems. However, MD studies have so far largely focused on single molecules or complexes without considering crowded environments, even as multimillion atom systems are now becoming feasible,[60−62] whereas questions in regard to crowding have been addressed mostly with simplified models.[10] This may reflect in part the computational costs involved with fully atomistic detailed models of crowded environments, but the more important constraint is that sufficient experimental data for building high-resolution models of biological environments has not been readily available. There has also been a lack of

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suitable experimental data that predictions from simulating such models could be compared to. However, there is a recent influx of data from high-throughput experiments that probe the composition of a cell,[63−65] and new experiments can directly probe macromolecule structure and dynamics in cellular environments.[16−22,25,32,37,40,41,44−46,49,54,56,66−85] This now enables and, we would argue, requires detailed computational studies of realistic models of crowded biological environments to connect with the newly emerging experimental data and generate new hypotheses.[86]

KEY QUESTIONS

The questions to be addressed here revolve around the structure, interactions, and dynamics of biomolecules, proteins, nucleic acids, and metabolites in cellular environments. Essentially, the key point is to determine to what extent the vast amount of knowledge about the biomolecular structure−dynamics−function paradigm generated during decades of structural biology and mechanistic analyses of biological function under *in vitro* conditions translates to real biological environments and ultimately what makes up life.[87] The end point is a fully integrated view of biology that scales from single molecules to whole-cell models[88,89] to explain life based on physical and chemical principles.[90]

Structure and Stability. Most of the knowledge about protein structure has been derived from crystallography where the environment, although relatively dense in terms of packing, lacks the heterogeneity of biological environments and where packing contacts are more likely related to artifacts than being representative of what would be seen in crowded biological environments. Structures based on nuclear magnetic resonance (NMR), on the other hand, are traditionally obtained under dilute conditions that are far from crowded cellular environments. Thus, it remains largely unclear whether and how the structure and stability of proteins, and more broadly the conformational energy landscape, is affected by crowding in the cell.[91] Simple arguments based on a volume exclusion effect due to crowding focus on a shift in populations toward compact states on an otherwise largely unchanged landscape,[11] but this view conflicts with recent experiments.[17,22,23,73,92,93] Largely unaltered conformations *in vivo* vs *in vitro* are seen with in-cell solution NMR[40,44] and solid-state (SS) NMR,[54] but other evidence from experiments and simulations indicates that cellular environments may remodel the folded or unfolded ensemble or both[16,17,49,71,94−100] with potentially varying effects in different cellular compartments.[82] This suggests a more complicated picture where it is unclear whether remodeling of the ensemble is again driven mostly by entropic volume exclusion effects or by more enthalpic effects due to interactions with protein crowders and other components of the biological environment.[16,17] Significant crowding effects are also expected for intrinsically disordered peptides (IDPs) where extended and dynamic ensembles have to be accommodated under crowded environments.[37,69,75,76,83,100,101]

Although proteins have received most of the attention, similar, less well-explored questions have been raised about how nucleic acid structures may act as crowders[43] or be affected by crowded cellular environments compared to what we know about DNA and RNA from crystallography and solution NMR studies.[102−109]

Dynamics. Biomolecular dynamics links structure with function, and it is equally unclear how it may be affected in the context of cellular environments. Conformational dynamics is a key mechanistic factor in most biological macromolecules, and any effect of cellular crowding on such dynamics would directly impact biological function. There is some insight into altered folding kinetics under crowded conditions,[48,56,80,82] and a few studies suggest that the internal backbone and side chain dynamics on sub-microsecond time scales remain largely unaltered.[78,110−112] On the other hand, only a few studies have examined how functionally relevant motions in the native state, e.g., dynamics related to allosteric mechanisms, ligand binding, or catalysis, and dynamics of intrinsically disordered peptides may change upon crowding.[113−115] This leaves many open questions yet to be addressed.

Diffusional motions of biomolecules in cellular environments, on the other hand, are somewhat better understood. Diffusion rates are a key determinant for the kinetics of many biological processes. Ligand diffusion is rate-limiting in many enzyme reactions, and protein diffusion is an important factor in most protein−protein and protein−nucleic acid interactions.[116−120] There is a general understanding that diffusion of proteins is slowed down by about 1 order of magnitude under crowded conditions due to increased viscosity and interactions with crowder molecules,[77,121−124] although this may not be generally true for natively disordered proteins.[122] Anomalous diffusion results in diffusion rates varying on different time scales due to confinement within cellular compartments and caging effects due to temporary encapsulation by larger crowders.[34,123,125−128] However, details of how diffusion is modulated by different cellular microenvironments and by the presence of DNA or membrane surfaces have remained unclear.[77] There is also an incomplete understanding of how smaller molecules, metabolites and other ligands navigate the dense cellular environments where available space is not just limited and solvent viscosity may be altered but where the various macromolecular surfaces also provide ample opportunities for distractions that could, for example, slow down diffusion toward an enzyme active site.[129]

Interactions. Cellular crowding undoubtedly increases the opportunities for frequent encounters of biomolecules due to weak interactions.[79,130,131] It is not clear, however, whether such soft interactions simply hinder diffusion and alter the conformational energy landscape as discussed above or whether interactions between biomolecules are altered with possible functional implications.[16,66,70,74,84,132] Crowding has been found to affect native complex formation[31,68,133−135] and aggregation,[136−138] while other evidence indicates enhanced formation of nonspecific complexes between proteins in the cellular milieu.[14] Weak interactions under highly crowded conditions may further lead to protein-enriched regions, forming distinct phases within the cell such as droplets with gel-like characteristics.[139,140]

Metabolite−protein interactions are also subject to crowding effects. Studies of substrate binding and product release during enzyme reactions under crowded conditions have suggested crowding effects.[129] Moreover, effective metabolite concentrations *in vivo* may be reduced as a result of metabolites being sequestered from bulk solvent by nonspecific binding to biomolecular surfaces,[141] whereas competition by promiscuous interactions with the environment, on the other hand, may modulate specific binding of ligands to target binding sites.[132,142]

Solvent and Membrane Environments. Finally, a fundamental question is how the solvent environment is altered in the presence of biomolecules at very high concentrations. Simple geometric considerations suggest that,

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although water still makes up about 70% of the cytoplasm, only a relatively small fraction is actually bulk-like, as defined by water molecules being several layers away from the closest macromolecule. This has been shown to impact water dynamics,[29,143] viscosity,[66,144,145] and dielectric properties,[26,146] which then in turn may further exacerbate crowding effects on the macromolecules compared to dilute environments.[29] While experiments have provided varying insights,[28,147] a more detailed analysis of the properties of aqueous solvent inside biological cells is desirable. There are also many unanswered questions about how membrane bilayers respond to crowding,[148] both inside the membrane by membrane proteins and outside by a crowded cytoplasm and how membrane surfaces modulate crowding effects in the cytoplasms.

A full review of all experiments, simulations, and theoretical studies that have looked at these questions is not possible here. Instead, the specific focus of this article is to highlight recent advances based on computer simulations of dense macromolecular systems in atomistic detail, and relate those findings to experimental studies, both past and future.

CELLULAR ENVIRONMENTS IN COMPUTER SIMULATIONS

Computer simulations have examined biological systems at a wide range of scales from full atomistic detail to very coarse representations. Most simulations of crowded cellular environments have involved simplified models where macromolecules, crowder molecules, and the solvent environment are represented with different degrees of coarse-graining.[95−98,127,149−152] For example, coarse-grained models of proteins in the presence of spherical crowders without any explicit representation of solvent are one avenue for studying the effect of crowding on protein folding or association equilibria,[30,153] whereas Brownian, Stokesian, or other stochastic dynamics simulations of rigid protein models at different levels of detail have offered insights into how crowding modulates diffusive properties.[127,151,154−158] Another possibility is postprocessing of trajectories using particle-insertion methods.[159] All of these approaches involve simplifications that are attractive for saving computational costs and have allowed the exploration of relatively long time scales, in the range of micro- to milliseconds. However, there are significant trade-offs as intermolecular interactions and solvent effects often do not fully reflect cellular environments and intramolecular dynamics may be neglected or not fully accounted for.

As with any modeling, the degree of detail should match the questions that are being asked and an *in silico* model of a complete cell does not necessarily have to include every atom that is present. However, we argue that the central questions that are being asked here, conformational sampling, stability, and dynamics of biomolecules in the cellular environment, require, at the minimum, a model with the following features: (1) Proteins, nucleic acids, and metabolites should be fully flexible with an interaction potential that is able to maintain native states under dilute conditions but that can also sample non-native states in response to interactions with cellular components. (2) Weak interactions between the cellular components are physically accurate and predictive without requiring previous knowledge about specific complexes, since nonspecific interactions dominate in cellular environments. (3) The essential characteristics of the solvent environment are captured including an ability to reflect altered solvent behavior under crowded conditions such as reduced solvent dynamics and hydrodynamics interactions.

These requirements are a tall order for most coarse-grained approaches that typically focus only on certain aspects while failing to capture other features. For example, Go models can describe the thermodynamics of protein folding and complex association assuming that the structures of the end states are known and may offer some insight into how crowding shifts populations between known states.[160−162] However, perturbations of the energy landscape toward unknown conformational states that may be induced as a result of crowding are difficult to capture with structure-based models. On the other hand, simulations of rigid or spherical biomolecules via Brownian and Stokesian dynamics schemes can describe diffusive properties well,[155,157,163,164] but these approaches neglect intramolecular dynamics and conformational sampling. More sophisticated physics-based coarse-grained models[165] such as PRIMO,[166] OPEP,[150] or COFFDROP[167] that are combined with implicit solvent and/or models for hydrodynamic interactions[156] are in a better position to satisfy these criteria, as the increased level of model detail requires fewer constraints and provides more transferability and ability to capture the various features outlined above. Such models can at least at a general qualitative level capture the main features of biomolecular structure and dynamics cellular environments.[149,168,169] Atomistic simulations, with explicit[26,143,170−176] or implicit solvent,[27,97,98] or multiscale models, where atomistic and coarse-grained resolutions are mixed,[169] provide even greater levels of detail and can, at least in principle, satisfy all of the requirements for modeling crowded cellular environments outlined above. However, because the balance between molecular stability, weak interactions, and solvent interactions in crowded environments depends on subtle shifts between enthalpic and entropic energy terms, the major challenge is an accurate interaction potential, both at the coarse-grained and atomistic level that can accurately reproduce both intra- and intermolecular interactions.[177−179] For example, just a slight unbalance between protein−protein and protein−water interactions is enough to lead to aggregation[177] and overcompaction artifacts[180,181] with significantly mispredicted diffusive properties being one consequence. The choice of the water model is also a concern, since the most widely used models (SPC/E[182] and TIP3P[183]) significantly mispredict viscosity and dielectric properties[26,184,185] which in turn impacts macromolecular properties.[185] Therefore, future efforts should continue the improvement of force fields[186−192] to better balance the interactions between the various cellular components.

Another issue is the high computational cost of the most detailed models that limits the time scales that can be accessed. Current computer hardware, especially GPUs and other many-core coprocessors such as Intel Xeon Phi and high-performance simulation software,[193−198] are now allowing for atomistic simulations of biomolecules in crowded cellular environments on time scales well into the microsecond regime with millisecond dynamics being within reach now[199] and probably becoming routine within a decade. While such time scales are short compared to most functional dynamics of biological processes, they cover the majority of diffusive processes within the cell for all but the very largest particles such as ribosomes based on the extrapolation of sub-microsecond diffusion rates in crowded environments estimated from simulation.[173] Therefore, the development of more accurate coarse-grained models that can describe biomolecular dynamics on millisecond

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Table 1. Simulations of Crowded Peptide and Protein Systems with Atomistic Detail

solutes[a]	solvent	sampling	year	group[ref]
Gly/Val/Phe/Asn (50−300 g/L)	expl. water	1 μs MD	2013	Elcock[178]
Gly/Ala/Val/Phe (50−300 g/L)	expl. water	10 ns MD	2015	Price[175]
$(GlySer)_2$ (9−560 g/L)	TIP3P water	8 μs MD	2014	Rao[29]
27 × $(Xxx^l)_4$ (40 mM; 10−30 g/L)	expl. water	100 ns MD	2016	Taiji[213]
Gly_5 (0.03/0.3 M; 10/100 g/L)	TIP3P water	100 ns MD	2014	Pettitt[176]
1 × Ala/Ala_{15}/melittin	GB[k] (ε = 2−80)	44−83 ns ReX[e]-MD	2008	Feig[27]
1 × Trp-cage/melittin + 8 × protein G (CG) (40% vol; 540 g/L)	GB[k] (ε = 80)	20 ns ReX[e]-MD	2012	Feig[169]
1 × chignolin, 0−8 × HS[c] (0−40% vol)	TIP4P water	1 μs MD	2012	Rajagopalan[215]
1 × Trp-cage + 8 × BPTI (7% vol; 95 g/L), 8 × HS[c] (7%/20% vol)	implicit	10^7 ReX[e]-MC moves	2015	Irbäck[98]
1 × Trp-cage/GB1m3 + 8 × BPTI (7% vol; 95 g/L), 8 × GB1[d] (7% vol; 95 g/L), 8 × HS[c] (20% vol)	implicit	10^7 ReX[e]-MC moves	2016	Irbäck[97]
4/8 × villin[b] (6/9.2 mM; 25/40 g/L)	SPC wat./NaCl	50−200 ns MD	2014	Zagrovic[177]
8 × GB1[d] (10−30% vol; 135−405 g/L), 4 × GB1[d]/8 × villin[b] (10−43% vol; 135−580 g/L)	TIP3P water	300 ns MD	2012; 2013	Sugita/Feig[26,171]
1 × CI2,[f] 8 × lysozyme (100 g/L), 8 × BSA[g] (100 g/L)	TIP3P water	100−250 ns MD	2012	Sugita/Feig[170]
1/4 × ubiquitin (16% vol; 216 g/L), 19/157 metabolites	TIP3P water, KCl, $MgCl_2$	200 ns MD	2011	Guallar[207]
1 × ubiquitin, glucose (325 g/L)	TIP3P water	500 ns MD	2013	Dal Peraro[112]
1−3 × ubiquitin, glucose (108/325 g/L)	TIP3P water	600 ns MD, 30−70 ns ReX[e]-MD	2014	Dal Peraro[208]
3 × ubiquitin, Glu/Arg/glucose (300 g/L)	TIP3P water	600−800 ns MD	2016	Dal Peraro[143]
1 × ACTR[h]/6 × NCBD[i]/1 × IRF-3[j] (175−296 g/L), 4 × ACTR[h]/24 × NCBD[i]/4 × IRF-3[j] (183 g/L)	TIP3P water	100 ns−3 μs MD	2016	Orozco[174]
8 × BSA[g] (400 g/L), 88 × GB1[d] (400 g/L), 132 × villin[b] (400 g/L)	TIP3P water	100 ns MD	2017	Sugita/Feig[172]
100s−1000s of proteins/RNA (300 g/L), 5000−40,000s of metabolites	TIP3P water	20−140 ns MD	2016	Sugita/Feig[173]

[a]Reported concentrations are underlined. [b]Villin headpiece. [c]Hard sphere. [d]B1 subunit of protein G. [e]Replica exchange sampling; given times are per replica. [f]Chymotrypsin inhibitor 2. [g]Bovine serum albumin. [h]Activator for thyroid hormone and retinoid receptor. [i]Nuclear coactivator-binding domain of CREB. [j]Interferon regulatory transcription factor. [k]Generalized Born implicit solvent. [l]Any amino acid.

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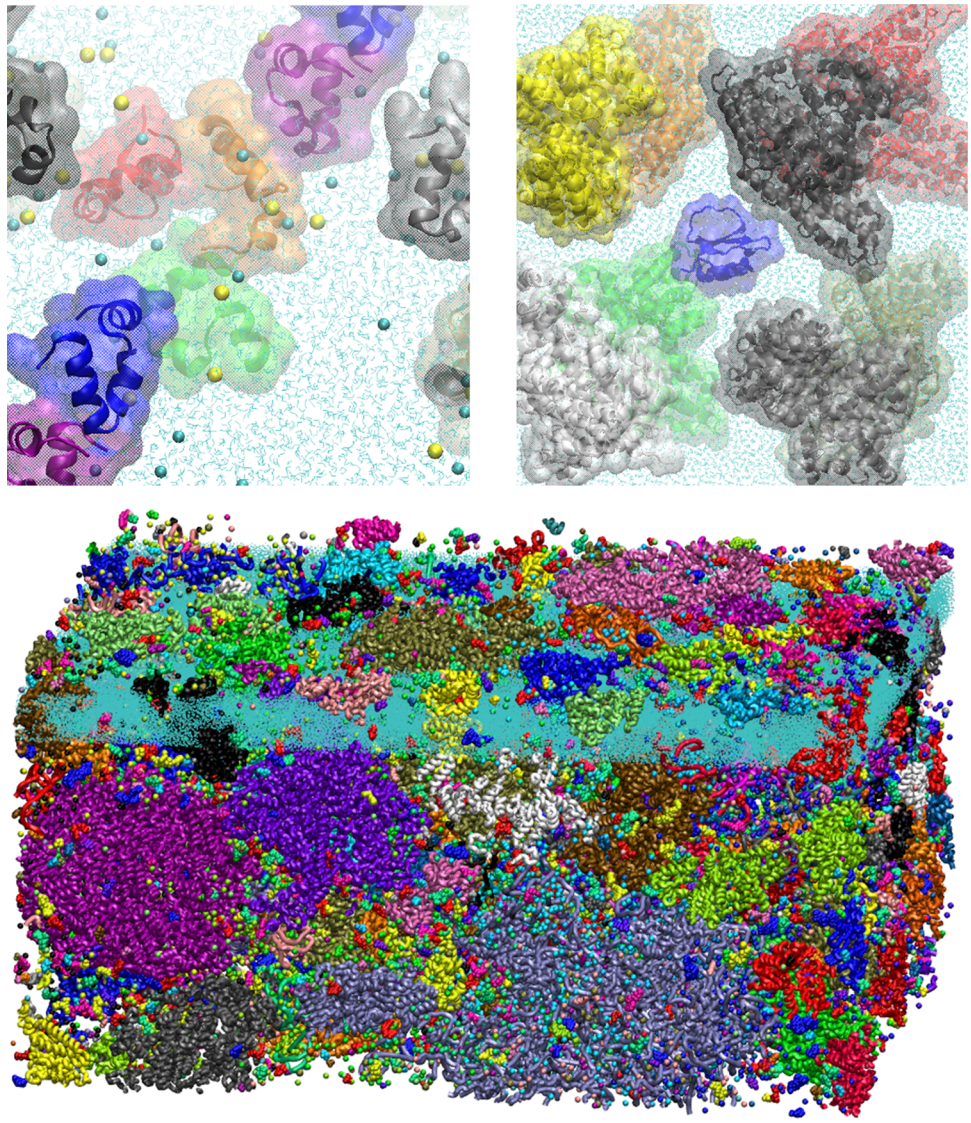

Figure 1. Crowded and cellular systems studied via molecular dynamics simulations: Concentrated solution of villin in explicit water and ions (top left); chymotrypsin inhibitor 2 (CI2; blue) in the presence of bovine serum albumin (BSA) crowders with explicit water (top right); model of a bacterial cytoplasm consisting of proteins, RNA, metabolites, ions, and water shown only for part of the system in cyan (bottom).

to second time scales remains an attractive goal in the foreseeable future. The length of the simulations also determines the degree of sampling and the statistical significance of properties determined via averaging. However, this is a minor issue, since parallel computing architectures facilitate replicate simulations and crowded systems with multiple copies of a given system of interest allow ensemble averaging in addition to time averaging.

One strategy for overcoming limitations in simulation times is the use of enhanced sampling techniques[200] such as umbrella sampling,[201] generalized-ensemble methods,[202,203] metadynamics,[204] λ-dynamics,[205] or accelerated MD,[206] just to name a few. These methods generally focus on specific processes where significant kinetic barriers hinder sampling, and they are usually used to extract thermodynamic quantities such as ligand binding affinities. So far, enhanced sampling methods have not seen extensive use, in part because there is still very limited knowledge about what states may exist under crowded conditions and enhanced sampling methods are less advantageous for exploratory simulations where little is known about a system's behavior. However, as more information is becoming available, the energetics of protein−protein or protein−ligand interactions or conformational dynamics between different states seen under crowded conditions are good problems for using enhanced sampling. Enhanced sampling methods are also less effective for accelerating processes that are limited by slow diffusion, a major factor in crowded cellular environments. Diffusion-limited processes can be accelerated, however, by using mean-field models of cellular environments,[27,103,152] although the implicit models have other drawbacks, as they may oversimplify cellular environments[169] and the extraction of kinetic properties is hindered. Developing enhanced sampling methods that target the sampling of diffusion-limited processes while fully capturing all components of cellular environments and allowing the extraction of thermodynamic and kinetic properties remains a significant challenge that we hope can be addressed in the future with new methods.

While it is clear that the simulation of crowded cellular environments is still in its infancy, the aim of this Feature Article is to review what has been reported so far from

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molecular simulations of proteins in dense cosolvents,[97,98,112,143,207,208] of dense peptide and protein solutions,[26,29,143,159,170−172,174,175,209−213] and of more complex models of cellular environments.[112,163,168,214]

ATOMISTIC SIMULATIONS OF CELLULAR CROWDING

Table 1 gives an overview of crowding simulations studied via atomistic models in recent years. We selected studies of peptides and proteins where at least the solute for which crowding effects are studied is represented in atomistic detail but allowed for crowders and/or solvent environments to be modeled in a coarse-grained or implicit fashion. We included simulations of dense amino acid and peptide systems, as they offer insight into protein amino acid interactions under crowded conditions. Despite some overlap, we did not consider simulations involving amyloidogenic peptides.[216] The concentrations of solutes in those studies are generally low compared to crowded environments, whereas their analysis primarily focuses on their aggregation propensities offering less insights into cellular crowding. In addition to studies of peptides and proteins, there are also a few studies involving nucleotides and nucleic acids under crowded and concentrated conditions,[103,217−219] but since general insights have remained limited, these studies will also not be discussed here.

As a representative overview of the kind of systems that are now being studied in atomistic detail via computer simulations, Figure 1 shows models that have been studied recently in our group. The systems range from mixtures of a few proteins at different concentrations to models of cytoplasmic environments with thousands of macromolecules. The number of atoms in these systems ranges from 50K to 100M atoms, and the time scales that were reached varied between tens of nanoseconds and microseconds. In all of these cases, all-atom force fields were used to describe the solutes, aqueous solvent, and any other molecules such as metabolites that were present, allowing for unrestrained dynamics of all components. On the basis of simulations of such systems, exciting new ideas have been found for how the stability and dynamics of proteins may be modulated by interactions with other molecules in the cell.

Solute Interactions. A key feature of crowded environments is the opportunity for frequent encounters between biomolecules. The well-understood volume-exclusion effect of crowding typically assumes crowder molecules that are repulsive or at least not attractive so that such interactions would be minimized to just unavoidable random collisions. However, atomistic simulations of crowded environments have shown many examples of attractive interactions between proteins[97,98,170,171,173,174,177] and between proteins and other molecules such as metabolites.[143,173,207] Such interactions are generally nonspecific; i.e., the formed complexes cover a broad range of arrangements and do not correspond to specific functional complexes, but they have the potential to significantly perturb protein structure and dynamics over solutes in dilute environments that are free from such contacts. Weak transient interactions, commonly termed quinary interactions,[2] nevertheless, do have the potential to be functionally relevant, for example, to bring enzymes involved in a common metabolic pathway into proximity to facilitate substrate channeling.[173] Whether such close interactions are formed depends greatly on the involved partners. For example, chymotrypsin inhibitor 2 (CI2) interacts extensively with lysozyme but hardly at all with bovine serum albumin (BSA), whereas BSA prefers to interact with other BSA molecules.[170] A similar finding was reported for Trp-cage in the presence of protein G vs bovine pancreatic trypsin inhibitor (BPTI) crowders, with BPTI more strongly self-interacting with other BPTI molecules.[97] Differential protein interaction propensities can be traced to the nature of the amino acids decorating the surface of different proteins that lead to charge differences[98] and hydrophobic patches[97,171] along with sufficiently good shape complementarity to allow close interactions. Simulations of dense solutions of amino acids and small peptides[29,143,175,176,178,213] have offered additional insights into which amino acids are more favorable to interact[178] and how such interactions are formed via hydrogen bonding,[176,213] aromatic ring interactions,[213] dipole−dipole interactions,[176] or mediation via water molecules.[29] Generally, the degree to which amino acid interactions were found to be favorable reflects established hydrophobicity profiles;[213] e.g., isoleucine, leucine, and valine readily interact due to their hydrophobicity, whereas basic and acidic residues strongly prefer to remain solvated.[143,213] There are differences that can be at least in part be explained by strong intermolecular self-interactions, e.g., between asparagine and glutamine side chains,[213] but variations were also seen as a function of the force field that was used.[178,213]

A concern that has been raised is whether the overall strength of peptide−peptide and protein−protein interactions in dense solutions is correctly calibrated in the current generation of force fields. On the basis of simulations of villin, it has been suggested that protein−protein interactions may be overall too strong relative to protein−water interactions.[177] This would also be generally consistent with the observation that intrinsically disordered peptides are too compact with most force fields[220−222] unless amino acid−water interactions are enhanced.[180,191] However, simulations of concentrated amino acids and peptides seem to come to the opposite conclusion that solubilities are significantly overestimated compared to experimental values.[176,178] This is clearly an area that will require further attention, but significant challenges exist in how to effectively compare with experimental measurements. For example, the extraction of solubilities that are comparable with macroscopic measurements, while possible in principle,[176] requires a system with a large number of solutes that are simulated over long enough times to overcome kinetic bottlenecks of aggregate formation. Another possibility is to match rotational and/or translational diffusion rates for concentrated solutions as the slow-down in diffusion upon crowding is expected to depend upon the extent of protein interactions[170] while deviations from nonideal behavior vary with the crowder type.[123] A meaningful comparison between experiments and simulations also requires long simulations as well as reliable force field parameters for solutes and solvent and faces potential experimental difficulties in analyzing highly retarded and likely complex dynamics in concentrated solutions, e.g., via nuclear magnetic resonance (NMR) spectroscopy.[81,175]

Apart from interactions between peptides and proteins, some studies have examined interactions with high concentrations of smaller cosolvents such as metabolites[173,207] or glucose[112,143,208] or osmolytes such as trimethylamine *N*-oxide (TMAO).[223] The general finding is that such molecules may interact extensively with protein solutes[172,207] or among themselves,[207] displace water molecules,[143] and further affect the structure and/or dynamics of proteins as a consequence of

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such interactions.[173] The simulations can be compared with experiments[224−227] by quantifying preferential solvation via the integration of MD-derived density fluctuation of water and cosolvents to obtain Kirkwood−Buff integrals.[228−232] While this approach can be readily applied for proteins in two-component, or at the most, three-component solvents with small cosolvent molecules, the extension to cellular environments where a large number of components are present and density distributions of larger molecules such as proteins do not converge well is not as straightforward.

Protein Structure Stability. A central question is whether crowded cellular environments affect the stability of protein native states and, more broadly, whether the conformational landscape is modulated over dilute conditions. As mentioned already above, one aspect of crowding, the reduction of accessible volume, is well understood and generally believed to stabilize the native state as the most compact state a given protein will assume.[233] Unfortunately, such a simple view is not borne out in the atomistic simulations of peptides and proteins under crowded conditions. Instead, the simulations suggest that crowded cellular environments may perturb the native state, either directly due to interactions with other solutes[170,173] or indirectly due to altered solvent properties (see example in Figure 2).[26,27,29,172] There is also evidence from simulation that

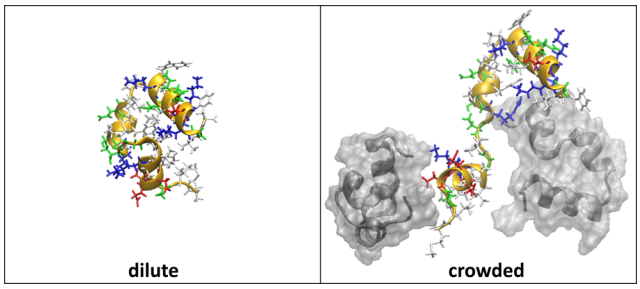

Figure 2. Villin native state destabilization under crowded conditions.

crowding may not just affect the native state but also folding intermediates[215] or the unfolded ensemble based on the analysis of an intrinsically disordered peptide.[174]

One theme for such native state perturbations is the competition between inter- and intramolecular interactions that can lead to a shift toward more extended states. Examples range from concentrated $(GlySer)_2$ solutions,[29] Trp-cage in the presence of protein G[169] or BPTI,[97,98] villin in villin/protein G mixtures,[171] pyruvate dehydrogenase α subunit in a cytoplasmic environment,[173] and ordered and natively disordered proteins in a heterogeneous environment.[174] The presumed general mechanism in all of these cases is that favorable intermolecular interactions with peptide or protein crowders combined with an overall reduction of solvent exposure due to complex formation can drive partial unfolding. However, specific interactions with crowders such as edge-to-edge β-sheet interactions may also have the opposite effect of stabilizing the native state, as demonstrated for the GB1m3 hairpin.[97]

A second theme is the modulation of secondary structure propensities as a result of crowder interactions. While this has been systematically analyzed using coarse-grained models,[95,96] atomistic simulations have provided evidence for an apparent stabilization of secondary structure elements, especially helices, as a result of crowding.[27,97,98,174] One way to understand that is via crowder amino acid side chain interactions that stabilize helices[95] or β-hairpin turns.[97,98] Another interpretation is based on a reduced dielectric response of solvent in crowded environments[26,27] where intramolecular hydrogen bonding would be strengthened as a result of reduced electrostatic screening.[27]

A third theme is a disruption of the hydrophobic core that would lead to a general destabilization of natively folded peptides.[27,29,169,174] This could simply be a corollary of a shift toward extended states driven by protein−protein interactions, but it may also be understood in the context of a reduced dielectric response of the solvent that would imply a weakened hydrophobic effect.[27]

Finally, a newly emerging theme is the role of metabolites in modulating protein structure. Functionally relevant ligand-induced conformational changes are well documented,[234] and it is easy to imagine that nonspecific metabolite interactions could have similar effects. Indeed, in a recent study of a model for a bacterial cytoplasm, nonspecific binding of negatively charged metabolites was found to modulate the structure of phosphoglucokinase and induce structural compaction via electrostatic screening.[173]

Experimental validation of the specific effects seen in the different simulations is largely missing so far and is therefore difficult to tell how real the specific effects seen in the simulations are. We imagine that it would be relatively straightforward to test the predicted effects of metabolites on protein structures, whereas in-cell NMR[24,38,41,42,44−46,115] and fluorescence techniques[56,58,235] should be able to test how real the crowding-induced perturbations of the conformational ensembles are. Specifically, the hope would be that the experiments could identify the presence of the predicted non-native subpopulations in the presence of certain protein crowders, but it may be challenging to discern minor populations of heterogeneous non-native conformations induced by crowding *in vivo*.[38] Other avenues for comparing the simulations with experiments would be to identify key residues based on the simulations that appear to play a role in facilitating crowding-induced structural changes and propose experimentally testable mutations that should be resistant to crowding effects on structural stability.

While the results from specific simulations are questionable without experimental validation, the overall picture from the simulations taken together is that the conformational landscape of proteins may become perturbed as a result of nonspecific weak interactions in cellular environments and a resulting competition between intra- and intermolecular interactions that can stabilize non-native states. The possibility of native-state destabilization *in vivo* is generally consistent with experiments,[22,23] but the simulations, furthermore, suggest specific mechanisms by which the energy landscape may be altered such as direct contacts with nearby crowder protein surfaces that shift the balance toward unfolded states and a role of altered solvent properties and metabolites. Nevertheless, much remains to be learned about more general principles that would, for example, allow predictions of how the structure and stability of a specific protein may be affected by cellular environments. Such insight is desirable to advance the fundamental understanding of biology, but it is also practically relevant in the context of protein design and therapeutic developments involving protein targets.

Protein Dynamics. Dynamics is what connects structure to function for all but a few proteins. The dynamics of proteins spans a wide range of time scales and covers internal,

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conformational rearrangements as well as diffusive motions. Internal dynamics may be further distinguished into fluctuations around the native state and conformational transitions between different states including the folding−unfolding transition. The available simulations suggest that fluctuations around the native state, as measured by S^2 order parameters or Debye−Waller *B*-factors, are only moderately affected by crowding.[97,98,143,174,208] Other results point at increased fluctuations as a result of protein−protein contacts in CI2 when interacting with lysozyme,[170] whereas somewhat reduced backbone dynamics were observed in other studies as a result of crowding.[112,174] Experiments also indicate moderate effects of crowding on fast internal motions but a stronger influence on slower conformational dynamics.[41,110,236] A more detailed understanding of how crowding may affect internal dynamics beyond these general observations can be gained from direct comparisons between MD and NMR,[143] but further studies are needed to address this question. A somewhat clearer picture exists for the kinetics of conformational transitions between different states. Crowding with either proteins[174] or glucose[143,208] led to a significant slowdown in conformational sampling. A similar conclusion was also reached for $(GlySer)_2$.[29] The slowdown in conformational transitions has been correlated with crowders, such as glucose, or water molecules becoming trapped between solutes and exhibiting slow diffusion and long residence times.[29,208] It remains difficult, however, to clearly separate cause and effect with respect to retarded solvation causing slow conformational dynamics or *vice versa*. In any case, as conformational transitions are essential in many biological mechanisms, understanding how much such kinetic processes are slowed down in cellular environments is critical for fully understanding biological processes.

Translational and rotational diffusion is well-known to be significantly slower under crowded conditions (by about a factor of 10).[237−240] Furthermore, experiments show varying degrees of deviations from nonideality with respect to how the observed diffusion slows down with increased viscosity as a function of crowder type (proteins vs nonproteins and also between different proteins) that are taken to indicate different degrees of protein−crowder interactions.[121−123] Diffusion rates from atomistic simulations under crowded conditions are generally consistent with these observations,[170,173,175,208] and simulations clearly show a slowdown in diffusion that strongly depends on protein−protein interactions.[170,173] This means in the context of a diverse cytoplasmic environment that different copies of the same protein may experience very different diffusion rates depending on the local environment. Or for a given molecule, diffusion may vary significantly on μs−ms time scales as different local environments are sampled as the molecule diffuses across a cell.[173] In addition, there is evidence for anomalous diffusion as a result of caging by crowders, especially when interactions with those crowders are weak.[170] One may take that argument a step further to postulate that a diffusing protein molecule in a heterogeneous cellular environment should experience a spectrum of diffusion time scales as crowders of different sizes and with different levels of attraction are encountered. This analysis informs dynamic models of processes at cellular levels that require diffusion rates as input.[241]

Taken together, new insight into the dynamics of biomolecules under crowded conditions from the simulations is a significant degree of heterogeneity in the diffusion on microsecond time scales, while other findings so far are largely confirming more general observations that are known already from experiments. We believe that simulations are especially valuable for describing how dynamics varies over different time scales in the presence of crowding. This will require that more efforts are made to extend future simulations of crowded systems well into the tens of microseconds regime which is technically becoming possible with specialized hardware such as the Anton 2 supercomputer.[242]

Solvent Properties. Most of the simulations discussed here involve explicit solvent. Therefore, the analysis of how crowding and cellular environments affects solvent properties is straightforward. The first realization from such analysis is that bulk-like water, defined here as water molecules beyond the second solvation layer from the closest macromolecular solute, only represents a small fraction under cellular conditions with macromolecular concentrations of 30−40% vol.[26] For concentrations beyond 40% vol, even water molecules in the second solvation layer become rare. This means that under crowded conditions most of the water is either in the first solvation shell in direct contact with a macromolecular solute or sufficiently close to still experience the electric field of a solute (see Figure 3). While this has only a minor impact on water structure,

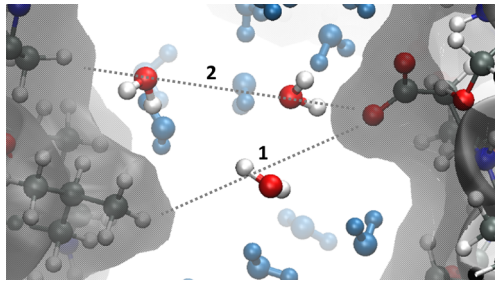

Figure 3. Interfacial water under crowded conditions with one (1) or two (2) layers of water between noninteracting proteins.

analyzed in terms of pairwise radial distribution and hydrogen bonding,[26,29] water dynamics has been found to be more strongly altered. According to the simulations, self-diffusion of water is reduced and residence times are increased significantly upon crowding.[26,29] Further analysis suggest that the crowding effects vary with the size of the protein crowders.[172] Smaller proteins led to a more pronounced slowdown in dynamics than large proteins at the same volume fraction, essentially as a result of larger protein surface areas for the smaller crowders.

As a further indication of reduced dynamics, the dielectric response of water is also lowered to values between 20 and 60,[26] in agreement with experimental measurements.[146] The dielectric response of the entire cellular environment is more difficult to assess. Polar and charged metabolites and ions may increase the polarizability,[207] while proteins with interior dielectric constants lower than pure water[243,244] would be expected to reduce the overall dielectric response. A net decrease in the dielectric response in cellular environment would also be consistent with a tendency toward native-state destabilization with a partial loss of the hydrophobic core and an increase in secondary structure formation as discussed above.

Taken together, the simulations suggest that solvent exhibits significantly altered properties in crowded environments which in turn is expected to affect biomolecular structure, dynamics, and function. The general idea is that crowding reduces water

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dynamics and lowers the dielectric response which in turn suggests a reduced hydrophobic effect in crowded cellular environments. There is limited experimental data consistent with this picture,[146] but further experimental probes that quantify solvent dynamics and describe electrostatic properties under crowded conditions are needed to validate the theoretical predictions.[32]

Limitations and Failures with Current Simulations. Since most simulations on crowded cellular systems lack direct experimental validation so far, it is difficult to assess how realistic the predictions made by the simulations really are. We expect that the overall picture of altered conformational landscapes and reduced diffusional dynamics as a result of intermolecular interactions with crowders is largely correct but there are indications that protein−protein interactions could be too strong[177] and that there is a bias toward the sampling of compact states vs more disordered states due to force field artifacts[180,191,220−222] limiting the ability to accurately describe conformational ensembles of peptides and proteins under crowded conditions. Ongoing force field improvements are aimed at addressing such problems.[186] The other major issue are the relatively short time scales in the atomistic simulations published to date that have largely limited the study of dynamics to sub-microsecond time scales. Although longer time scales can be reached with coarse-grained models,[154,155] such models do not provide as much detailed insight and a major challenge going forward is how to extend atomistic simulations of crowded systems further into the μs−ms regime. Quantitative studies of dynamics are also affected by the venerable three-site TIP3P and SPC/E water models in common use today that do not describe viscosity and dielectric properties of water as well as newer, more expensive four- and five-site models.[245,246] The use of the newer water models may appear straightforward, but the main challenge is that the current force fields will need to be reparametrized to be compatible with these water models while the higher computational costs due to the additional sites further limit the time scales that can be reached in simulations.

■ CONNECTIONS WITH EXPERIMENT

The recent detailed atomistic simulations of crowded cellular environments have been motivated and enabled by experiments in the growing field of cellular structural biology.[24,38,39,41,42,44−46,54,56,85,247] There are increasing opportunities now to advance the field by complementing the experiments with simulations,[143,170,171] where the role of simulations is ideally to provide more detailed insights and make predictions that can then be validated via additional experiments. Especially new NMR and fluorescence experiments[5,6,15,17,19,20,22,44,48−50,52,54,56,71,73,75,82,84,92,105,108,132,248] are well suited to connect simulations with experiments as the experimental observables can be extracted easily from simulations and the high resolution in both spatial and temporal scales, especially with NMR, matches the level of detail in MD results well. As the experiments offer insights into biomolecular structures under concentrated conditions *in vitro* and *in vivo*, simulations can provide additional details about the exact mechanisms by which the structure and dynamics of a given system may be altered as a result of quinary interactions with cellular environments. Simulations are in principle able to combine dynamic and structural analyses for a given protein in the presence of crowders and make a direct connection with how crowding interactions lead to a modified structural ensemble. One advantage of simulations is that even minor populations can be observed that could be more difficult to detect experimentally and simulations can, at least in principle, pinpoint and quantify which interactions with other molecules in a more complex cell-like environment are most relevant for explaining crowding effects observed, for example, via in-cell NMR experiments. It is also possible to consider a variety of mutants for both the protein that is studied and the crowder proteins to obtain a more detailed view of how individual residues contribute to crowding effects. The results of such simulations would then lead directly back to experimentally testable hypotheses with respect to mutant proteins predicted to exhibit altered susceptibility to crowding effects.

As new simulations and experiments of cell-like environments have just recently begun to emerge in parallel, there are not many examples of direct comparisons yet as different systems have been studied via experiment and simulation. Focusing both sides, experiments and simulations, on the same systems in the same environments will be necessary to lead to more productive and meaningful interactions. However, coordinating experiments and simulations is not without challenges. Experimental systems may involve large crowders such as bovine serum albumin (BSA) that are computationally costly to study via simulation or involve complex *in vivo* environments that are basically impossible to reproduce exactly *in silico*. On the other hand, some computational studies involve systems that are difficult to prepare or analyze experimentally such as highly concentrated protein solutions[171] or involve probe molecules at low concentrations in highly complex environments.[173] Nevertheless, there are questions raised by recent simulations that could be tested experimentally, such as, for example, the idea that the two domains of phosphoglucokinase are closer in cellular environments because of electrostatic effects,[173] and variations in secondary structure propensities as a function of crowder interactions.[95,96] On the other hand, recent experiments of altered protein stabilities of small proteins like CI2,[22] protein L,[73] protein G,[71] and SH3[48] in the presence of protein crowders as well as cosolute and crowding effects on the binding energetics of a dimer-forming protein G variant,[68] just to give some examples, are a strong motivation for simulations to determine the exact mechanisms by which destabilization occurs.

Finally, direct imaging methods are rapidly advancing in recent years and super-resolution microscopy[249] and high-resolution cryo-electron microscopy[250] are primed to study the structure and dynamics of cellular environments, especially if the resolution both in space and time can be further increased.

■ NEXT STEPS

Detailed simulations of crowded cellular environments have so far largely focused on proteins and protein crowders. There is also some insight into the role of metabolites as cosolvents under crowded conditions, but there is clearly room for additional studies to explore how metabolites may modulate biomolecular structure and dynamics in crowded environments. A related and possibly more important question is how metabolites behave in the presence of high concentrations of biomolecules where there are ample opportunities for nonspecific interactions that could reduce effective concentrations and distract from finding enzyme active sites. Insight into such questions would be especially relevant for understanding the dynamics of drug molecules where the kinetics of reaching a given target site is a key determinant of efficacy and important

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for understanding the mechanisms of unfavorable potential side effects.

Other largely unexplored questions revolve around interactions with nucleic acids, membranes, and cytoskeletal components. Some studies are hinting at crowding effects altering the structure and dynamics of DNA and RNA,[103,104] but how the presence of nucleic acids in the cellular interior affects proteins that do not specifically bind DNA or RNA is unclear. Chromosomal DNA fills a large part of bacterial cells[251−253] and most cytoplasmic proteins will not be able to avoid nonspecific interactions with the DNA, yet there is almost no insight, especially from simulation, how that may affect protein diffusion and/or stability. The major challenge in addressing such questions is the lack of realistic high-resolution molecular models of chromosomal DNA.

Membrane protein crowding has received some attention with limited insights from simulations.[148,254,255] However, interactions between membranes and crowded cellular interiors are unclear. For example, one may wonder how nonmembrane binding proteins that are pushed to interact with membranes simply by virtue of cellular crowding maintain their stability and whether they become kinetically trapped or gain fluidity by facing a membrane bilayer instead of a crowded cytoplasm. It is also possible that the structure and dynamics of membranes themselves are affected by the presence of a crowded cytoplasm, little of which has been explored so far. Clearly, this is a wide open area that is ripe for detailed atomistic simulations to generate insights and guide experiments.

The ultimate goal is a molecular-level understanding of entire cells. Fully atomistic whole-cell simulations, at least for bacterial cells, are not too far from reality. However, a larger impact will probably be realized by using the detailed insights from atomistic simulations of cellular environments to build more simplified models that capture the essential physics but scale to longer time scales and allow the exploration of biological questions by the broader community without requiring access to extreme high-performance computing environments. Computationally tractable, yet physically realistic *in silico* whole-cell models also offer much potential for transforming rational drug design protocols that up to now largely rely on single molecules and highly empirical approaches.[256,257]

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