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Title

Complementary Analyses of Fractal and Dynamic Water Structures in Protein-Water Mixtures and Cheeses

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Abstract

Water structures in protein-water mixtures and cheeses were examined by Time Domain Reflectometry (TDR) and Nuclear Magnetic Resonance (NMR). The relaxation time obtained from TDR increased and the diffusion coefficient obtained from NMR decreased with increasing protein concentration, even if those physical quantities and time scales of respective observation techniques were considerably different. The correlation of these dynamic behaviors obtained from TDR and NMR is also found for water structures in cheeses. Fractal analysis of protein solutions by TDR measurements suggested that water structures in both systems were same with usual dispersion systems much more than solution systems. The fractal analysis with the τ - β diagram using geometric characteristics of the whole diagram is effective to characterize various water structures.

Keywords

dielectric relaxation; nuclear magnetic resonance; water structure; protein solution; cheese

1. Introduction

Water is a typical hydrogen-bonding liquid consisting of molecules having hydroxyl groups with both donor and acceptor site of hydrogen bonding. Water molecules exhibit

Brownian movements, repeating breakup and recombination of hydrogen bonds. The breakup and recombination are considered as elementary processes of intermolecular correlations in the liquid structure, and interpret a model in which a concept of the dynamic cluster formed by 5-6 water molecules is often used today[1]. Generation and annihilation of such dynamic clusters of water molecules observed as a single relaxation process of Debye type by dielectric spectroscopy suggests why specific behaviors of the water are shown. The relaxation process observed for water indicates that the relaxation time τ is 8.27ps at 25°C [2-5]. The relaxation time is a characteristic time of rotational diffusion of molecules, while the presence of a small relaxation process reflecting more local dynamics has been reported in one or more decades higher frequency range of THz region. The elementary process of break up and recombination of hydrogen bonding may be closely related to this THz frequency process, the detailed discussion has not been completed yet.

A dynamic agglomeration structure of water molecules interacting with surrounding molecules identified by those dynamics behaviors is called water structure. In recent years, molecular mechanisms forming water structures has been extensively investigated in various polymer-water mixtures, and an importance to incorporate the handling of hydrogen bond network is pointed out in the interpretation of water structures. However, the dynamic range of molecular behaviors of water is extremely wide under various conditions, and those understanding have been delayed due to the dearth of enough wide time scale in conventional observation techniques. Then complementary experimental techniques of molecular dynamics in different time scales must be effective to discuss water structures in a wide time scale. The interpretation thus focused on the dynamic behavior of water molecules should be effectively employed in extensive area of applications as well as fundamental researches [6].

The relaxation time obtained for the relaxation process due to molecular dynamics of water increases and the distribution is broadened with increasing concentration in polymer-water mixtures. This behavior is called slow dynamics, and is an important concept to explain liquid structures, polymers, proteins, nucleic acids, and lipid molecules in biological systems, in which hierarchical structures of water molecules with solute molecules and dispersed particles appear. In order to characterize these hierarchical structures, fractal concept should be helpful because of the multi scaled treatment [7, 8]. It is reasonably expected that the fractal concept can be utility to characterize various water structures.

The fractal expression of the relaxation process for water molecules obtained by dielectric spectroscopy has been developed to apply to various aqueous systems [9, 10].

When exponential hyperbolic type dipolar correlation function corresponding to Cole-Cole equation [11] was compared with memory function, it was shown that exponent of Cole-Cole type and spatial-temporal fractal dimension are well corresponding. Cole-Cole equation is expressed as

$$\varepsilon^* - \varepsilon_\infty = \frac{\Delta\varepsilon}{1 + (j\omega\tau)^\beta} \quad (0 < \beta \leq 1) \quad (1)$$

where ε^* is the complex dielectric permittivity, ε_∞ is the high frequency limit of the dielectric constant, $\Delta\varepsilon$ is the relaxation strength, j is the imaginary unit, ω is the angular frequency, τ is the relaxation time, and β is the relaxation time distribution parameter. The space fractal dimension to scale the volume division size of the relaxation structure unit is obtained from Eq.1 with a relationship between, the relaxation time, and the exponential of Cole-Cole type relaxation time distribution through relaxation processes dependent on the water content.

In this study, we performed TDR measurements on protein-water mixtures and cheeses in order to investigate water structures. NMR measurements on diffusion process of water molecules are also performed to compare the results with the relaxation parameter obtained from TDR measurements. **It is expected that molecular dynamics related with the rotational and the translational diffusion, respectively, by TDR and NMR measurements with different characteristic time scales of observation are compared.** Fractal analysis using the τ - β diagram obtained from TDR measurements is also examined to discuss hierarchical structures of water and proteins. We focus on the space fractal dimension as physical quantity to characterize water structures of water mixtures.

2. Experimental

2.1. Sample Preparations

Albumin from bovine serum (BSA), Ovalbumin (OVA), and Lysozyme from Chicken Egg-White (CEWL) were purchased from Sigma-Aldrich. Each protein was mixed with distilled water (Millipore, ultrapure water with resistivity higher than 18.2 M Ω · cm). Each protein concentration was adjusted to 5, 10, 20, 30, and 40 wt%.

Various kinds of cheese available in market were purchased. Prepared in the present work were mozzarella cheese (KRAFT), camembert cheese (MEGMILK SNOW BRAND Co.,Ltd.), cream cheese (Bel Japon K.K.), processed cheese (MEGMILK SNOW BRAND Co.,Ltd.), gouda cheese (TOKYO DAIRY CO.,LTD), and red cheddar cheese (TOKYO DAIRY CO.,LTD).

Samples were kept in the refrigerator before measurements, and the sample temperature was kept at 25.0 $\pm$ 0.2 °C during both TDR and NMR measurements.

2.2. Time Domain Reflectometry Measurements and Fractal Analysis

Digitizing Oscilloscope Mainframe (HP 54120B) and its Four Channel Test Set (HP 54124A) was employed as the TDR system [12-16] to make dielectric measurements especially for a relaxation process due to molecular dynamics of free water in the frequency range from 0.1 to 30GHz. Reflected signal including information of the dielectric behavior of the sample responding to the incident step pulse was observed.

The complex permittivity of the sample can be expressed by the basic equation of TDR method [12-14] as

$$\epsilon^* = \frac{c'}{j\omega\gamma d} \frac{v_0 - r}{v_0 + r} X \cot X \quad (2)$$

where c' is the speed of propagation in a coaxial line, v_0 and r are the Laplace transforms of the incident and reflected pulse waveforms, $X \cot X$ is propagation and multiple reflections in the sample and coaxial line geometry, and γd is a value of corrective coefficient. Since accuracy would be better off using the difference method [12-14].

50Ω semi-rigid coaxial cable (SUHNER) with outer diameter $d = 2.2$ mm and electric length $\gamma d = 0.165$ mm is used for electrodes of the TDR measurement.

The τ - β diagram shows the characteristic behavior of slow dynamics for each material, and some tendencies were obtained for the type of polymers.

Ryabov et al. expressed the relationship between the Cole-Cole parameter β and the relaxation time τ in the fractal structure expression[9]

$$\beta = \frac{d_G}{2} \frac{\ln(\tau\omega_s)}{\ln(\tau/\tau_0)} \quad (3)$$

where τ_0 is the cutoff time of the scaling in time domain, d_G is the fractal dimension of the point set where relaxing units are interacting with the statistical reservoir,

$$\omega_s = 2d_E G^{2/d_G} D_s / R_0^2 \quad (4)$$

is the characteristic frequency of the self-diffusion process where d_E is the Eucidean dimension, D_s is the self-diffusion coefficient, R_0 is the cutoff size of the scaling in the space, and G is a geometrical coefficient approximately equal to unity.

2.3. Frequency Sweep Dielectric Measurements

Frequency sweep dielectric measurements were carried out using a Vector Network Analyzer (Agilent N5230C) with a dielectric kit probe (Agilent 85070E) in the frequency range from 0.5 to 50GHz.

VNA measurements offers the complex reflection coefficient $\Gamma^*(\omega)$, from which the complex dielectric constant $\epsilon^*(\omega)$ is obtained following the procedure reported by Bao et al.[17], through the relationship

$$\epsilon^*(\omega) = \frac{A_1^*(\omega)\Gamma^*(\omega) - A_2^*(\omega)}{A_3^*(\omega) - \Gamma^*(\omega)}$$

(5)

where $A_j(\omega)$ ($j = 1, 2, 3$) are frequency-dependent complex constants which can be obtained from calibration procedure performed with air, short connections, and water[18].

2.4. Pulse-Field Gradient Spin-Echo Method of Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) measurements were performed by ^1H 90 MHz, FT-NMR, JNM-EX90 (JEOL) for water proton translational diffusion coefficient. Pulse-Field Gradient Spin-Echo (PGSE) method of NMR was used to obtain the diffusion coefficient of water molecules with the gradient field applied parallel to z-axis of rectangle shape of length δ in a Spin-Echo pulse sequence.

In this method, we obtained echo signal at a number of different gradient pulse length, δ . The self-diffusion of molecules is calculated from the echo signal attenuation[19]. The echo amplitude is given by following equation[20],

$$\ln \left[\frac{A(\delta)}{A(0)} \right] = -\gamma^2 \delta^2 g^2 D \left(\Delta - \frac{\delta}{3} \right) \quad (6)$$

where A is the echo signal intensity, γ is the gyromagnetic ratio, g is the magnetic field gradient, δ is the field gradient pulse duration, and Δ is the separation of gradient pulses.

3. Results and discussion

Typical dielectric relaxation spectra obtained by TDR measurements for protein-water mixtures with various concentrations are shown in Fig. 1, where the real part ϵ' and imaginary part ϵ'' of the complex permittivity are plotted as a function of frequency f . The relaxation process observed around 10GHz is considered to be due to rotational diffusion process of water molecules from the relaxation time and the strength. The lower frequency processes reflecting ion behaviors, chain dynamics, and restricted water molecules are also usually shown for aqueous systems in lower frequency region below 1GHz. The lower frequency processes are larger for CEWL than those for BSA, because the ion concentration is probably larger. Fig.1 shows that dielectric constant is smaller with increasing protein concentration because of decreasing water content. Fig.2 shows the concentration dependence of the relaxation parameters obtained from fitting procedures of Eq.1 to experimental data shown in Fig.1. The relaxation time τ increased and the relaxation-time distribution parameter β and the relaxation strength $\Delta\epsilon$ decreased with increasing protein concentration. Since $\Delta\epsilon$ means a magnitude of orientation polarization by dipole moments of water molecules, the decrease in the relaxation strength accompanied by the increase in protein

concentration reflects decreasing number density of water molecules in the mixtures. The decrease in β reflecting broadening relaxation curves evaluates the relaxation time distribution, which is corresponding to increasing fluctuation of the dynamics of water molecules caused by larger interactions with protein molecules. The increase in τ shows that the mobility of water molecules decreases by increasing in between polymer chains and water molecules. In these behaviors, the relaxation time is given as a characteristic time of the dynamics of water molecules on the average.

Fig.3 shows the concentration dependence of the diffusion coefficient of water molecules obtained from PGSE-NMR measurements for protein aqueous solution. The diffusion coefficient of pure water is $2.32 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$. The diffusion coefficient decreases, as protein concentration becomes higher. This tendency of concentration dependence is corresponding to that obtained for the relaxation time of dielectric spectroscopy. In the relaxation profile obtained by TDR, the rotational diffusion process of water molecules and the distribution are ranging in time scale of picosecond order. On the other hand, the diffusion coefficient of the translational motion obtained by NMR measurement is ranging in millisecond time scale. Then it is suggested that both of rotational and translational diffusion processes are similarly restricted by increasing concentration, and the restriction also affects the mobility in the same manner in picosecond and millisecond time scales.

Dielectric relaxation spectra observed by VNA measurements for various kinds of cheeses are shown in Fig.4. The lower frequency processes are smaller than those observed in protein solutions. It is reasonable to consider that the ion mobility should be smaller in cheese than solutions. The relaxation parameters for the GHz frequency processes were obtained by the same procedures with the case of protein solutions, and are listed in Table 1.

Since there exist some difficulties in evaluation of exact water content of cheese because of the existence of water molecules which cannot be easily removed by usual procedure, the water content is represented by the relaxation strength in the present work. The relaxation strength dependence of the diffusion coefficient of water molecules for cheeses is shown in Fig.5. The diffusion coefficient tends to decrease with decreasing $\Delta\epsilon$ as shown in Fig.3 for protein solutions. The plot for camembert cheese is shown in the smaller side of the tendency. This deviation should be attributed to the heterogeneity of hydration.

Generally, aqueous systems show varieties in concentration dependences of relaxation parameters, and it is difficult to find out any universal explanations of water structures. The τ - β diagram created as a relaxation between the average characteristic time and its distribution of water structures is a possible tool as a universal treatment. In our

former reports, we indicated that solution systems and dispersed systems were classified and characterized by the locus plotted in the τ - β diagram [21, 22, 23]. Plots of the dispersion systems were shown in the diagram as extended lengthwise forms, and the value of the fractal dimension of the dispersion systems was smaller than 1.0. Any conventional techniques to classify solutions and dispersed systems with a common criterion have not been shown so far.

Fig.6 is τ - β diagram for protein solutions and cheeses. Plots for the present samples were shown in the diagram as extended lengthwise forms, and they are in the area for dispersion systems in Fig.6. Table 2 shows the space fractal dimension, the characteristic frequency of the self-diffusion process, and cutoff time that were obtained by the least-square fit method of Eq.3 with experimental data according the plot of the τ - β diagram. The values of the fractal dimension thus obtained were 0.03 ± 2.45 for BSA, 0.03 ± 1.70 for CEWL, and 0.22 ± 0.89 for OVA. These values are much smaller from those obtained for usual aqueous solution systems, and the error is much larger than usual solutions. Usual values of the fractal dimension obtained for water structures in aqueous polymer solutions are about 1.3-1.5, and error is 0.02-0.71. Water structures with the small fractal dimension give only small increase in the relaxation time with increasing concentration. Then, in order to determine the fractal dimension from the τ - β diagram, a sufficient high-concentration domain is necessarily required. However, it is fairly difficult to prepare high-concentration samples especially for dispersion systems, and the global image of trajectory of the plots cannot be obtained. Therefore the magnitude of error and the fractal dimension are correlated each other, and it is unavoidable to have a large error of the fractal dimension from the geometric condition for dispersion systems [24].

It turns out that physical sense of one or less value of the fractal dimension is proceeding agglomeration in dispersed system from knowledge of our geometric model calculation. The value of such a small fractal dimension is not unreal for dispersed system, and it is strongly dependent on the spatiotemporal scale of analyzing method [25]. The process of the slow dynamics reflects the conformation of polymer chains and those agglomeration structures, and also hydrogen bonding ability. Therefore, characterization of the slow dynamics is not determined only by the absolute value of fractal dimension but also by the geometric feature of the domain where the locus of a plot appears in the τ - β diagram. In the eyes of slow dynamics of water structures shown in the τ - β diagram, protein solutions and cheeses have quite similar water structures with the characteristics of dispersed systems.

The variations of plots obtained for the various kinds of cheese are also shown in Fig.6.

Even with the difference in different kinds of cheese, all plots for cheeses show the water structures of typical dispersion systems. Fig.7 shows plot of the relaxation-time distribution parameter against the inverse of the diffusion coefficient for various cheeses. Figs. 7 and 6 are resembled, and $1/D\beta$ plot might be also a candidate of a effective tool to characterize water structure. The Stokes-Einstein equation is described with the diffusion coefficient as

$$D = \frac{k_B T}{6\pi\eta R_h} \quad (6)$$

where k_B is Boltzmann's constant, T is the absolute temperature, η is viscosity of surrounding medium and R_h is hydrodynamic radius of the motional unit. The Debye-Stokes-Einstein equation is described with the relaxation time as

$$\tau_{rot} = \frac{V\eta}{k_B T} \quad (7)$$

where V is volume of the motional unit. Then the relaxation time corresponding to the inverse of the diffusion coefficient suggested by Eq.6 and Eq.7 support the accordance of Figs. 6 and 7.

Figs. 8 and 9 show the relaxation strength dependence of the relaxation time and its distribution parameter for cheese. The relaxation strength reflects the water content as expressed by equation as

$$C = \rho_w \frac{\Delta\epsilon}{\Delta\epsilon_w} \quad (8)$$

where ρ_w and $\Delta\epsilon_w$ are the density and the relaxation strength of pure water, respectively[23]. Considering that the relaxation strength is proportional to the water content, Figs. 8 and 9 can be treated as usual concentration dependence of physical quantities shown in Fig.2. Figs. 8 and 9 show the deviation of the plot for camembert cheese because of the heterogeneous hydration as shown in Fig.5.

The relaxation strength dependence of the relaxation time and its distribution parameter are also shown in Figs. 10 and 11 for protein aqueous solutions. These results indicate the similar tendency with Fig. 2. It is reasonable to consider that water structures interacting with protein structures included in cheese and protein solutions are not necessarily same, since the protein structures, denaturalization, and various additives affect water structures. Actually in the fractal analysis, the τ - β diagram shows different water structures with different values of fractal dimension, however, the water structures in various kinds of cheese and protein solutions are clearly classified in dispersion systems. Fractal analysis with the τ - β diagram using geometric characteristics of the whole diagram can be an effective tool to characterize various water structures.

4. Conclusion

It was found that the relaxation profile obtained from TDR measurements indicated that restricted water structures show typical slow dynamics in the picoseconds time scale. The slow dynamics observed for the diffusion process of water molecules observed by PGSE-NMR in millisecond time scale also shown the similar behavior, even if those physical quantities and the time scales of respective observation techniques were considerably different. Fractal analysis of protein solutions by TDR measurements suggested that water structures in both systems were same with usual dispersion systems much more than solution systems. The fractal analysis with the τ - β diagram using geometric characteristics of the whole diagram is effective to characterize various water structures.

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Captions

Fig. 1. Frequency dependence of dielectric constant and loss for 5($\circ$), 10($\square$), 20($\triangle$), 30($\diamond$), and 40wt%(∇) BSA and 5($\bullet$), 10($\blacksquare$), 20($\blacktriangle$), 30($\blacklozenge$), and 40wt%($\blacktriangledown$) CEWL aqueous solutions at 25°C.

Fig. 2. Concentration dependence of the relaxation strength $\Delta\epsilon$, the relaxation time τ and the relaxation time distribution parameter β for BSA($\circ$) and CEWL($\diamond$) aqueous solutions at 25°C.

Fig. 3. Concentration dependence of the diffusion coefficient for BSA($\circ$), CEWL($\diamond$) and OVA($*$) aqueous solutions at 25°C.

Fig. 4. Frequency dependence of dielectric constant and loss for various kinds of cheeses(mozzarella[$\blacktriangleleft$], camembert[$\blacktriangleleft$], cream[$*$], processed[$\blacktriangleright$], gouda[$\blacktriangleright$], and red cheddar[$+$]).

Fig. 5. Plots of the relaxation strength versus the diffusion coefficient for water($\times$), mozzarella($\circ$), camembert($\diamond$), cream($*$), processed($\square$), gouda($\triangle$), and red cheddar($+$).

Fig. 6. Diagram of the relaxation time versus the relaxation-time distribution parameter of Cole-Cole equation for water($\times$), BSA($\circ$), CEWL($\diamond$), OVA($\star$), PEG($\bullet$), PVP($\blacksquare$), PVME($\blacktriangledown$), PVA($\triangle$), PAIA($\square$), and PEI(∇). The inset is the τ - β diagram for proteins($\star$) and cheeses(mozzarella[$\blacktriangleleft$], camembert[$\blacktriangleleft$], cream[$*$], processed[$\blacktriangleright$], gouda[$\blacktriangleright$], and red cheddar[$+$]).

Fig. 7. Plots of inverse of the diffusion coefficient versus the relaxation-time distribution parameter of Cole-Cole equation for water($\times$), mozzarella($\circ$), camembert($\diamond$), cream($*$), processed($\square$), gouda($\triangle$), and red cheddar($+$).

Fig. 8. Plots of the relaxation strength versus the relaxation time for water($\times$), mozzarella($\circ$), camembert($\diamond$), cream($*$), processed($\square$), gouda($\triangle$), and red cheddar($+$).

Fig. 9. Plots of the relaxation strength versus the relaxation-time distribution parameter for water($\times$), mozzarella($\circ$), camembert($\diamond$), cream($*$), processed($\square$), gouda($\triangle$), and red cheddar($+$).

Fig. 10. Plots of the relaxation strength versus the relaxation time for water($\times$), BSA($\circ$), and CEWL($\diamond$) aqueous solutions.

Fig. 11. Plots of the relaxation strength versus the relaxation-time distribution parameter for water($\times$), BSA($\circ$), and CEWL($\diamond$) aqueous solutions.

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Table1. Relaxation parameters for water and various kinds of cheeses.

sample	$\Delta\varepsilon$	τ / ps	β
mozzarella cheese	38.5	11.8	0.88
camembert cheese	37.1	11.5	0.82
cream cheese	29.9	10.0	0.86
processed cheese	24.5	11.8	0.82
gouda cheese	18.71	13.5	0.80
red cheddar cheese	17.0	14.8	0.73
water[26]	73.2	8.27	1.00

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Table 2. Relaxation parameters obtained from the fitting procedures for the τ - β diagram.

Sample	dimension	Error	frequency	Error	cut off time	Error
	d_G	$\pm e_{dG}$	ω_s (Hz)	$\pm e_{\omega s}$	τ_0 (s)	$\pm e_{\tau_0}$
BSA	0.03	2.45	1.92×10^{40}	1.04×10^{44}	3.36×10^{-12}	4.91×10^{-12}
CEWL	0.03	1.60	4.88×10^{39}	1.52×10^{43}	2.67×10^{-12}	2.87×10^{-12}
OVA	0.22	0.89	1.93×10^{14}	6.71×10^{15}	3.66×10^{-12}	1.88×10^{-12}

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