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Andreas Janshoff · Stephanie Künneke

Micropatterned solid-supported membranes formed by micromolding in capillaries

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Abstract The formation of individually addressable micropatterned solid-supported lipid bilayers has been accomplished by means of micromolding in capillaries. Small unilamellar vesicles were spread on glass slides to form planar supported membranes along microscopic capillaries molded as trenches into a polydimethylsiloxane (PDMS) elastomer. PDMS provides an elastic and transparent carrier for microcapillaries molded from silicon wafers displaying the desired inverse trenches. The so-called master structure has been conventionally etched into silicon by photolithography. The cured PDMS elastomer was briefly exposed to an oxygen plasma, rendering the surface hydrophilic, and subsequently attached to a glass surface in order to form hydrophilic capillaries equipped with flow-promoting pads on either side. One flowpad acts as a reservoir to be filled with the vesicle suspension, while the other one serves as a collector to ensure a sufficient capillary flow to cover the substrate completely. Formation of planar lipid bilayers on the glass slide along the capillaries was followed by imaging the flow and spreading of fluorescently labeled DMPC liposomes with confocal laser scanning microscopy. By means of scanning force microscopy in aqueous solution the formed lipid structures were identified and the height of the lipid bilayers was accurately determined. With both techniques, it was shown that the patterned bilayers remain separated and persist for several hours on the substrate in aqueous solution.

Key words Microfluidic networks · Micromolding in capillaries · Scanning force microscopy · Soft lithography · Solid-supported membranes

A. Janshoff (✉) · S. Künneke
Institut für Biochemie,
Westfälische Wilhelms-Universität,
Wilhelm-Klemm-Strasse 2,
48149 Münster, Germany
e-mail: janshof@nwz.uni-muenster.de
Tel.: +49-251-8339765
Fax: +49-251-8333206

Introduction

The formation of individually addressable, patterned biomaterials on surfaces is of paramount interest for the development of biosensors, combinatorial libraries, and high-throughput systems for pharmacological screening (Fodor et al. 1991). Particularly, the combination of high-resolution scanning devices with structured biomolecules on surfaces is advantageous, if the amount of biomaterial is limited or the number of surface reactions is vast. Conceivable applications include the detection of pico- to nanomolar concentrations of bacterial toxins binding to cellular receptor molecules, localization of DNA hybridization, or screening of pharmacological relevant molecules.

As various reactions, i.e. ligand-receptor interactions, are located at biological membranes, solid-supported membranes (SSMs) are a versatile matrix for embedding and immobilizing natural and artificial receptor molecules such as functionalized lipids or proteins (Sackmann 1996; Steinem et al. 1996). As an artificial system imitating biological membranes in their physical and chemical structure, SSMs exhibit extraordinary properties such as lateral mobility, efficient suppression of nonspecific interactions, and high long-term stability (Kalb et al. 1992). Numerous preparation techniques are available to deposit lipid bilayers on surfaces, including fusion of vesicles on hydrophilic surfaces (Brian and McConnell 1984) and techniques based on self-assembly processes of thiols or organosilanes on gold and SiO_2 surfaces, respectively (Florin and Gaub 1993; Plant 1993; Terrettaz et al. 1993). It has been demonstrated that SSMs are well suited to study membrane-confined lipid-protein interactions, such as binding of lectins and toxins to gangliosides (Mou et al. 1995) or the influence of small membrane-active peptides on membrane structure by means of scanning force microscopy (Janshoff et al. 1999). Lipid-protein interactions do not only depend on the receptor type and its concentration within the membrane but are also strongly influenced by lipid

composition, fluidity and elasticity of the membrane, as well as lateral receptor clustering. Generally, systematic studies of these aforementioned aspects are very time and labor consuming and can be ambiguous if suitable controls cannot be realized in a single experiment.

This led us to the idea of forming microstructured membrane compartments, which are individually addressable on a micrometer scale. The membrane segments are designed to be accessible to microscopic techniques and surface analysis tools, allowing investigation of a vast number of different membrane-confined reactions quasi-simultaneously while varying the above-mentioned components on a single chip. In order to immobilize individually addressable membrane structures on a solid support, varying in their composition, we employed microfluidic networks (μFNs) as provided by micromolding in capillaries (MIMIC). This technique has been established by Whitesides and co-workers (Kim et al. 1995, 1996) and been applied by Biebuyck and co-workers (Delamarche et al. 1997, 1998) to form microstructured protein films on surfaces. Figure 1 outlines the principle of forming membrane patterns by MIMIC.

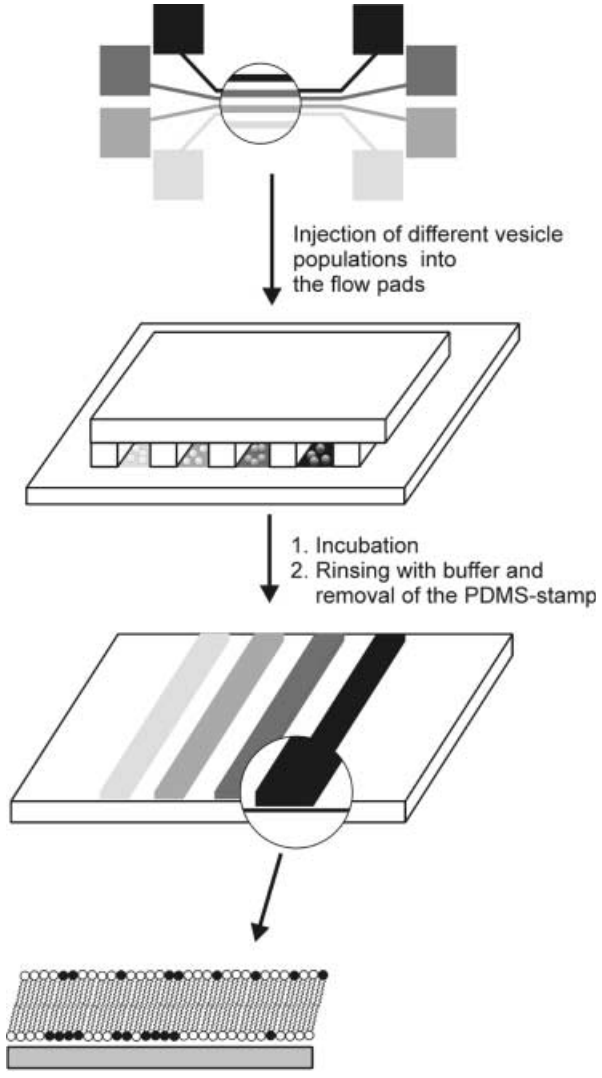

Fig. 1 Schematic outline of the procedure for generating individually addressable patterns of planar lipid bilayers. A suspension of vesicles is added to each of the flow promoting pads on the *left-hand side*. The *colors* indicate different types of receptor molecules in each of the liposome population. The flow pads on the *right-hand side* ensure a sufficient flow of liposomes to cover the substrate completely with solid-supported membranes

Materials and methods

Liposome preparation

Lipids were used as purchased to prepare films of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Avanti Polar Lipids, Alabaster, Ala., USA) doped with 1 mol% Texas Red-DHPE (Molecular Probes, Eugene, Ore., USA). Small unilamellar vesicles (SUVs) of DMPC were prepared as described by Steinem et al. (1996) using the extrusion method (100 nm nominal pore diameter) and subsequent sonification for 5 min by a tip sonifier.

Soft lithography

We developed a general procedure to generate patterned lipid bilayers by using a hydrodynamically coupled network of capillaries. The trenches were molded into a polydimethylsiloxane (PDMS) elastomer using a silicon wafer as the master structure. The silicon wafer displaying the desired inverse structure was obtained from conventional optical lithography of silicon wafers (IMSAS, Bremen, Germany). The wafer was covered by a thin layer of 1*H*,1*H*,2*H*,2*H*-perfluorodecyldimethylchlorosilane (ABCR, Karlsruhe, Germany), rendering the surface hydrophobic in order to facilitate removal of the cured polymer. For replication of the master structure a 10:1 mixture of Sylgard 184 and curing agent was cured at 60 °C for 2 h (Kim et al. 1995). Requirements for the structural design of microfluidic networks include a hydrodynamically coupled network of capillaries and the fulfillment of stability criteria for PDMS elastomers (Xia and Whitesides 1998). A sufficient material flow to compensate for material losses due to adsorption on the capillary walls, and sufficient hydrophilicity of the substrate and the PDMS walls to ensure adhesion and capillary flow, also needs to be considered (Delamarche et al. 1998).

Preparation and investigation of microstructured bilayers

The elastomer was exposed to an oxygen plasma, rendering the surface hydrophilic. Glass substrates were pretreated with H_2O_2/H_2SO_4 (1:3) followed by dipping them into a 1 M KOH solution for 30 s. The pretreatment of the PDMS stamp and glass substrate ensures a strong seal between stamp and substrate. Liposome suspension was applied to one flowpad using a syringe. Confocal laser scanning microscopy was performed using a MRC 600 unit (Bio-Rad, Munich, Germany). Images of solid-supported membranes spread on glass were obtained using a Nanoscope IIIa Bioscope microscope (Digital Instruments, Santa Barbara, Calif., USA) operating in contact mode using probes with a nominal spring constant of 0.06 N/m (Digital Instruments). The accurate spring constant was determined for each cantilever by the thermal noise method. Minimal load force (~200 pN) was employed to reduce the extent of bilayer deformation. Imaging was performed in 10 mM NaCl at a scan velocity of 80 μm/s.

Results and discussion

A simple capillary structure used to prove the concept of addressable membrane compartments is depicted in Fig. 2, displaying an array of several hundred capillaries

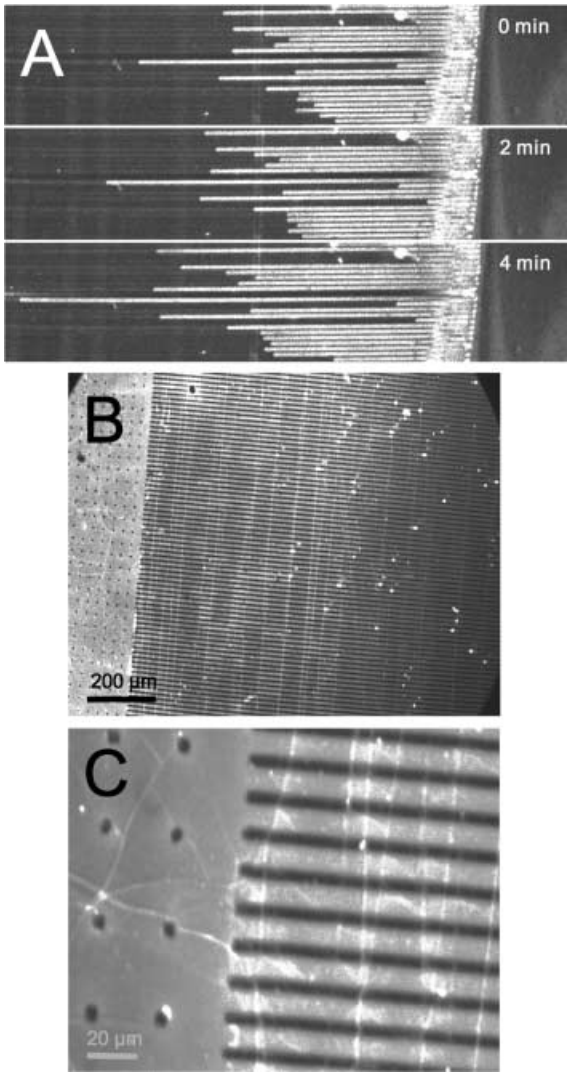

Fig. 2 **A** Fluorescence micrograph snapshots (confocal laser scanning microscopy) of Texas Red-DHPE labeled unilamellar DMPC vesicles ($c = 2$ mg/mL) moving along an array of capillaries. A droplet (6 µL) of vesicles in 10 mM NaCl is placed directly at the starting point of the capillaries on the *right-hand side*. The capillaries are filled with liquid within a few seconds, while the strong fluorescent bands carrying the liposomes travel much slower. The elastomer is maintained on a glass slide by mere adhesion with 300 parallel aligned capillaries, 10 µm in width, 4 µm spaced, and 3 µm in height, ending in a flowpad of 9.5 × 9.5 mm. The flowpad is equipped with regularly spaced 5 × 5 µm posts to prevent the elastomer collapsing and to ensure contact to the substrate. **B** After a few minutes the capillaries are entirely filled with liposomes. **C** is a magnification of **B**

being 3 mm long, 10 µm wide, 3 µm high, and separated by a barrier of 4 µm. Since depletion of lipids by vesicle fusion on the substrate has to be compensated by a large excess of material flowing through the capillaries, an extended flowpad at the end of the capillaries is added to maintain a continuous flow. The pad at the beginning of the capillaries serves as a reservoir to facilitate application of the vesicle suspension by a syringe. Although PDMS is not gastight, the capillary system was kept open to air on both sides to ensure continuous flow, which is solely driven by capillary forces. The liquid flow within the microcapillaries is laminar since it is characterized by a small Reynolds number ($Re < 1$). Pressure and viscosity dominate in this regime, while inertial forces may be neglected. The mean flow velocity, $\mathrm{d}x/\mathrm{d}t$, is directly proportional to the reciprocal length l of the capillary (Delamarche et al. 1998):

$$\frac{\mathrm{d}x}{\mathrm{d}t} = \frac{C_\mathrm{g}}{\eta}\frac{\Delta p}{l} = \frac{\frac{1}{8}\left(\frac{ab}{a+b}\right)^2}{\eta}\frac{P_\mathrm{c} + \Delta p_\mathrm{int}}{l} = \frac{\frac{1}{8}\left(\frac{ab}{a+b}\right)^2}{\eta}\frac{\gamma\left(\frac{\cos(\theta_\mathrm{glass})+\cos(\theta_\mathrm{PDMS})}{a} + \frac{2\cos(\theta_\mathrm{PDMS})}{b}\right)}{l} \quad (1)$$

where C_g/η is the dissipation term, in which C_g comprises the geometric boundaries of the capillary (a is the width and b the height of each capillary) and η is the viscosity of the liquid; P_c is the capillary pressure; θ_glass and θ_PDMS are the contact angles of the liquid with the respective walls of the capillary. The internal pressure drop Δp_int is zero if the capillaries are open at both ends.

Cleaned glass surfaces were used as hydrophilic substrates, while the elastomer was exposed to an oxygen plasma, rendering the polymer hydrophilic due to formation of a thin silicon oxide layer, thus providing strong adhesion on the glass substrate. A good seal between elastomer and surface is a prerequisite to ensure that leakage and material interchange between individual capillaries does not occur. Planar lipid bilayers were deposited by fusing unilamellar vesicles on the hydrophilic glass substrate (Brian and McConnell 1984), which is a rapid process due to the high surface energy of the substrate.

The liposome flow within the capillaries and the formation of planar bilayers was visualized by confocal laser scanning microscopy. Figure 2A shows the flow of DMPC SUVs doped with 1 mol% Texas Red-DHPE through a parallel capillary network. The temperature was kept well above the main phase transition temperature of DMPC (21 °C) to ensure fusion of the vesicles on the substrate. Within the first few seconds (approx. 5–10 s) the capillaries are rapidly filled with liquid, but they merely display a faint red fluorescence. The capillaries start to be filled with a strong fluorescent liquid after about 30 s, indicating the presence of labeled liposomes. Complete filling of the capillaries with the dye-containing vesicles occurs within a few minutes, as demonstrated in Fig. 2. Smaller liposomes travel a longer distance within the capillaries than larger vesicles, thus consuming more time to flow from one end of the capillary to the other. Therefore, it is conceivable that the faint fluorescence arises from the presence of larger vesicles being faster. In some cases, clogging of the capillaries is observed which might be due to the fact that the vesicle size distribution is rather broad and some multilamellar vesicles exceed the opening size of the capillaries. Moreover, different flow velocities were observed, which can be attributed to differences in hydro-

philicity of the channels leading to different contact angles of the liquid with the capillary walls.

After 5–15 min, most of the capillaries and the flowpad exhibit high fluorescence intensity, indicating that the vesicles are homogeneously distributed in the capillary network (Fig. 2B, C). At this stage, patterned planar lipid bilayers are formed and the elastomer is rapidly removed from the substrate, accompanied by vigorous rinsing. The resulting solid-supported bilayers exhibit a homogeneous fluorescence intensity well separated by 4 μm dark spacings without lipids over a millimeter scale, as shown in Fig. 3A. The newly formed lipid bilayers sustain many hours without lateral fusion of the segmented bilayer patches. We anticipate that, once the bilayers are formed, mixing of the lipid compartments does not occur, in accordance to the results obtained by Cremer et al. (1999), who could demonstrate that a mechanical scratch in a bilayer, removing lipids from the surface, results in an efficient diffusion barrier between two bilayer patches.

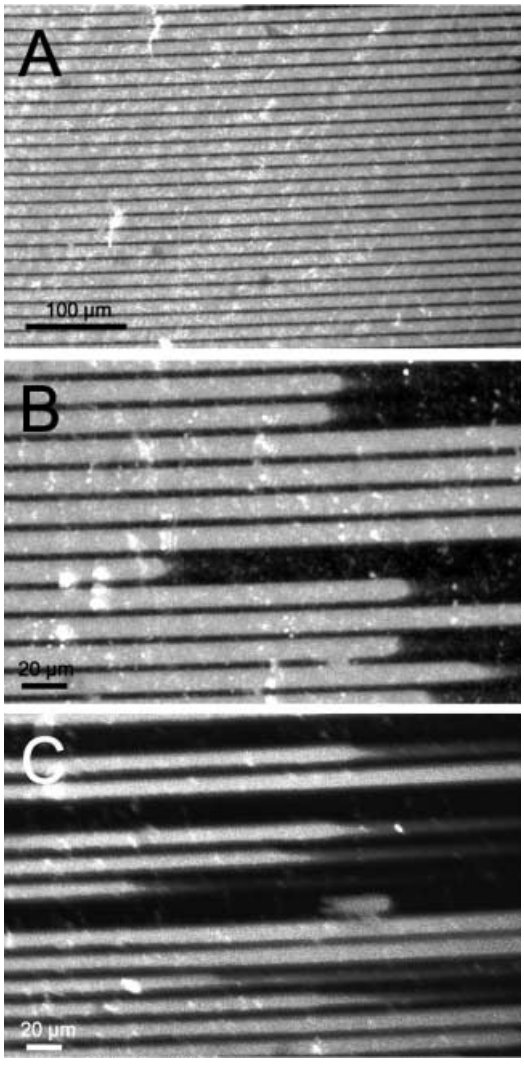

Fig. 3 **A** Fluorescence micrographs of the glass substrate displaying bilayer formation along the capillaries after removal of the elastomer while rinsing with 10 mM NaCl to remove excess liposomes. **B**/**C** Bilayer formation at the end of the capillaries is sometimes incomplete, probably owing to the depletion of lipids in the small volume of the capillaries

Figure 3B and C depict examples of capillaries in which the bilayers were not completely fused on the glass slide. The fluorescence intensity decreases gradually towards the end of the capillary, which is indicative of depletion of lipid material leading to an incomplete formation of bilayers. Small bright areas are characteristic for small bilayer patches in the capillaries formed by spreading of individual liposomes.

In order to confirm that a bilayer is deposited on glass substrates we performed scanning force microscopy (SFM) to identify the structure. Figure 4A and B displays SFM images employing contact mode in liquids, revealing that planar lipid bilayers have indeed been formed on the glass substrate. The average height of the structures is 4.2 ± 0.5 nm as deduced from height analysis (Fig. 4B). Notably, the spacings between the bilayer stripes are slightly smaller (approx. 3 μm) than compared to the original structure given by the PDMS elastomer. This might be due to a partly incomplete seal between PDMS and the glass substrate at the boundaries.

Because of the roughness of the glass substrate it would be more desirable to use an atomically flat surface like mica or silicon, which is better suited for SFM analysis. First experiments revealed that on mica surfaces the seal between substrate and polymer is rather weak, resulting in a considerable amount of interconnections between the bilayer channels. The conditions (r.f. plasma, wet etching, surface functionalization) necessary to improve the seal between an appropriate atomically flat surface and the PDMS elastomer remain to be elucidated.

Conclusions

In summary, the described procedure expands the merits of solid-supported lipid membranes to an array system that might be of interest for biosensor applications as a multi-spot analysis device. Limitations of this approach to form specifically addressable compartments of planar solid-supported lipid bilayers by MIMIC concerning lateral dimensions and barrier spacings remain to be elucidated. The technique is considered to become a starting point for the creation of networks that can be used to study the interaction of ligands from solution with different kinds of membranes, varying in composition and physical properties without using fluorescent labels to identify the membrane, and might allow study of a vast amount of interactions simultaneously on different surfaces. In particular, the combination of patterned membranes with SFM will provide the possibility to study morphological changes due to ligand binding while determining the surface coverage simultaneously. Furthermore, the study of reactions at bilayer edges as recently demonstrated by Gaub and co-workers (Clausen-Schaumann et al. 1998) could be facilitated since controlled bilayer edges are formed with this technique.

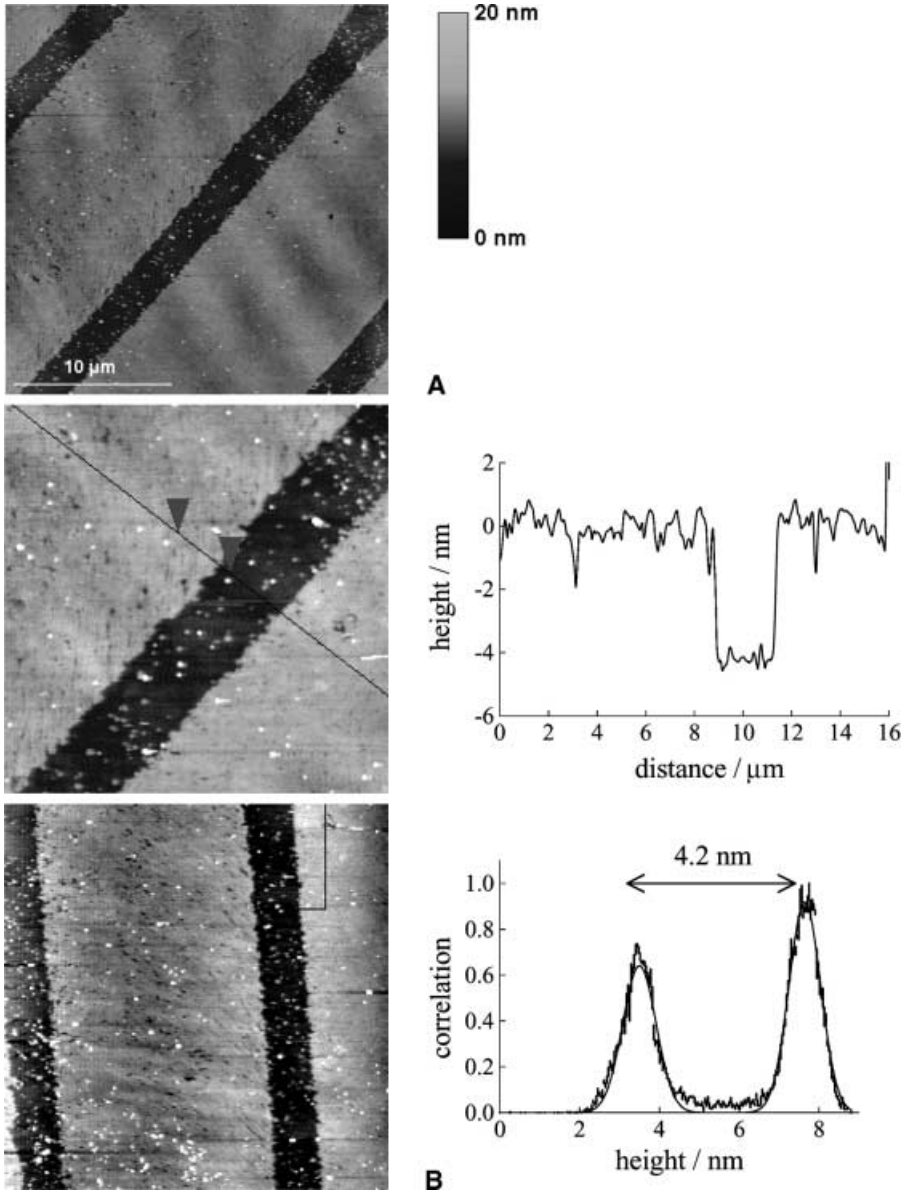

Fig. 4 **A** Scanning force microscopy images of planar membranes formed by vesicle spreading along the capillaries (contact-mode, scan velocity 80 µm/s). The spacings between the bilayer stripes are slightly smaller (approx. 3 µm) than given by the capillary design, which is probably due to a partly incomplete seal between elastomer and substrate. **B** The *top image* shows a height analysis of the structure presented in **A** (higher magnification) by means of section analysis, while the image at the *bottom* displays the average height difference by depth analysis. The average height of the structures is 4.2 ± 0.5 nm, consistent with the predicted height for DMPC bilayers on solid supports

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