Fabrication of Nanobiodevices that Utilize the Function of Membrane Proteins

Yoshiaki Kashimura, Azusa Oshima, and Koji Sumitomo

Abstract

Membrane proteins play vital roles in a wide variety of functions in living organisms. The development of nanobiodevices that fuse semiconductor nanotechnology and membrane proteins makes it possible to understand various biological processes as well as engineering applications. In this article, we introduce the fabrication of nanobiodevices that are functionalized by membrane proteins and designed to mimic the synaptic signal transmission mechanism in the nervous system.

Keywords: lipid bilayer, membrane protein, nanobiodevice

1. Introduction

Membrane proteins have attracted interest as a post-genome target since the mapping of the human genome was completed in 2003. Membrane proteins that are located in cell membranes perform a variety of functions vital to the cell membrane. There are several types of membrane proteins. Most of them are transmembrane proteins that span the membrane and in most cases work as gateways that permit the transport of specific chemicals across the membrane. Transmembrane proteins can be classified into three types based on their transport mechanisms: channels that form transmembrane passive pores and transport molecules and ions; transporters that require conformational changes when transporting substances; and pumps that move ions against a concentration gradient by consuming energy sources such as ATP (adenosine triphosphate).

Among channel proteins, ion channels that allow ions to flow into cells play an important role in the signal transduction in living organisms. The nervous system responsible for informational signal transmission consists of a network of neurons. A synapse is a connection that is formed at a narrow gap between neurons. At this connection, an electrical signal is converted into a chemical signal such as the release of a chemical called a neurotransmitter. The neurotransmitter is released from a presynaptic neuron and binds to ion channels called receptors located in a postsynaptic neuron. This causes the ion channels to form passive pores leading to an influx of ions into the postsynaptic neuron, and consequently induces a synaptic potential that spans other neurons. Thus, ion channels play pivotal roles in the signal transduction in living organisms. Therefore, a loss or a gain of a channel function can cause brain diseases such as depression, addiction, dementia, and anxiety disorders.

As described above, membrane proteins including ion channels are biomolecules associated with many physiological and behavioral functions such as drug response, immune response, and the outbreak of a disease. Membrane protein research will become increasingly important since it is known that more than 60% of commercially available pharmaceuticals are targeted at membrane proteins. The transport rate through an ion channel is typically 10^6 ions per second or greater. This is interesting from the viewpoint of technological applications because the channel gating is controlled solely by the attachment of a molecule to a channel. The fusion of semiconductor nanotechnology and membrane proteins should lead to further progress on diagnostic tools, the medical

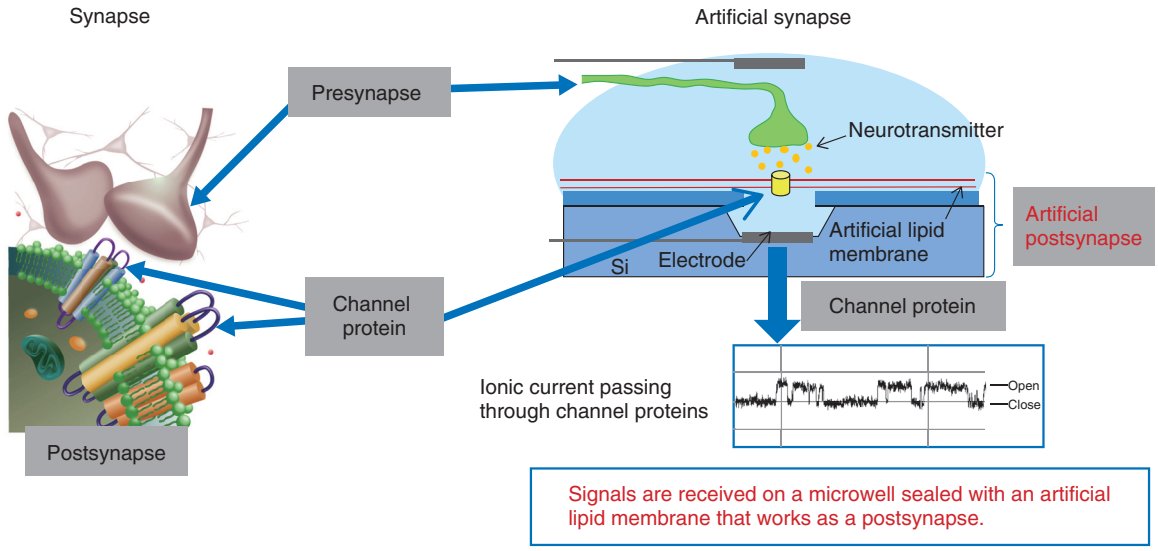


Fig. 1. Nanobiodevice that mimics the neuronal transmission mechanism.

treatment of diseases, and environmental applications in addition to basic research in nanobioscience.

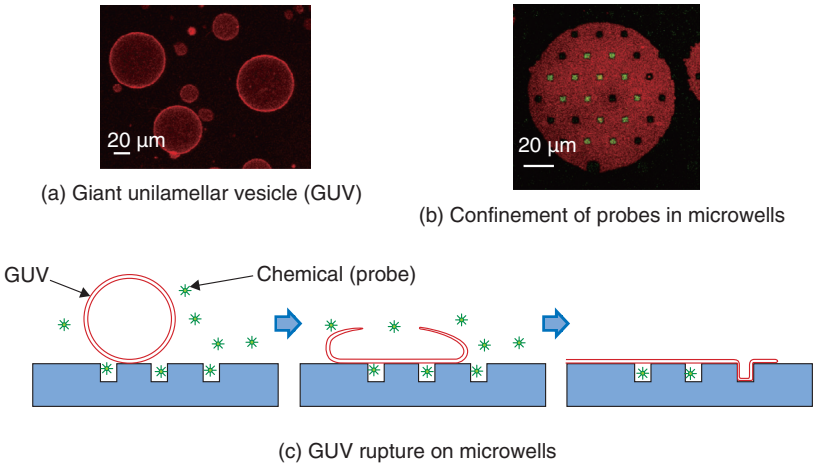
Despite its importance, research in this field remains a challenge. One reason is that membrane proteins are very difficult to handle compared with DNA (deoxyribonucleic acids), which has already been put to practical use. For example, membrane proteins denature on a semiconductor substrate or under atmospheric conditions. In this article, we introduce our efforts to overcome these hurdles and to create nanobiodevices that are functionalized by membrane proteins.

2. Construction of artificial cells

A schematic diagram of a nanobiodevice that utilizes membrane protein functions is shown in **Fig. 1**. We have designed a new type of nanobiodevice called an artificial synapse that mimics the structure of a postsynaptic neuron, which is the signal-receiving part of the synapse. One difficulty when using membrane proteins is that they can only function within lipid membranes. If the proteins are simply disposed on a substrate, they are deformed as a result of an unfavorable protein-substrate interaction, which leads to their denaturation. Membrane proteins account for half the weight of a cell membrane. The other main membrane component is a lipid molecule, which is an amphiphilic molecule containing both a hydrophilic head group and a hydrophobic tail group. In an aqueous solution, lipid molecules arrange themselves into topologically closed structures that protect the hydrophobic part from being exposed to the surrounding aqueous solution. This results in the formation of a lipid bilayer membrane, which is a fundamental component of a cell membrane.

Membrane proteins can diffuse laterally within a lipid membrane *in vivo*. Our first goal was to reproduce a biological cell-like environment on a semiconductor substrate. A promising way to achieve this is to use microwells on a Si (silicon) substrate sealed with a lipid membrane. A sealed microwell is comparable in size to a biological cell and has a suspended lipid membrane area that works as a cell-mimetic field on the substrate. When membrane proteins are reconstituted within a suspended lipid membrane, they can diffuse within the membrane and function in the same way as those *in vivo*. Thus, individual microwells can be considered the simplest model of a biological cell.

An example method to fabricate the above-mentioned artificial cell is illustrated in **Fig. 2**. First, lipid vesicles (or liposomes), which are one stable form of a lipid membrane, were prepared by electroformation [1]. We used giant unilamellar vesicles (GUVs) with diameters in the 10–100 μm range, which should be larger than those of the microwells. We used a lithographic technique to fabricate microwells (2–4 μm in diameter and 1 μm deep) on a silicon dioxide (SiO_2) substrate [2]. The microwells were sealed with lipid membranes by rupturing the GUVs in an electrolyte solution such as calcium chloride ($CaCl_2$). We



(a) Giant unilamellar vesicle (GUV)

(b) Confinement of probes in microwells

(c) GUV rupture on microwells

Fig. 2. Fabrication of microwells sealed with a lipid membrane.

confirmed that the microwells were sealed by confining fluorescent probes in them. A fluorescent image of a lipid membrane patch labeled with red fluorescent rhodamine B, where green fluorescent calcein was confined in the microwells, is shown in Fig. 2(b). The fluorescence was clearly observed from the calcein confined in the microwells. This result shows that the lipid membrane successfully seals the microwells. Depending on our target functions, other chemicals can be confined in the microwells using the same protocol instead of fluorescent probes.

3. Biodevice functionalized by membrane proteins

A functional analysis of ion channel activity requires first reconstituting the ion channels within a lipid membrane. To demonstrate this, we began by using α-hemolysin, which is widely studied and is known as a bacterial exotoxin with a membrane-damaging function [3]. We inserted α-hemolysin monomers into the lipid membrane and then oligomerized it to form a heptameric transmembrane pore through which ions or small molecules pass. The function of α-hemolysins is easily identified by their ability to pass ions through unregulated pores, thus providing a good prototype for confirming the operating principle of the device.

For this purpose, a calcium ion (Ca^{2+}) indicator (fluo-4) that emits green fluorescence in the presence of Ca^{2+} was confined in the microwells instead of calcein. Then, to analyze the Ca^{2+} transport through the ion channels, a Ca^{2+} concentration gradient between the outside and inside of the microwells was created by adding a $CaCl_2$ solution to the outer solution. When α-hemolysin was added to the outer solution, it diffused into the suspended lipid bilayer and formed ion channels. Time-lapse images of the fluorescent intensity of microwells containing fluo-4 after adding the $CaCl_2$ solution (t = 0) are shown in **Fig. 3**. The obtained images reveal that most of the microwells became bright after several minutes and their fluorescence intensity gradually increased. This result indicates that Ca^{2+} were transported from the outer solution to inside the microwells through the ion channels formed by α-hemolysin.

A fluorescence intensity analysis revealed that the detection limit for Ca^{2+} transport was estimated to be several tens of ions/s/µm^2, which is much smaller than the ion current in a standard electrophysiological measurement. This is because the small microwell volume, which is a minimum of several hundred attoliters, induces a large change in the ionic concentration even when the ion permeation is small. This demonstration enables us to mimic a local ion concentration change in the biological cells when we use more sophisticated membrane proteins.

4. Toward electrophysiological detection of ion channel activity on a device

The most widely used methods for studying ion channel functions are related to electrophysiological techniques that directly measure the ionic current through channel proteins. Among them, the patch clamp technique is the gold standard, and it can be

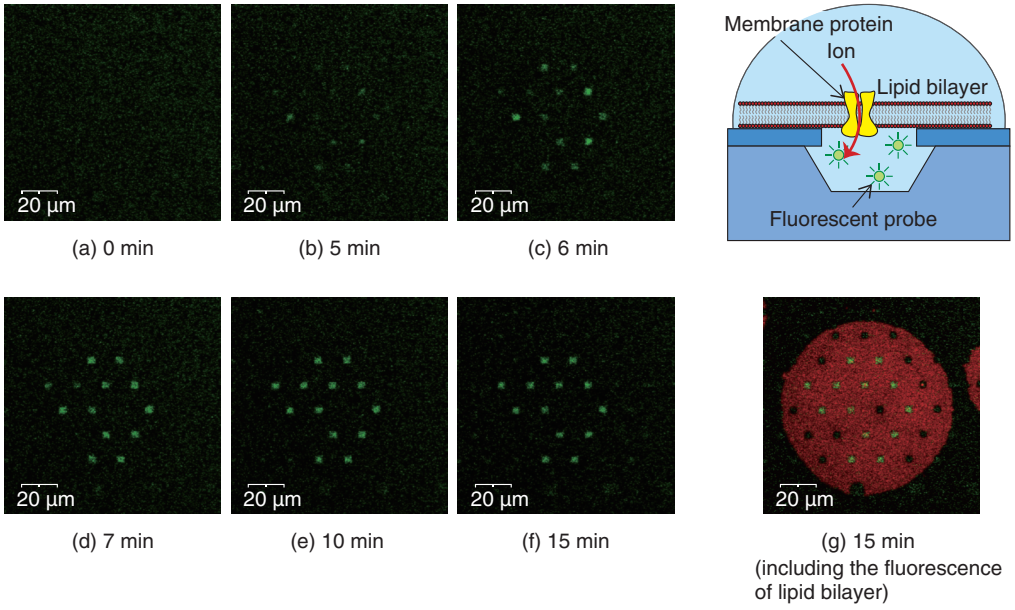


Fig. 3. Functional analysis of α-hemolysin with fluorescent microscopy.

applied to cells and tissues using a glass micropipette as a recording electrode. However, the use of an electrophysiological technique to detect an ion channel current on a semiconductor device still remains challenging. One difficulty is that the ion channel current is very low and comparable to the background noise (several pA). Although we succeeded in observing Ca^{2+} transport through ion channels with fluorescent microscopy using our proposed microwell device, when we try to achieve electrophysiological detection we are faced with the problem of ion leakage through the water layer between the lipid bilayer and the substrate, as shown in **Fig. 4** [4]. This phenomenon did not cause any serious changes in the optical measurements; however, it is not negligible in an electrophysiological measurement, which detects a very small signal.

To overcome this problem, we proposed a newly designed microwell structure that uses a self-assembled monolayer (SAM) on a gold (Au) surface to prevent ion leakage from/into the microwells, as shown in **Fig. 5** [5]. Microwells with a slightly offset Au ring were fabricated using a lithographic technique. A SAM of octadecanethiol, which has a similar structure to a lipid, was created on the Au surface. By rupturing GUVs on the structure, a lipid monolayer was formed with the hydrophobic tails of both molecules facing each other to minimize unfavorable interactions with the aqueous regions. A supported

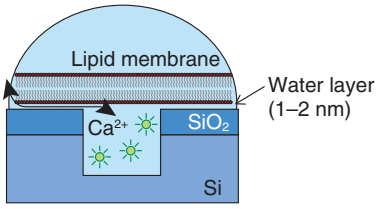


Fig. 4. Schematic illustration of microwell structure sealed by a lipid bilayer showing the passage of Ca^{2+} through the water layer.

lipid bilayer was formed in the SiO_2 regions of the surface, and a suspended lipid bilayer was formed at the microwell aperture.

The fluorescence from the rhodamine B-labeled patch of a lipid membrane suspended over the structure is shown in Fig. 5(c). The green fluorescence from the microwell indicates that fluorescent probes are successfully sealed with the lipid membrane. There is no fluorescence on the Au ring, probably owing to the energy transfer between dyes and gold. The fluorescence recovery after the photobleaching observation revealed that lateral fluidity is maintained across the Au ring. This result supports the structural geometry predicted in Fig. 5(a).

The time course of the fluorescence intensity of the Ca^{2+} indicators confined in the microwells when

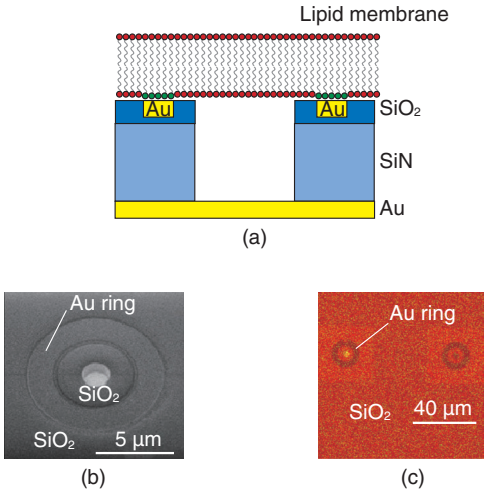


Fig. 5. (a) Schematic illustration of microwell structure. (b) SEM image of the Au ring substrate. (c) Fluorescence from the rhodamine-B labeled lipid membrane. The green fluorescent probe is confined in the microwell.

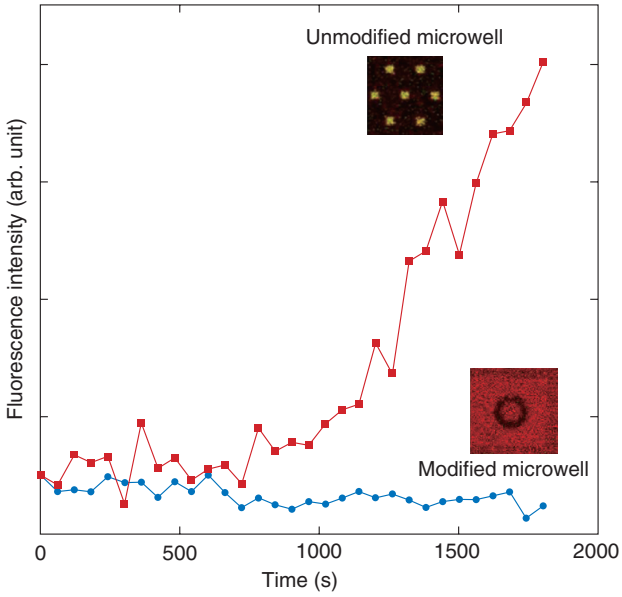


Fig. 6. Change in fluorescence intensity from fluo-4 confined microwells as a function of time.

$CaCl_2$ solution was added to the outer solution is shown in **Fig. 6**. Unmodified substrates (without a Au ring) caused a significant increase in the fluorescence intensity within 20 min. However, with a modified substrate (with a Au ring), there was no increase in fluorescence intensity during the observation time. Separating the microwells and the outer regions effectively reduced the ion diffusion through the water layer. Toward the aim of achieving the electrophysiological detection of ion channel activity, we estimated the membrane resistance by using a conventional patch-clamp amplifier. The membrane resistances for the modified microwells were one order higher than those of the unmodified microwells, leading to a reduction in background noise. The improved membrane resistance and background noise for the modified microwells resulted from the reduced ion leakage. The device structure used in this study therefore has the potential to be used as a platform for the very low signal-to-noise ratio measurement of ion channels.

In addition to the ion leakage problem, there are certain other hurdles to overcome such as the purification and alignment of membrane proteins. In our group, researchers from a variety of backgrounds including physics, chemistry, and medical engineering are working hard to overcome such hurdles in their efforts to achieve nanobiodevices.

5. Conclusion

Despite recent developments in biological information transmission, many important problems remain unsolved such as the synaptic plasticity associated with learning and the understanding of mechanisms at the neuronal circuit level. In addition, the mechanisms of neurological disorders such as Alzheimer's disease, which are caused by synapse abnormalities, have not yet been described in detail. One of our aims for our proposed nanobiodevice is to artificially replace synapses, which will allow us to access our information transmission system. By constructing a model synapse system in a controlled manner in an artificial environment, it will be possible to elucidate the mechanisms and functions in as simple a way as possible. The acquisition of information at a single protein level allows us to reveal the biological information transmission mechanism at the molecular level and, furthermore, to find ways of treating incurable diseases.

References

[1] D. S. Dimitrov and M. I. Angelova, "Lipid Swelling and Liposome Formation Mediated by Electric Fields," Bioelectrochem. Bioenerg., Vol. 19, No. 2, pp. 323–336, 1988.

[2] K. Sumitomo, Y. Tamba, Y. Shinozaki, and K. Torimitsu, "Confinement of Fluorescent Probes in Microwells on Si Substrates by Sealing with Lipid Bilayers," Appl. Phys. Express, Vol. 3, No. 10, pp. 107001-1–3, 2010.
[3] K. Sumitomo, A. McAllister, Y. Tamba, Y. Kashimura, A. Tanaka, Y. Shinozaki, and K. Torimitsu, "Ca^{2+} Ion Transport through Channels Formed by α-hemolysin Analyzed Using a Microwell Array on a Si Substrate," Biosens. Bioelectron., Vol. 31, No. 1, pp. 445–450, 2012.
[4] S. J. Johnson, T. M. Bayerl, D. C. McDermott, G. W. Adam, A. R. Rennie, R. K. Thomas, and E. Sackmann, "Structure of an Adsorbed Dimyristoylphosphatidylcholine Bilayer Measured with Specular Reflection of Neutrons," Biophys. J., Vol. 59, No. 2, pp. 289–294, 1991.
[5] R. Forbes, Y. Kashimura, and K. Sumitomo, "Hermetically Sealed Microwell with a Lipid Bilayer Created Using a Self-assembled Monolayer," Appl. Phys. Express, Vol. 8, No. 11, pp. 117201-1–4, 2015.

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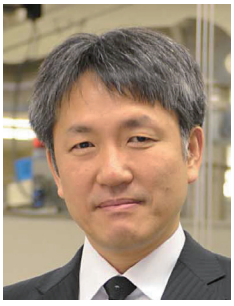

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