Multichannel incubation type planar patch clamp biosensor using plastic (PMMA) substrates and characterization by laser-gated ion-channel protein

Zhi-Hong Wang[1,2], Hidetaka Uno[1,2], Noriko Takada[3], Obuliraju-Senthil Kumar[1,2], Toru Ishizuka[2,4], Hiromu Yawo[2,4], Tsuneo Urisu[1,2]

[1]Research Center for Innovative Nanobiodevices, Nagoya University, Furo-cho, Chikusa-Ku, Nagoya 464-8603, Japan

[2]JST, CREST, K's Gobancho, 7 Gobancho, Chiyoda-ku, Tokyo 102-0076, Japan

[3]Institute for Molecular Science, Myodaiji, Okazaki 444–8585, Japan

[4]Department of Developmental Biology and Neuroscience, Tohoku University Graduate School of Life Sciences, 2-1-1 Katahira, Aobaku, Sendai, 980-8577, Japan

ABSTRACT

Recently we have developed 4 channel incubation type planar patch clamp biosensor with polymethylmethacrylate (PMMA) substrate by using both-side hot embossing, focused ion beam micro-fabrication and surface modification by extra cellular matrix. We have evaluated the performance by using the light-gated ion-channel protein, channelrhodopsin wide receiver (ChRWR) expressed on HEK293 cell. Observed laser-induced current profile and the membrane potential dependences well agreed to those observed by pipette patch clamp, suggesting its excellent possibility in important applications such as portable ion channel biosensors and high throughput screening with neural systems.

KEYWORDS

Patterned planar patch clamp biosensor, multichannel, PMMA, lithography, focused ion beam, HEK293, ion channel current

INTRODUCTION

Ion-channel biosensor should find its use in diverse applications from the detection of biological warfare agents to the high throughput screening devices for the pharmaceutical development, because of its extremely high selectivity and sensitivity. Pioneering works were done by pipette patch clamp. However, it is not suitable for the multipoint measurements which are essentially necessary for high throughput screening applications. Recently-developed planar patch clamp opened newly the way to the high throughput screening applications. Since long time incubation is impossible in the conventional planar patch clamp devices, application to the neuro-science and high throughput screening in neural system is hindered. Recently, our group have developed incubation type planar patch clamp ion channel biosensor by using Si substrates and succeeded in demonstrating the excellent performance equivalent to the pipette patch clamp concerning channel current waveforms and membrane potential dependences in the whole cell mode operations [1]. In our previous devices, however, device fabrication process was not suitable for extending to the multi-channel structures.

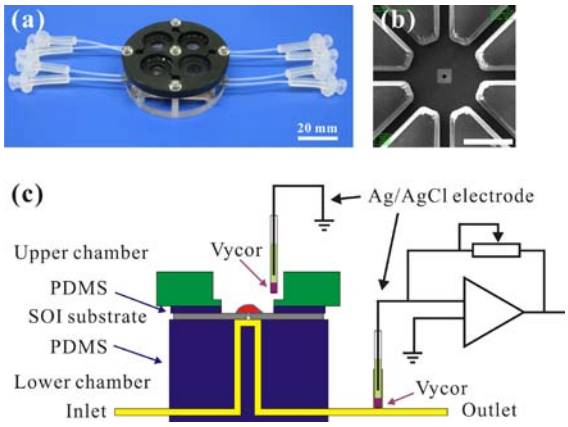

Figure 1: (a) Photograph of 4 channel incubation type planar patch clamp biosensor. (b)Cell trap pattern of PMMA substrate with micropore of 2 μm diameter at the center. (c) Schematic structure of single channel planar patch clamp biosensor.

In this work, we have newly developed the 4 channel incubation type planar patch clamp biosensor (Fig. 1a) with polymethylmethacrylate (PMMA) substrate (Fig. 1b) by using both-side hot embossing, focused ion beam micro-fabrication and surface modification by extra cellular matrix (ECM). We have evaluated the performance of the newly developed PMMA sensor chip for incubation type 4 channel planar patch clamp biosensor by using the light-gated ion-channel protein, channelrhodopsin wide receiver (ChRWR) expressed on HEK293 cell [2]. The HEK293 sensor cell on the micropore of the sensor chip was stimulated by 473 nm laser light (1.5 mW) focused to the beam diameter of about 10 to 50 μm under the fluorescence microscopy objective lens. Observed laser-induced current profile and the membrane potential dependences well agreed to those observed by pipette patch clamp, suggesting its excellent possibility in important applications such as portable ion channel biosensors and high throughput screening with neural systems.

EXPERIMENT

We formed the cell trapping pattern as shown in Fig. 2. on the bath-solution-side surface of the PMMA sensor chip using the Ni mold as shown in Fig. 2a. The pipette solution side (lower side of Fig.2b) was formed using the brass mold. To make the Ni mold for the cell trapping pattern formation, we first made the resist pattern by photolithography using positive resist AZP4903 (AZ Electronic Materials Ltd.) on the 40 mm×40 mm square and

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0.6 mm thick Si(100) substrate with a mirror polished surface. The surface of the Si substrate was exposed to the vapor of hexamethyldisilazane (Tokyo Chemical Industry Co., Ltd.) to improve the adherence of photoresist on the Si surface. The Ni mold shown in Fig. 2a was formed by electroforming (IKEX Industry Co., Ltd.) using this resist pattern as the master mold. The depth of the cell trapping pattern was 8 μm. The brass mold for the formation of the pipette solution well was made by ultrafine machinery (FANUC Ltd.). The sensor chip was formed using the both-sides hot embossing machine (Engineering System Co. Ltd.) using a 0.2 mm thick PMMA substrate. The thickness of the thin film region between the cell trapping round area and the pipette solution well was controlled to be about 10μm (Fig. 2b). The micropore with diameter of 1.5 μm to 2 μm was formed at the center of the round area of the cell trapping pattern by the FIB machine (Fig. 1b). Before the FIB processing, a Cu thin film was deposited on both surfaces of the PMMA substrate to reduce the charge-up effects during FIB processing. This Cu thin film was easily removed using diluted HNO_3 solution after the micropore formation.

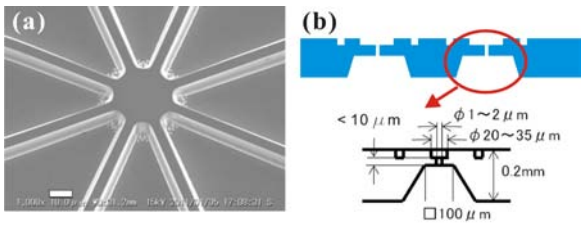

Figure 2: (a) Scanning electron microscope image of the upper Ni mold with cell trapping pattern. Scale bar: 10 μm. (b) Cross sectional structure of the PMMA sensor chip.

ChRWR is the name given to the chimeric molecule ChR(ABCDEfg) in ref. 2, and its plasmid construction is reported in detail in that reference. HEK293 cells, which were a generous gift from Minoru Wakamori of Tohoku University, were cultured at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co.) supplemented with 10 % fetal bovine serum (FBS, Biological Industries Ltd) and transfected using Effectene transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. After cloning twice with the addition of G418 (Gibco) in a 10 cm dish, single colonies with bright Venus fluorescence were selected by using a cloning cylinder IWAKITE-32 (Asahi Glass Co., Ltd.) and cultured in a medium containing G418 until they were confluent in the dish.

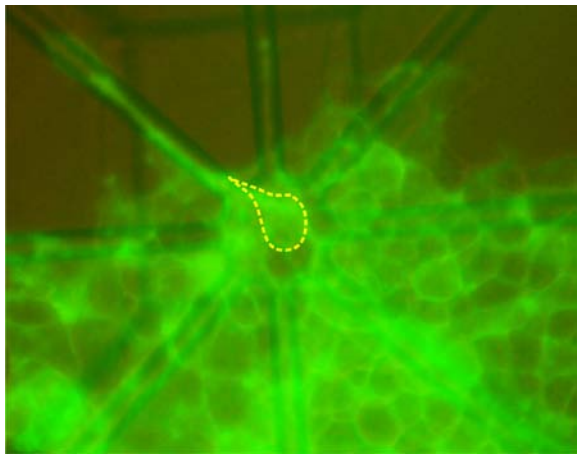

Figure 3: Fluorescence microscopy image of the ChRWR-expressing HEK293 cells. The monolayer colony was formed on the cell trapping pattern. The cell on the micropore is indicated by dotted line.

The surfaces of PMMA sensor chips were coated with ECMs of poly-L-lysine (PLL, Sigma-Aldrich Co.) for more than 1 days. After removal of excess solution, the substrate was rinsed with sterilized water, dried under a gentle nitrogen stream, and kept sterile until use. Cells were cultured in dishes filled with the medium under the conventional incubating conditions, i.e., 37°C and 5 % CO_2. After cells were detached from the culture dishes, the cell suspension was seeded at a density of 100–300 cells/mm^2 on the ECM-coated chip. Channel current was measured after several days of culturing, at which point about 70 % confluence was reached and the monolayer colony around the micropore was formed (Fig. 3).

The electrical measurement systems were the same as those used in our previous planar patch clamp experiments [1, 3, 4]. An ion-channel biosensor and preamplifier head stage were set inside the aluminum electromagnetic shield box. The culture medium was replaced with bath and pipette solutions for the upper and lower chambers, respectively. The bath solution (BS) in the upper chamber contained: 140 mM NaCl, 3 mM KCl, 10 mM 4-(2-hydroxyethyl)-1–piperazineethanesulfonic acid (HEPES), 2.5 mM $CaCl_2$, 1.25 mM $MgCl_2$, and 10 mM glucose at pH 7.4. The lower chamber solution (pipette solution (PS)) contained: 40 mM CsCl, 80 mM $CsCH_3SO_4$, 1 mM $MgCl_2$, 10 mM HEPES, 2.5 mM MgATP, and 0.2 mM Na_2EGTA. All data were recorded using a patch-clamp amplifier (Axonpatch 200B, Axon Instruments) and a data acquisition system (Digidata 1440A, Axon Instruments)with a whole cell mode at room temperature. Data were obtained using a 2 kHz low-pass filter and output gain of 1 mV/pA and analyzed using pClamp 10.2 software. For whole-cell current recordings, sub-nanometer 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 with the final concentrations of 100–200 μg/ml before use. The formation of the whole-cell arrangement was confirmed by the observation of a capacitance increase of about 10 pF from 5 to 10 min after the addition of the nystatin solution to the lower chamber. A laser beam with a 473 nm wavelength and 1.5 mW maximum output power was used (Sumitomo Osaka Cement Co., Ltd.) to irradiate the cell on the micropore. The focused spot size of the laser beam was about 50 - 100 μm.

RESULTS AND DISCUSSION

It took several days to form the monolayer colony around the micropore as shown in Fig. 3. A typical example of channel current measurement for one of the 4 channels was depicted as shown in Fig. 4. A rectangular pulse of a laser beam was used to irradiate the sensor cell after the monolayer colony had formed around the sensor cell. Very stable and sufficiently good signal to noise ratios were easily obtained as shown in Fig. 4a by using stable AgCl/Ag electrodes of the salt bridge type and doing measurements inside an electrically sealed box [5]. The observed channel current wave forms matched the reported form of ChR-WR (which correspond to the chimera molecule, ABCDEfg in reference [2]): a sharp rise with no clear desensitization, a rather rectangular wave form, and slightly slower fall. We observed a pulse train with a variable pulse interval. Attenuation of the second pulse, which has been observed in ChR-2 in dark surroundings, was not observed over the range of the pulse intervals larger than 500 msec. These are unique characteristics of ChR-WR [2], and shows that reliable channel current reflecting the molecular structure of the ion channel can be observed in the incubation type planar patch clamp.

The observed dependence of the channel current on the membrane bias (I-V characteeristics)(Fig.4b) shows characteristics of a cation channel, and it matches the reported I-V characteristics of ChR-WR reported in [2], where ChR-WR was expressed on HEK293, stimulated with a filtered Xe lamp light (477 ± 10 nm), and measured by pipette patch clamp under almost the same cation concentrations as with PS and BS. From the current profile and I-V relations, we conclude that the laser evoked channel current of ChR-WR expressed on HEK293 was correctly measured by our incubation type planar patch clamp device using PMMA sensor chip..

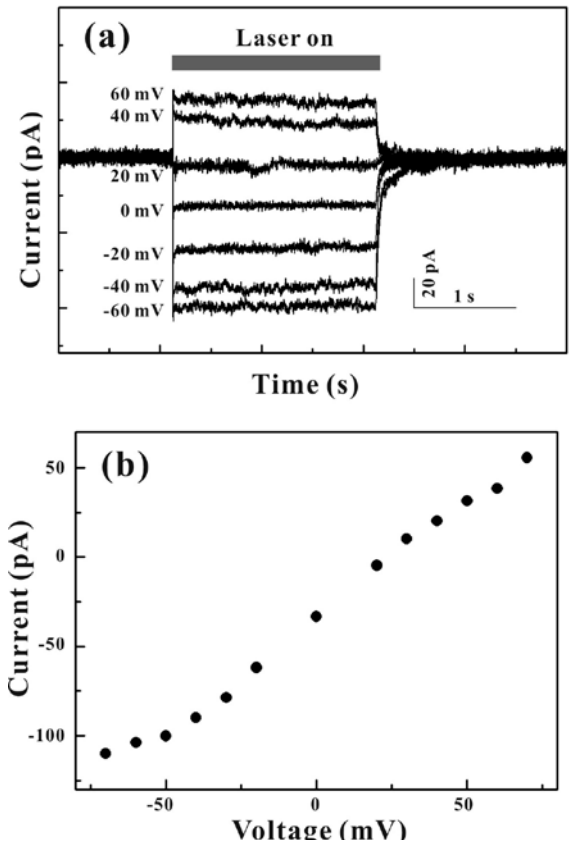

Figure 4: (a) Observed dependence of the laser-gated ion-channel current recordings on the membrane potential. (b) Dependence of the ion-channel current on the applied membrane potential.

CONCLUSIONS

The PMMA substrate has several attractive features: lower cost and easy microfabrication, which are important characteristics for multichannel device applications, small capacitance, which gives a high speed response time and transparency, which enables observation by inverse-type optical microscopy, which is especially useful in cell imaging. We have developed the 4 channel incubation type planar patch clamp biosensor with PMMA substrate by using both-side hot embossing, focused ion beam micro-fabrication and surface modification by ECM. We measured the laser-evoked channel current of a ChR-WR-expressing HEK293 sensor cell. The waveform unique to ChR-WR and its dependence on the membrane potential agreed closely with those observed under similar conditions using a pipette patch clamp, suggesting its excellent possibility in important applications such as portable ion channel biosensors and high throughput screening with neural systems.

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