

Sensitivity of Saturation Transfer Electron Spin Resonance Extended to Extremely Slow Mobility in Glassy Materials

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A novel extension of the saturation transfer (ST) ESR technique that enables the determination of extremely long rotational correlation times of nitroxide spin labels up to values around 10^4 s is proposed. The method is based on the observation that the integral of ST-ESR spectra is sensitive to the spin–lattice relaxation time of the electron of the spin label, which in turn is directly dependent upon the rotational correlation time. The method is applied to the spin label TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) in glycerol. From the known viscosity data and the related rotational correlation times of the TEMPOL spin label in glycerol, the rotational correlation times of unknown samples can be determined. The method is especially applicable to systems with a very high viscosity, such as glassy materials. The method is applied to a 20 wt% glucose–water mixture in the glassy state, giving a value for the highest limiting rotational correlation time of about 10^3 s at a temperature of 45 K below the glass transition temperature of this system. This is an extension by six decades for the rotational correlation time, as compared to the current application of ST-ESR.

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INTRODUCTION

During the past years there has been an increasing interest in the study of molecular motions in glassy food materials (1). Knowledge about the glass transition and physical properties of glassy materials is of crucial importance for the processing, quality, and storage stability of biomaterials, such as food and pharmaceuticals. Furthermore, below the glass transition temperature, the very low molecular mobility of the matrix would predict a good food stability (2, 3). Most of the basic work in this area has been carried out on sugar–water systems that are used as a model for food systems (1).

Electron spin resonance (ESR) spectroscopy and saturation transfer (ST) ESR have been shown to be suitable spectroscopic tools for obtaining information about the molecular mobility in sugar–water systems over a large range of tem-

peratures, using small nitroxide spin-labeled molecules as probes (4–6). Conventional ESR is sensitive in the motional region for values of the rotational correlation time τ_R between 10^{-11} and 10^{-8} s. In the motional region for $\tau_R > 10^{-7}$ s, ST-ESR is commonly used. ST-ESR is based on the diffusion and recovery of saturation between different portions of the spectrum (7, 8) in competition with field modulation. For the usual ST-ESR field modulation frequencies of around 50 kHz, the upper limit of sensitivity for rotational mobility is for $\tau_R \approx 10^{-3}$ s (7, 9), provided that there is sufficient recovery of saturation, which is determined by the spin–lattice relaxation time of the electron (T_1). At room temperatures, T_1 is about 1 μ s for nitroxide spin labels (10), which is in the right range for ST-ESR spectroscopy. Beyond the upper limit, the ST-ESR spectra are no longer sensitive for the diffusion of saturation, because the dominating effect is the recovery of saturation, as given by T_1 .

This article is based on the hypothesis that ST-ESR spectra are also sensitive to recovery of saturation with a limiting value of $T_1 \approx 10^{-3}$ s, and that T_1 is only dependent on τ_R for $\tau_R > 10^{-3}$ s. By recent work of Robinson *et al.* (11) on the spin–lattice relaxation of nitroxide spin labels in glycerol–water solutions, it is found that the spin–lattice relaxation time T_1 is proportional to $\tau_R^{1/8}$ for $\tau_R > 10^{-6}$ s, and it follows that T_1 is about 10^{-4} s at values for τ_R around 10^{-3} s. It may be expected that beyond the upper limit of sensitivity for rotational mobility ($\tau_R \approx 10^{-3}$ s), ST-ESR spectra will still be sensitive to saturation recovery given by T_1 , in competition with field modulation until T_1 has a value of 10^{-3} s. Therefore the dependence of T_1 with τ_R provides an indirect means for measuring molecular mobility in this motional region.

In this paper, we have exploited this idea by studying ST-ESR spectra of the spin label 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL) in glycerol in the temperature range from 140 to 280 K. From these experiments it is found that the ST-ESR spectra, as represented by the relative integrated intensity, indeed are sensitive to rotational correlation times $\tau_R > 10^{-3}$ s. From the known viscosity data and theoretically derived rotational correlation times of the

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TEMPOL spin label in glycerol, the relative integrated intensity of ST-ESR spectra of the TEMPOL spin label in glycerol can be used as a calibration for the rotational correlation times of unknown samples. The method is applied to a 20 wt% glucose–water mixture in the glassy state, giving a value for the highest limiting rotational correlation time of about 10^3 s at a temperature of 45 K below the glass transition temperature of this system. This method opens exciting new ways for obtaining information about rotational molecular motions in the glassy state. This is an extension by six decades for the rotational correlation time, as compared to the current application of ST-ESR.

MATERIALS AND METHODS

Preparation of Solutions

Glycerol was mixed with the spin label TEMPOL (obtained from Sigma) and dried over phosphor pentoxide under vacuum for at least 2 weeks. Glucose was obtained from Jansen Chimica. The glucose concentration was adjusted with water and an aqueous solution of the TEMPOL spin label. The final concentration of the TEMPOL spin label in the samples was 0.2–0.5 mg/ml. For the use in ESR and ST-ESR, the samples were sealed in 100- μ l tubes (Brandt). The sample height was 5 mm. The capillaries were placed in 4-mm quartz ESR sample tubes. For the ESR experiments, the sample was carefully placed in the center of the microwave cavity. All samples were rapidly cooled to a temperature of about 140 K and subsequently measured during stepwise warming up.

Spectroscopy

ESR and ST-ESR spectra were recorded on a Bruker ESP 300E ESR spectrometer equipped with a TMH (Bruker) cavity and nitrogen temperature control. The temperature was measured with a small CuCo thermocouple close to the sample. The temperature accuracy is ± 1.0 K. For conventional ESR the microwave power was set to 2 mW. The scan range, scan rate, time constant, and field modulation amplitude were adjusted so that distortion of the spectra was avoided. The rotational correlation time (τ_R) of weakly immobilized spin labels (10^{-11} s $<$ τ_R $<$ 10^{-9} s) was obtained from the relation (12)

$$\tau_R = 6.5 \times 10^{-10} \Delta B_0 (\sqrt{h_C/h_H} - 1), \quad [1]$$

where h_H and h_C are the heights of the high field and central lines in the ESR spectra, respectively. ΔB_0 is the linewidth of the central line in tesla (T). The rotational motion of the TEMPOL spin label is assumed to be isotropic.

For ST-ESR spectroscopy the second harmonic quadrature absorption signal was detected (10, 13). The phase was set with the self-null method (7).

The relation between the rotational correlation time τ_R and temperature of TEMPOL spin label in glycerol was determined in the following way:

1. Conventional ESR spectra of the TEMPOL spin label in anhydrous glycerol were recorded between room temperature and 373 K, and the rotational correlation time τ_R of the spin label was determined from the ESR spectra using Eq. [1]. It is generally assumed that the rotational behavior of the spin label can be described with a modified Stokes–Einstein equation, given by (4, 14–16)

$$\tau_R = (\eta V/k_b T)k + \tau_0, \quad [2]$$

where τ_R is the rotational correlation time, η is the solvent viscosity, k_b is Boltzmann's constant, V is the volume of the rotating molecule, T is the absolute temperature, and τ_0 is the zero viscosity rotational correlation time. The parameter k is a dimensionless interaction parameter, which is a measure of the coupling of the rotational motions of the spin label to the shear modes of the fluid. It has been found in many cases that the interaction parameter $k \ll 1$, and that k is independent of temperature and viscosity (4, 14, 17). For the TEMPOL spin label, the volume $V = 0.180$ nm³ (4), and for the spin label in glycerol it was found that $k = 0.09$ and that τ_0 is negligibly small. The data for the viscosity of glycerol were obtained from Ref. (18).

2. Since ST-ESR spectra were recorded in the temperature range from 140 to 280 K, the viscosities in this temperature range need to be known. However, viscosity data of anhydrous glycerol in the low-temperature region are published only for temperatures above 240 K (18). Therefore, the known viscosity data at temperatures from 240 to 300 K were fitted to the empirical Williams–Landel–Ferry (WLF) equation (19)

$$\log \frac{\eta}{\eta_g} = \frac{C_1(T - T_g)}{C_2 + (T - T_g)} \quad [3]$$

to provide the viscosities at lower temperatures. In Eq. [3], T_g is the glass transition temperature of glycerol, and η_g is the corresponding viscosity. It should be noted that Eq. [3] is valid only in the temperature range of about 100 K above T_g . The constants C_1 (–17.4) and C_2 (51.6 K) are universal constants of the WLF theory. Using Eq. [3], T_g was found to be 185 K, which is identical to the literature value (20, 21). This validates the use of the universal values for the constants C_1 and C_2 in Eq. [3]. The resulting value for the viscosity η_g at the glass transition is 9.4×10^{11} Pa·s. By using Eqs. [2] and [3], the rotational correlation time τ_R of the TEMPOL spin label in glycerol can be calculated as a function of temperature.

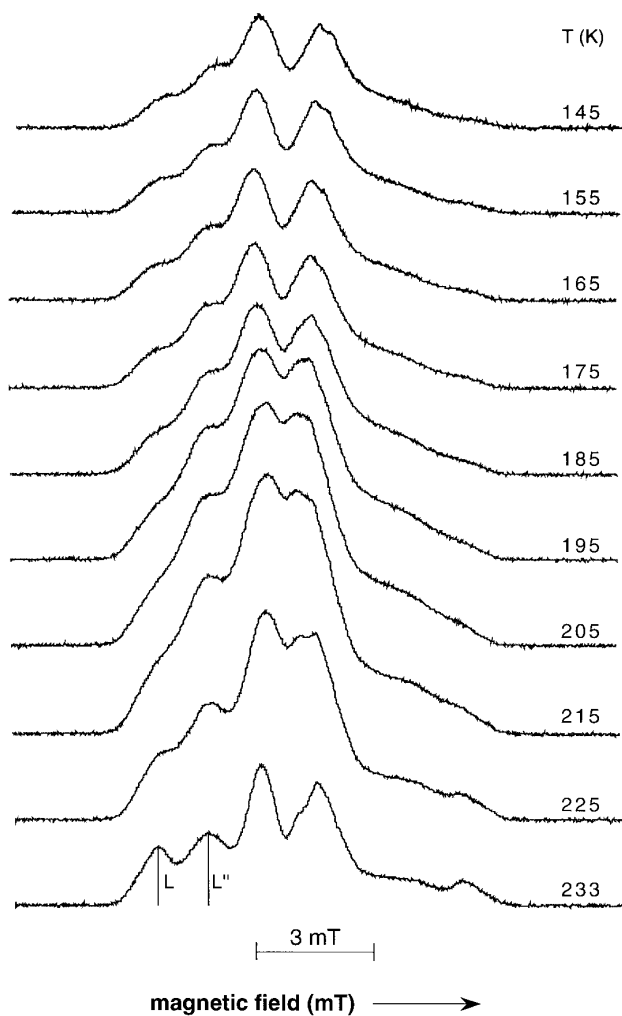


FIG. 1. ST-ESR spectra of the TEMPOL spin label in pure glycerol at different temperatures (in K). The lineheights L and L'' determine the characteristic low-field ratio L''/L . The second harmonic quadrature absorption signal was detected under the following conditions: field modulation amplitude 0.5 mT, microwave power 100 mW, and field modulation frequency 50 kHz. The phase was set with the self-null method (7).

RESULTS

ST-ESR spectra of the TEMPOL spin label in glycerol (Fig. 1) are measured as a function of temperature from 140 to 280 K. The low-field ratio (L''/L) of these spectra increases with decreasing temperature and reaches a maximum value of 2.2 at a temperature of about 200 K (Fig. 2A). The low-field ratio decreases again with a further decrease in temperature. A closer look at the spectra reveals quite a different lineshape at temperatures above and below 200 K (Fig. 1). On decreasing the temperature from room temperature, the lineshape changes toward an absorption-type ESR spectrum at a temperature of about 200 K in which the lineheights L'' and L cannot be well distinguished. On further decreasing the temperature, the lineheights L'' and L become

more distinguished again. In Fig. 2B, the relative integrated intensity I_{rel} of the ST-ESR spectra is shown as a function of temperature. To calculate the integrated intensity, the ST-ESR spectra in Fig. 1 were integrated by a computer. The relative integrated intensity is expressed as a fraction of the maximal integral. The curve in Fig. 2B shows a clear maximum at a temperature of 210 K. The temperature of this maximum shifts to a lower temperature if a field modulation frequency of 12.5 kHz is used instead of 50 kHz. Using a 100-kHz field modulation frequency, the maximum occurs at a slightly higher temperature (Fig. 3).

The lineheight ratio L''/L and relative integrated intensity I_{rel} are replotted as a function of the rotational correlation time τ_R in Fig. 4, by substituting the temperature for the rotational correlation time as described by Eqs. [2] and [3]. Because the viscosity of glycerol can be calculated only for temperatures above T_g (see Eq. [3]), the range of τ_R is from 10^{-6} to 5×10^4 s. Note that in Fig. 4A the lineheight ratio

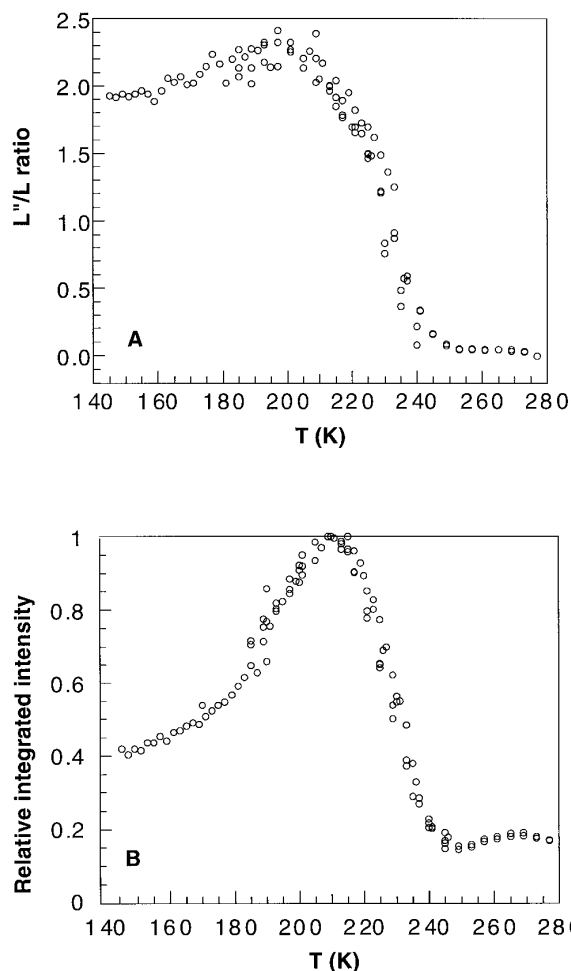


FIG. 2. The L''/L ratio (A) and the relative integrated intensity I_{rel} (B) of the ST-ESR spectra of Fig. 1. The values of τ_R are calculated from the temperature using Eqs. [2] and [3]. The results of four separate experiments are shown.

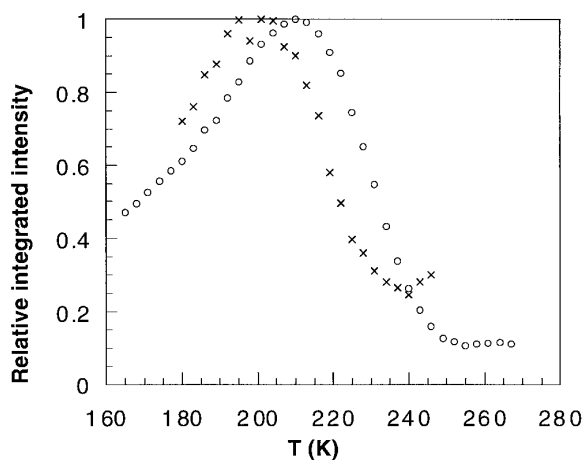


FIG. 3. The relative integrated intensity I_{rel} of ST-ESR spectra as a function of temperature at field modulation frequencies of 12.5 (×) and 100 kHz (○).

L''/L levels off and loses motional sensitivity for values of τ_R above 10^{-2} s, whereas the relative integrated intensity I_{rel} (Fig. 4B) still shows a peak around this τ_R value. However, for values of τ_R below 10^{-2} s, the slope is much steeper than for values above this value, indicating that the sensitivity of the relative integrated intensity I_{rel} for motion is decreased for very slow motions.

By using the ST-ESR data obtained from the TEMPOL spin label in glycerol as a calibration, the rotational correlation time of the TEMPOL spin label in a 20 wt% glucose–water mixture is determined as a function of temperature (Fig. 5). For comparison, the results arising from both the relative integrated intensity I_{rel} and the L''/L ratio of the ST-ESR spectra are plotted, showing that the relative integrated intensity I_{rel} is much more sensitive for long rotational correlation times than the L''/L ratio.

DISCUSSION

The goal of this study is to develop ESR methods that can be used to determine molecular motion in high-viscosity systems, such as glassy sugar–water systems and foods. Conventional ESR and ST-ESR spectroscopy using nitroxide spin labels have already been shown to be suitable methods for this purpose: the spin labels provide information about their local environment, and ESR spectroscopy is especially powerful in obtaining data about molecular mobilities that cannot easily be obtained in alternative ways (4–6, 22, 23). ST-ESR is based on rotational diffusion and/or recovery of saturation in competition with the field modulation. For ST-ESR, employing field modulation frequencies around 50 kHz, the upper limit of sensitivity for rotational mobility is usually found for rotational correlation times $\tau_R \approx 10^{-3}$ s (7, 9, 10). This article is based on the hypothesis that ST-

ESR spectra are also sensitive to recovery of saturation with a limiting value of $T_1 \approx 10^{-3}$ s, and that T_1 is only dependent on τ_R for $\tau_R > 10^{-3}$ s. Recently it has been observed that the spin–lattice relaxation time of the electron (T_1) of the TEMPOL spin label in glycerol–water mixtures increases with τ_R and has a value of 10^{-4} s at the limiting value of $\tau_R \approx 10^{-3}$ s (11). Thus it may be expected that in the motional region for $\tau_R > 10^{-3}$ s, ST-ESR spectra will still be sensitive to saturation recovery in competition with field modulation. This would provide a new indirect way of quantifying the rotational correlation time τ_R in this motional region.

To analyze ST-ESR spectra, a spectral characteristic sensitive for rotational diffusion should be related to calculated values of τ_R in a reference sample. In our study we have used the TEMPOL spin label in glycerol as a calibration system for carbohydrate–water systems. Glycerol has a close chemical resemblance to sugars, and it can be used as a model compound for glassy sugar–water systems. Further-

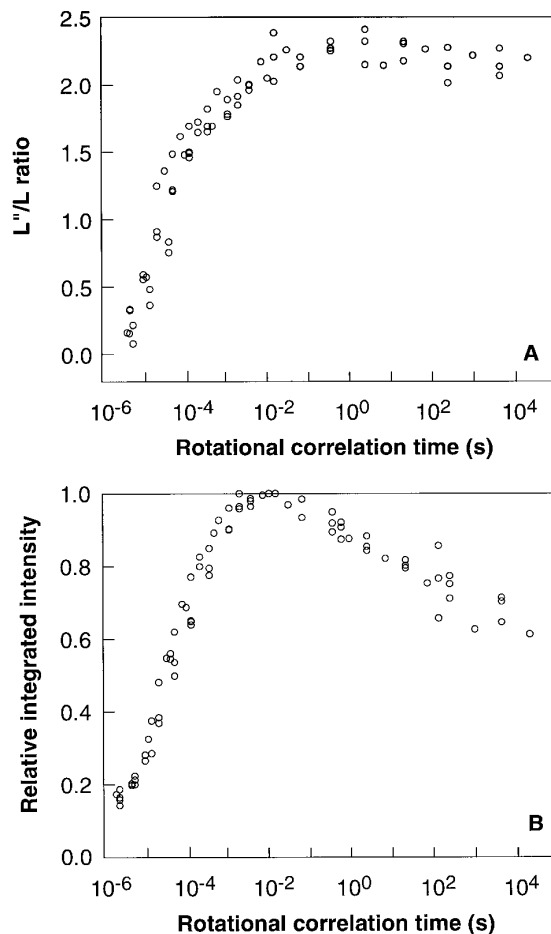


FIG. 4. The L''/L ratio (A) and the relative integrated intensity I_{rel} (B) of the ST-ESR spectra of the TEMPOL spin label in glycerol as a function of the rotational correlation time τ_R at a field modulation frequency of 50 kHz. The results of four separate experiments are shown.

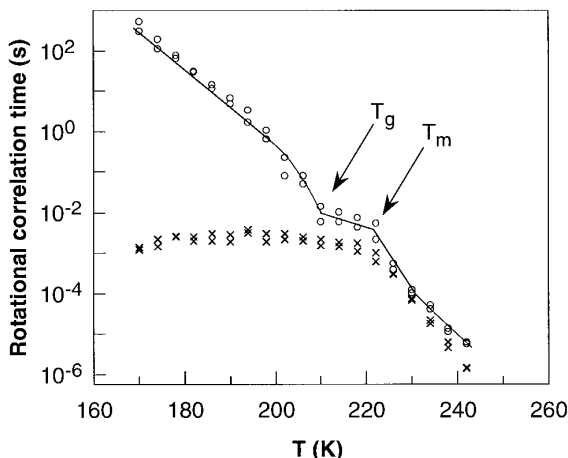


FIG. 5. Rotational correlation time τ_R of the TEMPOL spin label in a 20 wt% glucose–water system as a function of temperature. The values of τ_R are analyzed using the L''/L ratio (\times) and the relative integrated intensity I_{rel} (\circ) obtained in glycerol (see Fig. 4). The glass transition temperature T_g (210 K) and the melting temperature T_m (223 K) are indicated with arrows. The results of two separate experiments are shown. The field modulation frequency is 50 kHz.

more, the viscosity of glycerol is known over a large temperature range. We used a very broad temperature range to study the ST-ESR spectra of the TEMPOL spin label in glycerol (Fig. 1), to cover a large range of molecular motions and spin–lattice relaxation times. At temperatures below 210 K the characteristic transfer features of ST-ESR spectra disappear because the very slow rotational motions inhibit transfer of magnetization at the time scale of the modulation frequency. At these low temperatures overmodulated spectra of saturation recovery are measured.

The analysis of the ST-ESR spectra was carried out in two different ways, e.g., by using the low-field spectral ratio L''/L (7) or the integrated intensity (24–26). Both spectral characteristics have been shown to yield equivalent results in determining rotational correlation times τ_R up to $\tau_R \approx 10^{-4}$ s (24, 27). Our results are plotted in Fig. 2. On decreasing the temperature both the low-field spectral ratio L''/L (Fig. 2A) and the relative integrated intensity I_{rel} (Fig. 2B) reach a maximum, but after passing the maximum, the temperature dependence of the L''/L ratio is relatively small compared to the relative integrated intensity I_{rel} . It should be noted that around a temperature of 200 K, the L''/L ratio is difficult to determine from the ST-ESR spectra (Fig. 1), which is represented by the scattered values in this temperature range (Fig. 2A). Below a temperature of 200 K, the relative integrated intensity I_{rel} (Fig. 2B) has a more pronounced change with temperature and lacks the scattering problem. For this reason the integrated intensity has the best properties to be used as a spectral characteristic below a temperature of 200 K. Apart from a better reproducibility and a stronger temperature dependence, another advantage

of the integrated intensity is that possible line-broadening effects are not reflected in the relative intensity I_{rel} . Therefore, the relative integrated intensity I_{rel} is exclusively affected by τ_R and T_1 . It should be noted that a disadvantage of the use of the relative integrated intensity I_{rel} , which is normalized to the maximum, is that the whole temperature range must be studied.

Maximal effects of rotational motion and spectral recovery in ST-ESR spectra arise if the rotational correlation time τ_R , spin–lattice relaxation time T_1 , and angular field modulation frequency ω_m obey the equations (7, 8, 10)

$$\tau_{R,opt} \approx 1/\omega_m \quad \text{and} \quad T_{1,opt} \approx 1/\omega_m, \quad [4]$$

where $\tau_{R,opt}$ and $T_{1,opt}$ are the optimal values for τ_R and T_1 , respectively. The maximal sensitivity for motion at a field modulation frequency of 50 kHz, as used in our ST-ESR experiments, is then expected for a value of $\tau_{R,opt}$ of about 10^{-5} s. The sensitivity range is usually taken as a range plus or minus two decades, giving $\tau_R \approx 10^{-3}$ s as a higher limit. In Fig. 2B, a maximum is seen in I_{rel} at a temperature of 210 K. From Eqs. [2] and [3], it can be calculated that at around this temperature the rotational correlation time τ_R has a value of about 10^{-2} s. By extrapolating the T_1 data of the TEMPOL spin label in glycerol–water systems versus τ_R (11), it is then estimated that T_1 is about 10^{-4} s. At a field modulation frequency of 50 kHz, this is in the sensitivity range for T_1 , and relaxation processes will still affect the ST-ESR lineshape.

Following well-known theories of AC electric circuits and viscoelastic response, it can be anticipated that the out-of-phase response given by the relative integrated intensity I_{rel} of the ST-ESR spectra upon the angular field modulation frequency $\omega_m = 2\pi\nu_m$ and with a characteristic time T_1 can be described by

$$I_{rel} \propto \frac{\omega_m T_1}{1 + (\omega_m T_1)^2}. \quad [5]$$

In Eq. [5] it can be seen that effects of the relaxation time T_1 on I_{rel} will be most efficiently detected, if $\omega_m T_1 \approx 1$. This is reflected by the top of the curve in Fig. 2B. Using a smaller modulation frequency, the temperature of the maximum relative intensity decreases (Fig. 3), because the relaxation time T_1 increases with decreasing temperature (11).

Since it may be expected that T_1 continues to increase with decreasing temperatures (T_1 varies with $\tau_R^{1/8}$ for $\tau_R > 10^{-3}$ s (11)), the ST-ESR lineshape will change as a function of temperature until a final limit for sensitivity to T_1 is reached at a temperature somewhere below a temperature of 140 K. This effect of T_1 is illustrated by the decrease of the relative integrated intensity I_{rel} for temperatures below 210 K (Fig. 2B), as is predicted by Eq. [5]. The effect can also

be observed in the temperature dependence of L''/L (Fig. 2A), but it is less pronounced.

The lineheight ratio L''/L and relative integrated intensity I_{rel} can be directly related to the rotational correlation time τ_R , as is presented in Fig. 4. Because the viscosity of glycerol, as described by Eq. [3], can be calculated only for temperatures above the glass transition T_g of glycerol (185 K), the values for τ_R range from 10^{-6} to 5×10^4 s. From a comparison of Figs. 4A and 4B, it can be seen that the relative integrated intensity is much more sensitive for values of τ_R from 10^{-2} to 10^4 s, as compared to the L''/L ratio. Therefore, the relative integrated intensity I_{rel} as given in Fig. 4B is the best parameter to be used as a calibration for rotational correlation times τ_R of the TEMPOL spin label in unknown samples, as compared to the L''/L ratio. It should be noted, however, that because of the small slope of I_{rel} in Fig. 4B for values of τ_R above about 10^{-2} s, the errors in τ_R will be relatively large.

In Fig. 4 it is assumed that the rotational correlation time τ_R can be calculated from Eqs. [2] and [3]. This implies (1) that the viscosity follows the Williams–Landel–Ferry (WLF) theory and (2) that the modified Stokes–Einstein Eq. [2] is valid in supercooled liquids. It has been observed in glycerol that in general dynamical processes follow the WLF Eq. [3] (21, 28, 29). This is also true for the rotational correlation times obtained from NMR hole-burning experiments in glycerol (20). This means that the temperature dependence of the viscosity is well described by Eq. [3], and that the interaction parameter k in Eq. [2] is temperature independent.

It is interesting to compare our rotational correlation time data obtained with the TEMPOL spin label in glycerol with those of glycerol itself (20). For glycerol at the glass transition temperature T_g (185 K), it has been found that τ_R is 1.0×10^3 s (20). In our case, we calculate for the TEMPOL spin label a value for τ_R of 4.0×10^3 s, which is in the same order of magnitude. Using the molecular volumes of glycerol as given by Bondi (30), the interaction parameter k of glycerol can be calculated to be 0.03. For the TEMPOL spin label the interaction parameter k is a factor of 3 larger (0.09). This difference can be explained by the fact that a TEMPOL spin label is slightly larger than a glycerol molecule (the average radii are 0.35 and 0.27 nm, respectively). It has been observed that for small molecules with an average radius between 0.2 and 0.6 nm, the experimental rotational diffusion coefficient is systematically higher than calculated from the modified Stokes–Einstein; this discrepancy increases as the molecule becomes smaller (17).

To illustrate the possibilities of this ST-ESR method, the rotational correlation times τ_R of the TEMPOL spin label in glycerol determined from the L''/L ratio and the relative integrated intensity I_{rel} were used as a calibration to determine unknown τ_R values in a 20 wt% glucose–water sample

(Fig. 5). Upon cooling this sample a phase separation takes place, because ice is formed. Upon rewarming such a sample, the melting of ice starts at the melting temperature T_m . The process of ice formation upon cooling concentrates the remaining glucose solution containing the spin label, and finally the supersaturated solution undergoes a glass transition at a temperature T_g of 216 K (1). The low mobility below this glass transition temperature is expected to allow a prolonged storage stability of the glass, and therefore is an important object of study in food science.

From Fig. 5, it is seen that the glass transition temperature T_g (210 K) and the melting temperature T_m (223 K) are well identified as breaks in the plot determined from the relative integrated intensity I_{rel} . These values of T_g and T_m are in agreement with the literature (1). Furthermore, τ_R values can be determined at temperatures far below the glass transition temperature T_g , and τ_R can be determined in the glassy state up to a value of about 10^3 s. In contrast, the L''/L ratio levels off at a value of about 2.1, giving τ_R values around 10^{-3} s. At these values the limit of sensitivity for mobility for the L''/L ratio is reached. This effect can be explained by realizing that the L''/L ratio is almost insensitive to T_1 effects, and that changes in T_1 are the main cause for sensitivity for motion for $\tau_R > 10^{-3}$ s. This means that by using the relative integrated intensity I_{rel} of ST-ESR spectra, the range of sensitivity for mobility is extended with six orders of magnitude, as compared to using the L''/L ratio.

In conclusion, we have shown that small stable nitroxide spin labels incorporated in a glassy host material reflect the motional properties of the host. Until now, the motional sensitivity of ST-ESR was limited to slow molecular motions around a rotational correlation time of about 10^{-3} s. This value does not always reflect the glassy state of the host, and therefore limits the motional information that can be obtained about the glassy state. Here, we introduce a new approach of ST-ESR, which enables us to measure rotational correlation times of spin labels up to values of 10^3 s. This is a major breakthrough in the ST-ESR technique, giving us an increase by six decades as compared to previous methods. Taking into account that the lower limit of conventional ESR is for values of rotational correlation times τ_R of about 10^{-11} s, our experiments show that *a single set of equipment for ESR spectroscopy is able to cover an immense range of rotational molecular motions.*

This observation makes ESR spectroscopy an extremely versatile technique that enables one to provide motional knowledge about the glassy state. Since our work is aimed at the study of the stability and preservation of food and pharmaceuticals, such motional information might help technologists to develop better ways for storage and manufacturing. In addition, it could give some clues about the long-term stability of biological materials (i.e., seeds).

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