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A Cell-Culture-Type Planar Ion Channel Biosensor

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We have developed a new planar-type ion channel biosensor with a silicon-on insulator substrate and a cell culture function. Fibronectin is coated on the substrate surface to promote cell growth in this sensor. A transient receptor potential vanilloid type 1 (TRPV1) channel-expressing HEK293 cell is positioned on the micropore of the SOI sensor chip and incubated. Although the seal resistance was quite small, 10-20 MΩ, compared with that of the conventional pipette patch-clamp method, the signal-to-noise level was sufficiently high. However, a much lower noise level is required for observing the opening and closing of fewer than 30 channels.
Key words: Biosensor, Ion channel, SOI substrate, TRPV1

1. INTRODUCTION

Ion channels in the cell membrane play important roles in maintaining cell life functions. The function of the ion channel molecules can be directly measured using the patch-clamp method [1]. The activity of a single molecule or of several molecules is recorded by measuring the ion current flowing through the ionchannels. However, there are several problems with the conventional patch-clamp method using a pipette when it is applied to high-throughput screening and multi-point and multi-function simultaneous measurements of neural cell networks. In particular, miniaturization of the experimental apparatus is difficult, and operator must be highly skilled. The planar patch-clamp method [2-7] overcomes both problems. However, the lifetime of the cells in the measurement chamber is too short (less than 1 h) to be applied to cell function analysis.

We have developed a new planar-type ion channel biosensor that overcomes the problems of both the conventional pipette and planer patch-clamp methods. The biosensor has some advantages that significant miniaturization by combining with an Si integrated electrical circuit, which enables it to be applied to high-through put screening applications, that the operator does not need to be highly skilled. A cell culture function is incorporated into the extracellular solution chamber of the device, so the cells have a much longer lifetime.

2. MATERIALS AND METHODS

2.1 Sensor chip fabrication using SOI substrate

Previous, planar patch-clamp devices used Si, glass, plastic, and polydimethylsiloxane (PDMS) for the sensor chip [2-6]. However, Si induces high background current noise due to its low electrical resistance. We succeeded to reduce the noise current by using a P-type silicon-on-insulator (SOI) substrate with 50-75 Ωcm resistivity. The SOI substrate is composed 4 ± 0.2 μm SOI layer and 3 ± 0.5 μm SiO_2 layer [8]. The micro fabrication procedure for the substrate is shown in Fig.1.

1. A 1-μm thick SiO_2 layer is formed on the substrate surface by wet thermal oxidation using O_2+H_2O (95℃, bubbling) at 900℃ for 10 h.
2. A well 1 mm in diameter and 400 μm deep is formed on the backside by diamond grinding.
3. The well is enlarged so that it reaches the SiO_2 layer by anisotropic wet etching using 8% TMAH at 90 ℃ for 100 min.
4. A 1-μm thick SiO_2 layer is formed on the substrate surface by wet thermal oxidation

using O_2+H_2O (95℃, bubbling) at 900℃ for 10 h.

5. A micropore with a diameter of 0.7-1.5 μm is formed at the bottom of the well by focused ion beam (FIB, SII NanoTechology Inc.) milling.

Figure 1(b) shows a scanning electron microscope (SEM) image of a micropore. The wall is vertical and smooth.

A high-resistance seal is formed between the cell membrane and the sensor chip surface at their contact point to enable measurement of the small ion channel current. The sensor chip can be recycled by cleaning it, with acetone and methanol for 30 minutes, boiling it in piranha solution (H_2SO_4+H_2O_2[4:1]) for 30minutes, rinsing it with copious amounts of 18MΩ deionized water (mill-Q), and drying it by N_2 blowing. The chamber assembly with the integrated SOI substrate is schematically shown in Fig. 2.

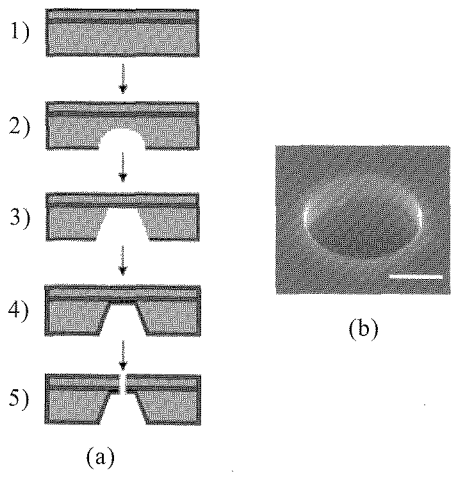

Fig. 1 (a) Schematic of sensor chip fabrication; (b) SEM image of micropore fabricated by FIB milling (scale bar 0.5 μm).

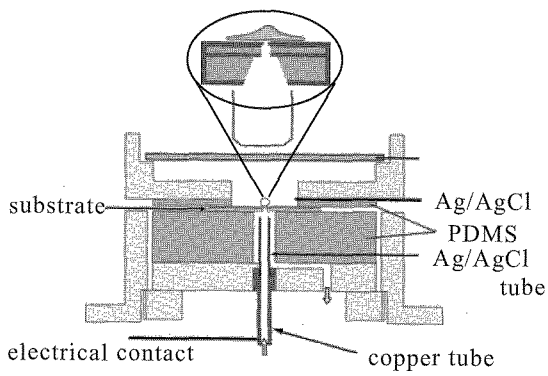

Fig.2 Schematic view of planar ion channel biosensor device with SOI substrate.

2.2 Fibronectin coating

The cell culture function incorporated into the extracellular solution chamber of the device enables cells to be cultured inside the device, so cell culturing and channel current measurement can be done sequentially. The device can thus be used for longer, time-lapse measurements.

Interaction between the cell adhesion membrane protein and the extracellular matrix is necessary for cell adhesion and growth. We coat the SOI substrate surface with fibronectin (FN), a cell adhesion glucoprotein, and culture the cell in the chamber. FN contributes to cell adhesion, proliferation, differentiation, and migration [9].

A tock solution of FN (F2006, Sigma) is prepared using deionized water at 1 mg/ml; it is diluted in phosphate buffered saline (PBS, WAKO) at 500 μg/ml. A droplet of FN solution with a density of 10-50 μg/cm^2 is placed on the SOI surface and incubated for 1 h at 37℃ under 5% CO_2. The substrate is rinsed with sterilized water, naturally dried, and kept it sterile before use.

2.3 TRPV1-transfected HEK293 cells

We introduce transient receptor potential vanilloid type 1 (TRPV1) channel-expressing HEK293 cells to evaluate the performance of oyr sensor (Fig. 2). TRPV1 is a ligand-gated nonspecific cation channel, which is mainly expressed in sensory nerves from the peripheral terminal to the central endings. It is activated by capsaicin, proton, or heat (≧43℃). We used capsaicin as the ligand molecule.

2.4 Cell culturing

HEK293 cells are cultured in culture dishes filled with culture medium (37℃, 5% CO_2). The culture medium is Dulbecco's modified Eagle's Medium (DMEM, SIGMA) with added 10% (v/v) fetal bovine serum (FBS, Biological Industries), 1% (v/v) Glutamax^{TM} (GIBCO), 0.5% (v/v) penicillin / streptomycin (GIBCO), and 0.1% (v/v) Geneticin® (G-418, GIBCO).

The culture medium is aspirated off and washed with PBS (WAKO) after 70-90% confluence. The n cells are then detached from the dish surface by agitating them several times with a pipette. Next, the cells are centrifuged at 190 ×g and resuspended in a mixture of DMEM at a concentration of 5×10^5-1×10^6 cells/ml. A cell suspension containing 3-5×10^4 cells is introduced into the upper chamber and cultured for 3-5 h in the sensor in accordance with the standard cell culture method. The FN-coated SOI substrate is set up beforehand in the chamber for the culturing and measurement.

2.5 Whole cell channel current measurements

The experimental conditions were similar to those used in conventional patch-clamp experiments. The extracellular solution in the upper chamber contained (in mM): 140 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, and 10 Glucose and had a pH of 7.4 (with NaOH). The intracellular solution in the lower chamber contained (in mM): 140 KCl, 5 EGTA, and 10 HEPES and had a pH 7.4 (with KOH). All the

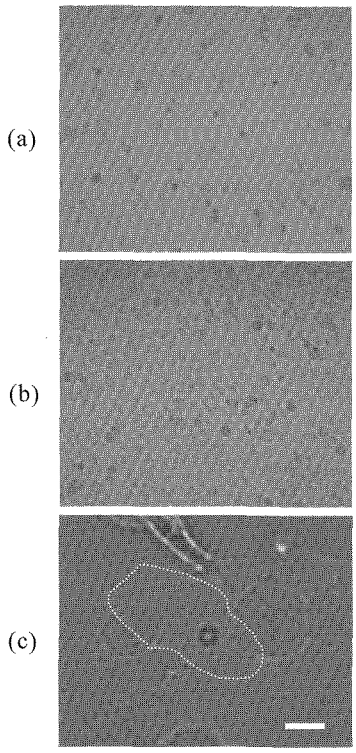

Fig. 3 Optical microscope images of cells on SOI substrate after 12 h incubation for a) non-coated and b) FN-coated substrates. c) Micropore covered by HEK-293 cell (scale bar 10μm).

chemicals were purchased from Sigma and were of analytical grade. The solutions were filtered through a 0.2-μm syringe filter before use to remove dusts. Ag/AgCl nonpolarizable electrodes were made by dipping Ag wire, 0.05 mm in diameter, into a sodium hypochlorite solution (commercial bleaching agent) for 5-6 h.

The electrical connection between the cell inside and the lower chamber electrode necessary for the whole-cell current measurements was formed by fabricating sub-nm conductive pores through the cell membrane by using nystatin. When an intracellular solution containing 100-200 μg/mlof nystatin was injected into the lower chamber, nystatin channels, which selectively transfer univalent cations such as K^+ and Na^+, formed in 5-10 min.

The lower chamber was connected to the input of a patch-clamp amplifier (Axopatch 200B), and the upper chamber was connected to the ground. Data were obtained with a 5 kHz cut-off frequency and an output gain of 5 mV/nA and were analyzed using pClamp9.2 software.

3. RESULTS AND DISCUSSION

Figure 3 shows the optical microscope images of the cells growing on SOI substrates with and without the FN coating. The FN coating clearly promoted cell growth. Although we did not control the cell positions, a cell was often positioned on the micropore, as shown Fig. 3(C).

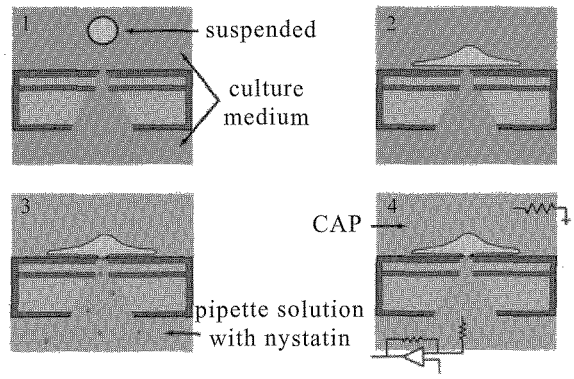

Fig. 4 Steps in planar ion channel current recording

Figure 4 shows the sequence of steps in the whole-cell current recording. The culture mediums in the upper and lower chambers were exchanged to the extracellular and intracellular solutions, respectively, for the ion channel current measurements.

A gentle suction, generated by a syringe pump in the lower chamber, was applied to the cell sited on the micropore to increase the seal resistance between the cell and the substrate surface around the micropore. The seal resistance before and after this suction was typically 6-8 and 10-20 MΩ, respectively. The intracellular solution was injected into the lower chamber. The nystatin caused the cell membrane contacting the micropore to perforate, as confirmed by an increase in the capacitance (5-10 pF) of the cell membrane, which was monitored while continuously applying a hyperpolarization pulse (100-200Hz, 5 mV)

The whole-cell current of the TRPV1, activated by capsaicin stimulation, was recorded, as shown in Fig. 5. The holding potential was set to -30 mV in the voltage-clamp. The decrease in the sensitivity unique to the capsaicin stimulation of TRPV1 with a Ca^{2+}-containing solution was observed [10, 11]. The observed seal resistance, 10-20 MΩ, is quite small compared to the corresponding value with the conventional pipette patch-clamp. The observed noise level, ~130 pA, was sufficiently small compared with the signal

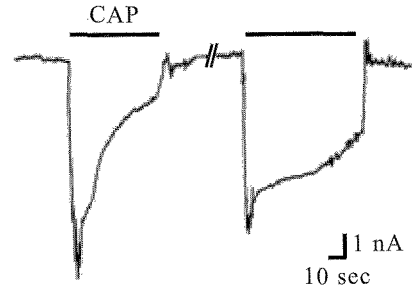

Fig. 5 Whole-cell channel current recording of TRPV1-transfected HEK-293 cell; current was activated by capsaicin stimulation.

level, ~7 nA. The observed current corresponds to the opening of about 1500 channels. This means that a much longer reduction in the noise level is necessary if we want to measure the whole-cell current corresponding to the opening of a few (<30) channels.

4. CONCLUSION

We fabricated a planar ion channel biosensor with a cell culture function and a silicon-on-insulator substrate. This greatly reduced size of the sensor, making it applicable to high-throughput screening. The cell culture function is incorporated into our biosensor and greatly lengthens the lifetime of the cells. As a result, this device can be applied for time-lapse measurements, which are difficult with the conventional patch-clamp method. The substrate surface is coated with fibronectin, which promotes cell adhesion and proliferation, and thus significantly improves the cell culturing. The electrical connection necessary for measurement is attained by perforating the cell membrane contacting the SOI substrate micropore by introducing nystatin solution into the lower chamber of the device. The signal-to-noise level was sufficiently high in the present observations. However, a much lower noise level is required if we want to observe the opening and closing of fewer than 30 channels.

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