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Selective Deposition of Native Cell Membranes on Biocompatible Micropatterns

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Abstract: We establish two methods to deposit native biomembranes (human erythrocyte membranes and sarcoplasmic reticulum membranes) selectively onto biocompatible microtemplates. The first method utilizes UV photolithography to micropattern the regenerated cellulose, while the second uses the "stamping" of protein barriers onto homogeneous cellulose supports. The relatively simple methods established here allow for the position selective spreading of three-dimensional native cells into two-dimensional films, retaining the orientation and lateral density of transmembrane proteins in their native state.

Introduction

Supported lipid membranes have been intensively and widely studied in the last couple of decades as a general model system of cell- and tissue surfaces.[1−3] Several methods have been developed for micropatterning solid supported membranes to manipulate and characterize each corral individually.[4−7] For example, the membrane patterns can be used as quasi two-dimensional fluid matrixes (instead of bulk three-dimensional matrixes such as gels) that allow for the accumulation and reorganization of functional molecules by electrophoresis.[8,9] The use of micropipettors will enable the parallel monitoring of interactions between the partitioned membranes and the analytes (antibodies, drugs, etc.) by spotting different analytes onto individual corrals. Moreover, the partitioning of membranes prohibits the diffusion of proteins across the separating barriers and, therefore, includes a large potential for complementary coupling of supported membranes and semiconductor devices[10−13] by matching of the lateral dimensions.

Incorporation of transmembrane (integral) proteins into the membrane introduces biospecific functions, which enables one to physically model cell adhesion.[14−17] One method is to reconstitute transmembrane proteins into artificial phospholipid vesicles (proteoliposomes) and to deposit them on solid surfaces; however, a fundamental drawback of this method is that control of the orientation and density of the proteins in vesicles is difficult. One of the most straightforward ways to overcome this issue is to use native cells as natural proteoliposomes. As generally known, the orientation of transmembrane proteins in cell membranes is stringently regulated by nature.[18] Along this line, several studies have been conducted to immobilize native cells (erythrocyte, HeLa cells, etc.) on several types of colloidal particles (Latex, silica, etc.).[19−21] Previously, we reported the spreading of native cell membranes (erythrocytes) onto planar solid substrate coated homogeneously with cellulose films.[22] Since no adhesion or rupturing of cells could be observed on bare glass substrates, the results suggested the potential for "printing" arrays of native cell membranes. Here we demonstrate direct spreading of native cells on biocompatible microtemplates, which has never been reported. In the following, two simple methods are described to process microtemplates of regenerated cellulose for local immobilization of native biomembranes on planar supports without losing membrane asymmetry.

Materials and Methods

Erythrocyte ghosts were prepared from freshly drawn blood as reported by Schwoch and Passow.[23] Orientation of the erythrocyte membrane was identified with two immune-fluorescence labels: (1)

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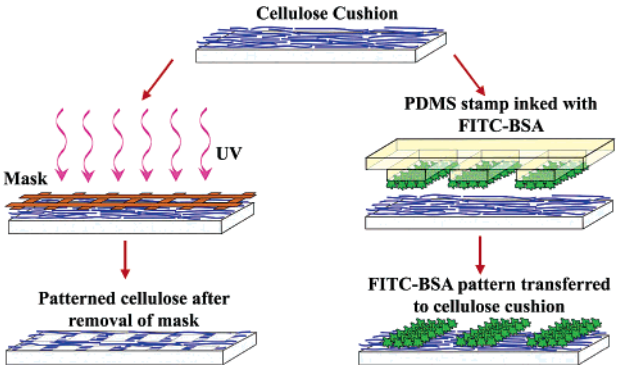

Figure 1. Preparation of cellulose microtemplates. The first method (a) uses deep UV photolithography to micropattern the cellulose, while the second (b) utilizes the "stamping" of protein barriers onto polymer supports.

the extracellular part of glycophorin could be labeled with a first monoclonal antibody (mouse IgG) and a second polyclonal antibody (goat anti-mouse IgG) with tetramethylrhodamine isothiocyanate (TRITC, outside label), while (2) the cytoplasmic domain of Band 3 could be recognized with a first monoclonal antibody (mouse IgG) and a second TRITC-labeled polyclonal goat anti-mouse IgG antibody (inside label). To gain the maximum immunofluorescence signals, the concentration, incubation time, and pretreatment with bovine serum albumin (BSA) were carefully optimized.[22] In brief: The first monoclonal antibody for the inside label was diluted 2500 times (by volume), while the one for the outside label was diluted 500 times. For both labelings, the second polyclonal antibody was diluted 150 times. The incubation time for each antibody labeling was about 30 min. Nonspecific adsorption of antibodies was avoided by treating the sample with a solution of bovine serum albumin (BSA, 30 mg/mL) for 1 h before the immunolabeling. Prior to the spreading upon cellulose microtemplates, we confirmed that intact erythrocyte ghosts retained their native orientation throughout the preparation, exhibiting fluorescence signals only from antibodies to the extracellular domain of glycophorin.

Ultrathin films of cellulose (thickness ~5 nm) were prepared by Langmuir−Blodgett deposition of synthetic trimethylsilyl cellulose (TMSC) onto hydrophobized glass substrates, followed by regeneration with HCl vapor.[10,22,24] Preparation of cellulose microtemplates was represented in Figure 1. The first method (a) used deep UV photolithography to micropattern the cellulose,[24] while the second (b) utilized the "stamping" of homogeneous protein barriers onto polymer supports.[25−27] Ni electron microscope grids (SCI Science Services, Munich, Germany, feature size: 40 μm, spacing between the grids: 60 μm) were used as photomasks. An EM grid was fixed onto the cellulose surface with a drop of chloroform, and the sample was illuminated for 30 min with a 500 W mercury arc lamp, using a thermal filter of fused silica (thickness: 6 mm, Melles Griot, Bensheim, Germany). The illumination with deep UV light (emmision lines at λ = 244, 194, and 185 nm) led to the ablation of cellulose backbones and alkylsilanes,[24,28] resulting in a hydrophilic glass surface. A poly(dimethysiloxane) (PDMS) stamp was formed by curing the two-part elastomer, Sylgard 184 (Dow Corning, Midland, MI), upon patterned photoresist at 70 °C for 1 h.[26] The photoresist was spun to a thickness of approximately 1.5 μm onto hexadimethyldisilazane (HMDS)-primed silicon wafers (3 × 3 cm^2) and was patterned with standard photolithographic techniques. These masters were used indefinitely. PDMS was cured on the masters and plasma-cleaned for 50 s to render the surface hydrophilic.

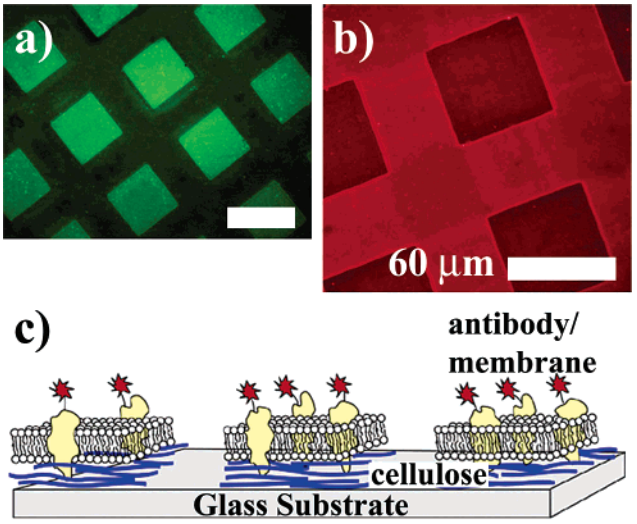

Figure 2. (a) Fluorescence image of cellulose micropatterns exposed to a solution of bovine serum albumin labeled with fluorescein isothiocyanate (FITC-BSA) after removing the photomask. FITC-BSA adsorbs only onto the ablated area (bare glass substrate). (b) Human erythrocyte membranes spread on cellulose microtemplates (without BSA treatment). After incubation, the cytoplasmic domain of Band 3 is identified with a monoclonal and a TRITC-labeled polyclonal antibody (inside label). (c) Orientation of the erythrocyte membrane after spreading. Erythrocyte ghosts selectively adhere and rupture only on the cellulose micropatterns, exposing their cytoplasmic side.

Results and Discussion

Figure 2a shows the fluorescence image of cellulose micropatterns exposed for 10 min to a solution of BSA (1 mg/mL) labeled with fluorescein isothiocyanate (FITC) after removing the photomask. FITC-BSA adsorbs onto the ablated area (i.e., bare glass substrate). Human erythrocyte ghosts are then incubated with the cellulose micropatterns at 37 °C for 2 h. After several washes to remove any unbound intact erythrocytes, the inside labels are applied (Figure 2b). A homogeneous fluorescence signal from the TRITC-labeled antibody confirms that the immobilized erythrocyte membrane exposes the cytoplasmic domain uniformly (Figure 2c). Here, the treatment with FITC-BSA is inserted only to visualize the patterns before incubation with the cells, but it does not influence the resulting patterns of erythrocyte membranes. It should be noted that the fluorescence signals in this image are from labeled transmembrane proteins and not from lipids. Outside labeling reveals no fluorescence signal up to our detection limit (data not shown), suggesting that all the adherent cells ruptured and inverted their orientation to "inside-out". Here, the treatment of the cellulose patterns with BSA solution has no influence on the selective spreading of cell membranes; the spreading of erythrocyte membranes took place only on the cellulose film but not on the glass surface.

Figure 3a shows the fluorescence image of "stamped" FITC-BSA patterns in an aqueous buffer. A poly(dimethysiloxane) (PDMS) stamp (feature size: 5 μm, spacing between the grids: 25 μm) is inked with a 1 mg/mL solution of FITC-BSA for 10 min and then placed upon a cellulose film for 10 min under a 30 g weight. Some bleeding of the BSA grids during stamping leads to a slightly larger transferred feature size, but the resulting

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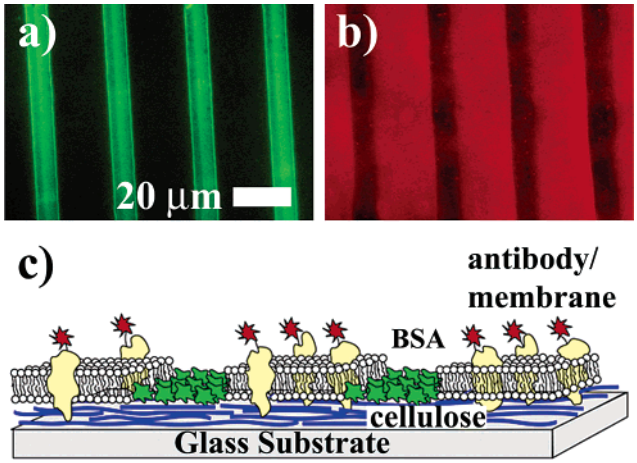

Figure 3. (a) Fluorescence image of "stamped" FITC-BSA patterns using a PDMS stamp. (b) Incubation of erythrocyte ghosts to the same microtemplates and subsequent inside labeling yield homogeneous fluorescence signals from areas with cellulose films. (c) "Inside out" orientation of erythrocyte membranes after deposition onto cellulose with FITC-BSA patterns. The protein patterns are stable after spreading and labeling.

micropatterns of FITC-BSA are stable for several days under water without any degradation. Incubation of erythrocyte ghosts to the microtemplates and subsequent inside labeling yields homogeneous fluorescence signals from areas with the cellulose cushion, but no fluorescence signal can be detected from areas with FITC-BSA. The fluorescence image with the FITC filter set is identical to that in Figure 3a, confirming that the protein barriers remain stable after treatment with cells and antibodies. Outside labeling of this system also exhibits no fluorescence signals, which verifies that spreading of cell membranes also results in an inside-out orientation (Figure 3c).

Immobilized erythrocyte membranes can cover macroscopically large surfaces homogeneously for both types of microtemplates. Immune-fluorescence labeling of transmembrane proteins exhibits no boundaries between the ruptured membrane patches, suggesting that the immobilized cell membranes fuse with each other and "wet" the surface continuously. Since the height deviation (~5 nm) is much smaller than the width of each pattern ($\geq 5\ \mu$m), the surface heterogeneity can be treated almost as a columnar one. Therefore, the spreading of cell membranes can be understood as an analogue of "complete wetting", characterized by a positive disjoining pressure and a positive spreading coefficient S:

$$S = \sigma_{SL} - \sigma_{SM} - \sigma_{ML}$$

where σ_{SL}, σ_{SM}, and σ_{ML} correspond to the tensions at substrate/liquid, substrate/membrane, and membrane/liquid interfaces, respectively. For example, strong electrostatic attractions between strong polycations and weak polyanions (glycocalix with sialic acid residues) destabilize the membrane and result in the formation of regions of tight local contact (de-wetting).[22] Cell membranes therefore prefer cellulose surfaces over bare glass and BSA-coated substrates to achieve a positive disjoining pressure and a positive spreading coefficient.

In addition to erythrocyte ghosts, sarcoplasmic reticulum (SR) vesicles are spread onto the similar cellulose micropatterns. SR vesicles, microsomes extracted from rabbit muscle as reported by de Meis and Hasselbach,[29] are purchased from Nimbus Biotechnologie GmbH (Leipzig, Germany). SR vesicles store

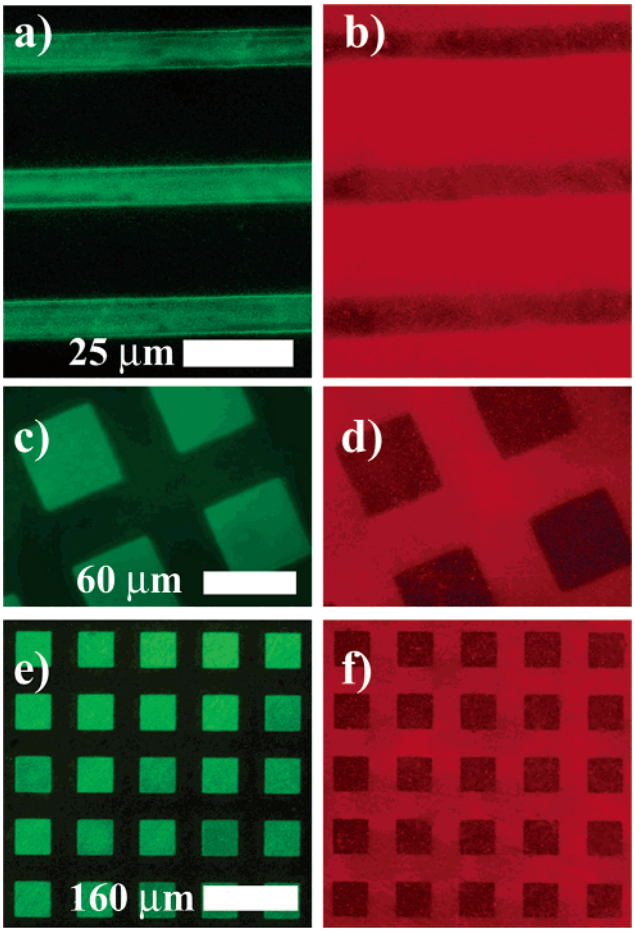

Figure 4. (a) Fluorescence images of the cellulose film with "stamped" FITC-BSA patterns. (b) After spreading of SR vesicles, the cytoplasmic head of Ca^{2+}-ATPase is labeled with a monoclonal and a TRITC-labeled polyclonal antibody. The protein patterns are stable after spreading and labeling. (c) Fluorescence images of the photostructured cellulose film treated with FITC-BSA solution. (d) Selective spreading of SR membranes onto cellulose micropatterns. The adsorbed FITC-BSA effectively prevents the spreading of SR membranes onto the bare glass substrate. (e and f) Fluorescence images of the same micropatterns taken with a lower magnification, demonstrating the uniform confinement of SR membranes over a macroscopically large area.

calcium ions in muscle fibers and contain two calcium ion transporters: the ryanodine receptor and Ca^{2+}-ATPase. The SR vesicles are disrupted through the preparation, and therefore, the membrane orientation cannot be controlled. After the vesicles are incubated with cellulose micropatterns at 37 °C for 2 h, the cytoplasmic head of Ca^{2+}-ATPase in the immobilized SR membrane is labeled with a first monoclonal mouse IgG antibody and a second TRITC-labeled polyclonal goat anti-mouse IgG antibody. When SR vesicles are incubated with the cellulose film with "stamped" FITC-BSA patterns (Figure 1b) and labeled with antibodies, we also observe clear patterns of TRITC-labeled Ca^{2+}-ATPase within the isolated grids (Figure 4, parts a and b). Parts d and f of Figure 4 show the TRITC-labeled SR membranes on a photostructured cellulose film (Figure 1a), which had been pretreated with 1 mg/mL of FITC-BSA solution before spreading membranes (Figure 4, parts c and e). In these series of experiments, treatment of the photolithgraphically patterned cellulose (Figure 1a) with FITC-BSA prior to incubation with the SR vesicles is necessary, since

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preliminary experiments showed that SR membranes readily spread not only upon cellulose films but also on other surfaces such as bare glass slides, poly(lysine), etc. (data not shown). This can be attributed to the fact that SR vesicles resemble artificial proteoliposomes more than native cells, because the extracted SR membranes are free of glycocalix and cytoskeleton and consist only of lipids and proteins. Similar to what is observed for erythrocyte ghosts, SR vesicles selectively spread on the prepatterned cellulose film, where Ca^{2+}-ATPase is homogeneously distributed. Fluorescence images taken at lower magnification (Figure 4, parts e and f) clearly demonstrate the uniform confinement of immobilized membranes over a macroscopically large area.

Fluorescence recovery after photobleaching (FRAP) experiments suggest no clear sign of protein diffusion in both of the native membranes,[30] which can be attributed to the following: (1) In intact erythrocyte membranes, Band 3 proteins are bound to the cytoskeleton (spectrin) via ankyrin. The cytoskeleton which remains after erythrocyte rupture as suggested by previous reports[19,21] acts as a net, preventing protein diffusion across spectrin network. The mesh size, which is less than 1 μm,[31] is smaller than the diameter of the bleached spot ($\sim$9 μm), and thus, diffusion is not observed. (2) Direct immune-fluorescence labeling of the proteins provides sufficient drag to protein diffusion. Two IgG antibodies (first monoclonal and second polyclonal) are used to label each protein. The total molecular weight of two IgGs is up to 300 kD, which is larger than that of the protein (about 50 kD for Band 3 and 110 kD for Ca^{2+}-ATPase). The diffusion of such "bulky" antibody complexes can significantly be impeded by the steric hindrance from the neighboring proteins (antibody complexes) and cytoskeletons. Thus, quantitative measurements of protein mole fractions and the use of smaller marker molecules will be necessary to gain further insight of the diffusion of proteins in the planar, native cell membranes.

(30) FRAP measurements were carried out by focusing a beam of an argon ion laser (Innova 70, Coherent, Santa Clara, CA) onto the sample (spot diameter 9.3 μm) through a microscope oil immersion objective (Fluar 100×, n.a. 1.3, Carl Zeiss, Göttingen, Germany). The dye molecules were bleached by a short laser pulse (200 ms), and recovery of the fluorescence intensity according to the diffusion of unbleached dyes was monitored by a photomultiplier (Hamamatsu Photonics, Herrsching, Germany). The lateral diffusion constant D and mobile fraction were calculated from the measured fluorescence recovery profiles, yielding the sensitivity limit of about $D = 0.01\ \mu m^2\ s^{-1}$. However, even the measurement in a long period (40 min) suggests no clear sign of protein diffusion out of the drift of the background signals.

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Conclusions

In this article, position and orientation selective spreading of native cell membranes (human erythrocyte membranes) on solid substrates has been demonstrated by the use of biocompatible microtemplates of regenerated cellulose films. In contrast to the previously reported techniques for the micropatterning of artificial lipid bilayers on bare solid substrates, the relatively simple microstructuring of biocompatible ultrathin films enables three-dimensional native cells to be transformed into two-dimensional films within confined geometry. Since the orientation and lateral density of proteins can be kept as in their native state, quantitative evaluation of the proteins' function on solid substrates is possible. These microtemplates can be further applied to immobilize microsomes from rabbit muscles (SR vesicles). Both types of membrane micropatterns retain their structure for more than a week, verifying their thermodynamic and mechanical stability. Thus, the processing methods established here allow for further manipulation by the addition of other native cell membranes to design heterobiofunctional surfaces to study the function of membrane proteins under more native environments.

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