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Interaction of MRI Gadolinium Contrast Agents with Phospholipid Bilayers as Studied by 95 GHz EPR†

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Interactions of two MRI gadolinium contrast agents, gadolinium ethoxybenzyl-diethylenetriaminepentaacetate (Gd-EOB-DTPA) and gadolinium *N*-pentyl-1,4,7,10-tetraazacyclododecane-*N'*,*N''*,*N'''*-triacetic acid (Gd-DOTA-P), with multibilayer phospholipid dispersions prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) have been investigated with high resolution EPR spectroscopy at 95 GHz. At a resonance field of 3.3 T, EPR spectra of a small nitroxide probe, 2,2,6,6-tetramethyl-1-piperidinyloxyl (Tempo), partitioned between aqueous and membrane phases are clearly resolved, making measurements of dynamics parameters of the probe accurate and unambiguous. Results show that although the presence of the more lipophilic contrast agent, Gd-DOTA-P, can be detected within the bilayer, the structural organization of the membrane remains unaffected even at physiologically high (5 mM) concentrations. The temperature of the main phase transition of the bilayer was also unaffected to within the 0.4 °C accuracy of its determination.

The rational development of new selective paramagnetic MRI contrast agents requires a detailed understanding of interactions of these agents with biological membranes and proteins. It also is important to determine the cellular location of these agents as well as their effects on the function and structure of cellular organelles, membranes in particular. NMR methods alone are often unable to answer all these questions.

Electron paramagnetic resonance (EPR) is an important experimental method for obtaining information on rotational and translational motion of spin-labeled molecules.[1,2] In addition, the relaxation of spin-labeled molecules [both the spin–lattice (T_1) and spin–spin (T_2) relaxation times] is also sensitive to spin–spin interactions such as Heisenberg exchange and dipolar interactions.[3–6]

In this report, we explore EPR spin probe/label methods that can be used to detect: (i) the presence and possible locations of lipophilic contrast agents in the membranes, (ii) the effects of MRI contrast agents on rotational dynamics of small solute molecules in the membrane, and (iii) changes in the local polarity of the bilayer and its phase behavior. These EPR studies are more informative if carried out at high frequencies (e.g., 95 GHz, W-band), which provides high *g*-value resolution and an increased range of measurable correlation times. Over the past couple of decades, high-frequency EPR spectroscopy has been carried out, and its special benefits demonstrated, by just a few research groups; see early examples from some of these groups.[7–10] Until recently, most applications of HF EPR were limited to samples of low dielectric loss. Developing high-quality-factor ($Q > 3700$) cylindrical resonators at 95 GHz and better sample handling techniques have allowed us to improve sensitivity for lossy samples and so to use HF EPR to study highly hydrated (80%) phospholipid dispersions.

Results and discussion

EPR studies of aqueous systems, including highly hydrated phospholipid bilayers, are usually carried out at 9.5 GHz (X-band). Some of these experiments utilize small nitroxide probes, such as Tempo or di-*tert*-butyl nitroxide (DTBN), which, on being partitioned between aqueous and membrane compartments, give rise to a characteristic splitting of the $m_I = -1$ nitrogen hyperfine component (Fig. 1A). This splitting commonly is characterized by an empirical partition parameter, which has been found to be useful to monitor phase behavior and fluidity of the phospholipid bilayer.[1]

Although X-band measurements may be very informative, a progressive increase of microwave frequency

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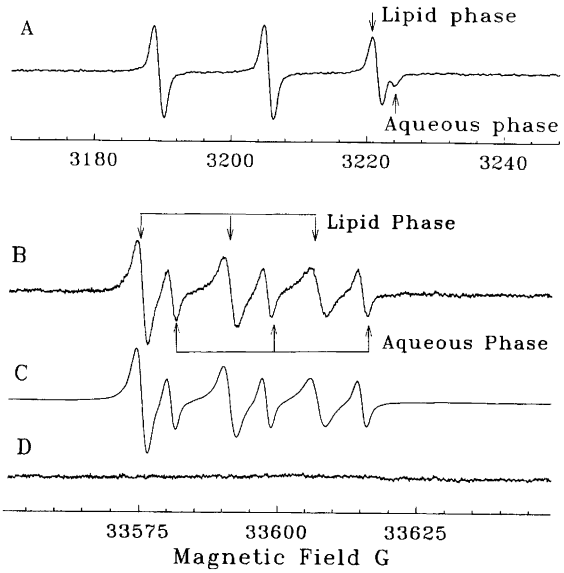

Fig. 1. (A) 9.5 GHz (X-band) EPR spectrum from spin probe Tempo (2,2,6,6-tetramethyl-1-piperidinyloxyl) partitioned into multilamellar DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine) liposomes at 43.8 °C. (B) 95 GHz (W-band) EPR spectrum from a Tempo/DPPC sample, showing a clear separation of signals originating from aqueous and lipid phases. (C) Least-squares simulation of experimental W-band spectrum. (D) Difference between experimental and simulated spectra (residual of the fit).

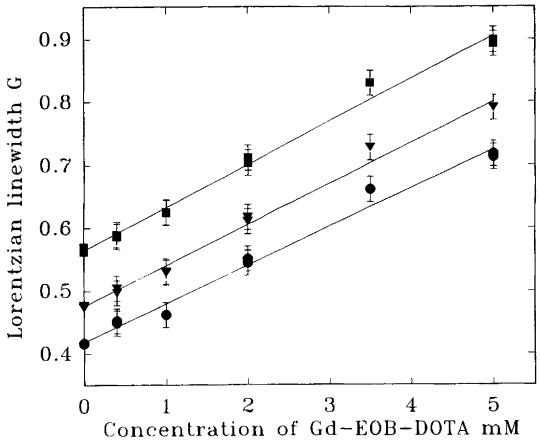

Fig. 2. Concentration effect of Gd-EOB-DOTA on 95 GHz EPR spectra from 1 mM Tempo aqueous solution at room temperature. Lorentzian linewidths of each nitrogen hyperfine component determined from least-squares simulations of experimental spectra[13] are displayed as follows: ●, $m_I = 1$; ▼, $m_I = 0$; ■, $m_I = -1$.

enhances both sensitivity to molecular motion and resolution between multiple membrane compartments. At 95 GHz, all three lines of the Tempo EPR spectrum from the lipid phase of DPPC membranes are clearly resolved from the signal originating from the aqueous phase (Fig. 1B). The spectrum corresponding to the lipid phase is shifted down-field because of the higher value of g_{iso} in the lipid phase compared with g_{iso} in the aqueous phase. It was shown earlier that such high g-value resolution is necessary to obtain a unique least-squares simulation of the Tempo/DPPC spectrum[11] (Fig. 1C). Here parameters of such least-squares simulations were analyzed to study effects of MRI gadolinium contrast agents on local microviscosity and polarity of the membrane and to extract the linewidth contribution arising from spin–spin interactions with the Gd^{3+} complexes.

The effect of spin–spin interactions with Gd^{3+} complexes on the 1 mM Tempo aqueous solution EPR spectrum is shown in Fig. 2. In the presence of Gd-EOB-DTPA (gadolinium ethoxybenzyl-diethylenetriaminepentaacetate), each of the three nitrogen hyperfine lines broadens equally at a rate of 65 ± 3 mG mM^{-1}. This can be explained by spin–spin interaction between Tempo and Gd-EOB-DTPA molecules during a bimolecular collision, in which a Tempo molecule can approach only the ligands. Since both electron spin exchange and dipolar interaction are effective over only a short distance, this observation suggests that the spin density on the ligands of Gd complexes is sufficient to shorten the electronic relaxation time of Tempo. This can be further exploited to measure the local concentration of Gd complexes by EPR and to probe selectively the relaxation properties of Gd complexes with respect to outer-sphere molecules.

In the fast tumbling limit, the homogeneous linewidth (T_2^{-1}) of a nitroxide probe can be expressed as eqn. (1),

$$T_2^{-1}(m_I) = A + Bm_I + Cm_I^2 \quad (1)$$

where m_I is the z component of the nitrogen nuclear spin quantum number. All linewidth parameters A, B, and C are functions of the rotational correlation time τ_R of the probe; however, parameter A also includes frequency-independent contributions to linewidth in addition to contributions from spin–spin interactions. If there is a paramagnetic metal ion in the vicinity of the probe, it will increase parameter A. Another informative linewidth parameter is B, which is independent of the spin–spin interaction and is proportional to the rotational correlation time of the probe. From 95 GHz experiments, linewidth parameter B [eqn. (1)] can be determined more accurately.[12,13]

In our experiments it was observed that addition of 10 mM Gd-EOB-DTPA did not affect, to within the experimental accuracy, the rotational correlation time τ_R of the Tempo molecule in the phospholipid phase of the membrane (plot of linewidth parameter B as a function of temperature is shown in Fig. 3A). Therefore, we conclude that when the membrane is in the fluid bilayer-structure phase (L_α), rotational dynamics of Tempo are unaffected by the presence of this Gd^{3+} complex at 10 mM. The changes in the temperature of the main phase transition (observed as a step change in τ_R) could not be detected. Thus, addition of Gd-EOB-DTPA at concentrations much higher than usually applied in MRI experiments (ca. $\leqslant 0.25$ mM) did not alter the phase behavior of the membrane. This result is in general

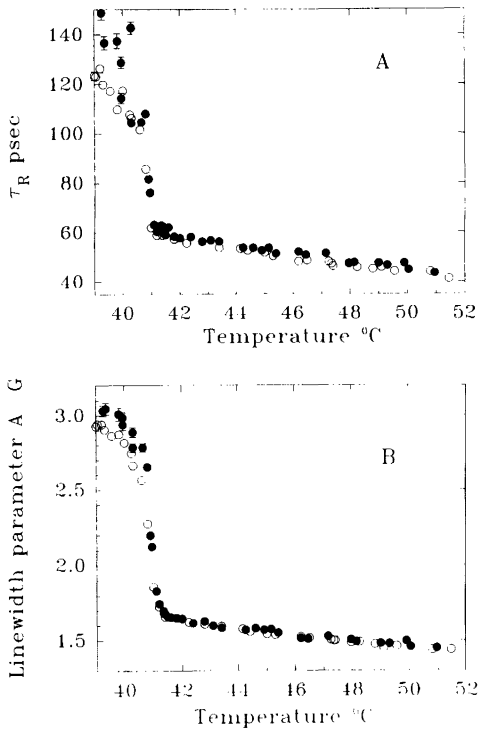

Fig. 3. (A) Rotational correlation time (τ_R) of Tempo molecules partitioned into the lipid phase of the bilayers as a function of temperature: ●, Tempo/DPPC; ○, Tempo/DPPC/Gd-EOB-DTPA. (B) Linewidth parameter *A* [eqn. (1)] as a function of temperature for a Tempo/DPPC control (●) and a sample containing 10 mM Gd-EOB-DTPA.

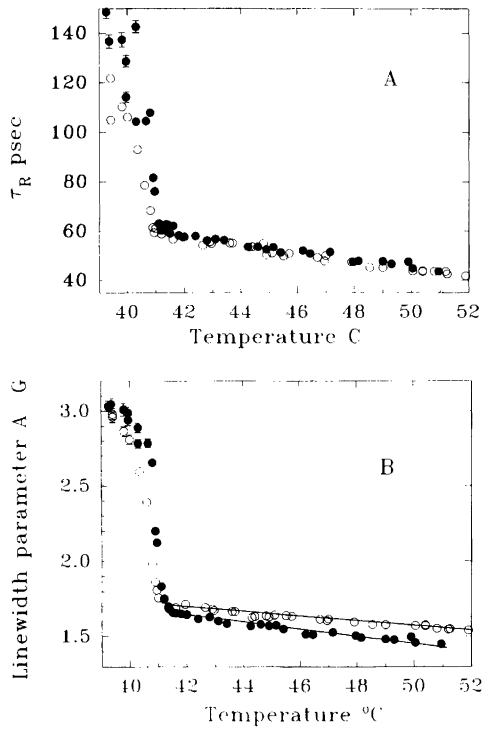

Fig. 4. (A) Rotational correlation time (τ_R) of Tempo molecules partitioned in the lipid phase of the bilayers as a function of temperature: ●, Tempo/DPPC; ○, Tempo/DPPC/Gd-DOTA-P. (B) Linewidth parameter *A* [eqn. (1)] as a function of temperature for a control Tempo/DPPC (●) and a sample containing 5 mM Gd-DOTA-P.

agreement with data from another EPR study in which changes in the cooperativity of the main phase transition of DPPC membranes were observed only at high electrolyte concentrations ($\geqslant$1 M).[14] Parameter *A* also was not affected by the presence of the Gd-EOB-DTPA (Fig. 3B). Because Tempo is distributed primarily in the polar head region of the bilayer,[15] the last result indicates that the presence of this contrast agent in the most polar part of the membrane is negligible.

Another example of an MRI contrast agent that we studied is a pentane derivative of Gd-DOTA, Gd-DOTA-P (gadolinium *N*-pentyl-1,4,7,10-tetraazacyclododecane-*N'*,*N''*,*N'''*-triacetic acid). We found that the phase behavior of the membrane and the rotational correlation time of Tempo were not affected when 5 mM Gd-DOTA-P was added (Fig. 4A). Therefore, even if the pentane group of this complex interacts and/or partitions within the membrane, the overall effect on the membrane phase behavior and structural organization of the polar head region (observed as changes in τ_R of Tempo) is rather small at concentrations of the complex up to 5 mM. While parameter *B*, and thus the rotational correlation time of the probe Tempo, was not affected, parameter *A* was increased at temperatures above the phase transition (L_α-phase; Fig. 4B). The difference in parameters *A* measured for the control sample and for the sample containing 5 mM of Gd-DOTA-P yields linewidth broadening [$\delta(\Delta B_{p\text{-}p})$, or $\delta(1/T_2)$] arising solely from spin–spin interaction between the contrast agent and the spin probe partitioned in the lipid phase of the bilayer. Since both spin exchange and dipole–dipole interactions have a short range (dipolar broadening has a $1/R^3$ dependence on the distance, *R*, from the bilayer surface if the paramagnetic relaxer is solely distributed in the aqueous phase), these data indicate a close proximity of Gd-DOTA-P to the lipid phase of the bilayer and, possibly, partitioning of some of the complexes into or close to the polar head region.

Analyses of the nitrogen hyperfine constant of Tempo EPR spectra corresponding to the lipid phase of the bilayer show that in the presence of 5 mM of Gd-DOTA-P this constant, and therefore the polarity of the membrane, was unaffected (data not shown).

Addition of the 10 mM Gd-EOB-DTPA to the aqueous lipid dispersion increased the nitrogen isotropic hyperfine splitting corresponding to Tempo molecules in the membrane lipid phase by a small, but measurable 12 ± 3 mG (Fig. 5A), indicating an increase in the membrane polarity, or, alternatively, a preferential distribution of Tempo molecules within the polar heads of phospholipid chains rather than within other parts of the membrane. A larger scatter in the hyperfine data at temperatures below the main phase transition is caused by a slower rotational diffusion of Tempo and a lower partition coefficient in

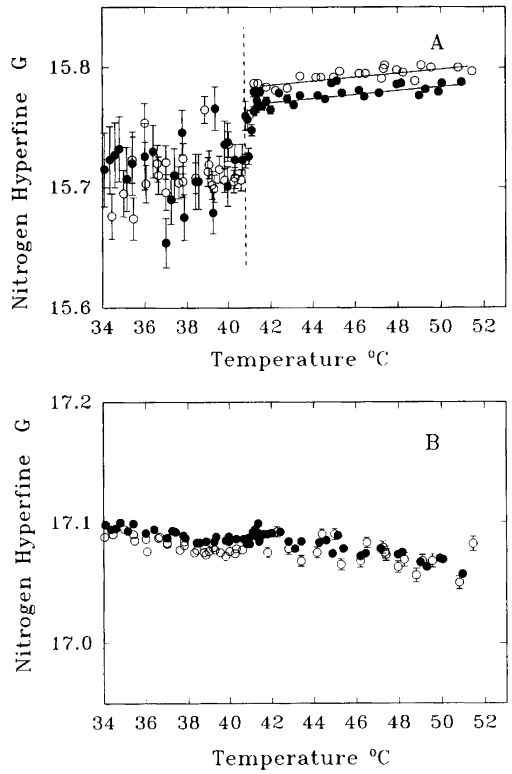

Fig. 5. (A) Temperature dependence of nitrogen hyperfine splitting of Tempo EPR signal corresponding to the lipid phase for a control sample (●) and for a sample containing 10 mM Gd-EOB-DTPA. In the presence of the contrast agent, the isotropic nitrogen hyperfine constant is increased by 12 ± 3 mG. Approximate temperature of the membrane main phase transition shown by vertical dashed line. (B) The corresponding plot for the signal corresponding to the aqueous phase. For the same two samples, the nitrogen isotropic hyperfine constant a^{N}_{iso} corresponding to Tempo molecules in the aqueous phase was not affected by either the main phase transition [appearing as a step change in a^{N}_{iso} at about 41 °C (A)] of the membrane or presence of the 10 mM Gd-EOB-DTPA.

the membrane when the membrane is in the gel phase. For comparison, the nitrogen isotropic hyperfine coupling corresponding to Tempo molecules in the aqueous phase was not affected by either the main phase transition of the phospholipids or the presence of the Gd-EOB-DTPA at 10 mM concentration (Fig. 5B) and shows only a monotonic temperature dependence.

The results of the 95 GHz EPR experiments presented here demonstrate that the more lipophilic Gd-DOTA-P penetrates into the phospholipid bilayer when the membrane is in the fluid bilayer phase. In contrast, there is no indication from these EPR data that the hydrophilic Gd-EOB-DTPA penetrates into the membrane. The results also show that Gd-DOTA-P and Gd-EOB-DTPA in concentrations of 5 and 10 mM, respectively, do not change measurably the rotational diffusion of small solute molecules partitioned in the membranes; thus, the structural organization of the membrane remains unaffected. The temperature of the main phase transition was also unaffected (within a 0.4 °C margin of accuracy).

Overall, the methodology presented here establishes a basis for further studies of interactions and spatial distributions of MRI contrast agents in complex biological systems – for example, by applying site-selective spin labeling of phospholipids or other biomolecules of interest (e.g., proteins). This kind of information is required for future rational development of new highly selective paramagnetic contrast agents for MRI. Another benefit of this high frequency EPR application is its high point sensitivity with ready accommodation of microsamples with volumes of 30–70 nl.

Experimental

Materials. Nitroxide Tempo (2,2,6,6-tetramethyl-1-piperidinyloxyl) was purchased from Aldrich (Milwaukee, WI). Chloroform solution of phospholipid DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Gadolinium contrast agents, Gd-EOB-DTPA and a lipophilic Gd-DOTA pentane derivative (Gd-DOTA-P), were kindly provided by Schering AG.

Preparation of multilamellar liposomes. Multilamellar liposomes were prepared from the phospholipid DPPC in a TRIS pH = 7.0 buffer as described previously.[11] The membranes were labeled with the nitroxide Tempo by addition of a stock 50 mM solution to give a final concentration of 1 mM. The final concentration of DPPC in aqueous media was 200 mg ml^{-1}. Gadolinium contrast agents from stock aqueous solutions were added to the membrane samples just before EPR experiments.

EPR experiments. Each DPPC/Tempo sample was drawn into a thin-walled quartz capillary (0.1 mm i.d., 0.15 mm o.d.; 25 mm long, Vitro Dynamics, Rockaway, NJ) and the ends of the capillary were sealed. The sample was transferred to a sample holder suitable for the TE_{012} resonator of the (95 GHz) EPR spectrometer designed and built at the University of Illinois EPR Research Center.[16] EPR spectra were taken over the temperature range 30–50 °C, which covers the temperatures of the main phase transition of the DPPC bilayer ($\approx$40 °C).

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