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学位（専攻分野） 博士（理学）

学位記番号 総研大甲第 1115 号

学位授与の日付 平成 20 年 3 月 19 日

学位授与の要件 物理科学研究科 構造分子科学専攻
学位規則第 6 条第 1 項該当

学位論文題目 Fabrication of incubation type planar ion-channel
biosensor using silicon-on-insulator substrate

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論文内容の要旨

Ion channels play key roles in many cellular processes. There are many distinct dysfunctions known as channelopathies caused by ion channel mutations. Therefore the investigation of ion channels is of great significance for understanding how they work and for drug discovery of channelopathies. Pipette patch-clamp technique has been proven to be a powerful technique for the investigation of fundamental ion channel biophysics and for drug discovery. This technique allows one to monitor the gating of ion channels under defined conditions and enables the coupling of functional and molecular studies on ion channels at the single cell level. However, this technique has some weaknesses, such as the requirements of precise micromanipulation and skillful experimenter, and electrode being an individual glass pipette, which are not suitable for the long time measurement of the cell function and the application to high-throughput screening. Recently planar patch-clamp method has attracted great attentions. Because it has some advantages compared with the pipette patch-clamp method, such as the miniaturization and parallelization of the planar substrates and the availability to combine with other physical probes. Many materials have been used to make the planar patch-clamp substrates, such as glass, Si, quartz and PDMS etc. It has been considered for Si that the background noise current is large due to the high density of free charge carrier in the substrate [Fertig et al., *Recept. Channels* 9(2003)29]. However, they have demonstrated that the noise current can be significantly reduced by using silicon-on-insulator (SOI) wafer. There are several other advantages in using SOI wafer: 1) the structure of the micropore through the substrate can be precisely controlled by using the large etching rate difference between Si and SiO₂ in both plasma and wet etching, and 2) it is possible to produce significantly miniaturized device by integrating the biosensor and Si electronic circuits of preamplifier into the same SOI substrate.

In the present study, he has fabricated the planar patch-clamp substrates using SOI wafer. A device, called conventional type planar patch-clamp biosensor, was assembled with the SOI-based substrate. He used this to demonstrate that SOI is a versatile material for planar patch-clamp substrate fabrication used in biosensor. However, the lifetime of the cell on the micropore is too short in the planar patch-clamp biosensor, which is a serious problem for measuring various cell functions. To elongate the cellular lifetime, the substrate in the conventional type planar patch-clamp biosensor was modified with fibronectin, an extracellular matrix protein, and cells were cultured under culture medium instead of buffer solution. By using this method, he has developed a new generation planar patch-clamp device, called incubation type planar ion-channel biosensor.

In the former half of his doctoral course research, he developed several elementary processes to fabricate the planar patch-clamp substrates using SOI wafer, which produce low access resistance and low capacitance.

Two procedures of planar patch-clamp substrate fabrication were developed. One is based on electron beam lithography (EBL) combined with reactive ion etching (RIE), the other is based on focused ion beam (FIB).

In the fabrication method with EBL combined with RIE, firstly circular patterns were made with EBL and RIE techniques. Then a SiO₂ layer with 1 μm thickness was grown at 900°C with thermal oxidation in which O₂ bubbled water vapor at 95°C was used as the reactive gas. After that large holes on the backside of the substrate were made with 1-mm-diameter diamond drill polishing, followed by 8% (v/v) TMAH etching at 90°C to the buried SiO₂ layer. Finally the buried SiO₂ layer at the bottom of the patterns was removed with 10% (v/v) HF solution from the topside of the substrate, followed by 1-μm-thick SiO₂ layer formation at 900°C with thermal oxidation in the presence of O₂ bubbled water vapor at 95°C. The scanning electron microscope (SEM) images indicate that the initially round pore becomes faceted after the thermal oxidation due to the crystallographic growth-rate dependence of single-crystal silicon, and the sharp edge at the rim of the pore becomes dull after the thermal oxidation. These are possibly unsuitable for the tight contact formation between the cell membrane and the substrate surface.

In the fabrication method using FIB, firstly a 0.2 μm thickness SiO₂ layer was formed on the silicon surface by thermal oxidation at 900°C with the water-saturated O₂ flow. Then large holes (~1 mm diameter and ~400 μm depth) on the backside were made by diamond drill polishing. Subsequently the pyramid-shaped holes were formed by 8% (v/v) TMAH etching at 90°C for about 40 min, which reached the buried SiO₂ layer. Finally micropores through the Si and SiO₂ layers were made by FIB milling from the backside. SEM images indicate that a round pore with sharp edge can be obtained with the FIB method. Therefore the planar patch-clamp substrates made with FIB were used to make the device. The substrate with 1.2 μm diameter pore was used to make the conventional type planar patch-clamp biosensor. The substrate was assembled into the microfluidic circuit. The human embryonic kidney 293 (HEK-293) cell transfected with transient receptor potential vanilloid type 1 (TRPV1) was positioned on the micropore and the whole-cell configuration was formed by suction. Capsaicin was added to the extracellular solution as a ligand molecule, and the whole-cell current of HEK-293 cell showing desensitization unique to TRPV1 in the extracellular solution containing Ca²⁺ [Caterina et al. *Nature* 389(1997)816] was measured successfully.

In the latter half of his research, to overcome the cellular short-lifetime problem, the incubation type planar ion-channel biosensor was developed. He modified the substrate of the planar patch-clamp biosensor with fibronectin, and cultured the cell positioned on the micropore under the culture medium instead of the buffer solution. Atomic force microscope (AFM) and cell spreading assays of the FN-coated substrates indicate that they are promising biomaterials for cell adhesion and spreading. After the cell attached and spread on the pore of the FN-coated substrate, the resistance of the

electrolyte in the cleft between the cell membrane and the substrate surface was evaluated based on a schematic model and its equivalent circuit. The obtained values of seal resistance quite well agree with the experimentally measured values. Using this method, the resistance of the electrolyte in the narrow space between the cell on the pore and all the contacting cells was evaluated. The whole-cell configuration of the HEK-293 cell spreading on the pore was obtained with nystatin perforation and the whole-cell current of TRPV1 was successfully measured. It is demonstrated that fibronectin modification of the substrate can make the cell live for a long time and the whole-cell current of the cell spreading on the pore can be obtained during cell culture. Moreover this planar ion-channel biosensor has high potential application to investigate the various cell functions and the neuronal signal transductions, because the cells can be cultured on this FN-coated planar ion-channel biosensor substrate.

論文の審査結果の要旨

イオンチャンネルは細胞の内部と外部との間の情報のやりとりに係わる膜タンパク質として、その構造や機能を知る事の重要性の他、中枢神経系疾患、神経変性疾患、心臓病等に密接に係わり、疾患の診断や創薬など、応用の観点からも重要なタンパク質で、イオンチャンネル電流を計測するバイオセンサーの開発が望まれている。技術が確立しているバイオセンサーとしてピペットパッチクランプは良く知られているが、これは長時間測定ができないことや、多点同時計測ができないなど、細胞、特に神経細胞ネットワークの機能を長時間にわたり計測することができない。この技術的欠点は、今後イオンチャンネルの機能の研究や神経細胞の細胞膜表面反応の研究など新しい分子科学を開拓する上で支障を来すと考えられる。本研究ではこれらの問題を解決するバイオセンサーとして培養機能をもったプレーナー型イオンチャンネルバイオセンサーの開発を行った。

第一章はピペットパッチクランプやプレーナー型パッチクランプの動作原理、長所短所などの基礎知識について、第二章は Si の酸化と各種の微細加工技術、など本研究で必要とされる実験技術について、第三章は、従来型のプレーナー型パッチクランプ素子をいわゆるシリコンオンインシュレーター(SOI)とよばれる基板を用いて作成し、イオンチャンネル電流の計測に成功した実験結果について、第四章は、動作寿命が短いことや動作が不安定であるといった従来型の欠点を克服した新しいタイプの、培養型プレーナーイオンチャンネルバイオセンサーを提案し、開発に成功した内容について記述してある。

プレーナー型パッチクランプ素子は欧米で活発な研究が展開されているが、Si を基板材料とすることについては電流雑音が大きいう理由で、多くの研究者が敬遠してきた事実があるが、本論文では、SOI 基板を用いることにより電流雑音を低減できるのみならず、さらに加工精度が高いという長所を生かせるということを実証している。また、この従来型のセンサー素子の研究をとおして、その欠点である、短寿命であることや動作が不安定という技術上の問題に気づき、その問題を解決する素子として、細胞外マトリックスの利用や、細菌汚染を防ぐ素子構造をもった、培養型の素子を世界に先駆けて提案し製作と動作確認に成功している。

第三章、第四章の成果はいずれも本研究の高い独創性と実験の信頼性を示す結果で、また、審査における質疑応答からは Zhang 氏の多大な努力と工夫が伺われた。イオンチャンネル分子の研究や神経細胞の機能の研究など、新しい分子科学を開拓する上で重要な方法論の開発の研究として、国際的にも高い水準の研究であると判定された。審査委員会は出願論文が博士(理学)の授与に値すると全員一致で判断した。