Surface characteristics and electrical properties of PMMA chips for incubation-type planar-patch-clamp biosensors

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Abstract

Polymethylmethacrylate (PMMA) plates were successfully applied as sensor chips in an incubation-type planar patch clamp (IPPC). Hot embossing both sides formed the PMMA plates, and a focused ion beam realized micropores. The low seal resistance of the IPPC was investigated by analyzing the surface roughness of the chips. Atomic force microscopy (AFM) showed that the chip surface had a roughness of several nanometers due to the molding process. Coating the molded surface with an anti-adhesive compound further increased the surface roughness of the PMMA chip because the anti-adhesive compound itself had a large roughness and in some case, the compound partially peeled off while detaching the mold. Similarly, coating a chip with extracellular matrix (ECM) poly-L-lysine (PLL) also increased the surface roughness. The measured seal resistance of the PMMA chip for an HEK293 cell was in the range of 4 to 15 M Ω . The low seal resistance was attributed to the sharp-edge structure of the micropore and the surface roughness of the chip. Nevertheless, the whole cell current was successfully recorded from HEK293 cells expressing channel rhodopsin wide receiver (ChRWR) using salt-bridge-type stable Ag/AgCl electrodes. Another advantage of the PMMA sensor chip was the small parasitic capacitance.

Keywords

PMMA, incubation-type planar-patch-clamp, seal resistance, whole-cell channel current, salt-bridge electrode, channel rhodopsin

1. Introduction

A pipette patch clamp is the gold standard for investigating ion-channel membrane proteins and performing subsequent drug screening. However, it is a tedious low-throughput technique. Recent advances in drug screening demand high-throughput device techniques, such as a planar patch clamp [1]. Planar patch clamps have rapidly improved in the past decade, and whole-cell recordings have been reported [2]. Unfortunately, conventional planar patch clamps cannot be applied to neural systems because they do not have a function to incubate cells on the chip's surface. Considering the strong demand for high-throughput screening devices with neural networks from the viewpoints of treatment and drug development with neural intractable diseases, such as neurodegenerative diseases, the development of an incubation-type planar patch clamp (IPPC) is an urgent problem.

In our research, we have developed an IPPC [3], which is extremely beneficial for long-term cultures and periodic investigations of neuronal functions. The critical problem with our IPPC is how to seed or manipulate cells over the micropore of the sensor chip, especially because cells often migrate from the micropore. Additionally, the extra cellular matrix (ECM) is prerequisite for cell incubation on a sensor-chip surface. For a conventional pipette patch clamp, a glass or quartz micropipette is used to select a specific cell by forming a seal resistance in the range of 10-100 G Ω . The seal resistance is mainly attributed to the dimensions and surface smoothness of the micropipette tip.

Despite our achievement, the nature of a tight seal is not fully understood. For a chip-based planar patch clamp, the probability of a high seal resistance remains low [4]. Several factors, such as the aperture dimensions, the non-smooth nature of the micropore side-wall, roughness of the chip surface [5], and the significant difference in the configurations between pipette- and planar-type clamps [6], have been cited as reasons for this inability.

Previously we reported whole-cell channel current recording by capsaicin stimulation from transient receptor potential vanilloid type 1 (TRPV1) channels expressed on human embryonic kidney 293 (HEK293) cells [7], where the system was a conventional patch clamp with a seal resistance of $\sim 300\text{ M}\Omega$. Recently, we have examined light-gated ion channels [channel rhodopsin wide receiver (ChRWR)] expressed on the HEK293 cells by a laser pulse with a wavelength of 473 nm using an IPPC [8]. In both cases, a silicon-on-insulator (SOI) substrate was used as a chip. The observed seal resistance in the IPPC case was 10-30 $\text{M}\Omega$ [8], which is very low compared to that of a “giga-ohm” seal, but is sufficient for whole-cell recordings because the signal-to-noise ratio of the system is reduced when a stable electrode with a salt bridge is used and the measurement system is contained inside an electromagnetically shielded box.

However, SOI chips have several disadvantages. First, the parasitic capacitance is high, typically in the 100-pF range. Second, the optically excited charge carriers cause unfavorable noise pulses when a laser stimulates the sensor cells with optical receptors. Micromolding has opened up the possibility of different types of materials as the chips for the planar patch clamp. Due to simple construction and ease of use, polymeric chips have received increase attention in the past decade. A planar configuration has been successfully achieved using poly(dimethylsiloxane) (PDMS) as an electrode material to replace the micropipette [9]. Polyimide films with micropore dimensions of 2 to 4 μm have been designed to investigate ion channel recordings via a planar patch configuration [10]. Recently, polymer chips, such as polyethylene glycol (PEG) and a hydrophilic polyethylene glycol diacrylate (PEGDA), have been successfully employed for ion-channel recordings [11,12].

Herein we employ polymethylmethacrylate (PMMA) as a chip due to its advantages (i.e., low parasitic capacitance, transparency, which is an important character in biomaterial observations using inverted optical microscopy, and the lack of a photoelectric effect). The channel current under a whole cell set up on the PMMA chip is successfully recorded using a

voltage clamp from the ChRWR-expressing HEK293 cells, even though the seal resistance is between 4-15 M Ω . We fabricated two types of chips:: with cell-trapping patterns and with a plain surface. For long-term cultures on the chip surface, the chip is coated with poly-L-lysine (PLL), which is a type of ECM (see Fig. 1). Then atomic force microscopy (AFM) is used to analyze the surface of the PMMA chip coated with ECM and to determine the origin of the low seal resistance.

2. Materials and methods

2. 1. Fabrication of PMMA chips

PMMA chips were fabricated by hot embossing both-sides. Figure 2 schematically depicts the processing steps and conditions. After the chips were properly aligned with the patterns, the chips were pressed on both sides with the molds. The cell-trapping patterns were formed using an upper nickel mold, which was made by electroforming via a photoresist pattern on a silicon substrate as the master mold. The pipette solution's well (on the lower side of the chip) was formed by ultrafine machining of a brass mold. Details of this fabrication procedure are presented elsewhere [13]. For a chip with a plain surface, a silicon wafer was used as the upper mold. After the typical hot-embossing process, micron-sized micropores (diameters of 1.7 to 2.3 μm) were created by a focused ion beam (FIB). In the FIB process, both sides of the PMMA chip were coated with a copper thin film to reduce the charge-up effects, and this film was removed using a diluted HNO₃ solution after the FIB process. The length of the pores ranged from 5 to 15 μm . The measured access resistances (R_a 's in Fig. 1) of the chips ranged from 1 to 2.5 M Ω .

2. 2. Surface-characterization process

All the topographic images were recorded by an XE-Bio atomic force microscope (Park Systems, Japan) fitted with an inverted optical microscope at ambient temperature in air. The microscope was equipped with a 12- μm independent z-scanner and a $100 \times 100\text{-}\mu\text{m}$ x-y scanner. Measurements were performed in the true non-contact mode, and all images were recorded at a scan speed of 0.3 Hz with a scan area of $5.0 \times 5.0 \mu\text{m}$. In some cases, high-resolution images were also recorded. A cantilever, which was composed of silicon with an aluminum coating, had a 4- μm thickness, 30- μm mean width, 120- μm length, 42-N/m force constant, and a 330-kHz resonance frequency.

2.3 Coating of the extracellular matrix

Commercially available 0.01% PLL was used as the ECM coating material for PMMA. Initially, the chips were cleaned with bleach and subsequently washed with Millipore water (Merck Millipore Japan) several times. Then they were treated by oxygen plasma induced in a Plasma Cleaner PDC-32G (Harrick Plasma) for 5 min under the following conditions: oxygen pressure of 500 mTorr, oxygen flow of 5 sccm, and output power of 18 W. The PLL was drop coated over the chip on a clean bench and incubated for two days. The chips were then washed with sterilized water thrice using a shaker and a one-hour interval between washings. To understand the nature of the chip's surface after each treatment, the water contact angle was measured (**Fig. S1**). **In the present experiments, the oxygen plasma treatment was essential for the PLL coating, and the cell incubation on the PMMA chip was impossible without PLL coating.**

2.4. *Cell cultures on PMMA*

HEK293 cells expressing light-gated ion channel ChRWR [14] were used in all experiments. The culture medium was composed of DMEM supplemented with 10% (v/v) fetal bovine serum (FBS, Biological Industries Ltd), 1% (v/v) Glutamax (Gibco), and 0.5% (v/v) penicillin-streptomycin (Gibco). The cellular seeding was achieved using the following procedure. First, nearly confluent cells on the petri dish were washed with phosphate-buffered saline (PBS, Wako). Second, 0.05% Trypsin-EDTA (Invitrogen) was added to peel off the cells, which took about 2–3 minutes. Third, a sufficient volume of the culture medium was added and centrifuged to collect the seeding cells. Fourth, PMMA chips were seeded with the cells at a density of 100-300 cells/mm². Finally, the cells were incubated until confluence over the micropore was achieved.

2.5 *Channel-current measurements*

A planar patch clamp was constructed as described in a previous report [8]. Figure 1 shows the structure of the biosensor and its equivalent circuit. A patch clamp amplifier (Axon Patch 200B, Molecular Devices) fitted with a data-acquisition system (Digidata 1440A) was used to record the channel current under the voltage clamp set up. All the data was acquired with a 1-kHz low pass filter. After confirming 70-80% confluence and the proper position of the cell over the aperture, the device was ready for whole-cell recording. The top of the device was filled with an external bath solution (BS), namely, a mixture of 2.5-mM CaCl₂, 1.25-mM MgCl₂, 10-mM D-glucose, 140-mM NaCl, 3-mM KCl and 10-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at a pH of 7.4. The lower part of the device was filled with an internal pipette solution (PS) containing 100-mM L-glutamine, 120-mM CsOH, 50-mM HEPES, 2.5-mM MgCl₂, and 1.25-mM Na₂EGTA at a

pH of 7.4. The PS duct was connected to a syringe pump and coupled to the head stage through an Ag/AgCl electrode in the branch channel. The circuit was closed using another Ag/AgCl electrode dipped in BS and connected to the earth of the head stage. Here we used a salt-bridge-type Ag/AgCl electrode, which significantly reduced the baseline fluctuation (Fig. 3) [13]. About 100 μg of nystatin in 1 ml of PS was used to create a perforated patch. Because the cells under investigation possessed light-gated ion channel ChRWR, a laser pulse with wavelength of 473 nm was used to stimulate the ion channels and record the channel current.

3. Results

3.1. PMMA for an incubation-type planar patch clamp

The use of PMMA as a planar patch clamp chip has advantages, such as mass production and hot embossing fabrication. Hot embossing is an efficient and cost-effective process to create microchannels on the PMMA [15]. Compared to a silicon-based device, a large-area and high-throughput PMMA device can be manufactured economically. In addition, PMMA is transparent and has a low capacitance. Figure 2 schematically depicts the hot-embossing process used in our laboratory.

Figure 4a, which shows a typical current-voltage curve for the signals from the PMMA chip, indicates that the chips' capacitances range between 1-2 pF, which is much lower than those in the case of silicon (around 100 pF). Due to the low-capacitance nature of the PMMA chip, a small increase in the capacitance due to nystatin perforation, which shows the whole cell mode arrangement, is easily identified. In addition, laser irradiation on the Si chip causes the transient parasitic-current pulses associated with a charge carrier excitation to appear (Fig. 4b). These transient parasitic-current pulses disturb measurements of the

laser-induced channel current of the ChRWR. However, PMMA does not exhibit any laser-irradiation effects.

The use of PMMA as a biochip has a few disadvantages, such as hydrophobicity mainly due to the absence of active functional groups. The typical water-contact angle of a pristine PMMA chip was around 60° (**Fig. S1a**). This hydrophobicity is unfavorable for the ECM coating and the subsequent interaction of the hydrophilic parts of the cellular membrane with the chips. So, generally, the chips are plasma treated with oxygen to improve their reactivity. The measured contact angle immediately after treatment was 20° (**Fig. S1b**). However, the ECM-coated surface became hydrophobic after two days. During the typical culture experiment, the observed water contact angle was 55° (**Fig. S1c**). This PLL coating was essentially necessary in our experiments for cell adhesion on the chip surface.

3.2. *Surface characteristics of PMMA*

The surface roughness, especially around a micropore, is responsible for the reduced seal resistance (R_j in **Fig. 1**) and is an important characteristic of the sensor chip in a planar patch clamp. Here the surface roughness was investigated by AFM. As previously reported [13], a chip with a cell-trapping pattern (**Fig. 2 of ref. 13**) can be fabricated using an upper nickel mold made by photolithography and subsequent electroforming. Any surface roughness on the PMMA should be translated from the mold during the molding and de-molding processes. Hence, the surfaces of the nickel mold before and after repeated use were investigated. Figures 5a and b show the observed topographic images before and after repeated use, respectively. The surface topography consists of short- and long-range roughness and many dot-like pits. The peak-to-valley values of the short- and long-range roughness (R_z) are about 2 nm before use. The origin of the roughness and pits may either be inherent or created during the electroforming process, which is commonly used to prepare

nickel molds.

Another problem is that the PMMA surface partially peels off and sticks to the surface of the mold as PMMA is removed from the mold. A mound of plastic residue and many small particles (like protrusions) on the nickel mold form after repeated use (**Fig. 5b**). Chip roughness (R_z) increases to about 10 nm. **Figures 6a** and **b** show the AFM images of the as-delivered PMMA substrate and that of the PMMA sensor chip with cell-trapping patterns, respectively. The R_z values are 12 and 4 nm, respectively. It should be noted that using a nickel mold decreases the surface roughness of the PMMA substrate. In general, the surface roughness of the PMMA sensor chip is greater than that of the nickel mold before use and similar to that of the nickel mold after repeated use. This increased roughness probably originates from both the roughness transferred from repeatedly using the nickel model and the voids creating upon releasing the chip from the mold.

To investigate the origin of surface roughness of the PMMA sensor chip, the roughness of the PMMA substrate surface after hot embossing (using a plain silicon wafer with a mirror-polished surface as the upper mold) was measured. Figure 7 a shows the AFM image of the silicon mold. The surface is very smooth (R_z is below 1 nm). However, the silicon surface is also deposited with PMMA after several runs of embossing (**Fig. 7b**), resulting in R_z of about 10 nm. In contrast, the PMMA sensor chip after embossing using the silicon mold increases R_z (namely, about 3 nm) (**Fig. 7c**), and it is slightly lower than that of the PMMA chip surface after embossing using the nickel mold (**Fig. 6b**). Thus, both the roughness of the mold surface and peeling during mold removal are responsible for the roughness of the PMMA chip.

To decrease the roughness from the peeling-off effect, the mold surface was coated with the fluorine-type anti-adhesive compound (Chukyo Kasei Kogyo Co., Ltd). Contrary to our expectations, the roughness of the PMMA chip surface increases after embossing because the thin film of the anti-adhesive compound itself has a large roughness and in some cases,

peels off partially upon detachment from the mold (**Fig. S2**).

Because our device is a culture-type, it is essential to use of an ECM coating to adhere the cells over the micropores. In the present investigations, PLL is used as the coating. Figure 7d shows the AFM images of the PLL-coated chip, which has a roughness of approximately 12 nm.

3.3. *Channel-current measurements*

ChRWR is a chimeric molecule derived from green algae and is known as channel rhodopsin 1 (ChR1) and ChR2. The N-terminal segments of ChR2 are replaced with ChR(ABCDEFg) in Ref. [14] and tagged with a green fluorescent protein derivative of Venus [14]. In our experiments, the channel current was measured after confirming growth of the seeded cells on and around the micropore, become confluent, and form a monolayer colony covering the micropore. The probability of successful measurements (namely, the number of trials where channel current is successfully measured / the total number of trials) depends on whether a healthy cell exists on the micropore without forming a multilayer.

Figure 8a shows a typical photograph of the cells on the micropore as a fluorescent image. In the channel-current measurements, the culture medium is replaced by the bath and pipette solutions, and the—pipette-solution-side AgCl/Ag electrode is connected to the amplifier. The design and properties of the electrode and the signal-to-noise ratio of the measurement system have been discussed previously [13]. The initial seal resistance prior to applying a negative pressure to the pipette solution side (suction) is always low, but increases to 8 to 15 M Ω after applying a negative pressure of 10 kPa. In the following, the seal resistance is the value measured after applying suction. To form a perforated patch, nystatin is introduced into the pipette-solution side, and in accordance with the laboratory protocol, the

patch is allowed to sit for 10 min. One interesting feature of the PMMA sensor chip is its low capacitance (**Fig. 4a**), which allows the formation of the whole cell mode arrangement to be easily confirmed using nystatin (Fig. S3). Figure 7d shows an AFM image of the PLL-coated chip. The roughness of the chips is around 12 nm.

Figure 8b shows the measured current signals under a voltage clamp, which has a current wave shape; that is, it rises quickly upward with a very small desensitization, and slowly decays. This shape agrees well with that measured by a pipette patch clamp [14]. The seal resistance in this case is 15 M Ω .

4. Discussion

The measured seal resistance in the developed IPPC is about two orders of magnitude lower than that of a giga-ohm seal obtained with a conventional pipette patch clamp. It is important to consider the reason for such a low seal resistance. In our previous work on a non-incubation-type planar patch clamp using a TRPV1-expressing HEK293 cell on a SOI sensor chip, a seal resistance of about 300 M Ω was easily obtained [7]. However, this value drastically decreases to 10 to 30 M Ω in the case of the IPPC with a PLL-coated SOI sensor chip. The decreased seal resistance is likely due to the PLL coating, which generates a very high roughness (R_z of about 12 nm; **Fig. 7d**), and the long incubation time. In the present IPPC using a PMMA sensor chip, the measured seal resistances were 4 to 15 M Ω for ChRWR cells expressing HEK293 with incubation times of four to nine days. Although the difference between the seal resistances between the SOI sensor chip and the PMMA chip was small, the difference was clear and reproducible. Because a micropore was formed by FIB in both cases, the micropores in the PMMA and SOI chips had almost identical structures. Thus, the difference in the seal resistance is attributed to the surface

roughness, namely, $R_z < 1$ nm in SOI (**Fig. 7a**) and ~ 4 nm in PMMA (**Fig. 6b**). **This conclusion is clearly supported by the seal resistance values obtained for several systems (Table 1).**

In the case of a pipette patch clamp, giga-ohm seals are obtained using glassy and smooth surfaces where the pipette is dipped into hexamethyldisilane to create a hydrophobic patching surface, which binds and denatures proteins. However, most studies on planar patch clamps have noted that obtaining a seal resistance greater than the giga-ohm level is difficult [4] due to the dimensions and/or shape of the micropore as well as the surface roughness and hydrophilicity of the planar chip [5]. Although the present study employs PMMA chips with various dimensions (diameters from 1.7 to 2.3 μm and depths from 5 to 12 μm), the value of the seal resistance is fairly constant, suggesting that the difference in the dimensions within this range is not correlated to the observed decrease in seal resistance (at least in our experiments).

For a standard planar patch clamp, the seal resistance significantly increases when an hourglass aperture is employed [16, 17]. Although we anticipate that applying an hourglass aperture to an incubation-type planar patch clamp will increase the seal resistance, we have yet to examine such a structure. To further investigate the seal-resistance problem, we measured the seal resistance of an incubation-type planar patch clamp by forming a neural network on a PMMA sensor chip using neurons from the hippocampi of the brains of 17-day-old rat fetuses. Once a dense network formed, the seal resistance was in the range 2 to 5 $\text{M}\Omega$ (Table 1), which was about half that in the case of HEK293 after 5 to 9 days of incubation. The HEK293 cells and rat neural cells have similar sized cell soma. It is possible that in the case of neurons, synapse formation connecting the axon and dendrite pull the soma and increase the size of the cleft between the cell membrane and the chip surface, and thereby decrease the seal resistance. In fact, some cells are removed from the chip surface after synapses formation. Additionally, it is possible that debris is formed under the cell soma

through cell division (of several kinds of glia cells) during the long incubation period, significantly decreasing seal resistance. Although no clear evidence is currently available, we believe that the low seal resistance is due to the long-term incubation itself. In the present experiments, however, whole cells are successfully recorded under loose patch conditions, which is definitely an attribute of a stable electrode with a salt-bridge structure.

5. Conclusion

Hot embossing both sides of a PMMA sensor chip realized an incubation-type planar patch clamp. AFM indicated that the surface roughness (R_z) of the PMMA sensor chips is about 4 nm, which is quite large compared to R_z of the SOI surface (<1 nm). A PLL coating on the chips increases the roughness. The rough and hydrophilic surface of the sensor chip after ECM coating is the main reason for the low seal resistance (i.e., 4 to 15 $M\Omega$) in the case of HEK293 cells expressing ChRWR. The debris formed during a long incubation period of the sensor cells also decreases the seal resistance. In the case of a neural network, the seal resistance was much smaller (i.e., 2 to 5 $M\Omega$). This difference in the seal resistance may be caused by an increase in the cleft thickness due to the tensile stress induced by synapse formation. Regardless of the low seal resistance, the whole-cell current can be successfully recorded by the patch clamp with a significant improvement in the signal-to-noise ratio of the system due to the stable salt-bridge electrode. As the next phase of our IPPC research, we plan to consider a micropore aperture with an hourglass structure to increase the sealing area and to improve seal resistance even in an incubation-type planar patch clamp.

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Figure captions

Fig. 1. Schematic structure of the incubation-type ion-channel biosensor and its equivalent circuit. PMMA: PMMA sensor chip, ECM: extracellular matrix, SE: salt-bridge-type stable electrode, R_j : seal resistance, C_s : capacitance of sensor chip, C_m : membrane capacitance, R_m : membrane resistance, R_a : series resistance, v_c : external command voltage, and I_t : current (channel current + leak current).

Fig. 2. Schematic of the fabrication process with the PMMA sensor chip. Upper mold for embossing both-sides is a nickel plate with a cell-trapping pattern or a 0.6-mm-thick silicon wafer with a mirror-polished surface. Lower mold is formed from the brass plate fabricated by ultra-precision machining. A micropore with a 1.7 to 2.3- μm diameter for the patch clamp is formed by FIB machine (Seiko Instruments Ltd). Acceleration voltage and current of the Ga ion beam are 30 keV and 50 pA, respectively.

Fig. 3. Comparison of the current noises through the micropore of the PMMA sensor chip between the salt-bridge-type and the typical AgCl/Ag electrodes. Resistance of the micropore is 1.8 M Ω , bias voltage is 5 mV, and low pass filter is 1 kHz.

Fig. 4. (a) Current-voltage signals obtained from the PMMA sensor chips with a HEK293 cell on the micropore. Sensor chips are set in the IPPC device shown in Fig. 1. External command V_c is 5 mV with 2 ms width. Seal resistance is 15M Ω . (b) Electric spikes from the silicon chips set in the device shown in Fig. 1 due to optical excitation of the charge carriers by laser light.

Fig. 5. (a) AFM topographic image of the nickel mold surface. (b) AFM image of the nickel mold with PMMA deposits after repeated use.

Fig. 6. (a) AFM image of a plain PMMA chip before embossing. (b) AFM image of a patterned PMMA chip after embossing.

Fig. 7. (a) AFM image of a mirror-polished surface of a silicon wafer. (b) AFM image of the wafer after several runs of embossing. (c) AFM image of a planar-surface PMMA after embossing using a silicon upper mold. (d) AFM image of a PLL-coated patterned PMMA chip.

Fig. 8. (a) Fluorescence microscopy image of the HEK293 cells expressing ChRWR on the micropore. (b) Typical light-gated channel-current recordings from HEK293 cells expressing ChRWR. Membrane voltages are given in the figure.

Graphical abstract

Table 1. Surface roughness and seal resistance

Chip	Surface Roughness (Rt)	ECM (PLL)	Pore diameter (μm)	Cell	Incubation time (days)	seal resistance (MΩ)
Si (SOI)	<1 nm	noncoating	2±0.3	HEK293	0	300
Si (SOI)	<1 nm	coated	2±0.3	HEK293	4-9	10-30
PDMAA (formed by Ni mold)	4 nm	coated	2±0.3	HEK293	4-9	4-15
PDMAA (formed by Ni mold)	4 nm	coated	2±0.3	neuron (rat cerebral cortex)	10-34	2-5

Fig. 1

Fig. 2

Fig. 3

Fig. 4

(a)

(b)

Fig. 5

(a)

(b)

(c)

(d)

Fig. 6

Fig.7

(a)

(b)

Fig. 8

Table 1. Surface roughness and seal resistance

Chip	Surface Roughness (Rz)	ECM (PLL)	Pore diameter (μm)	Cell	Incubation time (days)
Si (SOI)	< 1 nm	noncoating	2±0.3	HEK293	0
Si (SOI)	< 1 nm	coated	2±0.3	HEK293	4~9
PMMA (formed by Ni mold)	4 nm	coated	2±0.3	HEK293	4~9
PMMA (formed by Ni mold)	4 nm	coated	2±0.3	neuron (rat cerebral cortex)	10~14