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Substrate-Supported Lipid Nanotube Arrays

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Substrate-supported phospholipid bilayers are of interest for many reasons. First, these bilayers represent a convenient and versatile model of cellular membranes. Starting with the studies of McConnell and co-workers,[1,2] it is now well documented that phospholipid vesicles fuse spontaneously into planar membranes when incubated on treated surfaces.[3,4] In such planar assemblies, the lipids are mobile as in vesicles[4] and are suitable for incorporation of transmembrane proteins.[2] Second, many inorganic substrates can be functionalized by self-assembling lipid bilayers on the surfaces.[3] This substrate biofunctionalization is considered by many authors as one of the most attractive ways for building hybrid nanoscale devices and combinatorial assays to screen protein and phospholipid libraries for specific membrane−protein interactions.[3,4] Recently, Boxer and co-workers described spatially addressable libraries of chemically distinct phospholipids that can be used for such a screening and also for studying the mechanisms of such important intracellular processes as protein trafficking and cell activation.[4,5] Another potential use of the phospholipid arrays is for patterning of membrane proteins that would not require covalent attachment of proteins to the surface.

While several different approaches for building substrate-supported membranes are described in the literature,[3,4,6] essentially all of them are based on a planar design in which phospholipids are patterned/deposited on essentially flat surfaces. Planar bilayers can be assembled by covalently attaching the inner monolayer to the substrate. These substrate-supported bilayers are very stable but lack some phospholipid mobility that can be achieved when the entire bilayer is suspended from the substrate on an ultrathin 5−15 Å water and/or polymer layer. The latter planar bilayer exhibits a greater resemblance to cellular membranes.[4] In addition, suspended bilayers are more suitable for incorporation of membrane peptides and proteins because there is less steric congestion on the substrate side than for covalently attached bilayers. Spanning planar bilayers over the outside surface of anodically etched porous alumina might be helpful for decreasing the congestion for those regions of the bilayer that lay above the pores.[6]

Although planar phospholipid membranes are ideally suited for surface spectroscopy and imaging,[4,5,7] this technology has some limitations for building robust arrays of biosensors. Particularly, the planar lipid assemblies and protein-on-a-chip devices are very fragile because the entire surface of the chip is exposed to the environment. Even any minor mechanical perturbation or contamination of the surface such as, for example, an accidental touching or scratching, would be of a catastrophic consequence to the fragile phospholipid assembly. Also, to maintain the phospholipid order, the surface of such an array should be kept hydrated, and special care should be taken to keep this type of biochip from drying. In addition, the maximum number of lipid molecules and membrane proteins that could be deposited on such a chip is limited by the area of the planar supporting surface minus the total area of barriers.

Here, we report on an alternative approach to building substrate-supported lipid bilayers by self-assembling cylindrical phospholipid structures inside the nanopores. Initial experiments were carried out with nanoporous anodic aluminum oxide (AAO) disks because this well-studied material exhibits an aligned through-film porous structure that is suitable for designing vectorial transport assemblies.[8] The AAO pores are macroscopically homogeneous and hexagonally packed with the pore diameter tunable from ca. 4 to 200 nm.[9]

To characterize the structure of the phospholipids inside the nanopores, we have chosen spin-labeling EPR because this method is highly sensitive to the bilayer structure, can be used to study opaque and nontransparent materials, and causes minimal bilayer perturbation.[10] The method is based on labeling of a lipid molecule with a nitroxide, the spectrum of which reports on local dynamics, magnetic interactions, and molecular orientations.[10] This sensitivity of EPR to the orientation of a spin-bearing nitroxide moiety in the magnetic field is further enhanced at higher magnetic fields (>3 T, HF EPR), as illustrated in Figure 1, with an example of rigid-limit spectra from spin-labeled bilayers. At these fields, the nitroxide EPR spectra are dominated by *g*-factor anisotropy, making the contributions from all three orientations of the principal axes in the magnetic field easily identifiable.

DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was labeled with 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (5PC, both from Avanti Polar Lipids) in a 100:1 molar ratio as described previously.[11] Clean AAO disks (Whatman) were heated to ca. 650 K under <0.1 Torr vacuum to eliminate strong background EPR signal(s) presumably due to defects in the alumina. This treatment did not affect the average AAO pore diameter of 100 nm verified by SEM (Figure 2, top right). Lipids were deposited by exposing treated AAO disks from one side to a 20% phospholipid aqueous dispersion. After the deposition, an excess of lipid from the surface was carefully removed with Kimwipes EX-L. The samples, which were maintained fully hydrated, were characterized with conventional (X-band, 9.5 GHz) and high-field (95 GHz) EPR spectroscopy at various orientations of the supporting AAO chip in the magnetic field. Experimental details are given in the Supporting Information.

The lipids in fluid bilayers undergo a complex anisotropic motion including fast rotations around the long axis, lateral diffusion, and flip-flop. The local dynamics of the lipid fatty acid chains is varied across the bilayer and can be characterized by the local order parameter *S* which is known to decrease progressively toward the center from ca. 0.7 at the C-5 position to 0.1−0.2 at C-16.[12] Thus, the lipid with a nitroxide label at the position 5, 5PC, would be more informative for studying alignment of the self-assembled lipid structures. Dynamic lipid disorder and partial averaging of spectral anisotropies can be further reduced by taking EPR spectra at low

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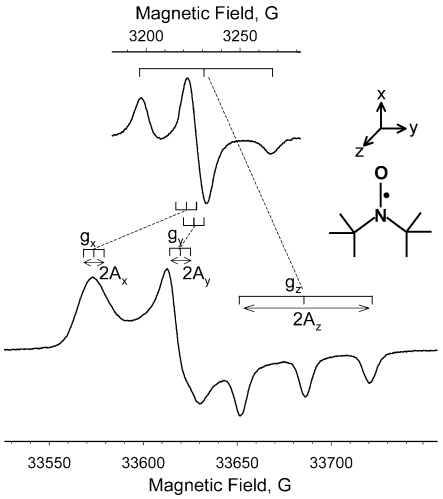

Figure 1. Comparative rigid-limit ($T = 100$ K) experimental EPR spectra of randomly dispersed DMPC:5PC (100:1 molar ratio) bilayers at 9.0 GHz (top) and 94.4 GHz (bottom) and orientation of the nitroxide magnetic axes with respect to the molecular frame.

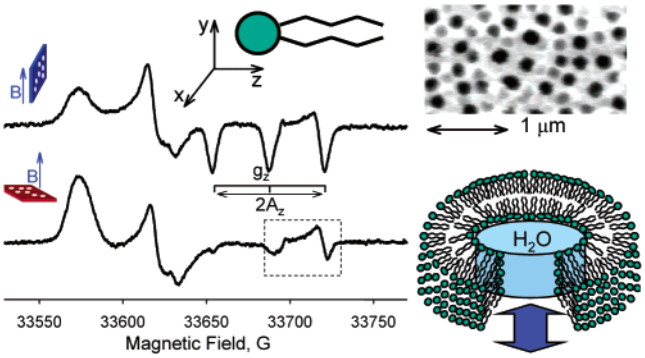

Figure 2. Left: Experimental rigid-limit ($T = 150$ K) high-resolution 94.4 GHz (W-band) EPR spectra of AAO substrate with deposited DMPC:5PC (100:1) at two orientations of the substrate surface in the magnetic field. A cartoon on the top shows orientations of the magnetic axes with respect to the phospholipid. Note that the bottom EPR spectrum has a low intensity in the g_z-region (the feature in the dashed box is due to a paramagnetic AAO impurity), indicating that at this substrate orientation the lipid chains are perpendicular to the magnetic field. Right: SEM of the substrate after heat treatment and a cartoon of a lipid nanotube formed inside the substrate nanopores.

temperature so the dynamics of the phospholipids is approaching the rigid limit (150 K).

The largest changes in 5PC spectra upon reorientation of the AAO substrate in the magnetic field were observed at 95 GHz (W-band) because of an enhanced angular resolution of HF EPR over conventional X-band. The relative intensities of characteristic peaks of these spectra, Figure 2 (left), are clearly different from those of randomly dispersed vesicles shown in Figure 1 (bottom). These changes in the relative intensities are indicative of lipid alignment. Particularly noticeable are the changes in the g_z-region (i.e., high-field component which spreads from ca. 33 850 to 33 940 G): when the surface of the AAO substrate is perpendicular to the magnetic field (bottom spectrum), the *z*-component almost completely disappears (the signals inside the dashed box are mainly due to paramagnetic impurities in the AAO substrate). This means that at this substrate orientation only a very small fraction of molecules have the *z*-axis of the N−O frame aligned with the external magnetic field. The orientation of the nitroxide magnetic axes of 5PC is such that the *z*-axis is approximately aligned along the phospholipid chain (Figure 2, cartoon in the right top corner). Thus, it must be concluded that a majority of the phospholipids inside the nanopores (because the surface phospholipids were mechanically removed during sample preparation) are positioned with their long axis perpendicular to the magnetic field and therefore perpendicular to the direction of pores. The AAO surface is known to be hydrophilic,[13] and, therefore, in fully hydrated samples the lipids are organized in bilayers rather than in monolayers. The bilayer lipid organization was further confirmed by experiments with the lipids labeled at the end of the acyl chain (16PC): these samples produced EPR spectra essentially identical to those from unsupported liposomes (not shown). This is consistent with a lipid nanotube geometry shown in Figure 2 (bottom right). The static order parameter of lipids in such a nanotube was determined by a "center of gravity" method[14] and was found to be exceptionally high, $S_{static} \approx 0.9$.

Room-temperature X- and W-band spectra of the AAO:DMPC:5PC (not shown) were also found to be orientation-dependent but to a lesser degree. The latter indicated that the lipids inside the AAO nanopores remained mobile. Moreover, the local dynamic order parameters deduced from X-band EPR spectra were within 5% of those for aqueous DMPC liposomes. This indicates that the mobility of the lipids in the nanotubes we describe is very close to that of unsupported bilayers.

Overall, we have demonstrated that lipid bilayers can self-assemble into lipid nanotubes inside the nanoporous AAO substrate. We propose that substrate-supported lipid nanotube arrays have potential for building robust biochips and biosensors in which rigid nanoporous substrates protect the bilayer surface from contamination. The total bilayer surface in the lipid nanotube arrays is much greater than that in the planar substrate-supported membranes. The lipid nanotube arrays seem to be suitable for developing patterned lipid deposition and could be potentially used for patterning of membrane-associated molecules.

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Supporting Information Available: Experimental procedures and room-temperature experimental spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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