Surface Modification of Cell Scaffold in Aqueous Solution Using TiO$_2$ Photocatalysis and Linker Protein L2 for Patterning Primary Neurons*

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Titanium dioxide (TiO$_2$) photocatalysis can be applied to pattern proteins and cells under aqueous solution. In this work, we extended the application of this technique to patterning primary neurons, a type of cell with relatively weak adhesibility. For this purpose, we employed ribosomal protein L2 (RPL2) that has high affinity toward silica and metal oxides, including TiO$_2$, to stably bind a neuronal adhesion protein laminin to the TiO$_2$ surface. We utilized two types of molecular recognition to achieve this—binding of anti-laminin antibody to its antigen (laminin) and binding of protein A to the antibody. We show that a protein complex consisting of laminin/anti-laminin antibody/protein A-RPL2 is spontaneously formed by simply mixing the precursor proteins in solution phase. We then show that the surface coated with the protein complex supports stable growth of rat hippocampal neurons. Finally, we show that the cells can be selectively grown on the protein complex patterned with the TiO$_2$-assisted method. The protocol established in this work is a unique combination of a top-down micropatterning of the surface using TiO$_2$ photocatalysis and a bottom-up self-assembly of biomolecules, which can be further applied to pattern a wide range of proteins and cells. [DOI: 10.1380/ejssnt.2015.213]

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I. INTRODUCTION

Adherent cells require appropriate scaffolds for growth and functioning [1,2]. When culturing the cells in vitro, the surface of the substrate must be treated to support cell adhesion by, for instance, coating the surface with extracellular matrix (ECM) proteins or cationic compounds. This is especially important when culturing cells with low adhesibility, such as primary neurons. When culturing neurons, surface of a polystyrene dish or a glass coverslip is usually coated with a cationic polypeptide, polylysine, in order to electrostatically promote cell-substrate interaction [3]. Another popular coating for neuronal culture is laminin (Ln), a heterotrimeric ~ 850 kDa protein that constitutes the ECM of the central nervous system and supports neuronal growth [4-7]. When coating the substrate with Ln or other ECM proteins, it is often combined with the cationic coatings in order to enhance the stability of their adsorption [8-12].

Such surface coatings can be combined with microfabrication technology to create a template surface for cell patterning [13,14]. The template is practically a two-tone pattern of cell-permissive and non-permissive coatings. The permissive area is coated with the abovementioned proteins or molecules. The non-permissive area can be created either by making the surface highly hydrophobic to allow strong binding of serum albumins that inhibit subsequent adsorption of adhesion-promoting proteins [15,16], or by creating hydrated surfaces and making the surface resistant to any protein adsorption [17,18]. The micropatterned template has been shown to be effective in creating cellular arrays for high-throughput screening [16,19], as well as in studying biological properties of the cells [20].

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Recent advancement in surface engineering further enabled us not only to array the cells on templates but also to manipulate the pre-defined pattern geometry while the cells are grown on the surface [14,21,22]. Such “in-situ” or “dynamic” patterning has been realized, for example, by using photoresponsive polymers [23,24] or laser ablation [25,26], just to name a few. We previously reported on the application of laser ablation phenomena to create growth pathways of neuronal processes (neurites) at sub-cellular resolution in situ [26]. Briefly, the working principle of the method is as follows: (1) Primary neurons are first grown on a micropatterned surface, (2) after supplementing the growth medium with Ln, focused femtosecond laser is scanned across the non-permissive area to ablate non-permissive compounds, (3) Ln adsorbs on the laser-scanned pathway, and finally (4) neurites elongate on the pathway. The method has been successful in guiding neurites of primary neurons in an arbitral direction and even making branches in the neurites, which allow us to freely construct networks of living neurons.

The laser ablation method, however, suffered from the low success rate of neurite guidance, which was primarily due to the mechanical damage to the neurons induced cavitation bubbles generated at the focal point of the laser and to the unstable adsorption of the scaffolding protein, Ln, to the laser-irradiated region [26]. To overcome the first issue, we very recently developed a novel surface modification method that takes advantage of titanium dioxide (TiO$_2$) photocatalysis to decompose non-permissive compounds upon irradiation of ultraviolet (UV) light [27]. This method does not require short pulse lasers and is free from generation of the cavitation bubbles.

In the current work, in order to solve the second issue, we used bottom-up self-assembly of biological molecules to enhance stability of Ln adsorption to the processed region. For this, we used in combination ribosomal protein L2 (RPL2), protein A, and anti-Ln antibody (immunoglobulin G; IgG) to enhance binding of Ln to the TiO$_2$ surface. RPL2, also called the “Si-tag”, is a protein purified from bacterial lysates that binds strongly to silica surfaces [28-30]. RPL2 has been successfully used as a tag to immobilize functional proteins on silica surfaces [28,29]. High affinity of RPL2 to silica is attributed to the attractive electrostatic interaction between RPL2 and silica that have net-positive and negative charges, respectively, at physiological pH and to the conformational flexibility of RPL2 [31,32]. Protein A is a protein that binds to the Fc region of mammalian immunoglobulins, with little interference with the antigen-binding sites of the antibody [29,33].

In this paper, we first show that RPL2 possesses a strong affinity to TiO$_2$ surface and that a protein complex of RPL2, protein A, anti-Ln antibody, and Ln actually serves as a growth scaffold of rat hippocampal neurons. We then show that the protein complex can be selectively adsorbed on the photopatterned region and that primary neurons can be selectively grown on that region. The novel surface modification method reported in this work should increase the success rate of in-situ neurite guidance experiments. This would facilitate fabrication of in vitro neuronal circuits with an arbitrary network topology, providing a simple platform for neuronal activity-based neural computation and drug screening for neuropsychiatric diseases. Our method also provides a stable Ln-based scaffold that can be widely used in primary neuron cultures, as well as a novel methodology to immobilize functional proteins on a substrate via molecular recognition.

II. EXPERIMENTAL

A. Photocatalytic decomposition of organosilane monolayers on TiO$_2$

An octadecyltrichlorosilane (OTS) self-assembled monolayer (SAM) or a methoxy-terminated polyethylene glycol (mPEG) SAM were deposited on TiO$_2$-coated glass coverslips. The method to prepare the TiO$_2$/glass coverslip modified with the organosilane SAMs was described elsewhere [27]. To remove the additional organosilane molecules, the coverslips were sonicated in toluene and ethanol, in that order. After rinsed in water, the coverslips were immersed in a neuronal plating medium [minimal essential medium (Gibco) + 5% fetal bovine serum (Gibco) + 0.6% D-glucose (Sigma)] overnight and then rinsed in a phosphate buffered saline (PBS) solution. The coverslip was then placed inside a culture dish and immersed in a PBS solution. UV light was irradiated from the backside to the coverslip immersed in the PBS solution. An inverted fluorescence microscope (Nikon Eclipse TE300) equipped with a 100 W mercury arc lamp, a UV laser (Oxxius LaserBoxx; 375 nm, 16 mW), a UV filter cube (EX 365/10 nm, DM 380 nm, BA 420 nm), and a 20× objective lens (0.45 NA) were used for the UV irradiation. When using the arc lamp, UV dose was altered from 60 to 1500 J cm$^{-2}$. When using the laser, scan speed and the intervals of scanning lines were set to be 1 μm s$^{-1}$ and 10 μm, respectively.

B. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Silica particles (1 mg) were mixed with a fusion protein of immunoglobulin-binding staphylococcal protein A and RPL2 (protein A-RPL2) (0.7 μg) [29,30], anti-Ln antibody (rabbit polyclonal; Millipore AB2034; 2 μg), and Ln (Sigma L2020; 4 μg) in 1 ml of PBS and incubated for 30 min at room temperature. The silica particles were precipitated by centrifugation at 12,000 g and washed 3 times with 1 ml of PBS. The bound proteins were released by boiling in SDS-PAGE sample buffer for 5 min and then separated by SDS-PAGE on a 12.5% acrylamide gel.

C. Fluorescence imaging

A fusion protein of green fluorescent protein (GFP) and RPL2 (RPL2-GFP) [28] was used to evaluate the selectivity and amount of RPL2 adsorption. After the UV irradiation, the OTS/TiO$_2$ sample was immersed in a PBS solution containing RPL2-GFP (10 μg ml$^{-1}$) for 30 min at room temperature, and then rinsed in a PBS solution.
FIG. 1. (a) Fluorescence images from RPL2-GFP and GFP adsorbed on the TiO$_2$-coated coverslips. The TiO$_2$ surface was exposed inside the octagon by the UV-irradiation at a dose of 600 J cm$^{-2}$. The outside of the octagon was covered with the organosilane SAM. Scale bar, 100 μm. (b) The fluorescence intensity from the RPL2-GFP adsorbed on the UV-irradiated region (TiO$_2$) relative to that from RPL2-GFP adsorbed on the outside (OTS SAM or mPEG SAM).

The fluorescence intensity from the RPL2-GFP adsorbed on the UV-irradiated region and un-irradiated region was measured by fluorescence microscopy (EX 470/20 nm, DM 500 nm, BA 515 nm; Hamamatsu ORCA-ER). As the control, the same routine was carried out with GFP without RPL2.

D. Cell culture

Timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories. Primary hippocampal neurons were obtained from embryonic day 18 rats. The dissociated neurons were plated at a concentration of $2.5 \times 10^4$ cells cm$^{-2}$ in the neuronal plating medium. After 3 h of culture, the medium was exchanged to a growth medium [Neurobasal medium (Gibco) + 2% B-27 (Gibco) + 1% Ghtamax I (Gibco)]. The neurons were then incubated in a humidified CO$_2$ chamber (5% CO$_2$, 37°C).

Primary neurons were cultured either on patterned or unpatterned substrate. To prepare the patterned substrate, a TiO$_2$-coated glass coverslip with the organosilane SAM pattern was immersed in a PBS solution containing protein A-RPL2 (63 nM), anti-Ln antibody (63 nM), and Ln (126 nM) for 1 h. For a control experiment, the substrate was immersed in a PBS solution containing poly-D-lysine (PDL) (50 μg ml$^{-1}$) overnight. The coverslips were then rinsed in PBS solutions twice, and immersed in plating medium overnight before plating the neurons. Unpatterned substrate was prepared by immersing a bare glass coverslip in a PBS solution of protein A-RPL2, anti-Ln antibody, and Ln, in that order, or in a PBS solution of PDL.

III. RESULTS AND DISCUSSION

We first studied the difference in affinity of the “Si-tag” RPL2 to TiO$_2$ and a cell-repellent (non-permissive) film, OTS SAM. First, an OTS SAM was deposited on a TiO$_2$-coated glass coverslip. Next, focused UV light was irradiated to the coverslip through the microscope objective at a dose of 600 J cm$^{-2}$. At around this dose, the SAM on the TiO$_2$ is completely decomposed [27]. Finally,
RPL2-GFP was applied to the surface, and GFP fluorescence was imaged to compare the affinity of RPL2 to the intact and UV-irradiated regions.

As shown in Fig. 1(a), bright GFP fluorescence was observed in the UV-irradiated region, only when the GFP was fused with RPL2. This indicates that RPL2 binds specifically to the TiO$_2$ surface that was exposed by the UV irradiation. Fig. 1(a) also shows that RPL2 binds selectively to the UV-irradiated region, since little GFP fluorescence was detected outside the UV-irradiated region. Results of the experiment using an alternative non-permissive film, mPEG SAM, are also shown in Fig. 1(a). In the case of the mPEG SAM, GFP fluorescence of RPL2-GFP was observed not only in the UV-irradiated region but also on the unirradiated region, indicating that RPL2 adsorbed non-specifically on the mPEG SAM. The selectivity of the binding was quantified by calculating the relative GFP fluorescence in the UV-irradiated region to that in the unirradiated region (Fig. 1(b)). As shown in Fig. 1(b), higher selectivity was achieved by using the OTS SAM, hence we used OTS SAMs as non-permissive coatings for the rest of the experiments.

Next we performed SDS-PAGE to study whether a protein complex comprising Ln and RPL2 (“RPL2-tagged Ln”) was appropriately formed. The protein complex we designed is schematically illustrated in Fig. 2(a). It consists of three precursor proteins—(1) a fusion protein of protein A and RPL2 (protein A-RPL2), (2) a rabbit polyclonal IgG to Ln, and (3) Ln—which were mixed in solution phase and used without further purification. The result of gel electrophoresis is shown in Fig. 2(b). Strong bands at $\sim$ 200 kDa, $\sim$ 60 kDa, and $\sim$ 50 kDa, corresponds to Ln subunits, protein A-RPL2, and IgG heavy chain [29,34]. As shown in Fig. 2 (b), Ln was more strongly detected in the presence of all three precursor proteins. A faint Ln band could be observed even when the anti-Ln antibody was absent from the mixture, most probably due to the non-specific adsorption of Ln to silica particles, but the band was much weaker compared to the sample that contained the antibody. This indicates that RPL2-tagged Ln (Fig. 2(a)) was properly obtained.

In parallel to the SDS-PAGE experiment, rat hippocampal neurons were cultured on the coverslip modified with RPL2-tagged Ln in order to confirm its suitability as a neuronal scaffold. For this experiment, a bare glass coverslip was sequentially immersed in a PBS solution of protein A-RPL2, anti-Ln antibody, and Ln. Then neurons were plated and cultured on the coverslip. As shown in Fig. 3(a) and (d), the neurons grew well on the coated coverslip, as can be seen from the uniform growth of processes on the surface and the un-aggregated cell bodies. The cells were comparable to that on conventional PDL-coated glass coverslips (Fig. 3(b) and (e)), indicating that the novel coating method can be used for the neuron cul-
Ln bound preferentially on the TiO$_2$ surface that was exposed by UV irradiation.

We also succeeded in using UV laser for surface modification. Fig. 4(b) shows neurons cultured on RPL2-tagged Ln patterned by scanning focused laser lines in a square geometry. As shown in the phase-contrast image, the neurons grew well inside the pattern, indicating that the cell-adhesive regions can be defined by the UV laser scan. Laser processing is convenient for the fabrication of arbitrary patterns.

Compared with a conventional patterning method based on photolithography that uses highly photosensitive resists, the throughput of the current method is low. Yet the un-necessity of resists can be an advantage in some applications, since residual photoresist can be cell toxic [35]. Most importantly, the method described here is potentially capable of processing surfaces in cell culture environment [27]. Use of the RPL2-tagged Ln is critically important for such an experiment since it has very little non-specific adsorption, in contrast to polylysine (Fig. 5). The patterning resolution can be reduced to ~ 3 µm (data not shown), which is enough to control the growth direction of individual neurites. Therefore, the results obtained in the present work open the new possibility of fabricating neuronal circuits with an arbitrary topology.

**IV. CONCLUSION**

We reported in this paper a surface modification technique that combined top-down photopatterning with TiO$_2$ photocatalysis and bottom-up molecular binding with a tag protein, RPL2. We showed that a neuronal adhesion molecule Ln can be easily tagged with RPL2, by simply mixing protein A-RPL2, anti-Ln antibody, and Ln in solution. The protein complex thus formed served as a scaffold for culturing primary neurons, and could be successfully patterned with the TiO$_2$-assisted surface patterning method. The novel process is expected to increase the success rate of in situ neurite guidance experiments, result of which will be reported soon.

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