THE JOURNAL OF PHYSICAL CHEMISTRY Letters

Letter

Cite This: J. Phys. Chem. Lett. 2018, 9, 6584−6592

pubs.acs.org/JPCL

How Molecular Crowding Differs from Macromolecular Crowding: A Femtosecond Mid-Infrared Pump−Probe Study

Pramod Kumar Verma,[†,‡,§,⊥] Achintya Kundu,[†,‡,#,⊥] and Minhaeng Cho*[,†,‡]

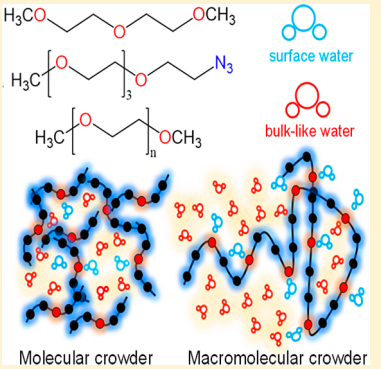
[†]Center for Molecular Spectroscopy and Dynamics, Institute for Basic Science (IBS), Seoul 02841, Republic of Korea

[‡]Department of Chemistry, Korea University, Seoul 02841, Republic of Korea

[§]Department of Chemistry, Institute of Science, Banaras Hindu University, Varanasi 221005, India

Supporting Information

ABSTRACT: Crowding is an inherent property of living systems in which biochemical processes occur in highly concentrated solutions of various finite-sized species of both low (molecular crowding) and high (macromolecular crowding) molecular weights. Is molecular crowding fundamentally different from macromolecular crowding? To answer this question, we use a femtosecond mid-infrared pump−probe technique with three vibrational probes in molecular (diethylene glycol) and macromolecular (polyethylene glycol) solutions. In less crowded media, both molecular and macromolecular crowders fail to affect the dynamics of interstitial bulk-like water molecules and those at the crowder/water interface. In highly crowded media, interstitial water dynamics strongly depends on molecular crowding, but macromolecular crowding does not alter the bulk-like hydration dynamics and has a modest crowding effect on water at the crowder/water interface. The results of this study provide a molecular level understanding of the structural and dynamic changes to water and the water-mediated cross-linking of crowders.



Downloaded via MAX BORN INST NICHTLINEARE OPTIK on November 6, 2018 at 09:00:14 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

In its natural state, a cell has a densely packed intracellular environment with a high concentration of macromolecules and cellular organelles.[1] In addition to these macromolecules, small molecules such as metabolites and osmolytes are present in high concentrations. For instance, mammalian kidney medulla cells contain urea concentrations of up to 5.4 M.[2] The high concentration (up to 400 g/L) of macromolecules and small molecules creates a crowded intracellular environment because, in general, while no single macromolecular or molecular species occurs at high concentrations, taken together they occupy a significant fraction (typically 40%) of the total volume.[3] This volume is thus physically unavailable to other species. This reduction in intermolecular space forces individual molecules to be constrained in a much smaller space compared to a more dilute solution.[4,5] This nonspecific steric effect is always present, regardless of any other attractive or repulsive interactions that might occur between solute molecules. This is known as the excluded volume effect, and it influences the diffusion rate, folded structure, structural stability, and interactions of biomolecules.[6,7] Organisms cannot avoid cellular crowding and thus require strategies to deal with it.

The effects of crowding on biomolecules such as proteins, enzymes, and nucleic acids have been studied in great detail.[8] Interestingly, it was observed that dissolved biopolymers, e.g., proteins, destabilize proteins in their native states via protein−protein interactions, which contrasts to the conventional notion that the exclusion volume effect by other proteins generally contributes to an increase of protein stability in its compact native state.[9] However, in other studies, it was shown that proteins are prone to form large aggregates as the extent of crowding increases.[10] However, it should be noted that these changes in protein structure and stability induced by crowding agents are likely to be correlated with water structure, but unfortunately there do not exist many reports on the time-resolved vibrational spectroscopic investigations of water dynamics in molecular crowding environments. Water is one of the most abundant cellular species.[11] Cellular components become tightly associated with water molecules, leading to the formation of hydration layers that are more ordered and osmotically inactive.[1,12,13] In a highly crowded environment, the hydration layers of biomolecules overlap by distances of several nanometers, and the fraction of interfacial water varies between 30% and 70% of the total water in a cell.[11]

Previous research has investigated the importance of intracellular water to cellular activity. For example, the growth rate of osmotically stressed *Escherichia coli* was found to be dependent on the amount of cytoplasmic water because of the enhanced cytoplasmic molecular interactions at increased osmolality.[14,15] In another study, it was concluded that there is a lower number of free water molecules in tumor cells than in normal cells.[16] This supports the notion that the volume of intracellular water is indeed correlated with the status and property of a cell.[17] More importantly, intracellular water has

Received: October 14, 2018
Accepted: October 31, 2018
Published: October 31, 2018

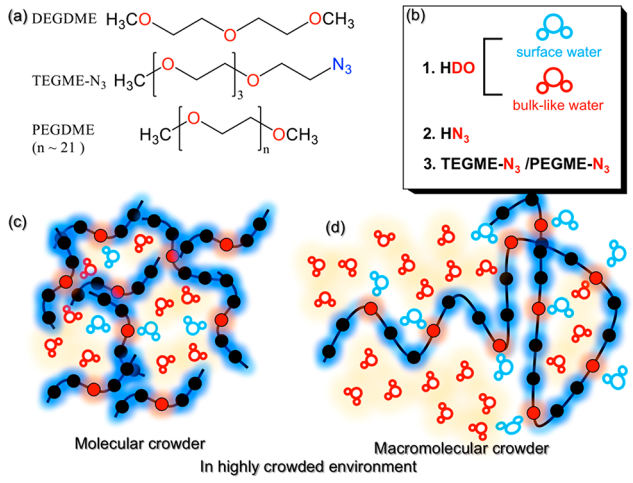
© XXXX American Chemical Society

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

much weaker dielectric properties than pure water. For example, the dielectric constant of a yeast cell is 50 or even less.[18,19] Molecular dynamics simulations predict values in the range of 30−50 in a crowded environment.[20] A decrease in the dielectric constant favors electrostatic attraction and H-bonding interaction and reduces the ability to sequester hydrophobic components away from water. Therefore, a question has arisen regarding to what extent the physicochemical properties of intracellular water differ from those of a dilute solution.

Polyethylene glycol (PEG) is, in this regard, one of the most extensively studied macromolecular crowding agents because of its high water solubility, low toxicity, biodegradability, and apparent inertness to proteins.[21,22] In our recent study on macromolecular crowding (PEGDME, Scheme 1), we found

Scheme 1. (a) Molecular Structures of Diethylene Glycol Dimethyl Ether (DEGDME), Its Azido Derivative, and Poly(ethylene glycol) Dimethyl Ether; (b) Three Independent IR Probes Used in This Study to Investigate the Water Structure and Dynamics in Molecular and Macromolecular Crowded Environments; Illustrations of the Structure of Molecular Crowders and Solvating Water (c) and That of Macromolecular Crowder and Bulk-like Water (d)



that interstitial water dynamics remains similar to that of bulk water even in a highly crowded environment but that the dynamics of surface hydration water molecules that are tightly associated with the ether oxygen atoms of PEG are slower than those in bulk-like water.[23] A question that remains unanswered, however, is how molecular crowding (i.e., cellular media with high concentrations of small molecules with a low molecular weight) affects the structural and dynamical properties of water and how this differs from macromolecular crowding. Therefore, in this study we focus on the water dynamics in the molecular crowded environment and compare it with the macromolecular crowded environment.

Unlike PEG, a molecular crowder does not have a large degree of conformational flexibility. PEG molecules can adopt multiple conformations (e.g., random coil and helical structure) depending on the composition of constituent molecules in solution and other parameters like temperature, pH, and so on. The conformational transitions occur via dihedral rotations along the C−C and C−O bonds of each O−C−C−O segment in PEG. In contrast, DEG, even though it can rapidly explore all the conformational space spanned by its internal degrees of freedom, does not have long-lived heterogeneous conformational states. PEG-based polymers thus have the unique ability to allow water to adopt constrained clathrate-like packing to avoid its hydrophobic ethylene units while also maintaining much of its hydrogen-bonding network due to favorable O−O spacing, which effectively matches water's H-bond geometry.[24] A comparative study of molecular and macromolecular crowding is important because, when contact occurs between PEG/DEG and a biological or chemical species, they share common hydration layers, and those water molecules in the layers play an important role in the function and stability of biological species or polymers. However, studying water alone would not provide a complete picture of the biologically important systems in a crowded environment. Thus, we focus on both the water and molecular crowders by attaching an infrared (IR) probe to the crowder, something which has not been the subject of research to date. In addition, we employ a third IR probe (HN_3) to identify changes in water density in the presence of molecular crowding.

The most important question has been whether there is a dynamic transition above a certain crowding threshold concentration. King et al.[25] carried out two-dimensional (2D)-IR experiments to study the spectral diffusion dynamics of a covalently linked metal carbonyl IR probe at the surface of a lysozyme at different concentrations of PEG400 (which has an average molecular weight of 400) and discovered a transition to the collective slowdown of water dynamics at around 60% PEG. Here, the collective slowdown means that the dynamics of most water molecules becomes slow as compared to that in bulk water and there is only a trace amount of bulk-like water molecules as the extent of crowding increases. A follow-up study by Hunag et al. attempted to answer the question of whether this transition is a general phenomenon or a protein-specific one. They used Overhauser dynamic nuclear polarization (ODNP) to probe the hydration dynamics at the surface of the wild-type human protein γS-Crystallin. The water molecules in the hydration shell of γS-Crystallin were found to behave like a bulk water (water interacting with other water molecules) even in highly crowded media, and no collective slowdown was observed.[26] Our previous study on macromolecular crowding also reported bulk-like behavior in highly crowded media, and no collective slowdown of water dynamics was found; however, the question about whether this will hold true in the presence of molecular crowding remains unanswered.

The molecular crowder investigated in this study is diethylene glycol dimethyl ether (DEGDME), which is shown in Scheme 1a. DEGDME does not have terminal hydroxyl groups, meaning complications do not arise from water−alcohol interactions. To gain a local and microscopic picture of the crowded system, we use three different IR probes (Scheme 1b) and employ the femtosecond (fs) mid-IR pump−probe (PP) technique that has been applied to the studies of local environments around IR probes.[27−35] One of the three probes is water itself, OD stretch vibration of HDO, that provides information on the local structure and dynamics of the water from the perspective of the water.[36−40] The other probes are the azido (NNN) stretch modes of both hydrazoic acid (HN_3) and TEGME-N_3 (O-(2-azidoethyl)-O′-methyl-triethylene glycol; Scheme 1b). The former provides information from the solute's perspective,[41,42] while the latter

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

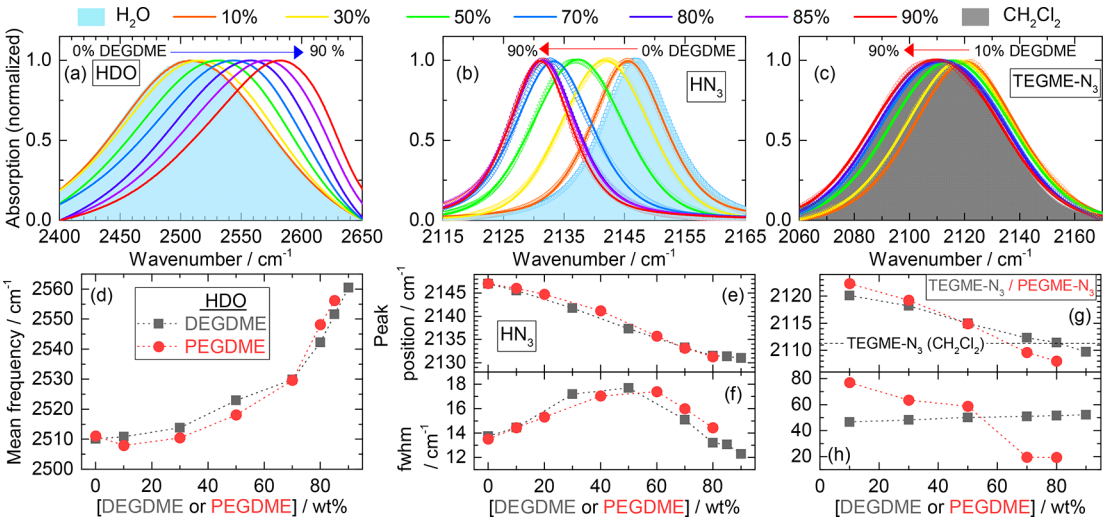


Figure 1. FTIR absorption spectra of the OD stretch of HDO (a) with the mean frequency (d) for different wt % of the molecular crowder. The absorption spectra of the azido stretch (b and c; the symbols represent the raw data, and the solid lines are obtained by fitting the FTIR data with a pseudo-Voigt function) of HN_3 and TEGME-N_3 along with their peak positions (e and g) and full-width at half-maximum (fwhm) values (f and h) as a function of the crowding. The blue shaded area is for neat water, and the gray shaded area is for dichloromethane. As a comparison, the mean frequency of the OD stretch of HDO (d) and the peak position (e and g) and the fwhm values (f and h) of HN_3 and PEGME-N_3 as a function of macromolecular crowding are presented as red circles.

is used to determine how the crowder itself responds to crowding stress. Our multiple-IR-probe approach provides a complementary stereoscopic view of water and the crowder, as well as any relationship that there may be between them.

Molecular crowding causes a blue-shift (i.e., a shift to a higher wavenumber) of the OD stretch IR spectrum from 2506 cm^{-1} (0 wt % DEGDME) to 2583 cm^{-1} (90 wt % DEGDME), a total of 77 cm^{-1} (Figure 1a). This large blue-shift indicates not only a redistribution of equilibrium H-bond numbers and H-bond strength but also the HDO molecules tightly associated with the ether oxygen of DEGDME, which is denoted as OD-e (Scheme 1).[38] The considerable narrowing of the red side of the IR spectrum indicates significant disruption to the extended H-bond network of water molecules. The significant IR absorbance on the blue side with an increase in crowding stress is attributable to an increase in the OD-e population. It should be noted that the reduced electric field along the OD bond of the HDO bound to the ether oxygen atom increases the OD stretch frequency in a similar way to HDO molecules bound to anions in salt solutions.[37]

Unlike the OD stretch mode, the azido stretch mode of both the solute (HN_3) and the crowder (TEGME-N_3) undergoes substantial red-shifts (i.e., a shift to a lower wavenumber) with increased crowding (Figure 1b,c). The asymmetric stretching vibration of azido is sensitive to the local density of water H-bond donors and not to H-bond strength or polarity.[42,43] As crowding increases, the water−ether population builds up at the expense of the water−water population and HN_3 encounters fewer water H-bond donors. What is more interesting is the increase in the line width (fwhm) of the azido band of HN_3 up to 50 wt % DEGDME, followed by a decrease (Figure 1f) with further crowding. This increase in width until 50 wt % means that the line is very broad and spans frequencies from more water-like solvent environments to those observed in water-poor solvents, indicating the presence of heterogeneous H-bond environments around the azido groups. However, when crowding becomes extreme (i.e., more than 50 wt %), the solute HN_3 experiences mostly water-poor environments. This disruption to the water H-bond network is further confirmed by the observation that the peak position of the azido stretch band of TEGME-N_3 in highly crowded media reaches a value that is close to that of TEGME-N_3 in dichloromethane (a waterless environment; Figure 1g). The fwhm of TEGME-N_3 gradually increases with crowding but does not show any sharp transition like PEGDME, which exhibits a crowding-dependent conformational transition at a macromolecular crowding of around 60 wt %.[23] Note that TEGME-N_3 is used as an IR probe instead of DEGME-N_3 because of the commercial unavailability of DEGME-N_3 (it is less stable in water) and because the number of EO units in TEGME-N_3 and DEGME-N_3 are two and one, respectively, excluding the terminal ether oxygen.

In time-resolved IR PP spectroscopy, vibrations are excited with an intense light pulse, and the relaxation of the excited vibrations back to the ground state is measured with a second, weaker probe pulse. The excited vibrations relax by transferring energy to its environment, generating lower-energy vibrations within the same molecule or surrounding molecules; thus, vibrational lifetime is highly sensitive to the local environment.[44] Ultimately, the energy of the excited vibrations is transferred into heat.

The isotropic IR PP spectra (Figures S1−S3 in Supporting Information) of HDO (water), HN_3 (water), and TEGME-N_3 (dichloromethane) consist of a positive (0−1 transition) ground-state bleach (GSB) and stimulated emission (SE) peaks at around 2520, 2147, and 2105 cm^{-1} and a negative (1−2 transition) excited-state absorption (ESA) peaks at 2430, 2116, and 2072 cm^{-1}, respectively. One of the representative isotropic IR PP spectra of the OD stretch mode of HDO in 90 wt % DEGDME solution is shown in Figure 2a.

The energy relaxation pathways for the excited OD vibration of HDO involve the high-frequency modes of water and other interacting molecules (e.g., the ether oxygen of DEGDME), such as HDO bends and low-frequency bath modes like intermolecular hindered transitional and librational modes.[44]

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

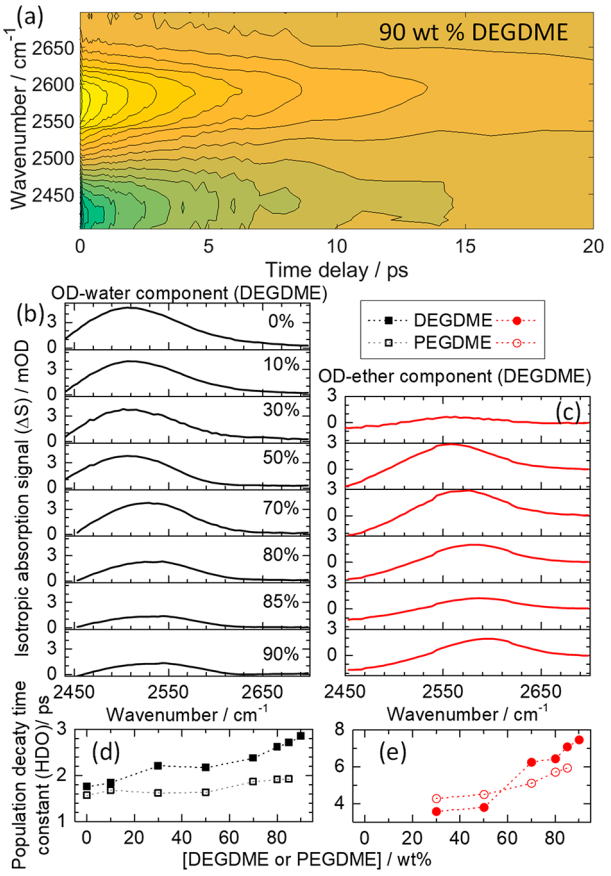


Figure 2. (a) Isotropic IR PP spectra of the OD stretch of HDO in 90 wt % of the DEGDME solution. Population relaxation is monoexponential below crowding of 30 wt %, above which it becomes biexponential. The OD-e and the OD-w components are shown in panels b and c, respectively. Vibrational lifetime (HDO) of the OD-w (d) and OD-e (e) components are shown as a function of molecular crowding (solid symbols) and macromolecular crowding (open symbols). Molecular crowding significantly increases the lifetime of both the OD-w and OD-e populations.

Under molecular crowding, HDO can interact with other water hydroxyl groups (OD-w) as well as with the ether oxygen of DEGDME (OD-e). However, the coupled energy acceptor modes of the OD group H-bonded to water are likely different from those of the OD group H-bonded to the ether oxygen of DEGDME.

In pure water, we measure a time constant of 1.6 ± 0.1 ps for the OD vibrational lifetime after fitting the isotropic PP data (see the Supporting Information for details).[37] In less crowded media (i.e., <30 wt % DEGDME), the population decay is monoexponential, with a time constant similar to that of HDO in water despite the fact that there are two subpopulations, OD-w and OD-e. The apparent monoexponential decay suggests that DEGDME molecules are segregated and surrounded by abundant water molecules in such a way that they become part of the extended H-bond network of water. At 10 wt % DEGDME, 67 water molecules are available per DEGDME (22 water molecules per ether).

When crowding is increased to 30 wt % and above, the population decay no longer fits a monoexponential function but instead fits a biexponential function well. The faster component (<2.5 ps) corresponds to bulk-like water (OD-w) (Scheme 1) and the slower component (>3.5 ps) corresponds to the OD-e population (Scheme 1). At 30 wt % and 50 wt % crowding, there are 17 and 7 water molecules per DEGDME, respectively (6 and 2 water molecules per ether), which reduces to three (one water molecule per ether) at 70 wt %. At 80 wt % and above, there is less than one water molecule available per ether. If all of the water molecules were associated with ether oxygen, there would be only OD-e water, which would result in a monoexponential decay at ≥80 wt % crowding. However, in crowding as high as 90 wt %, 36% of the relaxation occurs on a time scale consistent with the OD-w population, suggesting a nonuniform distribution of water molecules among the ether oxygen. The decomposed isotropic PP spectra of the OD-w and the OD-e are shown in Figure 2b,c.

The vibrational lifetime of the OD-w component gradually increases with crowding, and the increase in lifetime is significant after crowding of 50 wt % (Figure 2d). Similarly, the OD-e population exhibits a marginal increase in lifetime value up to crowding of 50 wt %, after which the lifetime becomes much longer (Figure 2e). Thus, crowding greater than 50 wt % strongly affects both the OD-w and OD-e populations and significantly slows their vibrational relaxations. Careful observation of the peak position of the OD-w component also reveals that the peak position is marginally blue-shifted up until 50 wt % crowding, but crowding of more than 50 wt % dramatically shifts the peak position to a higher wavenumber (Figure 2b,c).

A water molecule forms up to four H-bonds with an approximately tetrahedral geometry, and the ether oxygen of the ethylene unit ($-CH_2-O-CH_2-CH_2-$) of PEG-based polymers or DEGDME is capable of forming two H-bonds. In addition to this H-bonding, some water molecules have hydrophobic interaction with the terminal methyl groups and the ethylene segment. A water molecule near a hydrophobic surface forms fewer and energetically altered H-bonds compared to a bulk water molecule because the hydrophobic surface restricts the space available for other water molecules that are necessary for hydrogen bonding. However, DEGDME's oxygen is geometrically compatible with bulk liquid water's H-bonding network.[45] This specific arrangement helps the tightly associated DEGDME/water interface to achieve a well-structured hydration shell. Although water molecules in those hydration shells are dynamically constrained, they are fully capable of participating passively in the H-bonding network of the second hydration layer. In essence, all of the water molecules, except for the water molecules directly in contact with DEGDME, should experience little, if any, dynamical perturbation. This explains why the lifetime increases marginally up until 50 wt % crowding. At and below this point, there are more than two water molecules per ether oxygen.

When crowding, however, increases to 70 wt % and above, the overlap of hydration shells between DEGDME molecules becomes significant and the availability of water molecules per ether oxygen reduces to one or fewer. According to the Flory–Huggins theory of polymers, the interaction parameter χ defines these contacts: a χ less than zero favors polymer–solvent interactions more than polymer–polymer or solvent–solvent interactions, while a χ greater than zero leads to polymer–solvent interactions being favored over the other two interactions. The χ value becomes less negative with increasing polymer concentration.[46] In highly crowded media, the

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584–6592

DEGDME−water interaction is less favored, promoting DEGDME−DEGDEM and water−water contacts.

The population decay provides direct evidence of water aggregation and the truncation of the water H-bond network in highly molecular crowded media. Furthermore, the molecular crowder's water-mediated cross-linking slows the OD-e dynamics in highly crowded media. More evidence of the depletion of water from the surface of DEGDME is seen in the Fourier transform infrared (FTIR) spectra of TEGME-N_3 (Figure 1c,g).

The vibrational (-NNN) probe covalently attached to the molecular crowder (Scheme 1) provides a direct assessment of the crowder's behavior. In dichloromethane (a waterless environment), the excited azido stretch vibration in TEGME-N_3 decays with two time components: 1.3 ± 0.2 ps and 6.5 ± 0.3 ps (Figure 3c,d).[47] In dilute solutions and less

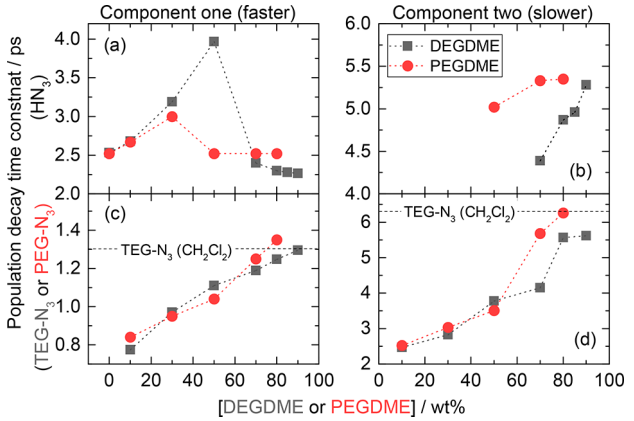


Figure 3. Population relaxation of the azido stretch excited state of HN_3 (a and b) decays monoexponentially until 50 wt % crowding, above which a slower component appears, representing HN_3 either residing at the crowder/water interface or at hydrophobic interstitial methylene units in between ether oxygen. Both TEGME-N_3 and PEGME-N_3 (c and d) exhibit two vibrational lifetimes, τ_1 and τ_2, in dichloromethane and at all crowding concentrations. With an increase in crowding, both lifetimes approach a value close to that in dichloromethane. Details of the heating correction process (if required) are presented in the Supporting Information.

crowded media, both the fast and slow time components accelerate, reflecting the sensitivity of the azido stretch mode to local water density.[34,47,48] At increased crowding, both lifetime components become progressively slower and reach values that are close to those of TEGME-N_3 in dichloromethane. This increase provides important evidence about local water density surrounding TEGME-N_3. In less crowded media, excluded-volume effects are predominantly intramolecular, so crowders and water interact to form an EO/water interface that allows the hydrocarbon groups to be placed in the interstitial spaces, and the EO unit remains extended and hydrated. As crowding increases, intermolecular excluded-volume interactions cause crowders to share their hydration shells with other crowders, thereby reducing their association with water. This increased polymer−polymer contact is reflected in the water clustering and dehydration of DEGDME, corroborating observations of the population decay of OD vibrations.

To determine how crowding affects a water-sensitive solute, the azido stretch mode of HN_3, which is an excellent vibrational probe that reports the number density of water in its immediate surroundings (e.g., the peptides and amino acids of biomolecules) is employed.[43,48−50] The vibrational lifetime of HN_3 is plotted in Figure 3a,b as a function of molecular crowder concentration (%). As the crowding increases from 0 wt % to 50 wt %, the lifetime of the azido vibration gradually increases from 2.5 ± 0.2 ps to 3.9 ± 0.2 ps. The fact that vibrational relaxation remains monoexponential until 50 wt % crowding (Figure 3a) strengthens the conclusion that DEGDME's first and second hydration shells are not very different from bulk-like water in less crowded media. It is important to note that the structured hydration shell at the DEGDME/water interface is formed because the O−O spacing of DEGDME matches with that between two H-bonded water molecules. The tightly associated water molecules mean that the majority of the water molecules, except for those in direct contact with the DEGDME, experience marginal dynamical perturbation in less crowded media. The increase in the lifetime until 50 wt % crowding is due to the disruption of water's extended H-bond network and heterogeneity in H-bonding (Figure 3a). DEGDME is the smallest PEGDME-based polymer; thus, it cannot form a highly extended structured hydration shells like PEGDME (vide infra). Consequently, at crowding of 70 wt % and above, intermolecular effects dominate. Molecular crowders are forced to share their hydration shell to cope with increased crowding stress. HN_3's lifetime no longer exhibits a single time constant but rather has two different time components: a faster component (≤2.5 ps) representing a bulk-like water environment (water aggregation) and a slower component (>4 ps) corresponding to a water-poor environment (water-mediated cross-linking).

In addition to vibrational population relaxation, polarization-selective IR PP measurements allow orientational relaxation to be analyzed. The orientational relaxation of water involves the concerted breaking and reforming of the H-bonds of a water molecule in a bifurcated H-bond configuration with two other water molecules (incoming and outgoing) based on the extended angular jump model proposed by Laage and Hynes.[51] Any hindrance to these jumps, such as the reduced number of H-bond partners or excluded volume effects, will slow the relaxation.[51] For HDO in water, the anisotropy, $r(t)$ decays monoexponentially with a time constant of 2.5 ± 0.2 ps (Figure 4a).[37]

Crowding affects anisotropy decay because it does not remain monoexponential. The anisotropy decay as a function of crowding wt % is plotted in Figure S4, and it is fitted with the equation $(r(t) = R_0 \exp(-t/\tau_r) + R_{slow})$, except for 0 wt %. The offset (R_{slow}) represents a slowly decaying component that is not recoverable within the lifetime of the OD vibrations. The slower component (R_{slow}) corresponds to the ether OD-e where the excluded volume plays a major role. The bulk-like water relaxation time constant τ_r (OD-w) shows a moderate dependence on crowding due to the truncation of the extended H-bond network of water. At 80 wt % crowding and above, τ_r (OD-w) remains unaffected and the offset value marginally decreases, suggesting water aggregation behavior.

Interestingly, the anisotropy decay of HN_3 remains unaffected by molecular crowding except the offset value. In less crowded media, the hydration shell of the DEGDME is geometrically compatible with the H-bonding of bulk water and thus τ_r (HN_3) relaxes on a time scale similar to that for pure water. In heavily crowded media, water molecules aggregate, and a third participant (a fraction of the HN_3

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

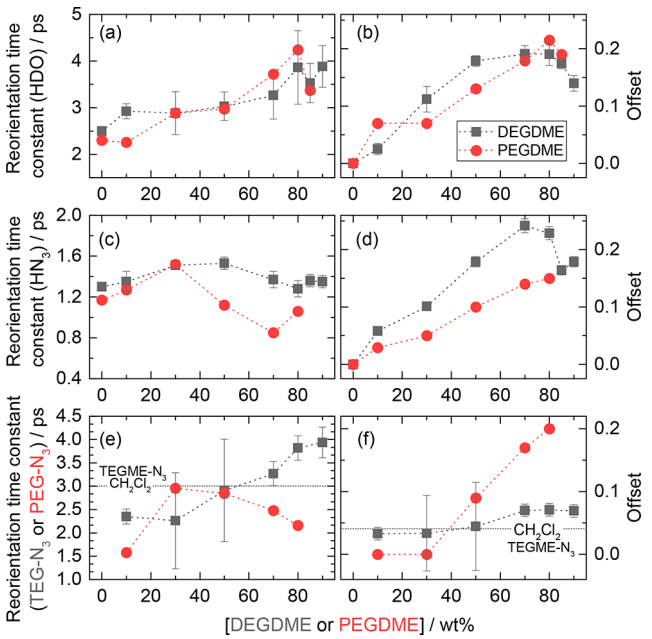


Figure 4. Reorientation time constants of HDO (a), HN_3 (c), and TEGME-N_3 (e) as a function of crowding. The anisotropy decays were fitted to a monoexponential function (τ_r) and an offset (R_{slow}), and the fits were carried out at the peak frequency of the corresponding vibrational band. All the anisotropy decays (HDO, HN_3, and TEGME-N_3) are depicted in Figures S4−S6. The error bars represent the error obtained from the fitting.

population in this case) encountering these water clusters does not exhibit a relationship with crowding. Unlike HDO, HN_3 generally forms a single H-bond with water; therefore, it is believed that the effect of H-bond rearrangement on the anisotropy decay of HDO will be larger than on that of HN_3. A fraction of the HN_3 molecules residing in water-poor environments are also affected because of the collectively excluded volume effect and the reduction in H-bonding partners as crowding increases. The population of this fraction rises with crowding, as evident from the increase in the amplitude of R_{slow} (Figure 3d).

We also measure the anisotropy of TEGME-N_3 in dichloromethane and find that it decays with a fast component (3.0 ± 0.2 ps) and an offset (R_{slow}) of magnitude 0.04. This biexponential decay indicates a wobbling-in-a-cone motion, where the fast component arises from the azido group, covalently attached to the tail segment of the molecular crowder, wobbling around within a limited cone of angles.[52] The restricted wobbling of the azido transition dipole contributes to anisotropy decay but does not result in complete decay. The offset corresponds to that incomplete randomization.

The anisotropy decay of TEGME-N_3 at crowding of 10 wt % and above consists of a fast component (τ_r) and an offset. Neither the fast component nor the offset exhibits any considerable dependence on crowding in media with low crowding (up to 50 wt %). The amplitude of the offset at 50 wt % is approximately the same as that in dichloromethane. Above 50 wt %, both τ_r and the offset increase. In highly crowded media, the presence of an offset value higher than that for dichloromethane is due to the water-mediated cross-linking of DEGDME and provides direct evidence of close contact between polyethers. The water-poor environment is responsible for the slower reorientation time in highly crowded media.

Overall, in less crowded media, molecular and macromolecular crowders behave similarly. In water-rich solutions, excluded volume effects are predominantly intramolecular, so crowders (molecular and macromolecular) and water interact to form a highly linked H-bond network that causes the hydrocarbon groups to be engaged in the interstitial spaces, while the DEG/PEG chain remains extended and hydrated. We believe that the possibility of water adopting constrained clathrate-like packing near hydrophobic ethylene units and maintaining its H-bond network because of geometrically favorable O−O spacing is less likely in DEGDME (n = 1) compared to PEGDME (n = 21) because the longer chain length of PEGDME provides a periodic arrangement of the oxygen atoms that constructively supports a bulk-like hydration interface. The outcome is a comparatively less stable hydration shell for DEGDME than for PEGDME. The effects are directly reflected in the vibrational lifetime of OD-w and OD-e in DEGDME and PEGDME (Figure 2c,d).

In highly crowded media, molecular crowding significantly slows the dynamics of both interstitial water and tightly associated ether water. In contrast, macromolecular crowding does not affect interstitial water dynamics but moderately slows the dynamics of tightly associated ether water. PEGDME responds to this high crowding by adopting a crystalline structure similar to solid PEG, in which its contact with water is minimized but optimized.[53−56] This enhanced intermolecular interaction favors the helical conformation of PEGDME and water pooling. Hence, the water dynamics remain unperturbed and the water associated with ether oxygen shows a marginal dependence on crowding due to conformational flexibility.

The molecular crowder (DEGDME) lacks the conformational flexibility to adopt various conformations around the C−C and C−O bonds of O−C−C−O unit segments because it has only two EO units. It therefore responds to high crowding by forming water-mediated cross-linking with neighboring DEGDMEs. This enhanced intermolecular interaction reduces the water consumed in the hydration of DEGDME and facilitates water aggregation. These water aggregates are highly constrained both dynamically and structurally because of the termination of the extended H-bond network. As a result, the interstitial water dynamics are significantly different from the bulk-like water dynamics. Water participating in the hydration of ether oxygen is also strongly affected as it mediates cross-linking in highly crowded media.

In summary, the use of three different vibrational probes and femtosecond mid-IR polarization-selective PP measurements in this study offers detailed insights into three important constituents of the intracellular environment: water, crowders, and water-sensitive molecules. Crowding is an inevitable characteristic of all cellular environments. The most important finding is that molecular crowding affects both interstitial water and water at the crowder/water interface in highly crowded media, but macromolecular crowding does not alter the bulk-like hydration dynamics and has only a modest crowding effect on water at the crowder/water interface, even in highly crowded media. This is because PEGDME's highly structured first hydration shell not only preserves water's H-bonding but also promotes and participates in the extended H-bond network of water. Even in highly crowded media, PEGDME (a long chain PEG polymer with 21 EO units) preserves

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

water's H-bond network by adopting a compact and presumably helical structure. For DEGDME (the shortest PEG, with only one EO unit), hydration dynamics depend strongly on crowding. Because of its low molecular weight, DEDGME facilitates water-mediated cross-linking and subsequent water aggregation in highly crowded media.

Our experimental data support the assertion that water in highly crowded solutions like the cytoplasm will be significantly affected by high molecular crowding but exhibits bulk-like behavior in the presence of high macromolecular crowding.[55,57] Furthermore, we did not observe any coalescing of bulk-like and surface hydration water with PEGDME or DEGDME, indicating that there is no abrupt change in the water dynamics associated with collective hydration. A far-reaching impact of this study is that molecular crowding has a considerable influence on water structure and dynamics, but there is still a fraction of the water population that keeps a certain proportion of water-sensitive solutes hydrated. This supports the notion that the effect of crowding depends on not only the stability of protein-specific hydration shells but also the type of crowding.[55] We anticipate that this comparative study of highly crowded media will enhance the understanding of the properties of water inside living organisms. In addition, this comparative study on the shortest PEG and a longer counterpart provides new information on PEG's biocompatibility and PEGylating of proteins (high molecular weight molecules) or therapeutics (low molecular weight molecules).

Although we used a femtosecond mid-IR pump−probe measurement method to study water dynamics in highly crowded solutions, local structural and dynamical heterogeneities need to be studied by employing 2D-IR spectroscopy and molecular dynamics simulation methods. Furthermore, it would be interesting to study the crowding effects on water structure and dynamics when the solutions contain highly concentrated monomeric molecules and their polymers, which is currently under investigation. Recently, we have shown that ions and polyols in water at high concentrations tend to form three-dimensionally extended network structures that are intertwined with water H-bonding networks, which could be one of the percolating phenomena.[58] Unlike ions and polyols, the ethylene glycols, e.g., PEG and DEG, have no H-bond donors like hydroxyl groups. Therefore, they cannot spontaneously form aggregates and network structures by making multiple H-bonds with each other without any bridging water molecules between them. Therefore, even if percolating (sample-spanning) aggregates of ethylene glycol molecules or polymers that are intertwined or intricately mixed with water H-bonding networks are formed in highly concentrated PEG and DEG solutions, they should involve water molecules as an adhesive agent. It would be interesting to investigate whether interfacial water molecules play a role as glue or just a solvent medium in such highly crowded solutions by carrying out molecular dynamics simulations in the future.

PEG is one of the most commonly and extensively studied macromolecular crowders, but because PEG's ether oxygen atoms (O−O spacing) are geometrically compatible with the bulk liquid water's H-bonding network, our conclusion drawn from the present experimental results for PEG and DEG solutions cannot be considered to be universal. We thus anticipate that systematic spectroscopic and comparative studies of other molecular and macromolecular crowders would provide crucial information on their different effects on water structure and dynamics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.8b03153.

Experimental methods including the fitting details and heat correction process, the details of the fitting of the FTIR data, representative isotropic PP data, and the anisotropy decay for various wt % of the crowder (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: mcho@korea.ac.kr.

ORCID

Pramod Kumar Verma: 0000-0001-8837-3167

Achintya Kundu: 0000-0002-6252-1763

Minhaeng Cho: 0000-0003-1618-1056

Present Address

#A.K.: Max-Born-Institut für Nichtlineare Optik and Kurzzeitspektroskopie, Berlin 12489, Germany

Author Contributions

⊥P.K.V and A.K. contributed equally to this work

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by IBS-R023-D1. All mid-IR PP measurements were taken at the Seoul Center of the Korea Basic Science Institute (KBSI). P.K.V. thanks the Department of Chemistry in the Institute of Science at Banaras Hindu University (BHU) for the use of its facilities. This Letter is dedicated to the memory of our wonderful colleague, Dr. Pramod K. Verma, who passed away recently.

■ REFERENCES

(1) Luby-Phelps, K. Cytoarchitecture and Physical Properties of Cytoplasm: Volume, Viscosity, Diffusion, Intracellular Surface Area. *Int. Rev. Cytol.* **1999**, *192*, 189−221.

(2) Rösgen, J.; Pettitt, B. M.; Bolen, D. W. Protein Folding, Stability, and Solvation Structure in Osmolyte Solutions. *Biophys. J.* **2005**, *89*, 2988−2997.

(3) Ellis, R. J.; Minton, A. P. Join the Crowd. *Nature* **2003**, *425*, 27−28.

(4) Minton, A. P.; Wilf, J. Effect of Macromolecular Crowding upon the Structure and Function of an Enzyme: Glyceraldehyde-3-Phosphate Dehydrogenase. *Biochemistry* **1981**, *20*, 4821−4826.

(5) Mikaelsson, T.; Ådén, J.; Johansson, L. B. A.; Wittung-Stafshede, P. Direct Observation of Protein Unfolded State Compaction in the Presence of Macromolecular Crowding. *Biophys. J.* **2013**, *104*, 694−704.

(6) Zhou, H.-X.; Rivas, G.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Annu. Rev. Biophys.* **2008**, *37*, 375−397.

(7) Minton, A. P. The Influence of Macromolecular Crowding and Macromolecular Confinement on Biochemical Reactions in Physiological Media. *J. Biol. Chem.* **2001**, *276*, 10577−10580.

(8) Nakano, S. I.; Miyoshi, D.; Sugimoto, N. Effects of Molecular Crowding on the Structures, Interactions, and Functions of Nucleic Acids. *Chem. Rev.* **2014**, *114*, 2733−2758.

(9) Harada, R.; Tochio, N.; Kigawa, T.; Sugita, Y.; Feig, M. Reduced Native State Stability in Crowded Cellular Environment due to Protein-Protein Interactions. *J. Am. Chem. Soc.* **2013**, *135*, 3696−3701.

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

(10) White, D. A.; Buell, A. K.; Knowles, T. P. J.; Welland, M. E.; Dobson, C. M. Protein Aggregation in Crowded Environments. *J. Am. Chem. Soc.* **2010**, *132*, 5170−5175.
(11) Ball, P. Water as an Active Constituent in Cell Biology. *Chem. Rev.* **2008**, *108*, 74−108.
(12) Garlid, K. D. The State of Water in Biological Systems. *Int. Rev. Cytol.* **1999**, *192*, 281−302.
(13) Garlid, K. D. The State of Water in Biological Systems. *Int. Rev. Cytol.* **1999**, *192*, 281−302.
(14) Record, T. M.; Courtenay, E. S.; Cayley, S.; Guttman, H. J. Biophysical Compensation Mechanisms Buffering E. Coli Protein-Nucleic Acid Interactions against Changing Environments. *Trends Biochem. Sci.* **1998**, *23*, 190−194.
(15) Cayley, S.; Record, M. T. Roles of Cytoplasmic Osmolytes, Water, and Crowding in the Response of Escherichia Coli to Osmotic Stress: Biophysical Basis of Osmoprotection by Glycine Betaine. *Biochemistry* **2003**, *42*, 12596−12609.
(16) Damadian, R. Tumor Detection by Nuclear Magnetic Resonance. *Science* **1971**, *171*, 1151−1153.
(17) Dupre, M. A.; Hempling, G. H. Osmotic Properties of Ehrlich Ascites Tumor Cells during the Cell Cycle. *J. Cell. Physiol.* **1978**, *97*, 381−395.
(18) Tanizaki, S.; Clifford, J.; Connelly, B. D.; Feig, M. Conformational Sampling of Peptides in Cellular Environments. *Biophys. J.* **2008**, *94*, 747−759.
(19) Asami, K.; Hanai, T.; Koizumi, N. Dielectric Properties of Yeast Cells. *J. Membr. Biol.* **1976**, *28*, 169−180.
(20) Harada, R.; Sugita, Y.; Feig, M. Protein Crowding Affects Hydration Structure and Dynamics. *J. Am. Chem. Soc.* **2012**, *134*, 4842−4849.
(21) Reid, C.; Rand, R. P. Probing Protein Hydration and Conformational States in Solution. *Biophys. J.* **1997**, *72*, 1022−1030.
(22) Hirano, A.; Shiraki, K.; Arakawa, T. Polyethylene Glycol Behaves like Weak Organic Solvent. *Biopolymers* **2012**, *97*, 117−122.
(23) Verma, P. K.; Kundu, A.; Ha, J. H.; Cho, M. Water Dynamics in Cytoplasm-Like Crowded Environment Correlates with the Conformational Transition of the Macromolecular Crowder. *J. Am. Chem. Soc.* **2016**, *138*, 16081−16088.
(24) Daley, K. R.; Kubarych, K. J. An "iceberg" Coating Preserves Bulk Hydration Dynamics in Aqueous PEG Solutions. *J. Phys. Chem. B* **2017**, *121*, 10574−10582.
(25) King, J. T.; Arthur, E. J.; Brooks, C. L.; Kubarych, K. J. Crowding Induced Collective Hydration of Biological Macromolecules over Extended Distances. *J. Am. Chem. Soc.* **2014**, *136*, 188−194.
(26) Huang, K. Y.; Kingsley, C. N.; Sheil, R.; Cheng, C. Y.; Bierma, J. C.; Roskamp, K. W.; Khago, D.; Martin, R. W.; Han, S. Stability of Protein-Specific Hydration Shell on Crowding. *J. Am. Chem. Soc.* **2016**, *138*, 5392−5402.
(27) Sengupta, N.; Maekawa, H.; Zhuang, W.; Mukamel, S.; Tobias, D. J.; Ge, N.-H. Sensitivity of 2DIR Spectra to Peptide Helicity: A Concerted Experimental and Simulation Study of an Octopeptide. *J. Phys. Chem. B* **2009**, *113*, 12037−12049.
(28) Maekawa, H.; Ge, N. H. Comparative Study of Electrostatic Models for the Amide-I and -II Modes: Linear and Two-Dimensional Infrared Spectra. *J. Phys. Chem. B* **2010**, *114*, 1434−1446.
(29) Maekawa, H.; Ballano, G.; Toniolo, C.; Ge, N. H. Linear and Two-Dimensional Infrared Spectroscopic Study of the Amide I and II Modes in Fully Extended Peptide Chains. *J. Phys. Chem. B* **2011**, *115*, 5168−5182.
(30) Müller-Werkmeister, H. M.; Li, Y. L.; Lerch, E. B. W.; Bigourd, D.; Bredenbeck, J. Ultrafast Hopping from Band to Band: Assigning Infrared Spectra Based on Vibrational Energy Transfer. *Angew. Chem., Int. Ed.* **2013**, *52*, 6214−6217.
(31) Van Wilderen, L. J. G. W.; Kern-Michler, D.; Müller-Werkmeister, H. M.; Bredenbeck, J. Vibrational Dynamics and Solvatochromism of the Label SCN in Various Solvents and Hemoglobin by Time Dependent IR and 2D-IR Spectroscopy. *Phys. Chem. Chem. Phys.* **2014**, *16*, 19643−19653.
(32) El Khoury, Y.; Van Wilderen, L. J. G. W.; Bredenbeck, J. Ultrafast 2D-IR Spectroelectrochemistry of Flavin Mononucleotide. *J. Chem. Phys.* **2015**, *142*, 212416−212421.
(33) Martin, J. P.; Fetto, N. R.; Tucker, M. J. Comparison of Biological Chromophores: Photophysical Properties of Cyanophenylalanine Derivatives. *Phys. Chem. Chem. Phys.* **2016**, *18*, 20750−20757.
(34) Schmitz, A. J.; Hogle, D. G.; Gai, X. S.; Fenlon, E. E.; Brewer, S. H.; Tucker, M. J. Two-Dimensional Infrared Study of Vibrational Coupling between Azide and Nitrile Reporters in a RNA Nucleoside. *J. Phys. Chem. B* **2016**, *120*, 9387−9394.
(35) Chalyavi, F.; Gilmartin, P. H.; Schmitz, A. J.; Fennie, M. W.; Tucker, M. J. Synthesis of 5-Cyano-Tryptophan as a Two-Dimensional Infrared Spectroscopic Reporter of Structure. *Angew. Chem., Int. Ed.* **2018**, *57*, 7528−7532.
(36) Lee, H.; Choi, J. H.; Verma, P. K.; Cho, M. Computational Vibrational Spectroscopy of HDO in Osmolyte-Water Solutions. *J. Phys. Chem. A* **2016**, *120*, 5874−5886.
(37) Omta, A. W.; Kropman, M. F.; Woutersen, S.; Bakker, H. J. Negligible Effects O Ions on the Hydrogen-Bond Structure in Liquid Water. *Science* **2003**, *301*, 347−349.
(38) Fenn, E. E.; Moilanen, D. E.; Levinger, N. E.; Fayer, M. D. Water Dynamics and Interactions in Water-Polyether Binary Mixtures. *J. Am. Chem. Soc.* **2009**, *131*, 5530−5539.
(39) Kundu, A.; Verma, P. K.; Cho, M. Effect of Osmolytes on the Conformational Behavior of a Macromolecule in Cytoplasm-like Crowded Environment: A Femtosecond Mid-IR Pump-Probe Spectroscopy Study. *J. Phys. Chem. Lett.* **2018**, *9*, 724−731.
(40) Kundu, A.; Verma, P. K.; Cho, M. Role of Solvent Water in the Temperature-Induced Self-Assembly of a Triblock Copolymer. *J. Phys. Chem. Lett.* **2017**, *8*, 3040−3047.
(41) Kundu, A.; Verma, P. K.; Ha, J. H.; Cho, M. Studying Water Hydrogen-Bonding Network near the Lipid Multibilayer with Multiple IR Probes. *J. Phys. Chem. A* **2017**, *121*, 1435−1441.
(42) Wolfshorndl, M. P.; Baskin, R.; Dhawan, I.; Londergan, C. H. Covalently Bound Azido Groups Are Very Specific Water Sensors, Even in Hydrogen-Bonding Environments. *J. Phys. Chem. B* **2012**, *116*, 1172−1179.
(43) Verma, P. K.; Lee, H.; Park, J. Y.; Lim, J. H.; Maj, M.; Choi, J. H.; Kwak, K. W.; Cho, M. Modulation of the Hydrogen Bonding Structure of Water by Renal Osmolytes. *J. Phys. Chem. Lett.* **2015**, *6*, 2773−2779.
(44) Oxtoby, D. W. Vibrational Relaxation in Liquids. *Annu. Rev. Phys. Chem.* **1981**, *32*, 77−101.
(45) Kjellander, R.; Florin, E. Water Structure and Changes in Thermal Stability of the System Poly (Ethylene Oxide)−water. *J. Chem. Soc., Faraday Trans. 1* **1981**, *77*, 2053−2077.
(46) Daoust, H.; St-Cyr, D. Microcalorimetric Study of Poly (Ethylene Oxide) in Water and in Water-Ethanol Mixed Solvent. *Macromolecules* **1984**, *17*, 596−601.
(47) Lee, K. K.; Park, K. H.; Joo, C.; Kwon, H. J.; Jeon, J.; Jung, H. Il; Park, S.; Han, H.; Cho, M. Infrared Probing of 4-Azidoproline Conformations Modulated by Azido Configurations. *J. Phys. Chem. B* **2012**, *116*, 5097−5110.
(48) Tucker, M. J.; Gai, X. S.; Fenlon, E. E.; Brewer, S. H.; Hochstrasser, R. M. 2D IR Photon Echo of Azido-Probes for Biomolecular Dynamics. *Phys. Chem. Chem. Phys.* **2011**, *13*, 2237−2241.
(49) Taskent-Sezgin, H.; Chung, J.; Banerjee, P. S.; Nagarajan, S.; Dyer, R. B.; Carrico, I.; Raleigh, D. P. Azidohomoalanine: A Conformationally Sensitive IR Probe of Protein Folding Protein Structure and Electrostatics. *Angew. Chem., Int. Ed.* **2010**, *49*, 7473−7475.
(50) Kim, H.; Cho, M. Infrared Probes for Studying the Structure and Dynamics of Biomolecules. *Chem. Rev.* **2013**, *113*, 5817−5847.
(51) Laage, D.; Hynes, J. T. A Molecular Jump Mechanism of Water Reorientation. *Science* **2006**, *311*, 832−835.
(52) Fujiwara, T.; Nagayama, K. The Wobbling-in-a-Cone Analysis of Internal Motion in Macromolecules. *J. Chem. Phys.* **1985**, *83*, 3110−3117.

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592

(53) Gemmei-Ide, M.; Miyashita, T.; Kagaya, S.; Kitano, H. Mid-Infrared Spectroscopic Investigation of the Perfect Vitrification of Poly(ethylene Glycol) Aqueous Solutions. *Langmuir* **2015**, *31*, 10881−10887.
(54) Gemmei-Ide, M.; Motonaga, T.; Kasai, R.; Kitano, H. Two-Step Recrystallization of Water in Concentrated Aqueous Solution of Poly(ethylene Glycol). *J. Phys. Chem. B* **2013**, *117*, 2188−2194.
(55) Smith, G. D.; Bedrov, D.; Borodin, O. Conformations and Chain Dimensions of Poly(ethylene Oxide) in Aqueous Solution: A Molecular Dynamics Simulation Study. *J. Am. Chem. Soc.* **2000**, *122*, 9548−9549.
(56) Shephard, J. J.; Bremer, P. J.; McQuillan, A. J. Structure and Conformation in Mixtures of Methyl-Terminated Poly(ethylene Oxide) and Water. Principal Component Analysis and Band Fitting of Infrared Absorptions. *J. Phys. Chem. B* **2009**, *113*, 14229−14238.
(57) Pollack, G. H.; Cameron, I. L.; Wheatley, D. N. *Water and the Cell*; Springer: Dordrecht, 2007.
(58) Choi, J.-H.; Lee, H.; Choi, H. R.; Cho, M. Graph Theory and Ion and Molecular Aggregation in Aqueous Solutions. *Annu. Rev. Phys. Chem.* **2018**, *69*, 125−149.

DOI: 10.1021/acs.jpclett.8b03153
J. Phys. Chem. Lett. 2018, 9, 6584−6592