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Perspective

Importance of Hydrogen Bonding in Crowded Environments: A Physical Chemistry Perspective

Xiao You and Carlos R. Baiz*

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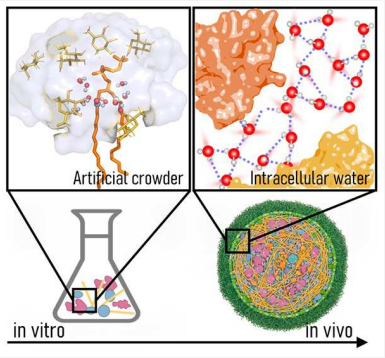
ABSTRACT: Cells are heterogeneous on every length and time scale; cytosol contains thousands of proteins, lipids, nucleic acids, and small molecules, and molecular interactions within this crowded environment determine the structure, dynamics, and stability of biomolecules. For decades, the effects of crowding at the atomistic scale have been overlooked in favor of more tractable models largely based on thermodynamics. Crowding can affect the conformations and stability of biomolecules by modulating water structure and dynamics within the cell, and these effects are nonlocal and environment dependent. Thus, characterizing water's hydrogen-bond (H-bond) networks is a critical step toward a complete microscopic crowding model. This perspective provides an overview of molecular crowding and describes recent time-resolved spectroscopy approaches investigating H-bond networks and dynamics in crowded or otherwise complex aqueous environments. Ultrafast spectroscopy combined with atomistic simulations has emerged as a powerful combination for studying H-bond structure and dynamics in heterogeneous multicomponent systems. We discuss the ongoing challenges toward developing a complete atomistic description of macromolecular crowding from an experimental as well as a theoretical perspective.



I. CELLS ARE CROWDED

A large fraction of the cell interior is occupied by biomolecules such as lipids, proteins, nucleic acids, and small organic compounds.[1] This high concentration of biomolecules produces a range of heterogeneous, complex, interfacial, and crowded environments where molecular interactions take place.[2,3] This increasingly complex environment (Figure 1) modulates the structure, stability, and dynamics of biomolecules inside cells.[4,5] Indeed, substantial differences have been observed in various biomolecular interactions in crowded environments compared to the same biomolecules in dilute solutions.[6]

Crowding is often interpreted as the combined outcome of "excluded volume" effects together with "soft interactions" (Figure 2). Excluded volume, often called "hardcore repulsion", is a simple size-dependent steric effect that results from the inaccessibility of certain regions of space due to the presence of neighboring macromolecules. This aspect of crowding is relatively well-characterized and generally agreed as favoring compact protein structures, increasing stability, and producing size-dependent effects.[7−10] However, this simplified model overlooks the significance of "soft interactions" in crowded systems.[11,12] "Soft interactions", including hydrophobic solvation, hydrogen bonding, and electrostatics, have been hypothesized to alter dynamics, modulate protein−protein interactions, and serve to spatially organize biomolecules in the cell.[13,14] Since crowding is a collective effect of thousands of biomolecules of all different sizes and composition, isolating the different contributions to the observed "crowding" effects remains challenging. Indeed, this understanding would be the first step toward developing artificial systems that accurately mimic biological environments.

Artificial crowders, such as polyethylene glycol (PEG), Ficoll, or dextran, are commonly used to mimic the cytosolic environment for in vitro studies and have become standard in biochemical experiments.[15] The impact of crowding on protein folding, stability, enzymatic activity, protein−protein interactions, and even membrane permeation has been of recent interest. Biophysical tools including molecular dynamics (MD) simulations,[16,17] nuclear magnetic resonance (NMR) spectroscopy,[18,19] and fluorescence-based techniques are commonly used to investigate crowding.[20] Initially, PEG and polysaccharides were used, as crowders, as the chemical nature of the crowder was not an important consideration. Recently, protein stability has been found to depend on the nature of the crowder, where more biologically relevant molecules may interact more strongly with proteins compared to artificial

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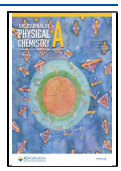

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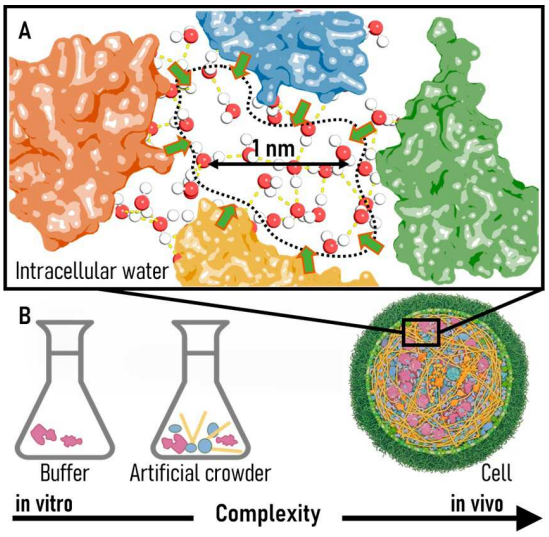

Figure 1. Illustration of molecular crowding. (A) Schematic representation of confinement effects on cytosolic water and the disruption of the water H-bond networks. (B) Schematic illustration of the complexity of standard solutions used in biochemical studies compared to the cell interior. The cell cartoon is adapted from an illustration by David S. Goodsell, The Scripps Research Institute. DOI: 10.2210/rcsb_pdb/goodsell-gallery-011.

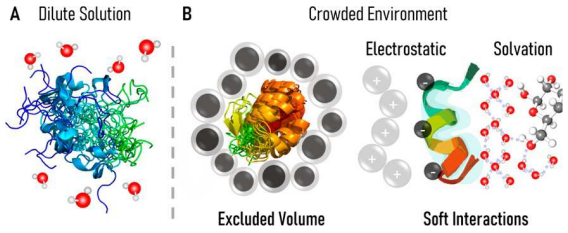

Figure 2. Crowding effects on proteins. (A) Crowding effects result in protein structural changes in dilute solution versus crowded environments. (B) Crowding effect is a sum of many interactions such as "excluded volume" and "soft interactions".

crowders.[21] The nature of the crowder may thus be important in modulating key biochemical interactions.

In-cell NMR studies have demonstrated that artificial crowders cannot replicate the structural dynamics of proteins in the cytosol.[22] The nature of the cytosolic environment, including H-bond networks, determines biomolecular interactions and, ultimately, the thermodynamics of all processes in the cytosol. Therefore, future studies should seek to arrive at a molecular description of how different crowders affect the solvent environment and, in turn, how these effects translate into the measured changes in protein thermodynamics. This requires a large repertoire of experimental and computational techniques that can disentangle the individual effects on the solvent environments and biomolecular interactions. Naturally, the importance of properly describing the solvent environment is well appreciated by the MD community, as different water models not only alter the computed physical properties of the liquid but also impact the structure and dynamics of biomolecules.[23−25]

Until now, efforts have been primarily focused on quantifying protein folding thermodynamics, and enzyme kinetics. Less emphasis, however, has been put on understanding the structure and dynamics of water in crowded systems, primarily because of the following reasons: (1) Water effects have been underestimated in favor of excluded-volume effects and nonspecific protein interactions. (2). Probing water dynamics in crowded environments remains challenging, and there are only a few select experimental techniques that can directly access dynamics within the relevant subpicosecond time scales. From a biophysical perspective, quantifying the H-bond ensembles and dynamics in biological environments is a natural starting point for describing crowding effects on the solvent.

In the remainder of this Perspective, we present an overview of the current ultrafast spectroscopy studies on H-bond structure and dynamics in various crowded environments and provide an outlook focused on ongoing obstacles to achieving a comprehensive, quantitative description of molecular interactions in crowded environments.

II. WATER IN CROWDED SYSTEMS

II.A. Confinement Effects on Water. In the cell, soluble proteins are primarily solvated by water, though nonspecific contacts with other biomolecules are also prevalent. To understand crowding beyond excluded volume effects, it is essential to first understand how biomolecules modulate their nearby environments. "Biological water" is a term frequently used to describe water molecules within the first or second solvation shell, and water directly bound to biomolecules is considered "hydration water". Biological water is hypothesized to be an active component of the cell, modulating the structure, dynamics, and interactions of biomolecules.[26] Extensive work has been dedicated to understanding the interplay between the dynamics of biomolecules and their local environment.[27,28] Though "hydration water" is not immobile like ice, its physical properties such as diffusion constants and dielectric properties are markedly different from bulk water.[29] Water molecules beyond the first two solvation shells are considered "free water". Compared to pure liquid water (bulk water), intracellular "free water" remains confined to nanometer-length channels between biomolecules.[30,31] Crowding and confinement at the molecular level alter the H-bond network structure and dynamics of water; in turn, the disrupted H-bond networks determine the stability, function, and dynamics of biomolecules within these environments.

In addition to crowding as an organizing principle, recent emphasis has been placed on the role of liquid droplets assembled by liquid−liquid phase separation (LLPS) as an additional mechanism for intracellular compartmentalization. The content of cells self-organizes into "biocondensates"; dense compartments composed of specific proteins and nucleic acids. These biocondensates result from a combination of interactions including strong electrostatic forces among charged moieties.[32] These interactions are sufficiently strong to assemble molecules, but not strong enough to freeze their dynamics or crash them out of solution. Despite the obvious role of water in mediating electrostatic interactions, confined water environments have not been investigated in sufficient detail within this context due to a number of challenges. For example, biocondensate simulations are often carried out using coarse-grained models in continuum solvent media to achieve sufficient conformational sampling.[33] However, it is clear that biocondensates contain significant amounts of water and that water is essential to mediating electrostatic interactions. For these reasons, it is important to work toward achieving molecular-level descriptions of interactions in these complex

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environments by studying the structure and dynamics of water and its unique solvation properties.

II.B. Describing H-Bond Networks in Water. The sp^3-hybridized oxygen molecular orbital dictates the geometries of water's H-bond networks in the solid and liquid phases. Water has an equal number of H-bond donors and acceptors, and each water molecule is capable of coordinating four others in a tetrahedral geometry (Figure 3A). In ice, each water molecule

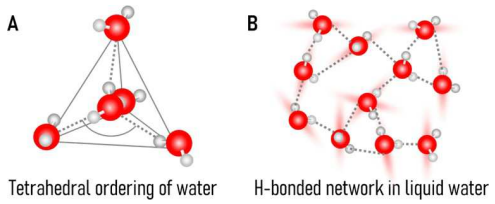

Figure 3. Diagrams of H-bond configurations. (A) Tetrahedral geometry of water H-bonds. (B) The extended H-bond network is the hallmark of liquid water.

forms approximately equal-length H-bonds with its nearest four neighbors at an angle of 109.5°, making a regular tetrahedral arrangement. Though not perfectly tetrahedral like ice, liquid water is characterized by a large fraction of the tetrahedral H-bond networks, retaining partial "ice-like" structures in the liquid. In essence, water can be viewed as a rapidly fluctuating matrix where molecules interact dynamically, forming a tetrahedral assembly but with short and long-range distortions that give liquid water its unique properties.

Water forms a dynamic H-bond network that undergoes continuous evolution as H-bond break, reform, and rearrange within the thermodynamic ensemble.[34] Describing the geometry is not straightforward; indeed, several order parameters have been suggested to quantitatively characterize local water structures, which have been especially useful in the context of molecular simulations. Laage and co-workers reviewed and compared five order parameters and concluded that angular distortions, in particular, significantly impact the water reorientation dynamics.[35] The geometric configurations of water are commonly described by the orientational tetrahedral order parameter (q) originally introduced by Debenedetti and co-workers to describe supercooled water.[36] The parameter q is calculated through the angles between a central water molecule and its four nearest neighbors (Figure 3A). It has a value of one for the perfect tetrahedral ice structure and zero for an ensemble of random configurations (i.e., an ideal gas, Figure 4).[37] The tetrahedral order parameter q has emerged as an effective way to describe the ordering of the H-bond network in aqueous systems, and quantify the effects of cosolvents on the tetrahedral structure of water.[36,38]

The presence of biomolecules leads to electrostatic interactions, and an imbalance of donors/acceptors, which all contribute to disrupting the extended H-bond networks.[39−43] Though cytosolic systems are difficult to probe directly, studies of cosolvent systems may offer some insights into the above-mentioned interactions. MD simulations predict local variations in the H-bond configurations in heterogeneous aqueous solutions.[44,45] These studies indicate that, on average, high-concentration solutions contain much more disordered or unstructured water configurations compared to pure liquid water.[45,46] However, surprisingly, certain crowders such as PEG induce long-range ordering, making the water outside of

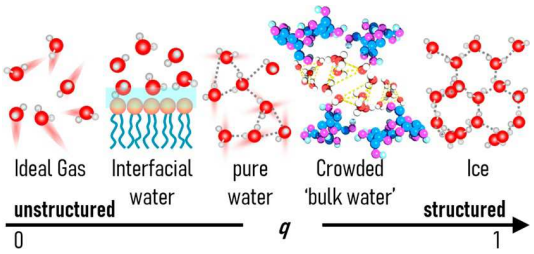

Figure 4. Schematic view of H-bond network ordering in selected systems. The H-bond network in ideal gas and ice depicts two limiting cases ice and an ideal gas. Interestingly, in crowded environments, certain ensembles can be more ordered than pure water, though H-bond networks remain significantly disordered at biological interfaces. The tetrahedral order parameter (q) is commonly used for describing the angular distortion in water, with a value of one for the perfect tetrahedral ice structure and zero for an ensemble of random configurations such as in an ideal gas.

the first two to three solvation shells (∼1 nm) more ordered in a crowded system compared to bulk water,[34] the implications of which remain to be fully understood.

II.C. Crowders Shape the H-Bond Network. Solvent-exposed regions of biomolecules, such as soluble proteins, lipids, or DNA, typically contain H-bond donors and acceptors, and these molecules can thus participate in the extended H-bond networks.[39] Common functional groups that participate in H-bonds include carbonyls, hydroxyls, phosphates, amines, and amides. As a consequence, the "soft interactions", which primarily consist of polar contacts and H-bonding, depend strongly on the chemical structure of a given biomolecule. For example, sugars containing large numbers of hydroxyl groups can become readily embedded within the H-bond network since the hydroxyl group can donate as well as accept H-bonds. Using monosaccharides as crowders results in more unstructured water in comparison to PEG (Figure 5A),

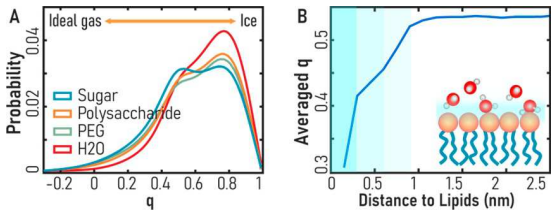

Figure 5. Crowding effect on orientational tetrahedral order parameter (q). (A) Histogram of tetrahedral order parameters (q) values for selected crowding agents. All crowders increase the population of unstructured water (lower q value). (B) Distribution of the order parameter as a function of distance to the lipid interface in a bilayer. The bilayer disorders water within ∼1 nm of the interface, but the effects are not propagated beyond this distance.

which can only accept H-bonds through the ether oxygens.[47] Phospholipids are another example of a biomolecule with phosphate and other polar groups which strongly interact with water. In membranes, the hydrophilic lipid headgroups disrupt the interfacial water H-bond network to a distance of ∼1 nm from the interface (Figure 5B), but surprisingly headgroup dipole orientation does not affect the local environments.[48,49]

Experimentally, several techniques can be used to directly probe H-bond interactions.[50−53] However, since each measurement provides a different description, it is important to define a fundamental, microscopic measure of H-bond interactions.

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The stability of the H-bond network is dictated by the donor−acceptor free energy landscape, which in turn is not only given by the chemical nature of the donor and acceptor, but by several factors including orientations and the delocalized interactions that define the H-bond networks. The H-bond switching process occurs along a reaction coordinate that involves at least three molecules.[54] Quantifying the temperature-dependent H-bond populations provides a direct way to determine the enthalpy and entropy change associated with H-bond rearrangements involving specific functional groups.[50] In applying this method, the temperature-dependent population changes reveal the donor−acceptor interaction energies, provided that there is a properly defined standard state. For example, the average observed rearrangement energy is 1.5 kcal/mol between the structured ("ice-like") and distorted ("broken-donor") local H-bond configurations.[53] Vibrational spectroscopy is a useful technique for quantifying H-bond populations and enthalpies since vibrational frequencies are sensitive to the number of H-bonds associated with a given functional group.[55,56]

Fourier-transform infrared (FTIR) spectra can directly measure H-bond populations for certain functional groups. The experimentally measured enthalpy is usually an average value of all the H-bonds involving a given functional group. In crowded solutions containing several donors or acceptor species, for example, −OH from waters, proteins, or sugars or C═O from proteins or lipids, measurements often cannot distinguish between the different H-bond donors or acceptors, and thus, the results must be interpreted with care. Such bulk quantities provide limited insight but are nonetheless useful benchmarks for modeling.[57] Simulation, on the other hand, can be used to disentangle the contributions from individual H-bond donors or acceptors and provide atomistic descriptions of local H-bond interactions.

III. CROWDING EFFECTS ON H-BOND DYNAMICS

In liquid water, the dynamics span time scales from subpicosecond in-place H-bond fluctuations to picosecond H-bond lifetimes and nano/microsecond diffusion rates.[58] Indeed, water dynamics are considered "fractal" in character; each time scale, no matter how short or how long, can be associated with a corresponding ensemble motion. This is particularly important in biomolecular systems, where the time scales of biomolecules can similarly span multiple orders of magnitude in time.[21,59,60] Several methods have been used to measure dynamics in crowded aqueous systems: Kerr effect[61] and THz spectroscopy[62] have been used to measure the low-frequency intermolecular vibrations (Figure 6). Correlation times associated with orientational and translational diffusion of the intracellular water have been studied using NMR and EPR spectroscopy.[60,63−65] Measuring the fast H-bond dynamics requires ultrafast spectroscopy approaches.

III.A. Femtosecond H-Bond Dynamics. Time-resolved vibrational spectroscopy probes the ultrafast motions of water molecules on a time scale of a few tens of femtoseconds to several picoseconds (Figure 6).[55] For example, reorientation dynamics, measured using IR pump−probe anisotropy, exhibit two relaxation times with time constants of approximately 700 fs and 13 ps.[66−68] Beyond time scales, the specific H-bond switching motions were elucidated using 2D IR anisotropy to measure the joint angular reorientation and OH frequency correlations, which has served as validation for the "jump reorientation" model.[69]

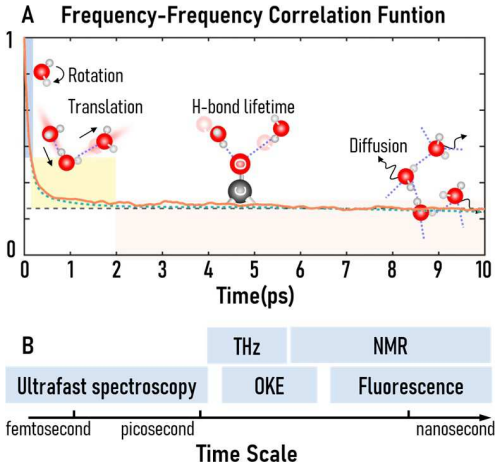

Figure 6. Multiscale dynamics of water and approximate time windows of selected spectroscopic techniques. (A) Frequency−frequency correlation function decay (orange curve), three-component exponential curve fitting (blue dotted curve), and time scales typically associated with specific water motions. (B) Common time-resolved characterization techniques. NMR: nuclear magnetic resonance. OKE: optical Kerr effect. THz: time-resolved terahertz spectroscopy.

Bulk measurements can be limiting in the case of complex systems, where either frequency alone is not sufficient to distinguish between ensembles, or where an ensemble interest encompasses a small fraction of the water molecules in the liquid. Time-resolved sum-frequency generation (SFG) spectroscopy enables selective measurements on interfacial O−H stretch modes at water−air or water−lipid membrane interfaces, directly accessing spectral diffusion and population relaxation rates of molecules at interfaces.[70−73] 2D SFG measurements showed strong frequency dependence of vibrational relaxation at the air−water interface compared to bulk water.[74,75] In a crowded environment, it is yet an ongoing challenge to selectively measure interfacial water around biomolecules, although some recent experiments have been able to reveal local structure, as well as the coupled dynamics of biomolecules and waters.[76−78]

III.B. Probing H-Bond Dynamics. Biological environments are characterized by molecular and spatial heterogeneity, raising an important challenge in studying solvation dynamics: it is difficult to isolate the response of water near biomolecules from the response of the molecules in bulk.[40] For instance, in lipid vesicles, water molecules at the interface experience different dynamics than water in a bulk environment.[48,79−81] Relying on the vibrational modes of water to probe heterogeneous dynamics may result in a multicomponent response that may be challenging to interpret, and in addition, if the fraction of water in interfacial environments is small, the majority of the contribution will arise from bulk water, making the overall response appear "bulk-like" in character.[66,82,83]

Specific modes in biomolecules can be used as intrinsic vibrational probes. For example, in phospholipids, ester carbonyls are precisely located in the ∼1 nm interface between the polar and nonpolar regions, and the carbonyl stretching frequency is sensitive to the local H-bond environment (Figure 7). In the absence of intrinsic vibrations, probes can be installed at precise locations to measure local dynamics.[84,85] Nitriles, including azides and thiocyanates, are common since the C≡N stretching mode appears in an uncongested region

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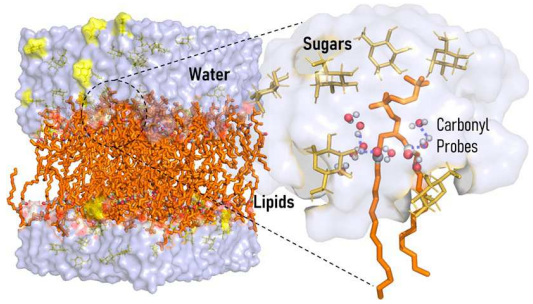

Figure 7. Schematic depiction of intrinsic vibrational probes in lipid interacting with interfacial water molecules. Zoomed in the structure of a phospholipid showing the carbonyl groups, located at the water−lipid interface, interacting with the water and sugar crowders through H-bonding.

of the spectrum and is highly sensitive to the environment.[46,86−88]

In the case of delocalized vibrations involving multiple oscillators, such as the amide carbonyls of proteins, the dynamics of the peaks can be affected by energy transfer, which can complicate the interpretation of spectral lineshapes. Isotope labeling techniques provide an effective way to reveal site-specific information in complex protein systems.[89,90] In conclusion, the combination of time-resolved IR spectroscopy and vibrational probes makes for a powerful approach to directly access H-bond dynamics in complex heterogeneous environments.

III.C. Atomistic Interpretation. Spectroscopy alone can only measure frequencies, amplitudes, and time scales; modeling is thus often required to obtain insight into the interactions that give rise to measured spectra. Molecular dynamics (MD) simulations provide a complete molecular description of a solvation system: the exact positions of all atoms as a function of time. Rich molecular information can be directly extracted from trajectories, including local properties such as the H-bond populations, and H-bond dynamics at selected positions, as well more global measures such as the temporal evolution of the H-bond network.[91] However, it is important to properly benchmark simulations against experiment to ensure that the models are sufficiently accurate to reproduce the molecular ensembles and dynamical time scales observed in experiments.[92] Vibrational maps are useful since they can be used to generate frequency trajectories for vibrational groups of interest.[85] The frequency−frequency correlation function (FFCF) extracted from MD trajectories can be directly compared to the FFCF measured using ultrafast vibrational spectroscopy.[46] In brief, the combination of ultrafast spectroscopy and MD simulation is ideal for measuring H-bond dynamics in complex systems and directly exploring the effects of crowding.[17,42,85,93]

IV. CHALLENGES

Ultrafast spectroscopy has been pivotal in providing an atomistic description of water dynamics in crowded solutions, but fully understanding the effects of crowding in realistic biological environments remains challenging.[28,94−96] Multiple constraints must be taken into consideration when designing experiments around vibrational probes. A suitable vibrational probe needs to minimally perturb the H-bond network while being sensitive to the local environment. The vibrational lifetime of the probe is another limiting factor, and significant efforts have been put toward synthesizing labels with long vibrational lifetimes.[97] In addition, the oscillator strength becomes an important consideration since labeled molecules must be dilute, particularly if a vibrational label is attached to a large biomolecule, where the biomolecule itself can become the crowder, but low probe concentrations result in weak signals.

Atomistic descriptions are often drawn from a combination of experiments and simulations as it is difficult to pinpoint the origin of the observed dynamics from experiments alone. Simulating large-scale systems at an atomistic level can quickly become unaffordable and coarse-grained simulations do not offer sufficient levels of detail given the local nature of H-bond interactions.[98]

V. SUMMARY AND OUTLOOK

Crowding effects in cell environments have drawn increasing attention, particularly concerning the stability, and assembly of biomolecules within living cells.[21,99] Researchers are beginning to explore crowding at the atomistic level. In this perspective, we argue that H-bonding is an important aspect of crowding which deserves significant attention. Over the past two decades, ultrafast spectroscopy has become a powerful tool to measure bond-specific picosecond dynamics, and spectroscopic methods can be further extended to probe crowded and otherwise complex environments. One exciting area of investigation is examining the role of water in biocondensates induced by liquid−liquid phase separation; however, there are many challenges ahead, primarily that biocondensates are highly complex multicomponent structures that only exist in the native environment of the cell[32,100] and that current model systems, such as polypeptide coacervates and synthetic polymers, may not recapitulate the environments present in intracellular condensates.[101−103] Nonetheless, there are exciting avenues to pursue in this area, and ultrafast spectroscopy is poised to make important contributions to this field.

We predict that future studies will explore the molecular origin and impact of crowding in systems with increased complexity that begins to mimic key aspects of the cytosol. Intracellular compartments, cell lysates, intact plasma membrane patches, or ultimately in-cell measurements would be logical systems for future studies. Liquid−liquid phase separation, as one of the important mechanisms for intracellular compartmentalization, will keep garnering attention. Site-specific labeling and incorporating vibrational probes within biological environments remains a critical challenge. There has been some excellent recent work dedicated to developing probes with a longer vibrational lifetime, brighter vibrational strength, and higher H-bonding sensitivity.[104−106] Ultimately, emerging technologies such as amber codon, biorthogonal chemistry, or native chemical ligation could be very beneficial for incorporating vibrational probes in fully native environments and assessing the effects of crowding.[107−109] Ultrafast spectroscopy, in combination with techniques such as NMR, EPR, and leading-edge MD simulations, will continue to advance our understanding of how water interacts with biomolecules in highly complex cellular environments.

AUTHOR INFORMATION

Corresponding Author

Carlos R. Baiz − *Department of Chemistry, The University of Texas at Austin, Austin, Texas 19104, United States;*

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orcid.org/0000-0003-0699-8468; Email: cbaiz@cm.utexas.edu

Author

Xiao You – *Department of Chemistry, The University of Texas at Austin, Austin, Texas 19104, United States;* orcid.org/0000-0002-0357-0311

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jpca.2c03803

Notes

The authors declare no competing financial interest.

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Biographies

Xiao You is a postdoctoral researcher at the University of Texas at Austin. She earned her B.S. degree in Macromolecular Science and Engineering from Fudan University in Shanghai, China, and a Ph.D. degree in Applied Physical Science from the University of North Carolina at Chapel Hill with a research focus on near-field microscopy and spectroscopy techniques. Her current research, mentored by Prof. Carlos Baiz, focuses on studying biophysics using time-resolved vibrational spectroscopy and molecular dynamics simulation.

Carlos R. Baiz is the W. T. Doherty Associate Professor of Chemistry at the University of Texas at Austin. His lab studies the biophysics of complex systems, including crowded environments, and heterogeneous lipid membranes, membrane proteins, and surfactants, using ultrafast 2D IR spectroscopy and MD simulations. He completed his Ph.D. in ultrafast spectroscopy working with Prof. Kevin Kubarych at the University of Michigan and did his postdoctoral training with Prof. Andrei Tokmakoff at MIT and the University of Chicago.

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REFERENCES

(1) Fulton, A. B. How crowded is the cytoplasm? *Cell* **1982**, *30* (2), 345–347.
(2) Ellis, R. J. Macromolecular crowding: an important but neglected aspect of the intracellular environment. *Curr. Opin Struct Biol.* **2001**, *11* (1), 114–9.
(3) Zimmerman, S. B.; Minton, A. P. Macromolecular crowding: biochemical, biophysical, and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22* (1), 27–65.
(4) Mukherjee, S.; Waegele, M. M.; Chowdhury, P.; Guo, L.; Gai, F. Effect of macromolecular crowding on protein folding dynamics at the secondary structure level. *J. Mol. Biol.* **2009**, *393* (1), 227–36.
(5) Chaplin, M. Do we underestimate the importance of water in cell biology? *Nat. Rev. Mol. Cell Biol.* **2006**, *7* (11), 861–6.
(6) Zhou, H. X.; Rivas, G.; Minton, A. P. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys* **2008**, *37* (1), 375–97.
(7) Hong, J.; Gierasch, L. M. Macromolecular crowding remodels the energy landscape of a protein by favoring a more compact unfolded state. *J. Am. Chem. Soc.* **2010**, *132* (30), 10445–52.
(8) Zimmerman, S. B.; Trach, S. O. Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli. *J. Mol. Biol.* **1991**, *222* (3), 599–620.
(9) Gnutt, D.; Gao, M.; Brylski, O.; Heyden, M.; Ebbinghaus, S. Excluded-volume effects in living cells. *Angew. Chem., Int. Ed. Engl.* **2015**, *54* (8), 2548–51.
(10) Minton, A. P. Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers* **1981**, *20* (10), 2093–2120.
(11) Guin, D.; Gruebele, M. Weak Chemical Interactions That Drive Protein Evolution: Crowding, Sticking, and Quinary Structure in Folding and Function. *Chem. Rev.* **2019**, *119* (18), 10691–10717.
(12) Speer, S. L.; Stewart, C. J.; Sapir, L.; Harries, D.; Pielak, G. J. Macromolecular Crowding Is More than Hard-Core Repulsions. *Annu. Rev. Biophys* **2022**, *51* (1), 267–300.
(13) Miklos, A. C.; Li, C.; Sharaf, N. G.; Pielak, G. J. Volume exclusion and soft interaction effects on protein stability under crowded conditions. *Biochemistry* **2010**, *49* (33), 6984–91.
(14) Miklos, A. C.; Sarkar, M.; Wang, Y.; Pielak, G. J. Protein crowding tunes protein stability. *J. Am. Chem. Soc.* **2011**, *133* (18), 7116–20.
(15) Rivas, G.; Minton, A. P. Macromolecular Crowding In Vitro, In Vivo, and In Between. *Trends Biochem. Sci.* **2016**, *41* (11), 970–981.
(16) McGuffee, S. R.; Elcock, A. H. Diffusion, crowding & protein stability in a dynamic molecular model of the bacterial cytoplasm. *PLoS Comput. Biol.* **2010**, *6* (3), e1000694.
(17) Feig, M.; Yu, I.; Wang, P. H.; Nawrocki, G.; Sugita, Y. Crowding in Cellular Environments at an Atomistic Level from Computer Simulations. *J. Phys. Chem. B* **2017**, *121* (34), 8009–8025.
(18) Schlesinger, A. P.; Wang, Y.; Tadeo, X.; Millet, O.; Pielak, G. J. Macromolecular crowding fails to fold a globular protein in cells. *J. Am. Chem. Soc.* **2011**, *133* (21), 8082–5.
(19) Latham, M. P.; Kay, L. E. Is buffer a good proxy for a crowded cell-like environment? A comparative NMR study of calmodulin side-chain dynamics in buffer and E. coli lysate. *PLoS One* **2012**, *7* (10), e48226.
(20) Yu, H.; Rathore, S. S.; Shen, C.; Liu, Y.; Ouyang, Y.; Stowell, M. H.; Shen, J. Reconstituting Intracellular Vesicle Fusion Reactions: The Essential Role of Macromolecular Crowding. *J. Am. Chem. Soc.* **2015**, *137* (40), 12873–83.
(21) Smith, A. E.; Zhou, L. Z.; Gorensek, A. H.; Senske, M.; Pielak, G. J. In-cell thermodynamics and a new role for protein surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (7), 1725–30.

(22) Inomata, K.; Ohno, A.; Tochio, H.; Isogai, S.; Tenno, T.; Nakase, I.; Takeuchi, T.; Futaki, S.; Ito, Y.; Hiroaki, H.; Shirakawa, M. High-resolution multi-dimensional NMR spectroscopy of proteins in human cells. *Nature* **2009**, *458* (7234), 106−9.
(23) Cisneros, G. A.; Wikfeldt, K. T.; Ojamäe, L.; Lu, J.; Xu, Y.; Torabifard, H.; Bartók, A. P.; Csányi, G.; Molinero, V.; Paesani, F. Modeling Molecular Interactions in Water: From Pairwise to Many-Body Potential Energy Functions. *Chem. Rev.* **2016**, *116* (13), 7501−7528.
(24) Levy, Y.; Onuchic, J. N. Water mediation in protein folding and molecular recognition. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 389−415.
(25) Onufriev, A. V.; Izadi, S. Water models for biomolecular simulations. *Wiley Interdisciplinary Reviews-Computational Molecular Science* **2018**, *8* (2), e1347.
(26) Ball, P. Water as an active constituent in cell biology. *Chem. Rev.* **2008**, *108* (1), 74−108.
(27) Colombo, M. F.; Rau, D. C.; Parsegian, V. A. Protein solvation in allosteric regulation: a water effect on hemoglobin. *Science* **1992**, *256* (5057), 655−9.
(28) Zhong, D. P.; Pal, S. K.; Zewail, A. H. Biological water: A critique. *Chem. Phys. Lett.* **2011**, *503* (1−3), 1−11.
(29) Nandi, N.; Bagchi, B. Dielectric relaxation of biological water. *J. Phys. Chem. B* **1997**, *101* (50), 10954−10961.
(30) Minton, A. P. Quantitative assessment of the relative contributions of steric repulsion and chemical interactions to macromolecular crowding. *Biopolymers* **2013**, *99* (4), 239−244.
(31) Elcock, A. H. Models of macromolecular crowding effects and the need for quantitative comparisons with experiment. *Curr. Opin. Struct. Biol.* **2010**, *20* (2), 196−206.
(32) Wang, B.; Zhang, L.; Dai, T.; Qin, Z.; Lu, H.; Zhang, L.; Zhou, F. Liquid-liquid phase separation in human health and diseases. *Signal Transduct Target Ther* **2021**, *6* (1), 290.
(33) Najafi, S.; Lin, Y.; Longhini, A. P.; Zhang, X.; Delaney, K. T.; Kosik, K. S.; Fredrickson, G. H.; Shea, J. E.; Han, S. Liquid-liquid phase separation of Tau by self and complex coacervation. *Protein Sci.* **2021**, *30* (7), 1393−1407.
(34) Myneni, S.; Luo, Y.; Naslund, L. A.; Cavalleri, M.; Ojamae, L.; Ogasawara, H.; Pelmenschikov, A.; Wernet, P.; Vaterlein, P.; Heske, C.; Hussain, Z.; Pettersson, L. G. M.; Nilsson, A. Spectroscopic probing of local hydrogen-bonding structures in liquid water. *J. Phys.: Condens. Matter* **2002**, *14* (8), L213−L219.
(35) Duboue-Dijon, E.; Laage, D. Characterization of the Local Structure in Liquid Water by Various Order Parameters. *J. Phys. Chem. B* **2015**, *119* (26), 8406−18.
(36) Errington, J. R.; Debenedetti, P. G. Relationship between structural order and the anomalies of liquid water. *Nature* **2001**, *409* (6818), 318−321.
(37) Ruocco, G.; Sampoli, M.; Vallauri, R. Analysis of the network topology in liquid water and hydrogen sulphide by computer simulation. *J. Chem. Phys.* **1992**, *96* (8), 6167−6176.
(38) Laage, D.; Elsaesser, T.; Hynes, J. T. Water Dynamics in the Hydration Shells of Biomolecules. *Chem. Rev.* **2017**, *117* (16), 10694−10725.
(39) Saenger, W. Structure and dynamics of water surrounding biomolecules. *Annu. Rev. Biophys. Biophys. Chem.* **1987**, *16* (1), 93−114.
(40) Laage, D.; Elsaesser, T.; Hynes, J. T. Water Dynamics in the Hydration Shells of Biomolecules. *Chem. Rev.* **2017**, *117* (16), 10694−10725.
(41) Persson, E.; Halle, B. Cell water dynamics on multiple time scales. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (17), 6266−71.
(42) Harada, R.; Sugita, Y.; Feig, M. Protein crowding affects hydration structure and dynamics. *J. Am. Chem. Soc.* **2012**, *134* (10), 4842−9.
(43) Pal, S. K.; Zewail, A. H. Dynamics of water in biological recognition. *Chem. Rev.* **2004**, *104* (4), 2099−123.
(44) Oh, K. I.; You, X.; Flanagan, J. C.; Baiz, C. R. Liquid-Liquid Phase Separation Produces Fast H-Bond Dynamics in DMSO-Water Mixtures. *J. Phys. Chem. Lett.* **2020**, *11* (5), 1903−1908.
(45) Oh, K. I.; Baiz, C. R. Crowding Stabilizes DMSO-Water Hydrogen-Bonding Interactions. *J. Phys. Chem. B* **2018**, *122* (22), 5984−5990.
(46) You, X.; Shirley, J. C.; Lee, E.; Baiz, C. R. Short- and long-range crowding effects on water's hydrogen bond networks. *Cell Rep. Phys. Sci.* **2021**, *2* (5), 100419.
(47) Zhou, T.; Wang, L.; Huang, X.; Unruangsri, J.; Zhang, H.; Wang, R.; Song, Q.; Yang, Q.; Li, W.; Wang, C.; Takahashi, K.; Xu, H.; Guo, J. PEG-stabilized coaxial stacking of two-dimensional covalent organic frameworks for enhanced photocatalytic hydrogen evolution. *Nat. Commun.* **2021**, *12* (1), 3934.
(48) You, X.; Lee, E.; Xu, C.; Baiz, C. R. Molecular Mechanism of Cell Membrane Protection by Sugars: A Study of Interfacial H-Bond Networks. *J. Phys. Chem. Lett.* **2021**, *12* (39), 9602−9607.
(49) Lee, E.; You, X.; Baiz, C. R. Interfacial dynamics in inverted-headgroup lipid membranes. *J. Chem. Phys.* **2022**, *156* (7), 075102.
(50) Guerin, A. C.; Riley, K.; Rupnik, K.; Kuroda, D. G. Determining the Energetics of the Hydrogen Bond through FTIR: A Hands-On Physical Chemistry Lab Experiment. *J. Chem. Educ.* **2016**, *93* (6), 1124−1129.
(51) Morton, J. G.; Joe, C. L.; Stolla, M. C.; Koshland, S. R.; Londergan, C. H.; Schofield, M. H. NMR Determination of Hydrogen Bond Thermodynamics in a Simple Diamide: A Physical Chemistry Experiment. *J. Chem. Educ.* **2015**, *92* (6), 1086−1090.
(52) Stoll, S.; Shafaat, H. S.; Krzystek, J.; Ozarowski, A.; Tauber, M. J.; Kim, J. E.; Britt, R. D. Hydrogen bonding of tryptophan radicals revealed by EPR at 700 GHz. *J. Am. Chem. Soc.* **2011**, *133* (45), 18098−101.
(53) Smith, J. D.; Cappa, C. D.; Wilson, K. R.; Messer, B. M.; Cohen, R. C.; Saykally, R. J. Energetics of hydrogen bond network rearrangements in liquid water. *Science* **2004**, *306* (5697), 851−3.
(54) Laage, D.; Stirnemann, G.; Sterpone, F.; Hynes, J. T. Water jump reorientation: from theoretical prediction to experimental observation. *Acc. Chem. Res.* **2012**, *45* (1), 53−62.
(55) Perakis, F.; De Marco, L.; Shalit, A.; Tang, F.; Kann, Z. R.; Kuhne, T. D.; Torre, R.; Bonn, M.; Nagata, Y. Vibrational Spectroscopy and Dynamics of Water. *Chem. Rev.* **2016**, *116* (13), 7590−607.
(56) Pazos, I. M.; Ghosh, A.; Tucker, M. J.; Gai, F. Ester Carbonyl Vibration as a Sensitive Probe of Protein Local Electric Field. *Angew. Chem., Int. Ed.* **2014**, *53* (24), 6080−6084.
(57) Zoranić, L.; Mazighi, R.; Sokolić, F.; Perera, A. On the Microheterogeneity in Neat and Aqueous Amides: A Molecular Dynamics Study. *J. Phys. Chem. C* **2007**, *111* (43), 15586−15595.
(58) Gomez, A.; Piskulich, Z. A.; Thompson, W. H.; Laage, D. Water Diffusion Proceeds via a Hydrogen-Bond Jump Exchange Mechanism. *J. Phys. Chem. Lett.* **2022**, *13*, 4660−4666.
(59) Caflisch, A.; Hamm, P. Complexity in Protein Folding: Simulation Meets Experiment. *Current Physical Chemistry* **2012**, *2* (1), 4−11.
(60) Persson, E.; Halle, B. Nanosecond to microsecond protein dynamics probed by magnetic relaxation dispersion of buried water molecules. *J. Am. Chem. Soc.* **2008**, *130* (5), 1774−87.
(61) Potma, E. O.; de Boeij, W. P.; Wiersma, D. A. Femtosecond dynamics of intracellular water probed with nonlinear optical Kerr effect microspectroscopy. *Biophys. J.* **2001**, *80* (6), 3019−24.
(62) Meister, K.; Ebbinghaus, S.; Xu, Y.; Duman, J. G.; DeVries, A.; Gruebele, M.; Leitner, D. M.; Havenith, M. Long-range protein-water dynamics in hyperactive insect antifreeze proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110* (5), 1617−22.
(63) Kaieda, S.; Setlow, B.; Setlow, P.; Halle, B. Mobility of core water in Bacillus subtilis spores by 2H NMR. *Biophys. J.* **2013**, *105* (9), 2016−23.
(64) Sunde, E. P.; Setlow, P.; Hederstedt, L.; Halle, B. The physical state of water in bacterial spores. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (46), 19334−9.

https://doi.org/10.1021/acs.jpca.2c03803
J. Phys. Chem. A 2022, 126, 5881−5889

(65) Fisette, O.; Päslack, C.; Barnes, R.; Isas, J. M.; Langen, R.; Heyden, M.; Han, S.; Schäfer, L. V. Hydration Dynamics of a Peripheral Membrane Protein. *J. Am. Chem. Soc.* **2016**, *138* (36), 11526−11535.
(66) Tros, M.; Zheng, L.; Hunger, J.; Bonn, M.; Bonn, D.; Smits, G. J.; Woutersen, S. Picosecond orientational dynamics of water in living cells. *Nat. Commun.* **2017**, *8* (1), 904.
(67) Fayer, M. D. Dynamics of water interacting with interfaces, molecules, and ions. *Acc. Chem. Res.* **2012**, *45* (1), 3−14.
(68) Woutersen, S.; Emmerichs, U.; Bakker, H. J. Femtosecond mid-IR pump-probe spectroscopy of liquid water: Evidence for a two-component structure. *Science* **1997**, *278* (5338), 658−660.
(69) Ramasesha, K.; Roberts, S. T.; Nicodemus, R. A.; Mandal, A.; Tokmakoff, A. Ultrafast 2D IR anisotropy of water reveals reorientation during hydrogen-bond switching. *J. Chem. Phys.* **2011**, *135* (5), 054509.
(70) McGuire, J. A.; Shen, Y. R. Ultrafast vibrational dynamics at water interfaces. *Science* **2006**, *313* (5795), 1945−8.
(71) Nihonyanagi, S.; Yamaguchi, S.; Tahara, T. Ultrafast Dynamics at Water Interfaces Studied by Vibrational Sum Frequency Generation Spectroscopy. *Chem. Rev.* **2017**, *117* (16), 10665−10693.
(72) Cyran, J. D.; Backus, E. H. G.; Nagata, Y.; Bonn, M. Structure from Dynamics: Vibrational Dynamics of Interfacial Water as a Probe of Aqueous Heterogeneity. *J. Phys. Chem. B* **2018**, *122* (14), 3667−3679.
(73) Vanselous, H.; Petersen, P. B. Extending the Capabilities of Heterodyne-Detected Sum-Frequency Generation Spectroscopy: Probing Any Interface in Any Polarization Combination. *J. Phys. Chem. C* **2016**, *120* (15), 8175−8184.
(74) van der Post, S. T.; Hsieh, C. S.; Okuno, M.; Nagata, Y.; Bakker, H. J.; Bonn, M.; Hunger, J. Strong frequency dependence of vibrational relaxation in bulk and surface water reveals sub-picosecond structural heterogeneity. *Nat. Commun.* **2015**, *6* (1), 8384.
(75) Inoue, K. I.; Ahmed, M.; Nihonyanagi, S.; Tahara, T. Reorientation-induced relaxation of free OH at the air/water interface revealed by ultrafast heterodyne-detected nonlinear spectroscopy. *Nat. Commun.* **2020**, *11* (1), 5344.
(76) McDermott, M. L.; Vanselous, H.; Corcelli, S. A.; Petersen, P. B. DNA's Chiral Spine of Hydration. *ACS Cent Sci.* **2017**, *3* (7), 708−714.
(77) Adams, E. M.; Lampret, O.; Konig, B.; Happe, T.; Havenith, M. Solvent dynamics play a decisive role in the complex formation of biologically relevant redox proteins. *Phys. Chem. Chem. Phys.* **2020**, *22* (14), 7451−7459.
(78) Xu, Y.; Havenith, M. Perspective: Watching low-frequency vibrations of water in biomolecular recognition by THz spectroscopy. *J. Chem. Phys.* **2015**, *143* (17), 170901.
(79) Flanagan, J. C.; Valentine, M. L.; Baiz, C. R. Ultrafast Dynamics at Lipid-Water Interfaces. *Acc. Chem. Res.* **2020**, *53* (9), 1860−1868.
(80) Valentine, M. L.; Waterland, M. K.; Fathizadeh, A.; Elber, R.; Baiz, C. R. Interfacial Dynamics in Lipid Membranes: The Effects of Headgroup Structures. *J. Phys. Chem. B* **2021**, *125* (5), 1343−1350.
(81) Venkatraman, R. K.; Baiz, C. R. Ultrafast Dynamics at the Lipid-Water Interface: DMSO Modulates H-Bond Lifetimes. *Langmuir* **2020**, *36* (23), 6502−6511.
(82) Daly, C. A.; Streacker, L. M.; Sun, Y.; Pattenaude, S. R.; Hassanali, A. A.; Petersen, P. B.; Corcelli, S. A.; Ben-Amotz, D. Decomposition of the Experimental Raman and Infrared Spectra of Acidic Water into Proton, Special Pair, and Counterion Contributions. *J. Phys. Chem. Lett.* **2017**, *8* (21), 5246−5252.
(83) Roget, S. A.; Piskulich, Z. A.; Thompson, W. H.; Fayer, M. D. Identical Water Dynamics in Acrylamide Hydrogels, Polymers, and Monomers in Solution: Ultrafast IR Spectroscopy and Molecular Dynamics Simulations. *J. Am. Chem. Soc.* **2021**, *143* (36), 14855−14868.
(84) King, J. T.; Arthur, E. J.; Brooks, C. L., 3rd; Kubarych, K. J. Crowding induced collective hydration of biological macromolecules over extended distances. *J. Am. Chem. Soc.* **2014**, *136* (1), 188−94.
(85) Baiz, C. R.; Blasiak, B.; Bredenbeck, J.; Cho, M.; Choi, J. H.; Corcelli, S. A.; Dijkstra, A. G.; Feng, C. J.; Garrett-Roe, S.; Ge, N. H.; Hanson-Heine, M. W. D.; Hirst, J. D.; Jansen, T. L. C.; Kwac, K.; Kubarych, K. J.; Londergan, C. H.; Maekawa, H.; Reppert, M.; Saito, S.; Roy, S.; Skinner, J. L.; Stock, G.; Straub, J. E.; Thielges, M. C.; Tominaga, K.; Tokmakoff, A.; Torii, H.; Wang, L.; Webb, L. J.; Zanni, M. T. Vibrational Spectroscopic Map, Vibrational Spectroscopy, and Intermolecular Interaction. *Chem. Rev.* **2020**, *120* (15), 7152−7218.
(86) Schmidt-Engler, J. M.; Blankenburg, L.; Zangl, R.; Hoffmann, J.; Morgner, N.; Bredenbeck, J. Local dynamics of the photoswitchable protein PYP in ground and signalling state probed by 2D-IR spectroscopy of − SCN labels. *Phys. Chem. Chem. Phys.* **2020**, *22* (40), 22963−22972.
(87) Schmidt-Engler, J. M.; Blankenburg, L.; Blasiak, B.; van Wilderen, L.; Cho, M.; Bredenbeck, J. Vibrational Lifetime of the SCN Protein Label in H2O and D2O Reports Site-Specific Solvation and Structure Changes During PYP's Photocycle. *Anal. Chem.* **2020**, *92* (1), 1024−1032.
(88) Chalyavi, F.; Schmitz, A. J.; Fetto, N. R.; Tucker, M. J.; Brewer, S. H.; Fenlon, E. E. Extending the vibrational lifetime of azides with heavy atoms. *Phys. Chem. Chem. Phys.* **2020**, *22* (32), 18007−18013.
(89) Marecek, J.; Song, B.; Brewer, S.; Belyea, J.; Dyer, R. B.; Raleigh, D. P. A Simple and Economical Method for the Production of 13C,18O-Labeled Fmoc-Amino Acids with High Levels of Enrichment: Applications to Isotope-Edited IR Studies of Proteins. *Org. Lett.* **2007**, *9* (24), 4935−4937.
(90) Flanagan, J. C.; Baiz, C. R. Site-Specific Peptide Probes Detect Buried Water in a Lipid Membrane. *Biophys. J.* **2019**, *116* (9), 1692−1700.
(91) Oh, K. I.; Baiz, C. R. Molecular heterogeneity in aqueous cosolvent systems. *J. Chem. Phys.* **2020**, *152* (19), 190901.
(92) Elcock, A. H. Models of macromolecular crowding effects and the need for quantitative comparisons with experiment. *Curr. Opin Struct Biol.* **2010**, *20* (2), 196−206.
(93) Nawrocki, G.; Im, W.; Sugita, Y.; Feig, M. Clustering and dynamics of crowded proteins near membranes and their influence on membrane bending. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (49), 24562−24567.
(94) Levy, Y.; Onuchic, J. N. Water mediation in protein folding and molecular recognition. *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 389−415.
(95) Laage, D.; Elsaesser, T.; Hynes, J. T. Perspective: Structure and ultrafast dynamics of biomolecular hydration shells. *Structural Dynamics* **2017**, *4* (4), 044018.
(96) Ostrowska, N.; Feig, M.; Trylska, J. Modeling Crowded Environment in Molecular Simulations. *Front Mol. Biosci* **2019**, *6*, 86−86.
(97) Levin, D. E.; Schmitz, A. J.; Hines, S. M.; Hines, K. J.; Tucker, M. J.; Brewer, S. H.; Fenlon, E. E. Synthesis and evaluation of the sensitivity and vibrational lifetimes of thiocyanate and selenocyanate infrared reporters. *RSC Adv.* **2016**, *6* (43), 36231−36237.
(98) Heo, L.; Feig, M. What makes it difficult to refine protein models further via molecular dynamics simulations? *Proteins: Struct., Funct., Bioinf.* **2018**, *86*, 177−188.
(99) Monteith, W. B.; Pielak, G. J. Residue level quantification of protein stability in living cells. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111* (31), 11335−40.
(100) Alberti, S.; Gladfelter, A.; Mittag, T. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. *Cell* **2019**, *176* (3), 419−434.
(101) Cinar, H.; Winter, R. The effects of cosolutes and crowding on the kinetics of protein condensate formation based on liquid-liquid phase separation: a pressure-jump relaxation study. *Sci. Rep* **2020**, *10* (1), 17245.
(102) Crowe, C. D.; Keating, C. D. Liquid-liquid phase separation in artificial cells. *Interface Focus* **2018**, *8* (5), 20180032.
(103) Cakmak, F. P.; Choi, S.; Meyer, M. O.; Bevilacqua, P. C.; Keating, C. D. Prebiotically-relevant low polyion multivalency can

improve functionality of membraneless compartments. *Nat. Commun.* **2020**, *11* (1), 5949.
(104) Ma, J.; Pazos, I. M.; Zhang, W.; Culik, R. M.; Gai, F. Site-specific infrared probes of proteins. *Annu. Rev. Phys. Chem.* **2015**, *66*, 357−77.
(105) Oh, K. I.; Choi, J. H.; Lee, J. H.; Han, J. B.; Lee, H.; Cho, M. Nitrile and thiocyanate IR probes: molecular dynamics simulation studies. *J. Chem. Phys.* **2008**, *128* (15), 154504.
(106) Rodgers, J. M.; Abaskharon, R. M.; Ding, B.; Chen, J.; Zhang, W.; Gai, F. Fermi resonance as a means to determine the hydrogen-bonding status of two infrared probes. *Phys. Chem. Chem. Phys.* **2017**, *19* (24), 16144−16150.
(107) Theillet, F. X.; Binolfi, A.; Bekei, B.; Martorana, A.; Rose, H. M.; Stuiver, M.; Verzini, S.; Lorenz, D.; van Rossum, M.; Goldfarb, D.; Selenko, P. Structural disorder of monomeric α-synuclein persists in mammalian cells. *Nature* **2016**, *530* (7588), 45−50.
(108) Frederick, K. K.; Michaelis, V. K.; Corzilius, B.; Ong, T.-C.; Jacavone, A. C.; Griffin, R. G.; Lindquist, S. Sensitivity-enhanced NMR reveals alterations in protein structure by cellular milieus. *Cell* **2015**, *163* (3), 620−628.
(109) Banci, L.; Barbieri, L.; Bertini, I.; Luchinat, E.; Secci, E.; Zhao, Y.; Aricescu, A. R. Atomic-resolution monitoring of protein maturation in live human cells by NMR. *Nat. Chem. Biol.* **2013**, *9* (5), 297−9.

https://doi.org/10.1021/acs.jpca.2c03803
J. Phys. Chem. A 2022, 126, 5881−5889