Molecular Self-Assembly, Chemical Lithography, and Biochemical Tweezers: A Path for the Fabrication of Functional Nanometer-Scale Protein Arrays**

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Exploration of the immensurable gene products of the ∼25000 human genes and mapping of dynamic protein interaction networks are key challenges in current biological research.1 Highly parallel protein chip technologies deliver promising tools for these challenges even down to the single-molecule level.2 Whereas DNA molecules are robust and easy to immobilize in a functional manner—as seen in the triumph of DNA chip technology3—their recognition with chemically heterogeneous entities denaturing rapidly in vitro. Using multivalency as a design principle, a chemical recognition unit with exceptionally high affinity for proteins was designed, while maintaining their functionality.4 These multivalent chelator heads consist of cumulated N-nitrilotriacetic acid (NTA) moieties, which provide chemical recognition for histidine-tagged proteins by complexation of transition metal ions, for example, Ni(II). These strong as well as specific binding capabilities can easily be switched on and off by addition or removal of Ni(II) ions. Such properties make tris-NTA chelators ideal “biochemical tweezers” for attaching proteins to surfaces and releasing them again. A tris-NTA unit coordinates six imidazole moieties and, thus, perfectly matches the coordination demands of a hexahistidine tag with binding affinities in the nanomolar range.5 For the generation of structured protein arrays, a lithography step is necessary. Both electron-beam lithography (EBL) or extreme UV interference lithography (EUV-IL) are promising for this task. EBL gives the largest variation of pattern shapes and lateral dimensions in a very controlled manner.6 The application of focused e-beams provides a chance to even generate structures capable of immobilizing single biological molecules. EUV-IL can create highly parallel patterns over large areas in a single processing step with the resolution approaching the EBL limit,7 which is of great potential for applications in nanotechnology. In this Communication we present a new path for the fabrication of solid templates for the laterally defined and functional immobilization of proteins. The technology is based on the combination of electron-induced chemical lithography with aromatic self-assembled monolayers8 (SAMs) and the NTA/His-tag concept,9 merging electron-beam/photon nanolithography (top-down) with molecular self-assembly (bottom-up) and chemical biology.

Scheme 1a presents the elemental steps of the protein chip assembly. First, a 4’-nitro-1,1’-biphenyl-4-thiol (NBPT) SAM on a gold surface is formed (step i). Then, e-beam or EUV lithography is used to locally reduce the terminal nitro groups into amino groups and to cross-link the underlying aromatic substrate (step ii). In the next step (step iii), multivalent chelators, cf. Scheme 1c, are grafted to the prepatterned amino-terminated areas. At this step the carboxylic functionalities of the NTA-groups are protected with tert-butyl groups. Then, their carboxyl functionalities are deprotected (step iv) and finally (step v), the areas of the pristine NBPT SAM are exchanged in solution with protein-repellent 16-mercaptohexadecanoic acid (triethylene glycol) ester (EG3-OH thiol), cf. Scheme 1b, to prevent unspecific adsorption of proteins. In the regions with the grafted multivalent chelators, the loading with metal ions results in the specific, high affinity, oriented and reversible immobilization of His-tagged proteins or protein complexes (vi).

To monitor and characterize the different steps of this molecular assembly, we choose X-ray photoelectron spectroscopy (XPS) because it provides a detailed analysis of the elemental composition of the chips’ surface. Figure 1a shows XP spectra of pristine, and Figure 1b of e-beam irradiated (40 mC cm⁻²) NBPT SAMs (steps (i)–(ii) in Scheme 1a). From the attenuation of the Au 4f signal (not shown), the thickness of the pristine SAM was calculated to (12.5 ± 0.8) Å. The S2p signal shows a doublet at binding energies (BEs) of 162.0 and 163.2 eV with a full-width at half maximum (FWHM) of 0.9 eV, which is characteristic for thiolate.10 The

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The C1s signal of the NBPT SAM consists of a main peak, C1s(I), at 284.2 eV (FWHM = 1.2 eV) that is assigned to the 10 adjacent carbon atoms in the aromatic rings; of a C1s(II) peak at 285.3 eV (FWHM = 1.4 eV) that originates from the carbon atoms in C–S and C–N bonds; and of aromatic shake-up satellites at 287.0 eV and 290.5 eV (FWHM = 1.7 eV). The N1s and O1s signals at 405.5 eV (FWHM = 1.4 eV) and 532.3 eV (FWHM = 1.7 eV), respectively, are assigned to the SAMs terminal NO2 group. From the XP spectra a nitrogen-to-oxygen ratio of ca. 1:2.5 was calculated, which is somewhat lower than the expected stoichiometry. This may be indicative for the formation of a co-adsorbate overlayer of water on the polar nitro groups during the air contact of the samples. The evaluation of the chemical composition of the monolayer and additional angle-resolved XPS measurements indicate the formation of a densely packed NBPT SAM on polycrystalline Au surfaces and the surface location of the nitro groups (Supporting Information (1)).

Irradiation of NBPT SAMs with electrons or EUV light results in the lateral cross-linking of adjacent aromatic molecules and in the conversion of the terminal nitro groups into the amino groups.[11,12] These changes can be seen in the XP spectra, Figure 1b. The N1s region shows an amino signal at 399.0 eV, accompanied with a low-intensity shoulder at 400.8 eV, and the nitro signal at 405.5 eV disappears completely. Similar to biphenylthiol SAMs, upon irradiation[13] a broadening of the C 1s signals by ca. 0.3 eV is observed. The S2p signal shows the formation of a second doublet at higher binding energies (ca. 163.5 and 164.7, FWHM ∼1.5 eV), indicative of the formation of sulfide or disulfide in the cross-linked SAM. The O1s signal is ca. 20 % of its initial value. As expected from the enhanced N:O ratio in the pristine SAM, the residual oxygen may result from co-adsorbates like CO2 or/and H2O. The thickness of the SAM decreases by ca. 1 Å, which is in agreement with the oxygen loss. It is interesting to note that
the air exposure of the irradiated NBPT SAMs results in an increase of their effective thickness by ca. 2 Å, which is accompanied also by increase the O1s signal intensity to ca. 70 % of its initial value, and a noticeable ca. 5 % increase of the carbon intensity. The nitrogen intensity is not affected by the air exposure, implying possible incorporation of the oxygen and carbon atoms into the carbon skeleton of the cross-linked SAM. This effect was taken into account for further evaluation of the data. The ARXPS show the surface location of the amino groups (Supporting Information (1)).

Figure 1c shows XP spectra of an EG3-OH thiol monolayer. The S2p signal is a thiolate doublet at 162.1 eV and 163.3 eV (FWHM = 0.9 eV). The carbon signal consists of three constituents: the C1s(I) peak at 285.1 eV (FWHM = 1.2 eV) accounting for carbon atoms in the alkyl groups; the C1s(II) peak at 287.0 eV (FWHM = 1.3 eV) accounting for carbon atoms in the tri(ethylene glycol) groups; and the C1s(III) peak at 289.4 eV (FWHM = 1.2 eV) reflecting the carbon atom in the carboxyl group. The O1s signal is a single peak at 533.3 eV (FWHM = 1.7 eV). The surface location of the tri(ethylene glycol) groups in the SAM is clearly shown from the C(I)/C(II) ratio, which is ca. 15:9, whereas the stoichiometric ratio is 15:6. The effective thickness of the EG3-OH SAM is (26.1 ± 1.1) Å. This value is lower than the thickness of an ideal EG3-OH monolayer with the helical conformation (ca. 28.5 Å) (evaluation has been made on the basis of the data presented in Ref. [14]), which may account for some structural defects in a real SAM.

To determine the positions of the characteristic XP peaks in tris-NTA units, Scheme 1c, we studied EG3-tris-NTA SAMs as a reference (Supporting Information (2)). For steric reasons the formation of a densely packed monolayer is not expected. Hence, the S2p spectrum contains the thiolate doublet and sulfur species at higher binding energies. The effective thickness of the EG₃-tris-NTA SAM corresponds to (26.8 ± 1.4) Å. The different XP peaks in Figure 1d are assigned to components in the tris-NTA units. The C1s signal at 285.1 eV (FWHM = 1.6 eV), 286.8 eV (FWHM = 1.7 eV) and 289.2 eV (FWHM = 2.0 eV) are attributed to alkyl groups, carbon in C–N bonds, and carboxyl groups, respectively. The higher FWHM values, in comparison to the similar signals in the EG3-OH SAM, result from the highly disordered structure of the EG₃-tris-NTA SAM. The O1s signal in the carboxyl groups is assigned to a BE of 532.4 eV (FWHM = 1.9 eV). The N1s signal in the amino groups of the NTAs units has a BE of 400.5 eV (FWHM = 1.5 eV), which is shifted by 1.5 eV to higher energies in comparison to the amino groups of the cross-linked aromatic SAM.

Using the above spectroscopic information, a detailed analysis of steps (iii) to (v) of the protein chip assembly is possible. Figure 2 presents XP spectra for the grafting of tris-NTA units (Fig. 1c) onto the amino-terminated surfaces of electron-irradiated NBPT SAMs. In view of the detailed spectroscopic information on the constituents of the tris-NTA units, their stoichiometry, the thickness of the grafted overlayer, Figure 2a clearly shows the coupling of tris-NTA to the cross-linked SAM. The stoichiometry of the grafted overlayer derived from the XP spectra within a statistical model [15] suggests a C:O:N ratio of 11.2:3.5:1, which corresponds very well with the expected value of 11.1:3.4:1. The intensity ratio, between

**Figure 1.** XPS spectra of the sulfur, carbon, nitrogen and oxygen regions of a) NBPT SAM, b) irradiated NBPT SAM (40 mC cm⁻²), c) EG3-OH SAM, and d) EG3-tris-NTA SAM.

**Figure 2.** Grafting of the multivalent chelators on the amino terminated area of the irradiated NBPT SAM (30 mC cm⁻²). a) A detailed fit of the XPS data. b) Electron dose-dependent thickness of the grafted monolayer.
the Ni signal in tris-NTAs and in the cross-linked SAM, shows that the coupling of the multivalent chelators occurs at about each tenth molecule of the SAM. That corresponds to the formation of a monolayer of multivalent chelators on top of the cross-linked SAM, as can be judged from the size of tris-NTA units. Above a dose of 10 mC cm\(^{-2}\) the thickness of the grafted layer was found to be ca. 6 Å (Fig. 2b). A dose-dependent growth of the overlayer correlates well with the generation of amino groups in the underlying SAM upon irradiation.

The chelation of Ni(II) ions to the deprotected NTA groups is confirmed by the appearance of a Ni2p\(_{3/2}\) signal at 856.3 eV. In comparison to metallic nickel, the peak position shows high resistance against exchange. The cross-linked SAM in solution at room temperature as a function of time in exchange experiments, which were conducted at room temperature, cf. Scheme 1a (v). The XPS data show that the pristine NBPT SAM can be completely exchanged in solution with the protein resistant EG3-OH SAM. The cross-linked SAM shows high resistance against exchange.

Figure 3 presents a summary of the “exchange experiments”, which were conducted at room temperature, cf. Scheme 1a (v). The XPS data show that the pristine NBPT SAM can be completely exchanged with the EG3-OH SAM. The cross-linked SAM shows high resistance against exchange.

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Figure 3. Exchange of the NBPT SAM with the protein resistant EG3-OH SAM in solution at room temperature as a function of time in exchange solution (h) and irradiation dose (mC cm\(^{-2}\)). The pristine SAM can be completely exchanged with the EG3-OH SAM. The cross-linked SAM shows high resistance against exchange.

The spectroscopic study of the elemental steps during protein chip fabrication confirms the efficiency of the presented new concept. Spatial patterns of ca. 2 μm circle were generated by electron-beam lithography in NBPT SAMs. The subsequent protein chip assembly steps were followed by atomic force microscopy (AFM) (Supporting Information (5)). We further studied the dependency of the chip assembly on the applied electron dose. In agreement with the XPS data, the best topographic characteristics of the fabricated chips are achieved for electron doses from 20 to 40 mC cm\(^{-2}\).

Figure 4 presents the functional and reversible immobilization of two different proteins on the same chip surface under physiological conditions. Proteins loading and regeneration cycles of the chip were repeated more than 10 times in a time interval of two months without showing its substantial degradation. The fluorescence pattern in Figure 4a and b, obtained by confocal laser scanning microscopy, demonstrates the immobilization of fluorescently labeled His\(_{10}\)-tagged maltose-binding proteins to the chip surface after activation of the multivalent chelators areas with Ni(II). The formation of specific and homogenous microarrays over large areas (ca. 10 mm\(^{2}\)) can be visualized. After imaging, the chip was regenerated with imidazole and EDTA, activated again with Ni(II) and inspected by AFM (non-contact mode). Similar to the freshly prepared chips surfaces, the areas with coupled multivalent chelators appear with an effective height of ca. 2–3 nm in comparison to the protein repellent EG3-OH areas, cf. Figure 4c. This effective height difference in buffer solution is slightly higher than the value expected from the UHV measurements, which is due to the complex nature of interactions between the AFM tip and chemically patterned surfaces. For further demonstration of the protein chip functionality we employed the barrel-shaped 20S proteasome complex from the archaea Thermoplasma acidophilum as a biological model system. This macromolecular protein complex has lateral dimensions of 11 nm × 15 nm and can be easily imaged by AFM.[10] Figure 4d shows a microarray of immobilized His\(_{10}\)-tagged proteasome complexes on the chip surface. Here, the His-tags of the proteins were engineered to result in an end-on orientation, cf. Scheme 1a (vi). The AFM data show specific and oriented immobilization of proteins on the chip surface. The section analysis along the immobilized protein arrays reveals an increase of the topographic features by ca. 15 nm, which corresponds well to an up-right orientation of proteins. After regeneration of the surface with imidazole, cf. Figure 4e, the topographic features remain as before the proteasome immobilization. After a second protein loading the surface displays almost an identical topography as after the first loading, showing a very good reusability of the chip.

For the fabrication of highly parallel protein nanoarrays we used EUV-IL lithography to introduce cross-linked amino-terminated areas in the NBPT SAM on Au-Pd substrates[12] in
comparison with the pure polycrystalline Au substrate they exhibit lower roughness, which enables detection of nano-objects. The subsequent steps of the protein chip assembly, Scheme 1, were the same as for the microarrays. Figure 5a presents an AFM image of 100 nm periodic proteasome line pattern immobilized over an area of ca. 1 mm². Demonstration of the specific orientation of proteasome from direct AFM imaging in non-contact mode is difficult, since proteins, nanopatterns, and AFM cantilever curvature (ca. 30 nm) have comparable dimensions. The application of higher forces in contact mode results in the removing of proteins from the chip surface, Figure 5b. The line profile over the region with proteasome and without proteasome reveals the high difference of ca. 20 nm, which is in good qualitative agreement with its specific up-right orientation. A somewhat higher height difference between the protein covered and protein free areas obviously results from the nanoshaving of the underlying SAM at higher cantilever forces. The scanning of the chip surface at the same parameters but without proteins removes the SAM from the substrate and form the topography features of ~ 5 nm height (Supporting Information (6)). The specific immobilization of proteins was also confirmed by complete regeneration of the chip surface after imidazole treatment.

In conclusion, we have introduced a methodology for the fabrication of large-area highly parallel protein chip templates with dimensions of periodic features from the micro- to the nanorange based on the combination of electron-beam or EUV lithography, molecular self-assembly, and the use of biochemical tweezers for highly specific affinity capturing of His-tagged proteins. The functionality of the protein chips has been demonstrated by specific, homogeneous, oriented, and reversible immobilization of His-tagged proteins. The high affinity of the protein arrays is achieved by multivalent interactions in NTA/His-tag pairs. The fabricated chips can be used in multiple loading and regeneration cycles with different His-tagged proteins. Since both electron and EUV lithography are suitable to fabricate SAM nanostructures with lateral dimensions down to 10 nm, this concept can be developed for the functional immobilization of structured proteins arrays on solid substrates down to single molecule resolution.
The electron doses in the range from 10 to 40 mC cm\(^{-2}\) were applied. The amino-terminated, cross-linked circular patterns were formed in tapping mode at resonance frequencies of around 20–30 kHz in HBS buffer and at drive amplitudes of 100 to 150 mV.

Proteins: The preparation of the recombinant 20S proteasomes and fluorescein-labeled maltose-binding protein was carried out as described \([19,20]\).

Fluorescence Microscopy: Patterned chips were imaged by confocal laser scanning microscopy to verify protein binding. After activating the surface for 15 min with 10\(\mu\)M NiSO\(_4\) at RT, Atto565-labelled, His\(_{10}\)-tagged maltose binding protein (\(^{14}\)C\(^{12}\)MBP-His\(_{10}\)) was immobilized specifically on the chip. After rinsing with HBS, the chip was immediately mounted into a self-built liquid cell and imaged using a Plan-Neofluor 63 \(\times\) oil-immersion objective (NA 1.4) and an inverted microscope set-up (Axiovert 200M, Carl Zeiss, Jena). The chip was kept wet at all times. A HeNe laser (543 nm, 1 mW) was used at 30 % maximal power output for excitation. Fluorescence image data were processed with LSM Zeiss software (Zeiss).

Atomic Force Microscopy (AFM): AFM experiments for surface characterization were performed with a NT-MDT NTegra atomic force microscope (NT-MDT, Moscow) in a commercial fluid cell. Protein binding studies were carried out in a Digital Instruments NanoScope IIIa SPM MultiMode atomic force microscope (Veeco Instruments, Santa Barbara, CA) in a commercial fluid cell. Protein binding studies were carried out in a Digital Instruments NanoScope IIIa SPM MultiMode atomic force microscope (Veeco Instruments, Santa Barbara, CA) in a commercial fluid cell. Protein binding studies were carried out in a Digital Instruments NanoScope IIIa SPM MultiMode atomic force microscope (Veeco Instruments, Santa Barbara, CA) in a commercial fluid cell. Protein binding studies were carried out in a Digital Instruments NanoScope IIIa SPM MultiMode atomic force microscope (Veeco Instruments, Santa Barbara, CA) in a commercial fluid cell.

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**Experimental**

Surface Preparation: 4'-nitro-1,1'-biphenyl-4-thiol SAMs (NBPT) on polycrystalline Au (30 nm thick Au layer thermally evaporated on 2.5 nm Cr primed Si(100) substrate; grain size \(30 \text{ nm}\), RMS value \(<1 \text{ nm}\)) and Au-Pd surfaces (30 nm thick Au\(_{60}\)Pd\(_{40}\) layer thermally evaporated on 2.5 nm Cr primed Si(100) substrate; grain size \(<30 \text{ nm}\), RMS value \(<0.6 \text{ nm}\)) were prepared as described elsewhere \([11]\). The synthesis of carboxyl derivative tris-NTAs, Figure 1c, tris-NH\(_2\) thiols, and matrix thiols (EG\(_3\)-OH), Figure 1b, is presented in Ref. \([4]\).

The coupling of tris-NTA units to amino terminated, cross-linked areas of e-beam patterned NBPT SAM was performed as follows. A mixture in the following sequence was prepared: 1.0 mL chloroform + 0.03 mL tert-butyl protected tris-NTA (10 mM) + 1.0 mL DIC. The mixture was preheated at 75 °C for 5–8 min. e-Beam patterned NBPT SAM chips were put into a glass vial and kept at 75 °C for 30 min. Rinsing 5 times with chloroform was followed by deprotection in TFA at room temperature for 3–4 h. The chips with deprotected tris-NTA groups were rinsed 10 times with Millipore water. Pristine NBPT SAMs on the chip surface have been exchanges against matrix thiols in 1 mM solution of EG\(_3\)-OH in ethanol or DMF at room temperature for 5 days. After that the chips were cleaned with ultrasonad in ethanol for 10 min, dried with nitrogen and stored under Ar until usage at RT.

**e-Beam Lithography:** Generation of the terminal amino groups and lateral cross-linking in NBPT SAMs were performed by a flood gun irradiation with 50 and 100 eV electrons in both high vacuum (\(p < 5 \times 10^{-7} \text{ mbar}\)) and ultra high vacuum (UHV) (\(p \sim 1 \times 10^{-10} \text{ mbar}\)) conditions. The electron doses in the range from 10 to 40 mC cm\(^{-2}\) were applied. The amino-terminated, cross-linked circular patterns were generated by electron irradiation of the SAM though a stencil mask (Quantifoil, Jena).

**EUV Interference Lithography:** EUV interference lithography exposures were performed at the XLII beamline of the Swiss Light Source. The wavelength and the spectral bandwidth of illumination were 92.5 eV and 2–3 %, respectively. EUV interference patterns were generated by defocusing beams with transmission diffraction gratings \([7]\). Two-beam interference schemes were applied to obtain 1D (line/space) type patterns respectively. The flux incident on the sample was 5–20 mW cm\(^{-2}\) depending on the particular gratings used in the experiment.

**XPS:** X-ray photoelectron spectroscopy (XPS) measurements were conducted in an UHV photoelectron spectrometer (Omiconron-Nanotechnology, Taunusstein). Monochromatic Al K\(_\alpha\) irradiation (300 W) was utilized. To compromise between the energy resolution and the signal intensities the former was set to 0.9 eV and an emission angle of 21° was used. Binding energies were referred to the Au 4f\(_{7/2}\) peak at 84.0 eV \([16]\). Thickness calculations were based on the attenuation of the Au 4f\(_{7/2}\) signal (\(\lambda \sim 36 \text{ Å}\)). The angle resolved XPS was conducted with an angular resolution of ca. 4°. X-ray damage of the molecular overlayers within the measurements has not been observed. For deconvolution of XP peaks a Shirley background and symmetric Voigt functions were employed \([14]\). All fits were self-consistent.

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**Figure 5.** Topographic AFM image of oriented and specifically immobilized proteasome complexes, period 100 (1 \(\times\) 1 \(\mu\)m\(^2\), z-scale corresponds to 7 nm). Removing of the proteins from the chip surface in AFM lithography mode (2 \(\times\) 2 \(\mu\)m\(^2\), z-scale corresponds to 35 nm).
Electron-induced chemical lithography combined with self-assembled monolayers and multivalent chelators for high-affinity capturing of His-tagged proteins are used to obtain specific, stable, highly parallel, and functional protein micro- and nanoarrays on solid substrates. The functionality of the generated large-area protein arrays is shown in situ via specific, homogeneous, oriented and reversible immobilization of His\textsuperscript{6}-tagged 20S proteasome and fluorescence labelled His\textsuperscript{10}-tagged maltose binding proteins.

**Arrays**


Molecular Self-Assembly, Chemical Lithography, and Biochemical Tweezers: A Path for the Fabrication of Functional Nanometer-Scale Protein Arrays