Well-Defined Microapertures for Ion Channel Biosensors

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Supporting Information

ABSTRACT: Gated ion channels are excitable nanopores in biological membranes. They sense and respond to different triggers in nature. The sensory characteristics of these channels can be modified by protein engineering tools and the channels can be functionally reconstituted into synthetic lipid bilayer membranes. The combination of the advances in protein engineering with electrical and/or optical signal detection possibilities makes ion channels perfect detection modules for sensory devices. However, their integration into analytical devices is problematic due to the instability of lipid bilayers. Here, we report on developing a stable sensory chip containing a mechanosensitive channel in a Si/SiO₂ chip with a 3 μm pore. Our new fabrication strategy was straightforward. It required only lithography and dry etching for the pore definition and membrane release and reduced the risk of membrane rupture in the fabrication process. A gated ion channel could be inserted, with the retention of its function, into the pores of Si/SiO₂ chips and be detectable at the single channel level upon activation. Excitable ion channels in stable small pores can serve as very sensitive detectors of specific molecules.

In recent years, there have been significant efforts on the use of synthetic or biological nanopores in single-molecule sensing platforms.1−3 The most attractive features of such systems are the ease of detection as the binding to or passage of analytes through the pores generates detectable changes in ionic pore current, no requirement of labeling or surface attachment of the analytes, and their costs.4 Among the nanopores, gated ion channels that are natural excitable nanopores embedded in biological lipid bilayer membranes stand out for their intrinsic high sensitivity for different stimuli and for functioning as “on/off” switches. Upon stimulation with various chemicals or electrical or mechanical triggers,5 these channels form temporary openings in the membrane and communicate with the other side of the membrane by allowing the rapid flux of ions ($10^6$–$10^7$ ions/s), which can be measured electrically with a millisecond time resolution.6−8 Furthermore, some of these channels stay functional in synthetic lipid bilayers. The combination of the advances in protein engineering for the modification of the sensitivity of gated ion channels with electrical and/or optical signal detection possibilities makes them perfect detection modules for sensory devices. However, the main challenge in integrating their excellent detection capacity into the sensory devices is in creating an environment suitable for the proper functioning of these channels, i.e., a lipid bilayer membrane. Lipid bilayers are not robust platforms for use in diagnostic devices. We developed a straightforward method to generate chips from silicon on insulator (SOI) wafers with microfabricated 3 μm apertures. We formed a stable, suspended artificial bilayer lipid membrane (BLM) across it and could reconstitute a gated-ion channel into the lipid bilayer. This method is suitable for generating wafers with closely positioned individual apertures that can accommodate ion channels and for volume production of devices, which could be important in future higher-level integrated sensory devices.

BLM has been used previously for ion channel activity measurements or receptor—analyte binding assays;9−11 however, they are not suitable for sensory devices because the conventional BLM cups have a large aperture (50–200 μm) and the lipid bilayer of about 5–20 nm thickness over this opening is very fragile. They easily break with mechanical and electrical disturbances.13 One of the solutions to obtain reproducible and stable BLMs has been forming the BLM over well-defined sub-10 μm diameter apertures in electrically inert materials.14−17 The approaches to generate such openings have been based on different combinations of silicon nitride and silicon oxide membranes where the pores have been created by the use of lithography followed by dry etching for pore definition and wet etching for membrane release or direct definition of the pores by ion beam milling. The major drawbacks of these approaches are the materials and fabrication processes. For instance, pinhole free silicon nitride films are challenging to make. Wet etching to release the membranes without membrane rupture is difficult because the membranes need to be very thin in order to make small apertures. Processes such as ion beam milling of the apertures are not suitable for
production of large numbers of devices. The aim and the novelty of our work is the introduction of a new micro-fabrication method for generating well-defined microapertures and showing its compatibility not only with self-inserting pore forming peptides but also with bigger, not self-inserting, gated-ion channels as sensory elements.

**RESULTS AND DISCUSSION**

To fabricate Si/SiO2 chips with microapertures, silicon on insulator (SOI) wafers were used. The fabrication of the devices was a three step dry etch process using UV-lithography on a SOI wafer with a 300 µm Si handle layer, a 500 nm buried oxide (BOX) layer, and a 5 µm Si device layer (Figure 1A).

First, a 3–30 µm hole was defined on the device layer by UV-lithography based on AZ5214E resist and transferred into the device layer by SF6/C4F8 Bosch dry etching using the BOX layer of the SOI wafer as etch stop (Figure 1B,C). Second, a 750 µm × 750 µm opening was defined on the handle layer side of the wafer using SF6/C4F8 Bosch dry etching (Figure 1D). The handle layer etch stopped at the BOX layer of the SOI wafer as well (Figure 1E). Third, the BOX was removed from the handle layer side of the wafer using a CF4/CHF3 based reactive ion etch (Figure 1F). Finally, a 150 nm thermal oxide was grown as electrical insulation. Representative SEM image of a 3 µm diameter aperture is given in G (Magnification is 15.00 KX). NOTE: In the drawings, the pore is represented by the slit.

Methods. The trans side of the aperture was formed from 0.8% agarose (in 1 M NaCl). After a drop of buffer (10 mM Tris, 250 mM NaCl, pH = 7.4) was placed on the cis side of the chip, a lipid bilayer across the 3 µm aperture was formed from Azolectine (20 mg/mL) lipids using the painting method. Alamethicin is an antimicrobial peptide that kills gram-positive bacteria by forming pores in their membranes. In this work, 2 µL of alamethicin from a 0.1 mg/mL stock (in methanol) was added into the buffer on the cis side of the bilayer. A few minutes after applying voltage across the bilayer...
(90 mV, sampling at 50 kHz and filtering at 5 kHz), distinct alamethicin channels could be observed (Figure 2B). A detailed conductance trace of alamethicin channels and the corresponding conductance histogram is shown in Figure 2C. The bilayers could be stored at ambient temperature (18 °C) for at least 2 days, and at the end of the period, they were still intact and could accommodate alamethicin pores.

Next, we tested the suitability of our setup for the reconstitution of a bigger, gated ion channel, MscL, which does not insert into the membrane by itself. In nature, MscL senses tension in a bacterial membrane.22 In its closed state, it does not allow even the passage of ions. However, upon sensing the membrane tension, it opens a temporary, large (∼3 nm in diameter),23 nonselective pore and allows the passage of not only ions but also small molecules and even small peptides.24 Previously, we have proven its potential as a remote-controlled nanovalve in liposomal drug delivery vehicles25 and in lipid−polymer hybrid nanocontainers.26 We also could modify its specificity toward different triggers. For instance, in one case, we made it recognize the ambient pH.27 In another case, we engineered it to respond to the wavelength of the light.28 The combination of its potential for sensing desired signals with its functioning in conventional BLMs29 and tethered lipid bilayer membranes30 makes MscL a suitable candidate for biosensors.

Figure 3A shows a schematic diagram depicting the procedure for reconstitution of MscL channels into preformed micro-BLMs on the Si/SiO2 chip. Since MscL does not insert into lipid bilayers spontaneously, it was first reconstituted into large unilamellar vesicles (LUVs) as explained in the Material and Methods. Proteo-LUVs (5 μL) were added into the cis chamber that had a low salt buffer (10 mM Tris, 250 mM NaCl, pH = 7.4). The fusion was facilitated by swelling of proteoliposomes with the help of an osmotic gradient between the inside and the outside of the proteoliposomes. Since MscL in LUVs were in their closed conformation, we could not detect the fusion events in real time. In order to test the functioning of
MscL in the bilayer, 5 min after the addition of LUVs, a positively charged, cysteine specific compound MTSET was added into the cis compartment (60 mM final concentration) as an analyte. Binding of MTSET into the Cys residues in the pore region of MscL opened the channel and allowed ionic conduction. The channel activity was recorded at −50 mV constant voltage. Signals were sampled at 50 kHz and filtered at 5 kHz. A representative current trace showing single MscL channel fluctuations are shown in Figure 3B,C. Bilayers alone did not give any signal upon treatment with MTSET (Supporting Information Figure S1). The all points histogram shown in Figure 3D indicated that, upon binding to MTSET, the channel started switching between on and off states as observed before on conventional patch clamp experiments.\textsuperscript{31,32} The activated channel mainly stayed open often for less than 1 ms and rarely 4 ms (Figure 3E).

In summary, we have developed a new, straightforward method in order to improve the stability of reconstituted micro-BLMs by generating microfabricated small apertures on Si/SiO\textsubscript{2} chips only from SOI wafers with no SiN deposition and with no risk of destroying the membranes in the release process. We could successfully demonstrate that these chips are suitable for accommodating not only self-inserting, pore-forming peptides but also bigger, selective, two-state (i.e., open/on and closed/off) gated ion channels. Sensory chips with gated ion channels that can be engineered to sense different analytes could offer opportunities ranging from very early detection of disease markers to environmental control.

\section{MATERIAL AND METHODS}

Si/SiO\textsubscript{2} Pore Chips. The devices containing the apertures in which the lipid bilayers are inserted were fabricated from silicon on insulator (SOI) wafers. The dimensions of the devices were 10 mm \times 10 mm with a 750 μm \times 750 μm Si/SiO\textsubscript{2} membrane area. The silicon thickness in the center was 5 μm. A single through hole 3−30 μm in diameter was situated in the center of this cavity. The device was covered on all edges by a 150 nm layer of high quality thermally grown SiO\textsubscript{2}.

Large Unilamellar Vesicle (LUV) Preparation. The LUVs containing nystatin were prepared as explained before.\textsuperscript{33} The mixture of azolectine (20 mg/mL) containing 20 mol % ergosterol and 25 μL/mL of nystatin (from a 2.5 mg/mL stock solution in dry methanol) in chloroform was vacuum-dried under reduced pressure. The dried thin lipid film was rehydrated in 2 mL of sodium phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, pH 8). The suspension was vortexed for 5 min and sonicated in a water bath for 2 min. The lipid vesicle suspension was subjected to six freeze−thaw cycles in liquid nitrogen and a 50 °C water bath, respectively. Finally, they were sized using a miniextruder (Avanti Polar Lipids) by eleven times passage through a polycarbonate filter with a pore size of 400 nm.

Reconstitution of Mechanosensitive Channel of Large Conductance into LUVs. MscL channels were isolated as explained before.\textsuperscript{34} Briefly, G22C MscL with C-terminal 6Histag\textsuperscript{35} was expressed in the mscL-knockout Escherichia coli strain PB104,\textsuperscript{36} using the pB10a expression vector.\textsuperscript{32} A 10 L culture was grown in Luria−Bertani (LB) medium containing 100 μg/mL ampicillin in a batch fermentor at 37 °C, and the protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma). The cells were passed twice through a French Press at 15 000 psi, and membrane fractions were isolated by differential centrifugation.\textsuperscript{35} Membrane vesicles (25 g wet weight) were suspended in 25 mM Tris−Cl, pH 8.0, and stored at −80 °C. For protein isolation, typically, 3 g wet weight of membrane vesicles were solubilized in 30 mL of extraction buffer (10 mM Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4}, pH 8.0, 300 mM NaCl, 35 mM imidazole, 2% (v/v) TritonX-100). The solubilized fraction was cleared by centrifugation (40 000 rpm, 45 min, 4 °C) and applied onto a nickel-nitriloacetic acid (Ni-NTA) metal-affinity column. After washing steps, the pure MscL was eluted with 10 mL of elution buffer (histidine buffer containing 235 mM histidine). Typical isolations yielded 3.4 mg of MscL protein (Bradford assay) which was >98% pure as analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

In order to reconstitute MscL into lipid membranes, LUVs were titrated with 0.1 volume of detergent Triton-X100. Detergent-solubilized pure MscL was added to LUVs at a protein−lipid ration of 1:75 (w/w), and this mixture was incubated at 50 °C for 30 min. After this period, one volume of 1.5 M glucose was added to the MscL−LUV mixture. The detergent was removed by incubating the mixture with 300 mg wet weight of Bio beads (Bio-Beads SM2, Bio-Rad) at 4 °C for overnight. This procedure generated uniform proteoliposomes with 100 nm average diameter containing 750 mM glucose.

Forming a Suspended Lipid Bilayer over a 3 μm Pore. A horizontal, custom-made transparent polymethyl metacrylate (PMMA) measurement chamber (75 mm \times 30 mm \times 10 mm) was used for the chips with a 3 μm hole. The chamber has a 10 mm \times 10 mm central indentation with a pore (7 mm in diameter) in its center. For BLM formation, the chip was glued to the center of the chamber at 60 °C, 1 h using polydimethylsiloxane (PDMS). Next, the chip was inverted, and the trans compartment was filled with 0.8% melted agarose (in 1 M NaCl). After the agarose was solidified, the holder was turned back. A lipid bilayer across a 3 μm hole was formed using the painting method.\textsuperscript{8} In these smaller pores, azolectine (20 mg/mL) was used as a lipid and 10 mM Tris, 250 mM NaCl, pH = 7.4, was used as the buffer on the cis side.

**Electrophysiology Measurements.** Ionic currents passing through the alamethicin or MscL channels were measured by a Warner Instruments planar lipid bilayer workstation. Data were amplified (BC-535D Bilayer Amplifier; Warner Instruments, Hamden, CT), digitized (DigiData 1440A; Axon Instruments, Foster City, CA), and stored on a computer using the Axoscope (version 10.2; Axon Instruments, Foster City, CA).

**ASSOCIATED CONTENT**

Supporting Information Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.
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