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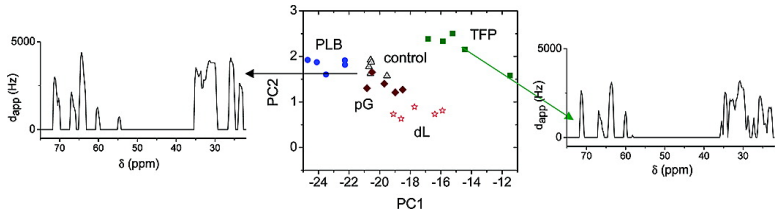

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Screening Molecular Associations with Lipid Membranes Using Natural Abundance ^{13}C Cross-Polarization Magic-Angle Spinning NMR and Principal Component Analysis

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Efforts to unravel the complex network of biomolecular interactions within the functioning cell have revealed that many proteins and drugs attain optimal activity by associating with cell membranes.[1] We report here an approach in which ^{13}C cross-polarization magic-angle spinning (CP-MAS) NMR spectroscopy[2] is combined with multivariate statistical analysis to detect and characterize the associations of molecules with hydrated lipid membrane preparations.

A molecule that associates with lipid bilayers may perturb the membrane host by altering the rates, amplitude and directionality of motion of the individual segments of the lipid molecules. Such effects have been observed in ^{2}H NMR studies of deuterated lipids,[3] in which the ^{2}H quadrupolar interaction is scaled by changes in molecular dynamics. Associations with a membrane will also scale the dipolar interactions between ^{1}H and ^{13}C in the lipid groups, although the measured changes in couplings will be relatively small ($\Delta\nu < 2$ kHz). It is possible to screen for, and characterize, interactions between additive molecules and membranes by measuring $^{1}H-^{13}C$ dipolar coupling constants (d_{HC}) for chemically distinct sites within membrane lipids. This is achieved by exploiting the NMR signal from the naturally abundant ^{13}C nuclei.

The experimental strategy relies upon the measurement of apparent H−C dipolar coupling constants d_{app} from natural abundance ^{13}C CP-MAS spectra of dimyristoyl-phosphatidylcholine (DMPC) in multilamellar vesicles, using the constant time DIPSHIFT experiment.[4] A curve-fitting procedure was used to convert the ^{13}C spectra into a dipolar profile, in which the intensity at each frequency point in the spectrum is replaced by a d_{app} value (Figure 1b). Coupling constants were regarded as apparent, rather than precise, values because of peak overlap and the scaling effect of homonuclear decoupling. The dipolar profile reflects the mobility of different regions of the lipid molecules and is a fingerprint of the state of the membrane.

Dipolar profiles were obtained for DMPC vesicles before and after adding aqueous solutions of the polybasic peptide decalysine (dL), the IgG binding domain of protein G (pG), and the drug trifluoperazine (TFP). Another DMPC sample contained the integral membrane protein phospholamban (PLB), which was incorporated after codissolution of lipid and protein in organic solvents. A 50-fold molar excess of lipid over the additives was used to ensure that no signal was observed from the additive species. To detect small, additive-induced changes above the random errors, measurements were obtained from sample groups of five replicates. Principal component (PC) analysis[5] was applied to the collective dipolar profiles to identify clusters of the sample groups. The conversion of raw spectral data into dipolar profiles removes any bias of the PC analysis toward the most intense peaks in the spectrum. Importantly, the presence of additives did not affect any of the chemical shifts for DMPC.

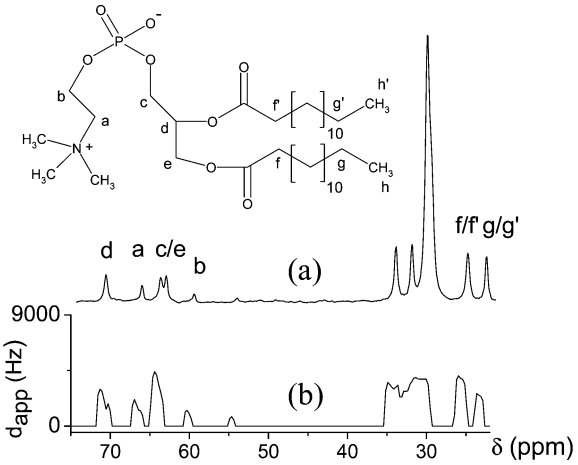

Figure 1. Summary of the procedure for calculating H−C dipolar profiles for lipid samples. The hydrocarbon region of a ^{13}C CP-MAS spectrum (at 100.13 MHz) of a DMPC vesicle suspension (30 mg of dry lipid in 100 μL of 10 mM phosphate buffer) is shown after 1024 scans (a). The spectrum was assigned according to ref 10, and the positions defined by labels a−g are shown in the inset. A series of $m = 13$ spectra were obtained using the ^{13}C CT-DIPSHIFT experiment,[4] where each spectrum was obtained for a different period t_1 (from 0 to 1 MAS cycle) of ^{13}C evolution under local proton fields. Peak intensities over one rotor period were measured at the same frequency point in the 13 spectra. The measured intensity values were compared with a library of curves calculated for different coupling constants to find the value giving the best agreement with the experimental data. When minimized χ^2 values of >0.05 were obtained in the fitting procedure, the points were regarded as noise and removed. This procedure was repeated for the 2048 points of the spectra to generate a profile of d_{app} values (b). Spectra were obtained at 303 K at a MAS rate of 4 kHz. The Hartmann−Hahn contact time was 2 ms.

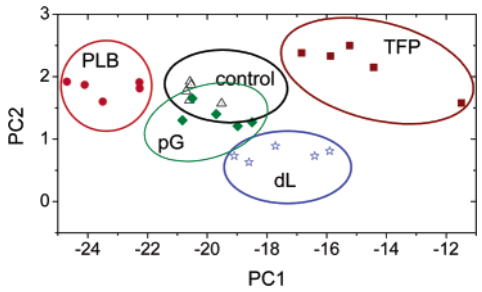

Figure 2. Scores plot of the first two principal components (PC1 and PC2) for dipolar profiles for DMPC membranes. Profiles were prepared from four sample groups ($n = 5$) of DMPC containing the various additives shown and a control sample group of pure DMPC. Principal components were obtained by diagonalization of the covariance matrix for the 25 combined profiles.

Figure 2 shows a scores plot of the first two principal components (PC1 and PC2) of the dipolar profiles for the sample groups. Scattering is present within each sample group, but clear, additive-

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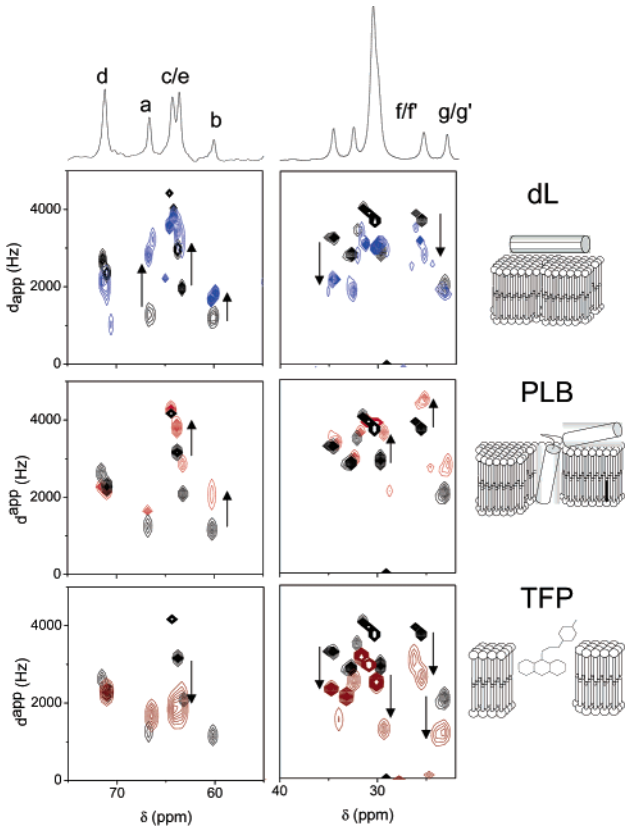

Figure 3. Two-dimensional plots constructed from dipolar profiles of DMPC. Contours can be seen for the lipid headgroup and glyceryl sites (left panels) and hydrocarbon methylene segments (right panels). Black contours show control membranes. Blue contours show samples containing dL. Red contours show samples containing PLB. Brown contours show samples containing TFP. Suggested modes of interaction between the lipid bilayer and each of the additives are shown on the right. A ^{13}C CP-MAS NMR spectrum of DMPC membranes is shown at the top with the labels and assignments as described in Figure 1. Nonoverlapping contours represent statistically significant differences in H−C couplings.

related clusters of points are observed. The data for the pG sample group (diamonds) are clustered together with the pure DMPC group (triangles), indicating that the membrane experienced little or no perturbation by pG. This protein does not contain any known membrane binding motifs, so the experimental data agree with the prediction that pG is membrane inactive. The clusters for the TFP (squares), dL (stars), and PLB (circles) are distinct from the control points and suggest that interactions occur between these additives and the membranes. Indeed, TFP is a psychotropic drug with highly lipophilic properties, and PLB is a transmembrane protein;[6] basic polylysines such as dL interact with the surfaces of anionic lipids[7] and, to a lesser extent, neutral lipids such as DMPC.[8]

The different positions of the PLB, TFP, and dL clusters in the PC plot suggest that the additives have quite distinct modes of association with the lipid membrane. Closer inspection of the dipolar profiles may give clues about these interactions. In Figure 3 are shown contour plots constructed from the combined dipolar profiles of different sample groups. The first dimension of the plots shows the ^{13}C chemical shift scale. In the second dimension, the contours are centered at the mean value of d_{app} for the sample group, with a Gaussian line width determined by the standard deviation of d_{app}. The contour levels were set to the corresponding peak intensities in the ^{13}C NMR spectrum.

The plots for control samples of pure DMPC are shown in black in Figure 3. Significant changes in contour positions were observed when dL was added to the membranes (Figure 3, blue). The values of d_{app} for some of the hydrocarbon chain CH_2 groups (22−40 ppm) are seen to decrease, whereas values of d_{app} for the choline headgroup (55−75 ppm) increase. Hence, dL appears to disorder the CH_2 segments of the fatty acyl chains and stabilize the polar headgroups. This dual effect is consistent with the propensity of polylysines to associate with the membrane surface.[6] A more uniform reduction in d_{app} was observed for DMPC in the presence of TFP (brown), which is consistent with membrane destabilization resulting from penetration of the drug into the bilayer interior. By contrast, the presence of PLB increases the d_{app} values in both the headgroup and acyl regions of the lipid bilayers (Figure 3, red contours). Positive changes in d_{app} values may be explained by a hydrophobic mismatch between the PLB transmembrane domain and the relatively short lipid chains (C14), which lead to local increases in bilayer thickness.[9] ^{2}H and ^{31}P NMR studies suggest that the transmembrane domain of PLB is better matched to longer chain lipids.[11]

In summary, we have reported a simple method for detecting the interactions between bioactive molecules and cell membranes. The method is attractive because it does not require isotope labeling and can be used with natural membranes. Although relatively large quantities of lipid (>10 mg) are required at the field strength used here, there is potential for method optimization to gain improvements in throughput, sample group size, and sensitivity. Applications, including characterization of drug lipophilicity and studies of membrane interactions involving signaling proteins, are under investigation.

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Supporting Information Available: Methods for conversion of NMR spectra into dipolar profiles and for principal component analysis of dipolar profiles, as well as Figure S1 showing the pulse sequence for the CT-DIPSHIFT experiment and Figure S2 showing a summary of the procedure used for preparing dipolar profiles (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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