Soft interactions and crowding

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Abstract The intracellular milieu is complex, heterogeneous and crowded—an environment vastly different from dilute solutions in which most biophysical studies are performed. The crowded cytoplasm excludes about a third of the volume available to macromolecules in dilute solution. This excluded volume is the sum of two parts: steric repulsions and chemical interactions, also called soft interactions. Until recently, most efforts to understand crowding have focused on steric repulsions. Here, we summarize the results and conclusions from recent studies on macromolecular crowding, emphasizing the contribution of soft interactions to the equilibrium thermodynamics of protein stability. Despite their non-specific and weak nature, the large number of soft interactions present under many crowded conditions can sometimes overcome the stabilizing steric, excluded volume effect.

Keywords Crowding · Excluded volume · Osmolytes · Protein stability · Second virial coefficient · Synthetic polymers

Introduction

For ideal solutions, the measured concentrations of species exactly match their chemical behavior (i.e., activity coefficients are unity), the enthalpy of solution is zero, and the interactions between any two species are identical. Under these conditions, only water–solute interactions are important and all solutes are invisible to each other. The cellular environment deviates markedly from ideality. It is heterogeneous and crowded by many different macromolecules. For instance, macromolecules in the *Escherichia coli* cytoplasm can reach concentrations of 300–400 g/L and occupy 30–40 % of the volume (Zimmerman and Trach 1991). In cells, solutes not only interact with water but also with the other cosolutes. Moreover, the interactions are neither chemically nor spatially similar. For example, any particular cellular protein can interact with other proteins, nucleic acids, and other biomolecules. Capturing the effects of this non-ideality demands that biological macromolecules be studied in cell-like environments.

Cellular interiors are generally mimicked by using various macromolecules as “crowders”. To understand the chemical nature of crowding effects, small cosolutes such as osmolytes are also often used. To facilitate observing the effect felt by one particular macromolecule in the crowded environment, we introduce the concept of the test molecule, a species whose concentration is insignificant compared to the total concentration of macromolecules. Thus, test molecules rarely interact with each other. To detect the test molecule in a sea of the other macromolecules, it must

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possess a unique probe, such as a fluorescent tag or isotopic enrichment.

Zimmerman and Trach (1991) showed that cytoplasmic conditions change the activity coefficients of test molecules. Thus, the equilibrium thermodynamic behavior of these molecules is expected to differ in cells compared to dilute solutions.

Here, we are mostly interested in the equilibrium stability of globular proteins with two states (Anfinsen 1973): the efficiently-packed (Richards 1977), biologically-active native state (N), and the inactive, denatured state (D). D comprises a large ensemble of conformations of the disordered protein (Fleming and Rose 2008), whereas N comprises a much smaller and more compact ensemble centered on the folded conformation. In other words, D is a thermodynamic state, while N is both a thermodynamic state and a well-defined structural state. Protein stability is defined as $\Delta G_{\text{den}}^{\circ}$, the modified standard state Gibbs free energy of D minus N. The stability at a given temperature is governed by the entropy and the enthalpy of each state, $\Delta G_{\text{den}}^{\circ} = \Delta H_{\text{den}}^{\circ} - T\Delta S_{\text{den}}^{\circ}$. N possesses the lowest free energy, but D is entropically favored because it is less ordered than N (Anfinsen 1973). Increasing $\Delta H_{\text{den}}^{\circ}$ and/or decreasing $\Delta S_{\text{den}}^{\circ}$ increases protein stability by increasing $\Delta G_{\text{den}}^{\circ}$.

Most mesophilic globular proteins are marginally stable in dilute solution near neutral pH at room temperature, possessing $\Delta G_{\text{den}}^{\circ}$ values of 5–15 kcal/mol (Creighton 2010). The stability arises from the difference between two rather large ($\sim 10^2$ kcal/mol) numbers, $\Delta H_{\text{den}}^{\circ}$ and $T\Delta S_{\text{den}}^{\circ}$. The cellular environment can affect stability by altering either or both of these terms. Part of the entropic contribution under crowded conditions arises from steric hard-core repulsions between the crowding molecules and the test protein. As pointed out by Minton (1981) in his ground-breaking work, these steric interactions are predicted to increase stability because N is more compact than D. Until recently, most efforts to understand crowding effects have focused on this entropic component.

Enthalpic contributions are more subtle because they depend on chemical interactions between the crowder and either or both D and N. Attractive interactions with D and non-specific attractive interactions with the protein in general (e.g., urea-induced denaturation) lead to destabilization (Makhatadze and Privalov 1992). On the other hand, attractive interactions with N (e.g., ligand binding) tend to be stabilizing. Stabilization can also arise when the free energy of transferring a peptide bond from a dilute aqueous solution to an aqueous solution containing the cosolute is unfavorable (Timasheff 1993). This preferential hydration of N is stabilizing because unfolding exposes more backbone to cosolutes (Street et al. 2006). In summary, unlike hard-core repulsions, which are always stabilizing, non-specific interactions can be stabilizing or destabilizing.

Excluded volume

The entropic and the enthalpic contributions to protein stability can be approximately dissected into hard and soft (also called chemical) interactions, respectively. The interplay of hard and soft interactions determines the excluded volume, a useful concept for understanding crowding effects.

The excluded volume, v , equals the negative volume integral of the Mayer f -function (Mayer 1942).

$$v = - \int \left(\exp \left[- \frac{U(r)}{kT} \right] - 1 \right) d^3r \quad (1)$$

$U(r)$ is the interaction energy, which depends on the distance r between the particles. At small values of r , the interaction is highly repulsive because of the difficulty in interpenetrating the electron shells of the two species. The interaction is often attractive at larger values of r , but eventually decays to zero.

A pictorial representation is shown in Fig. 1. The total shaded area is -1 times the excluded volume. With only hard-core repulsions, the excluded volume is just the volume that one crowder excludes from the test protein. If both particles are spheres, the hard-core excluded volume equals the co-volume; the volume of a sphere whose radius is the sum of the crowder and protein radii (Winzor and Wills 1995). Excluded volume arising from hard-core repulsion also increases with crowder size and concentration (Minton 1981).

Although we have focused on the simplest system, where both crowder and test proteins are hard spheres, even this type of excluded volume depends on both size and shape. This hard-particle excluded volume can be approximated by

Fig. 1 Normalized Mayer f function (f) as a function of the distance (r , in arbitrary units) between two spheres. The sum of the shaded regions defines the excluded volume. The excluded volume can be made positive or negative by adjusting the strength of repulsive (red) and attractive (blue) interactions. Adapted from Rubenstein and Colby (2003)

using two approximations: scaled particle theory and lattice theory. Scaled particle theory allows assessment of non-ideality for any hard convex particle in the midst of similarly shaped but differently sized hard particles (Lebowitz et al. 1965; Reiss 1966). Lattice theory, on the other hand, approximates the non-ideality of a hard particle of any shape in the midst of hard rectangular parallelepipeds (Minton 1981).

Soft interactions and excluded volume

Soft interactions can add to or subtract from the hard-core excluded volume. The idea that repulsive chemical interactions increase the excluded volume is easy to grasp. For instance, if the crowder and test protein have the same charge, the resulting repulsion increases the area below the x-axis in Fig. 1. That is, repulsive interactions make the crowder appear larger, increasing the excluded volume.

The idea that attractive non-specific interactions decrease excluded volume is less intuitive because it is difficult to picture how a particle can get smaller. Although its size does not change, the attractions increase the shaded area above the axis. The net result can be, as shown in Fig. 1, that attractive interactions balance the repulsive interactions leading to a small—or even negative—excluded volume (Rubenstein and Colby 2003). In summary, soft interactions can be attractive or repulsive, either diminishing or enhancing, respectively, the excluded volume.

Excluded volume, protein stability, and Le Chatelier's principle

The relationship between protein stability and the hard-core component of crowding is straightforward. Assuming that N and D are hard spheres, the fact that the radius of D is in some sense larger than that of N, means the volume excluded to D by crowding is larger than that excluded to N. Le Chatelier's principle tells us that the species taking up the least volume will be favored under crowded conditions, which means the protein is stabilized. This stabilizing effect is completely entropic because the model involves only the arrangement of molecules, not chemical interactions between them. Minton (1981) was the first to fully describe these ideas in terms of biology. Later, Zhou et al. (2008) also considered the equilibrium effects of crowding and confinement on biomolecular association, ligand binding, and protein stability with emphasis on steric, hard-core repulsions. They concluded with remarks encouraging the incorporation of non-specific chemical interactions. Minton (2013) also mentions the effects of hard and soft interactions on protein stability in his review on crowding and protein–protein interactions.

Non-specific, soft, attractive interactions between the crowder and the test protein tend to cancel this stabilizing

hard-core effect.¹ Urea provides an example of a small molecule that acts non-specifically. This cosolute destabilizes proteins because it interacts favorably with the protein backbone. This effect is stronger for D than it is for N because D offers up more backbone for interaction (Makhatadze and Privalov 1992; Schellman 2003). Applying Le Chatelier's principle leads to the conclusion that urea favors the denatured state because D maximizes the number of attractive, non-specific interactions. These attractive interactions overcome the hard-core repulsion, and the protein unfolds. In summary, the net effect of crowding depends on the winner of a battle between attractive and repulsion interactions.

Measuring excluded volume

Assessing hard and soft interactions via their effect on thermodynamic non-ideality is one way of predicting the behavior of a system. The non-ideality is defined by the virial expansion of the chemical potentials and the molar activity coefficients involving solute–solute, solute–cosolute, and cosolute–cosolute interactions. In this expansion, each component is identified by a subscript: 1 is the solvent, 2 is the test protein, 3, etc. are cosolutes (Scatchard 1946). B_{23} , B_{223} , B_{233} , etc., are cross-virial terms between the protein and the cosolutes. The latter two terms and others like them are unimportant in the studies discussed here because, by definition, the concentration of the test protein is small. The key term is ' B_{23} ', the second virial co-efficient, which measures the protein–crowder interaction. When no chemical interactions are present and both species are spheres, B_{23} is the excluded volume as represented by the co-volume (McMillan and Mayer 1945; Winzor and Wills 1995).

$$B_{23} = (4/3)\pi N(r_2 + r_3)^3 \quad (2)$$

where N is Avogadro's number. Thus, B_{23} measures the excluded volume as defined in Eq. (1), and its assessment can identify the source of crowding effects. Note that soft effects can be “fudged” by adjusting the size of the crowder in Eq. (2).

With the exception of Minton's (1995) seminal work on using osmotic pressure to define soft interactions involving Bovine Serum Albumin (BSA, 68 kDa), much of the work in this area has focused on the small molecule cosolutes. The Record group (Courtenay et al. 2000) used vapor pressure osmometry with the test protein BSA and six small cosolutes to obtain values of B_{23} . Their work focused on

¹ Non-specific attractive interactions are fundamentally different from ligand binding, because ligands bind only to N. Application of Le Chatelier's principle leads to the conclusion that ligand binding stabilizes proteins by favoring N.

correlations between protein–cosolute interactions and changes in water-accessible surface area. Similar studies probed the interactions between the test protein cytochrome *c* and small cosolutes (Weatherly and Pielak 2001) using sedimentation equilibrium analytical ultracentrifugation. Both studies showed that B_{23} decreases in systems where the small molecules interact favorably with the test protein and increases in systems when there is enhanced repulsion. A more detailed assessment of second virial coefficients for small osmolytes is given by Davis-Searles et al. (2001).

Crowding and assembly

Turning to the effects of macromolecular crowding on reactions, the self-association of the bacterial cell division protein, FtsZ, was studied by Rivas et al. (2001). Using cyanomethemoglobin and BSA as crowders and nonideal tracer sedimentation equilibrium ultracentrifugation for detection, they observed that crowding increases the weight average degree of the association of FtsZ. They concluded that pure hard-core excluded volume promotes the self-assembly. Hirota et al. (2010) examined the conversion of a linear cytochrome *c* trimer to the smaller cyclic trimer in a system crowded with polyethylene glycol, and observed that crowding shifted the equilibrium toward the cyclic trimer. Jiao et al. (2010) studied the effects of three synthetic polymers on the association of catalase and superoxide dismutase. They found that steric repulsions dominate at high temperatures, but protein–polymer non-specific interactions dominate at low temperatures.

Soft effects and the challenge of in-cell protein NMR

The ^{15}N - ^1H Heteronuclear Single Quantum Coherence (HSQC) experiment yields a protein's fingerprint (Rule and Hitchens 2006). The covalent bond between the backbone amide ^{15}N and its proton gives rise to a feature, called a crosspeak, at the chemical shift coordinates of the two nuclei. For this reason, the experiment is a powerful tool for obtaining residue-level information from ^{15}N -enriched proteins. Resonance broadening with the resultant loss of resolution and sensitivity, however, makes HSQC-based in-cell NMR a challenge for the study for globular proteins (Pielak et al. 2009; Barnes et al. 2011; Barnes and Pielak 2011; Crowley et al. 2011; Wang et al. 2011).

To obtain a high resolution HSQC spectrum, the test protein must have considerable rotational motion (Rule and Hitchens 2006). Increasing the molecular weight of the protein or the solution viscosity decreases its rotational motion, resulting in poor quality spectra. The cellular environment seems to increase the molecular weight of test proteins because poor or non-existent HSQC spectra are obtained for proteins that otherwise give outstanding dilute

solution spectra (Li et al. 2008). The apparent increase in molecular weight could be the result of increased viscosity in cells and/or the presence of numerous weak, non-specific attractive interactions between the test protein and other cellular macromolecules.

Soft interactions are important in cells

NMR studies of protein diffusion and dynamics can help differentiate weak non-specific interactions from increased viscosity under crowded conditions. Li and Pielak (2009) used an ^{15}N -relaxation experiment to identify the presence of attractive non-specific interactions involving the test protein Chymotrypsin Inhibitor 2 (CI2, 7.4 kDa) with BSA as the crowder. These interactions are also expected to affect diffusion. Wang et al. (2010) used ^{15}N relaxation to observe deviations in CI2 rotational diffusion under crowded conditions. These authors crowded the system with glycerol, polyvinylpyrrolidone, Ficoll, lysozyme, ovalbumin, BSA, and *E. coli* lysates. Rotational diffusion was impeded more in protein crowders than in synthetic polymers. These studies of nuclear relaxation showed that protein crowders and lysates interact favorably with CI2. In summary, attractive non-specific interactions increase the apparent molecular weight of globular test proteins in cells, impeding their rotational diffusion, which leads to poor quality, or non-existent, in-cell HSQC spectra.

Crowley et al. (2011) studied the interactions of GB1 (6.2 kDa, pI 4.5) and cytochrome *c* (11.5 kDa, pI 9.6) with cytosolic *E. coli* proteins by using size exclusion chromatography of cell lysates. GB1, which gives a good in-cell HSQC spectrum, elutes at a volume commensurate with its calculated molecular weight, showing that it does not interact strongly with cytosolic proteins. On the other hand, the highly positively charged protein cytochrome *c*, which does not give an in-cell HSQC spectrum, elutes at lower volume, implying stronger interactions. At least some of the effect is caused by charge–charge interactions because increasing the ionic strength or introducing charge-inverting mutations increased the elution volume of cytochrome *c*.

Interestingly, HSQC spectra of disordered proteins such as FlgM and α -synuclein are easier to observe in cells (Dedmon et al. 2002; Barnes et al. 2011). This increased detectability probably arises because disordered proteins possess much more internal motion than do globular proteins, and this internal motion is less affected by attractive interactions with other cellular macromolecules.

To determine why globular proteins give such poor in-cell HSQC spectra, the Gierasch group studied three small globular proteins, GB1 (6.2 kDa), NmerA (6.9 kDa), and ubiquitin (8.7 kDa) (Wang et al. 2011). They observed good quality in-cell spectrum of monomeric GB1, but a poor quality spectrum of NmerA and no signal from ubiquitin.

Their dimeric GB1 construct, although twice the size of NmerA, produced a reasonably high quality spectrum. They deduced that, if viscosity alone caused the broadening, folded proteins smaller than 13 kDa would give good quality in-cell spectra, which was not the case. They then set out to identify the interactions that result in poor or useless HSQC spectra.

NmerA and ubiquitin have the same number of surface hydrophobic residues, but they are localized in ubiquitin and dispersed in NmerA. Decreasing the surface hydrophobicity of ubiquitin and altering its distribution by changing three valine residues to alanines, yielded sharper spectra in lysates, indicating that the hydrophobic effect contributes to soft interactions in cells. Charge–charge interactions also play a role. The environment inside *E. coli* is anionic (Spitzer and Poolman 2009). GB1 has a negative charge at physiological pH whereas NmerA and ubiquitin have insignificant net charge, making the latter two more ‘sticky’. As discussed above, Crowley et al. (2011) have also pointed out the importance of charge.

In summary, the absence of signals in in-cell NMR experiments on globular proteins can be explained by weak attractive interactions, including charge–charge and hydrophobic interactions. Next, we turn to the effects of crowding on protein folding in cells and on the equilibrium thermodynamics of globular protein stability.

Protein folding in the cell

Schlesinger et al. (2011) tested the ability of the *E. coli* cytoplasm to fold a globular protein in cells. Wild-type Protein L (ProtL, 7.0 kDa) and a disordered variant were studied. As expected, the wild-type protein does not generate an in-cell HSQC spectrum. On the other hand, its Kx7E variant is disordered and, again as expected, yields a reasonable in-cell spectrum. Dilute solution studies showed that the variant required only 0.4–1.0 kcal/mol to fold (Tadeo et al. 2009). If macromolecular crowding effects in cells were predominately the result of hard-core repulsion, then the cellular environment would be expected to provide sufficient free energy to fold the protein. The protein, however, remained unfolded in cells. This observation suggests that soft interactions can overcome the stabilizing effects of hard-core repulsions.

In vitro effects on stability: crowding by synthetic polymers

With opposing phenomena playing key roles, it might be possible to predict the net effect of crowding given the details of the system. Hard-core repulsions will increase $\Delta G_{\text{den}}^{\text{O}^{\circ}}$ by shifting the equilibrium towards N, because N is more compact. This phenomenon has been studied theoretically and experimentally. Theoretical models have been successful in mimicking the hard-core repulsion effect (e.g.,

Berg 1990; Rivas et al. 2001; Zhou et al. 2008; McGuffee and Elcock 2010), and many ‘wet’ experiments show that crowding by synthetic polymers stabilizes proteins (e.g., Sasahara et al. 2003; Stagg et al. 2007; Charlton et al. 2008; Christiansen et al. 2010; Miklos et al. 2010; Benton et al. 2012). These observations are consistent with theoretical expectations in as much as the stabilizing hard-core repulsions seem to dominate, although significant attractive protein–crowder interactions have been noted for polyethylene glycol (Crowley et al. 2008; Hirota et al. 2010; Zhang et al. 2012) and the monomer of polyvinylpyrrolidone (Charlton et al. 2008).

We close this section with three caveats. First, synthetic polymers probably cannot often be approximated as impenetrable spheres (Pielak and Miklos 2010), making application of simple ideas such as co-volume problematic. An early simulation of excluded volume effects for polymer–protein interactions was performed assuming that polymers are flexible chains obeying Gaussian statistics (Hermans 1982). This assumption gave a more realistic picture. Second, crowding effects can be more complicated if the test protein is not spherical; Homouz et al. (2008) combined simulation with wet experiments to show that macromolecular crowding affects both the shape and stability of an aspherical test protein, VlsE. Third, the role of preferential hydration (Timasheff 1993) in the observed stabilization remains unclear.

In vitro effects on stability: crowding by proteins

Miklos et al. (2011) assessed CI2 stability in solutions crowded by BSA and lysozyme. In contrast to the stabilization observed with synthetic polymers, the protein crowders destabilized CI2. This observation suggests that the proteins interact favorably and non-specifically with the backbone of CI2 so as to overcome the stabilizing effect of hard-core repulsions. These authors also showed that a part of these weak non-specific interactions originate from charge–charge interactions, consistent with the in-cell experiments discussed above (Crowley et al. 2011; Wang et al. 2011). Molecular dynamic simulations showed that the backbone fluctuations of CI2 increase in lysozyme, which correlates with its destabilizing effect (Feig and Sugita 2012). Harada et al. (2012) used molecular dynamics to show that protein crowders reduce the dielectric constant of the solution. This decrease would diminish the hydrophobic effect while enhancing hydrogen bond strength. In summary, studies with protein crowders are consistent with the conclusions from in-cell studies: weak, non-specific, attractive interactions play important roles in determining the effects of macromolecular crowding.

We close this section by noting that few significant changes in the chemical shift of backbone amide protons and nitrogens are observed in these studies, suggesting that

even though the effect of non-specific interactions on stability can be considerable, they are individually transient and weak. This situation is unlike ligand binding, where specific binding to a particular domain of a protein can lead to large changes in chemical shift (Lian 2013).

Enthalpy and entropy

In an effort to understand the contributions of hard and soft interactions, several groups have measured the temperature dependence of protein stability to quantify the contribution of enthalpy and entropy to macromolecular crowding effects. Using a β -hairpin peptide as a test molecule, the Harries group (Politi and Harries 2010; Sukenik et al. 2012) observed stabilizing effects for small polyol osmolytes and the synthetic polymers, polyethylene glycol and dextran. The enthalpic components differed for the two classes of cosolute. Osmolyte-induced stabilization arose from an increased enthalpic contribution. Low concentrations of polymers also acted enthalpically, but higher polymer concentrations tended to drive the system entropically. Independent studies using larger test proteins with both synthetic polymers and proteins as crowders have also been reported. Both Wang et al. (2012), who studied his-tagged ubiquitin, and Benton et al. (2012) who used CI2, observed complicated relationships between the effects of crowding on $\Delta G_{\text{den}}^{\text{o}}$ and $\Delta H_{\text{den}}^{\text{o}}$. That is, an increased $\Delta G_{\text{den}}^{\text{o}}$ was not always associated with a decreased $\Delta S_{\text{den}}^{\text{o}}$ (and vice versa), plus the change in $\Delta H_{\text{den}}^{\text{o}}$ was non-zero. As pointed out by Benton et al. (2012), some of the complication observed for synthetic polymers may arise from the influence of preferential hydration (Timasheff 1993). In summary, the simplest ideas about macromolecular crowding predict entropically derived stabilization, but recent studies indicate that attractive, non-specific interactions complicate this interpretation.

Protein stability in cells

Interpretation of in-cell stability studies in terms of hard-core repulsions alone is problematic. Ghaemmaghami and Oas (2001) studied the stability of λ repressor protein (8.8 kDa) in *E. coli* by using amide proton hydrogen exchange and mass spectroscopy. Their main conclusion was that stability is unchanged in cells. The Gierasch laboratory fluorescently tagged cellular retinoic acid-binding protein I (16 kDa) in cells and measured stability by using urea denaturation (Ignatova and Gierasch 2004; Ignatova et al. 2007). They showed that the test protein was destabilized in cells. In summary, these seminal studies prove that crowding in cells involves more than just hard-core interactions.

Next, we turn to studies that did not use perturbing cosolutes. The inability of the cytoplasm to fold ProtL was

discussed above (Schlesinger et al. 2011). Inomata et al. (2009) used in-cell NMR to assess the amide proton exchange rates for ubiquitin in HeLa cells. The rates increased, suggesting destabilization of the protein. The Gruebele group studied the stability of the test protein phosphoglycerate kinase in human cell lines by using fluorescence spectroscopy (Ebbinghaus et al. 2010; Dhar et al. 2011; Guo et al. 2012). Their main conclusion was that the folding kinetics, stability, and dynamics are not only affected by crowding but also by the environment in different cellular compartments. Specifically, the protein folded faster and was stabilized in the nucleus compared to its behavior in the endoplasmic reticulum and the cytoplasm.

Modulating the strength of interactions is more straightforward in silico than it is in the laboratory, and combining the results from simulation with those from ‘wet’ experiments can yield important insight. A molecular model of the bacterial cytoplasm has been developed by McGuffee and Elcock (2010). It depicts an atomistic view of the macromolecules inside *E. coli*, and the authors implemented Brownian dynamics simulations to assess protein stability. (We refer readers to their chapter in this volume for details.). They utilized the model to simulate the systems described above (Ghaemmaghami and Oas 2001; Ignatova and Gierasch 2004) using four scenarios. The first scenario considered only hard-core repulsions and, as predicted, the result was stabilization. They then turned to a ‘full energy’ model, which incorporated electrostatic interactions and a Lennard-Jones potential to define steric, dispersion forces and hydrophobic interactions. The second scenario incorporated this full energy model for the cytoplasm, but used only hard-core repulsions for macromolecule-test protein interactions. A smaller stabilization effect was observed. The third scenario used hard-core repulsions between cytoplasmic proteins and the full model for macromolecule-test protein interactions. The result was destabilization. Lastly, they used the full energy model for both the cytoplasmic macromolecules and the test proteins, and found that their results matched the experimental data.

In summary, these detailed simulations showed the importance of incorporating both hard and soft interactions to obtain physiologically relevant information. Further experimental quantification of enthalpic and entropic contributions in crowded systems is expected to aid in the development of detailed and biologically relevant models that will be even more useful for simulating the biophysical effects of the crowded cellular environment.

Conclusions

Soft interactions are difficult to assess, yet it is essential that we understand them because they can diminish or even

reverse the effects of hard-core repulsions. There are several reasons for the difficulty. First, soft interactions can be attractive or repulsive. Second, they arise from multiple phenomena: charge–charge interactions, hydrogen bonding, the hydrophobic effect, etc. Third, although individually weak, their final effect depends on the sum of individual components. Fourth, their weak and additive (and probably synergistic), nature presents a major challenge to developing the force fields required to drive simulations of crowding effects. Fifth, resolving and identifying these individual components from wet laboratory data is expensive. These are the challenges in vitro, where reductionist approaches can be applied. It is even more difficult to identify soft interactions in the heterogeneous cellular environment where reductionist approaches are difficult or impossible to apply. Nevertheless, endeavors aimed at quantifying soft interactions are essential for producing a physiologically relevant understanding of biophysics. Once the details of soft interactions are known, it should be possible to tune them so as to obtain bespoke behavior in test tubes and in cells. In summary, biologists of all types must keep these interactions in mind when designing experiments to correlate in vitro studies with in vivo behavior.

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Conflict of interest Authors declare no conflict of interests.

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