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Scalable micro-cavity bilayer lipid membrane arrays for parallel ion channel recording

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ABSTRACT

A compact, scalable and high-throughput bilayer ion channel recording platform capable of simultaneous data acquisition from multiple bilayers is presented. Microfluidic chips house micro-cavities over which bilayers are made; each connected to a custom-made compact electronic readout circuit based on ASICs (Application-Specific Integrated Circuits). The micro-cavities are fabricated using a simple dry-film resist process on a glass wafer. Single 15 mm × 15 mm glass chips contain four separately addressable bilayers, each with integrated Ag/AgCl electrodes. The number of bilayers is scaled by increasing the number of ASICs and four-cavity chips. Each chip can be cleaned and re-used many times and the cavity-suspended lipid bilayers are stable for up to 10 days. System performance is demonstrated with simultaneous electrical recordings of the ion channels gramicidin A and α-hemolysin in multiple bilayers.

1. Introduction

Ion channel proteins play an important role in physiological processes and as drug targets they are of considerable interest to the pharmaceutical industry [1–3]. Electrophysiological analysis of these proteins provides detailed information about the function of ion channels and their modulation by pharmacological drugs. The two widely used systems for electrophysiological measurements are patch-clamped cells and aperture-suspended or supported lipid bilayers [4]. Patch clamp techniques are usually performed on living cells and require skilled manual operation. However, automated planar patch clamp techniques, based on microfabricated glass aperture chips rather than the conventional glass pipettes, have been developed and successfully commercialized in the past decade [5,6]. These instruments require a significant investment, due in part to the high cost of connecting each measurement site to a single commercial current amplifier, and are not configured for single-channel electrophysiology [5], which is related to problems with obtaining high quality GOhm seals between the cell and the glass aperture, which can lead to significant leakage currents that limit the signal quality [2].

Simplified biomembranes such as aperture-suspended lipid bilayers exhibit low leakage currents and are suitable for recording single-channel current events. The cost per data point is lower and throughput is higher than with patch clamp methods because cell culture and fluidic manipulation are not required [2]. Furthermore, it is relatively easy to obtain high quality single channel current recordings from artificial lipid bilayer systems. The principal limitations of lipid bilayer electrophysiology are (i) difficulty in forming stable long-lifetime bilayers and (ii) incorporation of purified vesicle-reconstituted membrane proteins into the bilayers. Recently it was demonstrated that the stability of artificial bilayer membranes can be significantly increased by using shaped apertures fabricated in negative resists such as SU8 [7], or complex (incline/rotation) exposure technique [8]. In order to increase throughput, bilayer arrays have been developed, for example using stereo-lithography to define chambers around a thin film of a hydrophobic polymer that includes an aperture for bilayer formation [9,10]. Both ease of bilayer formation and stability has been improved through the

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use of small cavities in the range 6–50 μm diameter [11–13]. However, small cavities have small in situ electrodes, which limits the chip lifetime or measurement duration [11,14]. Baaken et al. developed a 4 × 4 array of micro-cavities using a single layer of SU8 [11], and Ogier et al. fabricated micron sized cavities using a combination of SU8 and polymer films [12]. Suzuki et al. fabricated a 96 well planar bilayer structure using stereolithography, with apertures patterned in parylene [13]. Osaki et al. described a PDMS device with tens of micro-cavities as side chambers in a common microfluidic channel but only showed recording from one cavity [15]. Devices have also been made by bonding parylene film with pre-formed apertures to a backplane made using stereolithography [13]; parallel electrical recording was not possible and each well was sequentially switched to a recording amplifier [13]. Parallel recording using large commercial multi-channel (8 channel) amplifiers has been reported [9,11,16]. This approach is costly and the long wires increase electrical noise. To minimize parasitic capacitance a fully integrated platform has been described, which uses a custom made amplifier integrated directly beneath a solid-state nanopore [14]. Although this significantly reduces parasitic capacitance, thereby increasing the bandwidth, it is incompatible with the need for a disposable bilayer chip.

Previously we demonstrated a parallel recording platform that used small Application-Specific Integrated Circuits (ASICs) for continuously recording from several discrete bilayers made using the traditional vertical cup arrangement [17]. Both the digital control and analogue readout circuitry were fabricated on the same PCB, which led to an unacceptable level of noise due to crosstalk. The classical cup configuration also consumes comparatively large amounts of sample and imposes limits on scaling. To address this issue, we have designed a bilayer platform that uses a new design of ASIC and electronics, interfacing with a disposable glass microfluidic chip. The chip has four separate micro-cavities for four bilayers, each with its own integrated Ag/AgCl electrode, as shown in Fig. 1. The system can continuously record from multiple bilayers formed across apertures above cavities as shown in Fig. 1. The cavity has a diameter of 150–200 μm and is made using a layer of dry-film resist, with a thickness of ~55 μm. A second layer of dry-film resist partially covers this cavity, creating a smaller aperture of 20–100 μm diameter for the bilayer. This two-layer structure enables the bilayer and electrode size to be defined independently. A small aperture increases bilayer stability whilst a large cavity and electrode increases recording time. Each bilayer is connected via the integrated electrode to its own amplifier (ASIC) which sits directly next to the chip, interfaced using a simple edge connector. No wires are used to connect the bilayer chip to the electronics, and no electrical multiplexing is necessary, minimizing parasitic capacitance and electrical noise. In addition, the analogue and digital circuits are kept separate from each other, reducing electromagnetic interference and noise. The system is compact and has substantially less noise compared with other multiplexed bilayer platforms that use discrete off-the-shelf amplifiers [9,10]. The entire system is housed in an aluminium box and can be scaled as required; the design described here accommodates three chips (see Fig. 3) with a total of 12 bilayers, all of which can be recorded simultaneously.

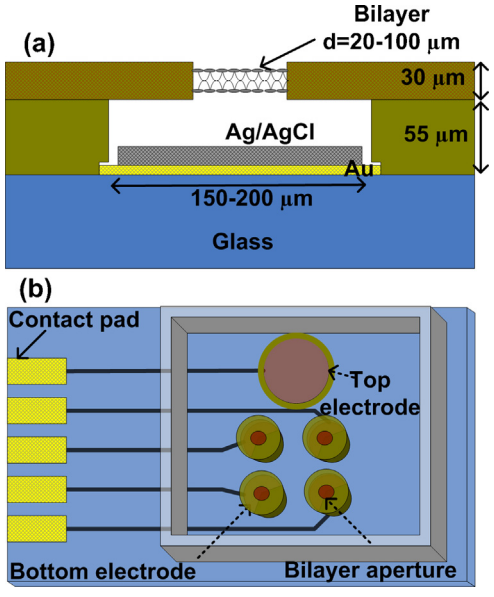

Fig. 1. (a) Schematic diagram of a micro-electrode cavity showing the large-area Ag/AgCl bottom electrode and the aperture for the suspended lipid bilayer (not to scale). (b) Diagram of a single chip with four separate cavities for bilayer formation, each with an integrated recording electrode. The chip also contains a common driven electrode that is in contact with the larger shared aqueous compartment on top of the bilayers. Each chip has five contact pads to interface with four ASICs and a common driven electrode.

2. Materials and methods

2.1. Fabrication of micro-electrode cavity chips

A four-mask process was used to fabricate the device according to the sequence shown in Fig. 2a. The substrate consisted of a 100 mm diameter 700 μm thick glass wafer. The first stage involved deposition and patterning of 200 nm gold (with 20 nm Cr adhesion layer) to define the electrodes and contact pads, using photoresist and wet etching. AZ9260 resist was spin-coated and patterned using a second mask to define areas for silver deposition. Subsequently, silver was electroplated onto the gold using a solution of 0.1 M $AgNO_3$ and 0.5 M NH_3. A two-electrode system was used with a large-area silver film as counter electrode. Electrodes were Ag plated for ~6 min at a current density of ~2 mA/cm^2, giving a silver thickness of 5–6 μm. After electroplating, a layer of AgCl was formed by immersion in $FeCl_3$ solution for 2 min [18].

After stripping the AZ9260 resist and cleaning, the cavity was defined from a single layer of TMMF S 2055 dry-film resist (S200 series, TOK, Kanagawa, Japan), laminated onto the wafer at 80 °C followed by soft baking for 5 min at 70 °C [19]. The protective foil was removed before soft baking and the resist exposed in a mask aligner (EVG 620) using an i-line filter for 40 sec at 11 mW/cm^2. Post exposure baking consisted of ramping to 90 °C for 2 min, with a hold at 90 °C for 3 min, then a ramp down to 30 °C for 5 min. The resist was developed in EC solvent for 8 min. A second layer of TMMF S 2030 was laminated over the first layer to define the aperture on top of the cavity. This was laminated at a temperature of 45 °C, the protective foil was removed from the resist, and the substrate was kept at room temperature overnight. The resist was then exposed for 27 s at 11 mW/cm^2 followed by a post exposure bake at 47 °C for 15 min in a convection oven with the resist facing downward, to prevent the unexposed resist falling into the cavity [20]. The resist was then developed in EC solvent for 7–8 min and the wafer was hard baked at 170 °C for 30 min.

Aperture-suspended bilayers require a hydrophobic support material. Therefore the surface of the chip was made hydrophobic by exposure to CF_4 plasma at 50 W for 2 min with 2% O_2 and 98% CF_4 at 50 mTorr pressure. Following this treatment, the TMMF contact angle increased to 115°. Fig. 2b and c shows optical and

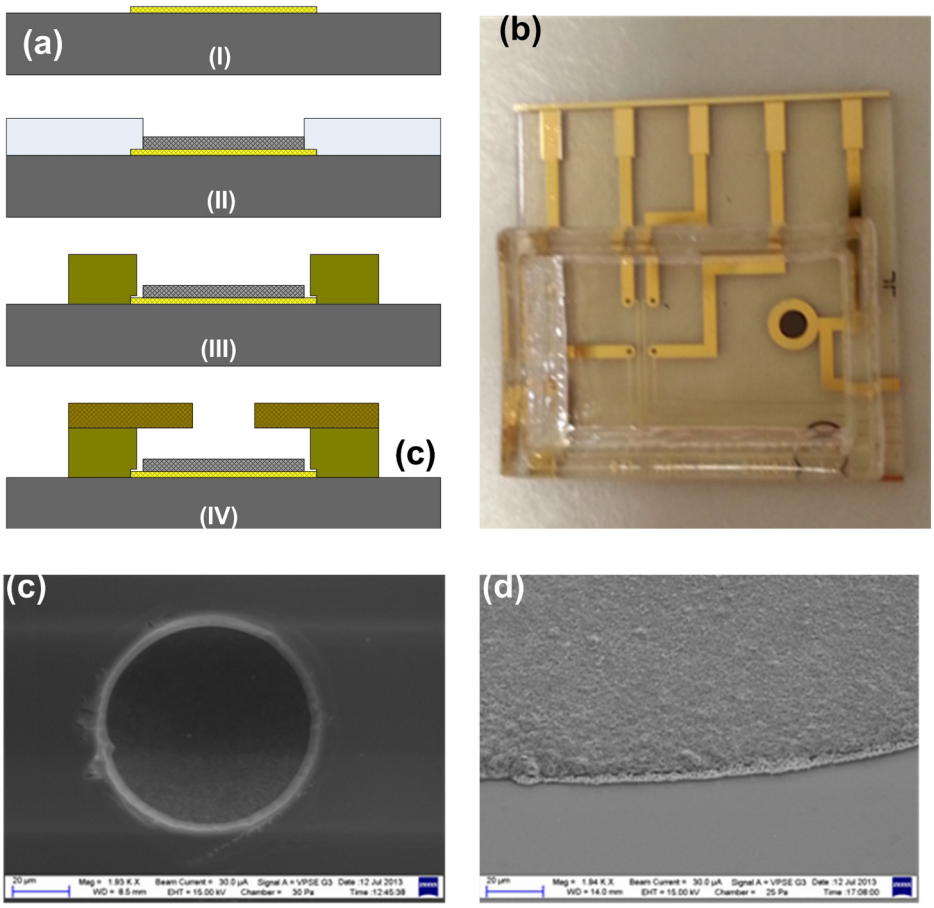

Fig. 2. (a) Fabrication sequence for manufacturing a micro-cavity from two layers of dry-film resist. (b) Optical image of the a single 15 mm × 15 mm bilayer chip. The four apertures can be seen together with the Ag/AgCl top electrode. (c) SEM image of a 75 μm diameter aperture made in TMMF dry film resist. (d) SEM image of the 1 mm diameter common Ag/AgCl electrode after electroplating and chlorination.

scanning electron microscope (SEM) images of a complete chip and bilayer aperture respectively, while Fig. 2d shows an SEM image of a chlorinated Ag/AgCl electrode.

2.2. Parallel ion channel recording

The circuits for digital control and analogue readout were made on two separate PCBs, a motherboard and daughterboard, respectively. The motherboard consists of an FPGA, power supply control circuits, connectors for daughter boards, and USB controller for data communications with a PC. Each daughterboard has four low-noise current amplifiers and interfaces with one bilayer chip. The separate digital and analogue front end minimizes electromagnetic interference from the digital circuitry. Each bilayer interfaces with a custom-made current amplifier and ADC (Analogue to Digital Converter), embedded into a single ASIC, made with CMOS 0.35 μm technology [17]. A photograph of a four-channel signal acquisition PCB, showing the four ASICs, is shown in Fig. 3a. Each ASIC has four different acquisition bandwidths, from 625 Hz to 10 kHz, and two different current ranges, ±200 pA and ±20 nA. The voltage stimulus is generated by the ASIC, with a range of ±450 mV, and the ASICs are individually programmable through a Serial Peripheral Interface (SPI). The signal acquisition PCBs and bilayer cavity

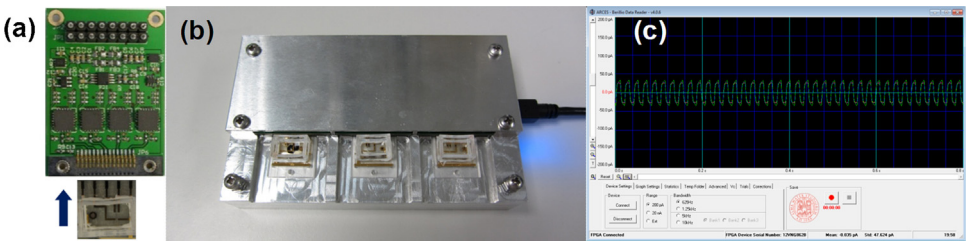

Fig. 3. (a) Four-channel signal acquisition PCB with four ASICs and an edge connector to interface with the bilayer chips. (b) Aluminium housing containing the motherboard, three daughter PCBs and three bilayer chips. This 12-channel recording platform has a dimension of 11.42 cm × 7.02 cm × 2.39 cm. (c) Screenshot of the Graphical User Interface for data display and storage.

chips are mounted on top of a motherboard that is housed in an aluminium casing, as depicted in Fig. 3b. The system can house 12 ASICs, interfacing with 3 bilayer chips, giving a total of 12 parallel channels. Digital data filtering and system control are performed with the FPGA on the motherboard, and the data is sent to a PC through a USB interface. A Graphical User Interface (GUI) in Visual Basic displays and stores data in real time, and is used to set the acquisition bandwidth, voltage stimulus and current ranges (Fig. 3c).

2.3. Reagents

All reagents except the phospholipids were obtained from Sigma–Aldrich (St. Louis, MO, USA). Electrophysiology buffers, either 150 mM KCl, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), pH 7.4, or 1 M KCl, 10 mM HEPES, pH 7.4, were prepared with deionized water. Stock solutions of α-hemolysin, prepared by solubilizing the protein in 150 mM KCl electrophysiology buffer to a concentration of 0.1 mg/ml, were aliquoted and stored at −80 °C. Stock solutions of gramicidin A (5 ng/ml) in ethanol were stored at −20 °C. The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG) and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and solubilized in chloroform (20/40 mg/ml). The appropriate amount of lipid–chloroform solution was dried in a desiccator to obtain a thin lipid film that was subsequently re-solubilized in decane to a concentration of 20 mg/ml. For mixed-lipid bilayers, the lipids were mixed in chloroform solution prior to drying and resolubilization in decane.

3. Results and discussion

3.1. Optimization of bilayer aperture edge smoothness

In order to facilitate bilayer formation and increase bilayer stability, it is important that the bilayer aperture wall is smooth. The protective foil on top of the dry film resist affects the smoothness of the aperture [19] because the foil scatters the exposure light causing roughness in the resist. In addition, the metal chuck of the mask aligner can also reflect light to some extent and may affect the smoothness of the aperture wall. Therefore, to determine the optimal process conditions the TMMF was exposed with and without protective foil under otherwise identical conditions. A non-reflective white paper was also used between the glass wafer and the metal chuck to improve the edge smoothness. Images of the aperture for all these different exposure conditions are shown in Fig. 4. It can be seen that the protective foil has a significant impact on the smoothness of the aperture wall. The non-reflective paper placed on the chuck has only minor effects, substantially less than the protective foil. In the fabrication process shown in Fig. 2a, the electrode lies directly below the aperture (for top side exposure) and therefore the white paper was not used. However, the protective foil was removed from the dry film resist after lamination, prior to exposure of the bilayer aperture.

3.2. Bilayer formation and stability

Bilayers were formed as follows. The top compartment of the chip shown in Fig. 1b was filled with electrophysiology buffer (150 mM or 1 M KCl, 10 mM HEPES, pH 7.4) and the chip was placed in a desiccator with mild vacuum to draw the buffer into the bottom

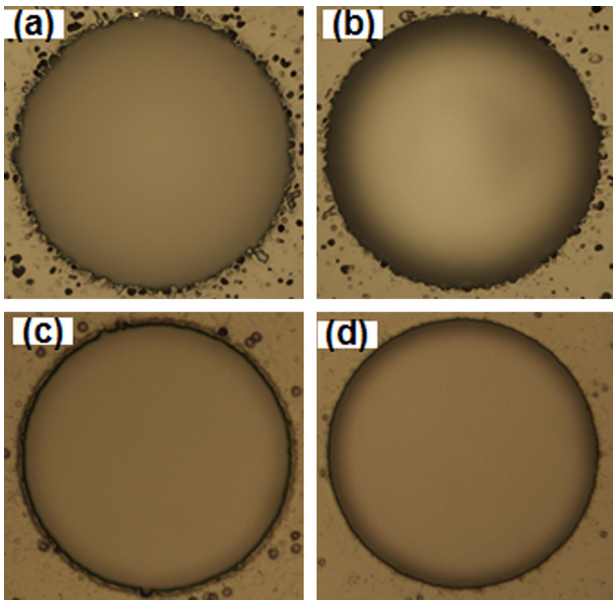

Fig. 4. Optical image of a 200 μm aperture made in TMMF S2030 photoresist for various exposure conditions. (a) With protective foil and without white paper on the chuck. (b) With protective foil and with white paper on the chuck. (c) Without protective foil and white paper on the chuck (d). Without protective foil and with white paper on the chuck. The process conditions are as described in the text.

compartment by displacing cavity-trapped air. The chip was then plugged into the recording electronics as shown in Fig. 3b. Bilayers were made by painting lipid dissolved in decane at 20 mg/ml over the micro-cavity apertures. Most of the time, bilayers formed immediately but occasionally the initial capacitance was small, 4–5 pF, implying insufficient thinning of the decane-lipid film. In this case, bilayers were quickly formed by removing and replacing the buffer solution using a disposable Pasteur pipette, which enables excess solvent to drain away [21]. The capacitance of a bilayer suspended across a 75 μm diameter aperture was typically 15–20 pF, with a background capacitance of ∼2–3 pF. Bilayers formed across these apertures were stable for several days. For example bilayers of DPhPC formed on 20 μm diameter apertures were stable for up to 10 days, evaluated by continuously recording the bilayer current with +100 mV applied potential across the bilayer, and regularly checking the bilayer capacitance. Buffer was topped up occasionally to compensate for evaporation. The DC current trace at day 1 and day 10 is shown in Fig. 5.

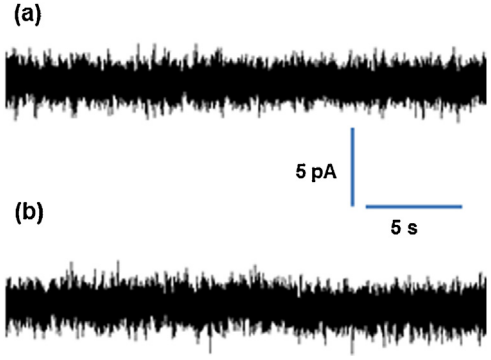

Fig. 5. DC current for bilayer stability measurements at (a) day 1 and (b) day 10 with a 100 mV DC potential applied continuously.

The variation of bilayer current remained below 3 pA. These bilayers could withstand potentials of up to 500 mV before rupturing.

3.3. Electrode stability and noise

The electrode stability was evaluated by measuring the DC current through the aperture without a bilayer for several days. The chip was filled with 150 mM KCl solution and a potential of 100 mV was applied to the electrodes in series with a 1 GOhm resistor to limit the current. The current was recorded for ten days (ID 526 BLM amplifier, Industrial Developments Bangor, UK) and was stable at 93.5 ± 1 pA for the entire time. This demonstrated that the large area integrated Ag/AgCl electrodes can sustain continuous recording for weeks at currents in the tens of pA range.

The electrical noise was evaluated with a bilayer of POPC:POPG (1:1) painted across a 75 μm diameter aperture (bilayer capacitance of ~17 pF) with an applied potential of +100 mV in 150 mM KCl. The current trace was recorded at 5 kHz bandwidth and filtered at 1 kHz, giving a noise of ~0.4 pA rms.

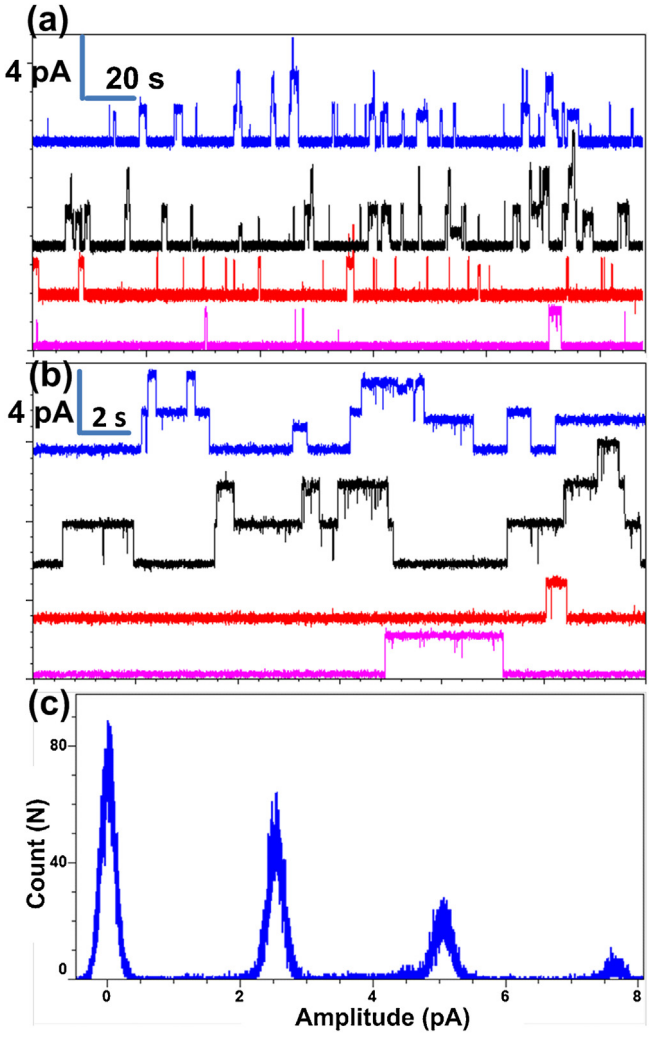

Fig. 6. Gramicidin ion channel current traces at applied potential of +100 mV, software filtered at 100 Hz of (a) 270 s duration and (b) 30 s duration, recorded simultaneously from four bilayers of DPhPC in decane on 75-μm diameter apertures. (c) The current histogram for a 30 s trace shows discrete current levels of ~2.5 pA. The buffer solution is 1 M KCl, 10 mM HEPES, pH 7.4.

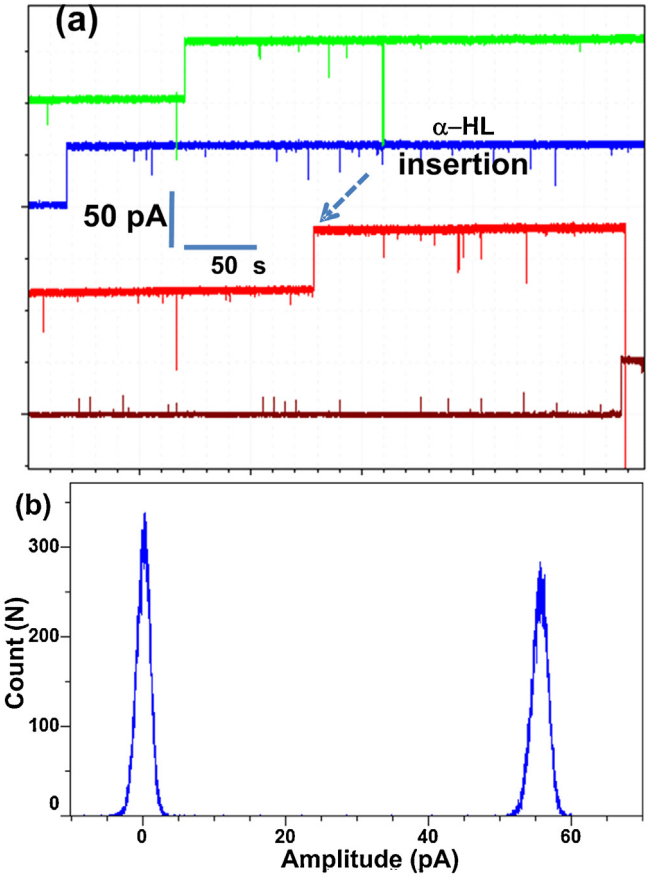

Fig. 7. (a) α-Hemolysin current traces recorded simultaneously from four bilayers of DPhPC across 75-μm diameter apertures, digitally filtered at 100 Hz. (b) The current histogram for a 60 s trace indicates a channel opening of 56 pA. The electrophysiology buffer is 1 M KCl, 10 mM HEPES, pH 7.4 and the applied potential is +50 mV.

3.4. Parallel ion channel recording

After the formation of bilayers by painting a DPhPC-decane solution over four 75-μm diameter apertures, an ethanolic solution of gramicidin was added to the top compartment, containing 1 M KCl buffer, to a final concentration of 50 pg/ml. The ASICs were used to apply a DC potential of 100 mV and to measure the current. The signal was digitized at an oversampling frequency of 1.25 MHz and filtered at 625 Hz using the built-in digital filter of the FPGA on the motherboard. Exported data was post-processed and low pass filtered at 100 Hz with Clampfit software (Molecular Devices, Sunnyvale, CA, USA). Fig. 6a and b shows gramicidin channel activity simultaneously recorded from the four bilayers, with a current histogram for a 30 s trace shown in Fig. 6c. The current amplitude at +100 mV is 2.5 pA, closely matching literature values [4,22,23].

Next, DPhPC bilayers were formed across four 75-μm apertures and α-hemolysin was introduced into the top compartment, containing 1 M KCl, 10 mM HEPES, pH 7.4, to a concentration of 2.5 μg/ml. Within 5 min, α-hemolysin pore insertion was observed in each of the four bilayers, as shown in Fig. 7a. Channel openings

at an applied potential of +50 mV were ~56 pA (Fig. 7b), typical for α-hemolysin nanopores [16,24,25].

4. Conclusions

We have developed a parallel scalable bilayer platform for ion channel recording from multiple bilayers. The system uses disposable glass chips interfaced directly with miniature discrete recording amplifiers, one per bilayer. The bilayers are made in apertures over cavities manufactured using laminated dry film resist. Two layers of laminated dry film are used to produce a large well with integrated Ag/AgCl electrode and apertures of variable dimensions, depending on the mask used.

To demonstrate the functionality of the device, simultaneous recording from four bilayers was made using gramicidin A and α-hemolysin channels. For both gramicidin A and α-hemolysin, it was observed that ion channels readily insert into all four bilayers simultaneously over a very short period of time, typically a couple of minutes or less. Previous reports of parallel bilayer platforms indicated channel insertion probability of typically 50% or less [11,13,16]. Additionally, larger diameter bilayers (>60 μm) routinely support ion channel recording from reconstituted protein such as KcsA and Na_V pore domain (data not shown).

The custom-made ASICs sit adjacent to the bilayer chips, reducing both the electrical noise and producing an extremely compact platform. The hydrophobic apertures made in the dry film resist facilitate easy formation of bilayers that are electrically and mechanically stable for several days. The Ag/AgCl electrodes can support a high current of ~100 pA for many days without noticeable degradation. The separation of digital signal processing and analogue front end provides the opportunity to assemble many daughter boards on the same mother board. This modular architecture can be easily scaled in multiples of four channels to produce compact platforms capable of recording from many bilayers simultaneously.

Acknowledgement

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Biographies

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