Deuterium NMR to study the surface of phospholipid bilayers

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Abstract: *Deuterons placed specifically in the head-groups of phospholipids in bilayers were studied by ^{2}H-NMR, which is a non-perturbing method for directly probing the conformation and dynamics of the deuterated segments at the bilayer surface. Information about the hydration properties of phosphatidylcholine bilayers was obtained by recording the spin-lattice (T_1) relaxation times of DOPC-d_9 and DMPC-d_9 as a function of water content. Head-group mobility was demonstrated to increase with the bilayer water content up to a limiting degree of hydration beyond which the bilayer dynamics remain unchanged as the water then forms a discrete bulk phase. The titration behaviour and the dissociation constants of the primary amino group and the phosphate group of phosphatidylethanolamine were characterized isothermally by recording the quadrupole splittings $\Delta\nu_Q$ of DMPE-d_4 as a function of bulk pH. Each deuterated segment was found to have a highly local sensitivity towards the small changes in head-group conformation occurring in response to the protonation/deprotonation of its adjacent ionizable group.*

Wide-line solid state ^{2}H-NMR examinations of specifically head-group deuterated lipids have provided structural and dynamic information about the surface of bilayers serving as models for biological membranes (Seelig & Seelig, 1980; Akutsu & Seelig, 1981; Sixl & Watts, 1982; Sixl & Watts, 1983; Seelig *et al.*, 1987; Watts, 1987; Seelig *et al.*, 1988; Watts, 1989). The labelled phospholipids are shown in Figure 1, where the arrows denote the sites of deuteration. Liquid crystalline dioleoylphosphatidylcholine (DOPC-d_9) and dimyristoylphosphatidylcholine (DMPC-d_9) as well an equimolar mixture of DMPC-d_9 with cholesterol were used in the study of bilayer hydration. For the characterization of the ionization states of the phosphate and the amino group in dimyristoylphosphatidylethanolamine (DMPE-d_4) this lipid was mixed with equimolar DMPC to ensure the formation of extended bilayers.

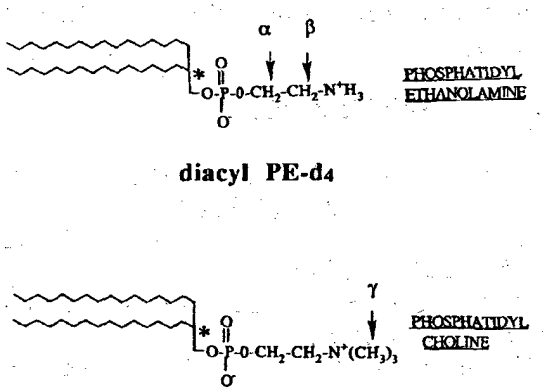

Figure 1: Phospholipids used in this work, with oleoyl ($C_{18:1}$) or myristoyl ($C_{14:0}$) acyl chains. The arrows denote the sites of specific deuteration.

Experiments were performed with a homebuilt spectrometer operating at a deuterium frequency of $\omega_0/2\pi$ = 55.27 MHz. The ^{2}H-NMR spectrum of a liquid crystalline multilamellar lipid dispersion in water has a characteristic lineshape, a "powder pattern", to which all orientations of the C-D bond contribute with spherically weighted intensities. The quadrupole splitting (arising from the interaction of the deuterium nuclear quadrupole moment with the electric field gradient of the C-D bond) between the lineshape maxima, $\Delta\nu_Q$, is sensitive to the orientation and conformation of the deuterated segment in the bilayer. The deuterium spin-lattice (T_1) relaxation time, measured by the standard inversion recovery experiment (π - τ - $\pi/2$) using 11 delay times τ, can be related to the rate of motion of the deuterated segment (Brown *et al.*, 1979). Both $\Delta\nu_Q$ and T_1 will be considered as qualitative experimental parameters whose changes are interpreted in terms of processes occurring at the bilayer surface, namely hydration and protonation/deprotonation of phospholipid head-groups.

A. Lipid bilayer hydration

When water is added to dry lipid, it is intercalated between the lamellae in immediate contact with the polar bilayer surface. The process of hydration may thus be expected to have a considerable effect on head-group mobility, which was studied by recording the deuterium spin lattice (T_1) relaxation times.

For an analysis of the relaxation behaviour, it was first necessary to characterize the angular dependence of the spin-lattice relaxation times. Using macroscopically oriented bilayers, it was demonstrated that relaxation of the DOPC-d_9 and DMPC-d_9 deuterons is essentially isotropic (within ± 3%), both for fully and partially hydrated samples as illustrated in Figure 2. This angular independence of T_1 is a consequence of the nearly isotropic distribution of the orientations of the terminal C-D bonds at the bilayer surface, as reflected in the very small quadrupole splittings (of the order of 1 kHz). The observation of isotropic spin-lattice relaxation times justified their subsequent determination from random lipid dispersion samples for which the degree of hydration was readily controlled over a wide range. Furthermore, a simple theory could be applied where relaxation occurs by a straightforward quadrupolar mechanism.

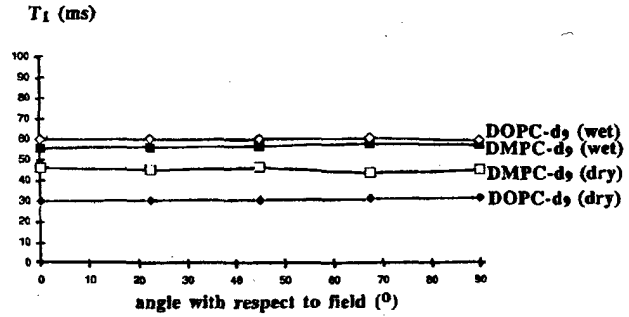

Figure 2: Deuterium spin-lattice (T_1) relaxation in oriented bilayers of liquid crystalline DOPC-d_9 and DMPC-d_9 at 30°C, both for fully and partially hydrated samples.

Figure 3 shows the relaxation data obtained for DOPC-d_9, DMPC-d_9 and DMPC-d_9/cholesterol dispersions at a series of different degrees of bilayer hydration, ranging from 4 H_2O molecules per phospholipid up to excess water. A single exponential decay was observed for the relaxation in all samples, and from the temperature dependence of T_1 it was confirmed that relaxation corresponded to the fast motional regime (where $\omega_0\tau_c << 1$). The following approximation thus applies (Brown *et al.*, 1979):

$$1/T_1 = [3\pi^2/2\ (e^2qQ/h)^2]\ \tau_c$$

where $(e^2qQ/h)^2$ is the static quadrupole coupling constant (170 kHz for methyl groups), and τ_c is the motional correlation time for the effective rotation of the deuterated methyl segments in the choline group (which was of the order of picoseconds at a temperature of 30°C). The rate of motion is thus directly proportional to the value of T_1, and Figure 3 may be regarded as a direct representation of the lipid head-group mobility versus bilayer hydration. The observed variation in the value of T_1 with increasing water content is significant, and it changes by more than a factor of four from dry to fully hydrated bilayers.

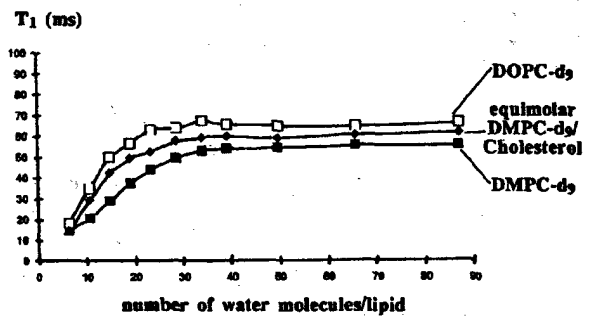

Figure 3: Deuterium spin-lattice (T_1) relaxation as a function of the degree of bilayer hydration at 30°C, for dispersions of liquid crystalline DOPC-d_9, DMPC-d_9 and equimolar DMPC-d_9/cholesterol.

In dry phosphatidylcholine multibilayers (i.e. the monohydrate), the coulombic and hydrogen-bonding interactions between neighbouring zwitterionic head-groups within one bilayer, as well as between adjacent bilayers, impose strong restrictions on their mobility. The incorporation of water into the bilayer surface region weakens these interactions between the head-groups and reduces their motional coupling. An approximately linear increase in T_1 is observed in Figure 3 as the head-groups attain greater mobility with increasing bilayer hydration. Although no relaxation time was measured for the original monohydrate (because the T_1 value would not be meaningful in terms of the the fast motional approximation), it is apparent from extrapolation to the dry system that the motions are then effectively inhibited.

With progressive hydration, all relaxation time profiles in Figure 3 reach a plateau (at greater than 30 H_2O molecules per phospholipid), with a T_1 value characteristic of the lipid type and the temperature. A bilayer may thus be considered as "fully hydrated" once the maximum head-group mobility is established. However a limiting number of water molecules per phospholipid required for full hydration cannot be clearly defined from this data because the plateaus are reached only gradually.

Uncharged multibilayers have a limited capacity for the uptake of water so that any excess water offered to the system forms a discrete bulk phase rather than being intercalated between adjacent bilayers (Small, 1967). This binary phase (> 35 H_2O molecules per phospholipid) appears to correlate with the high water content region in Figure 3 where the spin-lattice relaxation times remain constant on further addition of water. In a recent ^{2}H-NMR investigation (Ulrich *et al.*, 1989), phosphatidylcholine head-group mobility could be correlated with the restricted swelling of the bilayer surface region upon the addition of water as observed by neutron and X-ray small angle scattering.

Application of the ^{2}H-NMR method to the mixed lipid system of DMPD-d_9/CHOL has shown that the incorporation of a sterol into the bilayer does not have any significant effect on its hydration behaviour, since the profile in Figure 3 runs essentially parallel with those of the pure phospholipids. The present approach may be extended to study the effects of proteins and anaesthetics on the hydration state of membranes. The preliminary results here are also intended to emphasise the importance of controlling the degree of hydration of lipid samples, which becomes critical when temperature effects also have to be considered. Reproducible and biologically significant results are best obtained from fully hydrated membranes.

B. Ionization states of phospholipid head-groups

The process of protonation/deprotonation of ionizable groups on the bilayer surface was examined by pH titration of the phosphate and the primary amino group of phosphatidylethanolamine. The quadrupole splittings, $\Delta\nu_Q$, of specifically labelled DMPE-d_4 are sensitive to the surface ionization state of the the membrane (Poile & Watts, 1986), the deuterons being placed in the following positions in the head-group: -O-PO_2^--O-$CD_2(\alpha)$-$CD_2(\beta)$-NH_3^+ (see Figure 1).

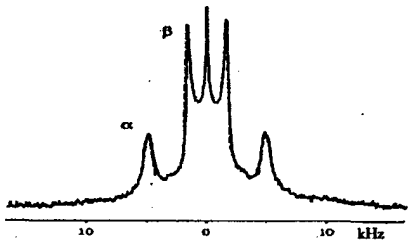

Figure 4: Typical ^{2}H-NMR spectrum of a random bilayer dispersion of liquid crystalline equimolar DMPE-d_4/DMPC at 57°C (pH 5.0), showing the resolution of the α- and β-deuterons.

Figure 4 shows a typical ^{2}H-NMR spectrum of equimolar DMPE-d_4/DMPC in a liquid crystalline bilayer dispersion, where the larger splitting corresponds to the α- and the smaller splitting to the β-deuterons, the central peak being due to residual HDO. On raising the pH, the outer resonances were observed to move further apart while the inner splitting decreased, which has been recorded as a function of bulk pH as illustrated in Figure 5a and b. The changes in $\Delta\nu_Q$ of the α-deuterons (Figure 5a) represent the deprotonation of the DMPE-d_4 phosphate group, which is directly connected to the deuterated α-CD_2 segment. A complete titration was not achieved because the sample decomposed below pH 3, however a pK_a value of the phosphate group was estimated near 3.5. Whereas $\Delta\nu_Q$ of the α-deuterons remains constant at high pH, the splitting of the β-deuterons in the same lipid head-group decreases by a factor of greater than 2. The curve fitted through the data points in Figure 5b represents the complete titration of the primary amino group in DMPE-d_4, i.e. the ionizable group directly adjacent to the deuterated β-CD_2 segment. The logarithm of the apparent dissociation constant for the lipid amino group, $pK_a \sim 9.6$, is lower than the value of 11.0 measured from the phase transitions of pure DMPE bilayers using fluorescence probes (Träuble & Eibl, 1974). This difference may be attributed to the presence of DMPC or to inherent differences in the experimental techniques, noting that the ^{2}H-NMR method is non-perturbing and can be performed isothermally.

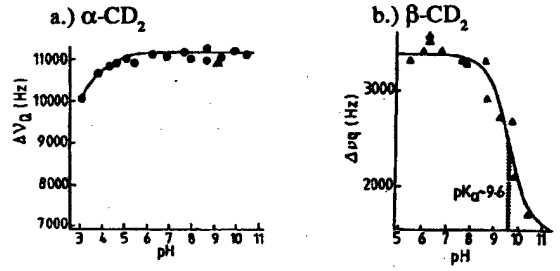

Figure 5: Deuterium quadrupole splittings of liquid crystalline DMPE-d_4/DMPC as a function of bulk pH at 57°C, showing (a) partial titration of the lipid phosphate group and (b) complete titration of the primary amino group.

Since the quadrupole splittings of the α- and β-deuterons vary independently of one another, they reflect the increase in bulk pH and are not caused by changes in the motional averaging or the chemical shift. Modification of the head-group conformation upon protonation/deprotonation occurs as a response to the presence of electric surface charges and dipole fields, whereby a small adjustment of the C_α-C_β torsion angle is sufficient to produce the observed effects (Seelig *et al.*, 1987).

The highly local sensitivity of the α- and β-deuterons in DMPE-d_4 towards protonation/deprotonation of the respective adjacent ionizable groups has been exploited to measure titration curves from which dissociation constants could be determined. This method may not ony be applied to various lipids but also to other molecules associated with a membrane, as has been successfully accomplished for the local anaesthetic tetracaine (Poile & Watts, 1986) and the small membrane-bound protein melittin (Lemon, Dempsey & Watts, to be published).

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