Picosecond orientational dynamics of water in living cells


DOI
10.1038/s41467-017-00858-0

Publication date
2017

Document Version
Final published version

Published in
Nature Communications

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Citation for published version (APA):
Cells are extremely crowded, and a central question in biology is how this affects the intracellular water. Here, we use ultrafast vibrational spectroscopy and dielectric-relaxation spectroscopy to observe the random orientational motion of water molecules inside living cells of three prototypical organisms: *Escherichia coli*, *Saccharomyces cerevisiae* (yeast), and spores of *Bacillus subtilis*. In all three organisms, most of the intracellular water exhibits the same random orientational motion as neat water (characteristic time constants ~9 and ~2 ps for the first-order and second-order orientational correlation functions), whereas a smaller fraction exhibits slower orientational dynamics. The fraction of slow intracellular water varies between organisms, ranging from ~20% in *E. coli* to ~45% in *B. subtilis* spores. Comparison with the water dynamics observed in solutions mimicking the chemical composition of (parts of) the cytosol shows that the slow water is bound mostly to proteins, and to a lesser extent to other biomolecules and ions.
Water plays a role in many cellular processes, ranging from protein folding to proton transport. Understanding the structure and dynamics of intracellular water is important, but to what extent these properties differ from those of bulk water is still debated. The high macroscopic viscosity of the cytoplasm (~10^5 times higher than water) is mostly due to the presence of biomacromolecules. When the intracellular viscosity is probed using small particles or molecules, the observed viscosity decreases rapidly with the probe size, with a sharp decrease below 50 nm, which can be regarded as the mesh size of the intracellular “gel”. But even the smallest fluorescent probes still show intracellular rotational and translational diffusion times that are slower than in normal water, indicating that at the molecular level intracellular water is different from bulk water. Some of these differences stem from the different dynamics of water surrounding biomolecules, but the spatial extent of the effect of biomolecules on water dynamics remains debated. In the rather dilute aqueous solutions of specific biomolecules studied to date, their effect on the water structure and dynamics is generally found to be short-ranged. However, such solutions are very different from crowded cells, where the high density of biomolecules might give rise to non-additive effects on the water dynamics.

Several methods have therefore been used to investigate the dynamics of cell water in vivo. Its low-frequency intermolecular vibrations have been studied using Kerr-effect and THz spectroscopy. The orientational dynamics of cell water has been investigated using nuclear magnetic resonance (NMR) from the frequency-dependent relaxation rate the distribution of rotational correlation times of the intracellular water can be determined, in particular for the water molecules exhibiting slow (>2 ns) dynamics. For water molecules exhibiting faster dynamics, averaged dynamical information can be obtained from NMR experiments. The distribution of ns reorientation times that underlie this average is difficult to access, and water molecules exhibiting picosecond orientational dynamics cannot be observed directly in NMR experiments. The rotation of such rapidly reorienting water molecules can be tracked in real time using ultrafast time-resolved infrared spectroscopy, which directly probes the random orientational motion of the water–OH bonds (or OD bonds in the case of deuterated water). This method has been used to investigate water dynamics in neat water and aqueous solutions of salts and biomolecules. Alternatively, the collective orientational motion of the dipole moments of water molecules can be probed by measuring the electric-field induced polarization of a sample as function of field frequency using dielectric-relaxation spectroscopy (DRS). Here, we combine these two spectroscopic methods to investigate the orientational dynamics of water in live cells of three prototypical species: a vegetatively growing bacterium (Escherichia coli) and a eukaryote (Saccharomyces cerevisiae, yeast) both living in aqueous environments, and Bacillus subtilis spores which can survive drought for many years and are filled with water, and in the spore sample mostly with air.

**Results**

*Time-resolved vibrational spectroscopy.* In the time-resolved infrared experiments we use the intramolecular vibrations of water to probe its rotational dynamics. To avoid coupling between the molecular oscillators and the OD-stretch vibration (at ~2500 cm^-1) of isotopically diluted (HDO:H2O) water. To ensure that the HDO:H2O isotope fraction is the same in the entire organism, the cells are grown or incubated in an aqueous environment containing isotopically diluted water (~5% HDO in H2O). We find that the small deuterium fraction has no effect on the biological properties such as cell division and sporulation. In Fig. 1 we show IR spectra of the organisms investigated, together with microscope images of the samples. In all samples the amount of extracellular water was kept as low as possible (this issue will be further discussed below). During the experiments the samples are kept between two CaF2 windows separated by a 25 μm teflon spacer. The OD-stretch mode of molecules other than water (such as sugars), and the ND-stretch mode (generated by NH/OD exchange) contribute negligibly to the absorbance (Supplementary Discussion 1). In all three organisms the OD-stretch absorption peak has the same shape and center frequency (within 1 cm^-1) as that of bulk HDO:H2O water. The other main features in the IR spectra (at 1650, 2100, and >2800 cm^-1) are due to H2O. The small peak at ~2350 cm^-1 in the E. coli sample is due to CO2, generated by the bacteria.

In the IR pump-probe experiments, a pump pulse preferentially excites ("tags") the stretching mode of OD bonds that are aligned along the IR polarization direction; the resulting anisotropic distribution of vibrationally excited OD groups is randomized by the random orientational motion of water molecules. This causes a decay in the anisotropy parameter.
Supplementary Discussion 1). In neat water, the anisotropy molecules contribute negligibly to the anisotropy decay, we compare the anisotropy decays of neat and intracellular experiment), and has been characterized in detail with NMR37 a broad range of time scales (that cannot be distinguished in our slow water fraction consists of water molecules with dynamics on denoted ensemble averaging61. We characterize the dynamics of the cell water with dielectric-relaxation spectroscopy (DRS). With DRS we probe the polarization of a sample induced by a dispersion in the real permittivity and a corresponding peak in the imaginary permittivity at ~20 GHz. These signatures are a combination of two Cole-Cole-type equations to the in-phase and out-of-phase (absorptive) components of the induced polarization61. The response is measured as a frequency-dependent complex permittivity, with the real and imaginary parts representing the in-phase and out-of-phase (absorptive) components of the induced polarization61. For pure water (dashed lines in Fig. 3) the spectrum is dominated by a dispersion in the real permittivity and a corresponding peak in the imaginary permittivity at ~20 GHz. These signatures are characteristic for a relaxation mode, that is due to random orientational motion of the dipolar water molecules62. The 20 GHz frequency of the dielectric relaxation corresponds to a relaxation time of $\tau_{\text{DRS}} \sim 9 \text{ ps}$ for neat water at 23 °C63. This value of $\tau_{\text{DRS}}$ can be related to the orientational correlation time $\tau_{\text{IR}} \sim 2 \text{ ps}$ observed in the IR experiments by taking into account that in the IR experiments we measure the second-order orientational correlation time (see previous paragraph), whereas DRS is sensitive to the first-order orientational correlation function64. In particular, the DRS spectrum is determined by the correlation function $\langle \mathbf{P}(t) \cdot \mathbf{P}(0) \rangle$, where $\mathbf{P}(t)$ is the total polarization of the sample (arising mainly from the rotation of the water molecules, which have a permanent electric dipole moment), and ... denotes ensemble averaging61. We find that the same 20 GHz relaxation also dominates the spectra of the organisms, with a somewhat reduced intensity (Fig. 3). Additionally, in cellular samples a low-amplitude relaxation is commonly observed at ~1 GHz (so-called $\delta$-relaxation)65, 66. This relaxation is not only due to the rotation of slow-down water molecules but also to polarization of polyelectrolytes, rotation of low-molecular weight solutes, and conformational dynamics of proteins65, 66.

To quantify the contribution of the dominant water relaxation we fit a combination of two Cole-Cole-type equations to the dielectric spectra (see Supplementary Table 3 for the fit parameters). These fits describe the spectra at frequencies ranging from 760 MHz to 70 GHz very well (solid lines in Fig. 3). The contributions of the two relaxation processes to the imaginary permittivity are shown as shaded areas in Fig. 3. From this fit we observe the dominant relaxation time $\tau_{\text{IR}}$ of the dominant water relaxation in all three organisms to be very similar to the ~9 ps relaxation time of bulk water63 ($E.\ coli: 8.4 \pm 0.2 \text{ ps}$, yeast: $9.1 \pm 0.2 \text{ ps}$, spores: $8.9 \pm 0.1 \text{ ps}$). Hence, most of the water in the investigated organisms exhibits picosecond dynamics that closely resembles the dynamics of neat water. The IR-anisotropy and DRS experiments thus give similar results, but it should be noted that these experiments probe different aspects of the water dynamics. Both experiments directly probe the random orientational motion of water molecules, whereas the IR-anisotropy experiment probes the motion of individual water molecules, DRS probes the collective motion of all water molecules, and therefore is more sensitive to collective water dynamics. Hence, the IR results demonstrate that the local orientational dynamics of cell water is similar to that of neat water, and the DRS results show that the longer-ranged, collective dynamics of cellular water also does not differ significantly from that of neat water.

**Dielectric-relaxation spectroscopy.** We also investigate the dynamics of the cell water with dielectric-relaxation spectroscopy (DRS). With DRS we probe the polarization of a sample induced by an externally applied oscillating electric field. The response is measured as a frequency-dependent complex permittivity, with the real and imaginary parts representing the in-phase and out-of-phase (absorptive) components of the induced polarization61. For pure water (dashed lines in Fig. 3) the spectrum is dominated by a dispersion in the real permittivity and a corresponding peak in the imaginary permittivity at ~20 GHz. These signatures are characteristic for a relaxation mode, that is due to random orientational motion of the dipolar water molecules62. The 20 GHz frequency of the dielectric relaxation corresponds to a relaxation time of $\tau_{\text{DRS}} \sim 9 \text{ ps}$ for neat water at 23 °C63. This value of $\tau_{\text{DRS}}$ can be related to the orientational correlation time $\tau_{\text{IR}} \sim 2 \text{ ps}$ observed in the IR experiments by taking into account that in the IR experiments we measure the second-order orientational correlation time (see previous paragraph), whereas DRS is sensitive to the first-order orientational correlation function64. In particular, the DRS spectrum is determined by the correlation function $\langle \mathbf{P}(t) \cdot \mathbf{P}(0) \rangle$, where $\mathbf{P}(t)$ is the total polarization of the sample (arising mainly from the rotation of the water molecules, which have a permanent electric dipole moment), and ... denotes ensemble averaging61. We find that the same 20 GHz relaxation also dominates the spectra of the organisms, with a somewhat reduced intensity (Fig. 3). Additionally, in cellular samples a low-amplitude relaxation is commonly observed at ~1 GHz (so-called $\delta$-relaxation)65, 66. This relaxation is not only due to the rotation of slow-down water molecules but also to polarization of polyelectrolytes, rotation of low-molecular weight solutes, and conformational dynamics of proteins65, 66.

Estimating the fractions of bulk-like and slow water. The relative amounts of bulk-like and slow water in the different samples can be estimated from the relative amplitudes of the decay and the residual in the time-dependent IR anisotropy50. Combining this information with the water-mass fraction in the samples (obtained by drying the samples completely after the experiments, and comparing their mass before and after drying), we determine the mass fractions of bulk-like water, slow water, and dry mass in each organism (blue, red, and green bars in Fig. 4). Similarly, from the DRS data we can determine the volume fraction of bulk-like water simply by determining the reduction in amplitude of the dominant water-relaxation mode in all three organisms to be very similar to the ~9 ps relaxation time of bulk water63 ($E.\ coli: 8.4 \pm 0.2 \text{ ps}$, yeast: $9.1 \pm 0.2 \text{ ps}$, spores: $8.9 \pm 0.1 \text{ ps}$). Hence, most of the water in the investigated organisms exhibits picosecond dynamics that closely resembles the dynamics of neat water. The IR-anisotropy and DRS experiments thus give similar results, but it should be noted that these experiments probe different aspects of the water dynamics. Both experiments directly probe the random orientational motion of water molecules, whereas the IR-anisotropy experiment probes the motion of individual water molecules, DRS probes the collective motion of all water molecules, and therefore is more sensitive to collective water dynamics. Hence, the IR results demonstrate that the local orientational dynamics of cell water is similar to that of neat water, and the DRS results show that the longer-ranged, collective dynamics of cellular water also does not differ significantly from that of neat water.
The extracellular part of the bulk-like fraction is indicated for the data obtained from the IR measurements by the light part of the blue bars in Fig. 4.

Discussion

When comparing the fractions of intracellular bulk-like and slow water (dark blue and red bars in Fig. 4), we see that in all three organisms most of the intracellular water is bulk-like: ~80% of the water in E. coli and yeast, and ~55% of the water in the bacterial spores. The ratio of slow water to dry mass is roughly similar in all three organisms (0.5 for E. coli, 0.4 for yeast, 0.3 for the spores). This similarity suggests that the slow water is immobilized because it solvates biomolecules and/or ions (the difference in the ratios could then be due to differences in solvent-accessible surface area per unit of dry mass). To confirm this idea we measured the water dynamics of a solution which mimics yeast cytoplasm by having the same protein-mass fraction and ionic concentrations. The dielectric-relaxation spectrum of the cytosol mimic is very similar to that of the cells (Fig. 3); the same holds for the anisotropy decay (gray data points in Fig. 2) which shows a residual anisotropy just like the cells. The ratio of slow water to dry mass obtained from the residual anisotropy is also similar to those of the cells (Fig. 4). In contrast, in a solution containing only the ionic species of the cytosol mimic and no protein, the residual anisotropy is reduced by a factor of ~3 (see Supplementary Fig. 1). These findings indicate that a large part of the slow intracellular water is in the hydration shells of proteins or buried inside them (our measurements cannot distinguish these two types of slow water, but NMR shows that the buried fraction is very small). Similar slowing down occurs in the hydration shells of other solutes such as osmolytes, DNA, and phospholipid. The slowing down of the orientational dynamics of water molecules associated with ions and biomolecules is a well-known effect, and is mainly due to hydrogen bonding, electrostatics, and confinement effects. These same short-range interactions can cause templating of water by certain proteins and charged planar membranes, an effect that is however limited to sub-nanometer length scales.

The most conspicuous difference between the water fractions in the different species is the lower fraction of bulk-like water in the spores. Bacterial spores can survive extreme conditions (heat, toxic chemicals, drought) for very long periods by effectively “shutting down” their biochemistry. To explain how this happens, it has been proposed that in the core of spores (which contains very little water) water might be in a glass-like state. However, NMR measurements indicate that this is not
resolved infrared pump-probe experiments were done using a setup described previously. Pump-beam scattering by the samples was eliminated by delaying every second pump pulse by half an optical cycle using a photo-electric modulator and averaging the signals at these two pump-probe delays. The thermal contribution to the pump-probe signals was taken into account in the data analysis using a procedure described previously.

Complex dielectric spectra of the samples were recorded in the frequency range from 0.76 to 70 GHz with a coaxial reflectometer based on an Anritsu Vector Star MS4647A vector network analyser with an open ended coaxial probe based on an 1.85 nm coaxial connector. To calibrate the instrument for directivity, frequency response, and source-match errors we used water, air, and conductive silver paste as calibration standards. The organisms were measured by putting small amounts of the sample on the probe head until the response plateaued upon addition of additional sample volume. The spectra of the cytosol mimic were measured by immersing the probe in the solution. Error bars were obtained from at least three reproduced experiments.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request.

Received: 20 April 2017 Accepted: 1 August 2017
Published online: 12 October 2017

References


Acknowledgements
We would like to thank Michiel Hilbers for taking the microscope pictures.

Author contributions
G.S., D.B., M.B., and S.W. conceived the experiments, L.Z. and M.T. prepared the samples, M.T. carried out the experiments, S.W. supervised the vibrational-spectroscopy experiments, and data analysis. J.H. supervised the dielectric-relaxation experiments and data analysis, G.S. supervised the biological and biochemical procedures, and analysis. All authors contributed to writing the manuscript.

Additional information
Supplementary Information accompanies this paper at 10.1038/s41467-017-00858-0.

Competing interests: The authors declare no competing financial interests.

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