Attaching Cells

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Switching On Cell Adhesion with Microelectrodes**

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Controlling the interfaces between cells and solid substrates is an important theme pertinent to a variety of research applications such as cell biology,[1] tissue engineering,[2] and cell-based sensors and chip devices.[3] In particular, the recent progress in the understanding of the molecular mechanism of cell adhesion has promoted the development of self-assembled monolayer (SAM)-based techniques for precise cell pattering, for example, microcontact printing (μCP).[4] SAMs that are terminated in short oligomers of the ethylene glycol (OEG) unit ($HS(CH_2)_{11}(OCH_2CH_2)_nOH$; $n = 3$–7) were most often used in these studies for resisting the nonspecific adsorption of proteins/cells, and are proven to be the best "nonadsorption" systems that are currently available.[5] However, conventional μCP methods produce static surface stimuli. Once cells are adherent, it is difficult to subsequently alter the matrix environment.[6]

Herein, we described a strategy for the real-time local manipulation of the cell-adhesive property of an OEG-terminated SAM substrate using ultramicroelectrodes (UME), which enables the template-free formation of cellular micropatterns by directing cell adhesion and growth in situ. The strategy is based on our finding that the cytophobic nature of OEG SAMs is rapidly switched to cell adhesive by exposure to some oxidizing agents, such as Br_2, which can be electrogenerated from Br^- in aqueous solution. By scanning a UME as a "pen" closely above the substrate, one can draw a cellular pattern. As illustrated in Figure 1, an OEG-terminated SAM substrate was formed by inserting a gold slide into an OEG-terminated alkanethiol solution for 12 h. Then, a microelectrode with a radius $r_T = 25$ μm (T = tip, of the UME) was brought to 5 μm above the substrate (Figure 1a). A 5 s potential pulse of 1.2 V (compared with an Ag quasi-reference electrode) was applied in a 0.1 M phosphate buffer solution containing 25 mM KBr (pH 7.4). The electrochemically generated Br_2 diffused to the substrate and quickly reacted with the OEG monolayer locally. The modification of the OEG SAM was confirmed by a scanning electrochemical microscopy (SECM) feedback image (Figure 1b). The contrast is based on the permeability difference of the OEG SAM and the Br_2-treated SAM. The bright parts in the image indicate higher reduction currents, corresponding to a higher permeability of the monolayer (see the Supporting Information). The treated substrate was then incubated in extracellular matrix (ECM) protein solutions, for example, fibronectin, fibrinogen etc. Proteins adsorbed exclusively on the electrochemically treated regions (Figure 1c). Figure 1d shows a confocal laser scanning fluorescent micrograph of ECM protein pattern formed after adsorption of a fluorescence-labeled protein (4 h in 100 μg mL^{-1} fibronogen-Alexa 488). During a subsequent seeding of human fibroblast culture on the substrate, the preadsorbed protein promoted the specific attachment of fibroblasts, resulting in the formation of a cellular micropattern on the surface

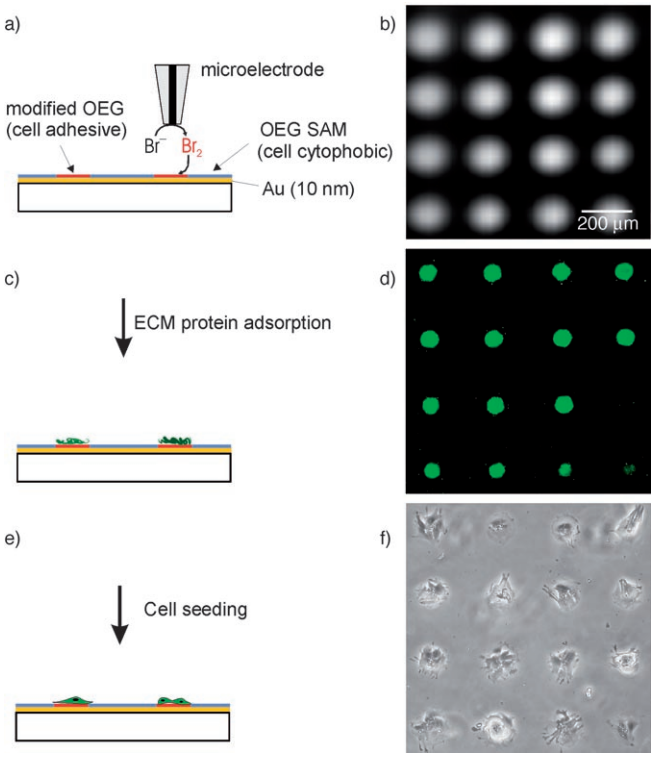

Figure 1. The use of a microelectrode to induce cell adhesion and growth onto an OEG SAM substrate. a) Microelectrochemical modification of the OEG monolayer by electrogenerated Br_2; b) SECM image of the modified OEG substrate; c) site-selective adsorption of fibronogen-Alexa 488 onto a modified region of the monolayer; d) confocal laser scanning fluorescence micrograph after (c); e) attachment of human fibroblasts; f) phase-contrast micrograph of the micropattern 24 h after immersion into the cell culture.

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(Figure 1e,f). Although spots could be formed as a result of holding the microelectrode in a fixed location above the surface, lines could be generated by translating the microelectrode horizontally while producing Br_2 (Figure 2). The

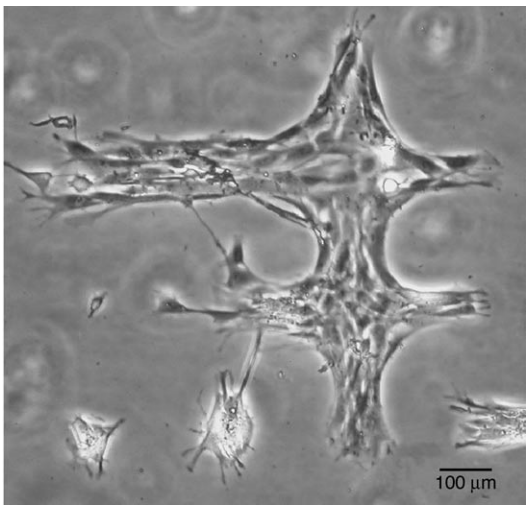

Figure 2. Phase-contrast optical micrograph of the linear cellular pattern fabricated by scanning the microelectrode ($r_T = 25$ µm) at a lateral speed of 5 µm s^{-1}. The microelectrode potential was kept at 1.2 V during "drawing" and at 0 V while not drawing.

size of the patterns depends strongly on the size of the microelectrode.[7] By using advanced approaches for microelectrode fabrication, the smallest pattern size generated by UME could be as low as approximately 50 nm.[8] No restriction exists on larger sizes. The throughput could be increased by the use of microelectrode arrays.

To control cell–substrate interaction dynamically, some groups have reported µCP-based electrochemical methods, such as electrochemically induced immobilization of peptide ligands by a Diels–Alder reaction to activate inert surfaces,[9] or electrochemical desorption of OEG SAMs to induce cell attachment to the SAM-desorbed area.[10] In this study, the UME has also been applied for the modification of prepatterned cellular substrates, which provides an alternative strategy to pattern multiple cell populations by sequentially switching the adhesivity of different regions of the substrate. As illustrated in Figure 3a, the UME was brought above the substrate onto which the first fibroblast population had been previously attached (labeled with a green fluorescent dye). Positioning the UME relative to the first cell pattern was achieved by a positioning system mounted on an inverted microscope. This allowed us to follow the movement of the UME to the desired location. A potential pulse was then applied to open a new region for the adhesion of a second fibroblast population (labeled by a red fluorescent dye, see Figure 3b,c). During this step, the growth of the Br_2 diffusion layer at UME was controlled by adjusting the UME size and potential pulse time to prevent the Br_2 from reaching the prepatterned cells, even though it has been recently reported by Matsue et al. that Br_2 and its subsequent degradation products do little damage to the preexisting cells.[11] Similar to

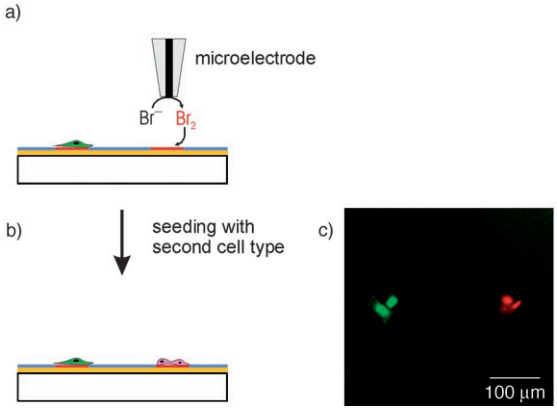

Figure 3. Modification of cellular substrate and formation of multiple cell cocultures by using microelectrodes. a) Microelectrochemical modification of the substrate prepatterned with fibroblasts; b) attachment of a second population of fibroblasts; c) confocal laser scanning fluorescence micrograph of a multiple cell coculture. The second population of fibroblast (red) was introduced onto the sample with the pattern of the first fibroblast population (green).

most lithrography-based methods,[9,12] our method relies on 100% occupancy of the first adhesive area for the first cell population. The second fibroblast population may also attach to the unoccupied region of the first cellular pattern (not shown). Therefore, formation of a confluent monolayer of the first cell population is necessary to obtain a well-defined coculture of different cells on the substrate.

Despite the immense practical importance of the protein/cell resistance effect of OEG SAMs, the underlying mechanisms are not completely clear.[13] Grunze et al. recently summarized the various factors that are critical for the functionality of the monolayer and suggested that only the combination of the three factors, the internal hydrophility, terminal hydrophobicity, and lateral packing density, allows the formation of a SAM that is fully protein/cell resistant.[14] When one of these factors is unfavourable or absent, the overall protein/cell resistance decreases. In this study, the detailed mechanism of the monolayer transformation is still under investigation. X-ray photoelectron spectra show that bromine is not incorporated in the SAM layer, in agreement with the observation that the procedure also works with oxidizers other than bromine. Polarization-modulated infrared reflection–absorption spectroscopy showed that during bromine treatment, the oligoethylene units of the monolayer are lost, whereas degradation of the alkyl part of the SAM takes considerably longer. This is consistent with our observation that bromine treatment of an OEG SAM leads to an increase in the terminal hydrophobicity. A detailed mechanistic study is still in progress.

In conclusion, we have described a simple microelectrochemical method for switching on cell adhesion and growth on OEG SAM substrates in situ. The method could be applied by using basic electrochemical instrumentation that is also employed in neurophysiological experiments (potentiostat, microelectrodes, and positioners). By using a microelectrode as a "pen", the method enables template-free drawing of cellular patterns and in situ modification of a

prepatterned cellular substrate. As stepwise site-directed introduction of different cell types onto the substrate is possible, the method could also facilitate the formation of micropatterned cocultures and, therefore, contribute to in vitro investigations of multicellular interactions and to tissue engineering. We believe that the application scope will be further enlarged by combining the in situ manipulation function with other microfabrication techniques, for example, microcontact printing that is capable of producing extended and complex patterns of cell culture in a highly parallel way.

Experimental Section

Substrates were prepared by the evaporation of chromium (5 nm) and then gold (10 nm) onto cleaned glass coverslips. The substrate was then immersed in OEG solution (3 mM) in ethanol to form the monolayer. The substrates were rinsed thoroughly with absolute ethanol and dried under a stream of argon.

The microelectrochemical modifications of the monolayer were performed on a Pt UME ($r_T = 25\,\mu m$) mounted on a home-built positioning system which was described previously.[15] A Pt wire as the auxiliary electrode and a silver quasi reference electrode, to which all potentials are referred, completed the electrochemical cell. The working solution was phosphate buffer solution (0.1M; pH 7.4) containing KBr (25 mM). The UME was brought close to the substrate and then a 5 s potential pulse of 1.2 V was imposed to generate Br_2. Electrochemically generated bromine reacts with the monolayer and quickly switched the monolayer from cell resistant to cell adhesive.

After the modification, the substrate was incubated in ECM (100 $\mu g\,mL^{-1}$) proteins fibrinogen-Alexa 488 in phosphate-buffered solution (PBS; phosphate (0.1M), NaCl (0.15M), pH 7.4) for 4 h before the substrate was examined by confocal laser scanning fluorescence microscopy (Leica TCS SP2/AOBS, Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). The dye was excited at a wavelength of 488 nm and the fluorescence was detected with a spectral range of 500–535 nm.

Human fibroblasts grown to confluency were labeled with CellTracker Green (25 μM) for 30 min. After removing the dye and repeated washing with serum-free minimum essential medium (MEM), the cells were trypsinized and resuspended in serum-free culture medium (40000 cells mL^{-1}) and then added onto a patterned monolayer precoated with fibronectin and allowed to attach for 4 h in a CO_2 incubator at 37 °C. The substrate was then transferred to a new culture dish containing complete medium and incubated for 24 h before examined by phase-contrast microscopy.

To prepare cocultures, the UME was then brought to the desired location under the control of the optical microscope and the potential pulse was applied to transform a new region. The substrate was then reseeded with the second population of fibroblasts that were prelabelled with CellTracker Orange at a concentration of 40000 cells mL^{-1}. The substrates were incubated at 37 °C for an additional 2 h before the substrate was examined by confocal laser scanning fluorescence microscopy. The two channels that were used to excite the dyes were 488 nm and 568 nm, and the spectral range of detectors was 500–535 nm (displayed in green) and 555–700 nm (displayed in red), respectively.

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