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The truncated driven NOE and ^{13}C NMR sensitivity enhancement in magnetically-aligned bicelles

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Abstract

The truncated driven nuclear Overhauser effect (NOE) sequence is examined as a means of sensitivity enhancement in ^{13}C NMR spectroscopy of magnetically-aligned bicelles consisting of 4.5:1 mixtures of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) plus DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine), with 1 mole% DMPE-PEG 2000 (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene glycol)-2000). Steady-state NOE enhancements were observed at all carbon segments except the lipid carbonyls, but full NOE enhancements were obtained only for the most mobile carbon segments, specifically the choline quaternary methyls and terminal acyl chain methyls of both DMPC and DHPC, as well as the ethylene oxide segments of the PEG head group of DMPE-PEG 2000. Other carbon segments exhibited NOE enhancements that scaled with mobility as determined by transient NOE measurements combined with spin–lattice relaxation measurements. We conclude that the truncated driven NOE provides sensitivity enhancement complimentary to that yielded by cross-polarization techniques and for mobile membrane-associated species may be preferred for its robustness and ease of setup.

Keywords: Truncated driven NOE; Carbon-13; Sensitivity enhancement; Bicelles; Magnetic alignment

1. Introduction

Bicelles are a novel model membrane system now widely employed in nuclear magnetic resonance (NMR) structural studies of membrane-associated amphiphiles, peptides and proteins [1–5]. First introduced by Sanders and coworkers [6,7], they consist of aqueous mixtures of short-chain amphiphiles, such as dihexanoylphosphatidylcholine (DHPC), and long chain amphiphiles, such as dimyristoylphosphatidylcholine (DMPC). At low lipid concentrations and low values of the ratio $q = \text{DMPC/DHPC}$, bicelles are discoidal with DMPC forming a planar lamellar bilayer disc body while DHPC is sequestered to the highly curved disc edges. Low q bicelles can be small enough to tumble isotropically in solution. Since they constitute a better approximation of natural membranes than do surfactant micelles, isotropic bicelles are ideally suited for solution-state NMR structural studies of membrane proteins, peptides and amphiphiles via measurements of isotropic chemical shifts, scalar couplings, and nuclear Overhauser effects (NOE). At higher lipid concentrations and higher q values, bicelles spontaneously align within the magnetic field of an NMR spectrometer. The resulting uniform alignment of bilayer director axes permits site-specific resolution of anisotropic NMR interactions, particularly in separated local field (SLF) experiments, thereby allowing solid-state NMR structural studies via measurement of residual orientation-dependent interactions such as dipolar or quadrupolar couplings, and chemical shift anisotropies.

Polarization transfer to enhance low-sensitivity nuclei is essential for both solution-state and solid-state NMR spectroscopy in bicelles. The INEPT (insensitive nuclei enhancement by polarization transfer) technique [8], and its variants, are suitable for isotropic bicelles, and for

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highly mobile segments in magnetically-aligned bicelles [9], but not for less mobile segments where the residual dipolar interactions are typically large relative to the J-couplings, resulting in complete loss of coherence in the transverse domain before polarization transfer can be achieved. Cross-polarization (CP) [10], on the other hand, is well suited for sensitivity enhancement of insensitive nuclei in situations where residual dipolar interactions are strong, and immediately upon its debut was applied to advantage in phospholipid bilayers [11]. CP is an essential component of SLF experiments such as PISEMA (polarization inversion spin exchange at the magic angle) [12–18], and its successors, employed for solid-state NMR structural studies of membrane proteins, lipids and membrane-associating ligands in magnetically-aligned bicelles. However, for small, mobile membrane-associated amphiphiles, or for mobile segments within a larger membrane-associated species, wherein dipolar couplings are reduced by varying degrees, care must be taken to avoid a mismatch of the Hartmann–Hahn matching conditions by using a ramp-CP sequence [19]. Even so, the eventual enhancement achieved can be limited due to unfavorably weak dipolar couplings and short $T_{1\rho}$.

A third approach, the heteronuclear NOE [20,21] is favorable when motional correlation times are short relative to the inverse of the resonance frequency. The considerable heteronuclear NOE enhancements achievable for phospholipids were recognized even in the earliest ^{13}C NMR studies of phospholipid bilayers [22], but received relatively little subsequent attention, due, in no small measure, to the debut of CP [10] and its successful application in ^{13}C NMR of phospholipid bilayers [11].

Recently, Warschawski and Devaux [23] compared all three polarization transfer methods for their efficacy in sensitivity enhancement of ^{13}C NMR spectra of phospholipid bilayers under magic angle spinning (MAS) conditions. They concluded that CP or ramped-CP could be advantageously replaced by refocused INEPT or NOE techniques under these conditions, since MAS suppressed the dipolar couplings. More recently, Dvinskikh et al. [24] compared CP, ramped-CP and NOE polarization transfer methods for achieving sensitivity enhancement in ^{13}C NMR spectra of magnetically-aligned bicelles specifically. These authors concluded that ''NOE is superior only for the most mobile groups'' [24], meaning principally the choline methyls and terminal acyl chain methyls of DMPC and DHPC.

Both of these comparative studies evolved the heteronuclear NOE using continuous ^{1}H RF (radio frequency) irradiation during the recycle delay to saturate the ^{1}H spins [23,24]. Of necessity, to avoid heating effects, the RF field strengths employed for NOE enhancement were relatively weak (1 kHz in both cases), particularly so in comparison to the RF field strength employed for cross-polarization or ^{1}H decoupling during ^{13}C signal acquisition (i.e. 20 kHz). This raises the possibility that in magnetically-aligned bicelles, where residual dipolar interactions remain substantial, saturation of the ^{1}H resonances for NOE purposes was achieved only for the most mobile groups.

An alternate means of evolving the heteronuclear NOE in solids and semi-solids was demonstrated by Findlay and Harris [25], and has been employed subsequently to good effect in polymeric and liquid–crystal systems [26–29], but not yet to lipid membranes or magnetically-aligned bicelles. As illustrated in Fig. 1, the so-called truncated driven NOE consists of a train of ^{1}H 90° RF pulses separated by a fixed delay, typically on the order of msec in duration. The use of sufficiently short ^{1}H 90° RF pulses alleviates concerns regarding uniformity of excitation, while the presence of the fixed delays keeps the duty cycle low, thereby minimizing sample heating. By varying the length of the ^{1}H pulse ''comb'' both the transient and the steady-state NOE may be evaluated from the ^{13}C NMR signal obtained under $\{^{1}H\}$ decoupling conditions subsequent to the ^{13}C excitation pulse.

In this report we describe enhancement of the ^{13}C NMR resonances of DMPC/DHPC magnetically-aligned bicelles via application of the truncated driven NOE sequence. It is demonstrated that significant sensitivity enhancements are achieved, not just for the most mobile chemical groups, but for most phospholipid ^{13}C NMR resonances, for both DMPC and DHPC. The positive correlation of the NOE enhancement with the mobility gradient across the

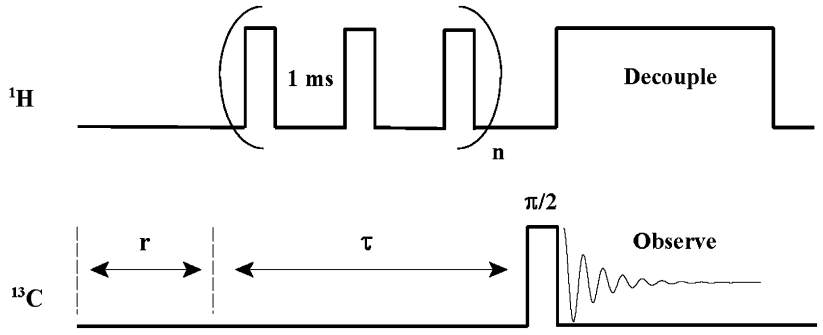

Fig. 1. Truncated driven NOE pulse sequence [25] consisting of a ''comb'' of ^{1}H 90° pulses separated by fixed delays (typically 1 ms), preceding excitation of the ^{13}C resonances, with ^{1}H decoupling during ^{13}C signal acquisition. For purposes of comparing spectral intensities with versus without the NOE, the recycle delay $(r + \tau)$ is kept constant.

phospholipid bilayer suggests that the truncated driven NOE provides a useful complement to CP for sensitivity enhancement in magnetically-aligned bicelles.

2. Results and discussion

Natural abundance ^{13}C NMR spectra of magnetically aligned $q = 4.5$ bicelles are compared in Fig. 2 for the case of single pulse excitation (SPE) and steady-state ($\tau = 4$ s) truncated driven NOE conditions. The DMPC resonances were assigned according to Shapiro et al. [30], taking account of the chemical shift anisotropies (csa) quantified by Braach-Maksvytis and Cornell [31], while the DHPC resonances were assigned according to Burns and Roberts [32]. The carbonyl resonances were assigned as described by Schmidt et al. [33]. A comparison of these SPE and NOE spectra demonstrates that most, if not all, ^{13}C resonances are considerably enhanced by the truncated driven NOE.

The spectra shown in Fig. 2 were acquired using SPINAL-64 (small phase incremental alternation) [34] proton decoupling during ^{13}C signal acquisition with a ^{1}H RF field strength of 13 kHz and the decoupler frequency set to the approximate center of the ^{1}H spectral envelope, i.e., the choline quaternary methyl resonance at ~3.2 ppm. A comparison of decoupling methods at this ^{1}H RF field strength revealed that SPINAL-64 decoupling yields resolution superior to that achieved with CW (constant wave), or WALTZ-16 (wideband alternating phase low-power technique for zero residue splitting) [35], or TPPM (two pulse phase modulation) [36] methods, as found by Dvinskikh et al. [24]. Only a modicum of additional resolution enhancement over that obtained using SPINAL-64 was achieved, however, upon implementing FLOPSY-16 (flip flop spectroscopy) [37] or COMARO (composite magic-angle rotation) [38] decoupling schemes under otherwise identical conditions of RF field strength, acquisition time, temperature, etc., and whatever improvement was achieved seemed confined to the glycerol backbone resonances. Thus, throughout the remainder of these studies SPINAL-64 decoupling was used exclusively.

Regardless of the decoupling scheme, resolution remained limited in crowded spectral regions such as that centered at 36 ppm, containing overlapping resonances from the DMPC acyl chain C4-11 methylene carbons, or that centered at 68 ppm, containing overlapping resonances from both DMPC and DHPC glycerol backbone and choline head group carbons. While a higher proton

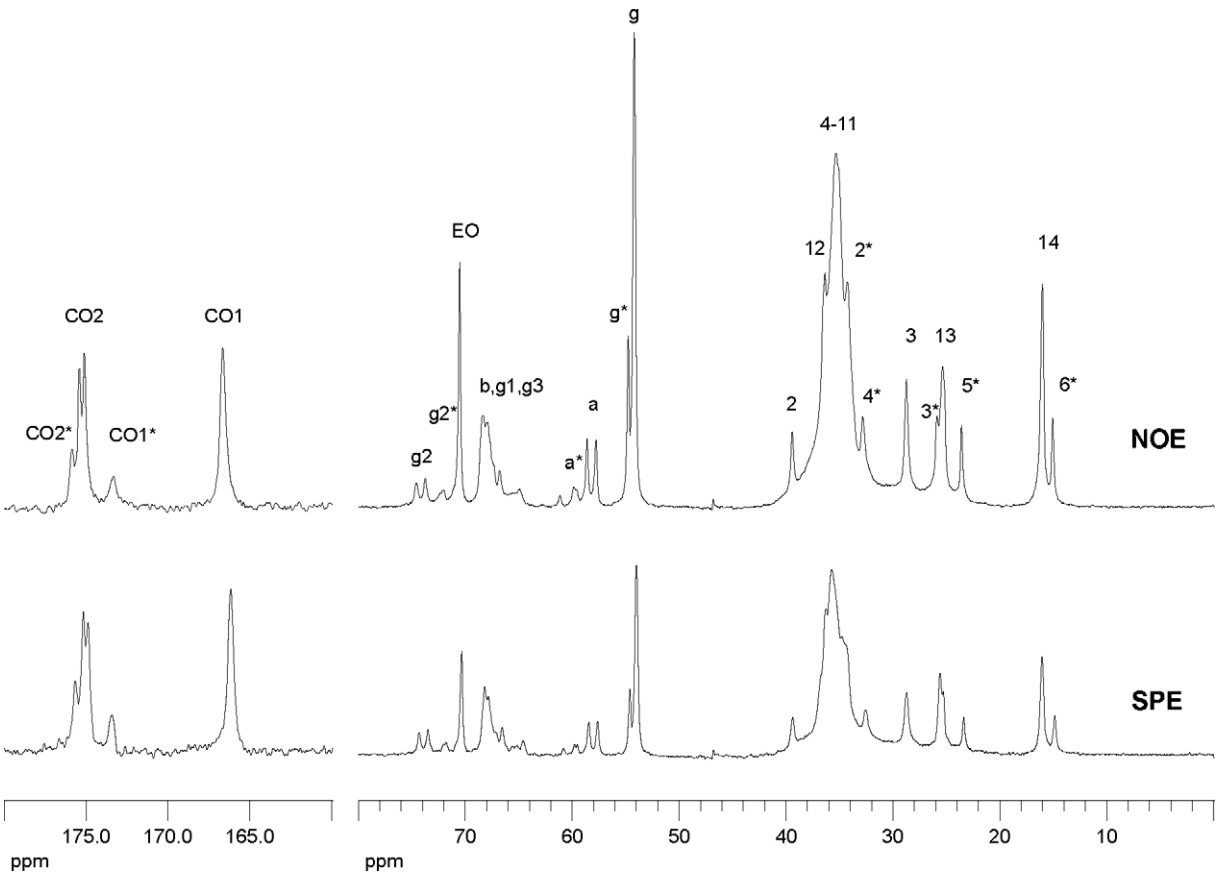

Fig. 2. Carbon-13 NMR spectra of magnetically-aligned bicelles (q = DMPC/DHPC = 4.5, 30 °C) acquired under single pulse excitation (SPE) and truncated driven NOE ($n = 4000$) conditions, with identical numbers of transients and recycle delays. Resonances were assigned as described in the text, with standard carbon segment numbering as shown in Fig. 3. Numbers with asterisks refer to DHPC carbons. EO refers to the ethylene oxide methylenes of DMPE-PEG 2000. The carbonyl regions are shown on an expanded horizontal and vertical scale.

decoupler RF field strength could have been employed, we wished to avoid excessive sample heating due to high RF decoupler fields. This is a particular problem for resolution in bicelles because the samples are salty and therefore highly susceptible to RF heating, which can degrade the quality of magnetic alignment. Often, resolution of DMPC and DHPC resonances relies on the residual chemical shift anisotropy being greater in the former than the latter. Consequently, higher temperature can actually degrade resolution. For example, the choline quaternary methyl resonances of DMPC and DHPC in the region of 54 ppm are separated from one another by 0.55 ppm at 30 °C, but only by 0.12 ppm at 37 °C. To maximize resolution of DMPC and DHPC resonances all the bicelle spectra reported here were acquired at 30 °C rather than the more commonly used 37 °C. Note that in Fig. 2 the resolution of these, and other, resonances has not been influenced by application of the truncated driven NOE.

The nuclear Overhauser enhancement factor (η) is calculated from the integrated signal intensity with (I_Z), versus without (I_0), the NOE,

$$\eta = \frac{< I_Z - I_0 >}{I_0} \tag{1}$$

and for a given carbon nucleus, having relaxation dominated by dipolar interactions with protons, ranges between 1.988 and 0.153 for the case $(\omega_C + \omega_H)^2 \tau_C^2 \ll 1$ and $\omega_C^2 \tau_C^2 \gg 1$, respectively [20,21], where ω is the relevant resonance frequency in rad s^{-1}, and τ_C is the motional correlation time. Maximum enhancement is obtained for highly mobile groups while lower mobility reduces the enhancement.

Fig. 3 illustrates the steady-state NOE enhancement as a function of carbon position within DMPC and DHPC phospholipids incorporated into magnetically-aligned bicelles, as derived from those resonances well-resolved in

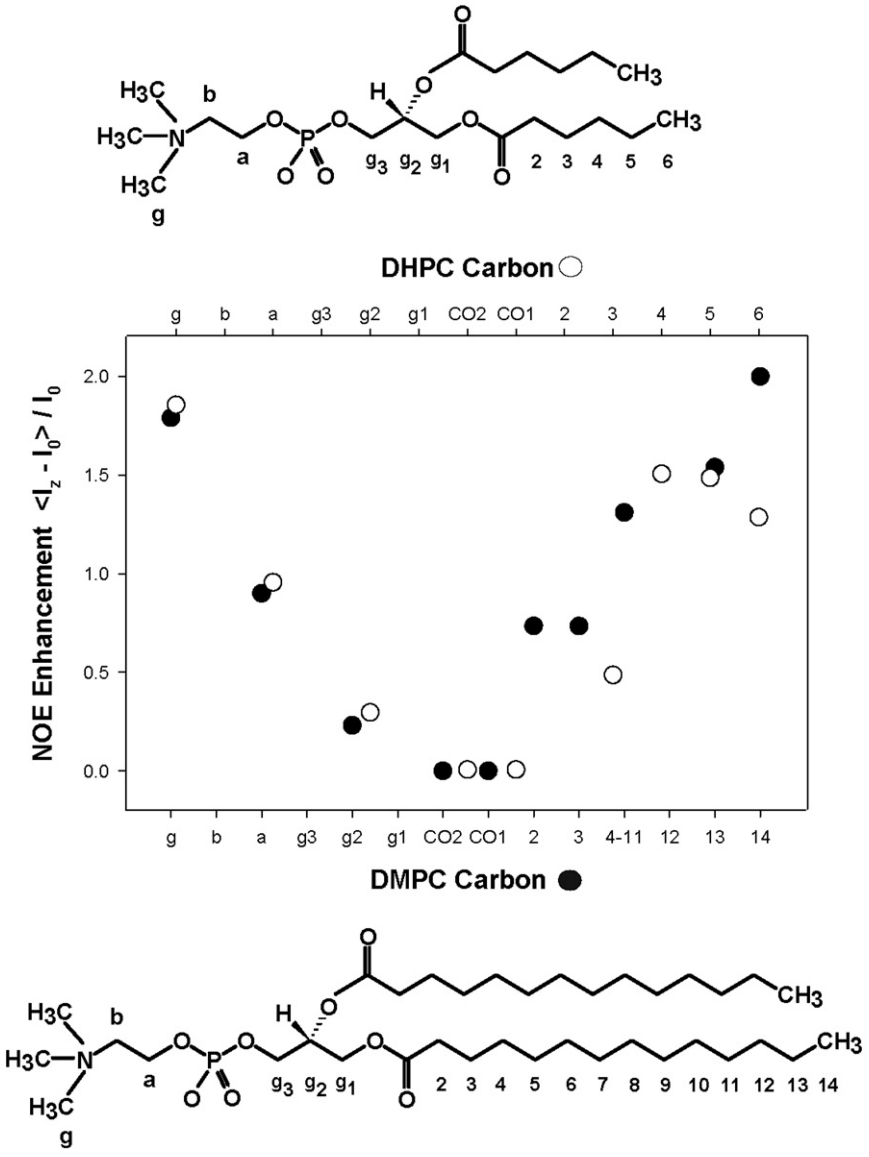

Fig. 3. Steady-state NOE enhancement η as a function of carbon segment in DMPC (●) and DHPC (○) in magnetically-aligned bicelles (q = DMPC/DHPC = 4.5, 30 °C).

the spectra in Fig. 2. It is evident that the full NOE enhancement is achieved at the extreme ends of the molecules, i.e., the acyl chain methyls and the choline quaternary methyls, indicating that for these carbons the short correlation time regime is in effect and that their relaxation is dominated by dipolar interactions with protons. With increasing proximity to the phospholipid's glycerol ''backbone'' the NOE enhancement is reduced progressively. For the glycerol g2 carbon, the NOE enhancement is only slightly greater than the expected minimum for long correlation times. A NOE enhancement is completely absent, however, only for the carbonyl carbons, due to their lack of directly attached protons. It is remarkable that DMPC and DHPC exhibit such similar dependence of the NOE enhancement on carbon position, despite their segregation into planar and curved regions of the bicelles, respectively, where one might expect quite different dynamics to pertain.

Further insight into the origin of the differential steady-state NOE enhancements may be obtained by examining the transient NOE enhancement as measured by varying the tau duration within the truncated driven NOE sequence. Fig. 4 illustrates the time course for the growth of the NOE for selected DMPC and DHPC resonances, where an exponential dependence on the tau duration is

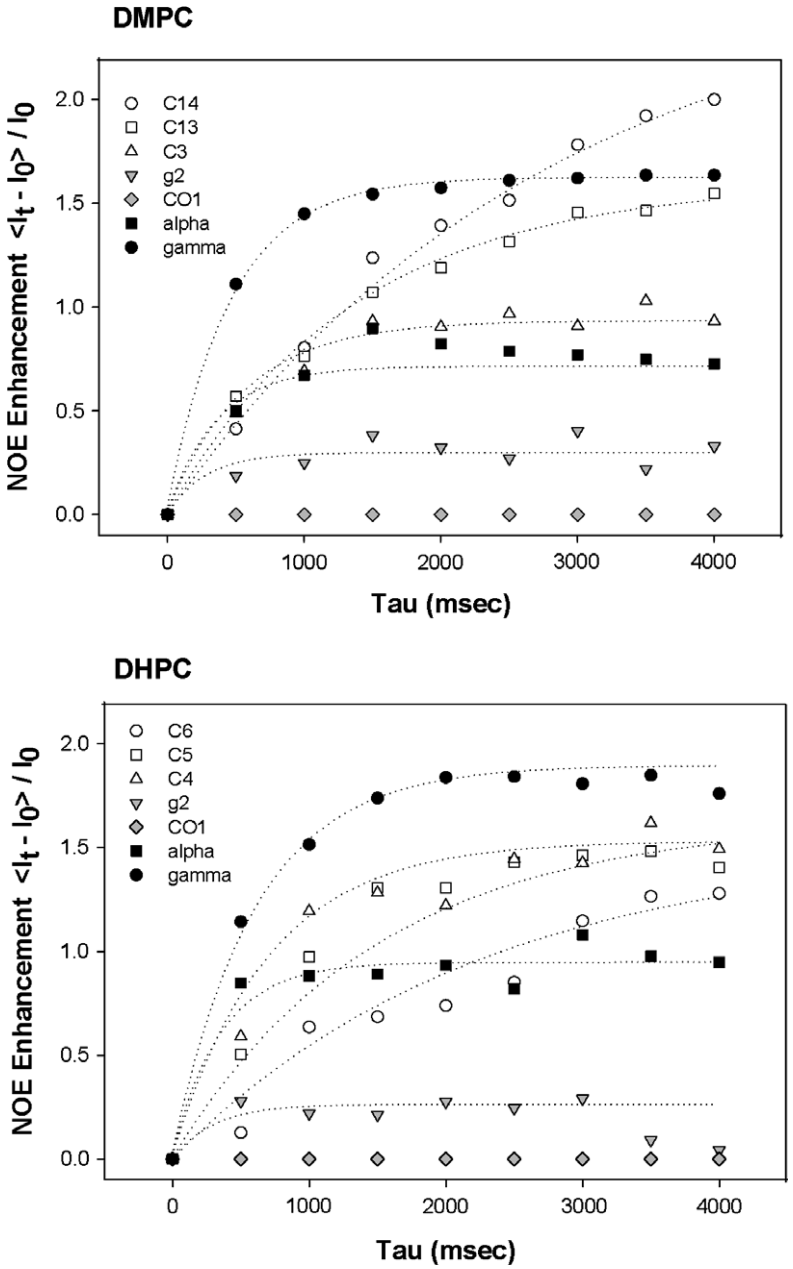

Fig. 4. Transient NOE enhancement η for selected carbon segments of DMPC (top) and DHPC (bottom) in magnetically-aligned bicelles (q = DMPC/DHPC = 4.5, 30 °C). Curves of best fit to Eq. (2). [27] are generated using fixed longitudinal relaxation rates determined in separate inversion recovery measurements and cross-relaxation rates varied to obtain optimal fits.

evident in all cases. For most carbons of DMPC and DHPC the steady-state enhancement was attained within 1–2 s.

The curves-of-best-fit shown in the figure were generated as described by White [27], where the time-dependence is expressed as

$$\eta = \frac{\sigma}{\rho}[1 - \exp(-\rho\tau)] \quad (2)$$

with σ being the cross-relaxation rate, and ρ being the ^{13}C spin–lattice relaxation rate. Fig. 5 shows the values of σ and ρ as a function of carbon position for DMPC and DHPC. The latter were measured in separate inversion recovery experiments, while the former were extracted from fitting the transient NOE enhancements in Fig. 4 to Eq. (2).

In bicelles DMPC remains confined to the bicelle's planar region. The $1/T_1$ profile versus carbon position for DMPC conforms with the long-established finding that in lipid bilayers the rate of T_1 relaxation decreases progressively with increasing separation from the glycerol backbone in the direction of either the choline head group [39] or the acyl chain methyl terminus [39,40]. This profile reflects the well-known segmental mobility gradient across a lipid bilayer, established, for example, from ^2H NMR studies [41]. In the case of ^{13}C NMR, the rate of longitudinal relaxation in lipid bilayers is modeled as a combination of contributions from slow collective motions (correlation times 10^{-6}–10^{-7} s) plus fast segmental motions (correlation times 10^{-11}–10^{-12} s) [42]. The contribution of the slow collective motions scales with the square of the segmental orientational order parameter S_{CH}, thereby producing the transbilayer variation in $1/T_1$. The cross-relaxation rates show an inverse dependence on the segmental orientational order parameter, in that, while remaining fairly constant across the acyl chain region, σ increases markedly across the head group region with increasing separation from the glycerol backbone. Thus one concludes that the transient and steady-state NOE enhancements observed here for DMPC conform well with expectations drawn from dynamic models of lipid bilayer structure.

DHPC in bicelles tends to favor regions of high curvature, but can also migrate into planar regions. Overall, therefore, it experiences more nearly isotropic motional averaging. Yet its profile of longitudinal and cross relaxation rates is remarkably similar to that exhibited by DMPC. One may understand this by analogy with relaxation of surfactants in micelles, which is modeled in terms of a sum of contributions due to fast local segmental motions and slower collective motions [43]. The latter correspond to rotation of the entire micelle plus diffusion of individual surfactants about the micelle's radius of curvature, and their contributions are scaled according to the square of the segmental order parameter. Thus, the rate of T_1 relaxation decreases with increasing separation from the polar interface of the surfactant micelle which anchors the surfactant. By analogy, similar, albeit not identical, consideration should influence T_1 of DHPC preferentially, but not exclusively, located within regions of high curvature and anchored by the glycero-phosphate group. This suggests that other small amphiphilic molecules associating with bicelles will likewise exhibit NOE enhancement profiles reflecting local internal ordering and that the NOE is an especially sensitive probe of such dynamics.

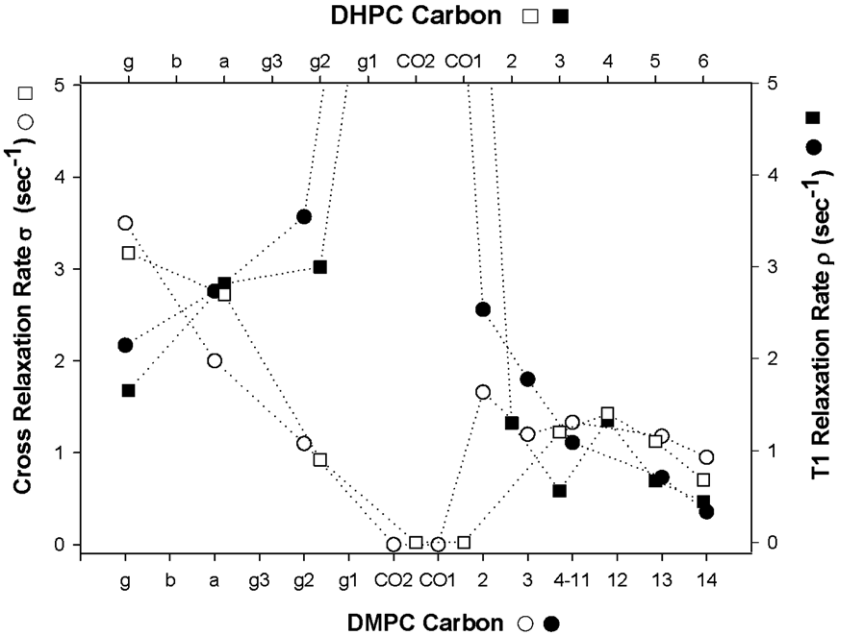

Fig. 5. Cross relaxation (open symbols) and longitudinal relaxation rates (closed symbols) as a function of carbon segment for DMPC (circles) and DHPC (squares) contained within magnetically-aligned bicelles (q = DMPC/DHPC = 4.5, 30 °C). ^{13}C T1 relaxation times were determined in separate inversion recovery experiments. Cross relaxation rates were estimated from fits of Eq. (2). [27] to transient NOE enhancement data such as shown in Fig. 4.

The ethylene oxide segments of DMPE-PEG 2000 have not yet been mentioned in any detail. PEG-lipids consist of a hydrophobic anchoring group, such as dimyristoyl phosphatidylethanolamine (DMPE), to which a polyethyleneglycol (PEG) group is covalently attached through the lipid's polar head group. The water soluble PEG becomes effectively "grafted" to the lipid bilayer surface through its DMPE hydrophobic anchor. In bicelles, a surface coating of PEG is used to enhance bicelle stability by virtue of steric stabilization [44].

The PEG group is well hydrated and enjoys considerable internal motional freedom. In fact, in magnetically-aligned bicelles subject to the refocused INEPT polarization transfer scheme, only the PEG ethylene oxide at 70.53 ppm survives, exhibiting fully the expected sensitivity enhancement (spectrum not shown). In this sense, the PEG group may be regarded as a proxy for highly mobile extramembranous domains, i.e., loop regions, of membrane bound proteins [9]. When subject to the truncated driven NOE sequence the ethylene oxide carbon resonance yields the full NOE enhancement of 1.988, as may be ascertained from Fig. 2.

3. Conclusions

In this study we examined the utility of the truncated driven NOE as a means of sensitivity enhancement in natural abundance ^{13}C NMR spectra of magnetically-aligned bicelles. Significant steady-state NOE enhancements were observed at most lipid carbon segments. The high correlation with local mobility indicates that as a means of sensitivity enhancement in magnetically-aligned bicelles the truncated driven NOE straddles the gap between cross polarization methods, which are best suited for examining more immobile segments, and INEPT-type enhancements methods, which are best suited for highly mobile segments. Its ease of set-up, robustness, and minimal heating effects suggest that the truncated driven NOE may be in fact a better alternative in particular cases. In the study of membrane proteins, in addition to sensitivity enhancement of mobile domains, the fact that the truncated driven NOE provides such considerable enhancement for methyl groups suggests the possibility of exploiting this property for probing methyl groups in membrane protein side chains to extract structural and dynamical information, in a manner analogous to that which has proved so useful in the study of large soluble proteins [45].

4. Experimental section

4.1. Sample preparation

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) and DMPE-PEG 2000 (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy(polyethylene glycol)-2000) were purchased from Avanti Polar Lipids, Alabaster, AL, USA. All other biochemicals and reagents were purchased from Sigma–Aldrich, Oakville, ON, Canada.

Bicelles were prepared to consist of 25 wt% lipid in 75 wt% aqueous 150 mM NaCl + 50 mM Tris–HCl, pH 7.4, in D_2O. The ratio q, being the proportion of long-to-short chain amphiphiles (DMPC/DHPC), was kept constant at $q = 4.5$. The long chain amphiphiles were taken to include DMPC plus 1 mole% DMPE-PEG 2000, the latter expressed as a percentage of the former. Since DHPC is extremely hygroscopic, an aqueous DHPC solution was prepared immediately upon opening the container received from the supplier, from which the necessary amount was added to the dry long-chain lipids. A sufficient volume of 150 mM NaCl was added to yield the desired lipid concentration. The lipid were hydrated by gentle mixing, followed by several cycles of heating to 40 °C and cooling to 4 °C. Macroscopic confirmation of bicelle formation was evidenced by the presence of an optically clear solution. Samples were stored at 4 °C prior to transfer to the NMR magic angle spinning (MAS) sample container. The latter accommodated a volume of approximately 100 μl, corresponding to 25 mg of phospholipid. Note that sample transfer is facilitated by maintaining the sample temperature at 4 °C due to the much lower sample viscosity at that temperature. Magnetic alignment of the bicelles generally occurred within 30 min of the sample being placed in the field of the NMR spectrometer and warmed to 30 °C, as assessed using ^{31}P NMR spectroscopy.

4.2. NMR measurements

All NMR spectra were recorded on a Chemagnetics/Varian Infinity 500 MHz NMR spectrometer using a Chemagnetics T3 5 mm triple resonance MAS probe under static conditions. All spectra were recorded at a sample temperature of 30 ± 0.5 °C. In separate experiments the temperature inside the MAS spinner was calibrated using the ^{207}Pb signal from $PbNO_3$ as an NMR thermometer [46] under MAS conditions, extrapolated to the static condition.

^{31}P NMR chemical shift spectra were recorded to assess the quality of bicelle alignment. Properly magnetically-aligned bicelles yielded two narrow resonances: one at −5.0 ppm (relative to 85% H_3PO_4) assigned to DHPC, and a second at −12.0 ppm assigned to DMPC, with relative integrated intensities of 1:4.5, as expected. DMPC-PEG 2000 could not be resolved given its low concentration.

^{13}C NMR spectra were recorded at 125 MHz using the truncated driven NOE pulse sequence [25] (see Fig. 1) with a ^{13}C 90° pulse width of 16 μs, a recycle delay of 5 s, a spectral width of 50 kHz and an 8 K data size, with SPINAL-64 $\{^1H\}$ decoupling during acquisition [34] as described in the text. The ^1H RF field strength during both the truncated driven NOE and decoupling periods was set to 13 kHz. Spectra were processed with an exponential multiplication

equivalent to 1 Hz line broadening prior to Fourier transformation, and were referenced to tetramethylsilane. ^{13}C T_1 relaxation times were measured using a standard inversion recovery protocol.

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