Neutravidin micropatterning by deep UV irradiation

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We describe a novel approach for directly patterning neutravidin protein by exposure to deep UV irradiation. Neutravidin is physically absorbed onto the glass or quartz substrate, dehydrated in acetone and air-dried. Dry neutravidin-coated samples are patterned either by top-side or back-side exposure to 185 nm UV. Subsequent introduction of fluorescent biotinylated proteins clearly demonstrates binding to the masked neutravidin regions and no binding to exposed areas. Patterned samples retain their protein affinity for at least three months of storage at room temperature.

Introduction

The ability to pattern proteins at microscale dimensions on surfaces is an important tool for cell adhesion investigations, for developing novel biosensors that incorporate immobilized enzymes or receptors, and for harnessing the transport abilities of biomolecular motors. To date, the three most common techniques for patterning proteins on planar surfaces are microcontact printing, lift-off processes, and photochemical methods.[1–3] However, each approach has its limitations, and to expand protein micropatterning as an enabling tool for fundamental and applied investigations requires novel approaches that are both straightforward and reproducible.

The use of ultraviolet (UV) irradiation for patterning proteins offers several advantages over microcontact printing and lift-off processes, including high spatial resolution, accurate alignment with existing patterns, and broad acceptance as a standard industrial practice. Here, we develop deep UV patterning as a tool for controlling the spatial organization of neutravidin on quartz and glass surfaces. Biotin–avidin chemistry is a popular and versatile approach for immobilizing antibodies, nucleotides, and other biomolecules on surfaces (streptavidin and neutravidin are functional analogs of avidin). Because of this utility, a number of approaches have been employed to spatially pattern avidin and biotin. Muthukrishnan employed both microcontact printing and parylene lift-off to pattern neutravidin for the purposes of immobilizing microtubules,[4] and Vail *et al.*[5] selectively capped carboxy groups on alkanethiol monolayers and coupled neutravidin to the remaining carboxy groups to create protein microarrays. Koyano *et al.* achieved streptavidin patterning by illuminating biotin-hydroxysuccinimide with 290 nm UV through a quartz mask, which caused the biotin groups to lose their affinity for streptavidin.[6] However, directly deactivating surface-adsorbed avidin provides a more straightforward and generalizable approach.

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The present study describes a novel approach for directly patterning neutravidin by exposure to deep UV irradiation. Neutravidin is physically absorbed onto the substrate, dehydrated in acetone and dried, and the neutravidin is selectively patterned by deep UV exposure. These patterned samples can be stored on the bench for months and upon rehydration used to selectively pattern biotinylated proteins on surfaces.

Experimental

Preparation of neutravidin and biotinylated proteins

Lyophilized neutravidin (Pierce Inc, IL, USA) was resuspended at 1 mg mL^{-1} in BRB80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9 with KOH). Tubulin was prepared and labeled by standard procedures,[7] and rhodamine-labeled biotinylated microtubules were polymerized as previously described using a ratio of 1 : 6 rhodamine : biotin tubulin.[8] Fluorescent biotinylated bovine serum albumin (BSA) was prepared by reacting 10 mg mL^{-1} BSA with 20-fold excess of both Biotin XX-SE (Invitrogen) and Rhodamine-NHS (Invitrogen) at room temperature for 2 h in BRB80. Excess dye and biotin were removed using a PD-10 desalting column (Amersham).

UV illumination tools

Four different UV light sources that differed in their output of 185 nm were used for patterning. A UVP PR100 photoreactor (UVP; Upland, CA, USA) with nitrogen purge provides high DUV output with 1.5 mW cm^{-2} at 185 nm and 15 mW cm^{-2} at 254 nm. A germicidal lamp (MCC-UV 254 nm lamp, Rexim, MA, USA) provides moderate DUV output with 4.8 mW cm^{-2} at 254 nm and low intensity at 185 nm. A Fisher Scientific UV crosslinker (FB-UVXL-1000) provides negligible 185 nm output and 3.5–4.5 mW cm^{-2} at 254 nm. A Karl Suss MA-55 aligner provides 15 mW cm^{-2} at the G-line (436 nm ± 10 nm).

Microscopy and image analysis

Fluorescent biotinylated microtubules and BSA were observed by epifluorescence microscopy (Nikon E600, 60× 1.2 N.A. water immersion objective). Images recorded on videotape were digitized using Scion Image (Scion Corporation). To

demonstrate neutravidin patterns, microtubules were counted on ten randomly selected 80 μm by 110 μm screens. Fluorescent biotinylated BSA was also imaged to show the clear outlines of neutravidin patterns.

Results and discussion

Neutravidin dehydration by acetone

Patterning proteins by UV irradiation in the dry state not only increases patterning efficiency but also enables patterning using standard cleanroom procedures. Further, absorption of aqueous solutions in the deep ultraviolet (<200 nm) is significant,[9,10] and air bubbles generated by heating can denature proteins on the surface. To enable patterning of dry proteins, we developed a simple acetone dehydration process that maintains the protein activity. Neutravidin was physically adsorbed by incubating the protein solution on pirhana-cleaned quartz and glass surfaces for 5 min. The sample was then immersed in acetone for 5 min and air-dried. To determine the effect of drying on neutravidin activity, neutravidin-coated coverslips were assembled into flow cells before and after drying, fluorescent biotinylated microtubules were flowed in for 5 min, and the number of bound microtubules were compared. Acetone-dried and reconstituted surfaces had 132 ± 26 microtubules per 80 μm by 110 μm video screen (mean ± SD; $n = 10$) compared to 163 ± 27 microtubules before treatment. Hence, the processed surfaces retain robust neutravidin activity.

Protein micropatterning

To pattern surface-adsorbed neutravidin, quartz masks were used to maximize transmission in the DUV, and a UV-blocking coating was used to mask the light from selected regions of the surface. Two geometries were used: top-side UV exposure from a quartz mask and back-side UV exposure through a quartz substrate.

The advantage of top-side exposure is that it can be used to pattern any flat surface. Neutravidin was selectively patterned on glass coverslips by DUV exposure through a quartz mask as shown in Fig. 1(a)–(c). The sample was assembled into a flow cell and rehdryated with a solution of 0.5 mg mL^{-1} casein in BRB80 to block nonspecific binding to the exposed regions. Fluorescent biotinylated BSA was then introduced and incubated for 5 min to assess the efficiency of patterning. The resulting neutravidin pattern, achieved with 10 min exposure from the germicidal lamp, is shown in Fig. 1(d). The clear pattern of biotinylated BSA demonstrates the precision and efficiency of this technique. The lateral resolution achieved here was roughly 5 μm, but for applications that require high lateral resolution there is significant room for improvement through optimization of the mask and improving contact between the mask and substrate.

The advantage of back-side exposure is that it eliminates the mask alignment step by instead using thin-film metal electrodes deposited on the surface to block the UV from selected regions of the surface. Because the protein pattern precisely matches the geometry of the pre-engineered metal electrodes, this approach is especially suitable for biosensor applications that use integrated electrodes. Electrodes were created by depositing a blanket layer of Cr (1000 Å) onto a quartz substrate and patterning the Cr

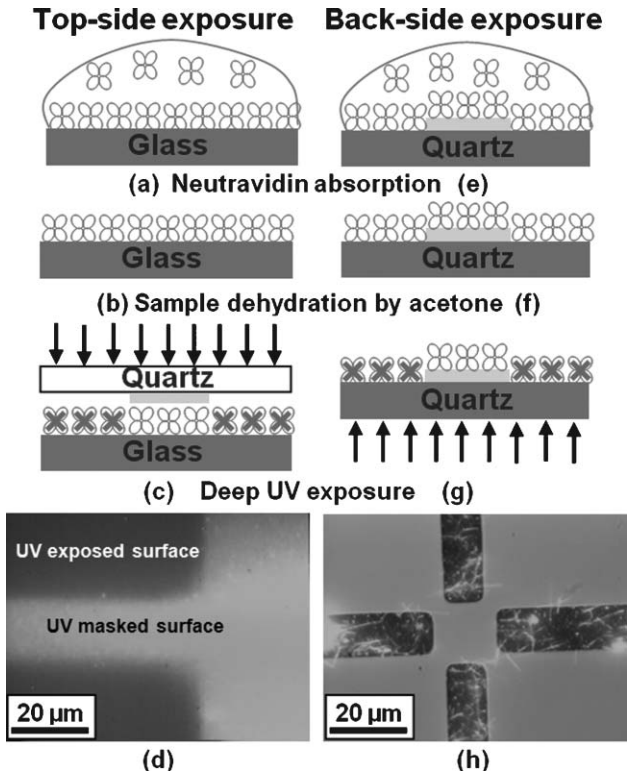

Fig. 1 Top-side exposure steps (a)–(c) and (d) after 10 min of DUV irradiation from the germicidal lamp. The resulting neutravidin patterns (UV masked surface) are shown by fluorescent biotinylated BSA. Back-side exposure steps (e)–(g) and the results after 60 s of exposure from the UVP photoreactor (h), as shown by binding biotinylated microtubules to the neutravidin pattern.

layer using photolithography and wet etching. Next, neutravidin was absorbed onto the substrate, dehydrated in acetone and dried. The neutravidin was then selectively patterned by DUV exposure from the back side of the sample with the electrode patterns serving as the mask (Fig. 1(e)–(g)) followed by rehdryation with a 0.5 mg mL^{-1} casin solution. Because the electrodes complicate the quantification of fluorescent BSA, biotinylated fluorescent microtubules were used to demonstrate the active neutravidin areas. The result following 60 s of DUV irradiation from the UVP photoreactor is shown in Fig. 1(h), demonstrating that the biotinylated microtubules only bind to the metal electrode surfaces where the neutravidin is still active.

Dependence on UV wavelength and exposure time

To maximze pattern definition and minimize loss of protein activity, UV wavelength and exposure times were optimized using back-side exposure of electrode-patterned quartz samples. As shown in Fig. 2(a), a 60 s exposure from the UVP photoreactor was sufficient to completely inactivate neutravidin in the exposed regions, while maintaining high neutravidin activity in the masked areas. This result demonstrates that the 185 nm UV effectively ablates neutravidin function. In contrast, the 254 nm illumination generated by the Fisher Scientific UV crosslinker required 60 min to inactivate neutravidin (Fig. 2(b)). Although clear protein patterns can be achieved in 60 min, the long exposure time and unavoidable sample heating limit

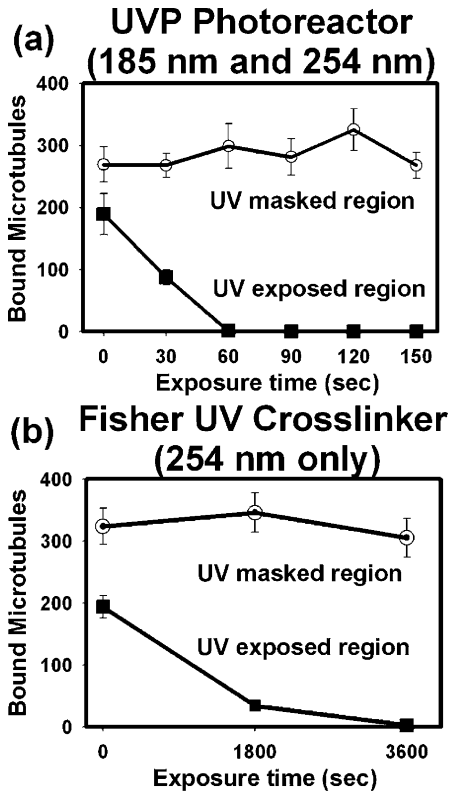

Fig. 2 Time dependence of neutravidin patterning. Six quartz samples from two experiments were used for each light source. Quartz samples were patterned with Cr electrodes, neutravidin was adsorbed and dried, and the samples were back-side exposed with (a) the UVP photoreactor or (b) the Fisher Scientific UV crosslinker. The samples were then rehydrated, assembled into flow cells, and incubated with biotinylated microtubules for 5 min to assess the patterning efficiency. The numbers of bound microtubules were counted in 5 randomly selected 80 μm by 110 μm screens from each of two flow cells for each condition. Data are plotted as mean ± SD ($n = 10$).

254 nm as an efficient wavelength for neutravidin patterning. The germicidal lamp, which generates a moderate 185 nm output also effectively patterned neutravidin (Fig. 1(d)), though it required a 10 min exposure. Finally, to clearly establish that DUV exposure is the key to neutravidin ablation, samples were exposed for 60 min to 436 nm light from the mask aligner. No measurable patterning was achieved (data not shown).

Vogel *et al.*[11] found that proteins have an absorption peak centered at 190 nm, corresponding to absorption by the peptide bond (O=C–N–H). Although it is difficult to rule out other mechanisms, we interpret the neutravidin degradation in our work as the breaking of peptide bonds in the polypeptide backbone, leading to protein denaturation. The photon energy of 185 nm UV (6.7 eV) and 254 nm UV (5 eV) are both greater than the dissociation energy of C–N (3.0 eV) or C–C (3.6 eV) bonds,[11] so it is reasonable that DUV absorption could rupture these bonds. However, in our work, 254 nm exposure alone is far less efficient than the combination of 185 nm and 254 nm UV irradiation, demonstrating that 185 nm UV plays a dominant role. Furthermore, the lack of neutravidin ablation we found from the 436 nm beam of the Karl Suss aligner is consistent with the fact that the 2.8 eV photon energy at 436 nm is smaller than the bond energy of the peptide bond.

Shelf life testing

A simple shelf life test was conducted to determine how long dehydrated UV-patterned neutravidin samples can maintain their protein activity. Two patterned neutravidin samples were stored in wafer boxes (Fluoroware Inc) and left on a bench top for three months without special attention. When microtubules were added, the patterning was similar to freshly prepared samples, demonstrating that these dry neutravidin-coated surfaces can be prepared in bulk and stored for later use without special care. Hence this technique is very practical and doesn't show the sensitivity of other surface chemistries such as SAMs or patterned hydrophobicity, that generally show significant pattern degradation over similar times.[12]

Conclusion

We present a novel technique for micropatterning neutravidin on glass and quartz substrates using deep UV irradiation. Top-side exposure through a quartz mask and back-side exposure through the electrode-patterned quartz substrate both fit well into standard cleanroom processes. Experiments demonstrate that 185 nm UV irradiation rapidly patterns the adsorbed protein, while 254 nm is also effective but considerably slower. The resulting protein patterns have high contrast and are stable for months. Because this approach can in principle be extended to virtually any protein, it provides a new tool for future tissue engineering, cell adhesion, and antibody patterning investigations.

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