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Protein Immobilization

Photochemical Surface Patterning by the Thiol-Ene Reaction**

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The immobilization of proteins on solid substrates while controlling the size and dimensions of the generated patterns is increasingly relevant in biotechnology.[1] Site-specific immobilization and thus control over the orientation of proteins is particularly important because, as opposed to nonspecific adsorption, it generates homogeneous surface coverage and accessibility to the active site of the protein.[2] Consequently, different types of bioorthogonal reactions[3] have been developed to attach proteins site-specifically to surfaces and to control protein patterning.[4] Herein, we report the photochemical coupling of olefins to thiols to generate a stable thioether bond for the covalent surface patterning of proteins and small molecules.[5] This reaction has been applied previously in solution for carbohydrate and peptide coupling.[6] The thiol-ene photoreaction proceeds at close to visible wavelengths ($\lambda = 365$–405 nm) and in buffered aqueous solutions. As a result of its specificity for olefins, this photoreaction can be considered to be bioorthogonal, unlike other photochemical methods used previously for protein immobilization.[7]

To adopt the thiol-ene reaction for the immobilization of biomolecules, surfaces functionalized with thiols and biomolecules derivatized with olefins were prepared (Figure 1). Polyamidoamine (PAMAM) dendrimers were attached covalently to silicon oxide surfaces.[8] An aminocaproic acid spacer was attached to the dendrimers to create distance from the surface.[8] Cystamine was coupled to the spacer, and subsequent reduction of the disulfide yielded the desired thiol-terminated surfaces.[8] A liquid layer of terminal-olefin-functionalized molecules dissolved in ethylene glycol was spread onto these wafers, which were then covered immediately with a photomask.[8] Subsequent irradiation[8] of the surfaces through the photomask led to patterning with adducts of covalently attached thioethers.

To establish the method, we photochemically attached the biotin derivative **1**[8] to a thiol-functionalized surface as described above (Figure 1). After the removal of unreacted biotin molecules, the surface was incubated with Cy5-labeled streptavidin (SAv) to produce a SAv-patterned surface. Fluorescence images of the resulting surface (Figure 1) demonstrated that lateral gradients and patterns with micrometer-sized features (5–100 μm) over areas of centimeters in width (Figure 1 A) were readily accessible. Figure 1 B,C and the fluorescence-intensity profile in Figure 1 D show that the patterns have a well-defined shape and are homogeneous over large distances. When prolonged sonication (4 h) and stringent washing were carried our after irradiation, SAv patterns with similar fluorescence intensities were observed, whereas control experiments with biotin that lacked the olefin linker showed no distinctive SAv patterns. These results indicate that the covalent attachment of biotin to the surface occurs specifically through the proposed thiol-ene reaction and that the nonspecific adsorption of biotin is insignificant.

Figure 1 E shows that the amount of material immobilized can be modified by changing the irradiation time. The procedure reproducibly requires a short irradiation time of 60 s to yield sufficient surface coverage for fabricating dense SAv patterns. To obtain homogeneous fluorescence signals of the patterns, the starting concentration of the solution that is drop cast onto the surface is also important. When the solution of **1** was diluted (to 1 mM), the Cy5-fluorescence intensity decreased considerably. Further dilution (below 500 μM) resulted eventually in disrupted SAv patterns. The application of more concentrated solutions of **1** (> 20 mM) resulted in the saturation of the fluorescence intensity of the SAv patterns. This behavior corresponds well with the effects observed upon varying the irradiation time. Longer irradi-

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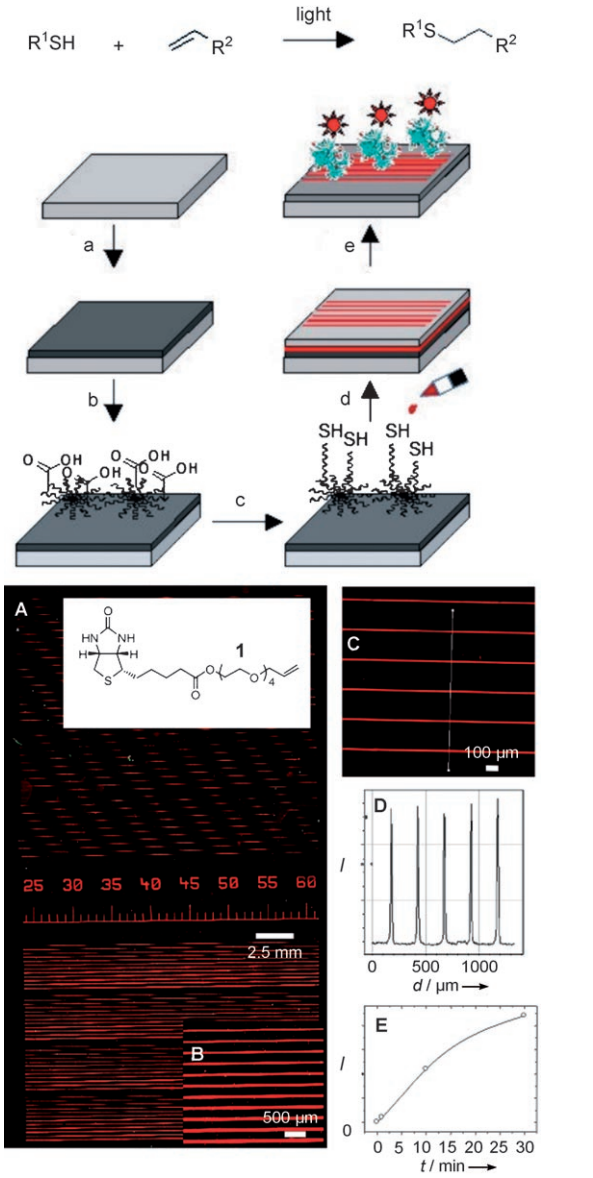

Figure 1. Schematic illustration of the surface-patterning technology. Surface modification: a) activation (plasma-enhanced chemical vapor deposition, silanization); b) optimization (aminocaproic acid linker, dendrimers); c) functionalization with thiols; d) drop casting of **1** (10 µL, 10 mM, ethyleneglycol) and immediate coverage with a photomask to prevent drying of the liquid film prior to irradiation (10 min, 365–405 nm); e) removal of the mask. Patterns of **1** are visualized by incubating the surface with SAv–Cy5 (100 nM). A) Large-area (3.5×2 cm^2) fluorescence image ($\lambda_{exc} = 632$ nm, $\lambda_{det} = 650$–690 nm). B) Magnified fluorescence image; line width: 20–100 µm. C) Magnified fluorescence image; line width: 5 µm. D) Fluorescence cross-section of the white line in (C). E) The amount of **1** immobilized is determined by the irradiation time.

ation times resulted in more-homogeneous patterns; saturation occurred at about 10 min. From a series of experiments in which the type and thickness of the silica coating on the wafers was varied, we concluded that a thickness of 500 nm of SiO_2 deposited on bare silicon by plasma-enhanced vapor deposition is optimal for the photocoupling efficiency.

Next, we applied the SAv patterns[7b] as templates for immobilizing biotinylated calf-intestine alkaline phosphatase (AlkPh). After the removal of unbound AlkPh, three assays were carried out to analyze the functionality of the immobilized AlkPh. The enzymatic activity of the AlkPh patterns was determined by using the substrate Vector Red, which produces a precipitating fluorescent dye upon dephosphorylation.[8] After incubation for 45 min, distinctive fluorescent patterns were observed on the surface in locations that corresponded precisely to the pattern of irradiation.[8] The use of the nonprecipitating fluorogenic AlkPh substrate Attophos[8] made it possible to record in real time the progress of the overall catalytic conversion of the substrate by the AlkPh-patterned surfaces. Thus, the AlkPh patterns were incubated with a solution containing Attophos. The dephosphorylation of Attophos by AlkPh gave a highly fluorescent soluble product ($\lambda_{em} = 555$ nm) in the solution above the immobilized AlkPh.[8] The curve followed pseudo-first-order kinetics as expected on the basis of the stoichiometry of the reaction, and the rate constant for dephosphorylation was calculated to be 1.4×10^{-4} s^{-1}. This behavior is in agreement with solution experiments with dissolved AlkPh (10 pM), which indicates the intactness of the surface-bound enzyme. An immunoassay was also carried out on the AlkPh-patterned surface. The microstructures were probed by sequential coupling of an AlkPh-specific primary antibody (mouse-anti-AlkPh-Ab) and a Cy5-labeled secondary antibody (goat-anti-mouse-Ab-Cy5) specific for the primary antibody (Ab). Images of the surfaces after Ab labeling revealed clear fluorescent signals from the bound Ab that matched the 50 µm wide lines of the AlkPh patterns (Figure 2A). No signal was detected when AlkPh immobilization was omitted. The results of these assays clearly demonstrate that the patterned biotin surfaces can be used as templates to generate biomolecular architectures.

To extend the use of SAv-patterned templates[7b] to protein–protein interactions, we used the small GTPase Ras.[9] The Ras-binding domain (RBD) of its effector molecule cRaf-1 was chosen as the binding partner.[9] The association constant of RBD with active guanosine-5′-triphosphate (GTP) bound Ras is approximately 160 nM, whereas for inactive GDP-bound Ras a value of approximately 50 µM is found.[9] Recognition of the Ras protein by RBD occurs through a protein–protein interface and requires the integrity of the tertiary structure of the immobilized Ras GTPase.[9] Biotinylated Ras complexes in both active and inactive form were immobilized on the SAv-patterned wafer, such that Ras:GDP and Ras:GppNHp, a constitutively active form of Ras, each covered half of the wafer. After washing, the entire slide was incubated with the RBD–YFP fusion protein as a probe to detect the Ras complexes (YFP = yellow fluorescent protein).[9] Subsequent to the removal of unbound RBD–YFP, clear YFP fluorescent patterns were observed in the area that contained Ras:GppNHp, whereas no detectable YFP patterns were revealed in the case of Ras:GDP (Figure 2B).

An antibody directed specifically against an amino acid sequence in a correctly folded helix close to the Ras protein active site[9] was used to detect the immobilized Ras proteins

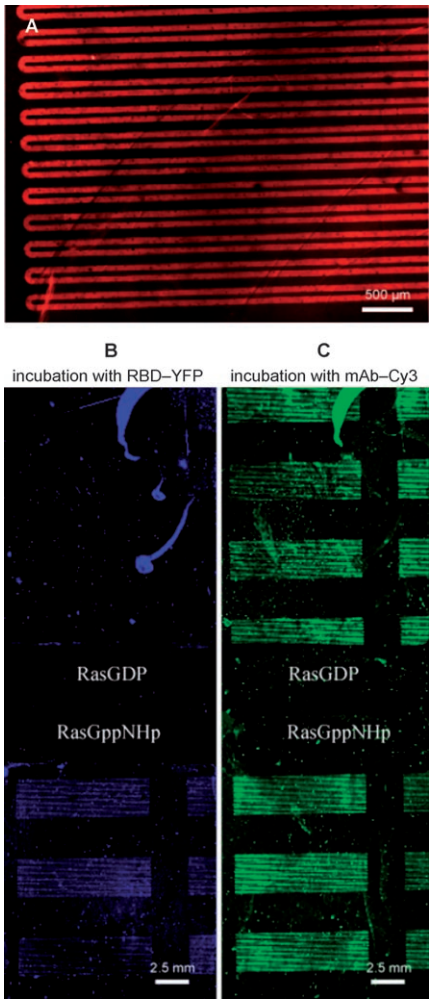

Figure 2. A) The SAv pattern was incubated with biotinylated AlkPh (1 μM). Fluorescence image ($\lambda_{exc}=632$ nm, $\lambda_{det}=650$–690 nm) showing specific tagging of immobilized AlkPh by fluorescently labeled antibodies. SAv patterns in (B,C) were incubated with either biotinylated Ras:GDP (1 μM; upper half of slides) or Ras:GppNHp (1 μM; lower half of slides). B) Fluorescence image ($\lambda_{exc}=488$ nm, $\lambda_{det}=510$–530 nm) after incubation with RBD–YFP shows RBD binding to patterned Ras:GppNHp. C) Fluorescence image ($\lambda_{exc}=532$ nm, $\lambda_{det}=565$–595 nm) showing immobilized Ras proteins after consecutive incubation with RBD–YFP and mAb–Cy3.

on both sections of the slide. Incubation of the aforementioned slide (without first drying the slide) in a solution containing the Cy3-labeled Ab (Y13-259-F5) led to the formation of distinctive fluorescent patterns as a clear indication of the presence of both Ras complexes in the protein patterns on the surface (Figure 2C). Thus, Ab–Cy3 binds to Ras regardless of its state of activity. This result also indicates that, in the case of Ras:GppNHp, Ab–Cy3 competes with the RBD as a binding partner. Although the Ab fluorescence intensities were comparable for the two types of Ras, it appeared that a portion of Ras:GppNHp still carried bound RBD–YFP. Probably, in those cases, Ab–Cy3 did not replace the RBD within the period of incubation. However, if the patterns were first incubated with Ab–Cy3 and only subsequently with RBD–YFP, no YFP emission was detected on the Ras:GppNHp patterns.[8] This result is in agreement with previous solution experiments which indicated that this specific Ras antibody could be used as an inhibitor for the binding of RBD to Ras.[9] Thus, this series of experiments clearly demonstrates the versatility of our method for the fabrication of biofunctionalized devices useful for studying protein–protein interactions.

To investigate whether the structural features of the patterns fabricated by using photomasks can be further miniaturized, we attempted to write patterns on surfaces by using a scanning 411 nm laser source combined with a confocal microscope. We applied a solution of the olefin-functionalized biotin derivative **1** onto a thiol-functionalized substrate and induced the photoreaction locally by scanning the surface with a laser in a square-wave pattern at a speed of 1 μm s^{-1} (Figure 3A). After incubation with SAv–Cy5, the

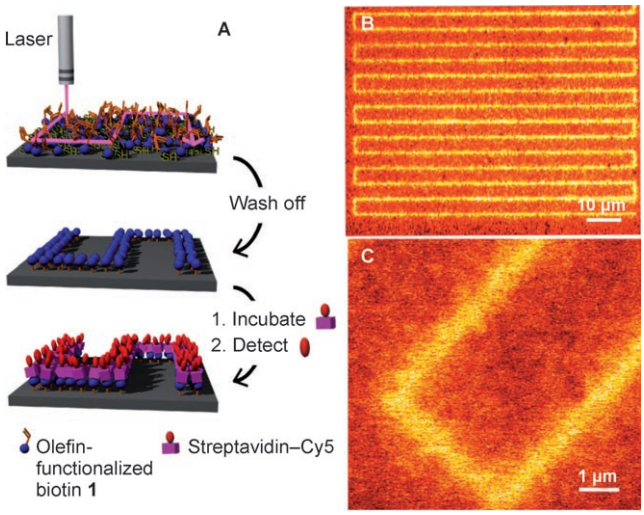

Figure 3. Photochemical nanopatterning. A) Olefin-functionalized biotin **1** is deposited onto a thiol-modified surface. After laser-assisted ($\lambda=411$ nm) nanopatterning in square-wave mode, the pattern is visualized by incubating the surface with SAv–Cy5. B,C) Scanning confocal fluorescence microscopy images of the nanopatterns ($\lambda_{exc}=630$ nm).

spatially controlled patterning of SAv was clearly visible by imaging the Cy5 emission (Figure 3B,C). Protein lines of 650 nm in width over an overall length of 1500 μm at a line spacing of 5 μm were produced readily.

Finally, we investigated the direct immobilization of highly functionalized biomolecules tagged with an olefin moiety onto surfaces to produce well-defined structures. We chose to immobilize a phosphopeptide[8] with an *N*-terminal olefin, a specific substrate for the protein tyrosine phosphatase PTP1B.[10] After wetting the thiol surface with an aqueous solution of this phosphopeptide and irradiation through a photomask, patterns of immobilized peptide were visualized by treating the surface with a Cy5-labeled anti-pY-Ab. The fluorescent patterns[8] clearly indicated the successful immobilization of the phosphopeptide, which is recognized by the pY-binding Ab. When these patterns where treated with PTP1B prior to treatment with this antibody, the fluorescence

intensity of the patterns decreased significantly, which indicates that the phosphopeptide was dephosphorylated by PTP1B. These results emphasize the broad scope of our photochemical method.[11]

In conclusion, we have developed a new photochemical method for the site-specific immobilization and patterning of proteins with retention of their structure and activity. The dimensions of the surface patterns span the centimeter to submicrometer range. We anticipate that this method offers great promise for the controlled fabrication of structured assemblies of proteins on surfaces.

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