

changes with changing temperature according to the Adam-Gibbs-Vogel (AGV) and Vogel-Tammann-Fulcher (VTF) equations below and above  $T_g$ , respectively.<sup>1</sup>

Investigations of the molecular mobility of freeze-dried formulations containing polymer excipients have demonstrated that glass transition occurs partially even at temperatures below  $T_g$ , enhancing molecular rearrangement motions and leading to decreases in the physical and chemical stability.<sup>2-6</sup> Changes in the temperature dependence of molecular rearrangement motions associated with the glass transition have been detected by measuring nuclear magnetic resonance (NMR) relaxation times such as laboratory and rotating frame spin-lattice relaxation times ( $T_1$  and  $T_{1\rho}$ ) and spin-spin relaxation time ( $T_2$ ) of  $^1\text{H}$  and  $^{13}\text{C}$ .<sup>7-12</sup> However, there is no clear explanation for the question why changes in the temperature dependence of molecular rearrangement motions with a relaxation time longer than 100 s at sub- $T_g$  can be detected by measuring NMR relaxation times such as  $T_1$ ,  $T_{1\rho}$ , and  $T_2$  of  $^1\text{H}$  and  $^{13}\text{C}$  that reflect molecular motions on the order of 1 MHz, 10 kHz, and >10 kHz, respectively. Thus, the aim of this investigation was to elucidate the relationship between molecular rearrangement motions and molecular motions reflected in NMR relaxation times in freeze-dried formulations containing polymer excipients. In an effort for this purpose, molecular motions reflected in NMR relaxation times were first compared with molecular motions reflected in dielectric spectra, which is capable of observing molecular motions ranging from 0.01 Hz to 100 kHz.

Dielectric relaxation spectroscopy (DRS) is a useful method of determining molecular mobility, as is NMR relaxation measurement. NMR measures the fluctuation of a certain atom, whereas DRS measures the reorientation of a molecular dipole. NMR allows determination of the molecular motion of a specific site in the molecule, whereas DRS reflects the mobility of molecular dipoles that are usually difficult to specify in the case of polymers. The correlation time, which represents the timescale of molecular motion, cannot be determined directly from the NMR relaxation time, because NMR relaxation time depends on the relaxation mechanism. Therefore, it is necessary to understand the relaxation mechanism in order to calculate the correlation time from the observed relaxation time. However, DRS is able to directly detect the timescale of molecular motions ranging from  $10^{-11}$  to  $10^3$  s.

DRS has been used to determine the molecular mobility of various amorphous pharmaceuticals and excipients.<sup>13-16</sup> The mobility of water coexisting with excipients has also been investigated using DRS.<sup>17-22</sup> Montes and Cavaille<sup>16</sup> used DRS to observe the  $\gamma$ - and  $\beta$ -relaxation of dextran film, which were assigned to the noncooperative motion of local segments and the cooperative motion of main chain segments, respectively, based on the observed activation energy. Noel et al.<sup>14</sup> observed the  $\alpha$ -relaxation of amorphous glucose corresponding to molecular rearrangement motions by DRS.

In this investigation, the molecular motions in freeze-dried dextran 40k, dextran 1k, isomaltotriose (IMT), and  $\alpha$ -glucose comprising a common unit but with different  $T_g$ 's, were first investigated by dielectric spectroscopy (DES). Then, the temperature dependence of the relaxation time determined by DES ( $\tau_{\text{DES}}$ ) was compared with that of the correlation time determined by NMR relaxation measurements ( $\tau_c$ ), in order to discuss the relationship between the molecular motions detected by DES and NMR. The  $\tau_c$  for the backbone carbon of freeze-dried IMT was calculated from the  $T_{1\rho}$  value measured in this study, and that of freeze-dried dextran 40k was calculated from the  $T_{1\rho}$  value reported previously.<sup>11</sup> Furthermore, the  $\tau_c$  and  $\tau_{\text{DES}}$  were compared with the timescale of molecular rearrangement motions measured calorimetrically, in order to discuss the correlation of molecular rearrangement motions and molecular motions reflected in the  $\tau_c$  and  $\tau_{\text{DES}}$ .

## EXPERIMENTAL

### Freeze-Drying

Freeze-dried samples for DES measurement were prepared as follows: each of dextran 40k (D-4133; Sigma Chemical Co.), dextran 1k (00268; Fluka Production GmbH), IMT (I-0381; Sigma Chemical Co.), and  $\alpha$ -D-glucose (anhydrous 158968; Aldrich) was dissolved in distilled water (5% w/w). Conversion of  $\alpha$ -D-glucose to  $\beta$ -D-glucose during the dissolution was prevented by cooling the solution with ice. Seven hundred microliters of the solutions was frozen in a polypropylene sample tube (17-mm diameter) by immersion in liquid nitrogen for 10 min, and then dried at a vacuum level below 5 Pa for 23.5 h in a lyophilizer (Freezevac C-1; Tozai Tsusho Co., Tokyo). The shelf temperature was between  $-35^\circ$  and  $-30^\circ\text{C}$  for the first 1 h,  $20^\circ\text{C}$  for the subsequent 19 h, and  $30^\circ\text{C}$  for the last 3.5 h.

**Table 1.**  $T_g$  of Freeze-Dried Cakes Determined using Differential Scanning Calorimetry

	Dry	23% RH	43% RH	60% RH	75% RH
Dextran 40k	230°C		80	58	35
Dextran 1k	167				
IMT	122	53	33	7	
$\alpha$ -Glucose	35				

To prepare freeze-dried samples for  $T_{1\rho}$  measurement, 400  $\mu$ L of IMT solution (2.5% w/w) was frozen in a polypropylene sample tube (10-mm diameter).

Freeze-dried samples with various water contents were obtained by storing the freeze-dried sample at 15°C for 24 h in a desiccator with a saturated solution of potassium acetate [23% relative humidity (RH)],  $K_2CO_3 \cdot 2H_2O$  (43% RH),  $NaBr \cdot 2H_2O$  (60% RH), or  $NaCl$  (75% RH). The  $T_g$  of samples was determined by differential scanning calorimetry at a scan rate of 20°C/min (2920; TA Instruments, New Castle, DE), and the results are shown in Table 1.

## DES

Dielectric measurements were performed with a dielectric analyzer (model 2970; TA Instruments) operating in the frequency range of 0.01 Hz to 100 kHz and in the temperature range of -20° to 200°C. Gold parallel plate electrodes (ceramic parallel plate, 25 mm) were used. Permittivity was measured with and without a thin Teflon sheet (0.2-mm thickness) inserted between the sample and the upper electrode. The Teflon sheet was used to inhibit charge transfer between the electrode and the sample, as reported for measurement of the Maxwell-Wagner process in a heterogeneous dielectric mixture.<sup>18</sup> Teflon was chosen because of the low permittivity and the lack of dielectric dispersion in the temperature and frequency range examined. The observed values of real permittivity ( $\epsilon'$ ) and imaginary permittivity ( $\epsilon''$ ) were relative rather than absolute, because the electrode area, which was larger than the sample area, was used for the calculation of permittivity. Therefore, the ratio of  $\epsilon''$  to  $\epsilon'$  ( $\tan \delta$ ) was calculated as a parameter independent of the surface area of the sample.

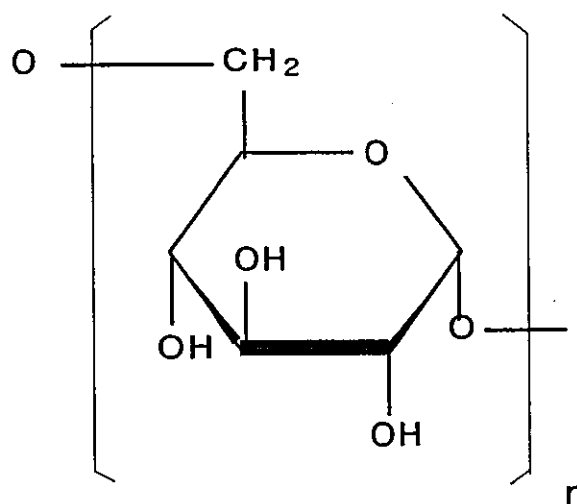
For measurement in the dry state, lyophilized cakes were placed on the lower electrode, and the chamber enclosing the sample was purged with nitrogen gas for 24 h at a constant temperature

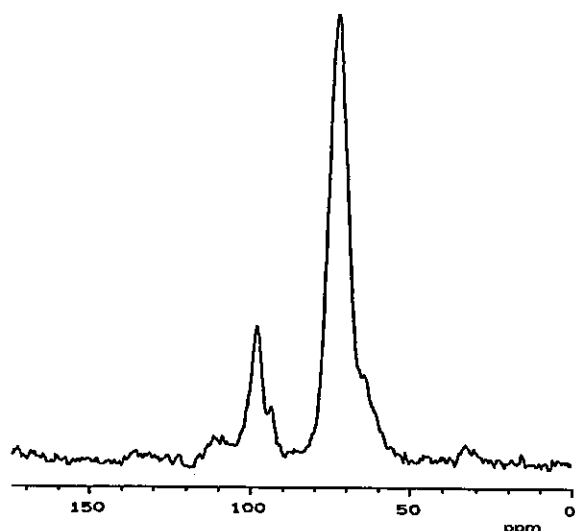
(120°C for dextran 40k, 100°C for dextran 1k and IMT, and 25°C for  $\alpha$ -glucose). A small container of  $P_2O_5$  was introduced into the chamber for measurement of  $\alpha$ -glucose samples. After removing moisture, samples were compressed with a force of 500 N, and permittivity was measured at a constant temperature as a function of frequency. Permittivity was also measured at a constant frequency of 1 Hz and 1 kHz as a function of temperature. Heating rate was 5°C/min for dextran 40k, dextran 1k, and IMT, and 20°C/min for  $\alpha$ -glucose (rapid measurement to prevent glucose crystallization during the measurement).

Permittivity of the samples containing moisture was measured at 25°C as a function of frequency, immediately after placing the cake (pre-equilibrated at 23%, 43%, 60%, or 75% RH) on the lower electrode (500 N).

## Determination of $T_{1\rho}$ by $^{13}C$ Solid State NMR

The  $T_{1\rho}$  of methine carbon in the IMT backbone (Fig. 1) was determined for the peak at 70 ppm (Fig. 2) at temperatures ranging from 5° to 65°C

**Figure 1.** Repeated unit of  $\alpha$ -glucose series.



**Figure 2.** NMR spectrum of lyophilized IMT equilibrated at 43% RH, measured at 25°C and 1 ms of spin-locking duration.

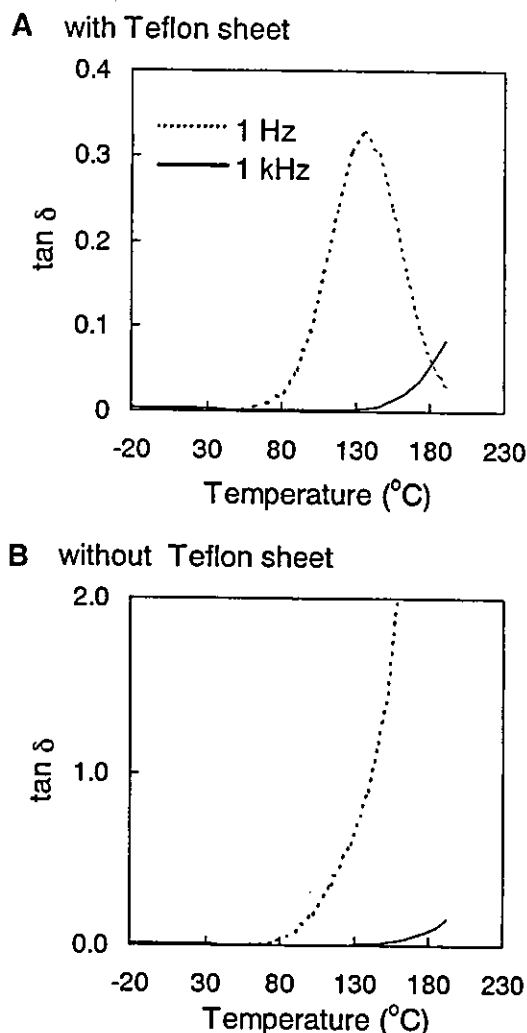
with freeze-dried IMT equilibrated at 43% and 60% RH, using a UNITY plus spectrometer operating at a proton resonance frequency of 400 MHz (Varian Inc., Palo Alto, CA), as previously described.<sup>11</sup> Spin-locking field was equivalent to 57 kHz. The rotor size was 7 mm and spinning speed was 4 kHz. Signal decay was describable with a mono-exponential equation.

## RESULTS AND DISCUSSION

### Molecular Motions Observed by DES

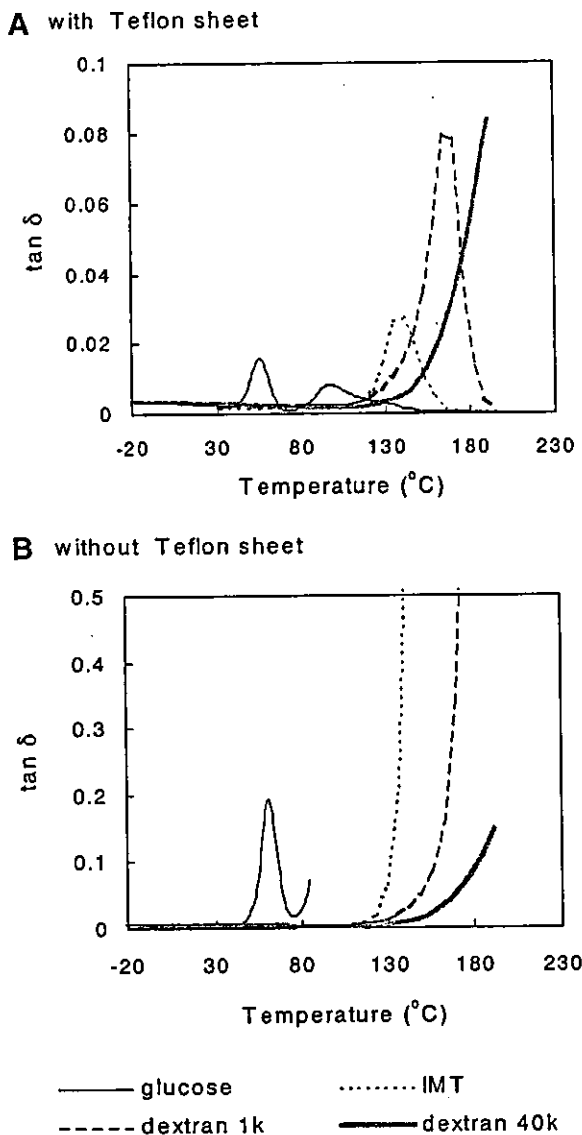
Figure 3 shows the temperature dependence of the  $\tan \delta$  observed for freeze-dried dextran 40k. The  $\tan \delta$  measured with a Teflon sheet inserted is also shown in Figure 3. The  $\tan \delta$  exhibited a peak around 130°C when measured at 1 Hz with a Teflon sheet inserted (Fig. 3A), but increases in  $\tan \delta$  were only observed in the absence of the Teflon sheet (Fig. 3B). The peak for  $\beta$ -relaxation of dextran 40k is expected to appear below 27°C at 1 kHz, because  $\beta$ -process was observed at 27°C for a film of a larger molecular weight dextran.<sup>16</sup> Therefore, the changes in  $\tan \delta$  observed at 1 kHz (Fig. 3A), the peak of which is expected to appear above 200°C, were attributed to another process that is slower than the  $\beta$ -process.

Figure 4 shows the temperature dependence of  $\tan \delta$  observed at 1 kHz for freeze-dried  $\alpha$ -glucose, IMT, and dextran 1k, together with that observed for freeze-dried dextran 40k. Freeze-dried IMT



**Figure 3.** Temperature dependence of  $\tan \delta$  measured for freeze-dried dextran 40k with (A) and without (B) a Teflon sheet at 1 Hz (dashed line) and 1 kHz (solid line).

and dextran 1k exhibited a peak with a Teflon sheet inserted, around 140° and 170°C, respectively (Fig. 4A). This peak was not observed in the absence of the Teflon sheet (Fig. 4B). The increases in  $\tan \delta$  with increasing temperature observed without the Teflon sheet may be attributable to the quasi-dc polarization (referring to low-frequency dispersion accompanied by abnormal increases in permittivity), as reported for hydrated granular or porous solids.<sup>23</sup> This was thought to be because both  $\epsilon'$  and  $\epsilon''$  measured as a function of frequency revealed a substantial increase in the low-frequency region (data not shown). Insertion of a Teflon sheet to inhibit charge transfer into the



**Figure 4.** Temperature dependence of  $\tan \delta$  measured for freeze-dried  $\alpha$ -glucose, IMT, dextran 1k, and dextran 40k with (A) and without (B) a Teflon sheet at 1 kHz.

electrode enabled the observation of quasi-dc polarization as a peak, in a similar manner as the Maxwell-Wagner process reported for a heterogeneous dielectric mixture.<sup>18</sup>

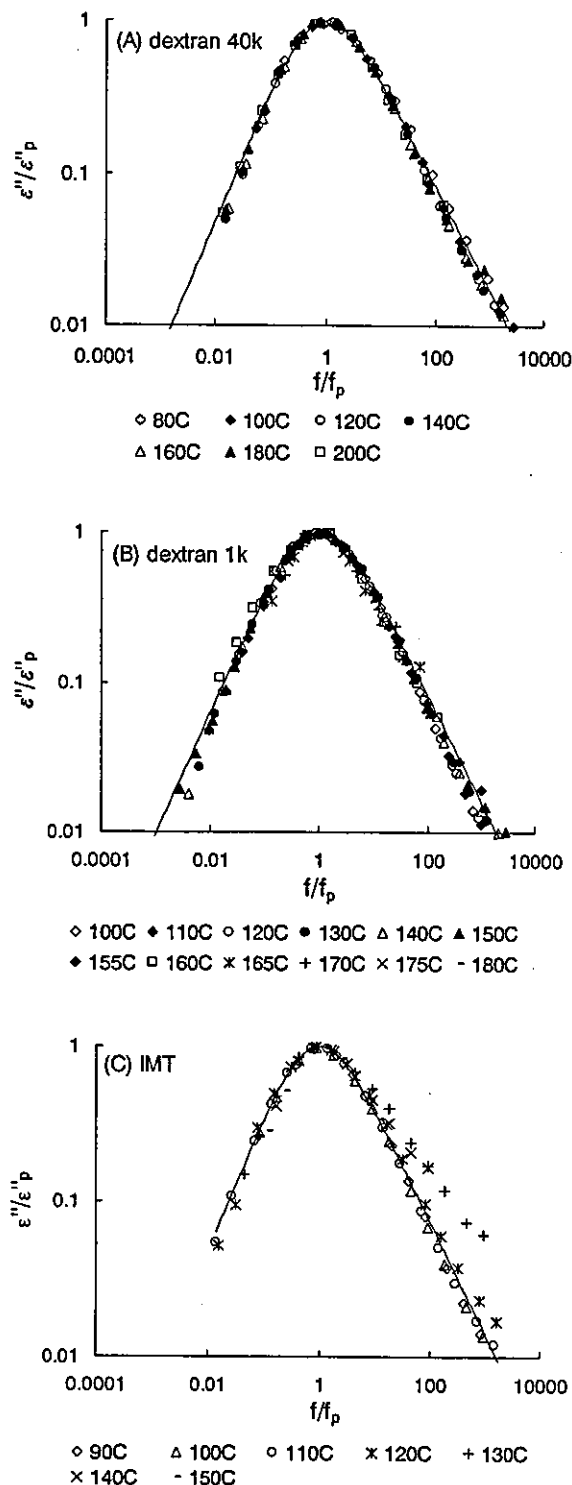
The mechanism of the observed quasi-dc polarization remains unclear. Quasi-dc polarization has been ascribed to diffusion of ions over the surface of the solid, as reported for hydrated mannitol,<sup>17</sup> or to the proton hopping reported for hydrated proteins.<sup>18,24,25</sup> However, the quasi-dc process observed for freeze-dried  $\alpha$ -glucose series

cannot be attributed to the hopping of protons in the ionizable carboxylic group as observed in a hydrated protein. A possible explanation for the observed quasi-dc polarization is that a proton-hopping-like process occurs in the hydrogen-bonded network constructed between hydroxy groups in the glucose unit in the form of clusters. However, there still seems to be a question whether such proton-hopping-like process is possible in the nearly dry state.

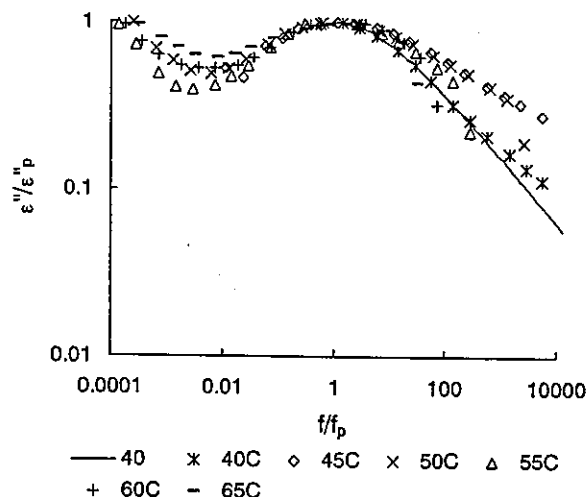
The frequency at which  $\epsilon''$  shows a maximum value depends on the thickness and permittivity of the sheet used, but can be regarded as a measure for comparing the mean relaxation time of the observed Maxwell-Wagner-like process, namely, proton-hopping-like process. In the freeze-dried IMT, dextran 1k, and dextran 40k, the  $\epsilon''$  value normalized to that observed at the peak ( $\epsilon''_p$ ) exhibited a similar shape of dielectric spectrum, as shown in Figure 5. The shape of dielectric spectrum did not vary significantly with changing the temperature both below and above the  $T_g$ . The dielectric spectra obtained for the freeze-dried IMT showed relatively large scattering.

Freeze-dried  $\alpha$ -glucose exhibited two peaks when a Teflon sheet was inserted (Fig. 4A). The peak for  $\tan \delta$  around  $100^{\circ}\text{C}$  was not observed in the absence of the Teflon sheet, in a similar manner as the peaks observed for IMT, dextran 1k, and dextran 40k (Fig. 4B). In contrast, the  $\tan \delta$  peak around  $50^{\circ}\text{C}$  was not affected by the presence of a Teflon sheet. The peak of  $\epsilon''$  corresponding to this  $\tan \delta$  peak was observed only at temperatures above  $T_g$ , when measured in the range from 0.01 Hz to 100 kHz, as shown in Figure 6. Furthermore, the distribution of the  $\epsilon''$  peak was larger than that of proton-hopping-like process observed for IMT, dextran 1k, and dextran 40k. These results suggest that the peak observed for  $\tan \delta$  is attributed to  $\alpha$ -relaxation—molecular rearrangement motions that are related to the instability of freeze-dried formulations. Noel et al.<sup>14</sup> observed the  $\alpha$ -relaxation of  $\alpha$ -glucose at 1 kHz and at  $70^{\circ}\text{C}$ , which was higher than the temperature reported herein. This difference in temperature may be attributable to the difference in the water content of the samples.

The other peak observed around  $100^{\circ}\text{C}$  (Fig. 4A) seems to be attributed to a charge transfer process, similar to the proton-hopping-like process observed for IMT, dextran 1k, and dextran 40k, because it was not observed without the Teflon sheet (observed  $\epsilon'$  values were negative at temperatures above  $85^{\circ}\text{C}$ ) (Fig. 3B). However, the



**Figure 5.** Normalized plot of imaginary permittivity for the Maxwell-Wagner-like process observed in freeze-dried dextran 40k (A), dextran 1k (B) and IMT (C) with a Teflon sheet inserted.



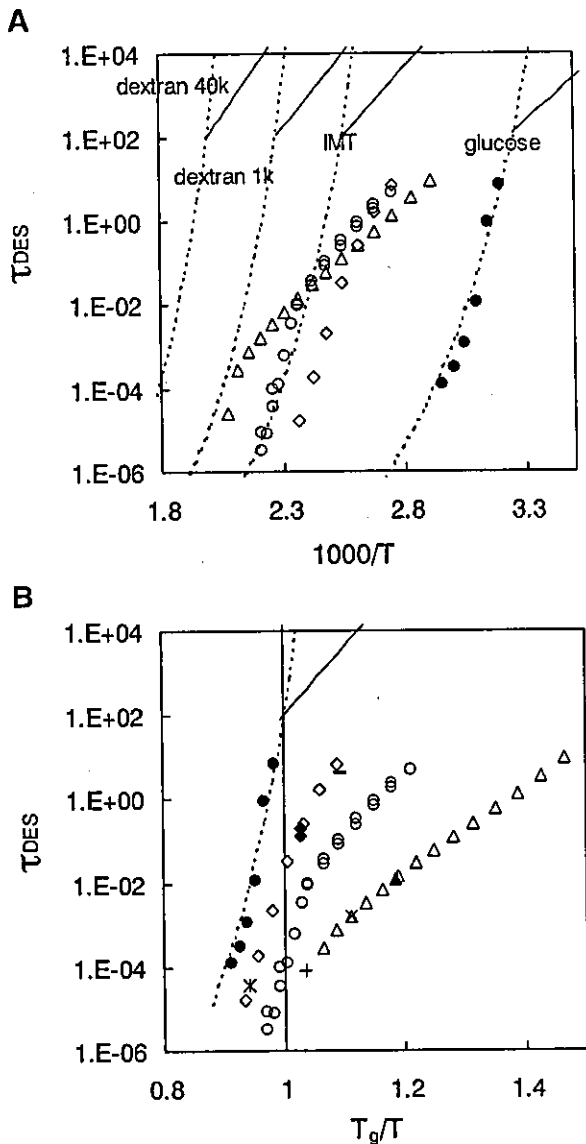
**Figure 6.** Normalized plot of imaginary permittivity for  $\alpha$ -relaxation observed in freeze-dried  $\alpha$ -glucose with a Teflon sheet inserted.

mechanism for this charge transfer process may be different from that for the proton-hopping-like process observed for IMT, dextran 1k, and dextran 40k. This was thought to be because this process occurred above  $T_g$ , whereas the proton-hopping-like process for IMT, dextran 1k, and dextran 40k occurred below  $T_g$ .

#### Temperature Dependence of Relaxation Time Determined by DES

Figure 7A shows the temperature dependence of the relaxation time of the proton-hopping-like process ( $\tau_{DES}$ ) observed for the freeze-dried dextran 40k, dextran 1k, and IMT, as well as that of the dielectric relaxation time of  $\alpha$ -relaxation observed for the freeze-dried  $\alpha$ -glucose. Figure 7A also shows the relaxation time of the molecular rearrangement motions in the freeze-dried  $\alpha$ -glucose series, calculated from the observed  $T_g$  values according to the AGV (solid line) and VTF (dotted line) equations below and above  $T_g$ , respectively. The assumption that the fictive temperature ( $T_f$ ) is equal to  $T_g$  (newly formed glass) and the fragility parameter ( $m$ ) is equal to 70 was made, as reported for amorphous indomethacin.<sup>26</sup> The calculated relaxation time below  $T_g$  was not affected by varying the  $m$  value from 40 to 100, whereas that above  $T_g$  was largely dependent on the  $m$  value.<sup>27</sup>

The dielectric relaxation time observed for the freeze-dried  $\alpha$ -glucose appeared to reach approximately 100 s at  $T_g$ , confirming that  $\alpha$ -relaxation is



**Figure 7.** Relaxation time of the proton-hopping-like process observed in freeze-dried dextran 40k ( $\Delta$ ), dextran 1k ( $\circ$ ) and IMT ( $\diamond$ ), and that of  $\alpha$ -relaxation observed in freeze-dried  $\alpha$ -glucose ( $\bullet$ ) determined with dry samples at various temperatures, plotted against  $1000/T$  (A) and  $T_g/T$  (B). Solid and dotted lines represent the relaxation time of molecular rearrangement motions calculated according to the AGV and VTF equations, respectively. (B) Also encloses the relaxation time determined with dextran 40k at 25°C and 43% RH ( $\blacktriangle$ ), 60% RH ( $\times$ ), and 75% RH ( $+$ ), and that determined with IMT at 25°C and 23% RH ( $-$ ), 43% RH ( $\blacklozenge$ ), and 60% RH ( $\ast$ ).

responsible for this process. This is further supported by the finding that the observed dielectric relaxation time was almost coincident with the relaxation time of molecular rearrangement motions calculated using the VTF equation, although the relaxation time is only an approximation due to the assumed  $m$  value.

The  $\tau_{DES}$  observed for the freeze-dried dextran 40k, dextran 1k, and IMT was shorter than the calculated relaxation time of molecular rearrangement motions (Fig. 7A), indicating that the proton-hopping-like process is faster than molecular rearrangement motions. A plot of the  $\tau_{DES}$  versus the temperature scaled to  $T_g$  in order to conceal the effect of differing  $T_g$  values (Fig. 7B), was not in good agreement with the data obtained from different molecular weights. As molecular weight increased, the  $\tau_{DES}$  at  $T_g$  decreased and the difference between the  $\tau_{DES}$  and the relaxation time of molecular rearrangement motions increased. These results suggest that the rate of the proton-hopping-like process is determined not only by the molecular mobility indicated by the  $T_g$  value. If the observed quasi-dc process is assumed to be due to the proton-hopping-like process occurring in clusters constructed between hydroxy groups in the glucose unit as described above, the rate of the process may be affected by the cluster size and distance between clusters, which may depend on the molecular weight. Thus, the difference in molecular weight may result in a difference in the rate of proton-hopping-like process at  $T_g$  in addition to a difference in  $T_g$  values.

Figure 7B also shows the  $\tau_{DES}$  observed for freeze-dried dextran 40k and IMT containing moisture. The temperature dependence of the  $\tau_{DES}$  was not affected by the presence of moisture, and changes in the  $\tau_{DES}$  were attributable only to changes in  $T_g$ . These findings suggest that moisture does not affect the rate of proton-hopping-like process itself, but enhances the process by increasing the molecular mobility as indicated by  $T_g$ .

As shown in Figure 7B, the slope of the  $\tau_{DES}$  versus  $T_g/T$  plot increased as the temperature approached  $T_g$  from a lower temperature. These changes in temperature dependence were similar to those observed for the relaxation time of molecular rearrangement motions (line in Fig. 7). This similarity in the temperature dependence suggests that the proton-hopping-like process is linked to molecular rearrangement motions such that changes in the temperature dependence of the latter process around  $T_g$  result in changes in the

temperature dependence of the former process. Thus, enhancement of molecular rearrangement motions may enhance the proton-hopping-like process. The close relationship between the proton-hopping-like process and molecular rearrangement motions seems to be confirmed by the finding that enhancement in the proton-hopping-like process upon absorption of moisture can be explained only by the decrease in  $T_g$ , as described above.

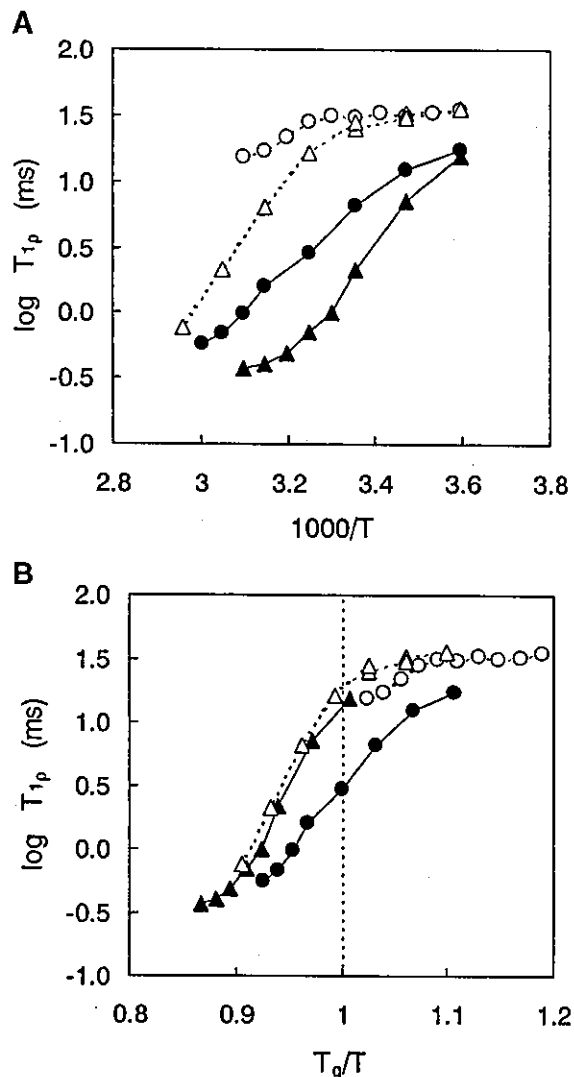
#### Temperature Dependence of Correlation Time Determined by NMR Relaxation Measurement

Figure 8 shows the temperature dependence of the  $T_{1\rho}$  measured for backbone carbon in the freeze-dried IMT. The  $T_{1\rho}$  for backbone carbon in the freeze-dried dextran 40k reported previously<sup>11</sup> is also shown in the figure.  $T_{1\rho}$  is plotted against  $1/T$  in Figure 8A and against the temperature scaled to  $T_g$  in Figure 8B. Decreases in  $T_{1\rho}$  with increasing temperature were observed for both freeze-dried IMT and dextran 40k even at temperatures below  $T_g$ . These shapes of temperature dependence suggest that  $T_{1\rho}$  can detect the enhancement of molecular rearrangement motions associated with glass transition partially occurring even at temperatures below  $T_g$ .

Correlation time  $\tau_c$  can be calculated from the observed  $T_{1\rho}$  according to eq. (1) if the minimum value of  $T_{1\rho}$  is known.<sup>28</sup>

$$\frac{1}{T_{1\rho}} = \frac{A\tau_c}{1 + 4\omega_1^2\tau_c^2} \quad (1)$$

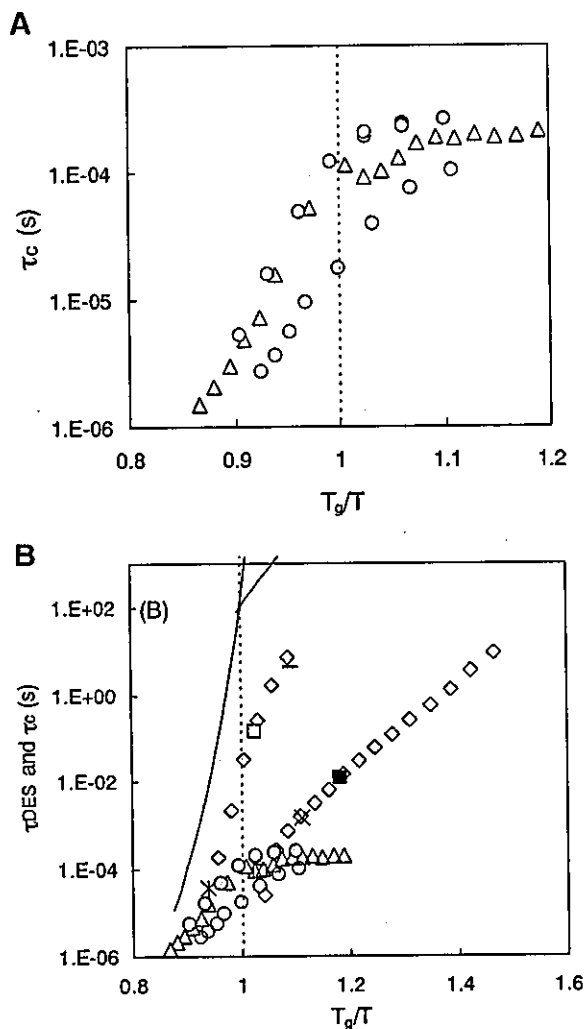
where  $\omega_1$  is the strength of spin-locking, and  $\omega_1^2\tau_c^2$  is equal to 0.5 when  $T_{1\rho}$  is the minimum. The change in  $T_{1\rho}$  for the freeze-dried IMT equilibrated at 60% RH decreased at high temperatures, and  $T_{1\rho}$  appeared to approach the minimum value. The value of  $A$  in eq. (1) was calculated assuming that the minimum value of  $T_{1\rho}$  can be approximated from the smallest value of  $T_{1\rho}$  measured at 60% RH, and used in the calculation of  $\tau_c$ . Because  $T_{1\rho}$  minimum was not observed for freeze-dried dextran 40k even at 75% RH, the value of  $A$  for freeze-dried dextran 40k was calculated from the minimum value of  $T_{1\rho}$  measured at 86% RH, which had been reported previously.<sup>11</sup> The  $\tau_c$  value calculated for dextran 40k and IMT is plotted against the temperature scaled to  $T_g$  in Figure 9A, and compared with the relaxation time of the proton-hopping-like process ( $\tau_{DES}$ ) in Figure 9B. The relaxation time of molecular rearrangement motions determined



**Figure 8.**  $T_{1\rho}$  of freeze-dried IMT equilibrated at 43% RH ( $\Delta$ ) and 60% RH ( $\blacktriangle$ ), plotted against  $1000/T$  (A) and  $T_g/T$  (B).  $T_{1\rho}$  of freeze-dried dextran 40k at 60% RH ( $\circ$ ) and 75% RH ( $\bullet$ ) reported previously<sup>11</sup> is shown.

calorimetrically is also shown in Figure 9B. The  $\tau_c$  value derived from  $T_{1\rho}$  was shorter than the relaxation time of molecular rearrangement motions for both freeze-dried IMT and dextran 40k.

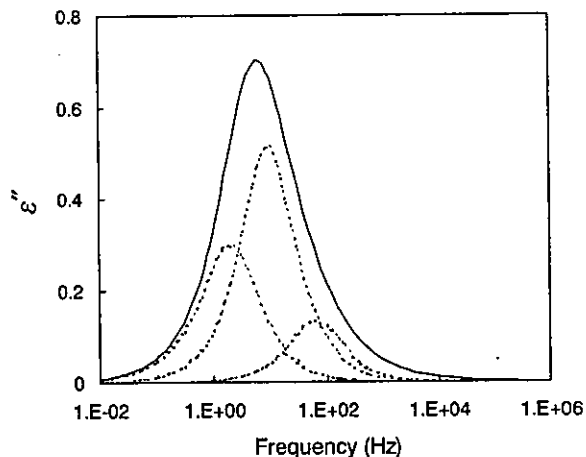
The  $T_{1\rho}$ -derived  $\tau_c$  for the freeze-dried IMT and dextran 40k was on nearly the same order as the  $\tau_{DES}$  around a  $T_g/T$  of 0.95 and 1.06, respectively, and was much smaller than the relaxation time of molecular rearrangement motions determined calorimetrically. As the  $T_g/T$  increased above 0.95 and 1.06, the  $T_{1\rho}$ -derived  $\tau_c$  for the freeze-dried IMT and dextran 40k, respectively, diverged significantly from the plots for the  $\tau_{DES}$ . A similar



**Figure 9.** (A)  $\tau_c$  for the methine carbon of dextran 40k at 60% RH ( $\Delta$ ), dextran 40k at 75% RH ( $\circ$ ), IMT at 43% RH ( $\circ$ ), and IMT at 60% RH ( $\Delta$ ) calculated from the observed value of  $T_{1\rho}$ . (B) Comparison of the  $\tau_c$  of methine carbon with the  $\tau_{DES}$  of the proton-hopping-like process for dextran 40k ( $\diamond$ ) and IMT ( $\diamond$ ) determined with dry samples at various temperatures, as well as  $\tau_{DES}$  determined for dextran 40k at 25°C and 43% RH ( $\blacksquare$ ), 60% RH ( $\times$ ), 75% RH ( $+$ ), and  $\tau_{DES}$  determined for IMT at 25°C and 23% RH ( $-$ ), 43% RH ( $\square$ ), and 60% RH ( $*$ ). The relaxation time of molecular rearrangement motion determined calorimetrically (solid line) is also shown.

divergence of  $T_{1\rho}$ -derived  $\tau_c$  from a DES correlation time at temperatures below  $T_g$  has been reported for glucose.<sup>15</sup>

To elucidate the reason for the divergence of the  $T_{1\rho}$ -derived  $\tau_c$  from the plots for the  $\tau_{DES}$ , the  $\tau_{DES}$  value determined from the dielectric spectrum



**Figure 10.** Dielectric relaxation spectrum of freeze-dried IMT at 120°C (solid line). Dashed lines represent three components of the Debye relaxation used for the transformation of  $\tau_{DES}$  to  $T_{1\rho}$ .

reflecting the proton-hopping-like process was transformed to the corresponding  $T_{1\rho}$  value and compared with the experimentally determined  $T_{1\rho}$ . The dielectric spectrum obtained for the freeze-dried IMT was first divided into three components of the Debye relaxation, and the three sets of  $\tau$  and its fraction ( $f$ ) were then calculated according to eq. (2).<sup>29</sup> An example of the imaginary permittivity ( $\epsilon''$ ) versus frequency plots used for the analysis is shown in Figure 10.

$$\epsilon'' = \sum_{i=1}^3 \frac{\Delta\epsilon_i \omega \tau_i}{1 + \omega^2 \tau_i^2} \quad (2)$$

$$\text{where } \Delta\epsilon_i = f_i(\Delta\epsilon_1 + \Delta\epsilon_2 + \Delta\epsilon_3)$$

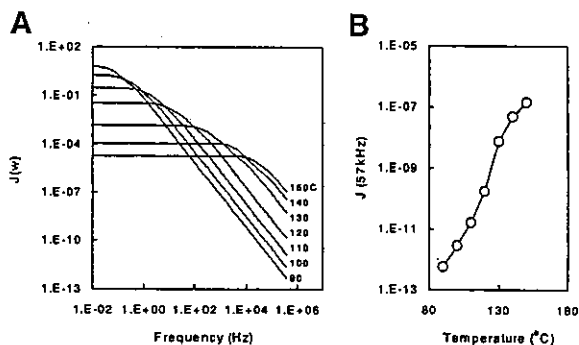
The three sets of  $\tau$  and  $f$  obtained were used to calculate the spectral density  $J(\omega)$  according to eq. (3).<sup>28</sup>

$$J(\omega) = \sum_{i=1}^3 \frac{f_i \tau_i}{1 + 4\omega^2 \tau_i^2} \quad (3)$$

Calculated  $J(\omega)$  at temperatures ranging from 90° to 150°C are shown as a function of frequency in Figure 11A. Figure 11B shows the temperature dependence of  $J$  at 57 kHz, which corresponds to the  $\omega$  value of the NMR instrument used in this investigation. The value of  $T_{1\rho}$  corresponding to the  $\tau_{DES}$  was calculated from the obtained  $J$  according to eq. (4),<sup>28</sup> and the result is shown in Figure 12A.

$$\frac{1}{T_{1\rho}} = AJ = \frac{1 + 4\omega_1^2 \tau_c^2}{T_{1\rho(\min)} \tau_c} \quad (4)$$

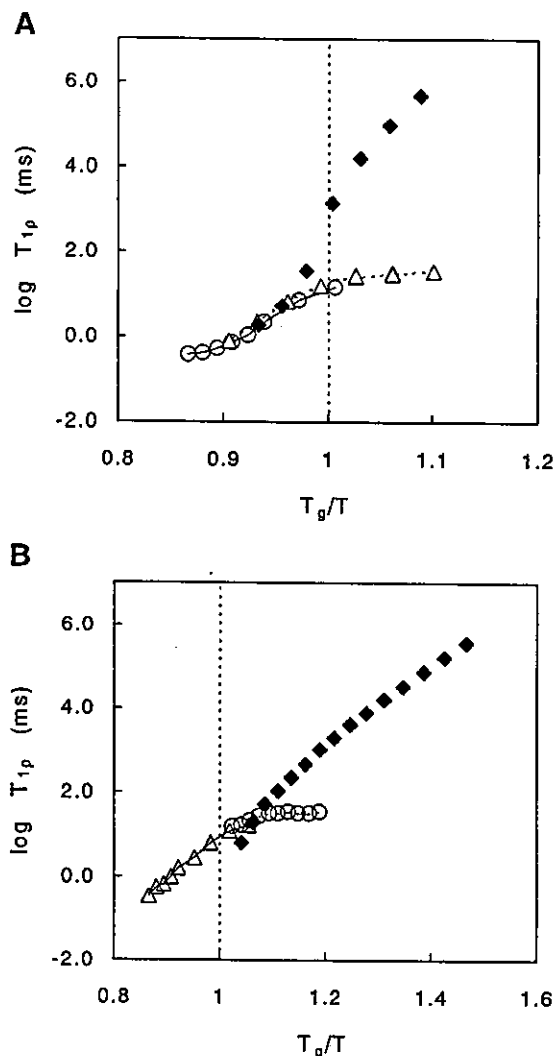




**Figure 11.** Spectral density calculated from dielectric relaxation spectrum of freeze-dried IMT as a function of frequency (A) and at 57 kHz as a function of temperature (B).

where  $\omega^2\tau_c^2$  was assumed to be equal to 0.5. The  $T_{1\rho}$  value corresponding to the  $\tau_{DES}$  for the freeze-dried dextran 40k was calculated from the dielectric spectrum in a similar manner as that of IMT. However, the dielectric spectrum was divided into two rather than three components, because the distribution of the spectrum for dextran 40k was smaller than that for IMT. The value of  $T_{1\rho}$  calculated from dielectric spectrum (DES-derived  $T_{1\rho}$ ) is shown in Figure 12B.

Figure 12 encloses the experimentally determined  $T_{1\rho}$  value compared with the DES-derived  $T_{1\rho}$  value. The measured  $T_{1\rho}$  was close to the DES-derived  $T_{1\rho}$  around a  $T_g/T$  of 0.95 and 1.06 for freeze-dried IMT and dextran 40k, respectively. However, as the  $T_g/T$  increased above these values, the measured  $T_{1\rho}$  diverged from the DES-derived  $T_{1\rho}$  and approached a plateau on the order of 10 ms. This plateau for the measured  $T_{1\rho}$  is thought to be due to static spin-spin interactions that affect  $T_{1\rho}$ . Namely,  $T_{1\rho}$  is determined predominantly by static factors rather than by molecular mobility at lower temperatures,<sup>30</sup> at which molecular mobility is very low. As temperature increases above a certain point, the contribution of molecular mobility to  $T_{1\rho}$  increases to a degree comparable to static spin-spin interactions, and begins to decrease the measured value of  $T_{1\rho}$ . The critical temperature at which the increase in molecular mobility begins to be reflected in  $T_{1\rho}$  is considered to be the  $T_{mc}$  defined previously as the  $T_g$  determined by the NMR relaxation time.<sup>9</sup> For freeze-dried dextran 40k, the  $T_g/T$  value of 1.06 was close to that of 1.07 calculated for a sample with a  $T_g$  of 58°C and a  $T_{mc}$  of 35°C (60% RH), and to that of 1.05 calculated for a sample with a  $T_g$  of 35°C and a  $T_{mc}$  of 20°C



**Figure 12.**  $T_{1\rho}$  observed at 43% RH ( $\Delta$ ) and 60% RH ( $\circ$ ) for freeze-dried IMT (A) and that at 60% RH ( $\circ$ ) and 75% RH ( $\Delta$ ) for freeze-dried dextran 40k (B), compared with  $T_{1\rho}$  estimated based on the spectral density calculated from dielectric spectrum ( $\blacklozenge$ ).

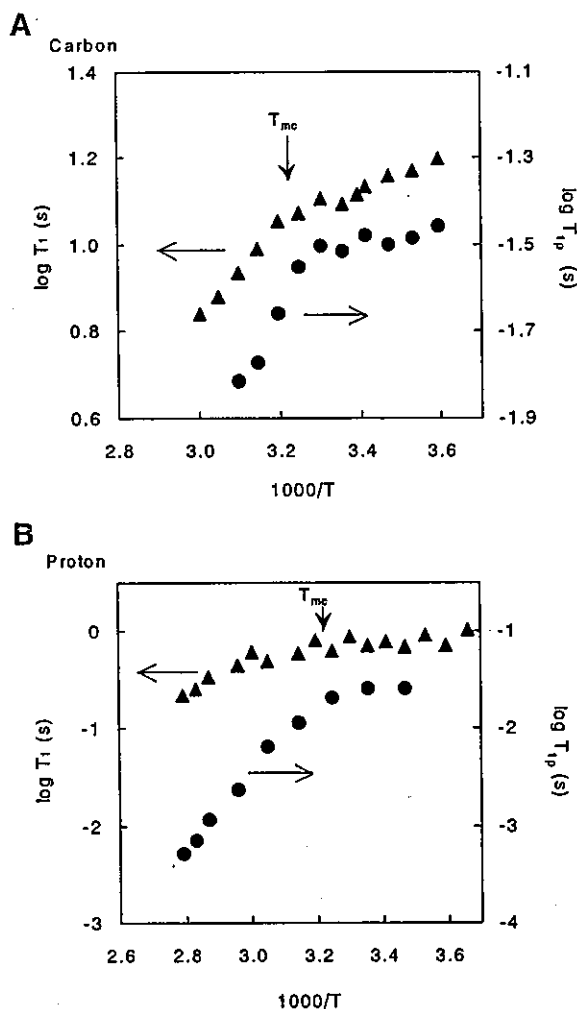
(75% RH). These findings suggest that  $T_{1\rho}$  cannot reflect molecular mobility at temperatures below  $T_{mc}$ .

In contrast to the freeze-dried dextran 40k, freeze-dried IMT exhibited a DES-derived  $T_{1\rho}$  much larger than the measured  $T_{1\rho}$  even at temperatures around  $T_g$ , at which  $T_{1\rho}$  is expected to reflect molecular mobility. This difference between IMT and dextran 40k suggests that a decrease in molecular weight decreases the rate of the proton-hopping-like process. Thus, even at  $T_g$ , the timescale of the proton-hopping-like process

in freeze-dried IMT may become on the order larger than 10 ms, at which  $T_{1\rho}$  cannot reflect the rate of process.

Molecular motions in freeze-dried IMT and dextran 40k were too slow to be reflected in  $T_{1\rho}$  at temperatures well below  $T_g$  such that the measured  $T_{1\rho}$  diverged significantly from the DES-derived  $T_{1\rho}$  (Fig. 12). However, the coincidence of the measured  $T_{1\rho}$  and the DES-derived  $T_{1\rho}$  around a  $T_g/T$  of 0.95 and 1.06 (although the range is very small) suggests that the timescale of molecular motion reflected in  $T_{1\rho}$  is on the same order as that of the proton-hopping-like process. As shown in Figure 7, the  $\tau_{DES}$  decreased rapidly as temperature approached  $T_g$ , showing a similar shape of temperature dependence to that of molecular rearrangement motions. The  $T_{1\rho}$ -derived  $\tau_c$  also showed a similar shape of temperature dependence (Fig. 9). The similarity of the temperature-dependence patterns suggests that the molecular motions reflected in  $T_{1\rho}$  are linked to molecular rearrangement motions, such that enhancement of molecular motions reflected in  $T_{1\rho}$ . Such linkage was also observed between molecular rearrangement motions and molecular motion reflected in  $\tau_{DES}$ , as described above (Fig. 7). Fujiwara and Nagayama<sup>31</sup> reported that the exact correlation functions expressed by multiexponential decay observed in the NMR relaxation of macromolecules were well approximated by a single exponential decay. This seems to support the interpretation that molecular rearrangement motions are linked to the molecular motions reflected in  $T_{1\rho}$  and  $\tau_{DES}$ . Because of this linkage between molecular motions, changes in the temperature dependence of molecular rearrangement motions related to the instability of amorphous formulations may be detected by measurement of  $T_{1\rho}$  and  $\tau_{DES}$ .

$T_{1\rho}$  of  $^{13}\text{C}$  in the polymer backbone was used as a measure of molecular mobility in the present investigation. However, previous investigations have demonstrated that changes in the temperature dependence for molecular rearrangement motions of freeze-dried dextran can be detected by the  $T_1$  of  $^{13}\text{C}$  in the polymer backbone,<sup>10</sup> as well as the  $T_1$  and  $T_{1\rho}$  of  $^1\text{H}$ .<sup>11</sup> Figure 13 compares the temperature dependence of  $T_{1\rho}$  of  $^{13}\text{C}$  in the dextran backbone described in this article with those of  $T_1$  of  $^{13}\text{C}$ , and  $T_1$  and  $T_{1\rho}$  of  $^1\text{H}$  reported previously. A change in the slope of the NMR relaxation time versus  $1/T$  plot was clearly shown around  $T_{mc}$  indicated by arrows. Thus, the shape of



**Figure 13.** Temperature dependence of  $T_1$  ( $\blacktriangle$ ) and  $T_{1\rho}$  ( $\bullet$ ) of methine carbon (A) and proton (B) in the freeze-dried dextran 40k observed at 60% RH.

temperature dependence of  $T_1$ , which reflects a smaller timescale of molecular motions than  $T_{1\rho}$ , was similar to that of  $T_{1\rho}$ . This finding suggests that molecular motion with a smaller timescale than the molecular motion reflected in  $T_{1\rho}$  is also linked to molecular rearrangement motions.

## CONCLUSIONS

DES was able to detect molecular rearrangement motions in freeze-dried  $\alpha$ -glucose. However, molecular rearrangement motions in freeze-dried dextran 40k, dextran 1k, and IMT were too slow to be observed by DES from 0.01 Hz to 100 kHz,

and proton-hopping-like process rather than molecular rearrangement motions was reflected in the dielectric spectra. The  $\tau_c$  for the backbone carbon of dextran 40k and IMT, calculated from the observed  $T_{1\rho}$  value, was found to be close to the relaxation time of proton-hopping-like process ( $\tau_{DES}$ ) at temperatures around  $T_g$ . The time-scales for molecular motions reflected in the  $\tau_c$  and  $\tau_{DES}$  were significantly smaller than that for molecular rearrangement motions, but the shapes of temperature dependence for the  $\tau_c$  and  $\tau_{DES}$  were similar to that of the relaxation time of molecular rearrangement motions. This suggests that molecular motions reflected in the  $\tau_{DES}$  and the  $\tau_c$  are linked to molecular rearrangement motions. Because of this linkage between molecular motions, changes in the temperature dependence of molecular rearrangement motions related to the instability of amorphous formulations may be detected by measurement of  $T_{1\rho}$  and  $\tau_{DES}$ .

This thought seems to address the question why changes in molecular rearrangement motions with a relaxation time longer than 100 s at sub- $T_g$  can be detected by measuring NMR relaxation times that reflect molecular motions on the order of 10 kHz and 1 MHz.

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## Effect of Freezing Rate on Physical Stability of Lyophilized Cationic Liposomes

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Factors affecting the storage stability of lyophilized cationic liposomes were investigated using liposomes prepared with various excipients and by different freezing rates, either quick freezing (freezing by immersion into liquid nitrogen) or slow freezing (cooling to  $-50^{\circ}\text{C}$  at a rate of  $-10^{\circ}\text{C}/\text{h}$ ). Increases in the particle size of cationic liposomes observed during freeze-drying were inhibited by the addition of sucrose, trehalose and sucrose-dextran mixtures (1:1 and 2:1 by weight). The storage instability of the formulations, as indicated by changes in particle size, was affected by their glass transition temperature ( $T_g$ ). Addition of high- $T_g$  excipients resulted in smaller increases in the particle size, indicating improvement of storage stability. The storage stability of cationic liposome formulations was also affected by freezing rate. Formulations prepared by slow freezing exhibited better stability. Longer shear relaxation times were observed for formulations prepared by slow freezing compared with those prepared by quick freezing. This indicates that formulations prepared by slow freezing have a lower matrix mobility, which may result in better storage stability.  $T_g$  or  $^1\text{H-NMR}$  relaxation measurements could not detect differences in matrix mobility between formulations prepared by different freezing rates. Shear relaxation measurements seem to be a useful method for evaluating the storage stability of cationic liposome formulations.

**Key words** cationic liposome; stability; lyophilization; shear relaxation time; molecular mobility

Cationic lipid has attracted much attention as a non-viral DNA vector. Cationic liposome-DNA complex (lipoplex), however, is usually unstable in solution and forms aggregates during long-term storage at room temperature, resulting in the loss of its transfection ability.<sup>1,2)</sup> Stability of lipoplex during freeze-drying and subsequent storage can be improved by lyophilization with lyoprotectants such as sucrose.<sup>3–5)</sup> The stabilizing effect of these compounds on liposomes during lyophilization has been attributed to incorporation of liposomes into glass matrices and hydrogen bonding between the excipient and the polar head group of lipids.<sup>6)</sup> Therefore, the storage stability of lyophilized cationic liposomes is expected to be improved by addition of excipients with higher glass transition temperature ( $T_g$ ), which yield glass matrices with a lower molecular mobility.

It is also known that process parameters such as freezing rate affect the stability of lyophilized formulations. It has been reported that the amount of drug entrapped in lyophilized liposomes is higher for formulations prepared by slow freezing than for those prepared by quick freezing.<sup>7)</sup> The effect of freezing rate depended on the lipid composition and was most pronounced for rigid liposomes. The storage stability of lyophilized tissue-type plasminogen activator was also affected by freezing rate.<sup>8)</sup> Faster freezing caused a larger surface area of the freeze-dried cake, and the storage stability of the incorporated protein was proportional to the surface area of the freeze-dried cake.

This paper describes the effects of excipients with different  $T_g$  as well as the effect of freezing rate on the storage stability of lyophilized cationic liposomes. Changes in the particle size of cationic liposomes were determined as a measure of storage instability.  $^1\text{H-NMR}$  relaxation time and shear relaxation time of the freeze-dried cakes were also determined. The storage stability of cationic liposomes is discussed in terms of the molecular mobility and visco-elastic property (matrix mobility) of the freeze-dried cakes.

### Experimental

**Materials** *N*-[1-(2,3-Dioleoyloxy) propyl]-*N,N,N*-trimethyl-ammonium chloride (DOTAP), cholesterol, sucrose, trehalose and dextran were purchased from Sigma (St. Louis, MO, U.S.A.).

**Preparation of Cationic Liposomes**<sup>3)</sup> DOTAP (250 mg) and cholesterol (125 mg) were dissolved in chloroform (about 5 ml). The chloroform was evaporated with a stream of nitrogen gas to make a thin film of the lipids. The film was further dried under vacuum for 1 h and hydrated in 50 ml of water. The lipid suspension was incubated at  $50^{\circ}\text{C}$  for 10 min and extruded through polycarbonate membranes (Millipore, Billerica, MA, U.S.A.) with pore sizes of 0.6 and 0.2  $\mu\text{m}$ , which were connected in series.

**Preparation of Lyophilized Liposome Formulations** Sucrose, trehalose and sucrose-dextran mixtures (1:1 or 2:1 by weight) were used as excipients for lyophilized liposome formulations. Equal volumes of excipient solution (10 w/w%) and liposome suspension were mixed. Aliquots (500  $\mu\text{l}$ ) of the mixture were frozen in polypropylene tubes by immersion into liquid nitrogen (quick freezing) or by cooling on the shelf of a freeze drier (Freezevac C-3; Tozai Tsusho, Tokyo) at a rate of  $-10^{\circ}\text{C}/\text{h}$  to  $-50^{\circ}\text{C}$  (slow freezing). Frozen samples were dried under a vacuum of approximately 5 Pa. The shelf temperature was maintained at  $-40^{\circ}\text{C}$  for 24 h,  $-20^{\circ}\text{C}$  for 16 h,  $0^{\circ}\text{C}$  for 6 h, and then  $20^{\circ}\text{C}$  for 6 h.

**Stability Study of Lyophilized Cationic Liposomes** The water content of the lyophilized formulations was adjusted by storage at  $25^{\circ}\text{C}$  and 23% RH for 1 d. The samples were stored at 40 or  $25^{\circ}\text{C}$  in a thermostatic chamber and withdrawn at appropriate intervals. The storage stability of liposomes was evaluated from changes in particle size. Particle size was determined at  $25^{\circ}\text{C}$  by dynamic light scattering with a DLS-7000 system (Otsuka Electronics Co. Ltd., Osaka). The viscosity of the rehydrated liposome suspension, required for the calculation of particle size, was determined with a model AR-1000 rheometer (TA Instruments, Inc., New Castle, DE, U.S.A.). The  $T_g$  values of the formulations are summarized in Table 1.

**Physicochemical Properties of Lyophilized Liposome Formulations.**  
**Determination of the Complex Shear Modulus** The visco-elastic property (matrix mobility) of the freeze-dried cakes was examined with the model AR-1000 rheometer. A freeze-dried cake was placed on the sample platform of the instrument and compressed to about 440  $\mu\text{m}$  thickness with a flat plate geometry (40 mm in diameter). An oscillating stress of 40 Pa was applied to the sample over a frequency range of 0.01 to 600 radian/s. The effect of sample size, which was smaller than the plate size of the geometry used, on the determination of shear relaxation time was considered to be negligible, since shear relaxation time was estimated from relative changes in the shear modulus determined as a function of stress frequency.

**$^1\text{H-NMR}$  Relaxation Measurements** In order to determine the molecu-

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lar mobility of the freeze-dried cakes, spin-lattice relaxation times in laboratory frame ( $T_1$ ) and rotating frame ( $T_{1\rho}$ ) were measured at 27°C with a model JNM-MU25 spectrometer (JEOL DATUM, Tokyo) operated at a  $^1\text{H}$  resonance frequency of 25 MHz. A freeze-dried cake was placed in an NMR tube (10 mm outer diameter) and dried at 25°C for 18 h under vacuum before measurement.  $T_1$  was measured by the inversion-recovery method. A spin locking field of 1 mT was applied to the samples for  $T_{1\rho}$  measurement.

**DSC Analysis** The  $T_g$  of freeze-dried cakes was determined with a model 2920 differential scanning calorimeter (TA Instruments, Inc.) equipped with a refrigerator cooling accessory. Temperature and heat flow calibration of the instrument was performed with indium. Approximately 3 mg of freeze-dried cake was weighed into a hermetic sample pan, which was stored at 25°C and 23% RH for 24 h and then sealed. Samples were heated at a rate of 20°C/min from -30 to 200°C. The DSC cell was purged with nitrogen gas at 30 ml/min during measurement.

**Water Vapor Sorption Measurements** Time profiles of water vapor sorption for lyophilized liposome formulations containing sucrose and trehalose were measured at 25°C and 10% RH with a GM-300 gravimetric sorption analyzer (VTI Corp., Hialeah, FL, U.S.A.). Approximately 25 mg of freeze-dried cake was placed in a sample holder and dried at 25°C under vacuum. When no change in the weight of the cake was observed over 5 min, the cake was considered to be in a dry state. The cake was exposed to water vapor equivalent to 10% RH. The weight of the cake was measured at intervals of 30 s.

## Results and Discussion

**Effect of Excipients on Stability of Lyophilized Cationic Liposomes** Figure 1 shows the effect of freeze-thaw and freeze-drying on the particle size of cationic liposomes. No significant difference in particle size was observed after the freeze-thaw cycle, regardless of the presence of excipients. Increases in the particle size, however, were observed after rehydration of liposomes lyophilized without excipient. On the other hand, liposomes lyophilized with excipients exhibited similar particle sizes before and after freeze-drying, indicating that all the excipients studied stabilized cationic liposomes against aggregation during drying.

Figure 2 shows the storage stability of cationic liposomes lyophilized with the excipients. Increases in particle size were observed during storage at 40°C except for the trehalose formulation prepared by slow freezing. The liposome

formulations containing sucrose, the  $T_g$  of which (32°C) was lower than the storage temperature, were least stable. The formulations containing trehalose, the  $T_g$  of which (49°C) was higher than the storage temperature, were more stable than the sucrose formulations. Addition of dextran to sucrose resulted in a higher  $T_g$  (43°C for 2:1 sucrose-dextran mixture and 60°C for 1:1 mixture) and increased the stability of the liposome formulations. These results indicate that the stability of cationic liposome formulations is closely correlated with the mobility of the formulation matrix as indicated by  $T_g$ . The formulation containing trehalose prepared by quick freezing exhibited increases in the particle size, but no significant increase was observed for the trehalose formulation prepared by slow freezing, indicating that the storage stability of lyophilized cationic liposomes is affected by the freezing rate.

Figure 3 shows a photograph of lyophilized cationic liposomes containing sucrose or trehalose stored at 25°C and 23% RH for 1 year. Shrinkage of the freeze-dried cake was observed for sucrose formulations. The extent of shrinkage was larger for the sample prepared by quick freezing. No significant shrinkage of freeze-dried cakes was observed for trehalose formulations and sucrose-dextran formulations (data not shown), which had  $T_g$  values higher than those of sucrose formulations. Figure 4 shows the particle size of liposomes after storage at 25°C and 23% RH for 1 year. The trehalose and sucrose-dextran formulations were stable, but an increase in the particle size was observed for sucrose formulations during storage at 25°C (approximately 8°C lower than the  $T_g$  of the sucrose formulation). These results indicate that the sucrose formulations have a sufficient degree of matrix mobility to cause liposome aggregation even at 25°C, a temperature lower than the  $T_g$  of the formulation.

### Molecular Mobility and Matrix Mobility of Cationic

Table 1.  $T_g$  of Lyophilized Cationic Liposome Formulations<sup>a)</sup>

Excipient	$T_g$ (°C) <sup>b)</sup>	
	Slow freezing	Quick freezing
Sucrose	32.3	31.9
Trehalose	48.4	49.0
Suc-Dex (2:1)	43.5	43.7
Suc-Dex (1:1)	59.7	59.9

a) Water content was adjusted by storage at 25°C and 23% RH for 1 d. b)  $T_g$  values reported were average of two determinations.

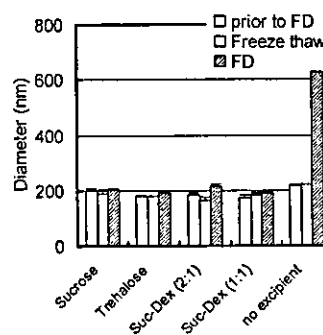


Fig. 1. Effect of Freeze-Thaw and Freeze-Drying on the Particle Size of Cationic Liposomes

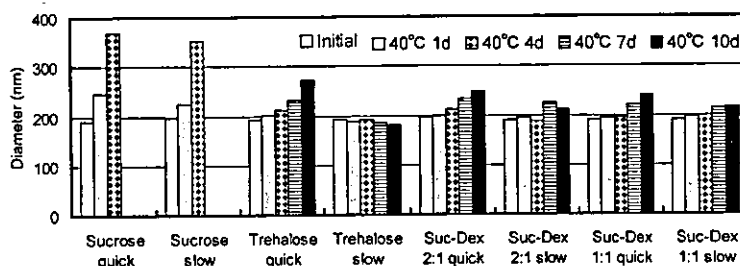


Fig. 2. Effect of Storage at 40°C on the Particle Size of Cationic Liposomes

**Liposome Formulations** The storage stability of lyophilized cationic liposomes was affected by freezing rate, such that formulations prepared by quick cooling were less stable than those prepared by slow cooling, as shown in Figs. 2—4. To gain an insight into the mechanism of the effect of freezing rate on the stability of cationic liposome formulations, the mobility of the formulation matrices was examined by <sup>1</sup>H-NMR relaxation and shear relaxation measurements. Table 2 shows the  $T_1$  and  $T_{1\rho}$  of cationic liposome formulations.  $T_1$  and  $T_{1\rho}$  are measures of molecular mobility on time

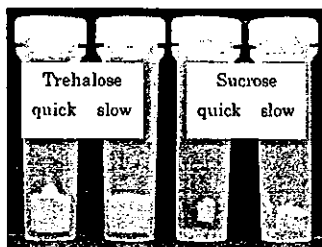


Fig. 3. Appearance of Freeze-Dried Cakes of Cationic Liposomes Containing Sucrose or Trehalose after Storage at 25 °C and 23% RH for 1 Year

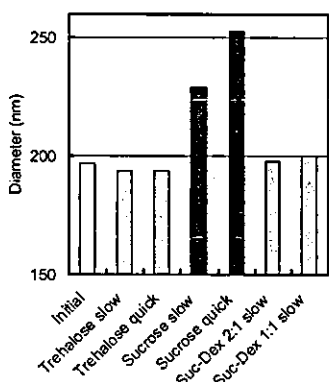


Fig. 4. Effect of Storage at 25 °C and 23% RH for 1 Year on Particle Size of Cationic Liposomes

Table 2. <sup>1</sup>H-NMR Relaxation Time of Cationic Liposome Formulations

Excipient		$T_1$ (s)	$T_{1\rho}$ (ms)
Sucrose	Slow	0.29	4.0
	Quick	0.28	4.0
Trehalose	Slow	0.28	4.4
	Quick	0.30	4.6

scales of the order of MHz and mid kHz, respectively.<sup>9)</sup>  $T_1$  and  $T_{1\rho}$  of the formulations did not change with freezing rate within experimental error, indicating that freezing rate has little effect on the molecular mobility reflected in  $T_1$  and  $T_{1\rho}$ .

The mobility of formulation matrices on longer time scales than those reflected in NMR spin-lattice relaxation times was determined by measuring the frequency dependence of the shear modulus of lyophilized cationic liposomes (the visco-elastic property of the formulations). The frequency-dependent shear modulus ( $G^*(\omega)$ ) of amorphous indomethacin has been shown to characterize the time scales of molecular motion of indomethacin in the amorphous state.<sup>10)</sup> The storage modulus ( $G'$ , a real part of  $G^*(\omega)$ ) of visco-elastic materials increases with increasing frequency of shear stress, and the loss modulus ( $G''$ , an imaginary part of  $G^*(\omega)$ ) exhibits a maximum. Visco-elastic materials behave as a viscous fluid and a rigid solid against low and high frequency stress, respectively. The shear relaxation time, the reciprocal of maximum frequency (in radian/s) of  $G''$ , can be used as a measure of mobility. Figure 5 shows the typical frequency dependence of the shear modulus of the cationic liposome formulation containing sucrose measured at 74 °C in the dry state. Figure 6 shows the temperature dependence of shear relaxation time for the cationic liposome formulations containing sucrose and trehalose measured under dry conditions. The relaxation time of the formulation prepared by slow freezing was longer than that of the formulation prepared by quick freezing. The effect of freezing rate on the shear relaxation time was also observed in the presence of moisture. Figure 7 shows the frequency dependence of  $G''$  for the cationic liposome formulation containing sucrose stored at 25 °C and 23% RH for 1 d. The formulation prepared by

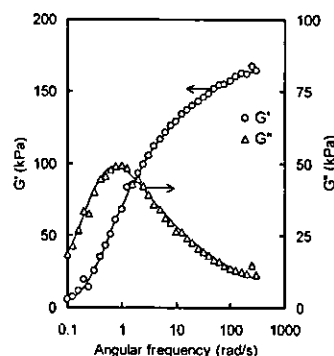


Fig. 5. Frequency Dependence of the Shear Modulus of a Cationic Liposome Formulation Containing Sucrose Measured at 74 °C in the Dry State

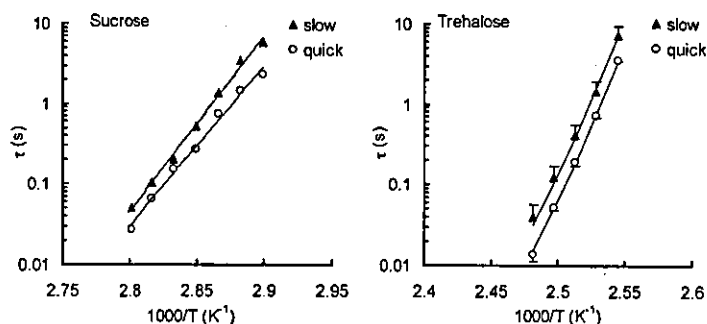


Fig. 6. Shear Relaxation Time of Cationic Liposome Formulations Measured under Dry Conditions

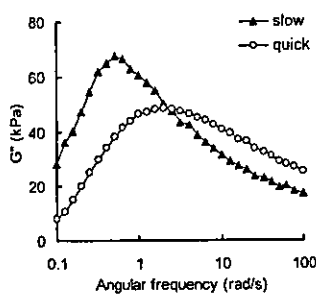


Fig. 7. Frequency Dependence of the Loss Modulus ( $G''$ ) of a Cationic Liposome Formulation Containing Sucrose at 30 °C

The water content was adjusted by storage at 25 °C and 23% RH for 1 d.

quick freezing exhibited a maximum at 2 radian/s, whereas the formulation prepared by slow freezing exhibited a maximum at 0.4 radian/s. These results indicate that formulation matrices prepared by slow freezing have a lower mobility than those prepared by quick freezing. Temperature dependence of  $G''$  supports this speculation.  $G''$  at a frequency of 0.1 radian/s showed a maximum at 21 °C for the sucrose formulation prepared by quick freezing, whereas 26 °C for the sucrose formulation prepared by slow freezing. The maximum temperature corresponds to the temperature at which relaxation time of 10 s (inverse of 0.1 radian/s) is observed. Therefore, the relaxation time at 25 °C is considered to be longer than 10 s for the sucrose formulation prepared by slow freezing. In contrast, shorter relaxation time than 10 s is expected for the sucrose formulation prepared by quick freezing. This difference in matrix mobility resulting from different freezing rates may cause a difference in storage stability. The difference in matrix mobility of cationic liposome formulations prepared by different freezing rates could be detected by shear relaxation time, but not by  $T_g$  and  $^1\text{H-NMR}$  relaxation measurements (Tables 1, 2). This finding suggests that the aggregation rate of lyophilized liposomes may correlate more closely with matrix mobility as measured by the shear relaxation time than with molecular mobility as measured by the spin-lattice relaxation time.

Figure 8 shows the time course of water vapor sorption for the cationic liposome formulations containing sucrose and trehalose stored at 25 °C and 10% RH. The water content at equilibrium was not affected by freezing rate. The weight of the formulations prepared by quick freezing, however, reached a plateau within 40 min, whereas it took more than 300 min to reach equilibrium for formulations prepared by slow freezing. The difference in water sorption rate indicates that the ratio of surface area to volume for the formulations prepared by quick freezing is larger than that for the formulations prepared by slow freezing. Similar differences in the surface area of freeze-dried cakes have been reported for lyophilized tissue-type plasminogen activator formulations.<sup>8)</sup> Such dependence of the surface area on freezing rate may cause the different mobility of the formulation matrices, as indicated by the different shear relaxation times. Formulations with larger surface area may be more susceptible to changes in the  $T_g$  upon local temperature fluctuations and/or local humidity fluctuations. Differences in the susceptibility may affect the shrinkages of freeze-dried cakes and the sta-

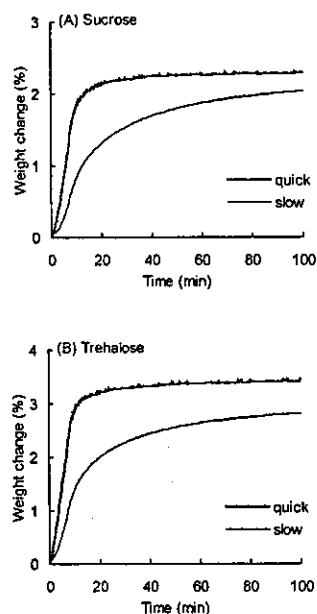


Fig. 8. Time Courses of Water Vapor Sorption of Cationic Liposome Formulations Stored at 25 °C and 10% RH

bility of liposomes during storage.

In conclusion, the storage instability of lyophilized cationic liposomes, as indicated by change in particle size, was affected by the  $T_g$  of the formulations. Formulations containing high- $T_g$  excipients exhibited better storage stability. The storage stability of lyophilized cationic liposomes was also affected by freezing rate. Longer shear relaxation times were observed for formulations prepared by slow freezing compared with those prepared by quick freezing, indicating that formulations prepared by slow freezing have a lower matrix mobility. The lower matrix mobility of the formulations prepared by slow freezing may result in better storage stability.  $T_g$  or  $^1\text{H-NMR}$  relaxation measurements could not detect these differences in matrix mobility. Shear relaxation measurements appear to be useful for evaluating the storage stability of cationic liposome formulations.

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# Ability of Polyvinylpyrrolidone and Polyacrylic Acid to Inhibit the Crystallization of Amorphous Acetaminophen

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**ABSTRACT:** The inhibition of crystallization of amorphous acetaminophen (ACTA) by polyvinylpyrrolidone (PVP) and polyacrylic acid (PAA) was studied using amorphous solid dispersions prepared by melt quenching. Co-melting with PVP and PAA decreased the average molecular mobility, as indicated by increases in glass transition temperature and enthalpy relaxation time. The ACTA/PAA dispersion exhibited much slower crystallization than the ACTA/PVP dispersion with a similar glass transition temperature value, indicating that interaction between ACTA and polymers also contributed to the stabilizing effect of these polymers. The carboxyl group of PAA may interact with the hydroxyl group of ACTA more intensely than the carbonyl group of PVP does, resulting in the stronger stabilizing effect of PAA. Dielectric relaxation spectroscopy showed that the number of water molecules tightly binding to PVP per monomer unit was larger than that to PAA. Furthermore, a small amount of absorbed water decreased the stabilizing effect of PVP, but not that of PAA. These findings suggest that the stronger stabilizing effect of PAA is due to the stronger interaction with ACTA. The ability of PAA to decrease the molecular mobility of solid dispersion was also larger than that of PVP, as indicated by the longer enthalpy relaxation time. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 93:2710–2717, 2004

**Keywords:** solid dispersion; crystallization; calorimetry (DSC); water sorption

## INTRODUCTION

The solubility and dissolution-related bioavailability of poorly water-soluble pharmaceuticals can be improved by preparing amorphous solids using spray drying, melt extrusion, melt quenching, milling, and so on. Because amorphous solids are physically unstable, various stabilizing methods have been reported, including the usage of polymer excipients having high glass transition temperature ( $T_g$ ). Crystallization of amorphous sucrose and indomethacin was inhibited by preparing solid dispersion with a small amount of polyvinylpyrrolidone (PVP).<sup>1–3</sup> Similarly, addition of alginate inhibited the crystallization of

amorphous lactose prepared by spray drying.<sup>4</sup> The stabilizing effect of these excipients was attributed to their antiplasticization effect as well as their ability to interact with sucrose, indomethacin, or lactose. Both antiplasticization and interaction effects of excipients were affected by water molecules.<sup>1–4</sup>

Drug–excipient interactions leading to the stabilization of amorphous drugs have also been reported for the systems of excipients with a pyrrolidone ring (PVP and polyvinylpyrrolidone-co-vinyl acetate) and drugs with hydrogen donor groups (indomethacin, lacidipine, nifedipine, and tolbutamide).<sup>5</sup> To elucidate how drug–excipient interactions contribute to the stabilization of amorphous drugs, it may be necessary to examine the effect of various excipients to stabilize a certain amorphous drug.

In this study, we examined the crystallization of acetaminophen (ACTA) in solid dispersions with PVP and polyacrylic acid (PAA) to gain further

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insight to the role of drug-polymer interaction in the inhibition of crystallization as well as the effect of moisture on the interaction. PVP and PAA, polyvinyl compounds with different side chains, may exhibit different modes of drug-polymer interaction, because the latter can be a proton donor as well as a proton acceptor. Different water-vapor sorption behaviors may also be expected between PVP having a pyrrolidone ring and PAA having a carboxylic group in the molecules. The effect of water on the crystallization of ACTA is discussed based on the physical properties of water absorbed into these polymers determined by dielectric relaxation spectroscopy (DRS) using time domain reflectometry.

## MATERIALS AND METHODS

### Materials

ACTA was obtained from Sigma Chemical Co. PVP with weight average molecular weight of 40,000, and PAA with weight average molecular weight of 25,000 were purchased from Wako Pure Chemical Industries Ltd. Other reagents were of analytical grade.

### Measurement of Isothermal Crystallization Rate of ACTA

Amorphous ACTA was prepared by melt quenching in a cell of a differential scanning calorimeter (DSC2920, TA Instruments) with a dry nitrogen gas purge at 20 mL/min. Indium was used to calibrate the cell constant and the temperature of the instrument. ACTA crystal (2–3 mg) in an aluminum pan was heated to 220°C at a heating rate of 20°C/min, kept at 220°C for 3 min, and then cooled to -70°C at a cooling rate of 35°C/min by pouring liquid nitrogen into the cooling jacket surrounding the cell. The samples were stored at a constant temperature (25–45°C) in desiccators containing P<sub>2</sub>O<sub>5</sub>. After certain periods of time, the sample pans were immediately sealed and the change in heat capacity ( $\Delta C_p$ ) at  $T_g$  was measured with a heating rate of 20°C/min. The ratio of amorphous form remaining at time  $t$ ,  $x(t)$ , was calculated according to eq. (1):

$$x(t) = \Delta C_{pt} / \Delta C_{p0} \quad (1)$$

where  $\Delta C_{pt}$  and  $\Delta C_{p0}$  are the changes in  $\Delta C_p$  at time  $t$  and initially, respectively. The decrease in  $x(t)$  as a function of storage time was analyzed according to the Avrami equation [eq. (2)] to

calculate the time required for 10% of amorphous ACTA to crystallize ( $t_{90}$ ).

$$x(t) = \exp(-kt^n) \quad (2)$$

where  $k$  is the crystallization rate constant, and  $n$  is the Avrami index related to the nucleation mechanism and the dimensionality of the growth process.

Amorphous ACTA/polymer solid dispersions were also prepared by melt quenching in a similar manner as amorphous ACTA samples. ACTA and polymer were dissolved in methanol, and the solvent was evaporated in a Teflon vessel by heating at 60°C on a hot-plate. The obtained mixtures were dried under vacuum at 60°C for 16 h, and then melted. Amorphous ACTA/polymer solid dispersions with moisture were prepared by melting and subsequently cooling the moistened mixture, which had been stored at 25°C under various relative humidities (RHs) for more than 72 h, as listed in Table 1. The aluminum pans containing the moistened mixture were crimped hermetically to avoid vaporization of sorbed water during the heating. The  $T_g$  values of the moist amorphous solid dispersions obtained were reproducible, as shown in Table 1.

Amorphous ACTA/polymer dispersions were stored at a constant temperature within the range of 25–60°C. The value of  $x(t)$  was calculated from  $\Delta C_p$  at  $T_g$ , then  $t_{90}$  was calculated by the curve-fitting of the  $x(t)$  time profiles in a similar manner as amorphous ACTA.

### Enthalpy Relaxation Measurements

Amorphous ACTA and amorphous ACTA/polymer solid dispersions were prepared in the same

Table 1.  $T_g$  of Amorphous ACTA/Polymer Dispersions

RH (%)	$T_g$ (°C)	
	ACTA/PVP (9:1)	ACTA/PAA (9:1)
0 (P <sub>2</sub> O <sub>5</sub> )	30.3 ± 0.3	31.3 ± 0.5
11 (LiCl · H <sub>2</sub> O)	23.7 ± 0.1	—
22 (CH <sub>3</sub> COOK)	—	16.5 ± 0.4
33 (MgCl <sub>2</sub> · 6H <sub>2</sub> O)	18.7 ± 0.1	14.2 ± 0.5
57 (NaBr · 2H <sub>2</sub> O)	13.8 ± 0.2	11.2 ± 0.3
76 (NaCl)	8.7 ± 0.3	—
86 (KCl)	3.2 ± 0.2	—

RH was controlled with salts described in parentheses. Data are mean ± SD ( $n > 3$ ).

manner as the samples prepared for the measurement of crystallization rates. Samples were then stored for 0–32 h at 6°C and 10°C (approximately 20°C below each  $T_g$ ), respectively. After cooling to approximately 100°C below the  $T_g$ , endothermic recovery was measured for the stored samples with a heating rate of 20°C/min. Crystallization of ACTA was not observed during the enthalpy relaxation measurement.

#### DRS of Water Absorbed in Polymers

DRS measurement was performed with lyophilized PVP and PAA. Two grams of 5–10% polymer solutions were frozen in polypropylene sample tubes (20-mm diameter) by immersion in liquid nitrogen for 10 min, and then dried at a vacuum level <5 Pa for 23.3 h in a lyophilizer (Freezvac 1CFS, Tozai Tsusho Co.). The shelf temperature was –40°C for the first hour, 20°C for the subsequent 19 h, and 35°C for the last 3.3 h. The lyophilized polymers were moistened in desiccators with a saturated salt solution (Tables 2 and 3) for >72 h, then the DRS was measured at frequencies between  $10^7$  and  $10^{10}$  Hz at 25°C by the time domain reflectometry method developed by Mashimo et al.<sup>6–9</sup> using a digitizing oscilloscope (54120B, Hewlett Packard). Complex permittivity was calculated using the software developed by Mashimo et al.<sup>6–9</sup> The water content of the samples was determined by the Karl Fischer method (Metrohm E684).

## RESULTS

#### Nonisothermal Crystallization of Amorphous ACTA and ACTA/Polymer Dispersions

The melting temperature ( $T_m$ ) of the ACTA crystal used was 167°C (Fig. 1a). Amorphous ACTA prepared by melt quenching showed a  $T_g$  at 26°C

(inflection point), an exothermic peak at approximately 90°C, and a melting endothermic peak at 155°C (Fig. 1b). These values of  $T_m$  and  $T_g$  were in agreement with data in the literature.<sup>10–12</sup>

A single  $T_g$  was observed in the amorphous solid dispersions prepared with PVP (Fig. 1c,d) or PAA (Fig. 1e,f,g), indicating complete miscibility of drug and polymer within the sensitivity limit of the differential scanning calorimetry (DSC) method. The exothermic crystallization peak observed for the ACTA/PAA dispersion disappeared in the presence of 5% PAA, whereas the ACTA/PVP solid dispersion showed an exothermic peak even in the presence of 10% PVP (at a heating rate of 20°C/min).

#### Isothermal Crystallization of Amorphous ACTA and ACTA/Polymer Dispersions

Figure 2 shows typical DSC thermograms of amorphous ACTA stored at 40°C. Three polymorphic forms have been reported for ACTA: form I with  $T_m$  at 168–169°C (stable form), form II with  $T_m$  at 157–158°C (metastable form), and form III which is unstable and easily transforms into form II at approximately 125°C.<sup>10–12</sup> The  $T_m$  of the ACTA sample stored at 40°C was 155°C, indicating that form II was formed. Similarly, the ACTA sample containing 10% PVP exhibited a  $T_m$  of 154°C corresponding to form II after storage at 40°C (data not shown). However, the amorphous solid dispersions containing 10% PAA exhibited two exothermic peaks (at approximately 100 and 150°C, respectively) and two endothermic melting peaks (127 and 163°C) after a sufficient period of isothermal storage, suggesting that forms III and I were formed. Decreases in both  $\Delta C_p$  at  $T_g$  and exothermic peak at the crystallization temperature indicate that crystallization occurred during storage. The crystallization rate was determined from the changes in  $\Delta C_p$  at  $T_g$ , and not from the

**Table 2.** Dielectric Relaxation Parameters for Water in PVP at 25°C

p/p <sub>0</sub>	Water (g/g polymer)	d (g/mL)	Low-frequency Process			High-frequency Process			$\epsilon_\infty$
			$\Delta\epsilon_1$	$\alpha$	log $\tau_1$	$\Delta\epsilon_h$	$\beta$	log $\tau_h$	
0.33 (MgCl <sub>2</sub> ·6H <sub>2</sub> O)	0.13	1.18	0.24	1.0	–8.9	0.53	1.0	–9.5	1.9
0.43 (K <sub>2</sub> CO <sub>3</sub> ·2H <sub>2</sub> O)	0.18	1.17	0.29	1.0	–8.8	0.63	1.0	–9.5	2.0
0.57 (NaBr·2H <sub>2</sub> O)	0.25	1.16	0.54	0.76	–8.7	1.3	1.0	–9.7	2.2
0.76 (NaCl)	0.38	1.14	2.1	0.59	–8.8	5.3	0.92	–9.7	3.5
0.86 (KCl)	0.50	1.13	4.6	0.24	–8.6	11	0.86	–9.8	3.8
0.98 (K <sub>2</sub> SO <sub>4</sub> )	0.82	1.11	7.0	0.25	–8.5	19	0.85	–10	3.6

RH was controlled with salts described in parentheses.

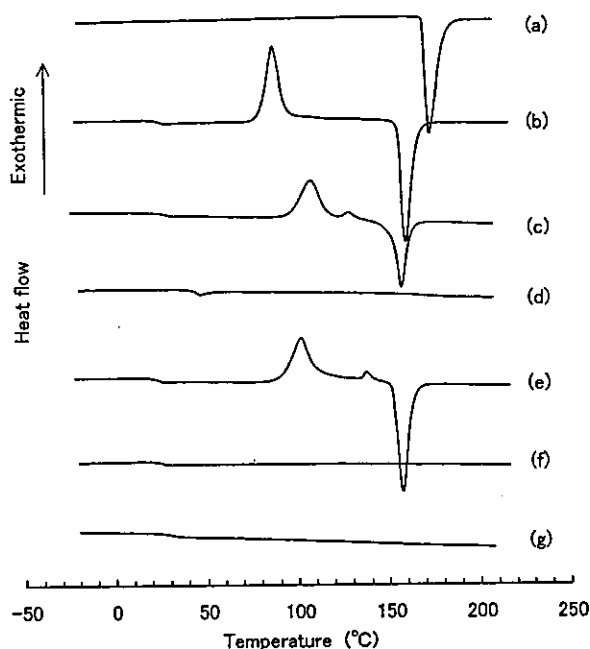
**Table 3.** Dielectric Relaxation Parameters for Water in PPA at 25°C

p/p <sub>0</sub>	Water (g/g polymer)	d (g/mL)	Low-frequency Process			High-frequency Process			ε <sub>∞</sub>
			Δε <sub>l</sub>	α	log τ <sub>l</sub>	Δε <sub>h</sub>	β	log τ <sub>h</sub>	
0.43 (K <sub>2</sub> CO <sub>3</sub> · 2H <sub>2</sub> O)	0.09	1.18	0.66	1.0	-8.6	0.53	1.0	-9.5	1.8
0.57 (NaBr · 2H <sub>2</sub> O)	0.13	1.18	1.0	1.0	-8.4	0.78	1.0	-9.5	2.2
0.76 (NaCl)	0.23	1.16	2.6	0.42	-8.5	2.4	0.79	-9.7	4.0
0.86 (KCl)	0.36	1.15	4.4	0.36	-8.6	5.0	0.57	-9.7	4.3
0.98 (K <sub>2</sub> SO <sub>4</sub> )	0.83	1.11	11	0.25	-8.7	14	0.49	-9.9	5.2

RH was controlled with salts described in parentheses.

heat of crystallization, because no crystallization exothermic peak was observed for the ACTA/PAA dispersion with higher contents of polymer (Fig. 1g).

Figure 3 shows the typical time profiles of ACTA crystallization in amorphous ACTA and amorphous dispersions of ACTA/PVP and ACTA/PAA. The profiles were analyzed according to the Avrami equation [eq. (2)]. All profiles obtained could be fitted with  $n = 3$ . Because the  $T_g$  of the moist solid dispersions was lowered with increasing extent of crystallization, the  $t_{90}$  was calculated by fitting the data showing  $x(t) < 0.3$ .



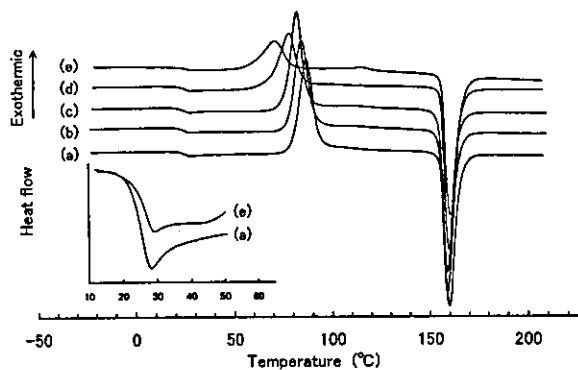
**Figure 1.** DSC thermograms for ACTA and ACTA/polymer dispersions. (a) ACTA crystal; (b) amorphous ACTA; (c) ACTA/PVP (9:1) dispersion; (d) ACTA/PVP (3:1) dispersion; (e) ACTA/PAA (199:1) dispersion; (f) ACTA/PAA (19:1) dispersion; (g) ACTA/PAA (9:1) dispersion.

Figure 4 shows the  $t_{90}$  versus  $(T_{exp} - T_g)$  plots according to the Williams-Landel-Ferry equation<sup>13</sup> ( $T_{exp}$  is the storage temperature). The amorphous solid dispersions with PVP and PAA exhibited a substantially larger  $t_{90}$  than the amorphous ACTA in the dry state (Fig. 4A). Addition of a greater amount of PVP and PAA increased the  $t_{90}$  of the solid dispersions. PAA increased  $t_{90}$  more effectively than PVP at the same polymer mass ratio. The  $t_{90}$  of the solid dispersions was decreased by the presence of moisture (Fig. 4B). The  $(T_{exp} - T_g)$  dependence of  $t_{90}$  for the ACTA/PAA dispersion obtained under a constant RH at various  $T_{exp}$ 's was different from that obtained under various RHs at a constant  $T_{exp}$ .

#### Enthalpy Relaxation of Amorphous ACTA/Polymer Dispersions

Figure 5 shows the enthalpy relaxation behavior of amorphous ACTA and ACTA/polymer solid dispersions observed at a temperature 20°C lower than the  $T_g$ . The fraction of glass relaxed at time  $t$ ,  $\phi(t)$ , was calculated by eq. (3):

$$\phi(t) = \Delta H_t / \Delta H_\infty \quad (3)$$



**Figure 2.** Typical thermograms of amorphous ACTA stored at 40°C for 0 h (a), 1 h (b), 6 h (c), 13 h (d), and 17 h (e).