Molecular Mobility of Protein in Lyophilized Formulations Linked to the Molecular Mobility of Polymer Excipients, as Determined by High Resolution ^{13}C Solid-State NMR

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Received April 14, 1999; accepted June 29, 1999

Purpose. The mobility of protein molecules in lyophilized protein formulations was compared with that of excipient molecules based on the spin-lattice relaxation time (T_1) of each molecule determined by high resolution ^{13}C solid-state NMR. The relationship between molecular mobility and protein stability is discussed.

Methods. Protein aggregation of lyophilized bovine serum γ-globulin (BGG) formulation containing dextran was measured by size exclusion chromatography. The T_1 of the BGG carbonyl carbon and dextran methin carbon in the formulation was determined by high resolution ^{13}C NMR, and subsequently used to calculate the correlation time (τ_c) of each carbon. The spin-spin relaxation time (T_2) of BGG and dextran protons was measured by pulsed NMR spectrometry, and the critical temperature of appearance of Lorentzian relaxation due to liquid BGG and dextran protons (T_{mc}) was determined.

Results. The τ_c of dextran methin carbon in BGG-dextran formulations exhibited a linear temperature dependence according to the Adam-Gibbs-Vogel equation at lower temperatures, and a nonlinear temperature dependence described by the Vogel-Tamman-Fulcher equation at higher temperatures. The temperature at which molecular motion of dextran changed was consistent with the T_{mc}. The τ_c of BGG carbonyl carbon exhibited a similar temperature dependence to the τ_c of the dextran methin carbon and substantially decreased at temperatures above T_{mc} in the presence of dextran. The temperature dependence of BGG aggregation could be described by the Williams-Landel-Ferry equation even at temperatures 20°C lower than T_{mc}.

Conclusions. High resolution ^{13}C solid-state NMR indicated that the molecular motion of BGG was enhanced above T_{mc} in association with the increased global segmental motion of dextran molecules.

KEY WORDS: lyophilized formulation; NMR relaxation time; high resolution ^{13}C solid-state NMR; molecular mobility; storage stability.

INTRODUCTION

The chemical and physical stability of solid pharmaceuticals is considered to decrease as their molecular mobility increases. Hydrolysis of pharmaceuticals (1,2) as well as crystallization of amorphous pharmaceuticals (3–6) were reportedly related to the molecular mobility as determined by the glass transition temperature (T_g). Protein aggregation in lyophilized formulations was also related to the molecular mobility as measured by enthalpy relaxation time (7).

We reported that aggregation of bovine serum γ-globulin (BGG) in lyophilized formulation is related to molecular mobility as measured by NMR relaxation-based critical mobility temperature (T_{mc}) and could be detected experimentally at temperatures above T_{mc}(8,9). In these studies, T_{mc} was defined as the critical temperature of appearance of the Lorentzian relaxation process due to liquid polymer protons determinced by solid-state pulsed NMR spectrometry. The T_{mc} obtained represents the molecular mobility of the polymer excipient, a main component of the formulation. However, this technique provides no information concerning the molecular mobility of protein present in these formulations.

In the present paper, molecular mobility was determined for each protein and excipient in lyopilized formulations using high resolution ^{13}C solid-state NMR in order to elucidate the relationship between molecular mobility and protein stability of lyophilized formulations in a more detailed manner. Lyophilized BGG formulation containing dextran was used as a model.

MATERIALS AND METHODS

Preparation of Lyophilized BGG Formulations

One gram each of dextran (D-4133, average molecular weight of 42,000, Sigma Chemical Co., Inc., St. Louis, MO) and BGG (G5009, Sigma Chemical Co., Inc.) were dissolved in 26.6 ml of distilled water. Three hundred microliters of the solution was frozen in a polypropylene sample tube (10 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), as previously described (8). The shelf temperature was between −35 and −30°C for the first 1 h, 20°C for the subsequent 19 h, and 30°C for the last 3.5 h.

Besides the BGG-dextran formulation described above, BGG formulation without dextran and dextran formulation without BGG were also prepared from each of 7.0% solutions of BGG and dextran, respectively.

Lyophilized formulations were stored at 15°C for 24 h in a desiccator with a saturated solution of NaBr $2H_2O$ (60.2%RH), and the water content was measured to be 0.154 g/g of solid by the Karl Fisher method (684 KF Coulometer, Switzerland).

Determination of T_{mc} by ^1H Pulsed NMR

The free induction decay (FID) of protons in lyophilized BGG formulations was obtained at temperatures ranging from 20°C to 60°C, using a pulsed NMR spectrometer (25 MHz, JNM-MU25, JEOL, Tokyo), and T_{mc} (the critical temperature of appearance of the Lorentzian relaxation process due to liquid polymer protons) was determined as previously reported (10).

Briefly, the T_2 and proportion of water protons were first calculated using the FID signals between 200 and 1000 μs according to the Lorenzian equation. Then, the T_2 of polymer protons was calculated from FID signals between 2.6 μs and 100 μs after FID signals due to water protons were subtracted using the T_2 and proportion of water protons calculated above. Two relaxation processes were assumed for polymer protons;

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0724-8741/99/1000-1621$16.00/0

a Gaussian-type relaxation process due to solid polymer protons and a Lorentzian relaxation process due to liquid polymer protons. Equation 1 representing the sum of the Abragam (11) and Lorentzian equations was used to estimate the T_2 of solid polymer protons ($T_{2(lm)}$) and the proportion of liquid polymer protons (P_{hm}). The T_2 of liquid polymer protons ($T_{2(hm)}$) and the constant c of the Abragam equation were assumed to be 20 μs and 0.11, respectively.

$$F(t) = (1 - P_{hm}) \exp(-t^2/2T_{2(lm)}^2)\sin(ct)/ct + P_{hm}(\exp(-t/T_{2(hm)})) \quad (1)$$

Determination of Molecular Mobility by High Resolution ^{13}C Solid-State NMR

The spin-lattice relaxation time (T_1) of each dextran methin carbon and BGG carbonyl carbon was determined at temperatures ranging from 5°C to 60°C using a UNITY plus spectrometer operating at a proton resonance frequency of 400MHz (Varian). The pulse sequence reported by Torchia (12) was used. Figure 1 shows the typical NMR spectra of the BGG-dextran formulation, BGG formulation, and dextran formulation, respectively. Peaks at 70 ppm and 180 ppm were assigned to the dextran methin carbon and BGG carbonyl carbon, respectively. The T_1 of each carbon was calculated from the signal decay due to spin-lattice relaxation (Fig. 2).

The correlation time (τ_c) of dextran methin carbon was calculated from the observed T_1 according to Eq. 2 by assuming that the dipole-dipole interaction between carbon and proton is predominant in the relaxation process, and that the relaxation time in the slow motional regime can be expressed by a single τ_c.

$$1/T_1 = (1/10)\gamma_C^2 \gamma_H^2 h^2(2\pi)^{-2} r_{C\text{-}H}^{-6} [1/(\omega_c - \omega_H)^2 + 3/\omega_c^2 + 6/(\omega_C + \omega_H)^2]/\tau_c \quad (2)$$

where γ_C and γ_H are the gyromagnetic ratios of ^{13}C and ^1H, respectively, h is the Planck constant, and ω_C and ω_H are the ^{13}C and ^1H resonance frequencies, respectively. $r_{C\text{-}H}$ is the C-H distance and the value of 1.2Å was used for the calculation.

The τ_c of the BGG carbonyl carbon was calculated from the observed T_1 using Eq. 3 on the assumption that the relaxation due to chemical shift anisotropy is predominant, and that the relaxation time in the slow motional regime can be expressed by a single τ_c.

$$1/T_1 = (6/40)\gamma_C^2 B_0^2 \delta_Z^2 (1 + \eta^2/3)(2/\omega_0^2 \tau_c) \quad (3)$$

where B_0, δ_Z and η are the static field, the chemical shift anisotropy, and the asymmetric parameter, respectively. δ_Z and η are defined in terms of three principal components (δ_{11}, δ_{22} and δ_{33}).

$$\delta_Z = \delta_{11} - \delta_0,\ \eta = (\delta_{22} - \delta_{33})/(\delta_{11} - \delta_0) \text{ when } |\delta_{11} - \delta_0| \geqq |\delta_{33} - \delta_0|$$

$$\delta_Z = \delta_{33} - \delta_0,\ \eta = (\delta_{22} - \delta_{11})/(\delta_{33} - \delta_0) \text{ when } |\delta_{11} - \delta_0| < |\delta_{33} - \delta_0| \quad (4)$$

where $\delta_0 = (\delta_{11} + \delta_{22} + \delta_{33})/3$. $\delta_Z^2(1 + \eta^2/3)$ was calculated for the 19 amino acids reported by Ye et al (13), and the average (3.21×10^{-8}) was used for the calculation of τ_c.

Determination of BGG Aggregation by High Performance Size Exclusion Chromatography

Protein stability in BGG-dextran formulation was determined at temperatures ranging from 20°C to 45°C. Protein aggregation during a 55-h storage period was measured based on the decrease in the peak height due to intact protein in size exclusion chromatograms (8). The column (Tosoh G3000SW, 30 cm × 7.5 mm, Tokyo) was maintained at 30°C and 200 mM phosphate buffer (pH 6.2) was used as the mobile phase.

Differential Scanning Calorimetry (DSC)

Thermograms of lyophilized BGG-dextran formulation was obtained at a scan rate of 5°C/min using a calorimeter (DSC 2920, TA Instruments, New Castle, DE), as previously described (8).

RESULTS AND DISCUSSION

Molecular Mobility of Lyophilized Formulation

Figures 3(A) and 3(B) show the observed T_1 and the calculated τ_c of the BGG carbonyl carbon in the BGG formulation and the BGG-dextran formulation, respectively. The τ_c of the BGG carbonyl carbon in the BGG formulation exhibited linear Arrhenius-like temperature dependence. In contrast, the τ_c of the BGG carbonyl carbon in the BGG-dextran formulation decreased significantly at temperatures above 308–313K (35–40°C), resulting in discontinuous temperature dependence.

The τ_c of the dextran methin carbon revealed a similar discontinuous temperature dependence at 35–40°C both in the dextran formulation and the BGG-dextran formulation, as shown in Fig. 4. The τ_c values of the dextran methin carbon were approximately 3 times larger than those of the BGG carbonyl carbon. This difference may be explained as below; although the τ_c of BGG carbonyl carbon was calculated by Eq. 3 assuming that chemical shift anisotropy is predominant in the relaxation process, the dipole-dipole interaction between carbon and proton may be involved in the relaxation process. Neglecting the contribution of the dipole-dipole interaction in the τ_c calculation for the BGG carbonyl carbon may result in the smaller τ_c values than those calculated for the dextran methin carbon.

The discontinuous temperature dependence observed for the τ_c of dextran methin carbon (Fig. 4) appeared to be due to phase transition accompanied by changes in the physical state. The temperature dependence of molecular mobility of amorphous materials at temperatures above T_g has been described by the Vogel-Tamman-Fulcher (VTF) equation, whereas the Adam-Gibbs-Vogel (AGV) equation has been used to describe the temperature dependence below T_g (14,15). These equations have been applied to the temperature dependence of the enthalpy, shear and dielectric relaxation time of amorphous solids (16–18). The VTF equation represents nonlinear relationship between the logarithm of relaxation time and the reciprocal of absolute temperature, whereas the AGV equation represents an Arrhenius-like relationship below T_g. The temperature dependence of the τ_c of dextran methin carbon (Fig. 4) appeared to follow the VTF equation at higher temperatures and the AGV equation at lower temperatures, suggesting that the discontinuous temperature dependence is due to a phase transition such as glass-rubber transition.

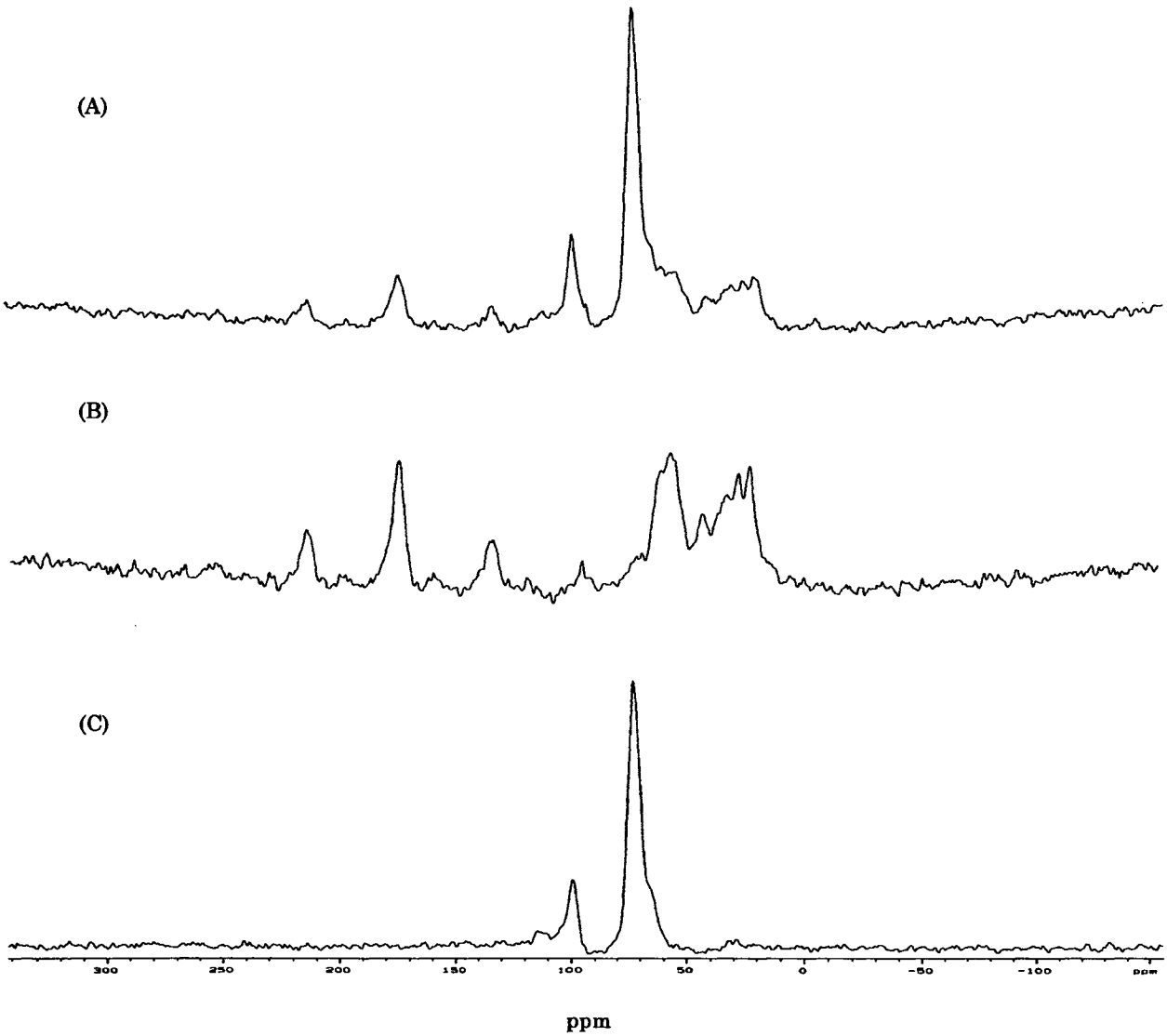

Fig. 1. NMR spectra of BGG-dextran formulation (A), BGG formulation (B), and dextran formulation (C) at 25°C.

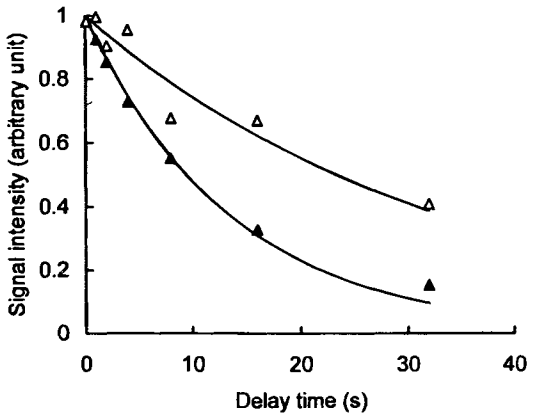

Fig. 2. Spin-lattice relaxation of BGG carbonyl carbon (△) and dextran methin carbon (▲) in BGG-dextran formulation at 25°C.

Spin-spin relaxation measurement by pulsed NMR showed the occurrence of Lorentzian relaxation due to liquid protons of BGG and dextran at 40°C in the BGG-dextran formulation. This observed T_{mc} (40°C) is consistent with the temperature at which the temperature dependence of the τ_c of dextran methin carbon changes (Fig. 4). The DSC thermogram of this formulation showed a single T_g at 50°C. The T_g of 50°C at a water content of 0.154 g/g of solid is much lower than the T_g value reported for pure amorphous dextran (229°C)(19), indicating that the BGG-dextran formulation is substantially plasticized by water absorption as expected from the Gordon-Taylor equation (20). The T_g of the ternary system containing dextran, BGG and water may be determined by the plasticizing effect of water as well as molecular interaction between these components (21). As reported previously (9), the BGG-dextran (1:50) formulation, which contained a larger ratio of dextran than the BGG-dextran (1:1) formulation studied in the present paper, revealed a T_g of 58°C at a water content of 0.187g/g of solid.

The finding that the temperature dependence of the τ_c of dextran methin carbon in the BGG-dextran formulation was discontinuous at around T_{mc} (Fig. 4) suggests that the type of

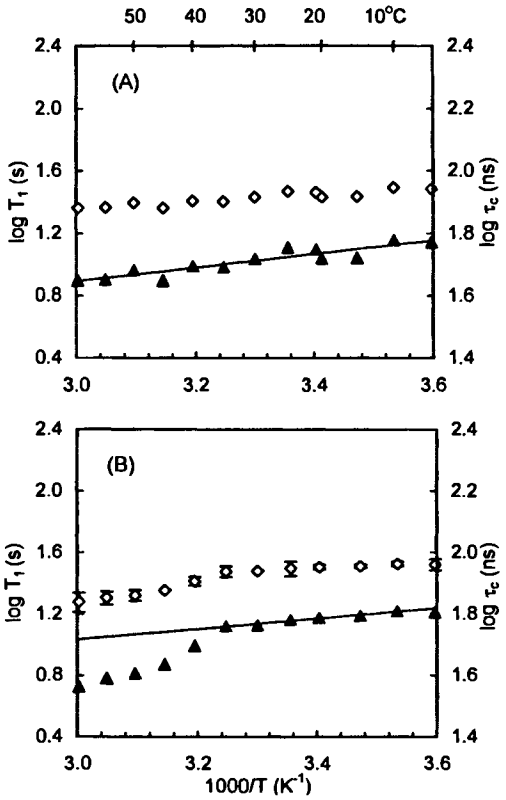

Fig. 3. Temperature dependence of T_1 (◇) and τ_c (▲) of BGG carbonyl carbon in BGG formulation (A) and BGG-dextran formulation (B).

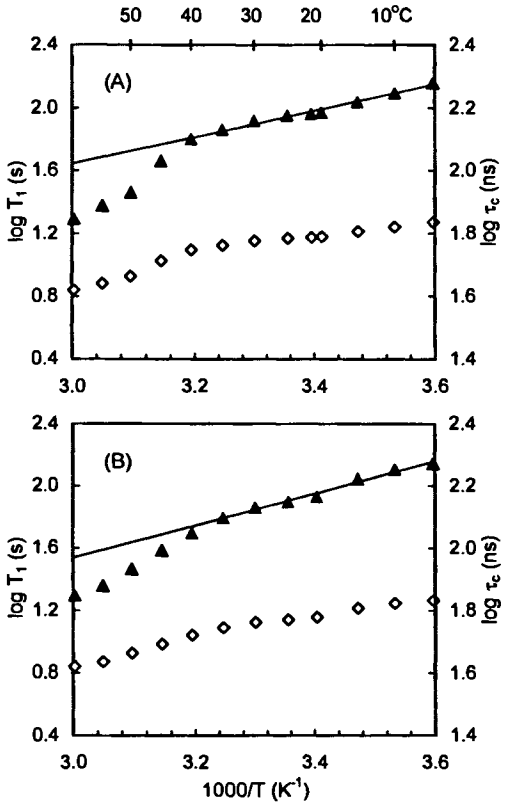

Fig. 4. Temperature dependence of T_1 (◇) and τ_c (▲) of dextran methin carbon in dextran formulation (A) and BGG-dextran formulation (B).

molecular motion at temperatures below T_{mc} is different from that above T_{mc}. Polymers such as dextran in lyophilized formulations can be considered to have local segmental motion as well as global segmental motion. At temperatures below T_{mc}, global segmental motion with a high degree of temperature dependence is very slow, thus local segmental motion with a lower degree of temperature dependence is predominant. The linear temperature dependence of the τ_c of the dextran methin carbon below T_{mc} indicates that local segmental motion becomes slower with decreasing temperature according to the AGV equation. At temperatures above T_{mc}, in contrast, global segmental motion becomes predominant, and exhibits a nonlinear temperature dependence described by the VTF equation.

As shown in Fig. 3, the τ_c of the BGG carbonyl carbon in formulations containing dextran exhibited a discontinuous temperature dependence at 35–40°C, similar to that observed for τ_c of the dextran methin carbon. This suggests that at temperatures above T_{mc}, the molecular motion of BGG is enhanced by the increased global segmental motion of dextran molecules.

Protein Stability of Lyophilized Formulations

Figure 5 shows the temperature dependence of BGG aggregation in the BGG-dextran formulation. The Williams-Landel-Ferry (WLF) equation (Eq. 5) describing the temperature dependence of viscosity (η) in polymers and glass forming liquids (15,22) was applied to the BGG aggregation.

$$\eta = \eta_g \exp[-C_1(T - T_g)/(C_2 + T - T_g)] \quad (5)$$

where η_g is the mean viscosity at T_g, and C_1 and C_2 are constants. In order to use the WLF equation to describe the temperature dependence of the BGG aggregation rate (k), Eq. 6 was derived:

$$k = k_g \exp[a\, C_1(T - T_g)/(C_2 + T - T_g)] \quad (6)$$

where k_g is the BGG aggregation rate at T_g, and a is constant. The line in Fig. 5 represent the curve derived by Eq. 6 using the universal WLF constants of 40.1 (C_1) and 51.6(C_2) as well as the observed value of T_{mc} (40°C) as the reference temperature. The constant a was estimated to be 0.276.

As shown in Fig. 5, the BGG aggregation rate could be described by the WLF equation that is a special case of the

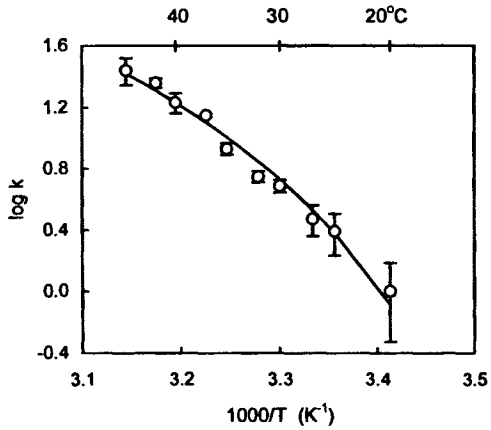

Fig. 5. Temperature dependence of BGG aggregation in BGG-dextran formulation. k represents percentage of aggregation during 55-h storage. Solid line represents curve derived by the WLF equation using T_{mc} (40°C) as reference temperature.

VTF equation (15). The WLF equation and hence the VTF equation appeared to be applicable to BGG aggregation even at temperatures 20°C lower than T_{mc}. However, conformity to the WLF equation cannot be assessed statistically since the temperature range studied is very limited (20–45°C) and the aggregation data at lower temperatures showed a larger deviation. The possibility that BGG aggregation below T_{mc} follows a linear temperature dependence described by the AGV equation cannot be excluded.

CONCLUSIONS

High resolution ^{13}C solid-state NMR analysis of spin-lattice relaxation of carbons in lyophilized BGG formulations containing dextran revealed that the molecular motion of dextran predominantly consists of local segmental motion with a linear temperature dependence according to the AGV equation at lower temperatures, whereas global segmental motion with a nonlinear temperature dependence described by the VTF equation at higher temperatures. The temperature at which molecular motion of dextran changed was consistent with the critical temperature at which the Lorentzian relaxation process due to liquid BGG and dextran protons occurs (T_{mc}), as determined by spin-spin relaxation measurement by pulsed NMR spectrometry. The molecular motion of BGG in lyophilized formulations exhibited a similar temperature dependence to dextran molecules and increased substantially at temperatures above T_{mc} when dextran was added in the formulations, suggesting that molecular motion of BGG is enhanced in association with the increased global segmental motion of dextran above T_{mc}.

The temperature dependence of BGG aggregation in the BGG-dextran formulations could be described by the WLF equation. The WLF equation appeared to be applicable to BGG aggregation even at temperatures 20°C lower than T_{mc}.

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