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A facile oxygen-17 NMR method to determine effective viscosity in dilute, molecularly crowded and confined aqueous media†

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We present an NMR method based on natural abundance ^{17}O relaxation of water to determine effective viscosity in biological aqueous samples. The method accurately captures viscosity of dilute and crowded protein solutions and offers a fairly simple way to quantify the internal fluidity of biological condensates formed through phase separation.

Life relies on the spatiotemporal arrangement and coordination of the components and activities of living systems.[1] In cells, the spatial arrangement is provided either through compartmentalization *via* membranous organelles, or alternatively, through a process termed liquid–liquid phase separation (LLPS) which leads to the formation of membrane-less bodies in cells.[2] Physical properties of the cellular bodies formed after LLPS exhibit remarkable diversity, ranging from liquid-like droplets to gel-like phases with different degrees of confinement.[3] Different micro-rheological and biophysical techniques based on fusion timescale or diffusion analysis are utilized to probe the viscoelastic properties of biological condensates.[4,5] Further progress in characterization of biological phase separation requires developments in experimental methods probing the highly crowded and confined environments inside biological condensates.[6]

Temporal coordination of biological activities depends on the characteristic times of various motional modes of biomolecules,[1] influenced potentially by local concentration and the crowding effect.[7] Protein dynamics generally occur at multiple time and length scales, which are best characterized through combination of several experimental techniques.[8,9] Due to differences in the inherent sensitivity of various experimental techniques, protein samples at different concentrations are often used for protein dynamical studies. It is therefore essential to quantify pure viscosity effects on protein dynamics before any reasonable comparison of experimental data obtained at different protein concentrations can be made.

It has been suggested that water dynamics could be used as a reliable proxy for the local viscosity.[10] One of the best methods to study water dynamics is ^{17}O NMR.[11] ^{17}O nucleus has spin quantum number I of 5/2 and natural abundance of 0.037%. The dominant NMR relaxation mechanism of ^{17}O is the anisotropic interaction between the quadrupole moment (Q) of ^{17}O and the electric field gradient present at the site of ^{17}O nuclei.[12] Here, we utilize ^{17}O longitudinal relaxation (R_1) rate of water, essentially a probe of water structure and dynamics, to determine effective viscosity in biological aqueous samples behaving as Newtonian fluids. Effective viscosity is a local microscopic property of solutions influencing Brownian diffusion of solutes. In non-homogeneous (crowded) solutions, the effective viscosity experienced by the rotationally diffusing solutes may be significantly different from the bulk viscosity.[13]

First, we investigated the viscosity dependence of ^{17}O R_1 rate of water in glycerol–water mixtures, for which the solution viscosities are well known[14] and diffusion obeys the classical Stokes laws.[13] The ^{17}O NMR experiments were performed for samples containing 0 to 34% (v/v) glycerol, in which the solution viscosity at 298 K varied from 0.89 to 3.01 cP. In 1D ^{17}O spectra, the intensity of water signal changed in proportion to water content (Fig. S1, ESI†). In addition, the signal linewidths rose from 46.5 ± 0.1 Hz at 0% glycerol to 101.8 ± 0.3 Hz at 34% glycerol, reflecting the increase in viscosity and its resultant signal broadening (Fig. 1a). Similarly, the ^{17}O R_1 rates followed an increasing trend from 145 ± 4 s^{-1} at 0% glycerol to 323 ± 9 s^{-1} at 34% glycerol (Fig. 1b and Fig. S2, ESI†). The value obtained at 0% glycerol is in excellent agreement with previous reports,[15] and implicates a rotational correlation time (τ_{rot}) of 1.54 ± 0.04 ps for water molecules in accord with earlier studies.[15–17]

Besides increasing viscosity and its resultant effect on τ_{rot} of water molecules, addition of glycerol may alter the hydrogen-bonded network structure of water and thereby modulate the quadrupole coupling constant (χ) and asymmetry (η) of water ^{17}O nuclei. To our knowledge, there is no report in the literature concerning the quadrupole parameters of water ^{17}O in glycerol–water mixtures, except in the frozen state, where ^{17}O parameters of water are not

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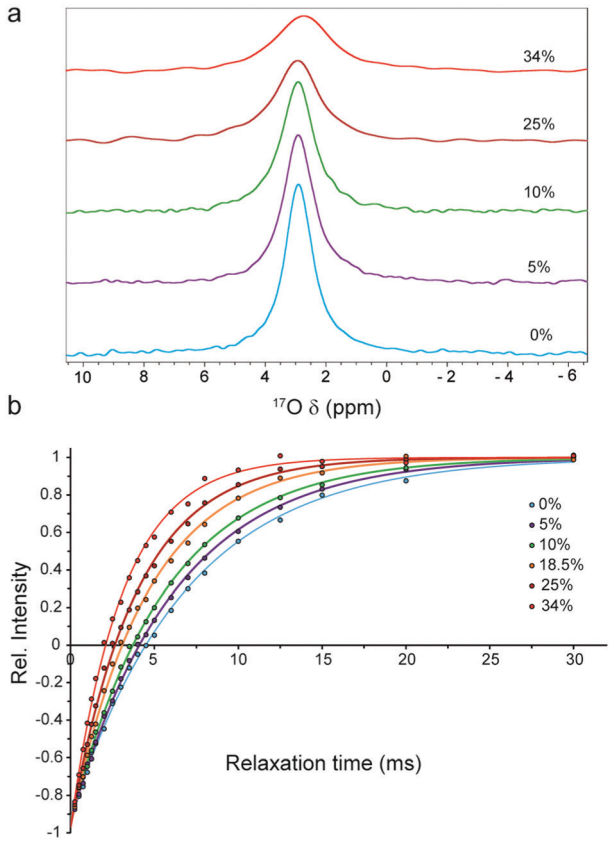

Fig. 1 ^{17}O NMR relaxation of water in water–glycerol mixtures, in dependence of glycerol concentration (v/v, %). (a) 1D ^{17}O spectra, showing glycerol concentration-dependent broadening of water ^{17}O signals. (b) ^{17}O longitudinal relaxation rate (R_1) of water, measured through inversion-recovery experiments. Relative intensities of water ^{17}O signals are shown as a function of recovery time. Faster recovery indicates larger R_1 rates.

significantly affected by 60% glycerol.[18] Thus, it has been shown that the quadrupole coupling factor, $\chi^2(1 + \eta^2/3)$, of water ^{17}O changes only minimally over a broad range of temperatures from 274 to 350 K (*ca.* 6%), where the hydrogen bond network of water undergoes significant alterations.[16] Consequently, we assume that the change in quadrupole coupling factor of water ^{17}O over the studied range of glycerol concentrations remains negligibly small for the purpose of current study. Based on this assumption, the τ_{rot} of water increases to ~1.7, 1.9, 2.3, 2.7 and 3.4 ps, respectively at 5, 10, 18.5, 25 and 34% glycerol concentration. It is interesting to note that the change in water dynamics upon addition of glycerol is less prominent when compared to solution viscosity (Fig. S3, ESI†). Contrary to the τ_{rot} of water molecules which is supposedly linearly related to ^{17}O R_1 rates, the solution viscosity *versus* ^{17}O R_1 data showed better fit to a quadratic function when compared to a linear function (p-value < 0.001). The obtained empirical function was:

$$\eta\ (\text{cp}) = 1.719\ (\pm 0.194) \times 10^{-5} \times (R_1)^2 + 0.004\ (\pm 0.001) \times R_1 - 0.065\ (\pm 0.017) \quad (1)$$

which can be used in determination of viscosity in aqueous solutions at 298 K. Similar quadratic relations were observed at 310 K, and to a less degree, at 288 K (Fig. S4, ESI†).

Next, the temperature dependence of ^{17}O R_1 rate of water was studied in pure water, for which the temperature dependence of viscosity is well established.[19] Upon a decrease from 310 K to 275.4 K, the ^{17}O R_1 rate increased from 107 ± 6 s^{-1} to 385 ± 14 s^{-1} (Fig. S5a, ESI†). The increase in ^{17}O R_1 rates upon cooling is expected because of the longer τ_{rot} of water at lower temperatures. This effect is however partially counteracted by the small albeit considerable drop in ^{17}O quadrupole coupling factor of water at low temperatures.[16] Taking the temperature dependence of the quadrupole coupling factor into consideration, the τ_{rot} of water changes from 1.12 ± 0.06 ps at 310 K to 4.16 ± 0.16 ps at 275.4 K. The relation between viscosity/temperature (η/T) ratio and ^{17}O R_1 rates deviated from linearity and was better represented by a quadratic function (p-value < 0.001, Fig. S5b, ESI†). The observed deviations from linearity, both in water–glycerol mixtures and in pure water over the studied temperature range, suggest that the effect of perturbation on solution viscosity cannot be well approximated to a first-order change in the dynamics of individual water molecules.

With the viscosity and temperature dependence of ^{17}O R_1 rate of water established, we employed ^{17}O R_1 rates to evaluate effective viscosity (henceforth viscosity) in a number of test cases. First, we investigated aqueous solutions containing high protein concentration, such as 4 mM ubiquitin and 3 mM GB3. At 298 K, the ^{17}O R_1 rates were 164 ± 3 and 153 ± 4 s^{-1}, respectively, which using eqn (1), corresponded to viscosities of 1.07 ± 0.03 and 0.96 ± 0.03 cP. The $\eta_{protein}/\eta_{water}$ ratio was ~1.20 ± 0.03 for 4 mM ubiquitin and 1.08 ± 0.04 for 3 mM GB3 solutions, in close agreement with the viscosity ratios of ~1.17 and 1.08 predicted on the basis of volume occupancies (see ESI†). Then, the rotational correlation time (τ_c) of ubiquitin and GB3 were determined through ^{15}N cross-correlated relaxation (CCR) rates. At 4 mM concentration, the average CCR rate of ubiquitin was 4.83 ± 0.05 s^{-1}, corresponding to a τ_c of 4.82 ± 0.04 ns. Compared to the τ_c of ubiquitin at much lower concentration of 0.37 mM (see below, also ref. 20), the τ_c of ubiquitin at 4 mM concentration exhibited an increase by a factor of 1.20 ± 0.02. The increase in τ_c of ubiquitin closely matches the observed viscosity ratio obtained above. This finding is interesting as it rules out significant dimerization of ubiquitin at such a high concentration, as previously suggested.[21] For GB3, the average CCR rate was 3.82 ± 0.03 s^{-1}, corresponding to a τ_c of 3.47 ± 0.04 ns (Fig. S6, ESI†), slightly larger than the value of 3.35 ± 0.03 ns reported at 2 mM GB3 concentration.[22] Considering the expected 8% change in viscosity, the τ_c of GB3 at infinite dilution would be ~3.23 ns, implying a rather small atomic effective radius (AER) value of ~2.5 Å for hydrodynamic calculations. Taken together, the ^{17}O R_1 measurement of bulk water allowed quantification of viscosity changes at high-concentration protein samples and enabled estimating alterations in the τ_c of proteins.

Second, we studied molecularly crowded aqueous solutions mimicking the environment of interior of cells.[7] To address how crowding agents alter the solution viscosity, we examined two commonly used neutral polymeric crowding agents: Ficoll 70 and sucrose. At 200 g L^{-1} concentration of Ficoll 70, the ^{17}O R_1 rate of water was 247 ± 5 s^{-1}, which using eqn (1), corresponded

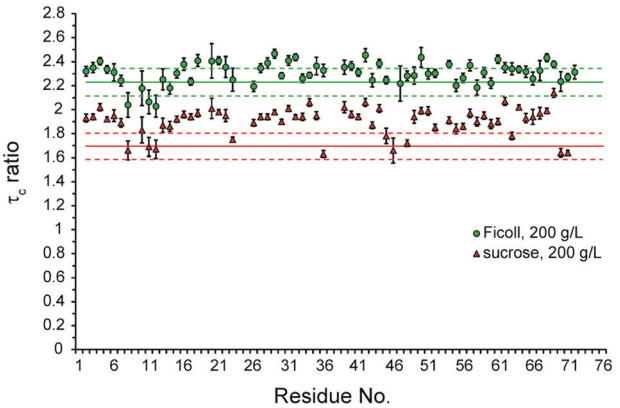

Fig. 2 Solution viscosity in crowded media, as probed by ^{17}O longitudinal relaxation rate (R_1) of water and rotational correlation time (τ_c) of ubiquitin. Residue-specific τ_c of ubiquitin in dilute and crowded solutions are reported as the ratio of $\tau_{c,crowded}/\tau_{c,dilute}$. The corresponding viscosity ratios obtained through ^{17}O R_1 of water are shown as solid (average) and dashed (±SD) lines.

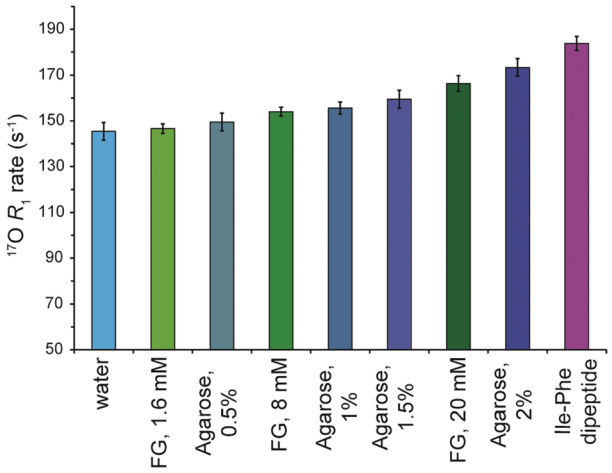

Fig. 3 Water dynamics in the confined media of biological hydrogels, as probed by ^{17}O longitudinal relaxation rate (R_1) of water. ^{17}O R_1 of water in agarose, Ile-Phe and FG peptide-based hydrogels are compared.

to a viscosity of 2.00 ± 0.07 cP and the $\eta_{Ficoll}/\eta_{water}$ ratio of 2.23 ± 0.12 (Fig. 2 and Fig. S7, ESI†). Similarly, the ^{17}O R_1 rate of water at 200 g L^{-1} sucrose was 207 ± 7 s^{-1}, corresponding to the viscosity of 1.52 ± 0.08 cP and $\eta_{sucrose}/\eta_{water}$ ratio of 1.70 ± 0.11. Assuming that the quadrupole parameters of water ^{17}O remained effectively unperturbed, the τ_{rot} of water molecules were 2.62 ± 0.04 ps in Ficoll 70 and 2.19 ± 0.05 ps in sucrose solutions. To further evaluate the ^{17}O R_1-based viscosities, we employed ^{15}N relaxation rates R_1 and R_2, as reported in ref. 20, and determined τ_c of the ^{15}N-labelled ubiquitin in dilute and crowded samples. The τ_c of ubiquitin calculated from residue-specific ^{15}N R_2/R_1 ratios were 3.98 ± 0.30 ns in dilute solution, 9.06 ± 0.84 ns in 200 g L^{-1} Ficoll 70 and 7.50 ± 0.69 ns in 200 g L^{-1} sucrose (Fig. S8, ESI†). Interestingly, the $\tau_{c,Ficoll}/\tau_{c,water}$ of 2.31 ± 0.05 was in agreement with the viscosity ratio of 2.23 ± 0.12 obtained through ^{17}O R_1 rates (Fig. 2). The $\tau_{c,sucrose}/\tau_{c,water}$ of 1.88 ± 0.04 was however slightly larger than the ^{17}O R_1-based viscosity ratio of 1.70 ± 0.11. Overall, the method based on ^{17}O R_1 rates of water provided a fairly accurate value of viscosity in crowded solutions.

Third, we examined confined aqueous media such as biological hydrogels. To investigate whether the ^{17}O R_1 of water could be used as a proxy to monitor the confinement level inside biological hydrogels, we first examined gels formed at different agarose concentrations. The ^{17}O R_1 rate of water increased from 150 ± 4 s^{-1} at 0.5% agarose gel to 156 ± 3, 160 ± 4 and 173 ± 4 s^{-1}, respectively, at 1%, 1.5% and 2% agarose gels (Fig. S9, ESI†). The τ_{rot} of water molecules is expected to undergo a similar rising trend from 1.59 ± 0.04 ps to 1.65 ± 0.03, 1.69 ± 0.04 and 1.84 ± 0.04 ps, in accord with the increasing level of confinement in dependence of agarose concentration.

Subsequently, we examined a thermo-responsive Ile-Phe dipeptide-based hydrogel.[23] Interestingly, the ^{17}O R_1 rate of water in the Ile-Phe gel was 184 ± 3 s^{-1}, even bigger than that of 2% agarose (Fig. 3). The large ^{17}O R_1 of water in Ile-Phe hydrogel may have its origin in the large level of confinement, or alternatively, be caused by the presence of residual alcohol (HFiP) within the gel and its impact on local viscosity.

Finally, we measured ^{17}O R_1 of water in a gel-forming FG-based peptide, part of the nuclear pore complex.[24] At 1.6 mM peptide concentration, the ^{17}O R_1 rate of water in the formed hydrogel was 147 ± 2 s^{-1}, between the values observed in water and in 0.5% agarose gel. The ^{17}O R_1 rate of water exhibited a concentration-dependent increase to 154 ± 2 s^{-1} at 8 mM and 166 ± 3 s^{-1} at ~ 20 mM FG peptide concentration (Fig. 3). The corresponding τ_{rot} of water were 1.55 ± 0.02, 1.63 ± 0.02 and 1.76 ± 0.04 ps, reflecting slight restriction of water dynamics within the hydrogel in dependence of FG peptide concentration. The level of mobility restriction in 1.6, 8 and 20 mM FG peptide concentration hydrogels is similar to <0.5%, 1% and 1.5–2% agarose gels, respectively. Taken together, the ^{17}O R_1 of water was capable of capturing differences in the confinement level of agarose and peptide-based hydrogels.

The ^{17}O R_1-based method presented above offers a simple and quick way for determination of viscosity in biological aqueous samples. Despite the very low natural abundance of ^{17}O and its low gyromagnetic ratio, the presented method is not particularly demanding in terms of sample requirements or magnetic fields. At 9.4 T magnetic field corresponding to proton Larmor frequency of 400.13 MHz, the ^{17}O R_1 experiment of a typical sample required 3–4 hours of measurement. This is because of the high concentration of water and fairly high mobility of water molecules, even in environments as confined as 2% agarose gel studied above. Compared to the alternative methods of viscosity determination in crowded cellular or confined media, such as NMR line width analysis,[25] or ^{19}F NMR- or fluorescence-based analysis of rotational and translational diffusion,[5,26] the method based on natural abundance ^{17}O relaxation of water is advantageous as it does not require additional labelling or external probes, and is relatively immune to transient protein–protein interactions ubiquitous in cellular media.

The ^{17}O relaxation rate of water is sensitive not only to the τ_{rot} of water molecules, but also to the quadrupole coupling factor of $H_2{}^{17}O$.[16] As a result, the use of ^{17}O R_1 rates of water in viscosity determination is in principle limited to the cases in

which changes in water structure and consequently in the quadrupole coupling factor of $H_2{}^{17}O$ are negligibly small. Theoretical and experimental investigations suggest that the above condition is indeed not very stringent and can be met over a wide range of temperatures and possible hydrogen-bonded network structures of water.[16] Another potential limitation arises when ^{17}O relaxation of water is altered by paramagnetic relaxation effects. Consequently, the presented method cannot be applied to samples in which the presence of a paramagnetic component (such as Cu^{2+} or Mn^{2+}) cannot be avoided. Moreover, to eliminate unwanted paramagnetic ions and dissolved molecular oxygen, it is essential to use deionized water and degas the samples before NMR experiments.

NMR spin relaxation is a powerful technique to probe protein dynamics at atomistic resolution. NMR-based dynamical studies are frequently performed at high protein concentrations, where concentration-dependent changes in viscosity influence the timescale of different motional modes of proteins. In particular, low-field proton relaxometry, a powerful technique monitoring slow reorientational dynamics in disordered proteins,[9] requires millimolar protein concentrations. As exemplified by the ubiquitin and GB3 data above, the viscosity determination through ^{17}O R_1 of water enables direct evaluation of the viscosity effects on protein dynamics and allows disentangling pure viscosity effects from potential concentration-dependent phenomena such as protein self-association events. In addition, NMR experiments in non-standard conditions such as crowded media, supercooled temperatures and high hydrostatic pressures can benefit from direct viscosity determination.[27]

The traffic between cell nucleus and cytoplasm is controlled *via* gel forming FG-rich domains of nucleoporins.[24] For the FG peptide used in this study, the hydrogel formed at low peptide concentration showed ^{17}O R_1 of water only slightly larger than the bulk water, suggesting that the dynamics of water within the channels of the FG hydrogel were largely unaffected. On the other hand, the ^{17}O R_1 of water in the high-concentration FG hydrogel was comparable to 1.5–2% agarose gel, indicating a considerable level of water mobility restriction. We suggest that the ^{17}O R_1 of water can be used as a proxy to monitor changes in the local environment of biological hydrogels.

In summary, a facile ^{17}O NMR method to determine effective viscosity in biological samples is presented. The method captures effective viscosity in dependence of protein concentration and crowding, and is capable of reporting the confinement level in biological hydrogels, therefore has the potential to be utilized in quantitative characterization of biological condensates in the rapidly growing fields of phase separation and peptide-based pharmaceutical hydrogels.

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Conflicts of interest

There are no conflicts to declare.

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