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Commentary and Perspective

Protein hydration and its freezing phenomena: Toward the application for cell freezing and frozen food storage

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Needless to say, water is an indispensable solvent for living things. ~60 % of our body is composed of water, the lack of which causes lots of fatal problems. It has also been known that protein function is performed only when it accompanies water molecules around the surface, i.e. hydration water molecules [1]. Therefore, it is essential to understand how water and biological component interact with each other in the view point of structure and dynamics. Freezing is a fundamental and simple phenomenon of water, and thus can be used as a "probe" for the purpose. Furthermore, preservation of cells and proteins under low temperature is crucial for numerous applications, which in turn triggers a myriad of undesirable consequences because of the freezing [2].

For these issues, we have a symposium at the 59th Annual Meeting of the Biophysical Society of Japan held in November 2021 inviting four speakers. At the symposium, the speakers review recent progresses on the understanding of the freezing phenomenon of water around cells (by Nakanishi), proteins (by Yamamoto), and model compound (by Nakagawa), which is linked to scrutinizing mode of action of biomaterials working for protecting biological specimens against freezing (by Rajan).

Water and ices in hydrated stratum corneum observed via dielectric relaxation spectroscopy

When pure water is frozen at ambient pressure, hexagonal ice I_h or cubic ice I_c is usually formed [3]. However, ice formed in complex materials is not as "simple" as pure ice because of complex interaction between water and surrounding matrices. In order to examine the complex interaction between water and matrices of biological material, stratum corneum (SC) is one of the best matrices. SC is about 10 – 40 μm thickness of the outermost layer of the skin, which plays a significant role in the separation of the body from the atmosphere [4]. It is composed of dead cells without nuclei nor cell organelles and the dimension of the cell is flattened typically less than about 1 μm of thickness and with about a 900 μm^2 area. SC contains a lot of keratin and possesses a great capacity for retaining absorbed water. These conditions are expected to yield a variety of interactions between water and SC. Hydration water and ices formed in SC were studied by means of dielectric relaxation spectroscopy (DRS). Samples of human SC were hydrated under equilibrium vapor pressure and weighed before and after the hydration. The hydration level, *h*, which is defined by mass of water per dried SC (g/g), resulted in 1.8–2.4.

Two temperature protocols, quenched and slow cooled, were employed for the DRS measurements. As shown in the inset of Figure 1, quenched hydrated SCs exhibited mainly two dielectric processes (at 156 K as example). The most prominent one was bimodal peak (β-process), which was decomposed into two processes denoted B1 and B2. As a high frequency shoulder of the β-process, another process to be attributed to hydration water was recognized. When the sample was slowly cooled, at less than 100 times slower, an additional process denoted H-process showed up at higher frequency

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than the β-process. As shown in the main part of Figure 1, relaxation times of these dielectric processes are plotted as Arrhenius representation, which gave activation energies of 43.8 kJ/mol for B1 and 42.8 kJ/mol for B2, respectively. These values are close to that of pure ice I_c (about 40 kJ/mol) reported by DRS [5]. On the contrary, the activation energy of H-process was 22.2 kJ/mol, which is close to that of pure ice I_h obtained by DRS [5]. Because this most typical form of ice is stable phase, it is consistent with the fact that this phase only appears under slow cooled condition. The result here indicates that the structure and dynamics of water molecules are apparently affected by cell surface as well as cooling conditions.

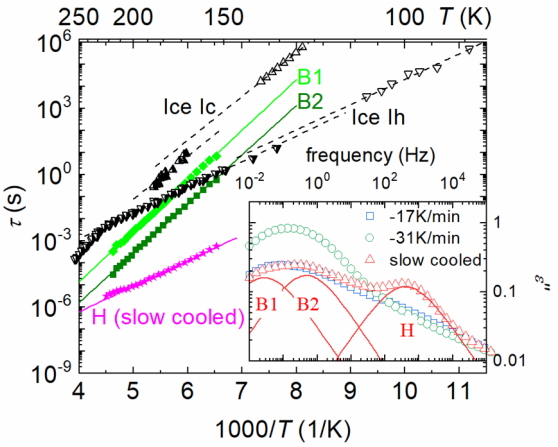

Figure 1 Temperature dependence of relaxation times of hydrated stratum corneum, and literature data of ice I_h and I_c. (inset) dielectric spectra of hydrated stratum corneum at 156 K.

Effect of hydration and its thermal energy on protein dynamics monitored by neutron scattering

It has been known that there are freezable and unfreezable hydration water existing on the protein surface by calorimetric study [6-7]. Recently, Yamamoto et al. observed some of hydration water freezes when the hydration degree is high [8-9]. Based on the observation, we tried to figure out how these freezable and unfreezable hydration water contribute to the activation of protein dynamics by using neutron scattering in picosecond timescale [10]. Freezing was monitored by q (the absolute value of scattering vactor)-dependent coherent scattering of ice whereas protein dynamics were evaluated using energy-dependent incoherent scattering. These evaluations can be achieved by hydrating protein with D_2O due to a negligible incoherent scattering of deuteron compared to that of hydrogen.

As shown in the inset of Figure 2, diffraction peaks of ice I_h were observed in the elastic scattering of a low temperature region at a high hydration level (h = 0.48), which are quite similar to those of ice I_h. On the other hand, such diffraction was not observed in a low hydration level (h = 0.30). The diffraction peaks possessed a temperature hysteresis phenomenon as shown in Figure 2, which is due to supercooling. However, the protein dynamics which was evaluated by inelastic incoherent scattering did not show any temperature hysteresis, and activation of protein dynamics at ~220 K compared to the dehydrated state was only observed (Figure 2). These results strongly indicate that unfreezable hydration water, and not freezable hydration water, contribute to activate protein dynamics in the picosecond timescale.

Unfreezable hydration water is expected to directly interact with protein surface via hydrogen bonds and electrostatic interactions. Whereas, the freezable hydration water molecules could be those interacting with unfreezable hydration water, and not the protein surface. In this situation, the dynamics of unfreezable hydration water couple with those of protein due to their strong interaction, so that unfreezable hydration water and protein behave as if they were one integrated structure. Due to their strong interaction, freezable hydration water would rather be independent from unfreezable hydration water or protein, and thus freezable hydration water would interact with unfreezable hydration water much more weakly. In this situation, the dynamics of freezable hydration water decouple from those of unfreezable hydration water, which could thus cause the dynamical decoupling of freezable hydration water from protein.

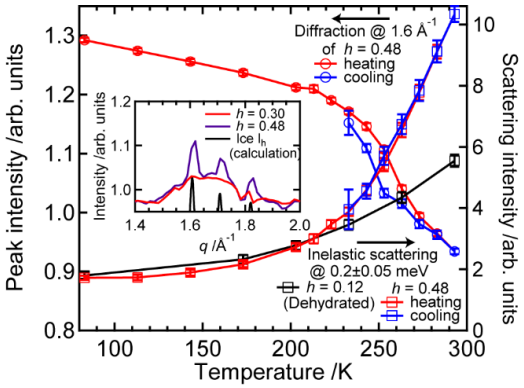

Figure 2 Temperature dependence of a diffraction peak intensity of ice I_h at 1.6 $Å^{-1}$ in elastic scattering and inelastic scattering intensity at 0.2±0.05 meV. The inset shows the elastic scattering profiles at 83 K.

Freezing, thawing and glass transition of biomolecules and water

Water activity (A_w) is a thermodynamic quantity defined as the ratio of the water vapor pressure of a sample to that of pure water at the same temperature. This value is known to be a reliable indication of the microbial growth, enzymatic activity, and preservation and quality of foods [11-12]. However, the molecular basis of A_w is under debate in the related disciplines. In this study, the diffusive dynamics of water were investigated with glycerol-water mixtures at various A_ws by incoherent quasi-elastic neutron scattering (IQENS) [13]. The difference spectra of deuterated glycerol in H_2O and deuterated glycerol in D_2O were analyzed to selectively observe the dynamics of water in aqueous glycerol solutions [14-15] . We note that the hydrogen atoms in the OH group of glycerol can dissociate and change to OD, but this has little effect on the estimate of the water diffusion coefficient. The energy resolution was about 106 μeV and q was 1.7 $Å^{-1}$. This corresponds to the observation of molecular motion on the time and space scales of about 10 ps and 3.6 Å, respectively. The resultant dynamics were compared with the differential scanning analysis (DSC). The following scattering function was used to analyze IQENS spectra of the mixture of glycerol and water.

$$I(q,\omega) = A(q)\text{-}\{EISF\text{-}\delta(\omega) + (1\text{-}EISF)\text{-}L(q,\omega)\} \otimes R(\omega) + B(\omega) \quad (1)$$

where $R(\omega)$ is the resolution function, $A(q)$ is the Debye-Waller factor, $EISF$ is the incoherent elastic scattering factor, $L(q,\omega)$ is the Lorentz function, and $B(\omega)$ is the background scattering. $\otimes$ is the operator of convolution. The apparent diffusion coefficient (D) of water was obtained from half-width-half-magnitude (HWHM) of $L(q,\omega)$ using HWHM = Dq^2.

As shown in Figure 3, the diffusion coefficient increases slightly with increasing A_w for $A_w < 0.7$, and increases sharply around A_w = 0.7-0.8. It was found that A_w dependent apparent diffusion coefficient of water is correlated with the water sorption isotherm. DSC showed that both freezing and melting of water are observed for the solutions at $A_w > \approx 0.8$, where water molecule exhibits highly diffusive character. Below $A_w \approx 0.8$, water dynamics are significantly suppressed, and freezing and/or melting do not exhibit. These results indicate that A_w should be related to the diffusive dynamics of water, which is also related to the physical properties of water, such as freezing and thawing.

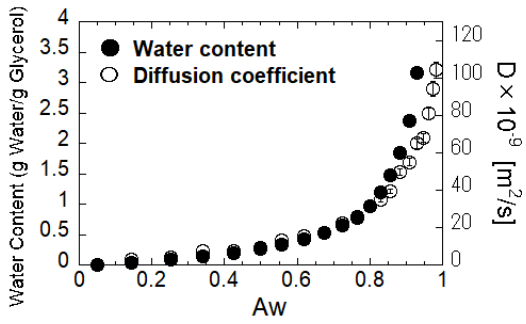

Figure 3 Water sorption isotherm (water content v.s. water activity (A_w)) and diffusion coefficient of water molecule as a function of A_w.

Polyampholytes for low-temperature preservation of cells and proteins

Freezing cells without any protective additive accompanies unwanted consequences owing to the formation of intracellular as well as extracellular ice. Cellular cryopreservation has often been done using membrane-permeable small molecules such as glycerol and dimethyl sulfoxide with good efficiency [16-17]. However, they possess a large number of drawbacks such as toxicity to cells, permeability to certain cell lines. Yet, they are still frequently used because of the lack of efficient alternatives [18].

Matsumura and co-workers have developed succinylated poly-l-lysine (PLLSA) based cryoprotective agent (CPA), which yielded remarkable results and showed excellent efficiency against a number of cell lines as well as for long-term preservation [19-20]. Although mechanistic studies using synthetic polyampholytes suggested that they protect the cell membrane from freezing-induced damages [21], a conclusive mechanism was unknown. In this study, we show molecular-level investigation of the mechanism of cryopreservation using solid-state NMR [22]. We investigated the soluble state as well as the molecular mobilities of the polymer chain, water, and salts at freezing temperatures and compared it with control samples to demonstrate polyampholytes' ability to prevent dehydration and decrease intracellular ice freezing during cryopreservation.

Using ^{23}Na NMR (Figure 4), it was found that a considerable reduction in the Na-ion peak intensity occurred when PLLSA solution is cooled, which indicates that mobility of Na ions decreases. This is possibly because polymer chains trapped the ion, thus reducing osmotic stress. Additionally, signal broadening in PLLSA was observed monitored by ^1H NMR. This clearly demonstrates that PLLSA loses its mobility at lower temperatures, probably due to the glass transition where a reversible network matrix containing water and solute molecules is formed. These studies unambiguously

demonstrated that polyampholytes cryopreserve cells by forming a reversible matrix, capable of trapping salts and water molecules, thus suppressing the osmotic stress and also inhibiting intracellular ice freezing.

We further exploited the protective properties of PLLSA for proteins against freezing-induced stress [23]. In this case, recrystallization takes place during freezing and subsequent thawing. Ice recrystallization is known to induce protein denaturation by introducing additional interfaces on which the protein can be absorbed. We incorporated polyvinyl alcohol (PVA) into PLLSA to develop several graft polymers (PVA-g-PLLSA) with different ratios of PVA and PLLSA as well as different chain lengths of PVA. As a result, it was clearly demonstrated that the graft polymer completely protects a protein activity even at a very low polymer concentration (5 mg/mL). These studies have opened a new avenue for the design of novel CPAs as well as understanding the protective mechanism of several other polymers.

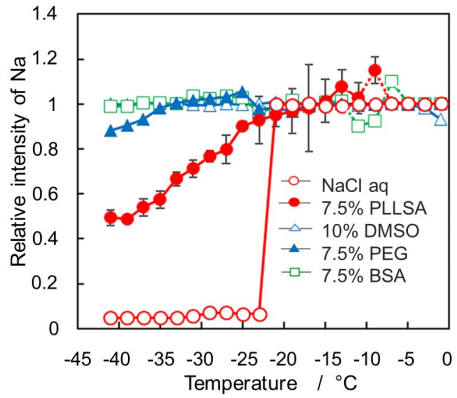

Figure 4 Temperature dependences of Na-ion signal intensities of solid-state NMR spectra in various CPAs.

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