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In situ generation of electrochemical gradients across pore-spanning membranes†

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Silicon substrates with cavities in the micrometre range were micro-fabricated and appropriately functionalized to allow for the generation of pore-spanning membranes (PSMs) sealing the pore cavities. PSMs were either formed by applying lipids dissolved in organic solvent (painting technique) on a hydrophobically functionalized silicon surface followed by 'solvent freeze-out', or solvent-free PSMs were prepared by spreading of giant unilamellar vesicles on hydrophilically functionalized substrates. The geometry of the silicon cavities in conjunction with three dimensional confocal laser scanning microscopy images enabled us to simultaneously monitor the PSMs and a pH-sensitive dye entrapped into the picolitre-sized cavities. The excellent sealing properties of both PSM types allowed an *in situ* generation of proton gradients across these membranes. In the presence of nigericin, a proton/potassium-antiporter, a preformed potassium ion gradient was transformed into a stable proton gradient across the PSMs, which was visualized by the pH-sensitive dye pyranine entrapped in the silicon cavities in a time resolved manner by means of confocal laser scanning fluorescence microscopy.

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Introduction

The eukaryotic cell contains a number of membrane-enclosed nanocompartments, which provide different (ionic) compositions leading to (electro)chemical gradients across the bilayers. Electrochemical gradients are of utmost importance for a variety of cellular processes, such as the generation of action potentials or the production of ATP in mitochondria.¹ They are generated by transmembrane proteins, which consume energy to establish the gradient. Examples are protein complexes I, III and IV from the respiratory chain, which require redox energy to produce a proton gradient across the inner mitochondrial membrane, or ATPases, which use the hydrolysis of ATP to generate an ion gradient across biological membranes.

To be able to establish stable ionic gradients across model membranes on a fast time scale, an insulating lipid membrane is required that separates pico- to attolitre-sized compartments from the surrounding medium. If picolitre-sized compartments could be optically addressed in a surface-confined and planar geometry, it would become possible to read out the establishment of electrochemical gradients in a spatially and temporarily dependent manner with the possibility to automatize the readout process. A large number of protocols have

been developed to generate model membranes on solid supports (for review see ref. 2–4). Such membranes are, however, not suited to establish ionic gradients, as the surface-facing compartment separating the bilayer from the substrate is very small or not even present. In contrast, membranes covering micro-fabricated pores or pore-arrays (pore-spanning membranes, PSMs, for review see ref. 5–9), would have the potential to cover picolitre-sized cavities. Several methods have been described to generate PSMs on single pores^{10–13} or open pore arrays.^{14–21} These PSMs have been applied to monitor the passive transport of ions mediated by pore-forming peptides like gramicidin and alamethicin.^{10–23} Among very few other examples,^{24,25} the most frequently reconstituted and studied protein in PSMs is α -hemolysin (for review see²⁶), a bacterial exotoxin expressed by *Staphylococcus aureus* as a water-soluble monomer, forming heptameric pores inside lipid bilayers.²⁷ The α -hemolysin mediated passive transport of ions and small molecules across PSMs has been studied by a number of methods such as fluorescence microscopy,^{28–33} chronoamperometry,¹⁶ voltage clamp,^{13,17,19–21,31,32,34–41} surface plasmon resonance⁴² and mass spectrometry.⁴³

While these transport processes are all passive in nature, the *in situ* generation of an electrochemical gradient by an active transport process across PSMs has not yet been demonstrated. To achieve electrochemical gradients on a fast time scale, membranes spanning micrometre-sized cavities are required instead of membranes spanning open pore arrays. Some examples are described in literature, where membranes span a pore that seals a micrometre-sized cavity.^{21,23,30,32,40,43} To prevent the membrane from aligning the internal surface of

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the cavity, a rather small pore is generally etched in a thin silicon layer covering a larger micrometre-sized cavity.²⁹ The reported pore diameters are typically in the sub-micrometre regime and thus the individual membrane, spanning the pore can barely be visualized by optical microscopy.^{28,44–46} As the membrane area that spans the pore is only in the $0.1\ \mu\text{m}^2$ range, a maximum of only a few 100 proteins could be reconstituted within a PSM covering the cavity. To be able to investigate transporter proteins, especially those with low turnover rates, a significantly larger number of reconstituted proteins per PSM would be desirable to obtain a good signal-to-noise ratio. Thus, the cavity as well as the PSM needs to be of micrometre dimensions to become visible in an optical microscope and to provide larger membrane areas. The preparation protocols for these membranes require that the membranes are hindered from aligning the inner pore walls but suspending the micrometre-sized compartment.

Here, we present two microcavity arrays in silicon with pore diameters in the range of $3.5\text{--}8\ \mu\text{m}$ that allow visualizing the PSM and the water-filled cavity underneath in one three dimensional confocal fluorescence microscopy image. Formation of PSMs generates well-defined picolitre-sized volumes separated from the bulk solution that can be filled with water-soluble pH-sensitive dyes such as pyranine (Scheme 1).^{47,48} The PSMs allow generating stable proton gradients as a result of an antiport of protons and potassium ions mediated by nigericin as shown by changes in the pH-sensitive fluorescence intensity of the entrapped pyranine.

Experimental section

Porous silicon substrates

To fabricate round-shaped pores on a Si-chip, we used a (100) oriented SOI wafer with a device layer thickness of $8\ \mu\text{m}$ and a handle layer thickness of $525\ \mu\text{m}$. First, a $1\ \mu\text{m}$ SiO_2 layer was deposited by wet thermal oxidation. The SiO_2 layer and the Si handle layer were structured by UV lithography (AZ1518, MicroChemicals GmbH, Ulm, Germany), reactive ion etching (STS MACS Multiplex RIE, UK), and deep reactive ion etching (STS MACS Multiplex ICP, UK) to pattern the pores.⁴⁹ It is noteworthy that the sidewalls of the pores have to be smooth and rectangularly shaped. For this purpose, we used the following etching parameters: SF_6 : 65 sccm, C_4F_8 : 55 sccm, $p_{\text{etch}} = p_{\text{pass}} = 10\ \text{mTorr}$; $t_{\text{etch}} = 8\ \text{s}$ and $t_{\text{pass}} = 7.8\ \text{s}$. The SiO_2 layer between the handle and device layer was used as an etch-stop for this process. After removing the photoresists, the sidewall roughness of the pores was reduced by thermal oxidation ($500\ \text{nm}\ \text{SiO}_2$) followed by wet-etching (BHF, $35\ ^\circ\text{C}$) to remove the SiO_2 again. To achieve a hydrophilic surface, the porous structure was covered with a SiO_2 layer ($500\ \text{nm}$) by thermal oxidation. In the final step, the wafer was diced into $25\ \text{mm}^2$ chips. The diameter of the pores was 3.5 , 4.5 and $5.5\ \mu\text{m}$, respectively, while the depth was $9\ \mu\text{m}$. The porosity of the substrates was varied from $P = 10$, 20 , 30 and 40% (round pores) and 65% (square-shaped pores). Before use, the substrates were sonicated in acetone ($2 \times 15\ \text{min}$) to remove the protective photoresist.

Square-shaped porous silicon substrates with an etch length of $8.0\ \mu\text{m}$ were a kind gift from Dr S. Schweitzer (University of

Scheme 1 Schematic illustration of a single lipid bilayer (red) spanning a pore with encapsulated dye (green). (a) Membrane formed via the painting method. The pore rims are functionalized with the cholesterol-derivative CPEO3 (brown), forming the lower leaflet of the hybrid bilayer. A magnification of the transition from the pore rims to the free-standing part of the membrane shows the formation of a solvent annulus (yellow, top) as discussed in the text. (b) Membrane formed via spreading of GUVs on a hydrophilically functionalized (blue) substrate. A continuous bilayer is formed that only weakly adheres to the pore rim area, covering the pore in a rather flat manner (top).

Halle/Wittenberg, Germany). All substrates were cleaned in argon plasma for 10 min. The top side was then sputter-coated with a thin layer of titanium (2.5 nm) followed by a gold layer (30 nm).

Preparation of giant unilamellar vesicles (GUVs)

GUVs were prepared according to the electrosweeling technique.⁵⁰ 25 μL of dissolved lipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), 9 : 1, 3 mg mL^{-1} in CHCl_3) were deposited onto indium tin oxide coated cover slips and the solvent was removed in vacuum overnight. GUVs were grown in a 0.3 M sucrose solution in ultrapure water using 3 V (peak-to-peak) and 5 Hz for 2 h.

Preparation of painted pore-spanning membranes

The gold-coated silicon substrate was functionalized with cholesterylpolyethylenoxythiol (CPEO3, 0.2 mM in *n*-propanol) at 4 °C overnight. Afterwards, it was rinsed with *n*-propanol, mounted into a petri dish or Teflon chamber and thoroughly rinsed with buffer. A small amount of lipids (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC)/1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (DPPA), 9 : 1, 20 mM in *n*-decane) was applied to the functionalized substrate surface by a paint brush. For 'solvent freeze-out', samples were stored at -21 °C for 15 min according to a procedure described by Ding *et al.*⁵¹

Preparation of solvent-free pore-spanning membranes

A gold-coated silicon substrate was functionalized with 11-amino-1-undecanethiol (1 mM in *n*-propanol) at 4 °C overnight. Afterwards, the functionalized substrate was rinsed with *n*-propanol, mounted into a petri dish or Teflon chamber and thoroughly rinsed with buffer solution. GUVs (10 μL) were carefully added and after 1 h of incubation at room temperature, the sample was rinsed with buffer to remove residual vesicles.

Determination of osmolarities

The osmolarity of all buffer solutions was determined with a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany).

Confocal laser scanning microscopy (CLSM)

Fluorescence images of PSMs and the entrapped water soluble dye pyranine were obtained with an upright confocal laser scanning microscope (LSM 710, Carl Zeiss, Jena, Germany) equipped with a water immersion objective W Plan-Apochromat 63 $\times$ /1.0 na VIS-IR (Carl Zeiss, Jena, Germany). Pyranine was excited at $\lambda = 458$ nm, BODIPY-PC at $\lambda = 488$ nm and Texas-Red DHPE at $\lambda = 561$ nm. Image analysis was performed using the ZEN software (Carl Zeiss, Jena, Germany) as well as ImageJ (V 1.41; <http://rsbweb.nih.gov/ij/>).

Scanning ion conductance microscopy (SICM)

Topographic information of the PSMs was obtained with an ICnano scanning ion conductance microscope from Ionscope (Melbourn, United Kingdom). The general procedure to image PSMs has been described previously.^{52,53} Nanopipettes were pulled from borosilicate glass (O.D. 1.00 mm, I.D. 0.58 mm,

length 80 mm, NPI Electronic GmbH, Tamm, Germany) using a P-1000 puller (Sutter Instruments Co., Novato, USA). Pipette resistances were >150 M Ω when filled with buffer (160 mM KCl, 10 mM TRIS/HCl, pH 7.3). For image recording, a constant potential difference of 150–300 mV was applied between two Ag/AgCl-electrodes. All measurements were performed in hopping mode.⁵⁴ Image analysis was performed using ScanIC Image software (V 1.0, Ionscope, Melbourn, United Kingdom) as well as Gwyddion software (V 2.20, <http://gwyddion.net/>).

Scanning electron microscopy (SEM)

SEM analysis was employed to examine the structure of the silicon substrates using a Supra 55 VP SEM instrument (Carl Zeiss, Jena, Germany) operated at 5 kV.

Proton transport

To induce proton transport across PSMs, nigericin (Applchem, Darmstadt, Germany) dissolved in ethanol was added to obtain the given concentrations.

Results and discussion

Formation of painted PSMs and the effect of 'solvent freeze-out'

To generate PSMs with large membrane areas, which serve as a permeability barrier and entrap small molecules such as fluorescent dyes, micro-fabricated silicon substrates with cavities with an edge length of $8 \times 8 \mu\text{m}^2$ and a depth of 12 μm (Fig. 1a) were used ($V = 0.8$ pL). The dimensions of each individual pore were chosen to allow for a simultaneous detection of the fluorescently labelled lipid bilayer with a large area of $64 \mu\text{m}^2$ and the underlying aqueous compartment by means of three dimensional confocal laser scanning microscopy images, taking the resolution of the optical microscope in the *z*-direction and the illumination geometry into account. PSMs were obtained by painting lipids (DPhPC/DPPA (9 : 1)) dissolved in *n*-decane and doped with BODIPY-PC (0.5 mol%) on the silicon substrates, whose top part was gold-covered and rendered hydrophobic by chemisorption of cholesterylpolyethylenoxythiol (CPEO3). For open pores or pore arrays it has been shown that the painted membranes 'thin out' rather quickly and spontaneously (see ref. 10,13,55 and references therein). However, in case of the closed cavities required in our setup, moderately thick lipid-solvent droplets are formed within the cavities, which can only be observed by taking *ortho* views of the functionalized pore arrays (Fig. S1 A, ESI†). A method to reduce the amount of residual *n*-decane in the membranes is to 'freeze out' the solvent^{19,51,56} at -21 °C for several minutes. *Ortho* views of the silicon substrates after this procedure clearly show a considerably reduced thickness of the BODIPY-PC labelled membranes (Fig. 2a) in the centre of the pores ($\sim 2 \mu\text{m}$), while they appear thicker at the pore edges ($\sim 4.4 \mu\text{m}$). As the resolution of the optical microscope is 1–2 μm in the *z*-direction, an apparent membrane thickness of about 2 μm indicates the formation of a rather 'solvent-free' membrane. The increased apparent thickness of the painted

Fig. 1 SEM images of silicon substrates with (a) square-shaped pores with dimensions of $8 \times 8 \times 12 \mu\text{m}^3$ and (b) and (c) round-shaped pores with a diameter of $4.5 \mu\text{m}$ and a depth of $9 \mu\text{m}$. Small fragments in (a) are due to the breaking of the substrate. A thin layer ($0.5 \mu\text{m}$) of SiO_2 is depicted as light grey line along the substrate surface in (b). Scale bars: $2 \mu\text{m}$.

membranes at the pore edges compared to the pore centre is interpreted as a solvent annulus of parent lipid solution (Plateau–Gibbs border), known from black lipid membranes.⁵⁷ The topology of the membranes as deduced from scanning ion conductance microscopy (SICM) images reveal that the membranes are located $(3.1 \pm 0.3) \mu\text{m}$ deep inside the cavities (Fig. 2b). In contrast, before ‘solvent freeze out’, a flat and featureless surface is monitored by SICM (Fig. S1 B, ESI†). The exact position of the membrane in *z*-direction varies by about 1

μm (standard deviation), which is the result of how much lipid dissolved in *n*-decane is exactly placed on the surface.

Three-dimensional confocal fluorescence micrographs have rarely been used to investigate the topology of lipid bilayers and in all these studies open pore arrays were used.^{18,32} However, the final appearance of these PSMs resemble the appearance we observed for membranes spanning picolitre-sized compartments supporting our interpretation of single lipid bilayers. The lateral mobility of the lipids within the PSMs was analysed by fluorescence recovery after photobleaching (FRAP) of the BODIPY-PC labelled lipids (Fig. 2c). Fast and almost full recovery with a mobile fraction of $M = 85 \pm 20\%$ ($n = 7$) of the fluorescence intensity demonstrates that continuous lipid membranes have been formed on the porous substrates with laterally mobile lipids that exhibit a diffusion coefficient of $D = 1.5 \pm 1.1 \mu\text{m}^2 \text{s}^{-1}$ ($n = 7$) characteristic for DPhPC lipids in solvent-free bilayers,⁵⁸ while for painted bilayers composed of DPhPC diffusion coefficients of $D = 6 \mu\text{m}^2 \text{s}^{-1}$ were reported.⁴¹

To investigate, whether the as prepared PSMs can serve as a permeability barrier for water-soluble molecules, we entrapped the water soluble and pH-sensitive fluorescent dye pyranine in the cavities. To simultaneously visualize the aqueous compartment with entrapped pyranine (green) and the membrane, the bilayers were labelled with Texas-Red DHPE (red) (Fig. 2d). In contrast to BODIPY-PC, Texas-Red DHPE strongly accumulated at the Plateau–Gibbs border, so that the pore-spanning part of the lipid bilayers appeared to be only weakly fluorescent. Part of the pores were filled with pyranine even after extensive rinsing with buffer, indicating that these pores were sealed with membranes, preventing the dye to leak out of the pores, which is a result of the direct interaction of the lipid layers with the hydrophobic self-assembly layer. Each individual pore with a volume of 0.8 pL and covered with a PSM was isolated from the neighbouring pores as shown by bleaching the fluorescence of the entrapped pyranine (Fig. S2, ESI†). On a time scale of 30 min, no recovery of the pyranine fluorescence was observed, demonstrating that there was no dye exchange between adjacent pores and that the pores were tightly sealed by the PSMs, which is prerequisite for the investigation of transport processes.

Establishment of a proton gradient across painted PSMs

We next addressed the question whether it is feasible to generate electrochemical gradients across the PSMs. With the pH-sensitive fluorescent dye pyranine entrapped inside the pores, it is possible to optically track the transport of protons across the PSMs. The active transport of protons was induced by nigericin, a well-known potassium/proton antiporter,⁵⁹ which transforms a pre-formed potassium ion gradient between the cavity and bulk solution into a proton gradient. Isoosmolaric buffer systems were used to generate the potassium ion gradient, *i.e.* potassium chloride containing buffer (160 mM KCl, 10 mM MOPS/KOH, pH 7.3) and sucrose buffer (320 mM sucrose, 10 mM MOPS/TRIS, pH 7.3). This gradient can be directed inside-out as well as outside-in with 0.5 mM pyranine added to the buffer encapsulated in the cavity. Acidification/deacidification of the cavity was monitored using the pyranine fluorescence intensity excited at 458

Fig. 2 (a) Ortho view of a confocal laser scanning micrograph of a porous silicon substrate ($8 \times 8 \times 12 \mu\text{m}^3$) with PSMs (DPhPC/DPPA/BODIPY-PC, 89.8 : 10.0 : 0.2). Scale bar: 10 μm , z -dimension: 7.9 μm ; $z = 0 \mu\text{m}$ defines the substrate surface (indicated by a white line), $z > 0 \mu\text{m}$ defines bulk solution and $z < 0 \mu\text{m}$ the pore interior. Buffer: 160 mM KCl, 10 mM MOPS/KOH, pH 7.3. (b) Scanning ion conductance micrograph and height profile of the PSMs (DPhPC/BODIPY-PC, 99.5 : 0.5). Buffer: 100 mM KCl, 20 mM TRIS/HCl, 1 mM CaCl_2 , 0.5 mM pyranine, pH 7.4. PSMs cover the pores with a depth of $z = -(3.1 \pm 0.3) \mu\text{m}$. (c) Fluorescence recovery after photobleaching (FRAP) curve obtained on the membrane shown in (a). From the monoexponential fit (red) a mobile fraction of $M = 94\%$ and a diffusion constant of $D = 1.24 \mu\text{m}^2 \text{s}^{-1}$ is deduced. Fluorescence images immediately after the bleach pulse ($t = 0$ s) and at $t = 30$ s are depicted as insets. The bleached area is marked by a white circle. Scale bars: 10 μm . (d) Ortho view of a confocal laser scanning micrograph of PSMs (DPhPC/DPPA/Texas-Red DHPE, 89.8 : 10.0 : 0.2, red) with entrapped pyranine (0.5 mM, green). Scale bar: 10 μm , z -dimension: 19.7 μm . Buffer: 160 mM KCl, 10 mM MOPS/KOH, pH 7.3.

nm, which corresponds to the absorption maximum of the deprotonated form of pyranine. If the pore interior contained potassium ions, while the bulk solution was potassium ion free, the addition of nigericin caused an influx of protons into the cavity, which was monitored by a strong decrease in pyranine fluorescence in a time-resolved manner (Fig. 3a). From the fluorescence intensity and under the assumption that the detected pyranine fluorescence intensity is proportional to the deprotonated pyranine concentration, we estimated the pH shift in the cavities taking the start pH of 7.3 and the pK_a value of pyranine of 7.22⁴⁸ into account (for further details see ESI†). Already after about 30 s, a constant proton gradient ($\Delta\text{pH} = 0.94$) has been established indicated by the constant pyranine fluorescence intensity. Injection of ammonium sulfate that is known to dissipate proton

gradients⁶⁰ partially let the pyranine fluorescence re-increase, demonstrating that the pH gradient indeed dissipates.

An initial potassium ion gradient in opposite direction caused a proton efflux after nigericin addition and, as expected, the pyranine fluorescence intensity increased translating into a pH gradient of $\Delta\text{pH} = 1.85$ (Fig. 3b). The addition of the protonophor FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone), which permeabilizes the membrane for protons, rapidly returned the pyranine fluorescence intensity nearly back to the original level.

Once established, proton gradients remained stable for up to several days, as indicated by the observation of a constant pyranine intensity (changes in intensity $\Delta I < 1.5\%$ in 2.5 h). From the initial slopes of the kinetics, a transport rate of $10^{14} \text{H}^+ \text{s}^{-1} \text{cm}^{-2}$ was roughly calculated after the addition of

Fig. 3 Time course of the pH within a single picolitre-sized cavity ($\lambda_{\text{ex}} = 458$ nm, $\lambda_{\text{em}} = 500\text{--}580$ nm). The cavities ($8 \times 8 \times 12 \mu\text{m}^3$, $V = 0.8$ pL) were covered with PSMs composed of DPhPC/DPPA (9 : 1) obtained by the painting technique and a potassium ion gradient was established. (a) 160 mM KCl, 10 mM MOPS/KOH, 0.5 mM pyranine, pH 7.3 (pore cavities); 320 mM sucrose, 10 mM MOPS/TRIS, pH 7.3 (bulk solution); 3.3 μM nigericin (1 mg mL^{-1} in EtOH) was added. 15 mM (NH₄)₂SO₄ (3 M in H₂O) was added after the proton gradient had been established. (b) 320 mM sucrose, 10 mM MOPS/TRIS, 0.5 mM pyranine, pH 7.3 (pore cavities); 160 mM KCl, 10 mM MOPS/KOH, pH 7.3 (bulk solution); 3.3 μM nigericin (1 mg mL^{-1} in EtOH) was added; 10 μM FCCP (1 mg mL^{-1} in EtOH) was added after the proton gradient had been established. At $t = 370$ s, the intensity drops to zero due to membrane rupture and release of pyranine, which is artificially calculated into a pH of 5.4. The time points of addition are indicated by arrows. Cartoons in the top right corner of (a) and (b) illustrate the direction of nigericin induced ion flux across the PSMs.

nigericin for both cases, the transport of protons inside the cavities as well as outside the cavities taking the buffer and pyranine capacity into account.

Control experiments ruled out that bilayer permeability was affected by the addition of any of the compounds. Bilayers treated with the same buffer composition inside and outside the pores did not show any significant changes in pyranine fluorescence intensity upon addition of the ethanolic nigericin solution. Moreover, addition of equivalent amounts of pure ethanol to PSMs with a pre-formed potassium ion gradient did not alter the pyranine fluorescence. In conclusion, the time dependent changes in pyranine fluorescence intensity clearly

demonstrate that a stable nigericin-driven proton gradient can be generated across PSMs.

Formation of solvent-free PSMs

One major drawback of PSMs obtained from spreading lipids dissolved in an organic solvent is the residual solvent that remains in the membrane. Even though such membranes are highly insulating and allow for single channel recordings (for review see ref. 6), the residual solvent might hamper the functional reconstitution of transmembrane proteins such as proton pumps, *i.e.* bacteriorhodopsin or ATPases. Thus, we asked the question whether solvent-free membranes can be prepared on silicon cavities and allow for the stable establishment of proton gradients. To establish a protocol to prepare solvent-free PSMs on porous silicon substrates, we made use of the strategy to spread giant unilamellar vesicles (GUVs) on an appropriately functionalized surface. To initiate spreading of GUVs, a strategy was pursued based on the electrostatic attraction of positively charged substrates and negatively charged GUVs. To achieve PSMs on silicon cavities, the upper part of the silicon was covered with gold and rendered positively charged at pH 7.3 by chemisorption of 11-amino-1-undecanethiol. GUVs with a negative surface charge density (POPC/POPS, 9 : 1) were prepared by the electroformation method. This method results in GUVs with an average diameter of roughly $10 \mu\text{m}$ (Fig. S6, ESI†). To ensure that the majority of GUVs are larger than the pore diameter of the cavities, silicon substrates were prepared with pore diameters of $d = 3.5 \mu\text{m}$, $4.5 \mu\text{m}$ and $5.5 \mu\text{m}$, all with a depth of $9 \mu\text{m}$ (Fig. 1b). These dimensions translate into nominal cavity volumes of 0.09 pL ($d = 3.5 \mu\text{m}$), 0.14 pL ($d = 4.5 \mu\text{m}$) and 0.21 pL ($d = 5.5 \mu\text{m}$). For the cavities with a diameter of $5.5 \mu\text{m}$ and taking the size distribution of the GUVs into account, about 80% of the GUVs are larger than the pore diameter, while for the $4.5 \mu\text{m}$ pores, about 90% and 100% for the $3.5 \mu\text{m}$ sized pores. Smaller pore diameters were not chosen as they do not allow to fluorescently access the interior of the pore cavities by more than $1.6 \mu\text{m}$, which is required to separate the bilayer fluorescence from that of the aqueous solution. Besides the pore diameter we also varied the porosity P of the substrate. We investigated whether the pore diameter as well as the porosity, *i.e.* the fraction of provided adhesion area for the GUVs, influences the spreading success of the GUVs. From our results we concluded that independent of the pore diameter and the porosity, all substrates are well-suited to prepare PSMs. However, in contrast to the PSMs generated by the painting technique, solvent-free PSMs did not cover the entire substrate. Instead, defined patches derived from single vesicle rupture were observed (Fig. 4a). This finding can be attributed to the different functionalization used to spread GUVs on the silicon surface compared to the painting technique. In case of the painting technique a hydrophobic functionalization strategy was pursued resulting in a bilayer covering the pore, while a hybrid bilayer composed of CPEO3 and a lipid monolayer is formed on the pore rims (Scheme 1a). GUV spreading, however, results in continuous bilayers throughout the substrate, which are electrostatically attached to the surface (Scheme 1b). If an individual GUV spreads and forms a planar lipid bilayer, the edges are folded so that fusion of

Fig. 4 (a) Ortho view of a confocal laser scanning micrograph of a porous silicon substrate (pore diameter $d = 5.5 \mu\text{m}$, depth $h = 9 \mu\text{m}$, porosity $P = 30\%$). GUVs composed of POPC/POPS/Texas-Red DHPE (89.8 : 10.0 : 0.2) were spread on an 11-amino-1-undecanethiol-functionalized surface forming a patch of planar PSMs (red) entrapping the water soluble fluorophor pyranine (0.5 mM, green). Buffer: 107 mM CaCl_2 , 10 mM MOPS/TRIS, pH 7.3. Scale bar: $10 \mu\text{m}$, z-dimension: $16 \mu\text{m}$; $z = 0 \mu\text{m}$ defines the substrate surface (indicated by a white line), $z > 0 \mu\text{m}$ defines bulk solution and $z < 0 \mu\text{m}$ the pore interior. (b) Top: Ortho view of a confocal laser scanning micrograph of a single PSM (DPhPC/DPPA/Texas-Red DHPE, 89.8 : 10.0 : 0.2, red) with entrapped pyranine (0.5 mM, green), prepared via the painting technique. Scale bar: $10 \mu\text{m}$, z-dimension: $19.7 \mu\text{m}$. Buffer: 160 mM KCl, 10 mM MOPS/KOH, pH 7.3. Bottom: Intensity profile of the membrane dye Texas Red DHPE (red) and entrapped pyranine (green). A Gaussian distribution was fit to the profile of the Texas Red DHPE fluorescence with a maximum at $z = -0.87 \mu\text{m}$ and a full width at half maximum of $FWHM = 2.7 \mu\text{m}$. The region of interest corresponding to the extracted intensities is depicted as white dashed rectangle. (c) Top: Ortho view of a confocal laser scanning micrograph of a single planar PSM ($d = 5.5 \mu\text{m}$, $h = 9 \mu\text{m}$, $P = 30\%$). GUVs composed of POPC/POPS/Texas-Red DHPE (89.8 : 10.0 : 0.2) were spread on an 11-amino-1-undecanethiol-functionalized surface forming a PSM (red) entrapping the water soluble fluorophor pyranine (0.5 mM, green). Buffer: 107 mM CaCl_2 , 10 mM MOPS/TRIS, pH 7.3. Scale bar: $10 \mu\text{m}$, z-dimension: $16 \mu\text{m}$. Bottom: Intensity profile of the membrane dye Texas Red DHPE (red) and entrapped pyranine (green). A Gaussian distribution was fit to the profile of the Texas Red DHPE fluorescence with a maximum at $z = -1.29 \mu\text{m}$ and a full width at half maximum of $FWHM = 2.6 \mu\text{m}$. The region of interest corresponding to the extracted intensities is depicted as white dashed rectangle. (d) Ortho view of a confocal laser scanning micrograph of a porous silicon substrate ($d = 5.5 \mu\text{m}$, $h = 9 \mu\text{m}$, $P = 40\%$). GUVs composed of POPC/POPS/Texas-Red DHPE (89.8 : 10.0 : 0.2) were spread on an 11-amino-1-undecanethiol-functionalized surface forming PSMs (red) entrapping the water soluble fluorophor pyranine (0.5 mM, green). Buffer: 107 mM CaCl_2 , 10 mM MOPS/TRIS, pH 7.3. Scale bar: $10 \mu\text{m}$, z-dimension: $10 \mu\text{m}$. Arrows point to a single planar PSM (1), a single highly curved PSM (2) and a patch of curved PSMs (3). (e) Three dimensional presentation of a scanning ion conductance micrograph (top) and height profile of the PSMs (bottom) (POPC/POPS/Texas-Red DHPE, 89.8 : 10.0 : 0.2) on a porous substrate ($d = 4.5 \mu\text{m}$, $h = 9 \mu\text{m}$, $P = 40\%$). Buffer: 160 mM KCl, 10 mM MOPS/KOH, 0.5 mM pyranine, pH 7.3. The dashed line indicates the shape of a membrane patch formed from single vesicle rupture, the arrow defines the line used for the height profile.

adjacent bilayer patches does not take place and thus the bilayer patches remain isolated.⁶¹ Despite the individual membrane patches, the dye pyranine was stably entrapped in pores that were covered with a lipid membrane suggesting

that the (electrostatic) interaction between the membrane and the self-assembled monolayer is sufficiently strong to isolate the pores from the bulk solution and at the same time does not disturb the integrity of the membrane. Z-stack images of

these membranes revealed a homogeneous Texas-Red DHPE fluorescence with an apparent thickness of 2 μm similar to that found for BODIPY-PC labelled painted PSMs in the centre of the pore after 'solvent freeze-out' (Fig. S3, ESI†) further supporting the notion of single lipid bilayer formation. The extended fluorescence in *z*-direction observed for painted PSMs at the pore edges was not present in case of the solvent-free PSMs corroborating the idea that this observed fluorescence is a result of a solvent annulus. Both, membranes prepared *via* painting technique after 'solvent freeze-out' and solvent free membranes obtained by spreading of GUVs, reveal an identical distribution of membrane fluorescence in *z*-direction and a well-separated fluorescence of the water soluble dye (Fig. 4b and c). Even though this additional sealing of the membrane by a solvent annulus is missing, the fluorescent dye was tightly entrapped in each individual pore as verified by bleaching entrapped pyranine in one pore. On an observation time scale of 32 min, no fluorescence recovery was observed, confirming isolation of each pore from the neighbouring one (Fig. S4, ESI†).

An interesting phenomenon that we observed when spreading GUVs was, that in some cases not planar PSMs were formed, but rather bulb-like protrusions (Fig. S5, ESI†). In this case, PSMs extended up to several micrometres into the bulk solution, still keeping the water soluble dye pyranine entrapped. Fig. 4d shows a fluorescence image of a mixed patch of hemispherical and planar PSMs. Only the *ortho* view clearly shows the two different geometries as indicated by arrows (single planar PSM (1), single strongly perturbed PSM (2) and a patch of hemispherical PSMs (3)), while they appear alike in the top view fluorescence micrograph.

SICM images confirmed the hemispherical structure of some of the PSMs (Fig. 4e, top). The height profiles (Fig. 4e, bottom) clearly allows to distinguish between non covered pores ($z < -6 \mu\text{m}$), planar PSMs ($z = -(1.3 \pm 1.1) \mu\text{m}$) and hemispherical PSMs ($+2 \mu\text{m} < z$). Both, SICM and confocal microscopy images indicate that for single patches, the hemispherical geometry is most frequently found in the centre of the patch, while the planar geometry is generally found at the edge of the patch. We attribute the formation of hemispherical PSMs to an osmotic pressure difference between the bulk solution and the solution inside the cavities. As GUVs are filled with 0.3 M sucrose to sediment on the substrate, spreading results in sucrose release, which can be entrapped in the pores, thus altering the composition of the encapsulated solution and generating an osmotic pressure that deforms the PSMs. Since the release of sucrose from the GUVs might be non-homogeneous along the area, the sucrose concentration in the pores varies and thus the height of the protrusion of the PSMs.

The mobility of lipids of solvent-free PSMs was investigated by FRAP experiments. Even though the conditions of an infinite reservoir of fluorescently labelled lipids is not fully met, since the bilayer patch is finite,⁶² the observed recovery of fluorescence intensity after photobleaching of a BODIPY-PC labelled PSM proves that the lipids are laterally mobile within the membrane with diffusion coefficients of about $1 \mu\text{m}^2 \text{s}^{-1}$ supporting the formation of a continuous lipid bilayer. The same results were observed for the hemispherical PSMs.

Establishment of a proton gradient across solvent-free PSMs

The active transport of protons was again induced by nigericin. In case of solvent-free PSMs two isoosmolaric buffer systems were used to generate the potassium ion gradient, *i.e.* potassium chloride containing buffer (160 mM KCl, 10 mM MOPS/KOH, pH 7.3) and a Ca^{2+} containing buffer (107 mM CaCl_2 , 10 mM MOPS/TRIS, pH 7.3) to keep the buffer conditions for GUV spreading. This gradient was established inside-out as well as outside-in with 0.5 mM pyranine added to the buffer encapsulated inside the pores. Addition of nigericin resulted in the establishment of a proton gradient similar to what has been observed for painted PSMs. Depending on the orientation of the initial potassium ion gradient, acidification (Fig. 5a) or deacidification (Fig. 5b) of the cavities upon addition of nigericin was observed as an increase or decrease in fluorescence intensity of the entrapped dye pyranine. Overall pH differences before and after addition of nigericin as well as the time dependent course of the pH change were similar to the results obtained for painted PSMs. Transport rates that were calculated from the initial slopes of the kinetics matched the values obtained for painted PSMs after 'solvent freeze-out' ($10^{14} \text{H}^+ \text{s}^{-1} \text{cm}^{-2}$). After acidification and deacidification, respectively, the fluorescence intensities remained stable (Fig. 5a and b), proving the formation of a stable proton gradient across solvent-free PSMs. As in case of painted PSMs, injection of ammonium sulfate (Fig. 5a) and FCCP (Fig. 5b) partially dissipated the proton gradient. The results clearly demonstrate that solvent-free PSMs are well-suited to generate stable proton gradients.

Conclusion

The formation of membranes spanning the cavities of silicon substrates allows for the entrapment of molecules and ions. The dimensions of the cavities have been chosen to provide a rather large membrane area, while the underlying aqueous compartment can be addressed individually and quasi-simultaneously by means of three dimensional confocal laser scanning fluorescence imaging. The highly ordered array of uniformly shaped pores would enable one to read out each compartment in a fully automated manner, which is a prerequisite for screening applications. This is highly advantageous compared to studies using individual, fluorescently labelled GUVs. Setups based on GUVs suffer from the heterogeneity of the size of the vesicles, as well as their random immobilization on a surface.^{63,64}

Our presented membrane-covered compartments have been demonstrated to be well suited to establish ionic gradients, whose development can be monitored *in situ* in a time resolved manner and which remain stable over hours. Such membrane system may offer an ideal setup to investigate electrochemical gradients generated by reconstituted protein pumps and may serve as a platform for large scale screening processes as they are readily prepared, long-term stable and accessible to fluorescence microscopy techniques.

Fig. 5 Time course of the pH within a picolitre-sized single cavity ($\lambda_{\text{ex}} = 458$ nm, $\lambda_{\text{em}} = 500$ –580 nm) covered with a PSM. The cavities ($d = 5.5$ μm , $h = 9$ μm , $V = 0.21$ pL, $P = 30\%$) were covered with PSMs (POPC/POPS/Texas-Red DHPE, 89.8 : 10.0 : 0.2) by spreading of GUVs and a K⁺-gradient was established. (a) 160 mM KCl, 10 mM MOPS/KOH, 0.5 mM pyranine, pH 7.3 (pore cavities); 320 mM sucrose, 10 mM MOPS/TRIS, pH 7.3 (bulk solution); 16.7 μM nigericin (5 mg mL⁻¹ in EtOH) was added. 15 mM (NH₄)₂SO₄ (3 M in H₂O) was added after the proton gradient had been established. (b) 107 mM CaCl₂, 10 mM MOPS/TRIS, 0.5 mM pyranine, pH 7.3 (pore cavities); 160 mM KCl, 10 mM MOPS/KOH, pH 7.3 (bulk solution); addition of 16.7 μM nigericin (5 mg mL⁻¹ in EtOH). 100 μM FCCP (10 mg mL⁻¹ in EtOH) was added after the proton gradient had been established. Additions are indicated by arrows. Cartoons in the bottom right corner of (a) and top left corner of (b) illustrate the direction of nigericin induced ion flux across the PSMs.

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References

- 1 P. Mitchell, *Nature*, 1961, **191**, 144–148.
- 2 A. Janshoff and C. Steinem, *Anal. Bioanal. Chem.*, 2006, **385**, 433–451.

- 3 E. T. Castellana and P. S. Cremer, *Surf. Sci. Rep.*, 2006, **61**, 429–444.
- 4 Y. Cheng, S. D. Ogier, R. J. Bushby and S. D. Evans, *J. Biotechnol.*, 2000, **74**, 159–174.
- 5 I. Mey, C. Steinem and A. Janshoff, *J. Mater. Chem.*, 2012, **22**, 19348–19356.
- 6 M. Zagnoni, *Lab Chip*, 2012, **12**, 1026–1039.
- 7 H. Suzuki and S. Takeuchi, *Anal. Bioanal. Chem.*, 2008, **391**, 2695–2702.
- 8 R. Peters, *Annu. Rev. Biophys. Biomol. Struct.*, 2003, **32**, 47–67.
- 9 E. Reimhult and K. Kumar, *Trends Biotechnol.*, 2008, **26**, 82–89.
- 10 M. E. Sandison, M. Zagnoni and H. Morgan, *Langmuir*, 2007, **23**, 8277–8284.
- 11 C. Schmidt, M. Mayer and H. Vogel, *Angew. Chem., Int. Ed.*, 2000, **39**, 3137–3140.
- 12 M. E. Sandison, M. Zagnoni, M. Abu-Hantash and H. Morgan, *J. Micromech. Microeng.*, 2007, **17**, S189–S196.
- 13 V. C. Stimberg, J. G. Bomer, I. van Uiter, A. van den Berg and S. Le Gac, *Small*, 2012.
- 14 S. Kresák, T. Hianik and R. L. C. Naumann, *Soft Matter*, 2009, **5**, 4021–4032.
- 15 E. K. Schmitt, C. Weichbrodt and C. Steinem, *Soft Matter*, 2009, **5**, 3347–3353.
- 16 A. Studer, X. Han, F. K. Winkler and L. X. Tiefenauer, *Colloids Surf., B*, 2009, **73**, 325–331.
- 17 B. Le Pioufle, H. Suzuki, K. V. Tabata, H. Noji and S. Takeuchi, *Anal. Chem.*, 2008, **80**, 328–332.
- 18 H. Suzuki, K. V. Tabata, H. Noji and S. Takeuchi, *Langmuir*, 2006, **22**, 1937–1942.
- 19 T.-J. Jeon, J. L. Poulos and J. J. Schmidt, *Lab Chip*, 2008, **8**, 1742–1744.
- 20 J. S. Hansen, M. Perry, J. Vogel, J. S. Groth, T. Vissing, M. S. Larsen, O. Geschke, J. Emneüs, H. Bohr and C. H. Nielsen, *Anal. Bioanal. Chem.*, 2009, **395**, 719–727.
- 21 G. Baaken, M. Sondermann, C. Schlemmer, J. Rühle and J. C. Behrends, *Lab Chip*, 2008, **8**, 938–944.
- 22 W. Römer, Y. H. Lam, D. Fischer, A. Watts, W. B. Fischer, P. Göring, R. B. Wehrspohn, U. Gösele and C. Steinem, *J. Am. Chem. Soc.*, 2004, **126**, 16267–16274.
- 23 P. V. Ganesan and S. G. Boxer, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 5627–5632.
- 24 G. Favero, L. Campanella, S. Cavallo, A. D'Annibale, M. Perrella, E. Mattei and T. Ferri, *J. Am. Chem. Soc.*, 2005, **127**, 8103–8111.
- 25 O. Gassmann, M. Kreir, C. Ambrosi, J. Pranskevich, A. Oshima, C. Röling, G. Sosinsky, N. Fertig and C. Steinem, *J. Struct. Biol.*, 2009, **168**, 168–176.
- 26 A. de La Escosura-Muñiz and A. Merkoçi, *ACS Nano*, 2012, **6**, 7556–7583.
- 27 E. Gouaux, *J. Struct. Biol.*, 1998, **121**, 110–122.
- 28 A. Kleefen, D. Pedone, C. Grunwald, R. Wei, M. Firnkies, G. Abstreiter, U. Rant and R. Tampé, *Nano Lett.*, 2010, **10**, 5080–5087.
- 29 K. Sumitomo, A. McAllister, Y. Tamba, Y. Kashimura, A. Tanaka, Y. Shinozaki and K. Torimitsu, *Biosens. Bioelectron.*, 2012, **31**, 445–450.
- 30 S. Ota, H. Suzuki and S. Takeuchi, *Lab Chip*, 2011, **11**, 2485–2487.

- 31 E. L. Chandler, A. L. Smith, L. M. Burden, J. J. Kasianowicz and D. L. Burden, *Langmuir*, 2004, **20**, 898–905.
- 32 R. Hemmler, G. Böse, R. Wagner and R. Peters, *Biophys. J.*, 2005, **88**, 4000–4007.
- 33 S. Ota, W.-H. Tan, H. Suzuki and S. Takeuchi, in *Proc. MEMS Conf.*, Tucson, AZ, 2008, pp. 18–21.
- 34 T. Osaki, H. Suzuki, B. Le Pioufle and S. Takeuchi, *Anal. Chem.*, 2009, **81**, 9866–9870.
- 35 M. C. Peterman, J. M. Ziebarth, O. Braha, H. Bayley, H. A. Fishman and D. Bloom, *Biomed. Microdevices*, 2002, **4**, 231–236.
- 36 N. Malmstadt, M. A. Nash, R. F. Purnell and J. J. Schmidt, *Nano Lett.*, 2006, **6**, 1961–1965.
- 37 I. van Uitert, S. Le Gac and A. van den Berg, *Biochim. Biophys. Acta, Biomembr.*, 2010, **1798**, 21–31.
- 38 M. Roerdink Lander, S. Ibragimova, C. Rein, J. Vogel, K. Stibius, O. Geschke, M. Perry and C. Hélix-Nielsen, *Langmuir*, 2011, **27**, 7002–7007.
- 39 R. Kawano, T. Osaki, H. Sasaki and S. Takeuchi, *Small*, 2010, **6**, 2100–2104.
- 40 T. Osaki, Y. Watanabe, R. Kawano, H. Sasaki and S. Takeuchi, *J. Microelectromech. Syst.*, 2011, **20**, 797–799.
- 41 N. Malmstadt, T.-J. Jeon and J. J. Schmidt, *Adv. Mater.*, 2008, **20**, 84–89.
- 42 H. Im, N. J. Wittenberg, A. Lesuffleur, N. C. Lindquist and S.-H. Oh, *Chem. Sci.*, 2010, **1**, 688–696.
- 43 G. Baaken, N. Ankri, A.-K. Schuler, J. Rühle and J. C. Behrends, *ACS Nano*, 2011, **5**, 8080–8088.
- 44 K. Buchholz, A. Tinazli, A. Kleefen, D. Dorfner, D. Pedone, U. Rant, R. Tampé, G. Abstreiter and M. Tornow, *Nanotechnology*, 2008, **19**, 445305–44510.
- 45 M. D. Mager and N. A. Melosh, *Adv. Mater.*, 2008, **20**, 4423–4427.
- 46 P. Jönsson, M. P. Jonsson and F. Höök, *Nano Lett.*, 2010, **10**, 1900–1906.
- 47 N. R. Clement and J. M. Gould, *Biochemistry*, 1981, **20**, 1534–1538.
- 48 K. Kano and J. H. Fendler, *Biochim. Biophys. Acta, Biomembr.*, 1978, **509**, 289–299.
- 49 F. Laermer, A. Schilp, K. Funk and M. Offenberger, in *Twelfth IEEE International Conference on Micro Electro Mechanical Systems*, Orlando, FL, USA, 1999, pp. 211–216.
- 50 L. Mathivet, S. Cribier and P. F. Devaux, *Biophys. J.*, 1996, **70**, 1112–1121.
- 51 L. Ding, J. Li, S. Dong and E. Wang, *J. Electroanal. Chem.*, 1996, **416**, 105–112.
- 52 I. Höfer and C. Steinem, *Soft Matter*, 2011, **7**, 1644–1647.
- 53 M. Böcker, S. Muschter, E. K. Schmitt, C. Steinem and T. E. Schäffer, *Langmuir*, 2009, **25**, 3022–3028.
- 54 P. Novak, C. Li, A. I. Shevchuk, R. Stepanyan, M. Caldwell, S. Hughes, T. G. Smart, J. Gorelik, V. P. Ostanin, M. J. Lab, G. W. J. Moss, G. I. Frolenkov, D. Klenerman and Y. E. Korchev, *Nat. Methods*, 2009, **6**, 279–281.
- 55 E. K. Schmitt, M. Vrouenraets and C. Steinem, *Biophys. J.*, 2006, **91**, 2163–2171.
- 56 S. H. White, *Biochim. Biophys. Acta, Biomembr.*, 1974, **356**, 8–16.
- 57 M. K. Jain, F. P. White, A. Strickholm, E. Williams and E. H. Cordes, *J. Membr. Biol.*, 1972, **8**, 363–388.
- 58 J. Lin, J. Szymanski, P. C. Searson and K. Hristova, *Langmuir*, 2010, **26**, 3544–3548.
- 59 B. C. Pressman, *Annu. Rev. Biochem.*, 1976, **45**, 501–530.
- 60 J. W. Hell, L. Edelmann, J. Hartinger and R. Jahn, *Biochemistry*, 1991, **30**, 11795–11800.
- 61 M. Kocun, T. D. Lazzara, C. Steinem and A. Janshoff, *Langmuir*, 2011, **27**, 7672–7680.
- 62 T. D. Lazzara, C. Carnarius, M. Kocun, A. Janshoff and C. Steinem, *ACS Nano*, 2011, **5**, 6935–6944.
- 63 A. Boll, A. Jatho, N. Czudnochowski, M. Geyer and C. Steinem, *Biochim. Biophys. Acta, Biomembr.*, 2011, **1808**, 2685–2693.
- 64 S. Bleicken, C. Wagner and A. J. García-Sáez, *Biophys. J.*, 2013, **104**, 421–431.