Tethered or Adsorbed Supported Lipid Bilayers in Nanotubes Characterized by Deuterium Magic Angle Spinning NMR Spectroscopy

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^{2}H solid-state NMR experiments were performed under magic angle spinning on lipid bilayers oriented into nanotubes arrays, as a new method to assess the geometrical arrangement of the lipids. Orientational information is obtained from the intensities of the spinning sidebands. The lipid bilayers are formed by fusion of small unilamellar vesicles of DMPC-d_{54} inside a nanoporous anodic aluminum oxide, either by direct adsorption on the support or by tethering through a streptavidin/biotin linker. The results support that the quality of the lipid bilayers alignment is clearly in favor of the tethering rather than an adsorbed strategy.

1. Introduction

Solid supported lipid membranes constitute a powerful approach to provide information on molecular processes occurring in biological membranes or some understanding of membrane−protein interactions.[1−3] The basic requirement of these biomimetic membranes is to obtain a single lipid bilayer in a fluid state. In our approach, this point is emphasized by tethering a phospholipid bilayer on a solid support allowing for an internal compartment, which will be promising for a future internal proteins insertion. Indeed, these biomimetic membranes are suitable for biophysical investigations as their robustness offers assay by various techniques such as surface plasmon resonance spectroscopy,[4] atomic force microscopy,[5,6] and small-angle neutron scattering.[7] These systems also offer potential for biosensor applications.[8] Supported lipid membranes can be formed either by lipid bilayers separated from the solid support by an ultrathin water layer or by lipid bilayers separated from the support by a discontinuous sublayer of a polymer.[9−11] Lipid adhesion depends crucially on the nature of the support, and bilayer formation requires hydrophilic surface.[12] In the tethered methods, the polymer is covalently linked to the solid support and to some of the lipids. The insertion of large proteins and particularly transmembrane proteins is then facilitated.[13,14] The methods to achieve the assembly call either upon vesicle spreading or film deposition. Whatever the strategy, the requirements are the fluidity of the lipid bilayer, a high level of hydration, and stability in order to be relevant of a biological state.

Solid-state nuclear magnetic resonance (NMR) is an adequate technique to investigate these molecular assemblies. Structural information can be obtained when using aligned biomimetics membranes specially designed for NMR studies,[15,16] like glass plates[17] or bicelles[18] allowing for high-resolution spectra. Recently, aligned bilayers inserted into the nanotubes arrays of anodic aluminum oxide (AAO) have been investigated by solid-state NMR.[19,20] An additional refinement consists of combining the oriented samples with the magic angle spinning technique (MAS) for better sensitivity and finest structural information.[21,22]

In a previous work, we have shown the formation of a complex phospholipid bilayer tethered inside the AAO support, obtained by fusion of small unilamellar vesicles (SUVs). The well-resolved ^{1}H MAS NMR spectrum seems to correspond to a fluid state of the lipids inside the nanopores. In addition, the effect of tethering on silica beads indicates no hindered motion compared to lipids simply adsorbed on this spherical support. However, the ^{31}P solid-state NMR experiments failed to prove the

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alignment of the phospholipid bilayers inside the nanopores.[23]

^{2}H NMR is often used on static samples to characterize lipid orientations.[24] In this study, ^{2}H solid-state NMR experiments were performed under magic angle spinning on lipid bilayers inside nanotube arrays to assess the geometrical arrangement of the lipids. Orientational information is obtained from the intensities of the spinning sidebands. The results support that the quality of the lipid bilayers alignment is clearly in favor of the tethering rather than an adsorbed strategy. The originality of this work lies in the tethering of the lipid bilayers as well as the stacking of AAO disks in the rotor oriented at the magic angle to determine the degree of alignment of the phospholipid bilayers. Using spinning rather than static samples allowed us to do so with a greater sensitivity and precision, even when samples were poorly oriented.

2. Material and Methods

2.1. Sample Preparation. The AAO Anodisc 47 disks (Whatman, Maidstone, England) with a pore diameter of 200 nm and a thickness of 60 μm have a porosity of 80% and a pore density of 2.54×10^9 pores per cm^2. The Anodiscs were cut into 5 mm diameter disks, and 60 disks were stacked into a 7 mm MAS rotor and filled with ^{2}H-depleted water.

The strategy of bilayer formation is based on vesicle fusion.[25−27] For the tethered method, amino groups were created at the surface of the aluminum oxide by reaction with a (3-aminopropyl)-dimethylethoxysilane. All the buffer solutions were prepared in deuterium-depleted water. In a next step, the supports were dipped for 45 min in a 2.1 mM NHS−biotin solution in a phosphate buffer in order to create a biotinylation of the surface amino groups. After extensive rinsing, the supports were dipped for 10 min in a 40 mg/mL streptavidin (Sigma, St. Louis, MO) solution in a PBS buffer (0.01 M phosphate buffer and 0.15 M NaCl). The biotin/streptavidin affinity is used to anchor the streptavidin sublayer. The supports were rinsed with an octylglucoside solution and then with the PBS buffer before mixing under agitation during 1 h with SUV mixture. The SUVs composed of chain-deuterated 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC-d_{54}) (Avanti Polar Lipids, Alabaster, AL) and containing 0.5% of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-biotinyl (Avanti Polar Lipids) in deuterium-depleted water are obtained by sonication of the mixture at a concentration of 5 mM. The high affinity of streptavidin toward the biotinylated lipids allows for the fixation of the SUVs. Fusion is achieved by four cycles of freeze−thawing and by rinsing. This final step leads to a lipid bilayer tethered through a spacer arm to the solid support (Figure 1B). A final phosphorus assay indicated that the bound quantity of phospholipids was 1.8 mg.[23] This value is consistent with the surface of one bilayer tethered inside each pore that leads to a calculated value of 1.7 mg. For the adsorption method, the SUVs were loaded into the AAO and fused as mentioned above. This last method is more difficult to achieve because, in most cases, the SUVs do not fuse.

2.2. NMR Experiments. The experiments were performed on a Bruker Avance DMX 400-WB NMR spectrometer (Wissembourg, France) with a ^{1}H frequency of 400.13 MHz and a ^{2}H frequency of 61.4 MHz. ZrO_2 rotors (7 mm) containing the samples were spun at 3 kHz in a 7 mm Bruker cross-polarized magic angle spinning probe, and the spinning frequency was controlled to within 15 Hz. The quadrupolar echo sequence[24] was performed with a 3.5 μs 90° pulse and a 250 ms delay between scans. A 333 μs interpulse delay has been used to synchronize the radio frequency pulses to the phase of the spinning rotor. Spectra were acquired at a temperature of 30 °C.

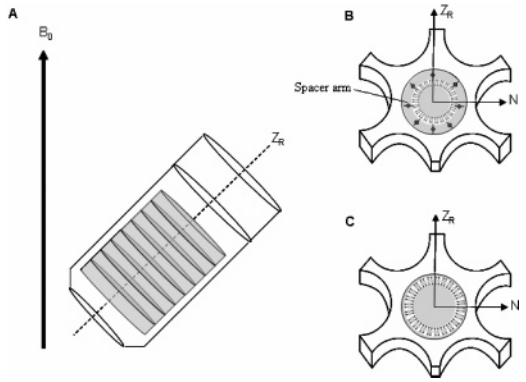

Figure 1. Geometrical arrangement of 60 disks stacked in the rotor filled with deuterium-depleted water (A). Orientation of the bilayer inside the pores, (B) for the tethered method and (C) for the adsorbed method.

2.3. Simulation of NMR Spectra. MAS spectra at 3 kHz of the AAO constructions were simulated in the time domain using an equivalent approach to those described by Glaubitz and Sizun and Bechinger.[21,22] The cylindrical distribution of lipids is simulated by a series of 360 crystallites describing a circle in the plane perpendicular to the rotor axis corresponding to the distribution of the membrane normal. To fit the experimental data, an additional Gaussian distribution of the crystallites' orientation is introduced to take into account for AAO surface irregularities or a mismatch between the lipid bilayer alignment inside the pore cylinder. The total free induction decay (FID) is calculated by adding the signal from the perpendicularly oriented crystallites, multiplied by the fraction of perfectly oriented lipids, and the signal from the Gaussian distribution of orientations, multiplied by the fraction of unoriented lipids. The simulations were calculated by taking into account that the z axis of the electric field gradient (EFG) tensor is parallel to the C−D bond in the rotor frame. The coordinate transformation from the rotor frame to the laboratory frame brings the cylindrical symmetry in the case of an ideal cylindrical distribution of lipids. The ^{2}H NMR quadrupolar splittings between 2.5 and 20 kHz corresponding to all the CD bonds of the fluid acyl chains are obtained from measurement on a multilamellar vesicles (MLV) sample (data not shown). A total of 360 crystallite orientations were considered, and 4096 steps with a dwell time of 10 μs were calculated. The resulting FID was finally multiplied by an exponential line broadening function of 200 Hz before Fourier transformation.

3. Results and Discussion

As shown in Figure 1A, AAO disks dipped in ^{2}H-depleted water are stacked in the rotor oriented at the magic angle. In both strategies, the lipid bilayers covered the inner cavity wall of the support either by adsorption or tethering. The AAO support is composed of parallel cylindrical pores,[28] and thus the bilayers are most likely oriented in the same way with a cylindrical geometry. Thus, the membrane normal (N) is perpendicular to the rotor axis (Z_R). In the tethered lipid bilayers (Figure 1B), a streptavidin/biotin spacer between the silanated aluminum oxide and the phospholipids allowed for a large water space (ca. 15 nm[26]) between the support and the lipid bilayer. In the adsorbed lipid bilayers (Figure 1C), an ultrathin water layer separated the support from the lipid bilayer.

To investigate the geometry of the obtained lipid bilayers inside the nanopores, experimental ^{2}H MAS NMR spectra, at a spinning rate which is small compared to the quadrupolar interaction, were compared to simulated

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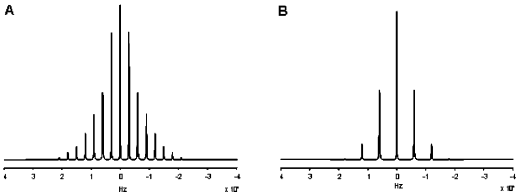

Figure 2. ^{2}H MAS NMR (3 kHz) simulated spectra of DMPC-d_{54} (A) for a random distribution and (B) for an ideal cylindrical phospholipid distribution.

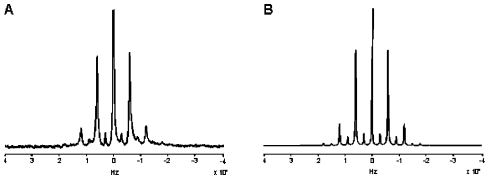

Figure 3. ^{2}H MAS NMR (3 kHz) spectra of the phospholipid bilayers (DMPC-d_{54}) tethered inside the nanopores: (A) experimental spectrum; (B) simulated spectrum with 89% of oriented lipids and a mosaic spread of 5°.

spectra taking into account the attempted geometry of these magic angle oriented spinning samples (MAOSS).

In the MAS NMR experiments at low spinning rates (3000 Hz), each sideband intensity is an orientational dependent function.[29,30] When the electric field gradient tensors are randomly distributed, the resulting ^{2}H MAS NMR spectrum is characteristic of an unoriented system like MLVs. The simulated spectrum is presented in Figure 2A and correctly resembles the fully disordered classical MLV spectrum. For lipid bilayers inside the cylindrical nanopores, the EFG tensors are distributed around a cylinder oriented at the magic angle. The theoretical approach of Glaubitz demonstrates that the simulated spectrum can be used to characterize this singular geometry. Indeed, the simulated spectrum leads to a striking zero intensity of odd-numbered spinning sidebands (Figure 2B).

For the tethered and adsorbed lipid bilayers in the AAO nanotubes, the spectra obtained at the magic angle with a slow spinning rate (3000 Hz) led to well-resolved spectra with narrow spinning sidebands (Figures 3A and 4A). The peak centered at 0 Hz is mostly due to residual D_2O from the ^{2}H-depleted water in all experiments. Indeed, in the tethered lipid bilayers, SUVs have completely fused[23] while in the adsorbed lipid bilayers, the isotropic resonance could also be caused by remaining SUVs. In both cases, the odd-numbered sidebands are still present on the experimental spectra. However, their intensities are lower than those in an unoriented spectrum. Beside the higher sensitivity, one advantage of ^{2}H MAS NMR is that sideband intensities can be exploited in the simulations for precise quantification of oriented vs unoriented lipids and mosaic spread. For the tethered lipids, the best agreement between the simulated and the experimental spectra is obtained for 89% of oriented lipids and a mosaic spread of 5° (Figure 3). For the adsorbed lipids, the best fit between the experimental and simulated spectra is obtained for 42% of oriented lipids and a mosaic spread of 18° (Figure 4). Whether by adsorption or by tethering, the AAO allows for a macroscopical orientation of lipid

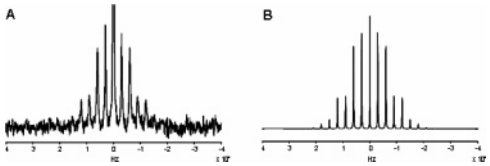

Figure 4. ^{2}H MAS NMR (3 kHz) spectra of the phospholipid bilayers (DMPC-d_{54}) adsorbed inside the nanopores: (A) experimental spectrum; (B) simulated spectrum with 42% of oriented lipids and a mosaic spread of 18°.

bilayers. Even after spinning at 3 kHz for 12 h, both biomimetic membranes have shown a high stability.

Our results clearly indicate that the molecular bridging permits a well-oriented biomimetic membrane. Indeed, the step-by-step strategy allows for a high control of the formation of a single tethered lipid bilayer.[25,27] After the fusion of the SUVs, the last step of extensive rinsing removed the remaining nonfused vesicles. The remaining fraction of unoriented lipids and the mosaic spread can be explained by the fact that all the cylindrical pores of AAO are not perfectly coaxial and that the pores are not perfect cylinders.

In the case of the adsorbed method on the same support, the geometrical considerations are similar but the results show that more lipids are unoriented. This stems from the fact that the spreading of SUVs does not allow for a high control of the formation of the bilayer. As the SUVs are loaded in the pores, there is no possibility of rinsing. In the analogous case of a biomimetic membrane obtained by a film deposited on a poly(ethylene terephthalate) polymer sheet wrapped cylindrically in the rotor, a better alignment is obtained but the percentage of oriented lipids ranges from 65% up to 75%, with a mosaic spread around 10°.[23] Furthermore, the results in terms of orientation vary from one experiment to another for these adsorbed methods, and the effect of prolonged spinning was not negligible. In contrast, there was no evolution of the signal when spinning during at least 2 days for the tethered samples.

4. Conclusion

In addition to providing an original, simple and efficient technique to characterize supported lipid membranes orientation, our results are very promising for further insertion of peptides or proteins in AAO as recently experimented by Lorigan et al.[19] as well as for biosensor development.[8] Tethering of the bilayer offers a high stability of a single fluid bilayer and a high control of lipid orientation. Furthermore, unlike bicelles, these bilayers allow for complex lipid mixtures in buffer. Similarly unlike glass plates, they allow for a fully hydrated system, analogous to a biological state. Finally, other nuclei can be observed by NMR for structural and dynamic MAOSS investigation of molecules in a tethered supported lipid bilayer, mimicking a simplified experimental model for internal membranes of chloroplasts or mitochondria for example.

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