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Review

Formation of protein complexes in crowded environments – From in vitro to in vivo

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ABSTRACT

Traditionally, biochemical studies are performed in dilute homogenous solutions, which are very different from the dense mixture of molecules found in cells. Thus, the physiological relevance of these studies is in question. This recognition motivated scientists to formulate the effect of crowded solutions in general, and excluded volume in particular, on biochemical processes. Using polymers or proteins as crowders, it was shown that while crowding tends to significantly enhance the formation of complexes containing many subunits, dimerizations are only mildly affected. Computer simulations, together with experimental evidence, indicate soft interactions and diffusion as critical factors that operate in a concerted manner with excluded volume to modulate protein binding. Yet, these approaches do not truly mimic the cellular environment. In vivo studies may overcome this shortfall. The few studies conducted thus far suggest that in cells, binding and folding occur at rates close to those determined in dilute solutions. Obtaining quantitative biochemical information on reactions inside living cells is currently a main challenge of the field, as the complexity of the intracellular milieu was what motivated crowding research to begin with.

1. Introduction

Virtually all aspects of cellular and multi-cellular activities involve the formation of protein complexes. These complexes can be transient or obligatory, they may assemble into homo or heteromers, and eventually create a wide range of entities, from simple dimers to large multi-component structures. Classic biochemistry deals with ideal solutions, in which solute molecules are assumed to have no volume and their concentration is so low that they do not influence the properties of the solution. Accordingly, quantitative biochemical studies have been performed in vitro, using purified components in dilute homogeneous solutions. But, unlike the experimental test-tube, the cellular environment contains high concentrations of finite-sized molecules, constituting 30–40% of the total cell mass [1]. These background molecules interact with each other and with the molecules under study via soft chemical interactions, namely electrostatic, hydrophobic and van der Waals interactions, as well as hard non-specific steric interactions. The recognition that this non-ideality might have a dramatic effect on biochemical processes in living systems set off the field of crowding research. In its traditional form, crowding relates primarily to the steric interaction of crowders with the molecules under study [2]. This is opposed to other, soft interactions, which may or may not occur, depending on the nature of the specific system [3].

An array of approaches has been taken to depict the effect of crowding on biochemical processes, mostly on the thermodynamics and kinetics of protein folding and binding. First, the theoretical framework had been established by the pioneering work of Lebowitz et al. [4] and Gibbons [5], and by the fundamental and extensive formalism of Minton [6,7]. These formalisms were complemented by detailed computer simulations and in vitro measurements. A number of instructive reviews summarizing theoretical aspects and computer simulations of reactions under crowd were published in recent years [8–10]. In this review we focus on the quantitative effects of crowding on protein–protein interactions, namely the stability and rate of complex formation. We will describe key theoretical predictions, summarize major finding from in vitro studies and computer simulations and refer to recent advances in quantitative cellular measurements.

2. Predicting the effects of crowded environments on protein complex formation

The most acknowledged feature of crowded environments is that the crowding molecules physically occupy a major fraction of the solution volume. Steric interactions of crowders among themselves and with the molecules under study give rise to the so-called excluded volume effect; since a considerable fraction of

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the volume is occupied, the position of each molecule is influenced by the position of other molecules, resulting in lower entropy of the system. Accordingly, states that exclude less volume would be thermodynamically favored [2,11]. Since steric interactions are a fundamental feature of crowded solutions, the term "excluded volume" is frequently and imprecisely used interchangeably with crowding. While theoretically, soft interactions can increase the excluded volume (for example, repulsion of a charged particle from a charged surface), these are usually ignored as they are less definite in their calculation than steric effects.

Minton [6,7] used the scaled particle theory to account for the influence of crowding on the thermodynamic activities of proteins. He showed that in a solution of inert crowders, binding constants are increased by orders of magnitude at physiological volume occupancy. By modeling the structure of proteins [9,12], crowders [13] and the solvent [12] more realistically, it was shown that the excluded volume effect is less salient than originally assumed, and that the association reaction is only marginally preferred in crowded solutions. The exact contribution of crowders depends on the exact shape and stoichiometry of the interacting molecules and on the shape and volume occupancy of the crowders.

Using transition-state theory, the scaled particle arguments can be extended to show that association is faster under crowded conditions. Furthermore, Asakura, Oosawa [14] and Vrij [15] proposed that an attractive force operates between interacting particles when they are close in space. This force, termed depletion force, can be described as an effective attraction between two spheres in a crowded solution, that is induced by the inability of the crowder molecules to enter the volume between the spheres (i.e. depletion of crowder) when the sphere separation is smaller than the crowder size. This region becomes a phase of the pure solvent, while the solution outside the spheres is little affected. Therefore, a force equivalent to the osmotic pressure of the solution of macromolecules acts between the two spheres. However, association involves diffusion and collision between the associating molecules, a process that is slower under crowded conditions due to high solution viscosity. Accordingly, in the crowd, diffusion-limited reactions should be slower and transition-state-limited reactions should be faster [16]. Alternatively, Zhou [8] introduced a kinetic treatment of binding under crowd. He suggests that for diffusion-limited reactions, the two opposing effects, namely depletion and diffusion, act in a concerted manner, resulting in a moderate effect on the overall binding rate. The relative size of background and tracer molecules affects both volume exclusion [17–19] and diffusion [20] (Fig. 1). It would take few copies of a large molecule to achieve the same volume occupancy achieved by many copies of a small molecule. Therefore, at a given volume occupancy, solutions containing larger background molecules would have larger voids, in which proteins interact as if they were in dilute solution.

3. In vitro crowding studies

While the biological justification of crowding research originates from the dense environment inside living cells, most of the experiments were performed in vitro. High concentrations of purified proteins or polymers like dextran, Ficoll and PEG were added as crowding agents to otherwise simple buffer solutions. The reactions were probed and compared to the same reactions taking place in dilute solutions with no crowding agents. Working with crowded solutions imposes technical challenges, primarily due to their higher viscosity and, depending on the type of measurement, due to large background signal. Yet, it provides a well-controlled environment where crowder type, size and concentration can be systematically changed.

Largely, in vitro studies confirmed theoretical predictions by showing enhancement of both stability and rate of complex formation. However, these reports applied to complexes containing many subunits (oligomers and polymers), while complexes with low number of subunits, and specifically dimers, were rarely reported to be enhanced in crowded solutions [9,21–23]. For example, the human mitochondrial co-chaperonin protein 10 (cpn10), a heptameric protein consisting of seven identical β-barrel subunits assembling into a ring, showed a 300-fold increase in the monomer–heptamer equilibrium constant in the presence of 300 g/L Ficoll 70, a highly branched sucrose polymer [24]. A large effect was observed also by Rivas et al. [25], who probed the formation of rod-like linear aggregates of the bacterial cell division protein FtsZ in the presence of two crowder proteins – hemoglobin and BSA. Decamers and higher oligomers, which were not observed in dilute solution, were proposed to account at least one-third of the total FtsZ population at a crowder concentration of 300 g/L. An even larger effect was reported for BPTI decamers. Using magnetic relaxation dispersion, Snoussi and Halle [26] were able to resolve and quantify the fraction of BPTI decamers and monomers in the presence of dextran, a branched glucose polymer. They found that BPTI self-association was strongly enhanced, with a five to six order of magnitude increase in the equilibrium constant at about 200 g/L dextran. Interestingly, for samples with higher dextran concentration, the monomer fraction appears to increase. This non-monotonic behavior hints towards the complexity of polymer solutions that deviates from the simplistic view of the scaled particle representation. Similar non-monotonic behavior was observed for the association between TEM1 and BLIP [27], where the effect of excluded volume was evident in PEG (polyethylene

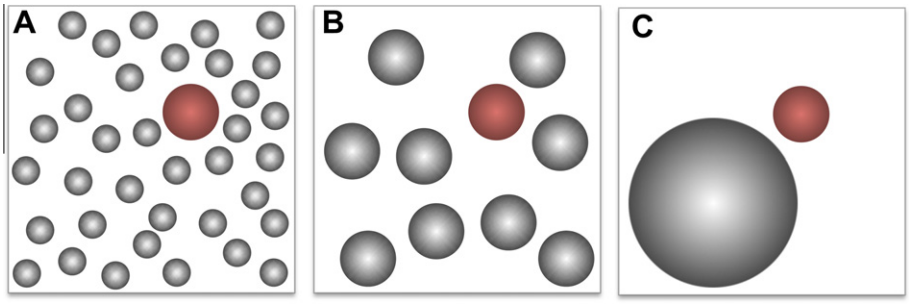

Fig. 1. Crowded solutions with varying size of background molecules. In all three cases the fraction of volume occupancy of the background molecules is 0.27. In (A), the background molecules (grey) are small relative to the tracer (red), imposing a strong excluded volume interaction (provided that the background molecules are larger than the solvent molecules). This solution is quite homogenous with respect to the tracer, and accordingly displays similar macro- and micro-viscosities. In (B), the background molecules are equal in size to the tracer. In (C), the background molecules are much bigger than the tracer and therefore the environment sensed by the tracer is heterogeneous. The micro-viscosity is lower than the macro-viscosity and approaches that of dilute solution.

glycol) solutions up to about 300 g/L, above which a repulsion between the proteins was inferred. Jiao et al. [28] assessed the equilibrium association between superoxide dismutase (SOD) and an undetermined number of binding sites on catalase in the presence of Ficoll, dextran and PEG. A maximal difference of about 50-fold in the equilibrium constant was reported in the presence of 200 g/L Ficoll 70 at 37 °C. However, it was found that lower temperature substantially modulated the effect of all three polymers on the affinity of SOD for catalase. At 8 °C there was no significant difference between the affinities under buffer and crowded conditions. This observation suggests that polymers and proteins do not interact exclusively via a non-specific steric interaction, but also via an attractive, temperature-dependent interaction that may modulate and even completely offset the effect of excluded volume. A recent study by Lee et al. [29] revealed the complex interplay between excluded volume and viscosity on the rate of amyloid formation and elongation. This study showed that in non-homogenous environments (akin to the cellular environment) the effect of crowd on fibrilization was less significant.

For specific dimerizations, most studies reported no effect or only modest increase in complex stability, and some studies even showed decreased rates of association under crowded conditions. The association rate constant of barnase with its inhibitor barstar did not change in solutions of Povidone (polyvinylpyrrolidone) [30], and was slightly reduced in the presence of PEG [31]. Moreover, at those conditions affinity of the complex was unchanged, implying a slightly reduced rate of complex dissociation [31]. Similarly, a lack of effect was reported for cytochrome *f*-plastocyanin interaction in Ficoll 70 solution [32] and for the SARS-CoV 3CL peptidase dimerization in PEG solution [33]. For TEM1 β-lactamase and its inhibitor protein BLIP, a two to fourfold decrease in association rate relative to buffer was reported for PEG, dextran and Ficoll 70 [27,31,34,35], again with a negligible effect on complex stability [31]. A mild stabilizing effect was reported for two hetero-complexes: the binding constant of the θ and ε subunits in *Escherichia coli* polymerase III was increased by up to fivefold in the presence of dextran and by less than twofold in the presence of Ficoll 70 [18]. Similarly, the heterodimer of xanthine oxidase with superoxide dismutase was stabilized in the presence of PEG, Ficoll and dextran, with an increase in binding constant of up to threefold [36]. The most substantial effect of crowd on dimerization was reported for the self-association of apomyoglobin in solutions of RNase A [37]. It was found that apoMb molecules, that are present as monomers at dilute solutions, self-associate at high RNase A concentration to yield a flexible dimer. A lower limit estimation of the equilibrium dimerization constant was $10^6\ M^{-1}$ at 200 g/L of RNase A. However, an equivalent mass concentration of HSA did not result in apoMb dimerization. The different effect exerted by the two crowders was much larger than that predicted based only on volume occupancy, indicating that additional, soft interactions between tracer and crowder may come into play.

Minton and others have provided a theoretical framework explaining why aggregation, or assembly of multimeric complexes, is greatly enhanced by crowders. The magnitude of the thermodynamic enhancement factor, which is the ratio of activity coefficients of products and reactants, is dependent on the molecular shape and weight of the associating proteins, and is increasing with the number of monomers in the aggregate [6,38]. The same phenomena is explained by Qin and Zhou by stating that while each binding event attributes only a small free energy towards the stability of the complex, the cumulative effect of multiple such events is significant [39].

To date, the aforementioned TEM1 and BLIP interaction is the only one in which the effect of crowd on the pathway of complex formation was experimentally explored [40]. Combined with a computational analysis, this study suggests that the pathway of complex formation does not significantly change under crowd, with the stability of the encounter complex, rate of final complex formation and the structure of the transition state being effectively the same as in dilute solution (Fig. 2). The apparent lack of change in the binding pathway despite slower translational diffusion of the proteins may be explained by a compensating excluded-volume attraction taking place at the encounter complex level.

Macromolecular crowding is often discussed solely in the context of volume exclusion, and as such, crowding agents are regarded as chemically inert with respect to the probed protein or system. But does this ultimate crowder exist? PEG, a widely used crowding agent, was criticized as not being an inert polymer [9], namely that it interacts with proteins not only through steric interactions. Indeed, some studies have suggested that PEG interacts favorably (although weakly) with proteins [28,41–44], but other studies have found PEG to behave as an inert polymer [31,45].

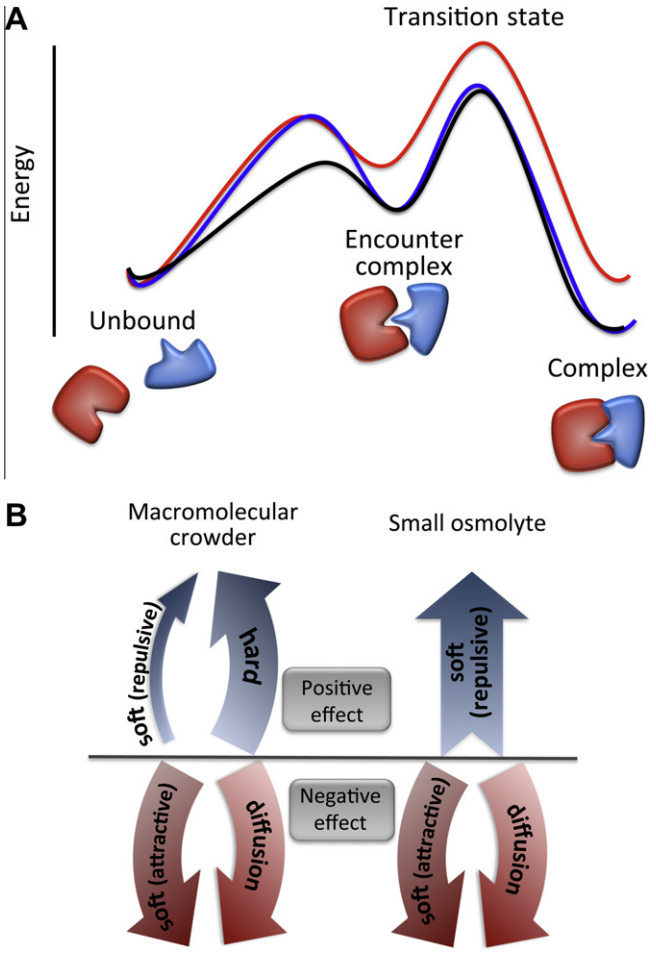

Fig. 2. Energy diagram demonstrating postulated effect of excluded volume and viscosity on the association pathway. In the presence of macromolecular crowders (blue), formation of the encounter complex would be slower compared with dilute solution (black) due to slow protein diffusion. Dissociation of encounter complex back to unbound proteins would be slower due to the depletion force, and thus encounter complex stability is similar to that in dilute solution. When the solution becomes viscose without being volume-occupied (red, as in the case of solutions containing small osmolytes), no stabilizing force counter-balance the reduced diffusion of proteins. Note that this diagram refers only to relative energy levels between states along the same association pathway, not to relative energy levels between the same states at different association pathways. The effect of hard (steric) interactions, soft (electrostatic, hydrophobic and van der Waals) interactions and diffusion on protein association rate is shown in (B). Attractive soft interactions between crowders and proteins may reduce the association rate, while repulsive soft interaction (e.g. electrostatic repulsion) may increase the association rate.

Other crowding agents are not inculpable: weak chemical interactions were reported between proteins and Povidone [46], Ficoll, dextran [28] and other proteins as crowders [37,47]. Another important aspect of soft interactions is the one of preferential binding [48]. At a given solution, the probed protein may have no preference in binding either the co-solute or water. However, a protein can preferentially bind the co-solute over the water molecules or vice versa – preferentially bind the water molecules over the co-solute (i.e. being preferentially hydrated). This may affect the behavior of proteins in crowded solutions even without direct interaction with the crowder. Either way, even if some crowding agents are regarded as inert for some model systems, no crowding agent can be claimed to operate exclusively through steric interactions for all possible proteins. While the use of crowded solutions in vitro have constructed and advanced our understanding in this field, none of these solutions can truly uncover the effect of excluded volume on protein biochemistry.

On the other hand, one can argue that as long as the use of crowded solutions is intended to uncover the effect of the cellular milieu on biochemical reactions, the request for inertness is counterproductive, since the cellular environment is certainly not inert. To better reflect the heterogeneity of the physiological environment, mixtures of crowding agents, both polymers and proteins, were used. These experiments showed that when comparing the sum of effects of each of the crowders alone with the effect of a mixture of crowding agents, the later have a synergistic effect on protein stability [49–52]. However, excluding a theoretical treatment [53], the effect of defined mixed crowding agents on association of protein complexes has not been reported. Along the same lines, cell extracts and bacterial lysates were used to achieve an improved representation of the in-cell composition of molecules. Pielak and co-workers showed that the effect of cell lysates and protein crowders on the diffusion of a tracer protein were similar, while the response to synthetic polymers was qualitatively different [54]. NMR measurements suggested that the source of this difference arises from weak interactions between the proteins present in the lysate and the tracer. This study underlines the importance of soft interactions and the possibility that synthetic polymers may not properly mimic the intracellular environment. Conversely, we recently showed that the association and dissociation rate constants of the TEM1–BLIP interaction in cell extract were similar in magnitude to those measured in dilute solutions and in solutions crowded by synthetic polymers [55]. The plot thickens when considering complex stabilities; on one hand, FRET measurements performed by You et al. [56] suggested that the affinity between the C-terminal SH3 domain from monocytic adaptor protein and the P2 peptide is increased by an order of magnitude in bacterial cell lysates. On the other hand, based on quantification of protein levels in cell lysates, and estimation of the cell volume, the binding affinity between capping protein and barbed ends of actin filaments was estimated to be two orders of magnitude lower compared with values determined in dilute solutions [57]. It should be noted here that there is a fundamental difference between concentrated solutions of synthetic polymers and concentrated solutions of proteins or cell lysates. While most proteins retain their tertiary structure at concentrated solutions, polymers create an entangled mesh rather then a semi-spherical shape [58]. Therefore, researchers should be cautious when using a spherical representation of the polymers to interpret the effects of crowded solutions.

While the effort to better reflect the cytoplasm by employing mixed crowded solutions and cellular extracts is valuable, it is inevitably inferior to measurements in actual intact cells. The complexity of the cellular environment stems from both the large number of chemically and structurally different macromolecules, and the structural division of the cell by membranes and cytoskeleton. We argue that only theoretical models and computer simulations can resolve the steric effect of crowded solutions, and that only in vivo measurements, performed directly inside cells and tissues, can uncover the actual effect of physiological environments on biochemical processes. This would be the focus of the next two sections.

4. In silico crowding studies

In silico studies of crowding may be roughly divided into two groups; those that aim to uncover the role of excluded volume by simplifying the structure of crowding agents and incorporating only steric interactions between crowder and proteins [39,59,60], and those that strive to reproduce a more realistic environment by modeling the crowders at some molecular details and including also soft interactions [61–63]. An excellent review on this subject was written by Elcock in 2010 [10].

In contrast to experimental studies, computer models of protein association under crowd tend to focus on dimerizations, probably because simulations in the presence of macromolecular crowding impose high computational cost. Zhou et al. [18,39] estimated the effect of excluded volume on the stability of two model hetero-dimerizations: barnase–barstar and polymerase III θ and ε subunits. Their calculations of the transfer free energies of the proteins from a dilute to a crowded solution suggested that the change in dimer binding constant was less than one order of magnitude for most volume occupancies and crowder sizes. Brownian dynamic simulations are often used to study the effect of excluded volume on association rates. Kim and Yethiraj [59] varied reaction probabilities following collision to model diffusion-limited and transition-state-limited interactions. The simulations nicely showed that reaction probabilities dictate the effect of crowding on association rates. For high reaction probabilities, that correspond to diffusion-dominated reactions, the rate constant decreased when the volume occupied by crowders increased. For low reaction probabilities, as in transition-state-limited reactions, the rate constant increased with increased volume occupancy, due to the increased probability of re-collisions between reactant pairs. For intermediate probabilities, the rate constant did not change much with respect to that in dilute solution. Similarly, Wieczorek and Zielenkiewicz [64] showed that the association rate was reduced for centro-symmetrically active particles (i.e. when the interaction rate is dictated by diffusion), but increased for a specific model system, where the association criteria were spatially restricted. The Caflisch group reproduced comparable outcomes for aggregation kinetics [65]. In this study, peptides were modeled as a string of beads that interact with each other through steric, hydrophobic and electrostatic forces. Crowders were modeled as spheres that interact with the peptides and with each other through Lennard–Jones potential. The influence of crowding on the self-assembly process was a combination of two competing effects: oligomer stabilization due to increased excluded volume, and reduced peptide mobility due to increased solution viscosity. Accordingly, the net effect depended on the aggregation propensity. For peptides with low aggregation propensity, crowding accelerated peptide assembly. For peptides with high aggregation propensity, the reduction in peptide mobility was more prominent than the thermodynamic stabilization of oligomers and thus crowding was less efficient in accelerating peptide self-association. Lee et al. [66,67] implemented a variation of Brownian dynamics that uses a probabilistic model of the diffusion process to achieve realistic Brownian particle trajectories in crowded environments with reduced computational cost. This model allows a systematic variation of multiple reaction parameters, including binding probability upon collision and dissociation rate constant. For high affinity reactions (i.e. high, diffusion-limited association rate and slow dissociation rate),

crowding had a moderate effect on both association rate and binding stability. Conversely, for low affinity reactions (i.e. slow, transition-state-limited association rate and high dissociation rate), binding stability was significantly enhanced [67]. Importantly, this study recognized that the effect of crowd on binding stability may be dictated by the affinity of the complex, as was previously suggested by us based on in vitro studies [31].

Studies that focus on steric interactions demonstrate successfully the importance of excluded volume, still, when dimer formation is considered, this effect is moderate. Steric-dynamic models are efficient in revealing the interplay between reaction probability and reduced diffusion. However, modeling only steric interactions between crowders and proteins is obviously inaccurate. Some studies attempt to correct this by incorporating soft interactions into the model. Studying the effects of attractive crowder–protein interactions on the stability of two hetero-complexes showed that while repulsive steric interactions with the crowder stabilized the complex, attractive interactions of the crowder with the unbound proteins favored stabilization of the unbound state [68]. By varying the attraction strength a critical point was reached, for which the excluded volume effect was counter-balanced by protein–crowder attraction and the binding free energy becomes identical to that in dilute solution. Most studies focus on the effect of interactions between crowders and the test proteins, while assuming only excluded volume interactions between the crowders themselves. Kim and Yethiraj [60] took a different approach; they modeled the proteins as hard sphere particles interacting with the crowding agents only via excluded volume interactions, and the crowders as hard spheres or chains of hard spheres (i.e. polymers) with additional attractive or repulsive interactions between them. In most cases the effect on complex stability was insensitive to the interactions between crowding agents, since it acts with similar magnitude on reactants and products.

A step towards a realistic physical representation of the cellular environment has been taken by Ridgway et al. [63]. Although only steric interactions were modeled and crowders were represented as spheres, the size distribution of the crowders, which dictate diffusion rates, was reproduced based on experimental data from *E. coli*. Analysis of barnase–barstar association kinetics revealed a phenomenon that is mostly overlooked by both experiments and simulations; while crowding generally decreases the association rate of this diffusion-limited reaction, at short time scales after initiation of the simulation crowding actually increases the association rate due to an increase in effective local concentration of the reactants. Thus crowding produces opposing effects which net impact depends on the timescale examined. An even bigger step toward realistic modeling of the physiological environments was taken by McGuffee and Elcock [62]. As a development of their previous work [69], they assembled an atomically detailed model of the cytoplasmic environment of *E. coli* by including 50 of the most abundant macromolecules at experimentally measured concentrations. Brownian dynamics simulations incorporating steric, electrostatic and hydrophobic interactions were used to calculate the stability of 11 homo-dimers and three homo-oligomers. When only steric interactions were modeled, dimers binding constants were increased on average by a factor of six compared to dilute solution. However, when electrostatic and hydrophobic interactions were considered, this effect was canceled. Interestingly, the larger stabilization of oligomers by steric interaction was attenuated, but not completely canceled, by taking into account the soft interactions.

By underlining the significance of soft interactions in modulating the excluded volume effect, in silico studies stress the importance of in vivo studies, where soft interactions between proteins and cellular components are ubiquitous.

5. In vivo crowding studies

Although the use of crowded solutions enables reproduction of major aspects of the in vivo environment, the cellular complexity cannot be achieved. The interior of a cell is not simply a viscous and crowded environment. Rather, it contains a unique and dynamic ensembles of molecules: small osmolytes, proteins, nucleic acids and fatty acids, which in eukaryotes, and to some extent also in prokaryotes, are structured in compartments and in specialized organelles. The cytoplasm itself is extremely heterogeneous, permeated by a network of cytoskeleton [70]. No crowding agent, not even a mixture of a few crowding agents, could reliably reconstruct this complex environment. Acknowledging this fact, researchers are starting to take the challenge and perform biochemical measurements inside intact cells and tissues [71]. Quantitative in-cell measurements pose technical difficulties. While for in vitro crowding measurements classical biochemical methods can be applied, in-cell measurements require the development of specialized experimental tools. For that reason, quantitative in vivo measurements of proteins are still rare.

One method to measure dissociation constants in living cells is by Fluorescence Cross-Correlation Spectroscopy (FCCS) [72]. This technique was applied by Wohland [73] to determine the affinity of Cdc42 to three effector-proteins. Compared to dilute solution, affinity of all three pairs was increased by ~twofold in CHO cells. The affinity in zebrafish embryos of Cdc42 to a fourth effector was not directly compared with dilute solution, but showed a 10-fold increase when compared with CHO cells [74]. Reaction rates inside cells displayed comparable values to those reported in dilute solutions [55]; Using fluorescence to determine concentrations, FRET to probe complex formation, and microinjection to initiate the interaction at a specific time point, we were able to calculate association rate constants of the TEM1–BLIP complex in HeLa cells. Similar to rates determined in crowded solutions in vitro, binding constants of the wild-type and mutant pairs wereless than twofold slower compared with dilute solutions. These finding are in line with phosphoglycerate kinase unfolding in cells that was ~twofold slower than in dilute solutions [75,76]. A FRET-based assay was also used to determine the dynamics of α_{2A}-adrenergic receptors with G proteins [77], but with no direct comparison to rates determined in dilute conditions. Campbell and Mullins [78] used time-lapse imaging to follow the intra-cellular dynamics of fluorescently labeled ParM, an actin-like protein from *E. coli*. Again, the polymerization rate in bacteria was similar to that measured in dilute solutions and the de-polymerization rate was less than twofold faster than in dilute solution.

Although not strictly quantitative, Burz et al. [79] presented in 2006 a promising method, called STINT-NMR, that has the potential to map the binding interface of interactions inside bacterial cells and to compare them to the same interactions probed in dilute solutions in vitro. The technique of in-cell NMR has since been applied to *Xenopus laevis* oocyte [80] and to human HeLa cells [81]. While there is evidence that protein-interaction interfaces in vitro and in vivo are similar [79,81], this method is yet to provide systematic and detailed information about the differences (if any) between binding interfaces in vitro and in vivo.

Although scarce, these in vivo measurements corroborate the in vitro and in silico data. Apparently, despite the complexity of the cellular environment, its effect on the strength and speed of interactions is moderate, usually in the range of twofold.

In cell measurements were criticized as being hard to interpretate. In their 2008 review, Zho et al. [9] discuss various concerns about this approach, including the impact of labeling on the spatial distribution of the tracer protein and the possible induction of artificial interactions with other cell components. They also questioned

the interpretation of the overall average signal of a test protein that is differently distributed in multiple micro-environments within the cell, and the effects of the inevitable stress that the measurement impose on living cells. The authors conclude that the influence of crowding on biochemical processes should be explored by a bottom-up approach, where features thought to be essential to the test proteins are incorporated systematically, from the most simple to the most complex. These are all valid concerns. The experimental limitations are not to be ignored and the validity of in vivo experiments is still to be proven. Yet, the value of these measurements could not be overestimated, and by no means should they be disregarded. We argue that, when feasible, a combined approach that encompasses both well-controlled in vitro measurements and native-like in vivo measurements should be applied. More importantly, when reactions are considered within their native environment, an attempt should be made to understand how different cell compartments [82] and physiological states [75] affect these reactions. Ultimately, this is the essence of crowding research.

6. Conclusion

Originating more than half a century ago as a theoretical field of research, advanced methodologies have brought experimental crowding studies to the mainstream. While intuitively, crowding might be conceived as a force that compacts any molecular system, the different branches of the field – theories, computer simulations and wet in vitro experiments – have converged to show that the truth is in the details; for some interactions and under some conditions, reactions can be only mildly affected or even hindered. Combining insights from in vitro studies and computer simulations, it is evident that retarded diffusion serves to balance the positive effect of crowding on association rate, while attractive soft interactions between proteins and crowders serve to modulate the positive effect of crowding on binding affinities. Crowding research is now facing the challenge of traversing from in vitro to in vivo measurements. This effort will tell whether reaction constants measured in the test tube under dilute conditions are reproduced inside living cells.

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