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Cryobiology 46 (2003) 271–276

CRYOBIOLOGY

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Freezing stresses and hydration of isolated cell walls☆

Yonghyeon Yoon, Jim Pope,[1] and Joe Wolfe*

School of Physics, University of New South Wales, Sydney 2052, Australia

Received 13 February 2003; accepted 22 April 2003

Abstract

The hydration of the cell walls of the giant alga *Chara australis* was measured as a function of temperature using quantitative deuterium nuclear magnetic resonance (NMR) of samples hydrated with D_2O. At temperatures 23–5 K below freezing, the hydration ratio (the ratio of mass of unfrozen water in microscopic phases in the cell wall to the dry mass) increases slowly with increasing temperature from about 0.2 to 0.4. It then rises rapidly with temperature in the few Kelvin below the freezing temperature. The linewidth of the NMR signal varies approximately linearly with the reciprocal of the hydration ratio, and with the freezing point depression or water potential. These empirical relations may be useful in estimating cell wall water contents in heterogeneous samples.

Keywords: Cell walls; Hydration; Freezing

How does the equilibrium water content of a cell wall vary with temperature in the presence of ice? Apart from its intrinsic interest, this question has practical importance for understanding some aspects of the behaviour of organisms with cell walls in response to freezing or to dehydration. In the presence of ice, cell walls may be dehydrated as the temperature falls. Water leaving the cell wall can then freeze, but the liberation of its latent heat slows the freezing rate [2]. Further, the mechanical deformation of the cell wall is likely to depend upon the extent of water loss. At sufficiently low temperatures, the water of hydration in cell walls (where present) also contributes to what is often[2] described as the 'unfreezable water' in a biological sample [16].

This paper answers this question using the freezing stress technique (FST), in which quantitative nuclear magnetic resonance (NMR) is used to measure the quantity of unfrozen water that is retained in colloidal phases [17,20]. Obtaining macroscopic, reasonably pure, but chemically and mechanically undamaged samples of cell wall is difficult for most species. For this reason, walls of

☆ Funded by the University of New South Wales.

* Corresponding author. Fax: +61-2-93854954.

E-mail address: J.Wolfe@unsw.edu.au (J. Wolfe).

[1] Current address: School of Physical and Chemical Sciences, Queensland University of Technology, Brisbane, Australia.

[2] We recommend against the use of the term 'unfreezable water,' because the quantity of water that does not freeze at a particular temperature may depend on the ingenuity and patience of the investigator.

0011-2240/03/$ - see front matter
doi:10.1016/S0011-2240(03)00043-9

the giant alga *Chara australis* were used in this study. Although *Chara* are found in a range of climates, even the freezing of the surface water usually does not expose these benthic organisms to freezing conditions [18]. The freezing sensitivity of this species did not influence our choice, which was made purely because of the possibility of obtaining almost uncontaminated cell wall samples. The *Chara* wall is intended as a model of cell walls with comparable composition.

The cell walls of *Chara* and other algae are reported to be composed of cellulose, non-methyl-esterified pectin, and hemicellulosic polysaccharides [1,6]. The cell walls have porous cellulose-based structure and ions can move into and out of walls. The walls also have fixed anionic charges, which are thought to arise mainly from the ionisation of carboxyl groups on pectinic acids present [4]. Dainty and Hope [3] suggested that the cell wall is an array of charged pores containing ions and water.

Fig. 1a shows a cartoon of the equilibrium between water in a fibrous phase and bulk water or ice. Above freezing temperature and in a saturated atmosphere, a bulk water phase can exist. (By bulk phase, we mean a phase whose dimensions are rather larger than the range of the colloidal forces between fibre surfaces.) If the fibrous phase is equilibrated with bulk water and/or a saturated atmosphere, we say that it is fully hydrated. The chemical potential of the water μ_w in the fibrous phase is then just the standard chemical potential of water μ_w^0.

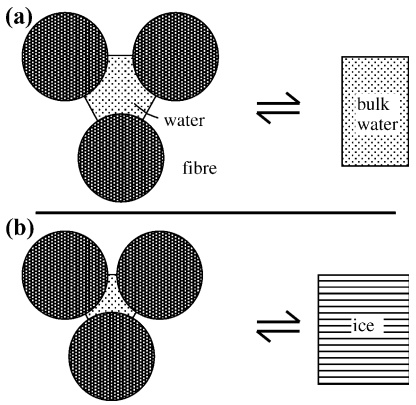

Fig. 1. Cartoon diagram of the phase coexistence between water in a fibrous phase and a bulk phase. The fibres are represented by long cylinders, shown here in section. (a) Water in the fully hydrated fibrous phase equilibrating with a bulk phase of pure liquid water at atmospheric pressure. (b) Equilibration between water in the colloidal, fibrous phase and ice. In each case a triangle has been drawn between the centres of the fibres. If the fibres formed a regular hexagonal array, this triangle would be repeated throughout the sample.

At sub-freezing temperatures, the chemical potential of ice is less than that of pure water by an amount that increases almost linearly with temperature. So, in the presence of ice and at sufficiently low temperature, water can leave the fibrous phase and freeze. This lowers μ_w in two ways. First, any solutes present in that phase become concentrated, increasing the osmotic pressure. Second, and more importantly, the hydrostatic pressure may fall below that in the bulk or reference phase. Suction in this phase can be supported by the fibres if the magnitude of the repulsive forces between them exceeds that of the attractive forces, which condition requires a smaller average distance between fibres (Fig. 1b). (Some authors combine subsets of the hydrostatic pressure and the osmotic pressure in a quantity called the matric potential. This is discussed by Passioura [8], who recommends against the practice.)

In this study, deuterated water D_2O is used to rinse and to hydrate cell walls. The deuterium NMR signal is used to quantify the fraction of unfrozen water.

Materials and methods

The *Chara* cells used in this study (*Chara australis*) were donated by Dr. Mary Beilby (Biophysics, UNSW). They were collected in rivers in South Eastern Australia and grown in tanks. Cell wall samples were prepared by removing the contents of the cell by squeezing them against a hard flat surface using the cylindrical surface on the back of a pair of tweezers. It is likely that this process does not remove the plasma membrane, but the wall thickness is much larger than that of the plasma membrane, so this contamination is minor. The chloroplasts are clearly removed because the final product is transparent. The chloroplasts in Chara form a layer at the perimeter of

the cell, so removal of the chloroplasts implies removal of the material lying inside the chloroplast layer. Thus, because of the large size of the cells, the contamination is small. The cell walls were then rinsed in excess D_2O and dried in open air. The dry cell walls were then rehydrated with excess D_2O and dried again in open air. The cell walls were further dried in the oven at 60 °C for several hours before the NMR sample preparation. This admittedly harsh treatment was considered necessary to remove all H_2O from the walls. The dried *Chara* cell walls were immediately transferred to pre-weighed NMR tubes (5 mm diameter, ~5 cm long) to minimise the adsorption of H_2O from the ambient atmosphere. The NMR tubes were then re-weighed and the amount of dry *Chara* cell wall was calculated. An appropriate amount of D_2O was added to the tube to give the desired the hydration level. The NMR tube was temporarily sealed with Parafilm. The sample end where the *Chara* cell wall and D_2O were located was frozen in liquid nitrogen. Then the other end of the tube was flame sealed to achieve the desired NMR sample size (~2 cm long) while keeping the sample end still in a frozen state. Four different samples were prepared with four different hydrations.

The quantitative NMR measurements were made on a Bruker MSL 200 spectrometer operating at 30.720 MHz. Temperature was controlled with a precision of 0.1 K by passing evaporated liquid nitrogen at controlled temperature around the sample tube, using a specially modified NMR probe. The sample was centred in an insulating jacket and the stream of cold gas passed between the sample and the jacket, over a thermocouple and then around the outside of the jacket, around which was wound the radio-frequency (RF) coil [19]. The solenoidal RF coil itself was wound from copper ribbon to improve dissipation of heat generated in it or conducted along the leads and a short sample was used to ensure temperature homogeneity. The spectral width employed was 10 kHz. A typical $\pi/2$ pulse length was ~8 μs, and typically 64–256 4 kilobyte samples were acquired.

Samples were cooled to a temperature of 253 K to initiate freezing. The hydration of the *Chara* cell wall was measured with increasing temperature and with equilibration at each temperature. In most cases, 20 min of equilibration per 1 K increase in temperature was sufficient to ensure that the signal amplitude did not change measurably with time. At some temperatures, the signal was monitored for several hours following this equilibration, and no further changes were observed. The process is described in more detail by Yan et al. [19] and Yoon et al. [20].

The temperature dependence of the gain of the induction coil and amplifiers was calibrated by measuring the total signal as a function of temperature in samples that do not freeze over the range of the experiment. One calibration used per-deuterated methanol. Another used pure, supercooled D_2O over a limited range of freezing temperatures. These calibrations were consistent. The temperature controller was calibrated by setting the measured equilibrium melting temperature of D_2O to 276.97 K [13]. The method is described in greater detail by Yan et al. [19]. Yoon et al. [20] used the method to determine equilibrium freezing point depressions for solutes and obtained good agreement with standard values.

Results and discussion

Fig. 2 shows a typical sample spectrum. A Lorentzian line shape with a line width of 240 Hz fits the central peak well and is shown for com-

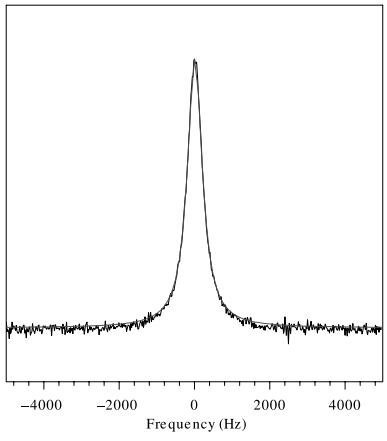

Fig. 2. A typical NMR spectrum of a cell wall-D_2O sample. A Lorentzian line shape (width 240 Hz) is superimposed for comparison (grey line).

parison. The observed line is attributed to the deuterons of unfrozen D_2O. The absence of a narrower line indicates that, at freezing temperatures, there is no free bulk D_2O in equilibrium with ice. The broader signal indicates unfrozen D_2O which is believed to be associated with the *Chara* cell wall. The absence of any measurable residual quadrupolar splitting indicates that the molecular motion of the unfrozen D_2O was fast and isotropic enough to reduce the quadrupolar splitting to a value small compared with the linewidth in D_2O ice. The spectrum of the deuterons in ice is so much broader (about 150 kHz) that it appears in the spectra studied here as the base line for the narrower spectra (full width at half heights less than 600 Hz) attributed to deuterons in the unfrozen water. The free induction decay NMR signal at any temperature is proportional to the number of deuterons that contribute to it. The number of deuterons contributing to the signal can therefore be determined once the temperature sensitivity of the spectrometer is calibrated as described above.

Four different samples were prepared with different total hydrations, as shown in Fig. 3. Hereafter, hydration is expressed as a hydration ratio: the mass of unfrozen water divided by the mass of cell wall dry weight. The four hydration ratios vs. temperature curves superpose at freezing temperatures. Once all ice has melted, their hydration is of course constant at the level determined by the sample preparation. The curves are nearly vertical just below freezing point. This suggests that the concentration of solutes in the water is low: very much less than one molar. If solutes were present in substantial concentrations, dehydration of the sample would concentrate the remaining solutes, gradually depressing the freezing point, as reported by Yoon et al. [20] for lamellar phases in the presence of solutes. Thus the freezing point depression is almost purely due to the hydration of the cell walls themselves. A consequence of this is the nearly vertical shape of this curve, which makes it impossible to estimate accurately the value of full hydration for a cell wall sample using this technique.

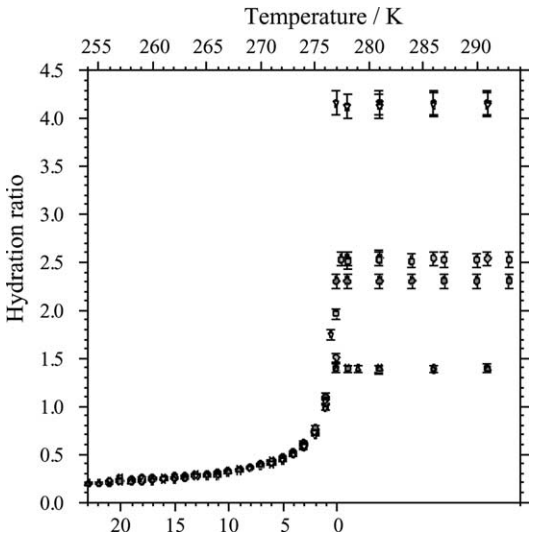

Fig. 3. The hydration ratio (the ratio of unfrozen water to dry weight) as a function of temperature and freezing point depression for four cell wall samples having different overall hydrations: 1.4, 2.3, 2.5, and 4.2. (Note that the melting temperature of D_2O exceeds that of H_2O.)

The shape of the hydration curves below freezing qualitatively resembles those of lipid lamellar phases [20], and may be qualitatively explained in the same way, although the different geometry and possibly different degrees of hydrophilicity give the curves quantitatively different shapes. As the temperature of the ice falls, the pressure in the water in the wall becomes increasingly negative, drawing the fibres closer and closer together until it is balanced by the short range repulsive force.

The water potential Ψ is defined as $(\mu_w - \mu_\omega^0)/v_w$, where v_w is the molecular or molar volume of liquid water. Under the approximation that v_w is constant, $\Psi = P - \Pi$, where P is the hydrostatic pressure and Π the osmotic pressure [10,14]. Fig. 4 plots $\ln(-\Psi)$ vs. the hydration ratio.

In the case of simple, known geometries, it is possible to use curves of hydration vs. Ψ or its components to determine the forces between macromolecules or other ultrastructural elements. For instance, Parsegian and colleagues [7] determined the forces between DNA in hexagonal crystals. We have used the freezing stress technique to determine force curves for bilayer membranes [20].

For planary geometries and at sufficiently low hydration, hydration forces usually dominate be-

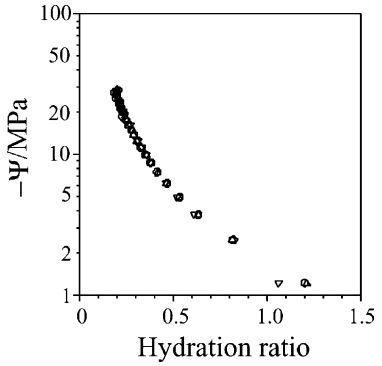

Fig. 4. A semi-log plot of the negative water potential, $-\Psi$ vs. hydration ratio for the samples shown in Fig. 3.

haviour in aqueous colloidal phases. Hydration forces are large (tens or hundreds of MPa) forces with short range exerted between hydrophilic surfaces. They decay approximately exponentially with separation with characteristic lengths of typically 200 pm. The thermodynamics of freezing of colloidal phases is described by Wolfe and Bryant [15], in which an appendix relates inter-molecular forces to hydration curves for a regular crystalline array. In the case of the cell wall, the structure is not known and may be insufficiently ordered to allow a comparable analysis. For instance, the diameter of cellulose microfibrils varies with hydration, and is possibly different among species [11]. Nevertheless, it is interesting to fit an exponential dependence to Fig. 4. The zero hydration intercept is typical of the values obtained for other hydrophilic surfaces studied [5,9].

Volke et al. [12] reported that the quadrupolar splitting is dependent on the activity of water, (and thus on Ψ). Using values of the D_2O quadrupolar splitting measured for phosphatidylcholine lipid bilayers at different water activities, they suggested that the quadrupolar splitting measurement may be used to infer hydration.

In the *Chara* cell wall/D_2O system, the Lorentzian line shape (Fig. 2) indicates that the quadrupolar splitting is essentially zero. However, it is interesting to see whether the linewidth might be used empirically as an indicator of water content or water potential. For this reason, we plot in Fig. 5 the linewidth (full width at half height) as a function of Ψ and also as a function of the reciprocal hydration ratio. In both cases, nearly linear relations are seen over the range investigated here. This linearity would facilitate the use of these parameters as empirical probes of water potential or freezing point depression.

Conclusions

Over the temperature range 23–5 K below freezing, the hydration of cell walls increases from about 0.2 to 0.4, then rises rapidly with temperature in the few Kelvin below the freezing temperature. Two empirical relations may be useful in estimating cell wall water contents: the linewidth of the NMR signal varies approximately linearly with the reciprocal of the hydration ratio, and with the freezing point depression or water potential.

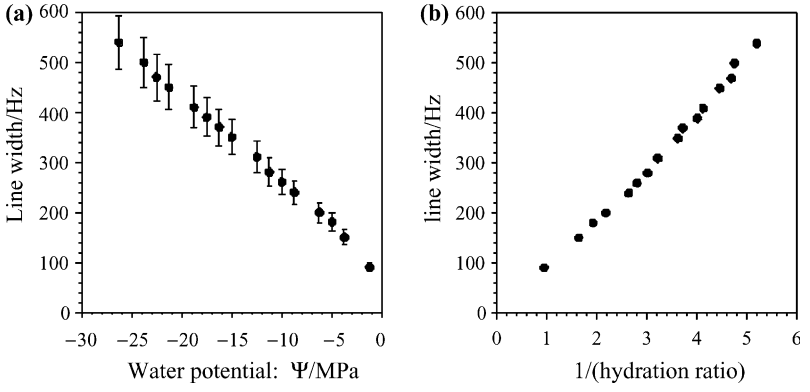

Fig. 5. (a) The signal linewidth vs. Ψ, (b) signal linewidth vs. the reciprocal of the hydration ratio.

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