Patterning of HeLa Cells on a Microfabricated Au-Coated ITO Substrate

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HeLa cells were patterned on a microfabricated Au-coated ITO substrate. Part of the Au film was dissolved by the electrochemical wet stamping technique, and the substrate was then immersed in a thiol-terminated methoxy-poly(ethylene glycol) (mPEG-SH) solution for further modification. Self-assembled monolayers (SAMs) on the gold island can form cell- and protein-resistant regions on the substrate, and living HeLa cells can adhere to the exposed ITO surface. Single cell patterns could be achieved when the size of a microstructure unit matched that of the cell. The fluorescence experiments further proved that living HeLa cells prefer to attach to the ITO substrate rather than the mPEG-SH-modified gold islands.

Introduction

The patterning process in living cells serves to locate the cell in a defined place and is able to integrate thousands of living cells together. This is a powerful tool in cell biology, wherein cell arrays are used to elucidate the factors that mediate migration, proliferation, and cell—cell interactions. Recently, indium—tin oxide (ITO) has been employed as an important kind of substrate for cell patterning in the study of the photoelectric behavior of mammalian cells or biochip devices; this is due to its favorable properties, including transparency, colorlessness, and good conductivity. The systems obtained by patterning of living cells on ITO have the potential for use in biochemical applications and the correlation of optical live cell imaging with applied illumination. Patternd substrates that allow cells to attach to the delimited regions can be prepared by a combination of self-assembled monolayer (SAM) and photolithography techniques or microcontact printing (μCP) methods. The photolithography techniques need a complex multistep process and can be applied for the patterning of Au films on the ITO substrate. Selective anodic dissolution can take place on the contact area owing to the constant supply of electrolyte from the agarose stamp to the interface. This assembly of patterned Au islands is then modified with cell-resistant SAMs (e.g., mPEG-SH molecules), which were most often used for resisting the nonspecific adsorption of proteins/cells. The exposed ITO part is used for the attachment of living cells. This electrochemical method proposed by us

Although these protein intermediate layers or SAMs help or prevent the subsequent immobilization of cells, the factors that control the transfer of protein or SAMs from the PDMS stamp to the substrate need to be investigated across substrate chemistry and surface energy to determine the limit of the μCP method. For instance, the hydrophobic PDMS surface makes the immobilization of biomaterials difficult, and hence the pretreatment of PDMS by oxidation has been done in order to improve the wettability. Therefore, it is desirable to develop a new technique to attach the cells to the ITO substrate with the desired pattern or the single cell array, which will help the study of the response of each cell to external stimulation.

Figure 1 illustrates the experimental setup and the procedure for the immobilization of living cells on ITO. The gold thin film, with a thickness less than 100 nm, is evaporated onto the ITO substrate by a magnetic sputtering. The electrochemical wet stamping technique (E-WETS) and can be applied for the patterning of Au films on the ITO substrate. Selective anodic dissolution can take place on the contact area owing to the constant supply of electrolyte from the agarose stamp to the interface. This assembly of patterned Au islands is then modified with cell-resistant SAMs (e.g., mPEG-SH molecules), which were most often used for resisting the nonspecific adsorption of proteins/cells. The exposed ITO part is used for the attachment of living cells. This electrochemical method proposed by us

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Preparation of Micropatterned Au-Coated ITO Substrate. The substrate is a conductive glass that is coated with a layer of 100 nm thick ITO giving a sheet resistance of 20 \( \Omega \cdot \text{cm} \). The ITO glass was then successively covered by a thin film of Au (thickness <100 nm) by magnetic sputtering. It was rinsed with ultrapure water and dried with N2 before the micromachining experiments. The electrochemical micromachining of Au/ITO was performed in a specially designed three-electrode electrochemical cell as shown in previously published work.\textsuperscript{12,13} The potential was controlled at 1.0 V versus saturated calomel electrode (SCE) for tens of seconds to dissolve the Au film without destroying the ITO layer beneath. The stamp was placed upside down at the bottom of the electrochemical cell filled with a known concentration of electrolyte. All the electrochemical measurements and the control of the working potential were performed using a CHI 631B electrochemical workstation. The microstructure of the mold and the etched workpiece were characterized using a confocal microscope (Olympus 2000).

Modified with Methoxy-poly(ethylene glycol) (mPEG) SAMs and Cell Culture. mPEG (0.088 g; CH\(_2\)O-PEG-SH, EG stands for an ethylene glycol unit, RAPP Polymere, \( M_w \) = 4870 Da) was dissolved in 10 mL of water, and the concentration of mPEG-SH solution was 1.8 mM. Before polymer coating, the substrates were further cleaned with ethanol to remove any organic contamination. The substrates were thoroughly rinsed with water and dried with a nitrogen gun. The mPEG-SH thiol solutions were prepared by dissolving powdered polymer in water. The gold-coated plates were immersed in the mPEG-SH solutions for 11–12 h at room temperature for subsequent polymer coating. Finally, the polymer-coated surfaces were washed thoroughly with ethanol to remove unbound polymer and dried under \( N_2 \). HeLa cells were trypsinized in a 0.25% trypsin solution and seeded (2 \( \times \) \( 10^5 \)) to the prepatterned Au/ITO plates in Petri dishes (Falcon) and then cultured in a RPMI1640 medium (Gibco) containing 10% fetal bovine serum (Gibco), 50 \( \mu \)g/mL penicillin, and 50 \( \mu \)g/mL streptomycin, in a humidified incubator (37 \( ^\circ \)C in an atmosphere of 5% CO\(_2\)). After washing, the cell pattern cultured for 8–10 h was characterized by microscopy (ZEISS Axiovert 200).

Fluorescence Experiments. The carboxyfluorescein diacetate succinimidyl ester (CFDA SE) probe can passively diffuse into cells and is therefore used as a probe in the dyeing experiment. The process is as follows: cells were cultured on the prepatterned Au/ITO inside a Petri dish filled with the appropriate culture medium. When the cells had reached the desired density, the medium was removed from the dish, and phosphate-buffered saline (PBS) containing 5 \( \mu \)M probe was added. The cells were incubated with the probe for 15 min at 37 \( ^\circ \)C, and a further 30 min in fresh, prewarmed medium without serum. The fluorescent image was also characterized by microscopy (ZEISS Axiovert 200). The approximate excitation and emission peaks of the probe after hydrolysis were 492 and 517 nm, respectively.

Results and Discussion

Figure 2 shows the oxidation process occurring on the gold substrate and the formation of Au–Cl complexes by the anodic oxidation of the Au electrode in the presence of...
The onset of gold dissolution, occurring at approximately 0.9 V versus SCE, predominantly forms \( \text{AuCl}_4^- \). The anodic dissolution of Au in contact with the agarose stamp starts at 0.9 V, as shown in the cyclic voltammogram of Figure 2. The current increased significantly as a result of the double layer charging and the formation of the chloricauric complex (\( \text{AuCl}_4^- \)). The sharp decrease of current at 1.2 V is attributed to the dissolution of Au on the ITO surface. Therefore, the chronoamperic current at an anodic potential of 1.0 V can be used as a sign of the finishing point of the electrochemical micromachining.

The fabricated Au microstructures of Figure 3c compared with the unit on the Si master (Figure 3b) showed that the lateral deviation between the master and the etched microstructure is around 2.47%. The average width of the Au straight line shown in Figure 3c is 21.7 \( \mu \text{m} \), and that of the structure on the Si master is 22.8 \( \mu \text{m} \). The deviation is calculated to be half of the difference between the Au microstructures and the convex straight line on the Si master. The small deviation is due to the lateral diffusion of KCl solution in the interface between the gel stamp and the substrate. Moreover, the distortion of the gel stamp might also affect the resolution of replication, and the distortion becomes more significant under the pressure of the Au/ITO workpiece when the feature size is decreased to a nanometer.

Several studies have demonstrated that mPEG-SH molecules can be tethered to surfaces via organosilane and organosulfur SAMs and other coupling agents to produce materials that reduce protein and cell attachment. Although theories have been proposed to explain the inertness of these mPEG-SH coated surfaces, the correlations between protein adsorption and cell adhesion are complex, and the underlying mechanisms remain unclear. The exposed ITO part is chemically compatible with biological media and optically transparent at visible wavelengths, allowing microscopic examination of cells. HeLa cells are attached to the exposed ITO region of the prepatterned Au/ITO substrate without additional modification of extracellular matrix proteins (fibronectin or other cell adhesive proteins). The ITO region has shown a high affinity for HeLa cells due to the adsorption of proteins of the cell culture medium before the cell adhesion. However, in the interface between the gel stamp and the substrate.
the gold islands on the ITO substrate are coated with 1.8 mM PEG for 12 h to prevent nonspecific protein adsorption and cell adhesion. This difference of adhesive property can be demonstrated in Figure 4. Panels b and d of Figure 4 are the magnified images of panels a and c. Since ITO is transparent and easy to observe with an optical microscope, Figure 4a,b clearly shows that multiple cells were aggregated and were immobilized onto the exposed ITO curved lines with a feature size of 74.0 $\mu$m.

When the size of a single unit of the microstructures matches that of the cell, a single cell pattern could be achieved. For squares with a side length of 25.0 $\mu$m, as shown in Figure 4c, the single cells were arranged one by one on exposed ITO lines. The size of the square unit shown in Figure 4d is 25.0 $\mu$m, which is equivalent to the size of a single HeLa cell; thus single-cell arrays were formed on the substrate. It was determined from the fluorescent image of Figure 4c,d that HeLa cells do not adhere to the mPEG-SH-modified Au islands. It should be noted that the stability of the surface grafted with mPEG-SH is highly dependent upon the solvent environment, and adsorbed mPEG-SH may be subject to desorption in a biological milieu. In the case of the higher density required for culturing, the HeLa cells form a continuous film and could not attach selectively on to the ITO surface. Much of the unoccupied ITO space resulted from the comparatively low cell intensity (200000–240000 cells/cm$^3$) for culturing in order to prevent the aggregation and differentiation of the living cells.

To verify the preference of ITO surface in the immobilization of HeLa cells, fluorescence experiments were performed. The probe CFDA SE is colorless and nonfluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. $^{22,23}$ The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well-retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and byproduct passively diffuse to the extracellular medium, where they can be washed away. The fluorescent image in Figure 5 shows that the HeLa cells are attached on the exposed ITO part of the hybrid substrate with different sizes. During this time, CFDA SE will undergo acetate hydrolysis, and the fluorescent image matches the image shown in Figure 4. To study the cell adhesion on the ITO substrate without the modification of SAMs, the comparative experiments (Figures 1S and 2S in the Supporting Information) have shown that the patterning of cell arrays on ITO surfaces failed without the modification of mPEG-SH molecules on Au microstructures.

**Conclusions**

In summary, we have shown a novel and facile fabrication method for a cellular micropatterned substrate. The electrochemical method has been applied to fabricate a micropatterned Au/ITO substrate for a cell array on an exposed ITO surface. The technical simplicity of the method allows rapid production of samples with different shapes and sizes. This method will contribute to the study of future photoelectric behavior of HeLa cells, and applications in medical devices, tissue engineering, and array technologies are easy to imagine.

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**Supporting Information Available:** Study of cell-resistant SAMs of mPEG-SH. This information is available free of charge via the Internet at http://pubs.acs.org.

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