Modulating Surface Density of Proteins via Caged Surfaces and Controlled Light Exposure

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ABSTRACT: We demonstrate the possibility of tuning the degree of functionalization of a surface using photoactivatable chemistries and controlled light exposure. A photosensitive organosilane with a protected amine terminal group and a tetraethyleneglycol spacer was synthesized. A o-nitrobenzyl cage was used as the photoremovable group to cage the amine functionality. Surfaces with phototunable amine densities were generated by controlled irradiation of silica substrates modified with the photosensitive anchor. Protein layers with different densities could be obtained by successive coupling and assembly steps. Protein surface concentrations were quantified by reflectance interference. Our results demonstrate that the protein density correlates with the photogenerated ligand density. The density control was proved over four coupling steps (biotin, SAv,-di, tris-NTA, MBP, or GFP), indicating that the interactions between underlying layer and soluble targets are highly specific and the immobilized targets at the four levels maintain their full functionality. Protein micropatterns with a gradient of protein density were also obtained.

1. INTRODUCTION

Photochemical stimuli are very useful in generating surfaces with tunable chemical states. There is a variety of molecular mechanisms by which the chemical function can be changed by light including (i) molecular isomerization (e.g., azo-derivatives), (ii) ionization (e.g., spirobenzopyran derivatives), (iii) dimerization (cinnamic acid derivatives), (iv) oxidation or removal of surface attached groups by light-induced redox processes, or (v) grafting additional molecular species via photogenerated radical cross-reactions at the surface (photoaffinity). Alternatively, light can be applied to surface layers containing chemical functionalities protected with a photocleavable group (caged surface). Light irradiation removes the cage and activates the functional groups at the surface (Scheme 1). This is a particularly flexible approach, since a good number of photoremovable groups are known that can be combined with the different organic functional groups and applied to generate surfaces with a great number of chemically tunable states. The cage can either be attached to the surface in a second step after its chemical modification with the reactive species (typically organosilanes or derivatized thiols for modification of silica or gold surfaces respectively) or be part of the surface coupling agent so that the reactive functionality and the cage are attached to the surface in a single step. The latter case offers greater control of the surface chemistry, since reactions carried out at the surface often suffer from low yields because of hindered access of the solution reagents to surface-attached reactive sites. As a consequence, reactive and caged species will always coexist at a surface in the former case.

Caged surfaces have mostly been used for the generation of chemical micropatterns. The intrinsic spatial resolution of light-induced processes allows site-selective cage removal and, therefore, the generation of caged and uncaged regions on a substrate (chemical lithography). In fact, using masks or arrays of micromirrors for site-selective irradiation, reactive sites down to submicrometer size can be activated and used for subsequent attachment of other species. This strategy has been exploited to directly synthesize biomolecules at surfaces in an array format (microarrays) by means of iterative light-activation and monomer coupling cycles. Microarrays of peptides, oligonucleotides, and peptides have been reported. High density oligonucleotide microarrays prepared by this method have also been commercialized (Affymetrix and Geniom biochips). Other species

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like metallic nanoparticles,\textsuperscript{12} polymer colloids,\textsuperscript{13} fluorescent dyes,\textsuperscript{14} biotinylated proteins,\textsuperscript{15} His-tagged proteins,\textsuperscript{16} or cells\textsuperscript{17} have also been patterned using caged surfaces.

Uncaging caged surfaces by light has an additional advantage for tuning surface functionalities. By controlling the irradiation dose, we can adjust the surface concentration of active functional groups. This property makes caged surfaces of great interest for biosensing, since patterns with reactive sites possessing different ligand concentration, or surface gradients of bound ligands could be readily fabricated. In this article, we demonstrate the possibility of using caged surfaces to control the density of surface-bound proteins. For this purpose a caged silane molecule has been synthesized (Scheme 1) and used to modify silica substrates onto which proteins have been immobilized. The silane carries an amine group caged with a nitroveratryl photoremoveable moiety. A tetraethyleneglycol spacer has also been included to minimize nonspecific interactions of the proteins with the substrate surface. Upon controlled light exposure the cage can be removed and a defined concentration of free amine groups can be generated at the surface and used for further chemical surface functionalization. Sandwich protein binding assays will be tested to proof the possibility to light-control protein density after up to four coupling steps. Protein patterns with concentration gradients will be demonstrated.

2. EXPERIMENTAL SECTION

2.1. Materials and Methods. Quartz substrates Suprasil were purchased from Heraeus Quarzglas (Hanau, Germany) and Quarzschmelze Ilmenau (Langewiesen, Germany). Silane 1 was synthesized as described previously.\textsuperscript{18} Maltose binding protein with a decahistidine tag (MBP-H10) and green fluorescence protein fused to an N-terminal hexahistidine tag (GFP-H6) were expressed in \textit{E. coli} and purified by immobilized metal ion chromatography and size exclusion chromatography by standard protocols. Biotinylated tris-nitriloacetic acid (BT-tris-NTA) was synthesized according to reference.\textsuperscript{19} Transducer slides for reflectance interference spectroscopy (10 nm Ta$_2$O$_5$ and 325 nm silica on a glass substrate $1 \times 1$ cm$^2$) were obtained from Analytik Jena GmbH, Jena, Germany, and from AMP Dünnschichttechnik GmbH, Tornesch, Germany. UV spectra were recorded on a Varian Cary 4000 UV—vis spectrometer (Varian Inc., Palo Alto, CA).

2.2. Surface Modification. Quartz slides (25 mm $\times$ 12 mm $\times$ 1 mm) were cleaned by soaking in freshly prepared Piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ 5/1) for one night (\textit{warning!} highly corrosive; eye and skin protection required), rinsing with deionized water and drying in vacuum at 90 °C for 1 h. Glassware was passivated prior to surface reaction by exposure in hexamethyldisilazane (HMDS) atmosphere at room temperature and in vacuum overnight. The conditions for surface modification experiment were selected according to previously published work.\textsuperscript{13,14} A 1% solution of silane 1 in THF with traces of NaOH (20 μL 1 N aqueous NaOH in 17 mL of THF) was prehydrolyzed for 2 h and filtered (0.2 μm PTFE filter) before the clean substrates were immersed for 24 h. Afterward, the substrates were washed with THF and Milli-Q-water, baked at 90 °C in vacuum for 1 h, sonicated in THF (three times for 2 min), washed with Milli-Q-water, and dried with a N$_2$ stream.

2.3. Light Exposure. Substrates were irradiated at 365 nm (1.1 mW cm$^{-2}$) using a Xe lamp coupled to a monochromator (Polychrom V, Till Photonics). Irradiation dose varied between 0 and 4.45 J/cm$^2$ (corresponding to exposure times between 1 and 70 min). The substrates were washed with THF after irradiation to remove the photolysis side products from the surface.

2.4. Determination of Amine Surface Density. The surface density of chromophore, \(\Gamma\) (molecules $\cdot$ cm$^{-2}$) was estimated from the UV absorbance using \(\Gamma = \frac{1}{\lambda} \left[A_\lambda \varepsilon_\lambda^{-1} N_\Lambda\right]\), where \(A_\lambda\) is the absorbance of the surface layer at a given wavelength, \(\varepsilon_\lambda\) is the molar decadic absorption coefficient of the chromophore in solution at \(\lambda\), and \(N_\Lambda\) is the Avogadro’s number.\textsuperscript{20} The factor \(1/\lambda\) refers to the fact that the quartz slides are modified on both sides. \(\Gamma\) values can be expressed in pmol/mm$^2$ by dividing the number of molecules by Avogadro’s number. The molar extinction coefficient of silane 1 in dichromethane was \(\varepsilon = 2214$ M$^{-1}$ cm$^{-1}$ at 300 nm. Note that this calculation assumes that the molar extinction coefficients of the chromophores in solution and at the surface are the same. This is only true if anchored chromophores at the surface are randomly oriented and if chromophore—chromophore—or chromophore—surface interactions are disregarded.\textsuperscript{20a} The surface density of amine groups, \(\Gamma_{\text{NH}_2}\) was obtained as the difference between the initial NVoc density and the NVoc density after each irradiation at 300 nm.
2.5. Surface Functionalization for Protein Studies. Substrates modified with silane were incubated in acetic anhydride at room temperature for 15 min and then thoroughly rinsed with THF and water and dried with nitrogen (substrates used in Figure 3). This treatment has been proved to be necessary to react free silanol groups and avoid their esterification with the biotin NHS-ester in the next coupling step. In the case of substrates used for immobilization of His-tagged proteins, a mixture of propionyl chloride and triethylamine for 5 min was used for the same purpose. This treatment reduced nonspecific binding to caged substrates before photoactivation and significantly increased protein binding signal on the irradiated samples. Treatment with propionyl chloride was proved to be more effective than acetic anhydride.

After UV exposure, the samples were incubated with biotinamido-hexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester (Sigma) in DMF (50 mg/mL) for 15 min (substrates used for His-tagged proteins) at room temperature by assembling the surfaces of two slides face to face. The excess reaction mixture was washed off with DMF and water. The transducers were then dried with nitrogen and stored at −20 °C.

2.6. Protein Binding Experiments. Protein immobilization and protein interactions were monitored in real time using reflectance interference (RII). This technique detects mass binding on the surface of a thin silica layer as a shift of the interference spectrum, which was determined at the minimum a 1.5 order. A shift of this minimum by 1 nm corresponds to a change in the optical thickness of the interference layer by 0.75 nm and to a surface mass change of ~1 ng/mm². All RII measurements were carried out in a flow chamber under continuous flow with a data acquisition rate of 1 Hz.

Measurements with SAv were carried out with a commercial setup (BIAaffinity from Analytik Jena AG, Jena, Germany). Binding assay on biotin functionalized surfaces was carried out using 70 μL SAv (188 nM) in PBS (pH 7.2) for 420 s. RII measurements with His-tagged proteins were carried out with a home-built setup consisting of a tungsten halogen source and a diode array spectrometer detector. These binding assays were carried out with HBS (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.01% Triton X100) as running buffer. The biotinylated surfaces were treated with 250 μL SAv 200 nM, followed by injection of 250 μL of tris-NTA (500 nM) loaded with nickel(II) ions, and then 250 μL of MBP-H10 (500 nM). MBP-H10 was eluted by injecting 250 μL of imidazole (500 mM) in HBS, and then the surface was loaded with 250 μL of GFP-H6 (500 nM).

2.7. Generation of Gradient Patterns. Quartz substrates were irradiated using a custom-built setup that allows automated writing of micrometric patterns with adjustable light intensity and selectable wavelength. The setup is based on an inverted microscope (Leica DM-IRM) fixed onto an optical table. Lasers of three wavelengths, violet (404 nm), blue (450 nm), and green (532 nm) (Z-laser Optoelektronik GmbH) are collinearly routed through a beam expander and a dual axis galvanometer based optical scanner (Edmund Optics GmbH) into the

Figure 1. (A) UV–vis spectra of a quartz substrate modified with silane 1 after irradiation at 365 nm with different doses and rinsing. (B) Same experiment but with no rinsing after exposure.
microscope through the side port. The scanner and other parts of the setup are controlled with custom developed software (Delphi) through
an AD/DA-IO card (BMC Messsysteme GmbH) and monitored with a
digital camera (Oscar, Allied Vision Technologies GmbH), allowing
automated irradiation of any chosen pattern in the field of view during
any selected time with any of the three wavelengths, at micrometer
resolution.

Squares (20 × 20 μm) were irradiated with the laser at 404 nm using
different light intensities on quartz substrates modified with silane 1. The
substrate was washed with THF and incubated with biotin N-hydro-
xysuccinimide ester (biotin-NHS) solution in DMF (60 mg/mL) for 90
min. The substrate was rinsed and sonicated in DMF and then rinsed
with THF and ultrapure water. Substrates were then incubated with
Cy5-labeled streptavidin (Natutec) in PBS (pH = 7.4) for 2 h and then
rinsed with PBS and ultrapure water. Fluorescent patterns were imaged
with a fluorescence microscope.

3. RESULTS AND DISCUSSION

3.1. Preparation of Substrates with Defined Surface Concentration of Active Groups by Controlled Exposure. Surface
layers of the photosensitive silane 1 were prepared and characterized by UV–vis spectroscopy.18 A representative UV
spectrum of a quartz substrate modified with silane 1 is shown in
Figure 1 (yellow curve). The surface density of the chromophore
can be estimated from the absorbance value, assuming that the
absorption coefficient of the chromophore at the surface is the
same as in solution (ε_{345} = 3028 M⁻¹ cm⁻¹). An average surface
density of ∼5 × 10^{12} molecules/mm² was obtained. This value is
comparable to the typical surface density of a SAM of thiols on
gold at maximum coverage (4.5 × 10^{12} molecules/mm²).24

Substrates modified with silane 1 were irradiated at λ_{max} = 365
nm with increasing doses. Figure 1A shows the UV spectra of the
substrate after irradiation and rinsing steps. The photolytic
reaction at the surface caused a clear decay of the UV absorbance.
This is as a consequence of the photochemical reaction and
cleavage of the o-nitrobenzyl group from the surface layer and the
removal of the photoproduct by washing (see Scheme 1).

Exposure doses higher than 2.23 J/cm² did not significantly
change the UV spectrum any further. A residual absorbance is
visible, indicating that part of the chromophore remains at the
surface layer. However, the profile of the UV spectrum after full
exposure is different from the initial spectrum. This means that the
remaining chromophore at the surface is not (only) the
original silane 1 molecule. We hypothesized that it could be the
nitroso photoproduct that reattached via the aldehyde group to
the surface by reacting with the free amines (see Scheme 1).

Figure 1B shows the UV spectra after irradiation but with no
rinsing. The maximum around 350 nm loses intensity, whereas
two new shoulders around 280 and 400 nm appear. These bands
should correspond to the nitroso photoproduct formed during irradiation and are also visible (but with less intensity) in the
rinsed substrate. These results confirm that part of the photolysis
byproduct remains attached to the surface layer, leaving a residual
absorbance on the substrate.

The residual absorbance of the byproduct makes it difficult to
extract the real photolytic conversion (and consequently the
density of free amine groups) out of the UV spectra, since both
ychromophores contribute to the absorbance spectrum. However,
Figure 1B shows that the absorbance at 300 nm remains constant
independently of the exposure time, indicating the presence of an
isosbestic point due to the presence of two species during the
irradiation process (nitro and nitroso derivative). This means
that the decay of absorbance at 300 nm after rinsing can be
 correlated solely to the removal of the NVoc cage from the
surface and, consequently, to the generation of free amine
 groups. Under this assumption the photoconversion at the
different irradiation doses was calculated (Figure 2), and from
these data (experimental errors within 15%), the amine density
was estimated. According to these results, ∼45% of the caged
groups were converted into free amines by photolysis after full
exposure. The reaction of the aldehyde byproduct has been
avoided in solution by adding carbonyl scavengers during
photolysis. This strategy has increased the yield of the photolysis
in solution up to 0.9,25 but it did not help in the photolytic
experiments on the surface. This suggests that the side reaction is
favored at the surface because of the high local concentration of
amine groups.

3.2. Protein Binding Studies. Substrates with predefined
amine densities were prepared by controlled light exposure and
then functionalized with biotin-NHS (also the nonirradiated
substrate) for protein binding studies (see Experimental Section
for details). Protein binding was monitored in real time using
label-free detection by reflectance interference (RIF).23 This
technique detects binding on the surface of a thin silica interference
layer. Binding curves were obtained from the shift of the
interference spectrum. A shift of 1 nm corresponds to 1 ng/mm²
protein on the surface.

In a first series of experiments Streptavidin (SAv) was
immobilized onto the substrates. Fast and stable binding values of
SAv was detected on all irradiated substrates, up to a saturation
value that correlated with the amine density: 0.2 nm (Γ_{NH2} = 0
pmol mm⁻²), 0.6 nm (Γ_{NH2} = 0.61 pmol mm⁻²), 1.6 nm (Γ_{NH2} =
2.12 pmol mm⁻²), and 2.0 nm (Γ_{NH2} = 2.62 pmol mm⁻²) (Figure 3).
The 0.2 nm signal observed in the absence of amines (no light exposure) indicates that SAv attachment is mostly
mediated by the biotin at the surface and there is little contribu-
tion of nonspecific interactions. From the SAv binding signal the
surface concentration of protein can be estimated (molecular
weight of SAv is 53 kDa). Figure 3b shows the linear increase of
total amount of SAv with surface amine density, proving that it is
possible to precisely control protein density on photosensitive
layers by tuning the exposure dose. In all cases protein coverage
was less than a monolayer (∼5 ng/mm² for a monolayer).
The ratio between surface bound SAv and amine groups at the surface indicates that on average only 1.4% of the amine groups participate in the immobilization of SAv (assuming that each SAv is coupled by two biotin molecules to the surface). This can be explained by the much bigger size of SAv with respect to the amine or biotin units. The ratio SAv/NH₂ remains constant with protein coverage, indicating that the crowding threshold has not been reached for surface loadings below 50% (i.e., below $\Gamma_{\text{NH}_2} = 2.62 \text{ pmol mm}^{-2}$).

We then tested site-specific protein capturing to SAv layers based on the stable, yet reversible interaction of oligohistidine tags with immobilized transition metal ions. For this purpose, SAv layers were loaded with biotinylated tris-nitrilotriacetic acid ($^{\text{BT}}$tris-NTA) and the interaction with two different His-tagged proteins was probed: decahistidine-tagged maltose-binding protein (MBP-H10) and hexahistidine-tagged green fluorescence protein (GFP-H6). Typical experiments are shown in Figure 4a: After SAv to biotin-functionalized surfaces were captured, $^{\text{BT}}$tris-NTA in the presence of Ni²⁺ cations was injected. Subsequently, binding of MBP-H10 to tris-NTA was probed, followed by regeneration with imidazole. Then, the same binding experiment was carried out with GFP-H6. Quantification of the binding amplitudes obtained for SAv, MBP-H10, and GFP-H6 confirmed an almost linear correlation with the densities of amine groups (Figure 4b). The nonirradiated substrates did not show significant protein binding, confirming high specificity of the His-tagged proteins to Ni-loaded NTA moieties. Moreover, quantitative elution of MBP-H10 and GFP-H6 with imidazole at physiological pH was observed, corroborating that coordinative interactions were responsible for protein capturing. In general, higher binding amplitudes were obtained for GFP-H6 compared to MBP-H10, which can be ascribed to the substantially faster association kinetics of GFP-H6 (cf. the binding curves in Figure 4a).

We have previously observed faster association of GFP-H6 compared to other His-tagged proteins, which could be explained by weak dimerization of this protein, which may promote capturing to the surface. The obtained ratio of surface bound GFP or MBP to SAv decreases when surface coverage increases and values are between 0.5 and 1.5 (see inset in Figure 4b). For the nonirradiated samples this ratio is higher (especially for GFP), indicating that there is some nonspecific interaction of the proteins with the aromatic units of silane 1 in the absence of the intermediate SAv layer.

3.3. Gradient Patterns. Microsquares with increasing exposure dose were written on substrates modified with silane 1. Afterward the substrates were incubated with the biotin/SAv complex, the streptavidin being labeled with dye Cy5. Figure 5a shows the fluorescence patterns. An increase in the fluorescence intensity with increasing irradiation dose is visible (see also profiles in Figure 5b), indicating that the extent of the photocleavage (and therefore the density of free amine groups) could be controlled and it determined the protein concentration in the...
The surface density of three different proteins (SAv, MBP, and GFP) has been successfully modulated using surfaces functionalized with precise regioselectivity and protein density at the four levels maintain their full functionality.

Our results demonstrate that caged surfaces allow great control of the surface concentration and distribution of active sites at surfaces, including the possibility of micropatterning compositional gradients. All of these could be achieved starting from a single surface composition, without the need of mixtures of surface coupling agents that may phase segregate at the surface and render inhomogeneous surface compositions. In addition, the photoactivation process can be performed in situ, opening interesting possibilities for a time-controlled assembly. These issues are relevant for biosensing and the generation of surfaces with precise and tunable compositions for cell studies. These are topics of current research in our group.

4. CONCLUSIONS

The surface density of three different proteins (SAv, MBP, and GFP) has been successfully modulated using surfaces functionalized with silane I after controlled light exposure. This modulation has been proved over four coupling steps (biotin, SAv, 111-tris-NTA, MBP, or GFP), indicating that the interactions between underlying layer and soluble target are highly specific and the immobilized targets at the four levels maintain their full functionality.

Our results demonstrate that caged surfaces allow great control of the surface concentration and distribution of active sites at surfaces, including the possibility of micropatterning compositional gradients. All of these could be achieved starting from a single surface composition, without the need of mixtures of surface coupling agents that may phase segregate at the surface and render inhomogeneous surface compositions. In addition, the photoactivation process can be performed in situ, opening interesting possibilities for a time-controlled assembly. These issues are relevant for biosensing and the generation of surfaces with precise and tunable compositions for cell studies. These are topics of current research in our group.

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Figure 5. (A) Fluorescence image of SAv patterns with di

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