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^{1}H NMR Dipolar Echo Decay Spectroscopy: A Sensitive Probe of Membrane Structure†

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ABSTRACT: The structural and motional properties of membrane lipids in various physical states and macroscopic organizations are elucidated by ^{1}H NMR dipolar echo decay spectroscopy (DECODE). Multilamellar lipid dispersions in the gel ($L_{\beta'}$) and liquid-crystalline (L_α) states and a nonbilayer, hexagonal (H_{II}) phase are readily distinguished, a dynamic profile within these phases is identified, and dipolar order parameters are obtained in the fluid phases. The method is suitable for any pulsed NMR spectrometer. DECODE provides the first depth-dependent assay of lipid order that does not rely on isotopic labeling or exogenous probe.

The architecture of lipids in biological membranes is vital to cellular function. Studies of lipid organization have traditionally utilized techniques such as electron spin resonance and fluorescence polarization, which rely on the behavior of exogenous perturbing reporter molecules. Of the nonperturbing techniques, nuclear magnetic resonance (NMR)[1] is most widely used. The high abundance, sensitivity, and ubiquity of protons in lipid systems suggested a promising avenue for ^{1}H NMR. Efforts to realize this potential were largely abandoned in the 1970s, mostly due to difficulties posed by broad, largely featureless line shapes induced by dipolar couplings. Subsequent studies by ^{2}H NMR proved valuable but sacrificed broad utility because of the requirement for isotopic substitution and more sophisticated instrumentation. Ultrasonic disruption preserved the advantages of ^{1}H NMR in the liquid-crystalline state but sacrificed dipolar information.

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; CL, 1′,3′-di-*O*-(3-*sn*-phosphatidyl)-*sn*-glycerol; DECODE, dipolar echo decay spectroscopy; DFT, delayed Fourier transform spectroscopy; NMR, nuclear magnetic resonance.

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Table I: Experimental Decays vs Predictions from Composition

symbol in Figure 2	lipid/moiety	T_{de} (μs)	% intensity predicted	% intensity observed	% "error"
●	DPPC—28 °C, gel				
	palmitoyl methylenes	52.8	70.00	71.4	+1.4
	terminal CH_3, choline CH_2, glycerol ^1H	119	18.75	15.9	−2.85
	choline methyl	387	11.25	12.7	−1.45
○	DPPC—48 °C, liquid crystal				
	plateau acyl (C2–C11)	153	50.00	51.2	+1.2
	nonplateau acyl, terminal CH_3, choline CH_2, glycerol ^1H	382	38.75	38.6	−0.15
	choline methyl	1580	11.25	10.2	−1.05
▲	CL—Na, liquid crystal ($\langle d \rangle$ = 230 nm)				
	preolefinic acyl, glycerol ^1H[a]	206	*a*	60.2	*a*
	postolefinic acyl (C9–C18)[a]	837	*a*	39.8	*a*
△	CL—Ca, hexagonal (H_{II})				
	preolefinic acyl, glycerol ^1H	275	51.1	50.2	−0.9
	postolefinic acyl (C9–C18)	1174	48.9	49.8	+0.9

[a] Small, motionally narrowed lamellar structures—see text.

We describe an NMR method, *d*ipolar *e*cho *de*cay spectroscopy (DECODE), that (i) preserves the unique advantages of proton observation, (ii) exploits dipolar measures of packing and structure, (iii) yields "high-resolution" dipolar edited spectra, (iv) is applicable to naturally occurring membrane lipids, and (v) is suitable for all modern pulsed NMR spectrometers.

The broad, largely featureless, super-Lorentzian line shapes, which have limited the application of wideline proton NMR studies of membranes and multilamellar lipid dispersions, are induced by strong homonuclear dipolar couplings. This "liability" can be exploited by the use of dipolar echoes (90_x–τ–90_y–τ–AQ) (Powles & Strange, 1963; Oldfield et al., 1971). Phospholipid molecules are composed predominantly of strongly coupled spin $^1/_2$ doublets (methylene) and spin $^1/_2$ triads (terminal methyl, choline methyl). The breadth of the isolated geminal methylene Pake doublet at the parallel edge is 63 kHz (r_{HH} = 0.179 nm), which is further broadened by the neighboring and next-nearest-neighboring *trans*-methylenes by approximately 24 kHz ($r_{HH} \simeq$ 0.248 nm) per proton (Feigenson & Chan, 1974), multiplied by 8 for a rigid midchain methylene. Fields of intermolecular origin yield additional broadening in the absence of rapid rotation about the bilayer normal and fast lateral diffusion.

The dipolar Hamiltonian of the system can be separated into two components (Boden & Mortimer, 1973; Boden & Kahol, 1983; Trahms & Boroske, 1979; Boden & Levine, 1975; Boden et al., 1975; Bloom et al., 1978):

$$\mathbf{H_d} = \mathbf{H_d}(\text{int}) + \mathbf{H_d}(\text{ext}) \quad (1)$$

In this approximation the former term is an inhomogeneous, pseudoquadrupolar interaction; consequently, the isolated methylene spin pair, in analogy to the deuteron, yields a Pake doublet (Higgs & Mackay, 1977; Spiess & Sillescu, 1981) with an exponential echo decay rate, $\exp(-t/T_{2e})$, that is independent of coupling in the rigid limit (Woessner et al., 1969). The latter term, in eq 1, describes the weaker couplings between spin systems that inhibit echo formation, approximated in a Gaussian manner ($\exp[(-t/T_{de})^2]$) (Boden & Mortimer, 1973; Boden & Levine, 1975; Boden et al., 1975). The resulting echo decay is sensitive to motions ($\leq T_{de}^{-1}$) that affect couplings between spin groups, such as (i) trans–gauche isomerism, which modulates fields from neighboring methylenes, (ii) axial rotation around the bilayer normal and rapid lateral diffusion, which modulate intermolecular dipolar fields, (iii) diffusion over curved surfaces, and (iv) motions on the order of the interpulse spacing, which modulate the fields of the spin pair or triads (Spiess & Sillescu, 1981).

DECODE is applied to multilamellar dispersions of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in the gel ($L_{\beta'}$) and liquid-crystalline (L_α) phases and 1′,3′-di-*O*-(3-*sn*-phosphatidyl)-*sn*-glycerol (cardiolipin, CL) in the L_α and hexagonal (H_{II}) phases. The decay of magnetization with dipole echo interpulse spacing provides inter alia (i) echo decay constants, T_{de}, which are very sensitive to the lateral packing and motional properties of the membrane, and (ii) high-resolution T_{de}-filtered spectra reminiscent of the "delayed Fourier transform" (DFT) method (Seiter et al., 1972).

Materials and Methods

Data were obtained in quadrature at 360 MHz on a Bruker AM spectrometer with 5-mm probe: spectral window 83 kHz; 9–10-μs 90° pulse; 4K data points; eight transients with modified cyclops phase cycling; HDO suppression by nonselective composite pulse inversion and null, postinversion delay of ≥4 s; no exponential filter or baseline flattening. Artifacts due to power falloff were deemed negligible. Lipids were obtained from Avanti Polar Lipids (Birmingham, AL), deemed pure by thin-layer chromatography, dried under nitrogen, evacuated overnight at pressures of ≤1 mT, and hydrated in excess ^2H_2O with vigorous vortexing at concentrations of 310 mM (DPPC) or 34 mM (CL).

Results and Discussion

In response to a pair of 90° pulses of orthogonal phase, an echo is formed, as shown for DPPC (inset in Figure 1A,B). The magnetization of the gel state (Figure 1A) decays rapidly, and echo formation is much less efficient than for the L_α state (Figure 1B). Fourier transformation of the decay (and its imaginary complement), beginning at 2τ, leads directly to the spectra shown. The initial DECODE spectrum (Figure 1B) is nearly indistinguishable from the Bloch decay (Figure 1C); however, as the interpulse spacing is increased the resonance is effectively narrowed as the broad (short T_{de}) components are filtered (see below).

In Figure 2, the integrated spectral intensities with a least-squares Gaussian fit are shown. The experimental decay parameters obtained from Figure 2 are presented in Table I. The predicted component intensities, shown in Table I, are based on the compositional considerations described below, with reference to the T_{de}-edited spectra. The difference between the experimental and predicted intensities is denoted as "error".

The observed intensities of DPPC correspond to the known composition when analyzed with a triple Gaussian fit. The choline *N*-methyl moiety comprises nine ^1H and corresponds

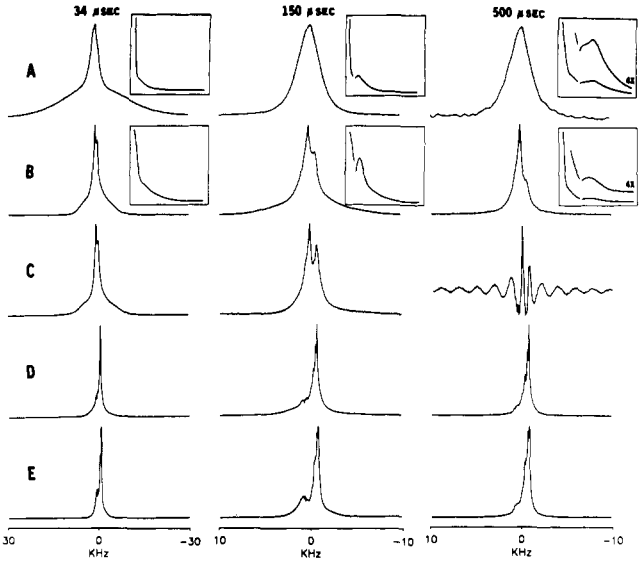

FIGURE 1: (A) Frequency and time domain (inset, 1 ms shown) DECODE spectra of DPPC in the gel state (28 °C) and (B) liquid-crystalline state (48 °C). Spectra obtained directly from the time domain signal without phase or baseline correction. (C) DFT spectra of (B). DECODE spectra of CL at 28 °C: (D) L_α, sodium salt; (E) H_{II}, calcium salt (Ca^{2+}/CL = 1.1). Shown as a function of time following the (initial) preparatory pulse (note change in frequency scale). The left-hand column represents the earliest acquisition following instrument dead time (34 μs, DECODE; 18 μs, DFT).

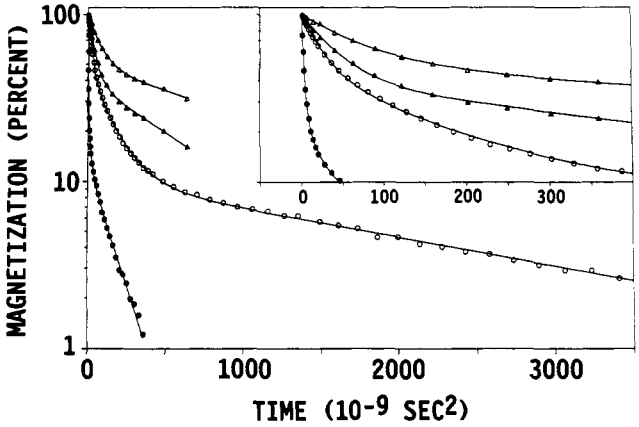

FIGURE 2: Integrated intensities of the dipolar echo transforms for DPPC at 28 °C (●) and 48 °C (○) and CL [sodium (▲) or Ca^{2+} (△; uppermost trace)], plotted semilogarithmically as a function of time squared following the preparation pulse [$(2\tau$ + pulse width)^2] and least-squares fit (solid line) to triple (DPPC) or double (CL) Gaussian decay (see text). Short time behavior is shown in the inset. Total experimental times ranged from 25 min (CL) to 50 min (DPPC L_α).

to the long-lived (narrow) component in both phases (experimental: 28 °C, 10.2 ^{1}H; 48 °C, 8.2 ^{1}H). The short-lived (broad) component corresponds to the palmitoyl methylenes, which comprise 56 ^{1}H in $L_{\beta'}$ (experimental: 57.1 ^{1}H). The short-lived component in the L_α phase corresponds to the motional plateau described by ^{2}H NMR (Seelig & Seelig, 1980) as extending through C_{11}, 40 ^{1}H (experimental: 41.0 ^{1}H). The intermediate component corresponds to the remaining species (terminal methyl, choline methylenes, glycerol backbone protons, and L_α nonplateau methylenes), which for $L_{\beta'}$ account for 15 ^{1}H (experimental: 12.7 ^{1}H) and for L_α account for 31 ^{1}H (experimental: 30.9 ^{1}H).

The isothermal calcium-induced L_α (Figure 1D) to H_{II} (Figure 1E) transition of the highly unsaturated bovine heart *cardiolipin [87% linoleate, 18:2(n–6); W. Shaw, personal communication]* not only demonstrates the sensitivity of DECODE to this transition but also emphasizes the utility of the method in cases where the line shape is highly asymmetric and moment analyses of time domain signals are problematic. A double Gaussian analysis of the decay behavior presented in Figure 2 and compiled in Table I is in agreement with the known lipid composition. The L_α phase exhibits a decay described by a double Gaussian fit: 60.2%, 206 μs; 39.8%, 837 μs. Echo formation is more efficient in the H_{II} phase: 50.2%, 275 μs; 49.8%, 1.174 ms. Assuming the entire acyl composition is linoleic, 48.9% of the CL protons originate at or after the first olefinic moiety (C9–C18), allowing assignment of the quickly decaying (broad) component to the plateau protons and demonstrating, once again, agreement between DECODE component intensities and chemical composition. The expected multicomponent behavior of the long T_{de} species was not resolved because distortions [perhaps originating from motions on the order of the interpulse spacing (Spiess & Sillescu, 1981)] precluded further analysis.

DECODE also provides dipolar edited high-resolution spectra, partly reminiscent of those obtained by the DFT method (Seiter et al., 1972). As shown in Figure 1, an increase in the interpulse spacing effectively narrows the resonances and increases the resolution and baseline definition. Comparison of the analogous DECODE (Figure 1B) and DFT (Figure 1C) spectra of L_α DPPC demonstrates that while the Bloch decay is dominated at long times by both the terminal methyl and the choline *N*-methyl, the dipolar echo is dominated at long times by the choline *N*-methyl. Not unexpectedly, the *N*-methyl exhibits greater isolation from external couplings. DECODE avoids the first-order phase distortions present in DFT spectra at long delays, as shown in Figure 1C, and does not require an external standard. The similarities, however, allow a DFT-like analysis of lipid perturbations in terms of intensity and line-width variations (e.g., between 28 and 48 °C at 2τ = 500 μs, the choline-centered intensity increases by 680%, from 1.9 to 13.0 protons, while the line width decreases from 2890 to 830 Hz). In systems of more complex acyl composition, such as CL, shown in panels D and E of Figure 1, DECODE exhibits increased resolution between the four spectrally resolved acyl (olefinic, interolefinic methylene, nonplateau methylene, terminal methyl) moieties (Janes et al., 1989). The smaller range of acyl intensities in the H_{II} phase, compared to that in the L_α, suggests a smaller range of external couplings, consistent with motional averaging about the H_{II} cylinders (see below). Thus, both the echo decay and the spectral intensity analysis exhibit striking changes through the phase transitions.

The present methodology is specifically adapted to treat chemically shifted species and their long time behavior, which is necessary to obtain T_{de} and T_{de}-edited spectra of lipids. This approach makes accessible generally neglected details of the line shape for analytical purposes. However, much is owed to the suggestion by Boden and co-workers (Boden & Mortimer, 1973; Boden & Levine, 1975; Boden et al., 1975), originally based on crystalline hydrates, that the reduced Van Vleck second moment (M_{2r}) between spin systems determines the decay, expressed here as $M_{2r}(\text{ext}) = C(8/T_{de}^2)$, $1 \leq C \leq 1.5$. Smaller values of C denote well-isolated spin systems (Boden & Levine, 1975). In the fluid phases, motional averaging allows the approximate simplification of eq 1 in terms of second moments (rigid limit, isolated, trans, M_2; averaged, M_{2r}) and dipolar order parameters (S) for a given spin system as follows (Boden et al., 1975; Bloom et al., 1978; Higgs & Mackay, 1977):

$$M_{2r} = M_{2r}(\text{int}) + M_{2r}(\text{ext}) \quad (2)$$

$$M_{2r} = M_2(\text{int})S^2(\text{int}) + \sum M_2(\text{ext})S^2(\text{ext}) \quad (3)$$

Assuming $C = 1$ and weighting T_{de} for L_α DPPC appropriately (Bloom et al., 1978), the palmitoyl M_{2r}(ext) is 2.457×10^8 s^{-2}, which is comparable to 1.54×10^8 s^{-2} obtained semiempirically (Bloom et al., 1978). The rigid M_2(ext) of the isolated *trans*-palmitoyl (Bloom et al., 1978) is 5.5×10^9 s^{-2}; therefore, S(ext) is 0.21. The mean acyl order parameter determined by ^2H NMR is slightly lower, 0.18 (Seelig & Seelig, 1980), as expected, since S(ext), unlike S(int), contains a time dependence on the internuclear distances (Boden et al., 1975), and is consistent with gauche conformers. Similarly,the nonacyl M_{2r}(ext) is 0.289×10^8 s^{-2}, and a reasonable fraction (44.5%) of the total experimental nonacyl M_{2r}, 0.65×10^8 s^{-2} (Bloom et al., 1978). Motional averaging about the H_{II} cylinders causes a scaling by 0.5 of the L_α ^2H order parameter (Seelig & Seelig, 1980). Diffusive motional averaging expected for the L_α CL presented here (median diameter 230 nm; freeze–fracture electron microscopy) negates a direct comparison. A comparison of the L_α DPPC and CL H_{II} plateau methylene T_{de}'s, however, indicates a scaling of S(ext) by 0.55. Although there is considerable uncertainty in relating T_{de} to M_{2r}(ext), the good correlation observed further solidifies the previous discussion on the origins of T_{de} and demonstrates the potential for more detailed moment analyses.

The foregoing results suggest that the utility of DECODE is due to the (1) sensitivity of the T_{de} to the details of motion and packing, (2) proton abundance, which precludes the need for isotopic enrichment, (3) facile acquisition on all modern pulsed spectrometers, (4) high receptivity of the proton, which allows rapid T_{de} data acquisition, (5) ability to obtain high-resolution T_{de}-edited spectra, (6) special need for analysis NMR methods to lipids lacking phosphorus, and (7) potential for order parameter and moment analysis.

In summary, the proton NMR line shape is very sensitive to the details of lipid packing and motion. The echo decay rates reveal subtle changes in the line shape that we attribute to behavior of different physical states of the lipid system. Lipid polymorphism, mixed-phase systems, and anesthetic–membrane interactions provide ideal subjects for study. As protein resonances are generally very broad and self-filtering (Seiter et al., 1972), considerable potential exists for studies of natural membranes and protein–lipid interactions.

Registry No. DPPC, 63-89-8.

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