Absence of Ethanol-Induced Interdigitation in Supported Phospholipid Bilayers on Silica Surfaces

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Membranes prepared by the adsorption of phospholipid vesicles on solid supports are much-used model systems in biomedical research. However, there is accumulating evidence that such membranes may not always be equivalent to the free-standing cellular membranes that they are modeling. In the present study, sonicated DOPC/DOPS (80/20 mol %) vesicles were adsorbed on hydrophilic silica surfaces, a system that has been demonstrated to produce confluent bilayers. In addition, pure DOPC and DLPC membranes were studied. It is demonstrated that ethanol-induced membrane interdigitation, as demonstrated for free-standing bilayers, does not occur in these supported membranes.

Introduction

The physiological effects of anesthetics and alcohols have been related to their effects on phospholipid packing in biological membranes. More specifically, it has been shown that alcohols in concentrations of 1−10 vol % may induce almost instantaneous overlap of the acyl chains from opposing monolayers (interdigitation) and increased tilt angles, causing membrane thinning and higher packing density.[1,2] In such interdigitated membranes, alcohol molecules are located in the chain region close to the phospholipid head groups.[3,4]

Membranes deposited on solid supports by the adsorption of unilamellar phospholipid vesicles have been used in many areas of biomedical research such as cell recognition,[5] membrane-mediated catalysis,[6] effects of anesthetics,[7] and antimicrobial peptides.[8] Recently, accumulating evidence has cast doubt on the validity of some of these preparations as cell membrane models (see Discussion), and because of its sensitivity to membrane properties, interdigitation seems to be a well-suited parameter for detecting support-induced membrane changes.

We therefore investigated the effects of ethanol on DOPC/DOPS membranes deposited on silica surfaces, which have previously been shown to consist of confluent bilayers,[9,10] as confirmed in the present study. Results were compared with membranes prepared from pure DOPC and DLPC. Membranes were studied by ellipsometry, a technique that is well suited to the detection of in situ changes in membrane thickness and packing.[9]

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Materials and Methods

Dioleoyl-phosphatidylcholine (DOPC), dioleoyl-phosphatidylserine (DOPS), and dilinoleoyl-phosphatidylcholine (DLPC) were from Avanti Polar Lipids (Alabaster, AL). HEPES buffer (10 mM, pH 7.4) was prepared with pure Milli-Q water (Millipore, Etten-Leur, The Netherlands) and contained 150 mM NaCl and 2 mM $CaCl_2$.

Silicon wafers (Aurel, Landsberg, Germany, n type, phosphorus-doped) were cut into slides of 4.0 × 0.8 cm^2 and thoroughly cleaned with detergent (Sparkleen, Calgon, Pittsburgh, PA) and water, and the silica (SiO_2) surface was made hydrophilic by 20 min of incubation in 30% chromic sulfuric acid (Merck, Darmstadt, Germany) at 80 °C.

Small unilamellar vesicles were prepared from phospholipids solvated in chloroform and dried under nitrogen. The dry films were resuspended in buffer, and the turbid suspensions, which were cooled by water, were sonicated to clarity in 20 min.

Supported membranes were formed by the addition of vesicles (20 μM total lipid) to the ellipsometer cuvette containing the silica surface in buffer. After membrane formation, the cuvette was flushed with 60 mL of pure water to remove remaining vesicles. Thereafter, ethanol was added to final concentrations of 1.6, 3.2, 6.2, and 11.7 vol %, each time followed by flushing with pure water. Experiments were performed at room temperature (21 °C).

Membrane properties were measured by ellipsometry as described.[9] This technique measures the polarization changes of monochromatic light (λ = 632.8 nm) after reflection from the silica surface in a 3 mL cuvette. Coverage of the silica surface with a membrane, as well as structural changes in such membranes, will change the readings of two polarizers (P and A), and from these changes, the optical properties of the reflecting surface can be determined. Results were calculated for an optical three-layer (silicon−membrane−medium) system.[9] Layer 1 is the medium and consisted of buffer, water, or a water/ethanol mixture with refractive index n_1. Layer 2 is the membrane, that is, the water/phospholipid mixture on the reflecting surface with thickness d_2 and refractive index n_2. Layer 3 is the silicon support with (complex) refractive index n_3.

The surface mass Γ of phospholipids was calculated from $\Gamma = 0.1d_2(L(n_2) - L(n_1)/(A/M - \upsilon L(n_1))$, with L defined as $L(n_i) \equiv (n_i^2 - 1)/(n_i^2 + 2)$.[11] If d_2 is expressed in nm, then Γ is obtained in μg/cm^2. A/M is the ratio of molar refractivity to weight, and υ is the specific volume of phospholipids. Values used for A/M and υ were 0.274 and 0.89 cm^3/g, respectively.[11]

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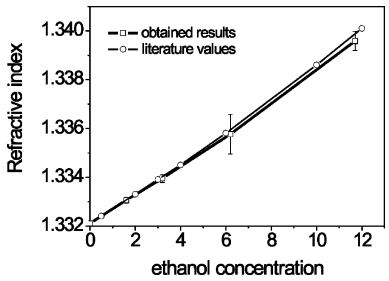

Figure 1. Average values of the refractive indices of water/ethanol mixtures (□) with standard deviations vs ethanol concentration compared with literature values (○). Averaged results of six experiments are shown.

Results

The refractive indices of water/ethanol mixtures were measured as follows. First, the n_3 refractive index of the silica surface was determined from a measurement in pure water with refractive index $n_1 = 1.33211$ at 21 °C.[12] Then ethanol was added, and using the earlier obtained value of n_3, refractive index n_1 of the ethanol/water mixture was determined. Average n_1 values (±SD) obtained for 1.6, 3.2, 6.2, and 11.7 vol % ethanol were 1.33306 ± 0.00012, 1.33396 ± 0.00016, 1.33577 ± 0.00082, and 1.33959 ± 0.00039, respectively. As shown in Figure 1, these results are in agreement with the literature.[12]

Using these n_1 values and the n_3 value obtained from a measurement in buffer ($n = 1.33344$) before vesicles were added, membrane thickness d_2 and refractive index n_2 were determined, and surface mass Γ was calculated. As shown in Figure 2, the effects of ethanol were reversible upon return to pure water. For DOPC/DOPS (80/20 mol %) membranes, vesicle fusion into bilayers was confirmed by a final surface mass of about 0.39 μg/cm^2 and a low thickness of about 6 nm.[9] For DOPC and DLPC, an average thickness of about 18 nm suggests adsorbed vesicles or mixed bilayer/vesicle membranes.

DOPC/DOPS bilayers did not show reduced thickness after the addition of ethanol, indicating that no interdigitation occurred. For DOPC and DLPC, membrane swelling was observed instead of the membrane thinning occurring during interdigiation.

Influence of Errors

Results were highly dependent on n_1 values. Using n_1 values of buffer or water instead of the correct values for ethanol/water mixtures, large spurious increases in surface mass and reductions in thickness were found. It was verified that taking a three-component (phospholipid/water/ethanol) mixture for the membrane instead of a two-component (phospholipid/water) mixture did not influence results up to 1:1 mol/mol ethanol/phospholipid or an ethanol content of 5% of the membrane mass, whereas no change in surface mass was observed upon addition of ethanol and the precision of the measurement is approximately 1% of the membrane mass.[9]

For silicon slides, an overestimation of d_2 will cause an underestimation of n_2 and vice versa.[9] Because Γ depends on the product of d_2 and n_2, the scatter in Γ is smaller than for d_2 and n_2, as shown in Figure 2. In spite of this larger scatter in d_2 and n_2, it was verified that for DOPC and DLPC increases in d_2 and decreases in n_2 after the addition of ethanol were significant for 11.7, 6.2, and 3.2% ethanol with $p < 0.001$ (Wilcoxon, two-sided). For DOPC/DOPS membranes, a small but significant similar effect was observed, but only for the highest ethanol concentration (11.7%).

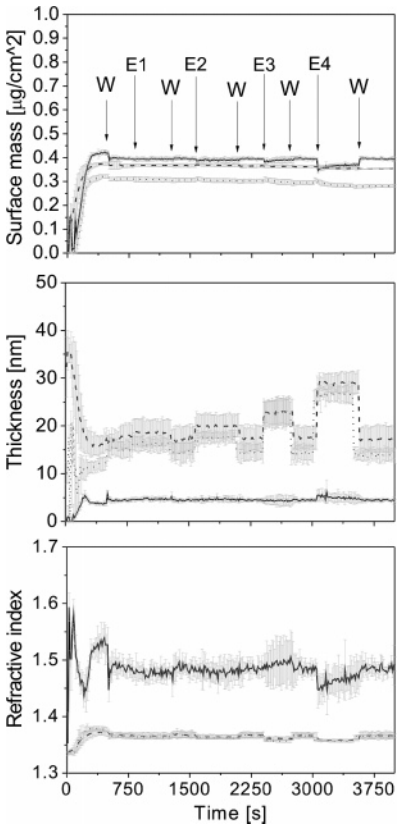

Figure 2. Surface mass, thickness, and refractive index for DOPC/DOPS (−), DOPC (---), and DLPC (···) membranes. Averaged results of six experiments are shown with standard deviations. Time points indicated by E1, E2, E3, and E4 indicate the addition of 1.6, 3.2, 6.2, and 11.7 vol % ethanol, respectively. W indicates flushing and the return to pure water.

Discussion

In the first application of vesicle-deposited supported membranes,[5] the absence of normal lateral mobility of a transmembrane protein was noted. Doubts about the validity of supported membrane models became stronger when it was shown that, depending on the support surfaces and phospholipids used, the resulting membranes often consisted of adsorbed vesicles or mixed vesicle/bilayer layers rather than confluent bilayers.[9,13−15] Recent evidence also indicates that membranes formed on supports, even when fused into confluent bilayers, may differ from free-standing bilayers in vesicles. For instance, PC/PS bilayers formed on mica showed the accumulation of PS in the leaflet facing the support[16] and lateral diffusion in PC bilayers on silica was 2 times slower than in giant vesicles,[1717] which was possibly caused by lipids sticking to the support.[18]

The present study for the first time shows real-time effects of ethanol on supported membranes and demonstrates that in such preparations interdigitation seems to be absent. This result seems

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to be at variance with a few AFM studies that claimed interdigitation in supported membranes.[7,19]

One of these studies[7] used dipalmitoyl-PC (DPPC), which has a high transition temperature of 41.6 °C. As a result, real-time transitions could not be measured, but preparations were heated to 60 °C, with and without ethanol, and measured after cooling down again. This procedure could freeze transitions that would not have occurred in fluid-state phospholipids as found predominantly in biological membranes and used in the present study. Another study[19] showed ethanol-induced changes in DOPC membranes deposited on mica. These membranes did not form confluent bilayers but consisted of isolated bilayer islands, separated by empty space. This absence of confluency may have been caused by the lack of calcium during membrane formation because it has been shown that, for DOPC on mica, calcium is required for vesicle fusion into bilayers.[9]

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