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Effect of Solutes and Matrix Structure on Water Mobility in Glycerol−Agar−Water Gel Systems: A Nuclear Magnetic Resonance Approach

Yang Huang,*[,†] Erika Davies,[†,‡] and Peter Lillford[§]

[†]CSIRO Food and Nutritional Sciences, North Ryde, NSW 2113, Australia

[‡]University of New South Wales, Sydney, NSW 2052, Australia

[§]CSIRO Food Futures Flagship Fellow, University of York, United Kingdom

ABSTRACT: Nuclear magnetic resonance spectroscopy (NMR) has been widely used to determine water molecular mobility in food systems. This study aimed to examine the effects of matrix structure and solutes on the dynamics of water molecules in model mixed systems, glycerol−agar−water gels, using low- and high-resolution NMR. Simple models to explain water relaxation rates and self-diffusion coefficients in mixed systems were developed using the experimental values obtained for the individual binary systems (glycerol−water solutions and agar−water gels). The spin−lattice relaxation of mixed systems was influenced by interactions of both glycerol and agar with water, while the spin−spin relaxation of mixed systems was dominated by the interaction of agar with water. Water diffusion was influenced by not only molecular interactions between all components but also the gel matrix structure. These models are able to differentiate the effect of solutes from that of matrix structure on water molecular dynamics.

KEYWORDS: ^{1}H Nuclear magnetic resonance, NMR ^{1}H relaxation rate, self-diffusion coefficient, agar−water gel, glycerol−water solution, glycerol−agar−water gels

INTRODUCTION

The state and content of water has a major impact on the microbial chemical and physical stability of food. Rather than simple water content, the activity of water (a_w) is routinely used to design or predict long-term stability of food. More recently, the dynamics or molecular mobility of water and substrates has been suggested to be more relevant.[1] Nuclear magnetic resonance spectroscopy (NMR) has been used to determine water molecular mobility in food, since it is capable of measuring rotational and translational correlation times at a wide variety of frequencies as well as self-diffusion over a variety of length scales.[2] Compared to ^{2}H NMR and ^{17}O NMR, ^{1}H NMR allows an economic means for nonintrusive measurement of food products. Simple benchtop NMR machines have been developed for this purpose. Unfortunately, there are many other proton species together with water in food products, which complicate data interpretation. For example, difficulties have been encountered because of not only the many molecular species containing protons but also the several mechanisms which may contribute to water proton relaxation, including intramolecular dipole−dipole interactions, exchange between water transiently associated with macromolecules, and chemical exchange between protons located on different molecular species.[2−6] Diffusion measurement by pulsed field gradient methods are more readily interpreted, since water is usually the dominant and the most mobile species providing the proton signal.

In this study we investigated mixed systems, glycerol−agar−water gels, which represent the simplest combination of molecular species in foods, being a soft solid structure comprising a macromolecular network, together with dissolved small solutes and water. This paper examines the experimentally determined relaxation rates and self-diffusion coefficient of protons, determined by low-resolution (23 MHz) and high-resolution (500 MHz) NMR measurements, in glycerol solutions, agar−water gels, and glycerol−agar−water gels. Additionally, we also examined glycerol−agar−water gels-N to which nutrients had been added, representing a microbiological medium, dichloran glycerol agar, commonly used for fungal growth.[7]

Attempts to predict the observed relaxation rates and diffusion coefficients of water protons of these mixed systems using those obtained for the individual binary systems are presented. Where appropriate, data from high-field measurements have been included to clarify and validate the data obtained from the low-resolution measurements.

MATERIALS AND METHODS

Reagents and Materials. Agar (LP0011, Oxoid Australia), glycerol (9326410004027, Chem-supply), Peptone (RM263, Amyl Media), KH_2PO_4 (A391, Ajax), and $MgSO_4 \cdot 7H_2O$ (230391, BDH) were used. The water used was Milli-Q grade.

Sample Preparation. Glycerol−water solutions were prepared by weighing water into 100 mL Schott bottles and then adding glycerol with gentle swirling. Samples were then poured into 5 mm Wilmad NMR sample tubes (535-PP-7, Sigma) for measurements on a Bruker Avance III 500 MHz spectrometer and 10 mm NMR sample tubes (Oxford Instruments, U.K.) for measurements on a Maran Ultra 23 MHz spectrometer (Oxford Instruments, U.K.).

Agar−water gels and mix systems, glycerol−agar−water and glycerol−agar−water-N gels, were prepared as follows. Water was weighed into 100 mL Schott bottles. The remaining components except for agar were added and dissolved in water by gentle swirling. Then agar was gradually added and allowed to wet thoroughly by gentle swirling. The mixtures were steamed for 4 h with agitation every 45 min and then poured into

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Table 1. Composition of Agar−Water Gel, Glycerol−Water Solution, and Mixed Systems (Glycerol−Agar−Water Gels without/with Nutrients)

systems	glycerol:agar:water (g/g)				
agar−water gel	0:0.026:1	0:0.053:1	0:0.081:1	0:0.111:1	0:0.143:1
glycerol−water solution	0.229:0:1	0.448:0:1	0.721:0:1	0.992:0:1	1.283:0:1
glycerol−agar−water	0.229:0.026:1	0.448:0.026:1	0.721:0.026:1	0.992:0.026:1	1.283:0.026:1
glycerol−agar−water-N[a] gels	0.229:0.053:1	0.448:0.053:1	0.721:0.053:1	0.992:0.053:1	1.283:0.053:1
	0.229:0.081:1	0.448:0.081:1	0.721:0.081:1	0.992:0.081:1	1.283:0.081:1
	0.229:0.111:1	0.448:0.111:1	0.721:0.111:1	0.992:0.111:1	1.283:0.111:1
	0.229:0.143:1	0.448:0.143:1	0.721:0.143:1	0.992:0.143:1	1.283:0.143:1

[a] N stands for nutrients, which were added at a constant percentage of total weight: 0.5% peptone, 1% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4$.

sterile Petri dishes and left to form gels. Strips of gels were excised using a scalpel and packed into 10 mm NMR tubes for measurements on a Maran Ultra 23 MHz spectrometer. Aluminum foil caps were loosely attached to the top of the NMR tubes, and tubes were steamed again for 30 min to allow the gel to settle to the bottom of the NMR tube to form a homogeneous gel. The NMR tubes were then sealed using film to control moisture loss. The NMR tubes were stored at 4 °C before measurements.

In order to compare mixed systems with binary systems, mixed systems (glycerol−agar−water and glycerol−agar−water-N gels) were made with the same ratio of solute to water as those in binary systems. The weight ratios of components are listed in Table 1.

Spin−Lattice Relaxation (T_1) Measurements. Spin−lattice relaxation measurements were performed on a Bruker Avance III 500 MHz spectrometer. The standard inverse recovery (INVREC) pulse sequence was used, and the water proton and glycerol hydroxyl signals were processed using the Bruker TOPSPIN 2.5b software. T_1 was derived from fitting integrated data to an exponential function, $y = A_1e^{(-x/T1)} + y_0$. Measurements were also made using a Maran Ultra 23 MHz spectrometer (Oxford Instruments, U.K.). The standard INVREC pulse sequence was also used, and apparent T_1 was automatically calculated using Maran Ultra RINMR software.

Spin−Spin Relaxation (T_2) Measurements. Spin−spin relaxation measurements were performed on the Maran Ultra 23 MHz spectrometer. The standard Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence was used, with a diffusion delay of 1 ms. Apparent T_2 values were obtained by fitting decay curves with Winfit software (Oxford Instruments, U.K.).

Diffusion Measurements. High-resolution NMR ^{1}H diffusion measurements were made on a Bruker Avance III 500 MHz spectrometer. A standard pulsed-field gradient stimulated echo (PGSE) pulse sequence was used. The PGSE sequence for water proton measurement consisted of a stimulated spin−echo sequence of RF pulses, consisting of two varied magnetic field gradient pulses with fixed duration (δ) and a fixed time interval (Δ) between pulses. The duration of gradient pulse ranged from 1.25 to 2.15 ms for water proton measurements and 1.7 to 3.7 for glycerol CH proton measurements . The time interval of (Δ) was 200 ms for all measurements.

Low-resolution NMR ^{1}H diffusion measurements were made on a Maran Ultra NMR spectrometer (Oxford Instrument, U.K.), with an operating resonance frequency (RF) of 23 MHz. A standard pulsed-field gradient stimulated echo (PGSE) pulse sequence was also used. The PGSE sequence consisted of a stimulated spin−echo sequence of RF pulses in which two magnetic field gradient pulses with a series of durations (δ) of 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, and 3.0 ms were applied, along the static magnetic field direction, z, after the first and second 90 °C pulse, and a time interval (Δ) of 200 ms between the two gradient pulses. The 90 °C pulse width was 3.45 μs. The relaxation delay time was set to 15 s.

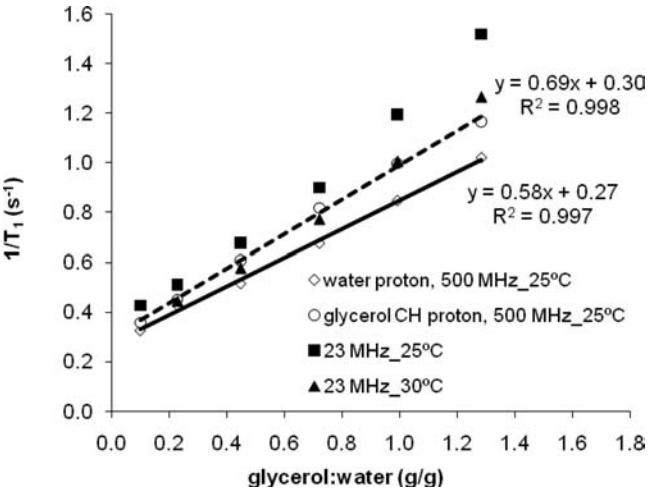

Figure 1. Spin−lattice relaxation rate of protons as a function of glycerol concentration at 25 °C, measured at 500 and 23 MHz. The lines represent least-squares fit for data measured at 500 MHz.

Values of D (m^2s^{-1}) were derived using the RI Diffusion software (Oxford Instrument, U.K.). In brief, the intensities of echoes (A) was plotted against $(\gamma G\delta)^2(\Delta - \delta/3)$, where the γ is the gyromagnetic ratio of ^{1}H (2.675×10^8 s^{-1} T^{-1}) and D was determined as the gradient of the best straight line fitted to the graph. D_0 was determined for a Milli-Q water sample under the same settings for the PGSE sequence.

RESULTS AND DISCUSSION

Spin-Lattice Relaxation of Glycerol Solutions. The relaxation rates ($1/T_1$) of protons are shown as a function of glycerol concentration (g/g) in Figure 1, measured at 500 and 23 MHz. As glycerol concentrations increased, the relaxation rates of protons increased.

At 500 MHz, water OH protons were separated from glycerol CH protons. As expected, the relaxation rate of CH protons was greater than that of water OH protons at all glycerol concentrations. The observed relaxation of water should be described by the Zimmerman Britten condition of rapid exchange between solvent water and water associated with glycerol, and decay plots should exhibit log/linear behavior.[2] This was observed in all cases. Thus

$$1/T_{1obs} = P_w/T_{1w} + P_b/T_{1b} \quad (1)$$

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where P_w and P_b are the weight fractions of water in the free and motionally modified states, respectively, and T_{1w} and T_{1b} are their relaxation times.

Alternatively

$$1/T_1 = (1 - Hc)/T_{1w} + Hc/T_{1b} \quad (2)$$

where c is the concentration of glycerol and H is the number of water molecules associated with each glycerol molecule.

We obtained an estimate of H/T_{1b} from the initial slope of Figure 1, giving a value of 0.58. If we assume that T_{1b} is comparable to the T_1 of nonexchangeable protons of glycerol at the same low concentration (approx 3 s), then H has a value of $3 \times 0.58 = 1.74$ g of water per gram of glycerol. Converting this to a molecular ratio, we obtain 8 mol of water per mole of glycerol. This value is sensible, since it relates to the two potential hydrogen bonding sites on each glycerol hydroxyl group, and comparable to the 16 mol of water/mol monosaccharide reported by Belton and Wright,[8] measured by ^{17}O relaxation.

Furthermore, the implication is that at high glycerol concentrations (glycerol/water > 0.6) all water molecules will interact with glycerol but are in fast exchange with each other. By inspection of the data, at these high glycerol concentrations, water T_1 relaxation times were slightly longer than those of glycerol CH protons, implying that the water associated with glycerol may have a slightly longer T_1 relaxation times than the CH protons, which is entirely reasonable.

All relaxation decays were log linear at 23 MHz, despite the fact that both nonexchangeable glycerol protons and water protons may be present within the decay. The relaxation rates measured at 23 MHz were similar at 25 and 30 °C and very close those of water proton measured at 500 MHz (Figure 1). We can therefore assume that the relaxation rates of protons in glycerol concentrations were dominated by water at low concentrations of glycerol but may be weighted slightly by glycerol at higher concentrations of glycerol. This resulted in a curved plot of relaxation rates of protons as a function of glycerol concentrations at 23 MHz (Figure 1).

Spin−Lattice Relaxation of Agar−Water Gel. Agar−water and agarose−water systems have been studied previously, and detailed models of relaxation behavior have been given.[9,10] Those models assume that T_1 of the agar−water system could be represented by fast exchange between "free" and transiently "bound" water.

Derbyshire and Duff[10] described the spin −lattice relaxation rate $(1/T)$ in agarose−water gels as

$$1/T = 1/T_a + [Hc/(1 - Hc)](1/T_b) \quad (3)$$

where T is the observable relaxation time and T_a and T_b are the relaxation times of the bulk and bound phases.

In a more recent study, Askin and Yilmaz[11] described the relaxation rates in agar−water solutions as

$$1/T_1 = 1/T_{1f} + Kc \quad (4)$$

where c is the concentration of agar, T_{1f} is the relaxation rate of free water as in pure water, and K is a constant.

This is essentially the same description of relaxation in terms of weight-averaged fast exchange as described in eq 3, and when (1-Hc) is close to unity then

$$K = H(1/T_b - 1/T_a) \quad (5)$$

Figure 2 shows that the plot of our observed $1/T_1$ versus agar concentrations gives a straight line, which can be expressed as

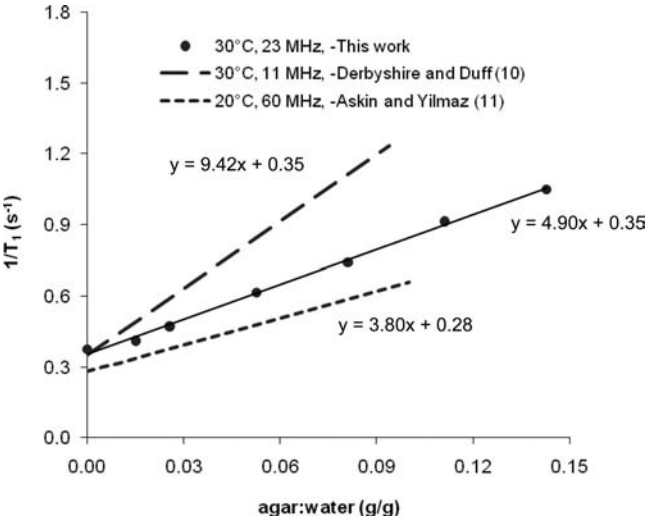

Figure 2. Spin−lattice relaxation rates of agar−water gels. The lines represent least-squares fit.

$1/T_1 = 0.35 + 4.9c$. The intercept (0.35) corresponds to the relaxation rate of agar-free water, whereas 4.9 denotes the slope of the line, K. However, when these data were compared with previous studies, while the intercept corresponded well with the relaxation rate of water, the slope K (3.8) was significantly different from those reported by Askin and Yilmaz[11] and Derbyshire and Duff,[10] whose work was, respectively, carried out at 60 MHz, 20 °C and 11 MHz, 30 °C (Figure 2).

Although samples and operating temperatures varied in these experiments, the dominant effect was a large and systematic dependence of K on measurement frequency. According to eq 5, the hydration of agar was changing, or the relaxation time of the motionally restricted water, or both. In fact, this phenomenon has been reported previously by Duff and Derbyshire,[12] who observed the same effect by direct measurements of the nonfreezable water in agar gels. They suggested that the simple model, where hydrating water has a unique correlation time, was an oversimplification. Instead, a distribution of correlation times of the hydrating species was present, and measurements at various frequencies sampled different parts of the spectral density function, lower frequencies being more sensitive to slower motions of protons associated with polymer. This implies that it is the measured relaxation time rather than the hydration state of agar which changes with frequency and further implies that comparative studies of spin−lattice relaxation of related systems need to be at a constant measurement frequency.

Spin−Lattice Relaxation of Mixed Systems. Comprehensive studies of spin−lattice relaxation were carried out on glycerol−agar−water gels with or without nutrients. All T_1 measurements gave log linear decay plots, and the behavior of the two systems was similar.

We can deduce from the previous studies that the relaxation measured in glycerol−agar−water gels was predominantly of water protons. The simplest model to assume is of fast exchange between all relevant water species, no matter to which site they are transiently attached. The observed relaxation time will therefore be represented as

$$1/T_{1obs} = (1 - P_a - P_g)/T_{1w} + P_a/T_{1a} + P_g/T_{1g} \quad (6)$$

where P represents the fraction of water and the subscripts w, a, and g represent free water, water associated with agar, and water

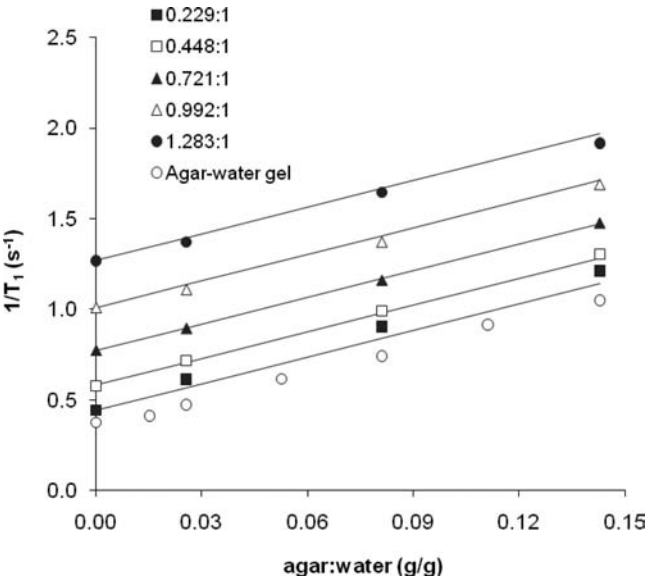

Figure 3. Fitted and observed spin−lattice relaxation rate of glycerol−water−agar gels at 30 °C, measured at 23 MHz. The ratios (g/g) are glycerol to water in glycerol−water−agar gels. The lines represent calculated fits using eq 8.

associated with glycerol respectively; T_{1w}, T_{1a}, and T_{1g} represent the relaxation times of each fraction.

Equation 6 can be rewritten as

$$1/T_{1obs} = (1 - P_g)/T_{1w} + P_g/T_{1g}) + P_a/T_{1a} - P_a/T_{1w} \quad (7)$$

If we assume that there is little change of the fractions of water interacting with solutes as they are mixed, then eq 7 is represented by

$$1/T_{1obs} = 1/T_{1obs} \text{ for glycerol solutions} + 1/T_{1obs} \text{ for agar} - \text{water gels} - 1/T_{1w} \quad (8)$$

where the last term is small and negligible with respect to the others.

The fitted data using eq 8 were plotted as the solid lines, superimposed on the measured data in Figure 3. The agreement between experiment and prediction was remarkably good, validating the assumptions made. Relaxation rates of glycerol−agar−water gels containing nutrient behaved similarly to those of glycerol−agar−water gels (data not shown).

Spin−Spin Relaxation of Glycerol solutions. Single-exponential relaxation decays were observed for each glycerol solution, measured at 23 MHz at 25 and 30 °C, using a CPMG sequence with a pulse spacing of 200 μs. The spin−spin relaxation rates ($1/T_2$) were plotted as a function of glycerol concentration (g/g) in Figure 4. The plots are curved and shown fitted as two lines but showed similar values and concentration dependence as the spin−lattice relaxation. Since the relaxation rates were significantly faster than in pure water, a fast exchange average between proton species can be assumed.

Fabri et al.[3] showed a dependence of T_2 on pulse spacing and interpreted the T_2 dispersion spectrum for small polyols in terms of proton exchange only. They have shown that exchange between OH protons of the solute and water can result in faster

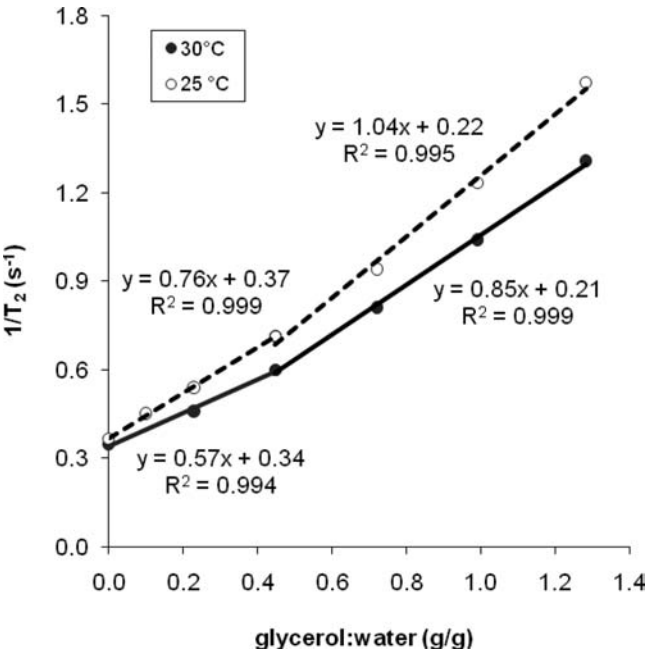

Figure 4. Spin−spin relaxation rates of protons in glycerol−water solutions against glycerol concentrations at 25 and 30 °C, measured at 23 MHz.

relaxation rates than pure water, particularly when the CPMG pulse spacing (tau) is extended to the order of milliseconds.[3,13] When the pulse spacing is long compared to the exchange rate, the exchange between the two chemically shifted sites causes dephasing of the spins, hence enhanced relaxation. When the pulse spacing is short compared to the exchange rate, the relaxation time would be long. Our data showed only a slight trend with tau spacing. In contrast, Lai and Schmidt[4] reported significant shortening of ^{17}O relaxation times in sugar solutions, where chemical exchange effects do not contribute. They reported T_2 values, at a concentration of 0.5 g sugar per gram water, to be one-half that of pure water. This is comparable to the effect shown above by adding glycerol, so exchange between free and motionally modified water molecules cannot be ignored under our experimental conditions. Similar results were reported by Belton and Wright.[8] We cannot clearly separate water or proton exchange in our experiments, since both would be fast. However, because of the small chemical shifts at low operating frequency and at the pulse spacing used in this study, we assumed that water exchange processes dominated the T_2 relaxation.

If we assumed there was only water exchange, then relaxation rate can be expressed as

$$1/T_2 = (1 - Hc)/T_{2w} + (Hc)/T_{2b} \quad (9)$$

where c is the concentration of glycerol, H is the number of water molecules associated with each glycerol molecule, and T_{2w} and T_{2b} are the spin−spin relaxation times for water in the free and motionally modified states, respectively.

Estimates of H/T_{2b} from the initial slopes of the graphs were 0.76 at 25 °C and 0.57 at 30 °C. If we assume that T_{2b} is comparable to the T_2 of nonexchangeable protons of glycerol at the same low concentration (approx 3 s), then H had values of 2.28 and 1.71 g of water per gram of glycerol at 25 and 30 °C, respectively, similar to results of spin−lattice relaxation as discussed previously. The small decline in hydrating water with increasing temperature is to

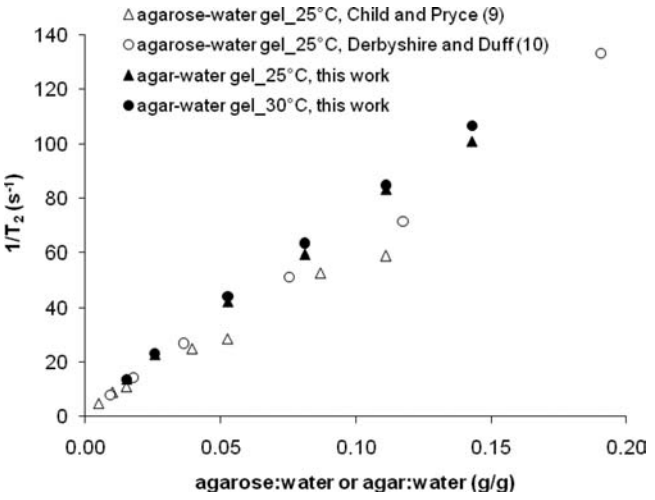

Figure 5. Comparison of ^{1}H spin−spin relaxation rate of agar gels at 25 and 30 °C with that of agarose gels at 25 °C. Data of agarose gels are from Child and Pryce[9] and Derbyshire and Duff.[10]

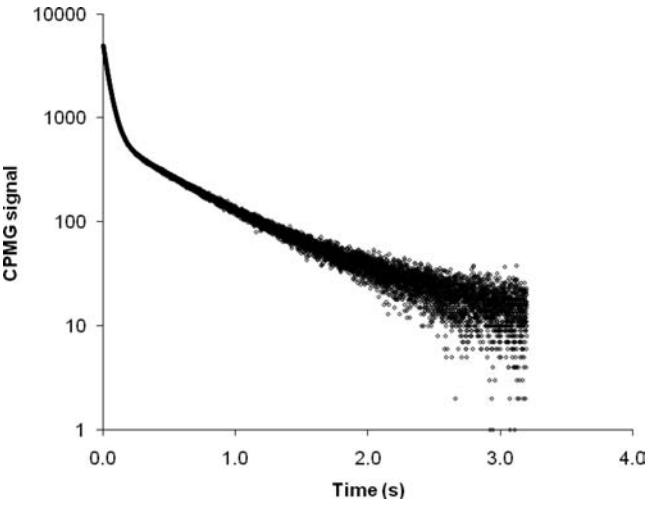

Figure 6. CPMG ^{1}H spin−spin relaxation decay of a glycerol−agar−water gel, glycerol:agar:water of 0.229:0.026:1, at 30 °C, measured at 23 MHz.

be expected. Converting these to a molecular ratio, we obtained 11.7 and 8.7 mol of water per mole of glycerol, which is again comparable to 16 mol water per mole of monosaccharide reported by Belton and Wright,[8] measured by ^{17}O relaxation.

Spin−Spin Relaxation of Agar Gels. Measured $1/T_2$ values (Figure 5) for agar−water gels were much greater than $1/T_1$ (Figure 1), as previous workers have also reported.[9,10,14,15] The observed spin−spin relaxation behavior was previously described by Ablett et al.[15]

$$1/T_{2\mathrm{obs}} = 1/T_{2\mathrm{a}} + hc(1/T_{2\mathrm{b}} - 1/T_{2\mathrm{a}}) + \gamma c/(1-\gamma c)(T_{2\mathrm{c}} + \tau_{\mathrm{ex}}) \quad (10)$$

where subscript a is free water, b is "bound" water, and c is a third proton species having an exchange rate comparable to its relaxation time, τ_{ex} is the exchange time of this c species with other components, and γ is a constant. The origin of this "c phase" is still ill defined for polysaccharide gelling systems. Ablett et al.[15] suggested that this phase could be assigned to hydroxyl protons on agarose exchanging with water but found no dependence on pH nor any dependence of T_2 on tau spacing, as would be expected for proton exchange. We observed similar result in this study, where T_2 of the agar−water gel (agar:water of 0.026) was ∼53 ms for tau values ranging from 100 to 1000 μs.

Since agar gelation involves both formation and aggregation of double helices, the possibility of a slowly exchanging water fraction remains a possibility. Its relaxation time could approximate that of agar protons, which we observed to be about 90 μs. On the other hand, Hills et al.[13] reported the relaxation behavior of a rehydrated crude cell wall extract with a proton relaxation time of 30 ms and suggested that this relaxation is dominated by the exchange between water and hydroxyl protons on the rigid cell wall polysaccharide, but these measurements were at high frequency, where proton exchange is more likely to dominate.

Spin−spin relaxation rate of agar−water gels was similar at 25 and 30 °C (Figure 5). This was expected since 25 °C is a temperature close to that where T_2 passes through its minimum value, reported by several other workers.[10,14,15]

Our results of T_2 relaxation rates of agar gels are comparable to those of agarose−water gels, despite the different measurement frequencies (Figure 5). This is because T_2 is always sensitive to low (zero frequency) modes of the spectral density function, and it is these motions that dominated the observed relaxation. Values of h or γ cannot be simply derived from these plots, because of the complexity of the relaxation equation and the absence of independent estimates of T_{2b} and T_{2c}. However, Derbyshire and Duff[10] obtained an estimate for h of 0.59 g per gram of agarose or 5 water molecules per saccharide residue, implying complete hydration of agar at a concentration of 66% by weight, but this was obtained by freezing studies of the simple gel.

Ablett et al.[15] showed that the low- temperature T_2 minimum characteristic of dilute gels was present in an agarose film of 66% agar in water. This supports the conclusion that the slow exchange is between a few immobile protons (water and/or hydroxyls) and a more mobile hydration water species while exchange between hydration and "free" water is fast. We made this assumption in further discussion and data fitting.

Spin−Spin Relaxation of Mixed Systems. Studies were carried out on glycerol−agar−water gels at both 30 and 25 °C. We observed nonlinear transverse relaxation decays. An example of the CPMG signal decay of the glycerol−agar−water gel of weight ratios 0.229:0.026:1 is given in Figure 6.

The origin of the nonlinear phenomenon for glycerol−agar−water gels will be discussed later, but for the purposes of comparing these mixed systems with data derived from binary systems, the dominant faster relaxation and the weighted mean relaxation times were used (Tables 2 and 3). The spin−spin relaxation of mixed systems showed a similar behavior to T_1 relaxation but with much shorter times, showing the dominance of agar in determining the T_2 relaxation rates of the mixed systems.

The weighted T_2 relaxation rates were plotted against agar concentrations, expressed relative to the total solvent (Figure 7). Regardless of the glycerol concentration, the weighted $1/T_2$ lay on the same line against agar concentrations, implying that the T_2 relaxation was dominated by agar, the exchange processes were relatively unaffected by the addition of glycerol, which acted simply as a solvent.

In the absence of the details of each of the relaxing proton species, we attempted a simple fitting procedure comparable to that used by Ablett et al.,[15] where it was assumed that the fast relaxing phase is represented by that of 66% agarose/water film.

Table 2. Observed Spin−Spin Relaxation Times (T_{2L} and T_{2S}) and Weighted Mean Relaxation Rate of Protons in Glycerol−Agar−Water Gels at 30°C

glycerol:agar:water	T_{2L} obs (ms)	%T_{2L} obs	T_{2S} obs (ms)	%T_{2S} obs	$1/T_2$_weighted (s^{-1})
0.229:0.026:1	583.1	12.59	46.9	87.41	18.9
0.229:0.081:1	423.4	12.64	15.9	87.36	55.3
0.229:0.143:1	349.4	12.17	9.7	87.83	90.6
0.448:0.026:1	488.4	20.90	60.3	79.10	13.5
0.448:0.081:1	380.8	18.82	19.5	81.18	42.1
0.448:0.143:1	299.4	19.71	12.1	80.29	67.3
0.721:0.026:1	415.0	28.65	66.4	71.35	11.4
0.721:0.081:1	325.2	24.83	22.5	75.17	34.2
0.721:0.143:1	260.0	25.36	13.3	74.64	57.00
0.992:0.026:1	354.9	34.66	71.50	65.34	10.10
0.992:0.081:1	291.0	29.87	24.5	70.13	29.7
0.992:0.143:1	233.9	29.74	15.4	70.26	46.8
1.283:0.026:1	292.0	39.29	67.5	60.71	10.3
1.283:0.081:1	224.3	34.47	24.9	65.53	27.8
1.283:0.143:1	188.4	32.64	15.2	67.36	46.1

Table 3. Observed Spin−Spin Relaxation Times (T_{2L} and T_{2S}) and Weighted Mean Relaxation Rate of Protons in Glycerol−Agar−Water Gels at 25°C

glycerol:agar:water	T_{2L} obs (ms)	%T_{2L} obs	T_{2S} obs (ms)	%T_{2S} obs	$1/T_2$_weighted (s^{-1})
0.229:0.026:1	463.4	13.31	49.5	86.69	17.8
0.229:0.081:1	341.0	12.80	16.9	87.20	51.9
0.229:0.143:1	287.8	12.71	10.4	87.29	84.4
1.283:0.026:1	225.8	44.00	69.5	55.60	9.9
1.283:0.081:1	172.2	35.96	26.8	64.04	33.5
1.283:0.143:1	157.8	34.40	16.0	65.60	43.3

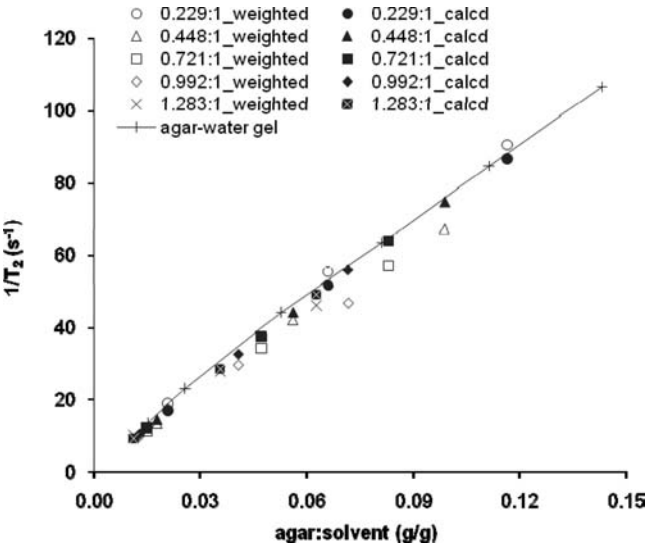

Figure 7. Weighted mean and calculated spin−spin relaxation rate of glycerol−agar−water gels at 30 °C, measured at 23 MHz. The ratios (g/g) are glycerol to water in glycerol−water−agar gels.

The fraction of fast relaxing phase (P_b) is then 0.5 times the concentration of agarose (g/g water). They assumed that this phase then exchanges with residual water, which had the same relaxation time as pure water. In our case, the solvent was not water but a glycerol/water mixture whose relaxation time has been independently measured here.

The observed relaxation time for any mixed system will then be given by

$$1/T_{2obs} = (1 - P_b)/T_{2solvent} + P_b/T_{2b} \qquad (11)$$

where T_{2b}, 0.6 ms, is the relaxation time of the film containing 66% agar at 30 °C, P_b is 0.5c, and c is the concentration of agar in the mixed system (g/g solvent).

The experimental results were in agreement with the calculated data using eq 11 (Figure 7). The observed relaxation times were dominated by agar, but the quality of the fitting of this simple model is quite remarkable. This implies that whatever the solvent composition, the system behaves as if it were a 66% agar film, simply diluted by solvent. Such fits would not be obtained if introduction of glycerol had a significant effect on the proton relaxation times of water associated with agar or the exchange rate of the "c phase". Further, this implies that agar gel structure and hence helical conformation and aggregation in the gels were not significantly influenced by the introduction of glycerol.

As a further check on the fit of this simple model, the temperature dependence of T_2 was measured for one mixed system, glycerol:agar:water of 1.283:0.143:1. This gel showed biphasic relaxation, so the weighted mean relaxation time was calculated at each temperature (Table 4). Both the dominant shorter relaxation time and the weighted average showed a minimum value as a function of temperature. For the weighted average T_2, this occurs at around 310 K (Figure 8), close to that of agarose in water shown by Ablett et al.[15]

Table 4. Temperature-Dependent Spin−Spin Relaxation Times (T_2, ms) of Glycerol−Agar−Water Gels (glycerol: agar:water of 1.283:0.143:1)

temperature	T_{2L} (ms)	% T_L	T_{2S} (ms)	% T_{2S}	T_2_mean (ms)
285 K	92.8	35%	22.1	65%	30.1
298 K	139.8	33%	16.9	67%	23.9
303 K	188.4	33%	15.2	67%	21.7
305 K	181.8	35%	14.1	65%	20.3
308 K	196.9	33%	13.8	67%	19.9
312 K	216.9	33%	13.6	67%	19.8
315 K	241.5	33%	13.4	67%	19.5
323 K	333.6	33%	18.3	67%	26.7
330 K	367.6	33%	22.0	67%	31.9
336 K	417.2	33%	27.0	67%	39.1

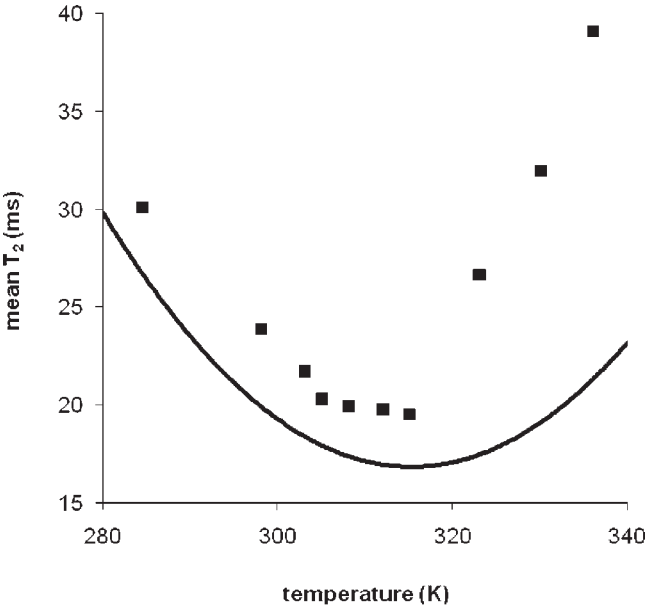

Figure 8. Temperature dependence of spin−spin relaxation times (T_2, ms) of a glycerol−agar−water gel (glycerol:agar:water of 1.283:0.143:1).

This data was then also modeled using the same approach described for the concentration plots above, using the temperature dependence of the 66% agarose film data from Ablett et al.[15] and the relaxation times of the solvents measured here. As above, the calculated data (shown as the solid line in Figure 8) were dominated by the term P_b/T_{2b}, so the temperature dependence of solvent relaxation has been ignored. The same agreement with experiment was found at temperatures below the T_2 minimum and the same deviation at higher temperature as were noted for simple aqueous agarose gels.[15] Considering the approximations made, the fit is remarkably good and provides further evidence that agar gels were not significantly disturbed by addition of high levels of glycerol.

All the same trends were observed for glycerol−agar−water gels containing nutrient at 30 °C (data not shown) and can be fitted by the same simple model.

Origins of Multiexponential Spin−Spin Relaxation in Mixed Systems. Multiple exponential relaxation behavior, as shown in Figure 6, may be explained by the slow diffusive exchange between water at different sites and results from the heterogeneity of the gels. Agar forms gels with a distribution of pore sizes and is therefore heterogeneous. Previous work has shown that if the heterogeneity is increased by freezing and thawing (when very large pores are created), then water proton spin−spin relaxations become nonexponential even in simple agar gels.[16] This is because water molecules sample nonuniform concentrations of agar during their own intrinsic relaxation time of several seconds. This behavior is not limited to gels but is found in many heterogeneous materials, for example, arrays of spun fibers and post rigor meat and fish.[16,17] Hills et al.[13] also reported complex transverse relaxation in skimmed milk powder and attributed it to slow diffusive exchange between the particulate and the solution phases. This behavior has been analyzed more completely in Hydrogel systems.[18,19]

In the mixed systems studied here, the greatest nonlinearity in decay curves was found at the highest glycerol and lowest agar contents, i.e., when diffusion was slowest, the largest pore sizes were present in the gels and when solvent relaxations were shortest. If we assume the diffusion of glycerol solution follows the Einstein relation, the space sampled by water protons in a glycerol solution within one relaxation time can be given by

$$d = (D\,t)^{1/2} \tag{12}$$

where D is the diffusion coefficient of water in glycerol solution and t is the water proton T_2 relaxation time. For a glycerol solution (glycerol:water of 1.283:1) at 25 °C, T_2 was measured to be 0.64 s and D from a pulsed field gradient experiment was found to be 4.3×10^{-10} m^2 s^{-1}. Therefore, d of the glycerol solution was 17 μm. This is 5 times smaller than its value in pure water, 100 μm.[16]

In other words, we suggest that as the glycerol concentration increases, the agar gel network retains similar dimensions and pore size distribution but the water protons sample smaller dimensions and are accordingly more prone to detect heterogeneities. This may be sufficient to cause multiexponential behavior. Chui et al.[19] displayed probability distribution functions of pore sizes in a series of aqueous agar gels. By inspection, if gel dimensions remain the same, a reduction of sampling dimensions by a factor of 5 would probably result in nonlinear decays. Since we have no direct data on pore size dimensions this data cannot be modeled. However, all the evidence presented above suggests that changes in gel network structure were not a major factor.

Diffusion in Glycerol Solutions. The results of diffusion measurements at 500 MHz on resolved signals of water OH protons and glycerol CH protons are shown in Table 5. Both proton species diffused slower when glycerol concentration was increased.

We observed that the self-diffusion coefficient of OH protons were at least two times greater than that of CH proton in all glycerol solutions at 500 MHz. Therefore, the faster diffusion of water protons should dominate the signal decay curves. This was true even at 23 MHz after observation time of 200 ms. The self-diffusion coefficient of protons in glycerol solutions was similar to those of water OH protons observed at 500 MHz (Figure 9).

Diffusion in Agar−Water Gels. The results for simple gels have been reported before.[20] The measured self-diffusion coefficients were markedly dependent on macromolecule concentrations but also showed a dependence on the observation time over which diffusion was measured. The observation time of 200 ms was shown to be long enough to minimize the effect of observation time. This restricted diffusion had not been reported previously for agar gels, but similar restricted diffusion has been reported in gelatin and starch gels.[21−23]

Table 5. Diffusion Coefficient of Water OH Proton and Glycerol CH Proton in Glycerol−Water Solutions Measured at 25°C, 500 MHz, Fixed Observation Time (Δ) of 200 ms

	water OH proton		glycerol CH proton	
glycerol:water	D_{water} (10^{-10} m^2 s^{-1})	δ (ms)	D_{water} (10^{-10} m^2 s^{-1})	δ (ms)
0.100	19.87	1.25	7.63	1.70
0.229	15.90	1.30	6.05	1.60
0.448	11.14	1.45	4.29	2.30
0.721	7.82	1.70	2.97	2.70
0.992	5.73	2.00	2.17	3.10
1.283	4.26	2.15	1.59	3.70

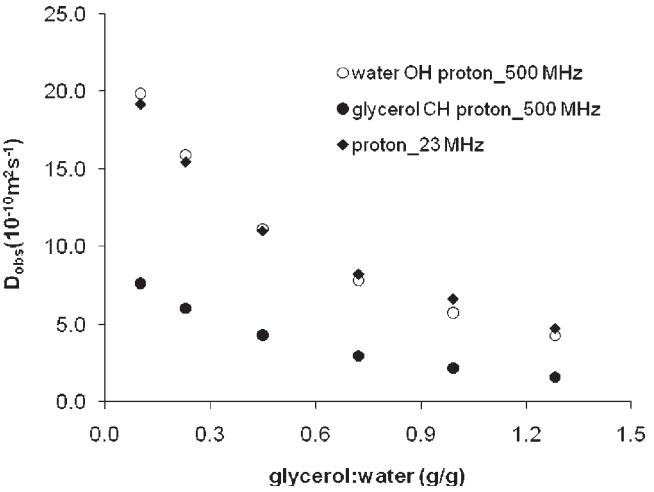

Figure 9. Self-diffusion coefficient of protons in glycerol solutions at 500 MHz and 25 °C.

It is noteworthy that this restriction effect was not reported in earlier studies of agarose gels,[10,14] guar solutions, and pectin gels, [24] presumably because short observation times were employed. For agarose, a two-phase diffusion model, in which the measured self-diffusion coefficient is a weighted average of free and polymer associated water was initially employed to explain the data, i.e.

$$D = D_w(1 - hc) + hc\,D_b \qquad (13)$$

where D_w is the self-diffusion coefficient of water, D_b is the self-diffusion coefficient of transiently attached water, h is the water associated with the polymer, and c is the polymer concentration.

This model could not adequately explain the observed reduction in the self-diffusion coefficient as agar concentration increased. Instead, a model following that of Wang[25] needed to be invoked, where freely diffusing water encountered hindrance from the hydrated polymer network. Wang's model implies that if all water molecules encounter barriers during the observation time of the experiment, then restricted diffusion would be detected, as found in this work. The diffusion behavior of water in these simple gels is crucial to the interpretation of the behavior of the mixed systems reported below.

Diffusion in Mixed Systems. Studies of proton diffusion were carried out on glycerol−agar−water gels with and without nutrients. A single value of Δ (200 ms) was used corresponding to an observation time where restricted diffusion was detected for

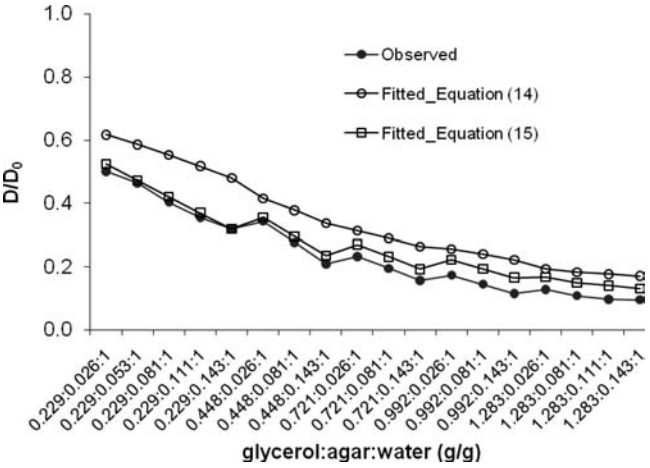

Figure 10. Observed and fitted ratio of apparent D of water in glycerol−agar−water gels to that of pure water measured at 23 MHz, Δ = 200 ms, and 25 °C. Fitted data were calculated using eqs 14 and 15.

binary agar−water gels.[20] Pulse echo decays were log linear over approximately one decade. Discussion of the results focuses on the glycerol−agar−water gels, though those containing nutrient behave the same.

We can deduce from the results of binary agar−water gels and glycerol−water solutions that the diffusing protons measured at 23 MHz were primarily those of water molecules. Even if protons from nonexchangeable CH protons of glycerol or exchangeable hydroxyls of glycerol or agar were included, they would have little weighting in the overall signals detected in these experiments. The results for glycerol−agar−water gels are shown in Figure 10.

In each suite of glycerol solutions there was a sudden reduction in the self-diffusion coefficient when agar was introduced into the system. This was the same behavior as for the binary agar−water gels and reflects the onset of restricted diffusion detected at the long observation interval (200 ms) used here. Glycerol−agar−water gels with nutrients give exactly analogous results (data not shown).

The simplest model needed to explain this behavior assumes that the observed self-diffusion coefficient in glycerol−agar−water gels (D_{obs}/D_0) can be represented by the product of the self-diffusion coefficient of water in glycerol−water solutions (D_{obs_g}/D_0) modified by the barrier effects induced by the agar gel (D_{obs_a}/D_0), shown as eq 14

$$D_{obs}/D_0 = (D_{obs\_g}/D_0)(D_{obs\_a}/D_0) \qquad (14)$$

This is the same model adopted by Farhat et al.[23] in their study of a starch−sugar−water system. The results of such a fit are compared with experimental data in Figure 10. The agreement is reasonable, but experimental results are systematically lower than predicted by this simple model, and at high glycerol and agar levels this deviation is as high as 50%. Our previous studies of relaxation behavior show that agar gel structure is not dramatically changed by addition of glycerol to the aqueous solution, so some other explanation is necessary.

The most obvious reason for discrepancy is that eq 14 assumes that only water will be slowed by barrier effects. However, we should assume that glycerol will also be slowed by the same barriers, and since water motion is coupled to glycerol during transport, it is incorrect to assume that the diffusion of the glycerol−water solvent in gelled mixtures will be the same as in free solution.[26] Corrections for this effect can only be approximated

from the available data, but if we assume glycerol is also slowed by obstruction effects to the same extent as water and that water diffusion is coupled to glycerol, then the self-diffusion coefficient of the glycerol water solvent in gelled matrices may be reduced by a similar factor, i.e., D_{obs_g}/D_0 (glycerol solution) in eq 2 should also be reduced by a scaling factor, which we estimate to be similar to D_{obs_a}/D_0 at any given agar concentration.

With these assumptions eq 14 becomes

$$D_{obs}/D_0 = \{(D_{obs\_g}/D_0)(D_{obs\_a}/D_0)\}D_{obs\_a}/D_0 \quad (15)$$

A significantly better fit is obtained (Figure 10). Brosio et al.[24] studied the mixed system pectin/sucrose/water and saw analogous effects. Restriction of diffusion was not observed, probably due to the shorter observation times (5−20 ms); however, increasing sucrose significantly reduced the diffusion rates.[24] Similarly to our studies above, diffusion rates were reduced by more than the simple additive effects of pectin and sucrose. This result was interpreted as the effect of salting out and gelation of pectin at high sucrose levels. Such an explanation cannot be applied to this work, since our mixed systems were all gels. Clearly the origin of reduced diffusion in these typical food model systems deserves further studies.

CONCLUSIONS

This study shows the first attempt to separate the effect of solutes from that of matrix structure on water dynamics, each of which are known to regulate the long-term microbial, chemical, and physical stability of food products.

We have shown that addition of a second solvent, glycerol, significantly alters both the spin−lattice and the spin−spin relaxation behavior of water protons in agar gels. Furthermore, it caused the multiexponential decay observed in spin−spin relaxation experiments. However, the use of simple models of fast exchange shows that the spin−lattice relaxation behavior can be explained by the similar magnitude of the effects of glycerol and agar, modifying water motion. The results imply that while glycerol had a significant effect on spin−spin relaxation times and diffusion rates, it had minimal effect on agar gel structure. In addition, the multiexponential behavior of spin−spin relaxation of mixed systems was not a result of the change of the gel structure but may be explained by the heterogeneity of agar gels.

Self-diffusion measurements of protons in mixed agar gel systems, at low field strengths, were dominated by water protons. In addition, the macromolecular gel matrix further limited the self-diffusion coefficient of water by obstruction and by significantly reduced diffusion path lengths. It is evident therefore that while T_1 relaxation times of water fortuitously correlate with glycerol concentrations in both small and macromolecular systems, the averaged T_2 and diffusion of water are dominated by the more immobile gel network.

Low-resolution NMR spectrometers are adequate to measure water mobility in complex systems; however, careful interpretation is required to give a highly detailed picture of the molecular motion of water.

AUTHOR INFORMATION

Corresponding Author

*To whom correspondence should be addressed. Address: Lumigenex (Suzhou) Co., Ltd., 218 Xing Hu Road, Biobay A3 Suite 224-225, Suzhou Industrial Park, China 215123. Phone: +86 512 80988092. Fax: +86 512 80988096. E-mail: yang.huang@lumigenex.com.

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