Supported Lipid Bilayer Membranes for Water Purification by Reverse Osmosis

Yair Kaufman,† Amir Berman,‡ and Viatcheslav Freger*†

†Zuckerberg Institute for Water Research—ZIWR, The Jacob Blaustein Institutes for desert Research, Environmental engineering unit, Ben-Gurion University of the Negev, Sede Boqer Campus, Israel, and
‡Biotechnology engineering department, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Received November 21, 2009. Revised Manuscript Received December 23, 2009

Some biological plasma membranes pass water with a permeability and selectivity largely exceeding those of commercial membranes for water desalination using specialized trans-membrane proteins aquaporins. However, highly selective transport of water through aquaporins is usually driven by an osmotic rather mechanical pressure, which is not as attractive from the engineering point of view. The feasibility of adopting biomimetic membranes for water purification driven by a mechanical pressure, i.e., filtration is explored in this paper. Toward this goal, it is proposed to use a commercial nanofiltration (NF) membrane as a support for biomimetic lipid bilayer membranes to render them robust enough to withstand the required pressures. It is shown in this paper for the first time that by properly tuning molecular interactions supported phospholipid bilayers (SPB) can be prepared on a commercial NF membrane. The presence of SPB on the surface was verified and quantified by several spectroscopic and microscopic techniques, which showed morphology close to the desired one with very few defects. As an ultimate test it is shown that hydraulic permeability of the SPB supported on the NF membrane (NTR-7450) approaches the values deduced from the typical osmotic permeabilities of intact continuous bilayers. This permeability was unaffected by the trans-membrane flow of water and by repeatedly releasing and reapplying a 10 bar pressure. Along with a parallel demonstration that aquaporins could be incorporated in a similar bilayer on mica, this demonstrates the feasibility of the proposed approach. The prepared SPB structure may be used as a platform for preparing biomimetic filtration membranes with superior performance based on aquaporins. The concept of SPBs on permeable substrates of the present type may also be useful in the future for studying transport of various molecules through trans-membrane proteins.

1. Introduction

Presently, the most economic way to desalinate water is by a reverse osmosis process, whereby water is selectively passed through a semipermeable polymeric membranes using mechanical pressure as a driving force. Despite the significant progress in development of polymeric membranes, recent reports show that there is room for major improvements through incorporation of nanostructural and bioinspired elements with unique characteristics, such as aligned carbon nanotubes,1–3 block copolymers,4 or proteins.5 The expected improvements, however, mostly concern permeability, whereas comparison with biological membranes shows that the latter are far superior compared to polymeric ones, both in terms of permeability and, in particular, selectivity.6 Water transport in biological membranes is carried out via highly selective membrane proteins aquaporins. Aquaporins are a group of trans-membrane proteins (∼120 kDa large) that are ubiquitously expressed in animals and plants cells and allow selective transport of water molecules and rejection of all ions.7 A single aquaporin is a tetramer, made of 4 equal units, often referred to as channels. Reported osmotic permeability of a single water channel is in the range $6 \times 10^{-14}$ to $24 \times 10^{-14}$ cm$^3$ s$^{-1}$, corresponding to $2 \times 10^9$ to $8 \times 10^9$ water molecules per second. It may then be expected that a phospholipids bilayer with ~75% surface area occupied by aquaporins, corresponding to lipid to protein ratio (LPR) smaller than 50, may have a hydraulic permeability as high as $2.5 \times 10^{-11}$ m s$^{-1}$ Pa$^{-1}$, which by an order of magnitude exceeds the permeability of typical reverse osmosis (RO) membranes along with much higher selectivity. Apart from complete rejection of ions, different aquaporins selectively reject many different solutes, such as urea, that readily pass polymeric membranes.9 Utilizing the superior performance of biological membranes and, in particular, aquaporins for water purification is therefore highly attractive from a technical point of view, yet it presents a serious scientific and technological challenge. This article describes a part of the ongoing effort by our group toward meeting this goal.

Biological membranes usually rely on the concentration-dependent, i.e., osmotic part of the chemical potential, to create a driving force for water transport. On the other hand, in industrial or laboratory applications the use of hydraulic (mechanical) pressure as a driving force for filtration is easier and more efficient. The ultimate challenge of the current study is to design and prepare an assembly that will allow artificial biomimetic membranes with embedded aquaporin proteins to sustain hydraulic water pressure gradients without losing its integrity and performance. The approach explored in this study was to place the selective protein/lipid layer on a mechanically robust water permeable substrate.

The approach of supporting the selective thin film is widely employed in commercial composite RO membranes; however, its realization for biological-like lipid membranes or analogous lipid films poses a few serious challenges. First, no use of water-permeable substrates for lipid layer deposition has been reported so far. Vesicle fusion was reported as a possible preparation route for forming supported phospholipid bilayer (SPB) on solid substrates such as mica, silicon, silica, etc. These inorganic substrates are well-defined, rigid, smooth, and nonfluorescent, and they are benchmark substrates in fundamental studies. However, their impermeability renders them unsuitable for the present purpose. On the other hand, the conventional supports for RO membranes are made of a porous polymer that is water-permeable. While they support well the rigid polymer top-layer of RO membranes, they cannot stop lipids and proteins. Therefore, for “soft” not-covalently bonded substrates in fundamental studies. However, their impermeability renders them unsuitable for the present purpose. The new approach introduced and explored here is to support the bilayer with a dense water-permeable film. It is proposed to use a commercial nanofiltration (NF) membrane for this purpose for the following reasons: (1) NF composite membranes are identical in their structure to RO membranes, yet their water permeability is higher and commensurable with biological membranes; still they totally retain proteins and, to a large extent, phospholipids. This guarantees that bilayer attachment to the support be visualized and quantified from the molecular up to the macroscopic scale relevant to filtration. The results reported below have employed a range of characterization techniques in order to get the full picture of the state of the lipid layer at the membrane surface. This was mostly focused on conditions ensuring most efficient coverage of the supporting NF membrane with a lipid bilayer, whereas incorporation of aquaporins into such supported lipid films will be subject of a separate report.

2. Experimental Section

2.1. Materials and Solutions. Lipids: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Sigma-Aldrich. 1,2-Dimyristoyl-3-trimethylammonium propane (DMTAP) (Fluka) and mixed with DMPC to give a mixture of 1.5 mM DMPC and 1,2-dimyristoyl-sn-glycero-phosphoethanolamine-N-(lissamine-Rhodamine B sulfonyl) (ammonium salt), Rh-PE, were purchased from Avanti-Polar lipids. The chemical structure of these lipids is shown in Figure 1.

NF membranes: flat sheet samples of NF-270 membrane (Dow-Filmtec) and NTR-7450 membrane (Hydranautics/Nitto Denko) were kindly supplied by the manufacturers. The top layer of NF-270 is composed of polyamide and that of NTR-7450 composed of sulfonated polyether-sulfone.

Aquaporin solution: (protein PM28: integral protein of spinach leaf plasma membrane) 10 mM potassium phosphate buffer (pH 7.5), 150 mM NaCl, 10% (vol.) glycerol, 1% (w/v) 1-n-octyl-β-D-glucopyranoside (OG, purchased from A. G. Scientific) and 8.41 mg/mL protein (according to the Bearden test) kindly provided by Professor P. Kjellbom and Dr. U. Johanson from Lund University (Sweden).

MPCTAP solution: DMTAP was dissolved in chloroform (Fluka) and mixed with DMPC to give a mixture of 1.5 mM DMPC with 20% (mol.) DMTAP. The chloroform was evaporated at 40 °C in vacuum for 1 h to ensure complete evaporation. The dry lipid powder was hydrated in aqueous solution of 150 mM NaCl, 20 mM MgCl2, 1 mM Tris (HCl) at pH 7.8; the solution was incubated for 30 min at 40 °C and then extruded 10 times through a polycarbonate track-etched membrane with 100 nm pores (SPI Pore) at 30 °C.

MPC solution: the same as MPCTAP except DMTAP was not added during lipid dissolution in chloroform.

MPCTAP-Rh solution: the same as MPCTAP except 0.5% (mol.) Rh-PE was added during lipid dissolution in chloroform.

NPCTAP-Rh solution: the same as NPCTAP except 0.5% (mol.) Rh-PE was added during lipid dissolution in chloroform.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

2.2. Methods. SPB on mica: freshly cleaved mica substrate of diameter 9.9 mm (Grade V-1 Muscovite SPI) was covered with 50 μL of a respective solution, and equilibrated for 30 min at room temperature followed by filtration. After filtration, the sample was incubated at 40 °C for an additional 60 min, washed with double-distilled water (DDW) and dried under nitrogen for further investigations.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.

Preparation of proteoliposomes: MPCTAP + 20 μL aquaporin solution (lipid/protein ratio (LPR) = 3600) + 1% (w/v) OG. The solution was dialyzed for 2 days in 6–8 kDa molecular weight cutoff dialysis bags (Spectra Por) against 150 mM NaCl + 20 mM MgCl2 aqueous solution at room temperature followed by proteoliposomes extrusion as described.
temperature in air. The samples were gently rinsed with DDW to ensure removal of unbound lipid solution from the surface. The samples were scanned using AFM (Nanoscope 3D Multimode, Veeco) using DNP-S cantilevers (Veeco) with spring constant 0.06 N/m in DDW using tapping mode. The temperature of the sample chamber was controlled and monitored during the scans.

**Force vs Distance Measurements** were carried out by AFM using DNP-S cantilevers. The tip velocity was kept constant (1 μm/sec) during all measurements. The sensitivity was measured on freshly cleaved mica in DDW keeping the laser in the same position during all measurements.

Phospholipids (PL) coverage on NF: NF-270 and NTR-7450 membranes were sonicated in 50% (vol.) ethanol in water for 10 min and then washed for 5 min in DDW. Deposition of a PL layer was carried out by the method of vesicle fusion on the NF membrane. The pH of the solutions was adjusted by addition of HCl or NaOH solutions and then 50 μL of the appropriate solution was used to cover 1 cm² of NF membrane and let equilibrate for 10 min and then washed for 5 min in DDW. Deposition of a PL membrane samples after lipid filtration were dried in vacuum (Pike). For quantifying the amount of lipids in the solutions the calibration data was recorded.

**Fluorescence Images** were acquired using an Axio Imager A1 M upright microscope (Zeiss) equipped with a filter set 20 (Zeiss) (excitation 546/12; beam splitter 560; emission 575–640 nm) and an AxioCam MRM camera (Zeiss) using ×10 objective.

**Attenuated total reflection Fourier transform infrared (ATR–FTIR)** spectra were recorded on a Vertex 70 IR spectrometer (Bruker) equipped with a Miracle ATR attachment with a KRS-5 ATR window element protected with a diamond layer (Pike). For quantifying the amount of lipids in the solutions the spectra were recorded, for solutions, after covering the window with 50 μL of solution or, for supported lipid layers, by pressing a dry substrate with a deposited layer onto the window using a dedicated clamp. The resulting spectra were analyzed using the QUANT 2 tool of the OPUS 6.5 software (Bruker) that is based on a chemometric algorithm.

For quantifying the amount of lipids in solutions, calibration solutions of 4 different known concentrations of lipid were employed. For NTR-7450 membrane samples, the calibration was accomplished by using lipid-covered membrane samples of three known concentrations of the lipid. The samples for calibration were prepared by filtering aqueous feed solutions of 3 different lipid concentrations (5 mL each) through three different NTR-7450 membranes of net filtration area 346 mm² (diameter 21 mm) under pressure 10 bar until the entire solution volume passed through that membrane. The lipid concentration in the feed and permeate solutions was determined using ATR–FTIR. The amount of the deposited lipid per unit membrane surface area or surface concentration was then calculated for each sample by subtracting the amount of lipids in the permeate from that initially present in the feed and relating it to the known membrane area. The membrane samples after lipid filtration were dried in vacuum at 40 °C for 3 h in order to minimize IR absorbance by water and ATR–FTIR spectra of dry membranes were recorded. The chemometric analysis showed a very good linear correlation (R² = 0.99) between IR absorbance and lipid surface concentration of the samples used in calibration. Homogenous surface coverage was verified by blending the lipid in the feed solutions with 0.5% (mol) Rh-PE and examining the membrane surface by fluorescence microscopy (not shown here).

Measurements of surface coverage of identical substrates (NTR-7450) using ATR–FTIR were performed after vesicle fusion, for which purpose 100 μL of appropriate vesicle solution were applied to 1 cm² of NTR7450 for 3 h. Thereafter, the samples were thoroughly rinsed with DDW to ensure absence of free lipid unattached to the membrane surface and dried at 40 °C in vacuum for 3 h. The dried samples were pressed onto the crystal window and the IR absorbance spectra were recorded and converted to surface concentration using the calibration data. The conversion to the number of equivalent phospholipid bilayers was based on the phospholipid (DMPC) headgroup area 0.7 nm² × molecule⁻¹ or 4.75 × 10⁻¹⁰ mol × cm⁻² per equivalent bilayer.

**Fluorescence recovery after photobleaching (FRAP) Measurements** were performed using a confocal laser scanning microscope (CLSM) LSM510 META (Zeiss) with ×63 objective as follows. 50 μL of MPCTAP-Rh were applied on freshly cleaved mica substrate for 30 min and gently rinsed with DDW. 50 μL of NPCTAP-Rh, adjusted to pH 2 with HCl, were applied to NTR-7450 (1 cm² sample) for 3 h, followed by gently rinsing with DDW. A 308 μm large circular spot on the sample was photo-bleached by illuminating with the 561 nm laser beam at full power through the microscope optics. The required time for complete photobleaching was 9.3 s on mica and 32 s on NTR-7450. The excitation wavelength was set to 561 nm and emission intensity was read in the range 593–604 nm, characteristic of Rhodamine B. To follow the recovery, a sequence of images was acquired using an identical pixel exposure time (1.27 μs).

**Flux Measurements** were carried out without stirring at 30 °C using a dead-end filtration cell equipped with a thermal jacket. The sample had a net filtration area of 346 mm² (diameter 21 mm). Prior to measurements, a sample of NTR-7450 membrane was pretreated as follows. First, the permeability to water (DDW) was measured for clean NTR-7450 at a pressure 10 bar. Then the cell was filled with NPCTAP solution at pH 2 (same as in the AFM section) and 3 h were allowed for vesicle formation. Residual dissolved lipids were removed by carefully sucking off 90% of the liquid volume from the cell and refilling it with DDW water. This procedure was repeated five times taking care to keep the membrane wet throughout. After repeated dilution the residual amount of lipid in the solution left in the cell was 2 orders of magnitude smaller than the estimated amount of lipid covering the NF surface, assuming formation of a SPB. The cell was then filled with DDW and pressurized to 10 bar with nitrogen to measure the flux and calculate the hydraulic permeability. After applying the pressure, 10 min were allowed for stabilization and then the flux was measured by continuously collecting and weighing permeate using an analytical balance for 5 min.

### 3. Results and Discussion

#### 3.1. Preparation of SPB with Embedded Aquaporins on Mica

Although the ultimate purpose was to demonstrate formation of SPB on a permeable membrane substrate, it was considered essential to first examine its main aspects on mica as a benchmark substrate for the following reasons: (1) vesicle fusion procedures for SPB preparation on mica are well-known and understood; (2) mica is rigid, atomically smooth and non-fluorescent and hence is ideal for direct observations using AFM, fluorescent microscopy etc.; (3) adhesion of positively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction. Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction. Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction. Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Phospholipids were applied to NTR-7450 membranes of net filtration area 346 mm² (diameter 21 mm). Prior to measurements, a sample of NTR-7450 membrane was pretreated as follows. First, the permeability to water (DDW) was measured for clean NTR-7450 at a pressure 10 bar. Then the cell was filled with NPCTAP solution at pH 2 (same as in the AFM section) and 3 h were allowed for vesicle formation. Residual dissolved lipids were removed by carefully sucking off 90% of the liquid volume from the cell and refilling it with DDW water. This procedure was repeated five times taking care to keep the membrane wet throughout. After repeated dilution the residual amount of lipid in the solution left in the cell was 2 orders of magnitude smaller than the estimated amount of lipid covering the NF surface, assuming formation of a SPB. The cell was then filled with DDW and pressurized to 10 bar with nitrogen to measure the flux and calculate the hydraulic permeability. After applying the pressure, 10 min were allowed for stabilization and then the flux was measured by continuously collecting and weighing permeate using an analytical balance for 5 min.

For quantifying the amount of lipids in solutions, calibration solutions of 4 different known concentrations of lipid were employed. For NTR-7450 membrane samples, the calibration was accomplished by using lipid-covered membrane samples of three known concentrations of the lipid. The samples for calibration were prepared by filtering aqueous feed solutions of 3 different lipid concentrations (5 mL each) through three different NTR-7450 membranes of net filtration area 346 mm² (diameter 21 mm) under pressure 10 bar until the entire solution volume passed through that membrane. The lipid concentration in the feed and permeate solutions was determined using ATR–FTIR. The amount of the deposited lipid per unit membrane surface area or surface concentration was then calculated for each sample by subtracting the amount of lipids in the permeate from that initially present in the feed and relating it to the known membrane area. The membrane samples after lipid filtration were dried in vacuum at 40 °C for 3 h in order to minimize IR absorbance by water and ATR–FTIR spectra of dry membranes were recorded. The chemometric analysis showed a very good linear correlation (R² = 0.99) between IR absorbance and lipid surface concentration of the samples used in calibration. Homogenous surface coverage was verified by blending the lipid in the feed solutions with 0.5% (mol) Rh-PE and examining the membrane surface by fluorescence microscopy (not shown here).

Measurements of surface coverage of identical substrates (NTR-7450) using ATR–FTIR were performed after vesicle fusion, for which purpose 100 μL of appropriate vesicle solution were applied to 1 cm² of NTR7450 for 3 h. Thereafter, the samples were thoroughly rinsed with DDW to ensure absence of free lipid unattached to the membrane surface and dried at 40 °C in vacuum for 3 h. The dried samples were pressed onto the crystal window and the IR absorbance spectra were recorded and converted to surface concentration using the calibration data. The conversion to the number of equivalent phospholipid bilayers was based on the phospholipid (DMPC) headgroup area 0.7 nm² × molecule⁻¹ or 4.75 × 10⁻¹⁰ mol × cm⁻² per equivalent bilayer.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.

Coverage of negatively charged phospholipids on mica is apparently nonspecific and is due to mostly electrostatic interaction.
Figure 2. AFM topography images of two different SPBs on mica. (A) SPB prepared from MPC solution (DMPC only) on mica at 30 °C. The black spots are 4–6 nm deep holes in the lipid bilayer. The height difference between the white and gray area is less than 1 nm. (B) SPB prepared from the proteoliposomes solution (DMPC/DMTAP mixture + aquaporins) at 28 °C. The protruding white spots in B are presumably aquaporins and their aggregates. Inset C shows a magnified image of the designated square area in B (500 × 500 nm², z-scale 10 nm) showing aquaporin aggregates at 28 °C. The largest aggregate marked with an arrow protrudes 1.5 nm from the surface. The brighter and darker colors of the lipid matrix in both images correspond to different phases in SPB.

with supported lipid membranes: (1) presence of pinhole defects in the SPB seen in black in images A and (2) poor dispersion and aggregation of the protein observed in images B and C. The domains of different height on the lipid surface in all images can be assigned to two coexisting phases in the lipid bilayer. Both bilayer phases provide an effective barrier to the nonselective transport bypassing aquaporins therefore their coexistence does not present a problem in terms of filtration.

Parts B and C of Figure 2 show no hole-like defects in the SPB. This was achieved by using the method proposed by Zhang et al., who showed that formation of holes in DMPC SPB may be eliminated by blending DMPC with DMTAP. Zhang et al. postulated that defects in pure DMPC bilayer were promoted by electrostatic repulsion between aligned phospholipid heads. Mixing with a cationic lipid, such as DMTAP, would reduce repulsion and favor a more compact structure without holes, which was indeed observed. Along with 20% (mol.) DMTAP the lipid compositions included embedded aquaporins PM-28 (proteoliposomes solution). As parts B and C of Figure 2 show no defects in the bilayer similar to those observed in Figure 2A, it was concluded that the mechanism proposed by Zhang et al. could operate in the presence of aquaporins just as well.

The observed inclusions in Figure 2, parts B and C, are presumably aggregated aquaporin molecules. The smallest inclusions protruding by about 0.5 nm from the thinner phase of the bilayer, presumably, the liquid lipid phase, are probably single aquaporin molecules, which are known to be about 8 nm large. However, most inclusions are larger, up to 70 nm, and the magnified image in the inset shows that aquaporins aggregate. The largest aggregate in part C protrudes from the surface by 1.5 nm. The typical height of aquaporin is about 6 nm (e.g., AQPZ), which is consistent with aquaporins aggregating laterally rather than “piling up” on top of each other. The propensity of membrane proteins to aggregation is well-known and is often utilized for preparation of two-dimensional protein crystals. Aggregated aquaporins within supported layers often preserve their native configuration and are quite densely packed; therefore, it is unclear whether absence of protein aggregation is as vital for functioning in water filtration as absence of holes in the lipid layer.

3.2. Lipid Bilayer on NF Membranes: Formation and Structure. The study used two different commercial NF membranes, NF-270 and NTR-7450. The former is representative of the majority of commercial NF membranes; its active layer is composed of cross-linked semiaromatic polyamide also containing weakly acidic COO⁻ groups and some amine groups. NTR-7450 has an active layer made of sulfonated polysulfone that contains strongly acidic SO₃⁻ groups. Both membranes are therefore negatively charged at neutral pH, as mica, but NF-270 is amphoteric and its surface charge is reversed as pH is lowered below 4 (see below), whereas NTR-7450 retains its negative charge in a wide pH range. NTR-7450 is somewhat more hydrophobic and is also more open and has a higher cutoff than NF-270, however, the top layer in both membranes is dense and virtually impermeable to lipids and proteins. The NTR-7450 surface is noticeably smoother and more homogeneous than NF-270, yet both are rougher and not as homogeneous as mica hence the characterization of SPB on NF is less straightforward to carry out and interpret. Keeping in mind the nonideality of the membrane surface and large areas necessary for the projected application, characterization of the NF surface at all relevant

(30) Schuring, S.; Ringer, P.; Borgnia, M.; Stahlberg, H.; Muller, D. J.; Agre, P.; Engel, A. EMBO J. 1999, 18, 4981-4987.
3.2.2. Quantitative Assessment of Lipids Coverage: ATR–FTIR. Since fluorescence yields only qualitative indication of coverage, quantitative assessment of the actual amount of DMPC per unit area of NTR-7450 was obtained by ATR–FTIR. The calibration and analysis of the ATR–FTIR spectra using the built-in chemometric routine of the OPUS software (see Experimental Section) utilized the bands at 2925 and 2853 cm⁻¹ attributed to methylene and methyl stretch of the lipids aliphatic tails and 1733 cm⁻¹ attributed to the carbonyl group, as shown in Figure 4.

The analysis indicated that after 3 h of vesicle fusion at pH 2, NTR-7450 became covered with an amount of phospholipids equivalent to about 4 bilayers of phospholipids. Admittedly, the accuracy of these measurements was not high, however, it indicates that surface coverage by lipids was most likely in the range of a few equivalent bilayers. In contrast, the amount of lipids on the NF-270 surface was below the detection limits of the instrument in all conditions; i.e., no noticeable difference from the spectrum of the bare membrane was found after vesicle fusion.


\[
f(t) = \frac{F(t) - F(0)}{F(\infty) - F(0)} = \exp \left( -\frac{2\tau_0}{t} \right) \left[ I_0 \left( \frac{2\tau_0}{t} \right) + I_1 \left( \frac{2\tau_0}{t} \right) \right]
\]

Here, \( F(t) \) is the average emission intensity of the bleached area, \( F(\infty) \) the intensity of the recovered bleached area after a long time (\( t \gg \tau_0 \)), \( F(0) \) is the average intensity immediately after photobleaching, \( I_0 \) and \( I_1 \) are the modified Bessel functions, \( \omega \) the diameter of the bleached circular area, and \( \tau_0 = \omega^2/4D_\text{mica} \). Assuming full recovery, \( F(\infty) \) should be equal to the intensity before bleaching.

The fitting yields the diffusion coefficient for DMPC bilayer on mica \( 2.20 \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \), which is within the range of 3 \( \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \) reported for DMPC bilayers on mica above 24 °C and close to 3.50 \( \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \) for DMPC on glass. The lipid composition usually does not play a major role in varying lateral mobility within the layer, and the present result on mica suggests that the effect of DMTAP on lateral diffusion of Rh-PE within the mixed bilayer is not substantial. On the other hand, on NTR-7450 the diffusivity was found to be 1.60 \( \times 10^{-10} \text{ cm}^2 \text{s}^{-1} \), which is relatively lower than that of DMPC on mica. This indicates that NTR-7450 exhibits a higher lateral mobility than DMPC on mica, as reported by Garcia-Manyes et al.

3.2.2.2. Quantitative Assessment of Lipids Coverage: ATR–FTIR. Since fluorescence yields only qualitative indication of coverage, quantitative assessment of the actual amount of DMPC per unit area of NTR-7450 was obtained by ATR–FTIR. The calibration and analysis of the ATR–FTIR spectra using the built-in chemometric routine of the OPUS software (see Experimental Section) utilized the bands at 2925 and 2853 cm⁻¹ attributed to methylene and methyl stretch of the lipids aliphatic tails and 1733 cm⁻¹ attributed to the carbonyl group, as shown in Figure 4.

The analysis indicated that after 3 h of vesicle fusion at pH 2, NTR-7450 became covered with an amount of phospholipids equivalent to about 4 bilayers of phospholipids. Admittedly, the accuracy of these measurements was not high, however, it indicates that surface coverage by lipids was most likely in the range of a few equivalent bilayers. In contrast, the amount of lipids on the NF-270 surface was below the detection limits of the instrument in all conditions; i.e., no noticeable difference from the spectrum of the bare membrane was found after vesicle fusion.

3.2.3. Lipid Dynamics on NTR-7450 Surface: FRAP. Additional insight into the state of mixed DMPC/DMTAP phospholipids in the immediate proximity of NF membrane surface during vesicle fusion is gained using FRAP. Phospholipids exhibit enhanced lateral diffusion while in a bilayer, whereas in more spatially confined formations, such as vesicles, the lateral diffusion is slower. Figure 5 shows the FRAP kinetics for two supported bilayers, one prepared from vesicles composed of DMPC with 20% (mol.) DMTAP and 0.5% (mol.) Rh-PE on mica at pH 7 and another one on NTR-7450 at pH 2. (Measurements in other conditions and for NF-270 could not be carried out since the fluorescence intensity was insufficient for CLSM.) The time dependence was fitted to the following analytical solution with the lateral diffusion coefficient \( D \) as a parameter.

\[
f(t) = \frac{F(t) - F(0)}{F(\infty) - F(0)} = \exp \left( -\frac{2\tau_0}{t} \right) \left[ I_0 \left( \frac{2\tau_0}{t} \right) + I_1 \left( \frac{2\tau_0}{t} \right) \right]
\]

Here, \( F(t) \) is the average emission intensity of the bleached area, \( F(\infty) \) the intensity of the recovered bleached area after a long time (\( t \gg \tau_0 \)), \( F(0) \) is the average intensity immediately after photobleaching, \( I_0 \) and \( I_1 \) are the modified Bessel functions, \( \omega \) the diameter of the bleached circular area, and \( \tau_0 = \omega^2/4D_\text{mica} \). Assuming full recovery, \( F(\infty) \) should be equal to the intensity before bleaching.

The fitting yields the diffusion coefficient for DMPC bilayer on mica \( 2.20 \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \), which is within the range of 3 \( \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \) reported for DMPC bilayers on mica above 24 °C and close to 3.50 \( \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \) for DMPC on glass. The lipid composition usually does not play a major role in varying lateral mobility within the layer, and the present result on mica suggests that the effect of DMTAP on lateral diffusion of Rh-PE within the mixed bilayer is not substantial. On the other hand, on NTR-7450 the diffusivity was found to be 1.60 \( \times 10^{-10} \text{ cm}^2 \text{s}^{-1} \), which is relatively lower than that of DMPC on mica. This indicates that NTR-7450 exhibits a higher lateral mobility than DMPC on mica, as reported by Garcia-Manyes et al.

3.2.2. Quantitative Assessment of Lipids Coverage: ATR–FTIR. Since fluorescence yields only qualitative indication of coverage, quantitative assessment of the actual amount of DMPC per unit area of NTR-7450 was obtained by ATR–FTIR. The calibration and analysis of the ATR–FTIR spectra using the built-in chemometric routine of the OPUS software (see Experimental Section) utilized the bands at 2925 and 2853 cm⁻¹ attributed to methylene and methyl stretch of the lipids aliphatic tails and 1733 cm⁻¹ attributed to the carbonyl group, as shown in Figure 4.

The analysis indicated that after 3 h of vesicle fusion at pH 2, NTR-7450 became covered with an amount of phospholipids equivalent to about 4 bilayers of phospholipids. Admittedly, the accuracy of these measurements was not high, however, it indicates that surface coverage by lipids was most likely in the range of a few equivalent bilayers. In contrast, the amount of lipids on the NF-270 surface was below the detection limits of the instrument in all conditions; i.e., no noticeable difference from the spectrum of the bare membrane was found after vesicle fusion.

3.2.3. Lipid Dynamics on NTR-7450 Surface: FRAP. Additional insight into the state of mixed DMPC/DMTAP phospholipids in the immediate proximity of NF membrane surface during vesicle fusion is gained using FRAP. Phospholipids exhibit enhanced lateral diffusion while in a bilayer, whereas in more spatially confined formations, such as vesicles, the lateral diffusion is slower. Figure 5 shows the FRAP kinetics for two supported bilayers, one prepared from vesicles composed of DMPC with 20% (mol.) DMTAP and 0.5% (mol.) Rh-PE on mica at pH 7 and another one on NTR-7450 at pH 2. (Measurements in other conditions and for NF-270 could not be carried out since the fluorescence intensity was insufficient for CLSM.) The time dependence was fitted to the following analytical solution with the lateral diffusion coefficient \( D \) as a parameter.

\[
f(t) = \frac{F(t) - F(0)}{F(\infty) - F(0)} = \exp \left( -\frac{2\tau_0}{t} \right) \left[ I_0 \left( \frac{2\tau_0}{t} \right) + I_1 \left( \frac{2\tau_0}{t} \right) \right]
\]

Here, \( F(t) \) is the average emission intensity of the bleached area, \( F(\infty) \) the intensity of the recovered bleached area after a long time (\( t \gg \tau_0 \)), \( F(0) \) is the average intensity immediately after photobleaching, \( I_0 \) and \( I_1 \) are the modified Bessel functions, \( \omega \) the diameter of the bleached circular area, and \( \tau_0 = \omega^2/4D_\text{mica} \). Assuming full recovery, \( F(\infty) \) should be equal to the intensity before bleaching.

The fitting yields the diffusion coefficient for DMPC bilayer on mica \( 2.20 \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \), which is within the range of 3 \( \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \) reported for DMPC bilayers on mica above 24 °C and close to 3.50 \( \times 10^{-8} \text{ cm}^2 \times \text{s}^{-1} \) for DMPC on glass. The lipid composition usually does not play a major role in varying lateral mobility within the layer, and the present result on mica suggests that the effect of DMTAP on lateral diffusion of Rh-PE within the mixed bilayer is not substantial. On the other hand, on NTR-7450 the diffusivity was found to be 1.60 \( \times 10^{-10} \text{ cm}^2 \text{s}^{-1} \), which is relatively lower than that of DMPC on mica. This indicates that NTR-7450 exhibits a higher lateral mobility than DMPC on mica, as reported by Garcia-Manyes et al.
Langmuir 2010, 26(10), 7388–7395

Kaufman et al. Article

much slower than on mica, yet significantly faster than vesicle diffusion on glass, $1 \times 10^{-17}$ cm$^2$ s$^{-1}$. Thus, the reduced diffusivity of lipids on NTR-7450 is likely to point to presence on the surface of a mixture of different formations, e.g., a bilayer and vesicles, possibly including flattened disk-like vesicles or a fragmented multilayer. Some factors could contribute to slowdown of the lateral diffusion, such as the roughness or surface heterogeneity of NTR-7450, which are apparently not very large (see Figure 6A) but exceed those of mica, or some specific non-electrostatic attraction (e.g., hydrophobic) between the NTR-7450 top-layer and the first lipid leaflet. Thus, hydrophobic interactions might strip the phospholipid head groups off the lubricating layer of water present on mica. Even if such interactions could not create energy barriers in the lateral direction sufficient to directly slow down the diffusion, they might affect continuity of the bilayer and thus stabilize the aforementioned confined formations.

3.2.4. Formation and Structure of the Lipid Layer at Nanoscale: AFM. The resolution of AFM allows visualization of vesicle fusion and bilayer formation at nanoscale. In the case of NF-270 no change was observed after vesicle fusion at all examined pH. On the other hand, marked changes were seen for NTR-7450. Figure 6 presents topographic images of its surface in solution of DMPC with 20% DMTAP (NPCTAP) at pH 2 for different elapsed times. They were obtained in the tapping mode under liquid at the same location within a few micrometers drift. Figure 6A displays the NTR-7450 membrane before exposure to lipid solution. In Figure 6B, vesicles are seen to attach to the surface; i.e., the first stage of vesicle fusion occurs. In comparison to vesicle fusion on mica or SiO$_2$ surfaces, this stage seems to be relatively slow on NTR-7450. On SiO$_2$ surface, for instance, vesicle fusion monitored by quartz crystal microbalance takes 5–10 min,$^{42,43}$ while on NTR-7450 it seems to take up to 40 min to cover the surface by vesicles. It was presumed that at this stage the unruptured vesicle on the surface, possibly having a flattened disk-like shape,$^{14}$ may coexist with the phospholipid bilayer that may also still contain some defects (holes).

After 120 min of vesicle fusion, the surface becomes flatter (Figure 6C). It was assumed that the vesicles may have fused and

Figure 4. ATR–FTIR spectra of clean NTR-7450 (light grey line) and NTR-7450 covered with lipids (dark grey line). The inset magnifies the 2925, 2853, and 1733 cm$^{-1}$ bands assigned to the lipids.

Figure 5. FRAP results of DMPC + 0.5% (mol.) Rh-PE on mica and NTR-7450 and model fits. A and B show CLSM fluorescence images on mica and NTR-7450 immediately after bleaching, scale bar 20 μm. The fitted lateral diffusion coefficients were $2.20 \times 10^{-8}$ cm$^2$ s$^{-1}$ on mica and $1.60 \times 10^{-10}$ cm$^2$ s$^{-1}$ on NTR-7450. The diameter of bleached was 308 μm$^2$.

settled on the surface and formed a continuous bilayer. In contrast to vesicle fusion on mica or SiO₂, on NTR-7450 even after 2 h there are still vesicles that did not rupture. This appears to be consistent with the FRAP results that indicate coexistence of a bilayer and vesicles after 3 h of vesicle fusion on NTR-7450 based on the value of the diffusion coefficient.

Figure 6D shows AFM force–distance curves that were measured on freshly cleaved bare mica and on mica and NTR-7450 covered with SPB. The results for bare NTR-7450 (not shown in the figure) are similar to bare mica. However, both surfaces covered with a SPB show a different pattern, whereby the AFM tip seems to puncture the bilayer when the repulsive force exceeds about 5.5 nN. The tip passes a distance of 6–8 nm from the first rise in force when it encounters the soft bilayer and starts penetrating it with a nearly constant force down to the point where it bumps into the rigid mica or polymer surface. This distance well agrees with the thickness of the DMPC/DMTAP bilayer, which indicates that a lipid bilayer was indeed formed on NTR-7450 surface, along with some vesicles that did not coalesce.

3.3. Hydraulic Permeability of Supported Lipid Bilayers.

Hydraulic permeability through the SPB is the key characteristic for its functioning in a desalination membrane, since the bilayer is supposed to effectively block nonselective transport of water and salts that may bypass aquaporin channels. The hydraulic permeability, \( L_p \), the volume flux normalized to the trans-membrane pressure, was measured directly by filtration of pure water through 346 mm² large membrane after vesicle fusion. These measurements represent the largest spatial scale of the employed characterization.
Using eq 2, this yields an equivalent osmotic permeability $P_{w}$ can be converted to the osmotic permeability $P$ and vice versa by using the following relation:

$$P = \frac{RT}{V_w} L_p$$

where $V_w = 18 \text{ mL} \times \text{mol}^{-1}$ is the molar volume of water.

Figure 7 shows that fusion of DMPC vesicles on NF-270 did not change the hydraulic permeability of the membranes significantly, whereas for NTR-7450 it dropped dramatically after vesicle fusion by more than an order of magnitude from $2.9 \times 10^{-11}$ to $2.3 \times 10^{-12} \text{ m s}^{-1} \text{ Pa}^{-1}$ for pure DMPC bilayer and to $8.3 \times 10^{-13} \text{ m s}^{-1} \text{ Pa}^{-1}$ for DMPC/DMTAP lipid mixture. These results are fully consistent with fluorescent images in Figure 3 indicating superior coverage of NTR-7450 and with the fact that addition of DMTAP helps to eliminate defects in the DMPC bilayer. These results are fully consistent with fluorescent images in Figure 3 indicating superior coverage of NTR-7450 and with the fact that addition of DMTAP helps to eliminate defects in the DMPC bilayer. Since the NF membrane and the bilayer on top are hydraulic resistances-in-series, the own permeability of the DMPC/DMTAP layer on NTR-7450 membrane is estimated as $1.3 \times 10^{-12} \text{ cm s}^{-1}$. For comparison, the reported osmotic permeability of lipid bilayers usually varies between $1 \times 10^{-13}$ and $1 \times 10^{-12} \text{ cm s}^{-1}$. The permeability measured here thus falls slightly off this range, which could be due to deviations from the pure diffusive mechanism embedded in eq 2 that ignores possible contribution from a convective transport in the pressure-driven mode.

The water permeability for NTR-7450 covered with a lipid bilayer sustained repeated filtration experiments. Though no surface characterizations were performed thereafter, the stable permeability seems to indicate that the phospholipid layer withstood the hydraulic pressure and was not affected by the water flow and pressure gradient.

4. Conclusions

As a first step toward the assembly of a biomimetic membrane with embedded aquaporins for possible application in water purification, the feasibility of covering a NF membrane with a continuous phospholipid bilayer using the vesicle fusion approach was studied. The comprehensively characterized bilayers and its formation on relatively rough surface of NF membranes a combination of techniques were used to ensure that a bilayer was indeed formed on the surface and fully covered it.

Fluorescence images revealed that tuning electrostatic interactions was crucial for successful vesicle fusion. The best coverage was achieved for the NTR-7450 membrane at pH 2 and low ionic strength, in which NTR-7450 and the vesicles are oppositely charged. ATR-FTIR measurements showed that an amount of DMPC deposited on the NTR-7450 membrane after 3 h of vesicle fusion was of the order of a few equivalent bilayers. This finding and the relatively low value of diffusion coefficient of the lipids on NTR-7450 measured by FRAP suggested that after 3 h of vesicle fusion vesicles still did not fully coalesce and other, confined or multilayer formations could coexist with the bilayer. This conclusion, as well as much slower formation of a bilayer on NTR-7450 surface, as compared to mica, was also consistent with direct imaging by AFM.

The combination of the above results suggests that the continuous lipids bilayer formed under optimal conditions extends over fairly large area and is likely to efficiently cover the surface. This was in agreement with the hydraulic water permeability measurement following vesicle fusion, which showed that the membrane was essentially “sealed” by the lipids and, in the optimal case of NTR-7450 covered with a mixed DMPC-DMTAP bilayer, approached the reported permeability of a lipid bilayer.

Therefore, it was concluded that through appropriate choice of membrane, lipid composition and deposition conditions, it is possible to uniformly cover the surface of a NF membrane with a lipid bilayer with only few defects and, possibly, a small fraction of unruptured vesicles. The optimized system shows promise to become a suitable platform for incorporation of aquaporins for selective filtration. The feasibility of incorporating aquaporins into a bilayer of identical lipid composition was demonstrated on mica and our efforts presently focus on preparation and characterization of a functioning prototype membrane utilizing high permeability and selectivity of aquaporins for water filtration. The concept of SPBs on permeable substrates of the present type may also be useful in the future for studying transport of various molecules through trans-membrane proteins.

Acknowledgment. This work was financially supported by the European Commission, FP6-NMP program MEMBAQ project, contract NMP4-CT-006-33234. The authors are grateful to Per Kjellbom and Urban Johanson (Lund University, Sweden) for supplying aquaporins for this study.