INCUBATION TYPE PLANAR PATCH CLAMP BIOSENSOR
Basic Performances

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Keywords: Planar patch clamp, Ionchannel, Biosensor, HEK293, TRPV1, PMMA.

Abstract: The biosensors based on the incubation type planar patch clamp method was developed and the basic properties were investigated. Usefulness of light-gated ionchannel method on the performance of the device was confirmed. The excess current noise and the thermal noise due to the micropore resistance and the seal resistance were the main sources of the noise, and the noise level of the developed biosensor was 7 pA at the 1 kHz low pass filter. This value is slightly larger than the single ionchannel current level (~4pA) of TRPV1. We consider that the developed device has a sufficient performance for the whole cell measurements, and extremely suitable for the high throughput screening application with neural network, in which incubation function is essentially necessary.

1 INTRODUCTION

Although the patch-clamp method using the pipette is now in practical use, it is not suitable for miniaturization and high throughput screening applications, since the measurement system is large and requires high level of skills for operations. It is expected that the breakthrough for these technical problems can be realized by the planarization of the device. For the planar typed ion channel biosensor, glass (Fertig, 2002), Si (Sordel, 2006, Matthews, 2006, Pantoja, 2004), quartz (Sett, 2003) and a silicon elastomer PDMS (Li, 2006), etc. have been reported as the substrate materials. And for Si, it has been considered that the background noise current is large due to the free charge carrier density in the substrate. However, we have recently demonstrated that the noise current can be significantly reduced by using silicon-on-insulator (SOI) or polymethyl methacrylate (PMMA) substrate.

Commercialized planer patch clamp devices, however, can not be used in a system that requires long incubation periods. New functional analysis and/or screening devices could be realized by adding an incubation function to the planar patch clamp method, and these would be especially useful in applications such as in vitro systems of neurons and neural networks using dissociated cultured neurons (Tao, 2000, Taylor, 2010, Reska, 2008, Erickson, 2008). Moreover, the planar patch clamp method enables simultaneous measurement of multi-point ion channel currents and advanced 2-D bio-imaging. We have developed an incubation type of planar patch clamp device and demonstrated its operation using TRPV1-expressing HEK293 cells and capsaicin as a ligand molecule. However, detailed investigation about the basic properties have not yet been done.

The recently developed light-gated ion-channel method is extremely suitable for the investigation of neural cell and/or neural network functional analysis due to its excellent time and space resolutions (Petreanu, 2007). It is also suitable to the application for the investigation of the basic property of the ionchannel biosensors. Concerning the application of light-gated ion-channel in the planar patch clamp method, however, no investigation has been done, in spite of its extreme importance.

In this work, we have investigated the basic properties such as noise and sensitivity of the

Urisu T., Uno H., Wang Z., Obuliraj S., Takada N., Aoyama M., Suzui M., Asano T., Ishizuka T. and Yawo H..
INCUBATION TYPE PLANAR PATCH CLAMP BIOSENSOR - Basic Performances.
DOI: 10.5220/0003764301430148
In *Proceedings of the International Conference on Biomedical Electronics and Devices* (BIODEVICES-2012), pages 143-148
ISBN: 978-989-8425-91-1

incubation type planar patch clamp biosensor. Usefulness of light-gated ionchannel on the incubation type planar patch clamp method was also confirmed. Furthermore, the excellent performace of this biosensor has been examined by detecting capsaicin using TRPV1-expressing HEK293 cells.

2 MATERIALS AND METHOD

2.1 Fabrication of Biosensor Chip

2.1.1 Si Chip

Si on insulator (SOI) substrates were used to make the Si sensor chip s. The fabrication process of the chip is shown in Fig. 1. A thermal oxidation layer of about 1μm thickness was formed on the substrate surface by the wet thermal oxidation at 900°C using a water-saturated O_2 flow, after which a large well on the reverse surface was made by diamond drilling. A pyramid-shaped hole that reached the buried SiO_2 layer was formed by etching in 8% (v/v) tetramethylammonium hydroxide (TMAH) at 90°C for about 40 min. A micro-pore with a diameter of 1 ~ 2 μm was made on the silicon membrane by focused ion beam (FIB) milling from the reverse side, as shown in Fig. 1.

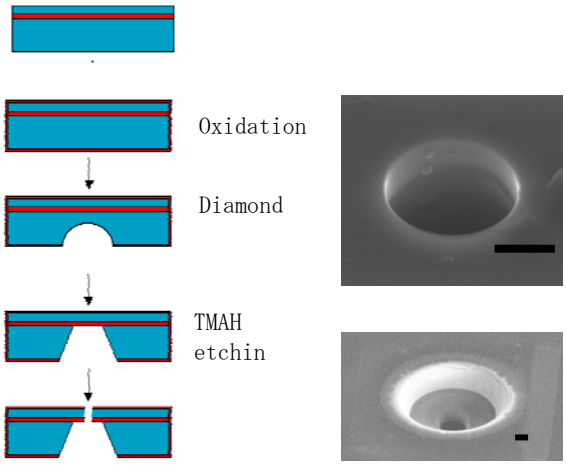

Figure 1: Fabrication process of the Si chip (left) and the top side (right upper) and back side (right lower) view of the chip. Scale bar is 0.5 μm.

2.1.2 Plastic (PMMA) Chip

Plastic substrate has several advantages such as lower cost, easiness in 3D micro structure formation and in surface chemical modifications. We have used PMMA for the substrate material of multi-channel devices. Fabrication process and the cross sectional view of the chip are shown in Fig. 2a and b, respectively. The basic structure of the substrate was formed by both side hot embossing, and the micropore was formed by FIB. The micro fluidic structure was formed on the upper surface of the substrate, and the pipette solution well was formed at the lower side.

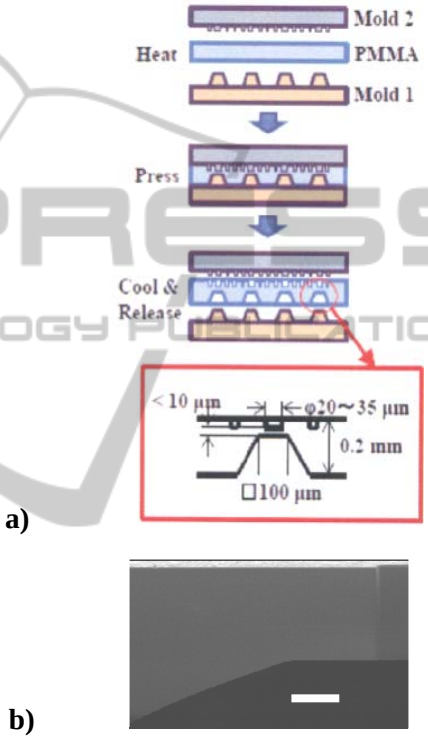

Figure 2: Fabrication process of PMMA chip (a) and the cross sectional view of the thin film region of the pipette solution well part observed by scanning electron microscopy (b). Scale bar is 5 μm.

The mold of brass for forming the pipette solution wells (Mold 1 in Fig.2a), by which thin film structures with 5~10 μm thickness were formed, was fabricated by ultra-precision machining equipment of Dr Omori's group, RIKEN Japan . Upper mold of nickel (Mold 2 in Fig.2a) for the micro fluidic structure formation was fabricated by electroforming, for which the master mold was formed by the photolithography using positive resist on the Si substrate.

2.2 Biosensor Chamber and the Stable Electrode

The 7 x 7 mm^2 (Si) or 11 x 11 mm^2 (PMMA) chip was sandwiched by PDMS plates, and the sensor structure was constructed as is shown in Fig. 3. We fabricated the stabilized AgCl/Ag electrodes, where the AgCl/Ag wire was inserted into glass tube of 1 mm inner diameter filled with the saturated KCl and AgCl solution and the top of the glass tube was sealed by the Vycor glass (Corning). We used these stabilized electrodes for both upper side (bath solution , ground) and lower side (pipette solution) electrodes of the biosensor.

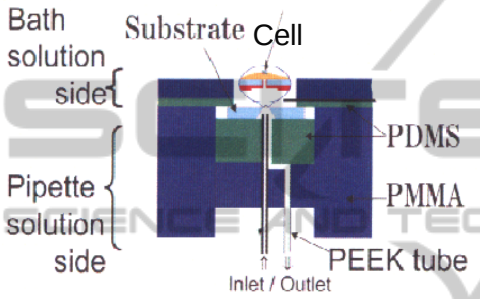

Figure 3: Schematic structure of the ion channel biosensor.

2.3 Expression of ChR2 and ChRWR

We used in this work channelrhodopsin 2 (ChR2) and the chimeric channelopsin between chop1 and chop2, which we call channelrhodopsin/wide receiver (ChRWR), as light-gated ionchannel molecules. HEK293 cells, which were a generous gift from Mr. Minoru Wakamori at Tohoku University, were cultured at 37℃ and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and transfected with cDNA of channelrhodopsin-Venus using Effectene transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. After cloning twice with the addition of G418 in a 10 cm dish, single colonies with bright Venus fluorescence were selected by using a cloning cylinder IWAKITE-32 (Asahi Glass Co., LTD, Japan) and cultured in a medium containing G418 until they were confluent in the dish.

2.4 Culture in Biosensor

The surface of the sensor chip was coated with extracellular matrixes (ECMs), collagen type 4 (BD), which was diluted using 1 mM HCl to 100 μg/ml. 50 μl of the solution was dropped on the substrate surface, followed by incubation for 2 ~ 4 h at room temperature. At this stage, the surface densities of the ECM were about 3 ~ 5 μg/cm^2. After removing excess solution, the substrate was rinsed with sterilized water, dried under a gentle nitrogen stream, and kept sterile before use. HEK293 cells were cultured in dishes filled with the medium under the conventional incubating conditions, i.e., 37°C and 5% CO_2. The culture medium was supplemented with DMEM to which 10% (v/v) FBS, 1% (v/v) GlutamaxTM (Gibco), and 0.5% (v/v) penicillin/streptomycin (Gibco) were added. After cells were detached from the culture dishes, the cell suspension was seeded at a density of 100 ~ 300 cells/mm^2 on the chip coated with ECM. Channel current was measured after about 70% confluence was reached in the biosensor.

2.5 Measurement of Light-gated Ionchannel Current

The electrical measurement systems were almost the same as those used in conventional pipette patch-clamp experiments. The culture medium was replaced with buffer. We used several of the buffer solutions reportedly used in experiments on ChR2. A typical bath solution in the upper chamber contained (in mM): 140 NaCl, 3 KCl, 10 4-(2-hydroxyethyl)-1–piperazineethanesulfonic acid (HEPES), 2.5 $CaCl_2$, 1.25 $MgCl_2$, and 10 glucose at pH 7.4 (with HCl). The lower chamber solution (pipette solution) contained (in mM): 40 CsCl, 80 $CsCH_3SO_4$, 1 $MgCl_2$, 10 HEPES, 2.5 MgATP, 0.2 Na_2EGTA, (pH 7.4). All data were recorded using a patch-clamp amplifier (Axopatch 200B) at room temperature. Data were obtained at a 1-kHz cutoff frequency and an output gain of 1 mV/pA, and they were analyzed using pClamp 9.2 software. For whole-cell current recordings, sub-nm conductive pores through the cell membrane, which electrically connected the inside of the cell to the lower chamber, were formed by applying nystatin (Sigma) solutions to the lower chamber. The nystatin stock solution was prepared by dissolving nystatin in 1 ml of methanol and successively adding 45 μl of HCl (1 M) and 45 μl of NaOH (1 M), which was then diluted with the lower chamber solution to final concentrations of 100-200 μg/ml before use. The formation of the whole-cell arrangement was confirmed by there being a capacitance increase of about 6 pF in a time interval of 5-10 min after addition of the nystatin solution to the lower chamber. The laser beam from the semiconductor laser with a 473-nm peak wavelength and 3.2-mW

maximum output power (Sumitomo Osaka Cement Co.,Ltd) was guided by optical fiber and focused with a micro lens with a 26.5 mm focusing length under the fluorescence microscope's objective lens (OLYMPUS). The diameter of the laser beam at the focusing point was 30 - 100 μm.

3 RESULTS AND DISCUSSION

3.1 Noise Properties of Incubation Type Planar Patch Clamp Biosensor

We consider the noise properties using the equivalent circuit of the planar patch clamp biosensor shown in Fig.4. The first noise source is the current noise (I_h) resulting from the interaction of the head-stage input voltage noise (e_n), the input capacitance (C_t) and the frequency bandwidth of the circuit (B), described by eq. (1) (Mayer, 2003). In the following, all current noises are given by root-mean-square (rms) values.

$$I_h^2 = (4/3)e_n^2 \pi^2 C_t^2 B^3 \quad (1)$$

$$C_t = C_m + C_s \quad (2)$$

We used a value of e_n = 2.3 x 10^{-9} V $Hz^{-0.5}$ reported for the cooled type capacitor feedback patch clamp amplifier (Axopatch 200B). C_t is the total input capacitance given by eq. (2), C_m is the membrane capacitance and Cs is the capacitance of the substrate. The thermal voltage noises (Johnson voltage noise) due to the access resistance R_a and seal resistance R_j cause the current noise, I_{Ra} and I_{Rj}, respectively as shown in eq. (3) (Mayer, 2003). The resistances of the ion channels, R_m and R_p are usually sufficiently larger than R_a and R_j, thus the contribution to the thermal noise can be ignored. In the incubation type planar patch clamp, contribution of the seal resistance to the thermal noise often can't be ignored.

$$I_R^2 = 4kTB/R \quad R = R_a \text{ or } R_j \quad (3)$$

where, k is the Boltzmann constant (k = 1.38 x 10^{-23} $J K^{-1}$), T is the absolute temperature.

In the case of the low seal resistance region, 1/f noise called excess noise, which possibly is generated by the current flowing at the narrow micro pore region, becomes important. The spectral density of the excess noise current, S_{ex}^2 , is given by

$$S_{ex}^2 = KI^2/fR_a^2 \quad (4)$$

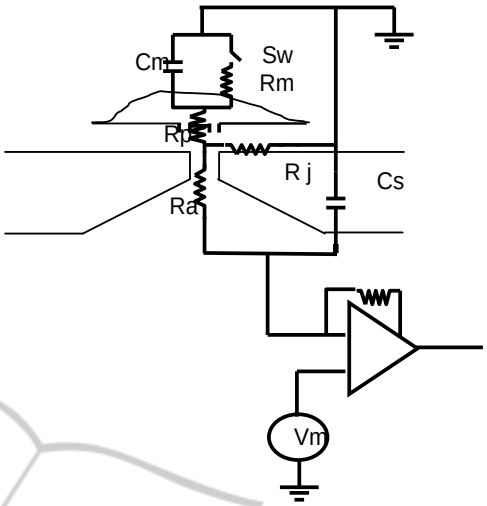

Figure 4: Equivalent circuit of the planar patch clamp biosensor. C_m : cell membrane capacitance, Sw: ionchannel, R_m, R_p: resistance of corresponding ion channel, R_j: seal resistance, R_a: access resistance, C_s: capacitance of the substrate, V_m: applied membrane voltage.

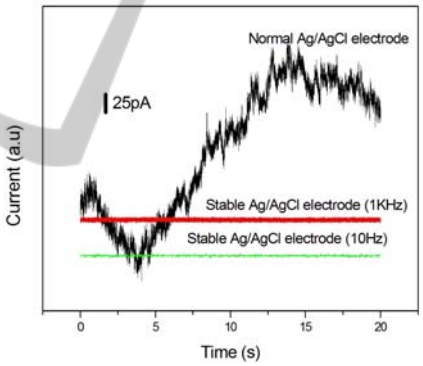

Figure 5: Observed current noise in the biosensor system shown in Fig. 3 using PMMA substrate, for normal and stabilized AgCl/Ag electrodes.

Where K is a constant and I is total current which is approximately given by $V_m/(R_j + R_a)$. The total rms value of the current noise (I_t) can be calculated using eq. (5).

$$I_t = (I_h^2 + I_{Ra}^2 + I_{Rm}^2 + I_{ex}^2)^{1/2} \quad (5)$$

Other than these intrinsic noise, fluctuation of the offset voltage (ΔV_m) of the electrode, which often becomes larger than 1 mV, causes the significant fluctuation of the base line. So it is often important to use the stablized electrode in the incubation type planar patch clamp as shown in Fig. 5. Here we calculate the noise current for the typical cases, C_t = 20 pF, B = 10^3 Hz, R_a = 2 MΩ and R_j =

10 MΩ, I_h = 0.005 pA, I_{Ra} = 2.8 pA, and I_{Rj} = 1.2 pA are obtained. So, if I_{ex}^2 is ignored, the total rms value of the current noise is evaluated to be I_t = 3.1 pA. This value is slightly smaller than the observed value ~ 7 pA (1kHz) shown in Fig. 5. The difference, ~ 4 pA, may be due to the excess noise, since 1/f noise is dominant in the present seal resistance region (data are not shown).

From these analysis, it is concluded that the measurement of the whole cell mode which usually gives the current level of several tens pA or larger can be easily attained by using the stable electrode. It is, however, necessary to increase the seal resistance by about one order of magnitude (~100 MΩ) to realize the single channel recordings in our system.

3.2 Light-gated Ion Channel Method in the Incubation Type Planar Patch Clamp Biosensor

The recently developed light-gated ion channel method has brought a significant progress into the neural network analysis field due to its temporal and spatial high resolutions. Since the in vitro neural network analysis and its application to the high throughput screening is an important application of the incubation type planar patch clamp method, here we investigate the basic property of the light-gated ion channel on the incubation type planar patch clamp biosensor.

ChRWR-expressing HEK293 cells were seeded on the chip of the incubation type planar patch clamp biosensor. After the cells became almost completely confluent, we observed the characteristics of the laser stimulated channel currents as shown in Fig. 6. The observed channel current pulse signals were similar in shape and signal to noise ratio to those observed in the pipette patch clamp method, although the seal resistance was much smaller than a giga-ohm. These data suggest that light-gated method is useful not only in the neural network analysis but also useful in the performance test of the biosensor due to its simplicity in handling.

3.3 Biosensor Operation using TRPV1-expressing HEK293 Cells

The unique points of ion channel biosensor is highly selective direct responses to various kind of ligand molecules and also sensitive responses to physical stimuli. Ion channels are also important drug targets and the biosensor is a potentially unique device for high throughput drug screening. It also finds its application in the detection of biological warfare agents (Bayley, 2001).

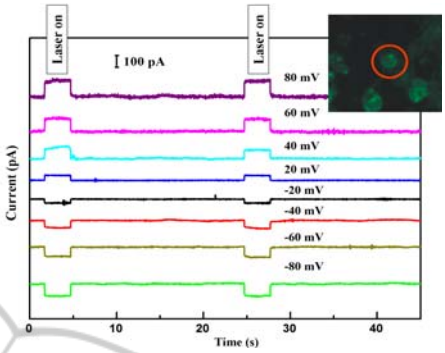

Figure 6: Observed ion channel current under voltage clamp of 473-nm laser irradiation with ChRWR-expressing HEK293. Ion channel current wave forms depend on the applied membrane potentials.

TRPV1 receptor, a nonspecific cation channel with preference for Ca^{2+}, is mainly expressed in sensory nerves from peripheral terminal to central endings, which can be activated by vanilloids such as capsaicin. Capsaicin is the pungent ingredient of hot pepper, which elicits a sensation of burning pain by selectively activating sensory neurons to transfer the noxious stimuli to the central nervous system (Caterina, 1997).

We have constructed ionchannel biosensor using TRPV1-expressing HEK293 cells (gift from Prof. Tominaga at National Institute for Physiological Sciences) and applied to the capsaicin detection.

The surface of the SOI sensor chip were coated with collagen type 4. After the cell covered on the pore and spread, the resistance R_j of the cleft between the cell membrane and substrate surface was measured (10.2 MΩ), then the perforated whole-cell configuration was formed by the application of nystatin to the pipette solution side. Then, the whole-cell current of TRPV1-expressing HEK 293 cell activated by capsaicin application was recorded as shown in Fig. 7.

The concentration of capsaicin was 3.3 μM. The desensitization was observed in the second injection of capsaicin (Caterina, 1997). The noise level of our experimental station was 7 pA using 1 kHz low pass filter (Fig. 5). The magnitude of the single channel current of TRPV1 is observed by pipette patch clamp method to be about 4pA at the membrane voltage +60 mV (Caterina, 1997). So we think that single channel recording maybe realized even in our incubation type planar patch clamp system by several times increase of the seal resistance.

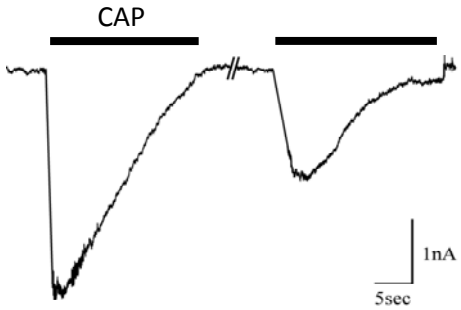

Figure 7: Whole cell channel current record of TRPVI expressed on HEK293 cell by capsaicin stimulations measured by ion channel biosensor based on the incubation type planar patch clamp method.

4 CONCLUSIONS

Ion channel biosensor based on the incubation type planar patch clamp method was developed and the basic properties were investigated. Due to the existence of ECM protein at the cleft between the cell membrane and the substrate surface near the miropore, it is not easy to realize the high seal resistance (giga-ohm seal). In the present case using collagen 4 as ECM, the seal resistance was usually about 10 MΩ, and the noise level was 7 pA with the 1 kHz low pass filter. The main noise sources were excess current noise and the thermal noise generated at micro pore resistance (R_a) and the seal resistance (R_j). All these noises can be reduced by increasing the seal resistance. Operation of the light-gated ion channel, ChRWR, was investigated by the incubation type planar patch clamp method using laser (λ = 473 nm) stimulations. The channel current profile and its membrane potential dependence well agreed to the reported data measured by pipette patch clamp method. So we think that light-gated method is also useful in the neural network function analysis and high throughput screening application based on the incubation type planar patch clamp method, and also useful in the simple performance check of these devices. The biosensor operation was examined using TRPV1-expressing HEK293 cells. Quite high sensitivity was confirmed. But for the single channel recording, more than several times improvement of the seal resistance is required.

ACKNOWLEDGEMENTS

We appreciate Dr. Hitoshi OHMORI and Mr. Yosuke HACHISU at RIKEN for their support in making brass mold by ultra-precision machining equipment.

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