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Communication

^{15}N and ^{31}P solid-state NMR study of transmembrane domain alignment of M2 protein of influenza A virus in hydrated cylindrical lipid bilayers confined to anodic aluminum oxide nanopores

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Abstract

This communication reports the first example of a high resolution solid-state ^{15}N 2D PISEMA NMR spectrum of a transmembrane peptide aligned using hydrated cylindrical lipid bilayers formed inside nanoporous anodic aluminum oxide (AAO) substrates. The transmembrane domain SSDPLVVA(A-^{15}N)SIIGILHLILWILDRL of M2 protein from influenza A virus was reconstituted in hydrated 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine bilayers that were macroscopically aligned by a conventional micro slide glass support or by the AAO nanoporous substrate. ^{15}N and ^{31}P NMR spectra demonstrate that both the phospholipids and the protein transmembrane domain are uniformly aligned in the nanopores. Importantly, nanoporous AAO substrates may offer several advantages for membrane protein alignment in solid-state NMR studies compared to conventional methods. Specifically, higher thermal conductivity of aluminum oxide is expected to suppress thermal gradients associated with inhomogeneous radio frequency heating. Another important advantage of the nanoporous AAO substrate is its excellent accessibility to the bilayer surface for exposure to solute molecules. Such high accessibility achieved through the substrate nanochannel network could facilitate a wide range of structure–function studies of membrane proteins by solid-state NMR.

Keywords: Solid-state NMR; Membrane proteins; Bilayer alignment; PISEMA; Nanoporous substrate; Anodic aluminum oxide

1. Introduction

Solid-state NMR spectroscopy has emerged as a powerful structural tool to study membrane proteins [1,2] that cannot be easily crystallized for high resolution X-ray diffraction nor examined by solution NMR methods. While spectral resolution in solid-state NMR is generally more limited because of broader lines, several methods have been developed to selectively average certain spin interactions while revealing the dipolar couplings of interest. One such experiment is polarization inversion spin exchange at the magic angle (PISEMA) that correlates anisotropic dipolar and chemical shift interactions [3]. To achieve adequate spectral resolution for many sites the sample for PISEMA experiments should be uniformly aligned with respect to the magnetic field axis. Typically, such samples of membrane proteins are prepared using aligned lipid bilayers formed on planar solid substrates [4] or by using magnetic forces to

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align bicelle discs in the external magnetic field [5,6]. In all these assemblies the lipids serve both to align the proteins and to provide an excellent mimetic environment of cellular membranes to ensure the native protein conformation. For substrate-supported bilayers the lipid composition and other experimental conditions [4,7–9], such as pH and temperature, can be adjusted over a wider range than for bicelles. Thus, the former method has generally wider applicability for studies of membrane proteins. In current experimental implementation of this method an NMR sample for the PISEMA experiment consists of a stack of 30–120 μm thick glass plates that sandwich many thousands of planar lipid bilayers containing membrane peptides or proteins between the plates. While a number of methods have been developed to improve the protein alignment, stability, and physical properties of samples during long—many hour—NMR experiments [10–12], the preparation of well-aligned bilayers continues to be a challenging task.

Here, we describe the use of nanopore-supported cylindrical lipid bilayers formed inside anodic aluminum oxide (AAO) substrates to align the transmembrane domain SSDPLVVA(A-^{15}N)SIIGILHLILWILDRL of M2 protein (M2 TMD) from influenza A virus [13]. Previously, self-assembly of phospholipids into well-aligned cylindrical nanotubular bilayers inside nanoporous AAO substrates has been characterized by spin labeling high field EPR [14]. More recently, the alignment of phospholipids and a transmembrane peptide supported by AAO has been observed using 1D ^{2}H solid-state NMR spectra [15]. Properties of the single lipid bilayers supported in AAO nanopores have been also studied by multinuclear NMR [25]. Here, we demonstrate the first example of high resolution 2D PISEMA NMR spectra of the M2-TMD using this new type of substrate-supported bilayers. We compare ^{15}N and ^{31}P NMR data from these samples with those obtained by alignment utilizing glass slides, and discuss the differences of the two alignment schemes.

The high resolution NMR structure of tetrameric M2-TMD was reported recently [13,16]. When reconstructed in hydrated 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) bilayers that were microscopically aligned between the glass plates, the peptide was found to have a tilt of $37° \pm 3°$ with respect to the bilayer normal [13,17]. The TM domain of this H^+ channel forms a symmetric tetramer [18] yielding a single set of NMR resonances [10] that has been characterized [16]. We use the M2-TMD to benchmark the new type of substrate-supported bilayers for high resolution 2D solid-state NMR studies of proteins aligned by nanoporous substrates.

2. Experimental

The ^{15}N-Ala-30 M2-TMD was prepared by solid-phase peptide synthesis and co-dissolved in trifluoroethanol (TFE) with DMPC in a 1:16 molar ratio. The solvent was removed by a rotary evaporator and then by drying under vacuum overnight. The lipid film was rehydrated with a 5 mM phosphate buffer at pH 8.0 containing 10 mM amantadine, the antiviral drug, forming multilamellar liposomes containing the M2-TMD in a tetrameric state. The liposomes were then pelleted by ultracentrifugation at 104,000*g*. The pellet was collected and homogenized by bath sonication.

For conventional glass-supported samples, the pellet was deposited on thin, 30 μm, 5.7×12.0 mm glass strips (Matsunami Trading, Osaka, Japan). The strips were stacked and then sealed in a square sample tube to maintain a constant hydration level during NMR experiments.

Before lipid/peptide deposition, Anodisc 47 membranes (Whatman, Maidstone, UK) were cut into 5.7×12.0 mm strips. These commercial AAO substrates are 60 μm thick and have a well-aligned through-film porous structure. The manufacturer specifies that these nanoporous substrates have a 20 nm filtration diameter cutoff. Examination of these Anodiscs with a JEOL 6400F Field Emission Scanning Electron Microscope (JEOL, Japan) indicated that the pores in this substrate have a diameter of 177 ± 12 nm for one side of the film, but for the other side the pores branch out into 28 ± 8 nm narrow channels for a thin, 1–2 μm, filtration layer. Because of the small thickness of the filtration layer it is expected that only a negligible amount of lipid will be confined there. Bilayers from the pellet were pipetted onto the AAO strips and were rapidly absorbed by the nanoporous substrate. Once deposited and stacked, both the AAO and glass strips were incubated at 45 °C and 93% relative humidity for 24 h.

For NMR PISEMA experiments it is important to achieve the best possible filling factor without compromising sample alignment. Thus, the AAO sample preparation protocol was optimized. Initially, the AAO-supported samples were prepared with an amount of bilayer material so that the lipid phase of the sample would occupy only 20–25% of the void volume of the nanopores preventing any possible pore blockage. The volume of deposited bilayers was then increased to ca. 80% of the void volume without any significant change in the ^{15}N NMR spectra (not shown) except for increased signal intensity. For the latter samples the signal-to-noise (S/N) ratio was found to be comparable to that of a sample prepared on a conventional glass support.

Hydration level is another important factor affecting line width in NMR experiments with membrane proteins. Typically, the optimal hydration level for conventional substrate-supported samples is achieved by incubating the entire stack of glass strip-supported bilayers for 24 h at 45 °C and 93% relative humidity. For the first set of the AAO-supported samples we followed this procedure. We will refer to these samples as

obtained through a "normal hydration procedure." For the second set of samples, the hydration of the bilayers deposited inside the nanopores was further improved by placing open rectangular NMR sample tubes containing the AAO stacks into a buffer solution at 45 °C for 24 h. Following this incubation, the excess buffer was removed and the tube was resealed. We will call these samples "fully hydrated."

1D and 2D ^{15}N NMR experiments were performed with Bruker data acquisition systems at 9.4 and 14.1 T equipped with an in-house ^{1}H/^{15}N double resonance static probe utilizing rectangular ~600 μL sample coils [19]. Such a configuration permits sample tube rotation by 90° with respect to the magnetic field. ^{31}P ^{1}H-decoupled spectra were accumulated with a Bruker data acquisition system at 7.0 T with an in-house engineered ^{1}H/^{31}P double resonance static probe [19]. All ^{15}N and ^{31}P chemical shifts are presented relative to the resonance of a saturated solution of $^{15}NH_4NO_3$ and 85% solution of $H_3{}^{31}PO_4$, respectively, at 0 ppm. All spectra were acquired at 25 °C with the DMPC bilayer in a liquid-crystalline phase.

3. Results and discussion

The major difference between the two methods of bilayer alignment for solid-state NMR protein studies—by glass plates and by nanoporous AAO—is illustrated by the ^{31}P NMR spectra of the lipids. Fig. 1 shows ^{31}P ^{1}H-decoupled spectra of hydrated DMPC bilayers containing M2-TMD deposited on glass slides and into nanoporous AAO. The spectra are compared for two orientations of the substrate planes in the magnetic field, B_0. For lipid molecules undergoing fast tumbling about their long axis coinciding with the bilayer normal, the ^{31}P chemical shift anisotropy tensor, σ, is known to be axially symmetric. Thus, when the glass plate and therefore the bilayer surface is either parallel or perpendicular to B_0, the resulting ^{31}P resonance is dominated by a sharp line centered at $\sigma_\perp$ or $\sigma_\parallel$, respectively (Fig. 1A). When lipids are confined to AAO nanopores, the bilayers take on a cylindrical configuration with the bilayer normal parallel to the substrate outer surface. Therefore, when the AAO surface is perpendicular to B_0, the resulting ^{31}P spectrum is dominated by a resonance line at the $\sigma_\perp$ frequency, as illustrated in Figs. 1B and C (solid lines). The spectra at this orientation of the AAO strips are similar to those obtained for the glass-aligned sample when the glass surface is parallel to B_0 (Fig. 1A, dashed line). When the AAO plate surface is parallel to the B_0, the resulting signal arises from lipids in the nanopores having a cylindrical distribution about an axis perpendicular to the field. Such a distribution results in a powder pattern with peaks at both $\sigma_\perp$ and $\sigma_\parallel$ (Figs. 1B and C, dashed lines).

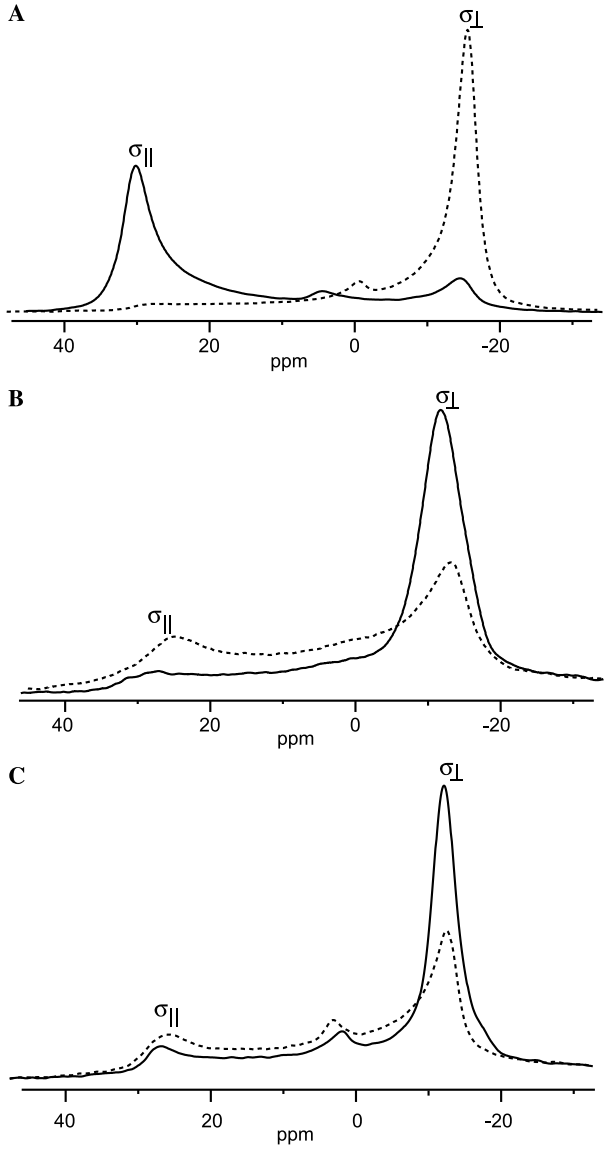

Fig. 1. Superimposed 1D ^{31}P ^{1}H-decoupled spectra of DMPC lipid bilayers containing single site ^{15}N-labeled Ala-30 M2-TMD at two orientations of the outer substrate surfaces with respect to the magnetic field: dashed line—the surface is parallel to B_0; solid line—the surface is perpendicular to B_0, (A) glass-supported sample, (B) AAO-supported sample obtained through normal hydration procedure, and (C) fully hydrated AAO sample.

Hydration effects on AAO-supported samples are illustrated in Figs. 1B and C. The spectra shown in Fig. 1B were taken from an AAO-supported sample prepared following the same hydration procedures as for glass-supported samples. The ^{31}P lines for this sample, and particularly, the width of the $\sigma_\perp$ feature are significantly broader than those observed for a fully hydrated AAO-sample (Fig. 1C). Thus, the modified hydration procedure resulted in significant narrowing of the ^{31}P lines indicating an improvement in the lipid alignment.

Note, that the susceptibility effects [20,21] are significant and may be, in part, responsible for both the line

width and the changes in the chemical shifts of ^{31}P spectra by a few ppm (Fig. 1). Fig. 1 also indicates that apparent anisotropy of the chemical shift ($\sigma_{\parallel}$–$\sigma_{\perp}$) for AAO-supported samples (Figs. 1B and C) is smaller than that for the glass-supported sample (Fig. 1A). One possible explanation is that the lipids in AAO-supported sample could be more mobile and this would result in a partial averaging of the spectral features.

One could argue that a partial disorder in the orientations of the individual nanopores and/or lipid bilayer surfaces could be another contributing factor to a decrease in the chemical shift anisotropy observed for the AAO-sample. However, one of the first manifestations of such a disorder would be an increase in the line width. From a comparison of the ^{31}P spectra for the fully hydrated AAO-sample in the $\perp B_0$ orientation with that of the glass-supported bilayers in the $\|B_0$ orientation (solid line in Fig. 1C and dotted line in Fig. 1A, respectively), no significant changes in width or shape are observed suggesting that the lipid bilayers are comparably well-aligned in both samples [22]. ^{31}P spectra from the AAO-sample also demonstrate that most of the lipid bilayers are assembled into lipid nanotubes that are stabilized inside the nanoporous substrates and that the fraction of lipids in other configurations, such as, for example, between the AAO strips, is negligibly small.

Examination of 1D ^{15}N NMR resonance lines for the same set of samples indicates large effects of sample hydration on the line width. Specifically, for the fully hydrated AAO-supported sample the width was significantly narrower than that for the sample prepared under conventional hydration protocol (i.e., 250–350 Hz vs. ca. 1500 Hz, Figs. 2C and A, respectively). No significant loss of NMR signal was observed even after subsequent multiple incubations of the AAO strips in buffer solutions. This indicates that the vast majority of lipid bilayers are tightly confined and bound to the AAO nanopores. It is worthwhile to note that the width of the ^{15}N NMR resonance for fully hydrated AAO sample was narrower than that for the glass-supported sample (solid line Fig. 2B). The broadening of the latter spectrum could be caused by a partial dehydration of the sample that is difficult to avoid in NMR experiments at high magnetic fields.

Fig. 3A shows a comparison of PISEMA spectra of a single site ^{15}N-labeled Ala-30 M2-TMD for different DMPC sample preparations. The top (A) spectrum was obtained from AAO-supported lipid nanotube array sample when the outer substrate surface was parallel to B_0 (only spectrum from a fully hydrated sample is shown). The PISEMA spectra from M2-TMD/DMPC sample aligned with the help of conventional glass slides at two orientations of the glass surface with respect to B_0 are shown at the bottom (Fig. 3B). To facilitate comparison of the all three spectra the PISEMA from the AAO-supported sample is indicated by dotted lines in (B). The

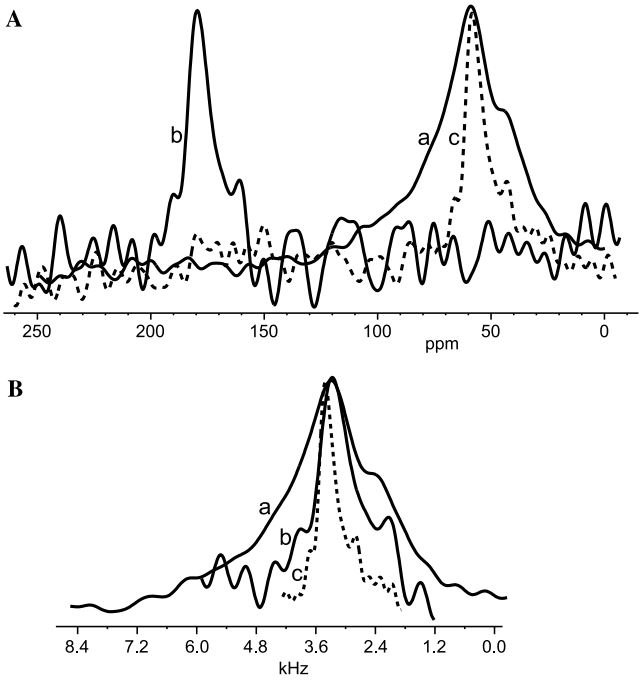

Fig. 2. Comparison of 1D ^{15}N NMR spectra of ^{15}N-labeled Ala-30 M2-TMD in DMPC bilayers for different sample preparations. The same spectra are shown using a ppm scale over the entire range of ^{15}N CSA (A, top) and also a frequency scale over a narrow range to illustrate line width difference (B, bottom): (a), (solid line) AAO-supported sample obtained through the normal hydration procedure with the lipid bilayer surface parallel to B_0 (20 mg, 16,384 scans, 14.1 T, 300 Hz line broadening); (b), (solid line) glass-supported sample obtained by the normal hydration procedure with lipid bilayer surface perpendicular to B_0 (20 mg M2 TMD, 224 scans, 14.1 T, 200 Hz line broadening); and (c), (dashed line) fully hydrated AAO-supported sample with the lipid bilayer surface parallel to B_0 (20 mg, 2048 scans, 9.4 T, 150 Hz line broadening). We estimate that about 50% of 20 mg of M2 TMD was lost during the AAO sample preparation.

comparison of the corresponding ^{15}N PISEMA spectra for the AAO- and the glass-slide-supported samples shows a similarity in both the dipolar coupling and the chemical shift values. We anticipate that some minor differences in the PISEMA spectra from these two samples arise from slight changes in dynamics, hydration levels, and/or surface and susceptibility effects. However, if there are any changes in the dipolar coupling between the two samples, those cannot be differentiated because of a limited sampling in the dipolar dimension. Both the ranges of chemical shift (CS) and dipolar coupling are scaled down by a factor of two for the AAO-sample as compared to the conventional glass-supported sample based on the orientation of the bilayer normal in the respective samples. For the former sample the NMR line widths should also be reduced by approximately the same factor providing potentially similar spectral resolution to that of the conventional glass support method. It is worth noting that the spectra for the glass-supported sample were recorded at 14.1 T whereas the spectrum on

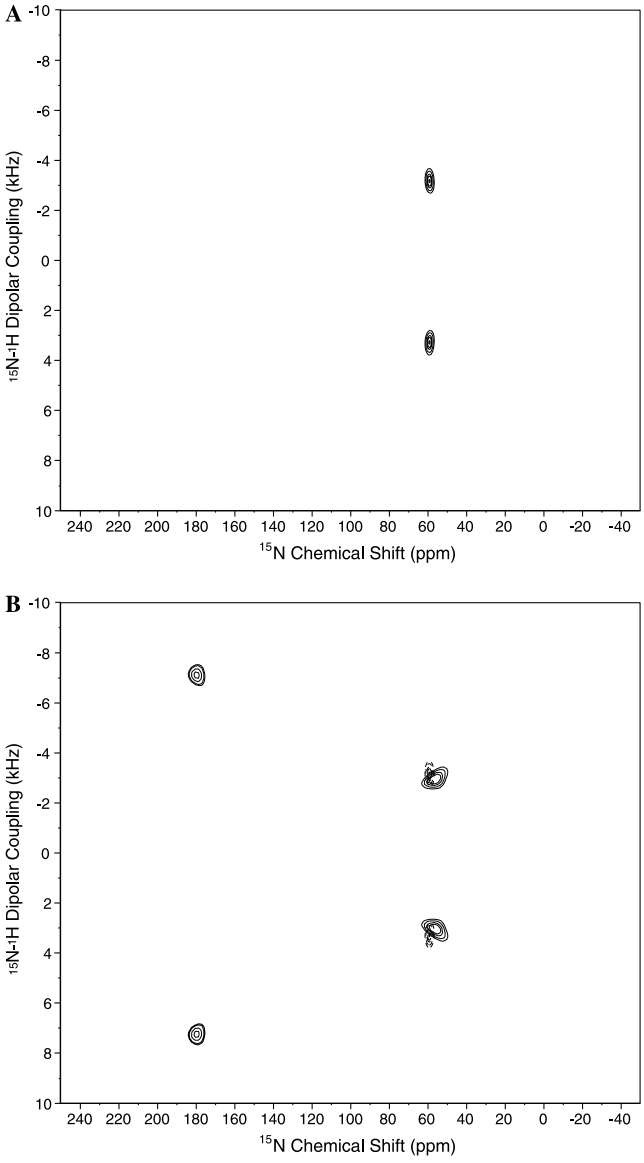

Fig. 3. Comparison of PISEMA spectra of a single site ^{15}N-labeled Ala-30 M2-TMD for different DMPC sample preparations. (A, top): AAO-supported lipid nanotube array sample with the outer substrate surface parallel to B_0 (doublet at $\sigma(^{15}\mathrm{N}) \approx 60$ ppm); experimental parameters: approximately 10 mg of the M2-TMD was deposited, 9.4 T (400 MHz), 32 t_1 increments with 1088 transients each, 4 s recycle delay, B_1 ^{1}H (decoupling) = 50 kHz, B_1 cross-polarization = 50 kHz, Lee–Goldburg = 35 kHz). (B, bottom): solid lines—lipid bilayer sample with the glass strip surfaces perpendicular (doublet at $\sigma(^{15}\mathrm{N}) \approx 180$ ppm) and parallel (solid lines, doublet at $\sigma(^{15}\mathrm{N}) \approx 60$ ppm) to B_0; experimental parameters: 20 mg of M2-TMD was deposited, 14.1 T (600 MHz), 16 t_1 increments with 256 transients each, 6 s recycle delay, B_1 ^{1}H (decoupling) = 40 kHz, B_1 cross-polarization = 35 kHz, Lee–Goldburg = 25 kHz. To facilitate comparison of the all three spectra the PISEMA from the AAO-supported sample is indicated by dotted lines in (B). For all the spectra the dipolar axis was adjusted to account for the scaling factor of 0.81 arising from the application of phase-alternated Lee–Goldburg homonuclear decoupling [23,24].

the AAO support was obtained at 9.4 T, i.e., with somewhat lower (in ppm) resolution in the CS dimension. Even in this case the line width corresponding to the AAO support is narrower when compared with the glass-supported sample.

4. Conclusion

Here we demonstrate the first example of high-resolution 2D NMR spectra of membrane proteins aligned with the help of a nanoporous AAO substrates. While the single site ^{15}N-labeled 25-residue transmembrane protein domain chosen for this study represents a relatively simple case and the high resolution NMR spectra could be obtained at only a single orientation of the substrate in the magnetic field, this work demonstrates several potential benefits for this new alignment method and this substrate over conventional protein alignment methods.

One of the advantages arises from a high—by 20- to 30-fold—greater thermal conductivity of anodic aluminum oxide when compared to glass ($25 \div 35$ vs. 1.1 W m^{-1} K^{-1}, respectively [26]) This higher conductivity is expected to suppress thermal gradients across the sample. For glass-supported samples these gradients arise from uneven radio frequency heating and heat dissipation of the hydrated lipid bilayers. This problem becomes more severe at high magnetic fields with higher NMR proton frequency. Also, the diameter of the nanopores is smaller than the distance between the glass strips and this decreases thermal gradients even further.

Another important advantage of the nanoporous AAO substrate is its excellent accessibility to the bilayer surface for exposure to solute molecules. This makes it possible to expose membrane protein samples to different solution media and to repeat this procedure multiple times with the sample. In particular, the kinetics of hydrogen–deuterium exchange may be able to be followed with much higher temporal resolution using the AAO substrates. In addition, fully hydrated membrane proteins could be exposed, for example, to buffers at various pH, ion, and drug concentrations facilitating a wider range of structural and functional studies in the native-like environment by means of solid-state NMR. Furthermore, as little as a single lipid bilayer could be stabilized inside the nanopore and diameter of this nanopore could be varied from ca. 10 to 200 nm providing a unique opportunity to study such structures by high resolution solid-state NMR.

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