Pore-Spanning Lipid Bilayers Visualized by Scanning Force Microscopy

Christian Hennesthal and Claudia Steinem*

Institut für Biochemie, Westfälische Wilhelms-Universität Wilhelm-Klemm-Strasse 2, 48149 Münster, Germany

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Suspended bilayers, a new class of artificial membrane systems, which bridge the gap between solid supported membranes (SSMs) and black lipid membranes (BLMs), have been developed on the basis of bilayers spanning the pores of porous alumina substrates. SSMs are among the most versatile mimics of biological membranes[1] and can be assayed by such sensitive surface analysis tools as scanning probe microscopy, ATR-infrared-, surface plasmon resonance spectroscopy or ellipsometry. Conventional methods for lipid immobilization exploit gold-thiol, capping of OH-groups by silanes, and electrostatic interactions. The resulting bilayers exhibit long-term stability, but their close surface proximity (typically 0.2−2 nm) limits lateral lipid mobility and incorporation of large transmembrane proteins or the establishment of chemical and electrochemical transmembrane gradients.[2,3] Such limitations preclude study of such critical biological systems as ion pumps, and ligand- or voltage-gated ion channels, which each require a membrane-surface separation of at least 5−10 nm. Several approaches to extending bilayer surface distances have been employed, including lipids with long hydrophilic spacers,[4] polymer cushions between substrate and membrane[1,5] and patterns with varied thiol-components that increase lateral mobility and free volume.[6] In contrast to SSMs, black lipid membranes span narrow apertures in partitions between two solutions. Although this eliminates the steric congestion, the lack of solid support limits bilayer stability.

Herein we report on an alternative solution to the problem of close membrane-surface contact and concomitant limited lateral lipid mobility. Bilayers immobilized on porous surfaces combine regions anchored to the surface that resemble SSMs and pore-spanning regions similar to free-standing BLMs. Where the membrane covers surface pores, femtoliter-sized compartments underneath provide free volume and ensure free ion flux across the bilayer (Figure 1A).

Extensive study of anodically etched porous alumina over the last five decades makes it an ideal mesoporous material for development of new bilayers.[7,8] In particular, pore sizes are readily adjustable by the applied cell voltage using either sulfuric, oxalic, or phosphoric acid.[9] In this study, porous alumina surfaces were obtained from aluminum foils of high purity (99.999%, 0.5 mm thick, Goodfellows), which were tempered for 3 h at 500 °C in air. The foils were then anodically electropolished in a sulfuric/phosphoric acid mixture at 20 V and 80 °C until a well-polished surface was achieved. The desired pore diameter of approximately 50 nm was accomplished by an anodic electrochemical etch at 40 V in 0.3 M oxalic acid at 2 °C for 6 h followed by a chemical etch to widen the pores in 0.3 M oxalic acid at 30 °C for another 3 h. Surfaces of the porous alumina layers were characterized by scanning electron microscopy (SEM) after evaporation of a 10 nm gold layer (Figure 1B) and scanning force microscopy (SFM) (see Supporting Information). A homogeneous distribution of pores with an average pore diameter of 60 ± 10 nm was obtained. Surface porosity of the substrates was determined to be 16 ± 2% as deduced from pixel analysis.[10]

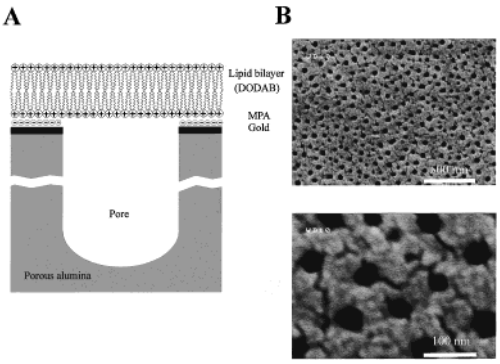

Figure 1. (A) Schematic representation of a pore, anodically etched into an aluminum foil. By covering the porous alumina with a thin gold layer, a self-assembled monolayer of 3-mercaptopropionic acid (MPA) can be deposited to functionalize the upper surface while the inner rims of the pores remain unfunctionalized. By spreading positively charged vesicles of *N*,*N*-dimethyl-*N*,*N*-dioctadecylammonium bromide (DODAB) onto the negatively charged MPA monolayer a lipid bilayer is suspended across the pore. (B) SEM images of a porous alumina surface etched at 40 V in 0.3 M oxalic acid at 2 °C for 6 h followed by a chemical widening of the pores for 3 h (0.3 M oxalic acid, 30 °C). Gold (10 nm) was evaporated on the upper surface.

To ensure that planar bilayers are formed that span the holes of the porous material instead of covering entire pores we separately modified the upper surface of the porous alumina by evaporating a 10 nm gold layer on top of the substrate. Functionalization of the gold surface was achieved by incubating it in 3-mercaptopropionic acid (MPA, 1 mM solution in H_2O), which chemisorbs on gold via self-assembly of the thiol group. Prior to incubation the substrate was exposed to an argon plasma for 5 min. At slightly basic pH (10 mM Tris, pH 8.0) the surface is negatively charged, allowing adsorption of positively charged lipids such as *N*,*N*-dimethyl-*N*,*N*-dioctadecylammonium bromide (DODAB) via electrostatic interactions.[11] We followed the strategy of fusing vesicles on the negatively charged surface instead of detergent micelles since vesicles, which are larger than the mean diameter of the pores, are excluded from the holes. Moreover, in contrast to other self-assembly techniques using a first hydrophobic monolayer chemisorbed on gold, this method utilizes a hydrophilic (negatively charged) surface forcing the spreading of unilamellar vesicles on the surface, which will lead to the formation of a lipid bilayer in one step. Fusion of DODAB vesicles[12] on MPA monolayers occurred within 1−2 h leading to bilayer structures as supported by in situ SFM studies and

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(10) Without chemically widening the pore diameters appear to be only ∼20 nm on top of the surface with a surface porosity of merely 9 ± 1%.
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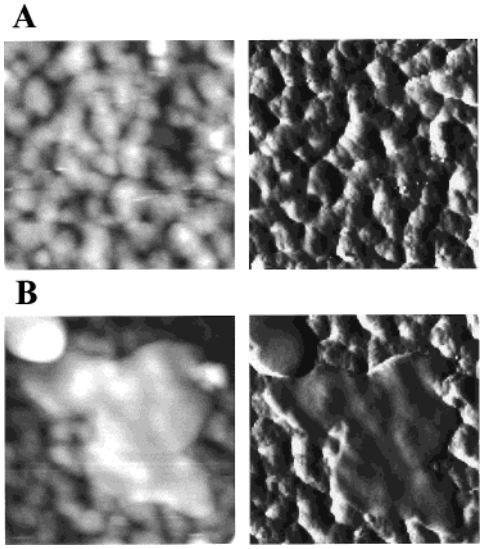

Figure 2. (A) SFM images of a porous alumina surface covered with a 10 nm gold layer in buffer. (B) SFM images of a porous alumina surface covered with a 10 nm gold layer after spreading of positively charged DODAB vesicles to form planar bilayers on a negatively charged self-assembled MPA monolayer. All images are 1×1 μm^2. At left, topographic images with a height scale of 50 nm are displayed; at right, deflection images with a scale of 10 nm are shown.

impedance analysis on gold evaporated on ordinary glass slides (see Supporting Information).

To visualize lipid bilayers on porous alumina surfaces and prove whether they are suspended across the holes of the substrate we performed SFM using contact mode in buffer solution (Si_3N_4 tips, spring constant ~0.02 N/m, Nanosensors). Figure 2A shows SFM-images (topography and deflection) of a porous alumina surface covered with 10 nm gold in buffer before spreading of vesicles. Due to a convolution between the tetrahedral SFM tip and the sample the pores are less clearly discernible compared to the SEM images, and their size appears reduced to an average diameter of 50 ± 10 nm. After incubating the surface with DODAB vesicles (1 mg/mL) for 2 h at room temperature and rinsing thoroughly with buffer, structures are formed as shown in Figure 2B, indicating the formation of lipid bilayers.

These bilayer patches cover the porous material so that the channels are no longer visible, indicating that the lipid membranes span the holes. Surface roughness analyses of various 200×200 nm^2 areas were performed, leading to a mean surface roughness of 4.2 ± 0.9 nm on the porous surface and a considerably smaller roughness of 1.6 ± 0.2 nm on the bilayer patches. The bilayers could be mechanically manipulated by SFM if a high load force was applied, resulting in a reversible indentation of the lipid membrane into the channels (see Supporting Information).

The estimation of the average height difference between the top of the bilayer and the porous surface was cumbersome due to the roughness of the surface. We performed depth analysis of different areas before and after fusion of DODAB vesicles on at least 10 spots. Before vesicle spreading, depth analysis of the surface displays only a single height distribution. Surfaces partly

(12) Unilamellar vesicles were prepared from multilamellar vesicles according to the extrusion method using polycarbonate membranes with nominal pore diameters of 100 nm.

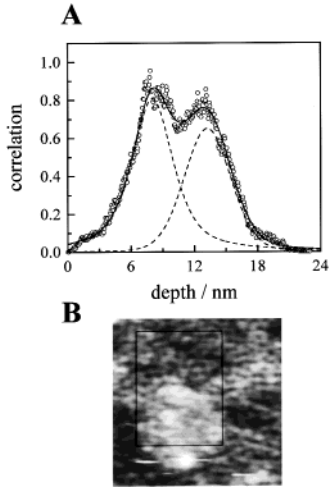

Figure 3. (A) Height analysis of a DODAB bilayer immobilized on a pre-functionalized porous alumina support. Two well-separated height distributions are decomposed by fitting mixed Lorentzian/Gaussian functions to the data. The sum and the component functions are shown as solid and broken lines, respectively. The difference between the peak maximums is 5.4 nm. (B**)** SFM image used for depth analysis (image size: 1×1 μm^2). The rectangle marks the area that was analyzed.

covered with planar bilayer patches, however, show two separated height distributions (Figure 3). Peak decomposition was accomplished by fitting mixed Lorentzian/Gaussian functions to the data. The difference between the peak maximums was 5.8 ± 0.8 on average indicating that planar DODAB bilayers were formed. The height is similar to that obtained from DODAB bilayers immobilized on mica (see Supporting Information).

The present work describes a first step toward the realization of a new kind of artificial lipid membrane system. The channels of porous alumina are utilized as a support for small nanometric BLMs and provide the required free volume between substrate and lipid bilayer as well as a second compartment accessible to sensitive surface analysis tools. The size of the confined compartment can be tuned to simulate cellular dimensions. Whether these nanometric BLMs will allow the establishment of electro-/chemical gradients across the spanning membrane remains to be elucidated. Experiments performed by Nollert et al.[2] indicated that solid supported lipid membranes immobilized on glass surfaces are permeable to small molecules such as dithionite. With BLMs, however, ionic gradients can be established and maintained. In general, suspended bilayers may provide a versatile method for the increasing needs of nanoscopic applications.

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Supporting Information Available: SFM images and height analysis of DODAB bilayers immobilized on mica surfaces, on gold surfaces evaporated on glass slides and on porous alumina surfaces along with a sequence of SFM images showing the impact of load force on the suspended bilayers as well as impedance spectra of the MPA-DODAB system on an evaporated gold surface (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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