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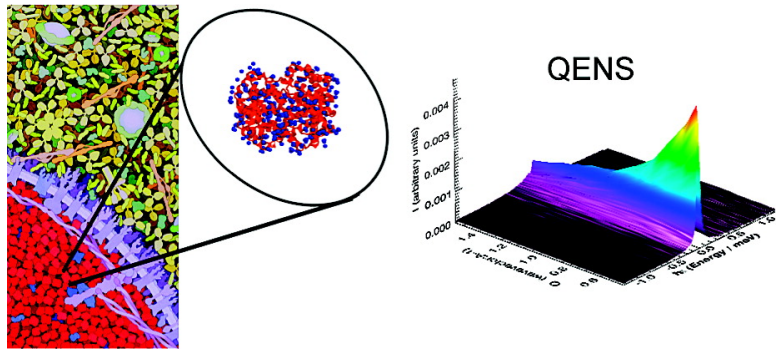

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Cytoplasmic Water and Hydration Layer Dynamics in Human Red Blood Cells

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Water is essential to life, and a major scientific interest lies in a detailed understanding of how it interacts with biological macromolecules in cells. The cellular environment is extremely crowded with macromolecular concentrations up to 400 mg/mL.[1] Distances between macromolecules are on the order of 1 nm, which corresponds to only a few layers of water. Water that is in close contact with hydrophilic or hydrophobic protein surfaces[2] or which is trapped in surface cavities[3] is shown to have significantly different behavior than bulk water. Recent studies point out that a major fraction of water in cells and bacteria shows bulk-like dynamics.[4,5] We measured quasielastic incoherent neutron scattering (QENS) of H_2O in red blood cells (RBC), *in vivo*. The data revealed two populations of water in RBC: a major fraction of ~90% which has translational properties similar to those of bulk water (time scale ~ps) and a minor fraction of ~10% which is interpreted as bound hydration water with significantly slower dynamics (time scale ~40 ps).

QENS is a well-suited technique to study water dynamics in the ps−ns and Å time and length scale, respectively. Incoherent neutron scattering predominantly detects the motion of hydrogen nuclei, which have an incoherent scattering cross section which is more than 40 times larger than all other elements that occur in biological matter, and deuterium.[6] By subtracting experimental data of natural abundance RBC in D_2O buffer from natural abundance RBC in H_2O buffer, the cytoplasmic dynamics of H_2O was separated from membrane and macromolecular dynamics. RBC consist mostly of H_2O and hemoglobin at a concentration of ~330 mg/mL.[7] Samples of human venous blood were prepared in H_2O and D_2O HEPES buffer (see Supporting Information). Extracellular water content was found to be less than 10% of the total water content. We estimate that the incoherent cross section of the intracellular H_2O is more than three times that of the macromolecules. In D_2O, the incoherent cross section of water is 8% that of the macromolecules. The difference is therefore dominated by the water signal, and we neglected changes in membrane and macromolecular dynamics due to isotope effects. Energy resolved neutron scattering was measured on the spectrometers IRIS at ISIS, FOCUS at PSI and TOFTOF at FRM-II. A neutron spectrometer is characterized by its energy resolution and scattering vector (q) range, which determine the observable time and length scales of hydrogen motions. The energy resolution of the instruments IRIS, FOCUS, and TOFTOF was 17, 50, and 100 μeV, respectively, corresponding to longest observable time scales on the order of 40, 13. and 7 ps. The theoretical scattering function of water dynamics is well described by a narrow and a broad Lorentzian for translational and rotational diffusion and a delta function for the fraction of slow hydrogen motions that appear localized on the time scale corresponding to the energy resolution of the instrument.[8] The dependence of the half widths at half-maximum (HWHM) of the narrow Lorentzian as a function of q yields information about the atomic scale translational diffusion process[8] (see Supporting Information).

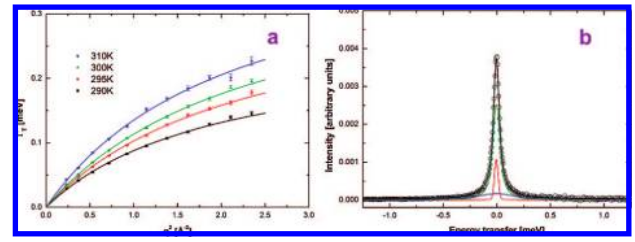

Figure 1. (a) Half widths at half-maximum of the translational diffusion process of cytoplasmic H_2O in RBC measured on IRIS at different temperatures. Solid lines represent fits according to a jump-diffusion model. (b) QENS spectrum on FOCUS of cytoplasmic H_2O in RBC at 290 K and $q = 0.55$ Å^{-1}. Symbols show measured data; the black line presents the result of the fit. The components correspond to the immobile fraction (red line), the narrow Lorentzian (green line), and the broad Lorentzian (blue line).

The HWHM of cytoplasmic H_2O measured on IRIS are shown with fits according to a jump-diffusion model in Figure 1a; in Figure 1b a measured spectrum on FOCUS together with the fitted components is given.

Figure 2a reports the obtained translational diffusion coefficients D of H_2O buffer and cytoplasmic H_2O as a function of temperature measured with IRIS, FOCUS, and TOFTOF. The data demonstrate that the translational diffusion coefficient of cellular water is nearly identical to that for H_2O buffer on all spectrometers. The dashed line indicates normal temperature behavior of H_2O buffer following an Arrhenius law and serves as a reference. The measured diffusion coefficients of intracellular water with the instrument IRIS show perfect agreement with the reference; the results of TOFTOF and FOCUS deviate only slightly above and below, respectively. Residence times were found to be larger in intracellular than in bulk water (see Supporting Information). The results indicate that translational diffusion of water in RBC behaves similarly to that of bulk water with increased residence times. A QENS study on translational and rotational diffusion of water in *Escherichia coli* concluded that the main fraction of intracellular water shows similar dynamic behavior as bulk water with residence times twice as long.[4] NMR work on rotational diffusion of cytoplasmic water found that ~85% of cell water in *Escherichia coli* has bulk-like dynamics.[5]

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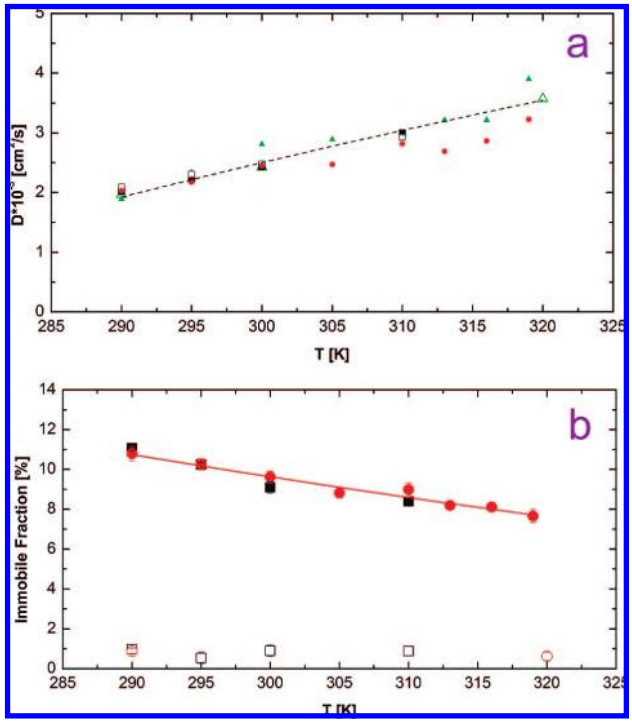

Figure 2. (a) Translational diffusion coefficient D of H_2O in RBC measured on the instruments IRIS (filled black squares), FOCUS (filled red circles), and TOFTOF (filled green triangles); H_2O buffer was measured on the instruments IRIS (empty black squares) and TOFTOF (empty green triangles). The dashed line indicates normal temperature behavior of H_2O buffer. (b) Percentage of immobile fraction of H_2O in RBC measured on IRIS at $q = 0.61$ Å^{-1} (filled black squares) and FOCUS at $q = 0.55$ Å^{-1} (filled red circles) are compared to values of H_2O buffer measured on IRIS (empty black squares) and FOCUS (empty red circles). The straight red line represents Arrhenius behavior.

We could identify a significant fraction of immobile water in RBC that is absent in H_2O buffer. The immobile water fraction was determined by the amplitude of the elastic peak divided by the integrated total intensity. Experimental data are presented in Figure 2b for the smallest accessible scattering vector of $q \approx 0.6$ Å^{-1} which reports on all movements up to an ~10 Å real space distance. On this length scale confining effects of protein surface cavities or boundaries on water become observable. From the q dependence of the elastic intensity we estimated root-mean-square displacements $\langle u^2 \rangle^{1/2}$ of ~2.3 and ~2.1 Å for IRIS and FOCUS, respectively, which are smaller than the mean distance between water molecules (~3 Å) and emphasize the localized nature of immobile water. Immobile water correlation times need to be longer than ~40 ps which is the time resolution of the high resolution spectrometer IRIS.

The obtained immobile water fraction of the RBC sample varies between 11% and 8% in the investigated temperature range. The uncertainty due to background subtraction was estimated to be below 2%. In contrast, the immobile fraction of H_2O buffer is below 1% at all temperatures. The temperature dependence of the immobile fraction of H_2O in RBC follows Arrhenius behavior with an activation energy of $E_a = 2.15 \pm 0.10$ kcal/mol.

Hydrodynamic calculations of hemoglobin needed to include a bound water coverage of ~50% of the first hydration layer to predict quantitatively experimentally measured hydrodynamic parameters.[9] We estimated that ~2300 water molecules are in the first hydration layer of hemoglobin (see Supporting Information). A study with High-Frequency Dielectric Spectroscopy (between 45 MHz-20 GHz) reported a value of 1030 ± 70 dynamically bound H_2O molecules on the surface of hemoglobin with highly restricted rotational dynamics[10] corresponding to an ~50% occupation of the first hydration layer. Site-directed tryptophan mutations combined with femtosecond spectroscopy identified two water populations in the protein hydration layer with fast (~1−8 ps) and slow (~20−200 ps) dynamics.[11] Assuming a cellular hemoglobin concentration of ~330 mg/mL, ~28% of all cellular water molecules are in direct contact with the surface of hemoglobin. Considering half of the first hydration layer to be bound, roughly 14% of cytoplasmic water would be bound in the protein hydration layer. This value is remarkably close to our results of the immobile water fraction. We therefore conclude that there exist two populations of water in RBC: a major fraction of ~90% which has translational properties similar to those of bulk water and a fraction of ~10% of immobile water which could be identified as dynamically bound water in the hydration layer of hemoglobin, *in vivo*. This bound hydration water should have important properties concerning protein stability and interactions in living cells.

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Supporting Information Available: Details on sample preparation protocols, neutron scattering experiments, data analysis, and surface area calculations. This material is free of charge via the Internet at http://pubs.acs.org.

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