Protein Patterns

DOI: 10.1002/anie.200900950

Making Protein Patterns by Writing in a Protein-Repelling Matrix**

*Nirmalya Ballav, Heidi Thomas, Tobias Winkler, Andreas Terfort, and Michael Zharnikov**

One of the challenges of modern nanotechnology is the development of reliable, efficient, and flexible methods for the fabrication of ordered and complex patterns of proteins. Such patterns are of importance for biology and medical science: examples are proteomics, panel immunoassays, cell research, pharmaceutical screening for potential drugs, medical diagnostics, and encoding directional biological information. An essential element of almost all the available techniques[1–9] is a protein-repelling background matrix which surrounds the active protein-adsorbing areas and prevents adsorption of proteins beyond these areas. Such a matrix is usually comprised of oligo- or poly(ethylene glycol)-based materials, polymers, or self-assembled monolayers (SAMs), and is generally prepared by a backfilling procedure after the fabrication of the protein-attracting patterns. Herein we present an alternative approach, showing that the protein-repelling films, both SAM- and polymer-like, can be used as a primary matrix for direct electron-beam writing of both non-specific and specific protein patterns of any shape, including gradient ones, on a variable length scale. These factors make the approach quite flexible, which is additionally strengthened by the intrinsic versatility of electron-beam lithography (EBL), a wide range of suitable electron energies, the broad availability of commercial oligoethylene glycol (OEG) compounds, variable substrate material, and the wide choice of the target proteins.

The approach is schematically illustrated in Figure 1. We used protein-repelling SAMs of OEG-substituted alkanethiols, $HO(CH_2CH_2O)_n(CH_2)_{11}SH$ with $n=3$ (EG3) and 7 (EG7), on evaporated Au(111) substrates.

Generally, the first step (or steps) to fabricate a protein pattern is to prepare a SAM-based chemical template. Such templates can be made by a combination of direct writing (molecules with specific binding groups to attract or bind proteins or intermediate moieties) and backfilling (OEG-based molecules) as in microcontact printing or dip-pen lithography.[1,4,7] In EBL, fabrication of a chemical template suitable for protein adsorption can be performed either by transformation of specific tail groups of an aromatic SAM[9,10] or by the irradiation-promoted exchange reaction (IPER) between a primary aliphatic SAM and a molecular substituent.[11] The transformation of specific SAM tail groups however, requires an additional exchange-reaction-mediated backfilling of non-irradiated areas by OEG-based molecules, which is a slow process.[9] The possibilities of IPER are limited as well, because of its low efficiency in the case of long-chain OEG-based SAMs.[12] Therefore, only inverse protein patterns (protein-repelling features on a protein-adsorbing background) have been fabricated by the IPER method to date.[11]

In view of these problems, patterning aliphatic SAMs directly, similar to the aromatic films, could be considered. However, in contrast to aromatic films, a tail group of an aliphatic SAM usually cannot be specifically modified by electron irradiation without severe damage to the entire film, which deteriorates the overall quality of the template.[13] We found, however, that this behavior does not occur in the case of OEG-terminated SAMs. According to X-ray photoelectron spectroscopy (XPS) data (see Figure 2a and Figure SI1 in the Supporting Information), the OEG part of such films is extremely sensitive to electron irradiation (similar behavior was previously observed for UV-light exposure).[14] It is modified to a severe extent even at very low doses ($\leq 1\ mC\,cm^{-2}$), but both the aliphatic part and thiolate anchor of the SAM remain mostly intact, maintaining a thorough coupling of the molecules to the substrate.[13] As a result of the electron-induced decomposition of the OEG chain, the effective thickness of the OEG SAM progressively decreases in the course of irradiation (Figure 2b).

Along with the thickness reduction, the cleavage of the C–O bonds within the ethylene glycol (EG) units leads to the generation of chemically active sites for subsequent non-specific binding of different moieties. The amount of adsorbate is governed by the density of these sites, that is, by the primary irradiation dose. As shown in Figure 2b (see also Figure SI2 in the Supporting Information), progressive irradiation of the EG7 and EG3 SAMs results in a progressive increase in protein affinity until saturation (an affinity which is 100% that of a dodecanthiolate (DDT) SAM) at higher doses. Extensive adsorption of proteins occurs even at small thickness reduction, especially for EG3/Au, thus it is the newly formed chemically active sites that are responsible for the protein attachment and not "holes" in the primary film which occur during the thickness reduction.[15] The selection of an appropriate dose allows a precise tuning of the protein coverage from zero to the values typical for surfaces with high protein affinity (DDT SAMs).

By combining this approach with lithography, it is possible to fabricate any desired protein pattern, including gradient-like ones. An example is given in Figure 3a, where an AFM image of a gradient-like fibrinogen pattern surrounded by the

[*] Dr. N. Ballav, Prof. Dr. M. Zharnikov
Angewandte Physikalische Chemie
Universität Heidelberg, 69120 Heidelberg (Germany)
Fax: (+49) 6221-546-199
E-mail: michael.zharnikov@urz.uni-heidelberg.de
H. Thomas, Dr. T. Winkler, Prof. Dr. A. Terfort
Institut für Anorganische und Analytische Chemie
Goethe-Universität Frankfurt, 60438 Frankfurt (Germany)

[**] This work has been financially supported by DFG (ZH 63/9-3 and Te 247/6-2). We thank M. Grunze for support of this work and S. Schilp for the help with e-beam writing.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200900950.

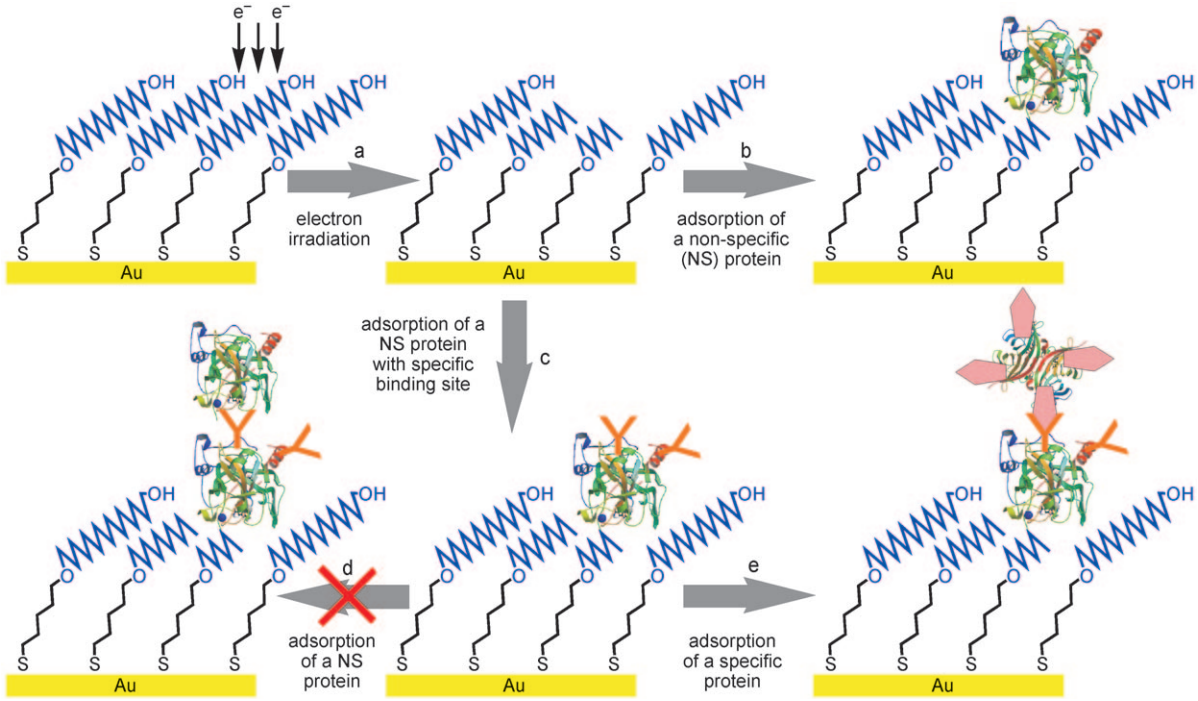

Figure 1. Illustration of the approach. A chemical template for the protein pattern is directly written by EBL in the primary OEG-based SAM or polymer film (a). This template can be either directly used for non-specific attachment of any proteins (b) or exposed to a protein carrying a specific binding site for subsequent specific adsorption of another protein (c). The specifically modified template does not adsorb any proteins (d) except for the target one (e). In all cases, the extent of protein adsorption is governed by the primary irradiation dose. As an example, a thiolate SAM on Au substrate is shown; however, the approach is believed to be applicable to any OEG-based SAM- or polymer-like film on any substrate as long as the primary film is protein repelling.

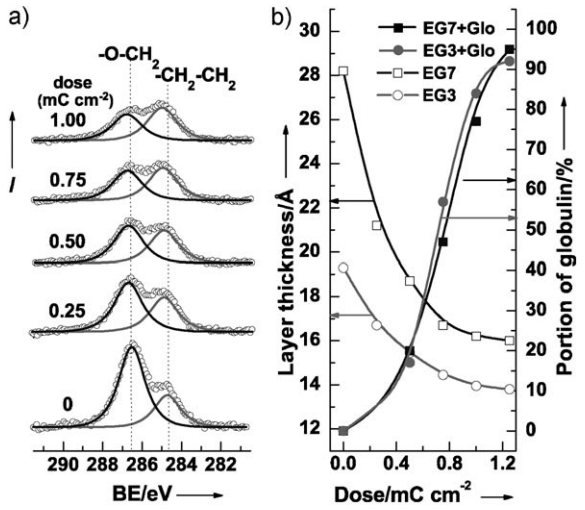

Figure 2. a) C1s XPS spectra of the pristine and electron-beam-modified EG7/Au SAMs. The spectra are deconvoluted in two peaks related to the OEG (black) and alkane (gray) parts of the EG7 molecules. The dose is marked at the spectra. b) Thickness of the EG7/Au (black) and EG3/Au (gray) films (open symbols) and portion of the adsorbed globulin on the electron-beam-modified EG7/Au (black) and EG3/Au (gray) SAMs (filled symbols) as functions of irradiation dose. The globulin coverage was referenced to the 1 monolayer (ML) film on a DDT/Au SAM, which was set as 100%.

pristine protein-repelling EG7 matrix is shown; the height profiles along the stripes (Figure 3b) reflect different variations of irradiation dose. According to these profiles, all three gradients are well defined and the protein coverage correlates precisely with the irradiation dose. The gradient profiles agree well with the protein coverage curves in Figure 2b, reproducing for example, the specific, slow onset at low doses. In addition, protein coverage varies gradually along the stripe I, but achieves saturation for the steeper gradients, stripes II and III (see Figure 3).

The saturation dose is approximately 0.5 mC cm^{-2}; the difference from the analogous value in Figure 2b (ca. 1.25 mC cm^{-2}) is related to the different kinetic energies of electrons used for the large-area spectroscopic experiments (10 eV) and patterning (1 keV).[16] Note also that in contrast to many other techniques,[17–19] not only linear or radial gradients but gradient-like patterns of any shape can be prepared by EBL.[20] Simple, array-like patterns can be prepared as well. An example of such a pattern is given in Figure 3c in which the respective optical micrographs are shown. This pattern was written in a EG7/Au SAM, which was afterwards exposed to the AlexaFluor 488 conjugate of human serum albumin (HSA).

A chemical OEG-based pattern fabricated by EBL can be directly used for the non-specific adsorption of any protein. In addition, the pattern can be easily transformed to a specific template by attachment of a protein carrying a binding site for subsequent specific adsorption of another protein. To demonstrate this concept we took the well-established biotin–avidin combination which is frequently used in screening assays.[21] The respective data are shown in Figure 4a. To

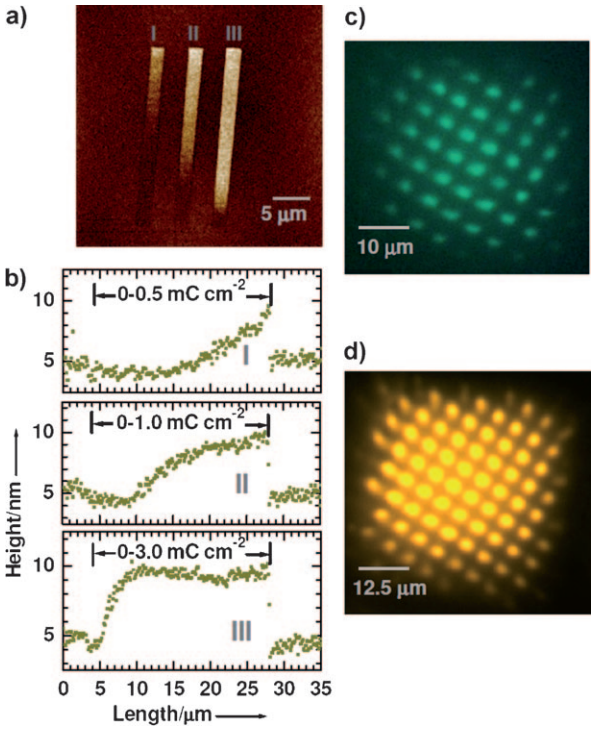

Figure 3. a) An AFM image of the protein gradient pattern taken after the adsorption of fibrinogen on the electron-beam-engineered EG7/Au SAM. The irradiation dose along the stripes was gradually varied from 0 to 0.5, 1.0, and 3.0 mC cm^{-2} for the stripes I, II, and III, respectively. b) Height profiles along the stripes. The extent of the fibrinogen adsorption clearly follows the primary irradiation dose. c) An optical micrograph of the dot pattern formed after adsorption of an Alexa-Fluor488/HSA conjugate on the electron-beam-engineered EG7/Au SAM. d) An optical micrograph of the dot pattern formed after adsorption of AlexaFluor546 conjugate of streptavidin onto the above template treated preliminary with Biotin-BSA.

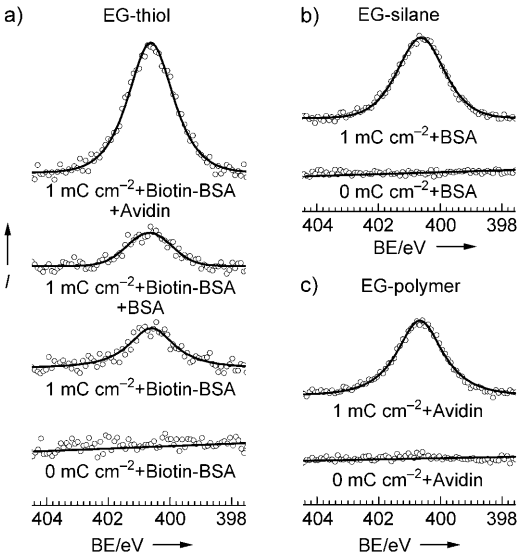

Figure 4. a) N1s XPS spectra after either direct or sequential adsorption of Biotin-BSA, BSA, and avidin on the pristine and electron-beam-modified EG7/Au SAM. b) N1s XPS spectra after adsorption of BSA on the pristine and electron-beam-modified EG-silane/Si film. c) N1s XPS spectra after adsorption of avidin onto pristine and the electron-beam-modified EG-polymer/Si film.

monitor protein adsorption we measured the N1s XPS peak characteristic of proteins. As a primary matrix, EG7/Au was used; it shows zero affinity to all the proteins used in our study. In the first step, EG7/Au was irradiated with electrons (1 mC cm^{-2}) and exposed to biotin-labeled bovine serum albumin (Biotin-BSA), which resulted in a characteristic N1s XPS signal. A subsequent exposure of the modified template to a non-specific protein, such as for example, bovine serum albumin (BSA), did not lead to any change of the XPS spectrum, which means, as expected, that no further protein adsorption occurred. In contrast, the immersion of the same template into the solution of a specifically binding protein (avidin) resulted in a significant increase of the N1s intensity, showing the specific adsorption event. This result is supported by the fluorescence microscopy data in Figure 3d, in which a dot pattern of an AlexaFluor 546 conjugate of streptavidin on the Biotin-BSA-EG7/Au template is shown. Note that this selective attachment can also be performed the other way around, that is, non-specific adsorption of avidin and subsequent specific adsorption of Biotin-BSA (Figure SI3 in the Supporting Information).

The approach is not limited to SAMs on gold substrates but can also be applied to any OEG-based films, including SAM-like and polymer-like films on silicon or glass. To demonstrate this, we took films of OEG-substituted trichlorosilanes with OEG parts of different lengths. In one case (EG6-silane), this part contained only six EG units; the film was SAM-like and repelled only some proteins, including BSA, which was therefore chosen for the experiments (for a silicon substrate more than six EG units are necessary for complete repelling of proteins).[22] In another case (EG-polymer), the OEG part contained about 2000 units so that the film can be considered to be a polymer film rather than a SAM. This film repelled all proteins we tested, including avidin which was then used as a test molecule. As shown in Figures 4b and c, electron irradiation of both EG6-silane and EG-polymer films promoted protein adsorption: while no characteristic N1s XPS signal was observed for non-irradiated films exposed either to BSA or avidin, a significant signal appeared after the electron irradiation. This suggests that all above results for the EG3/Au and EG7/Au SAMs are also applicable to other OEG-based films on different substrates. Such general behavior is a clear indication that proteins bind directly to the modified OEG matrix; this binding is presumably mediated by chemically active sites created by electron irradiation. The bonding is quite robust; the protein patterns remain intact after extensive washing and subsequent handling.

In conclusion, we have presented a universal and simple approach to the preparation of protein patterns. It involves one-step fabrication of a chemical template for non-specific protein adsorption—by direct electron beam writing in an OEG-based, protein-repelling primary film. This film can be both SAM- and polymer-like, with a broad choice of substrates. The required irradiation dose is smaller by two orders of magnitude than for an alternative, multi-step EBL

approach.[9,10] The non-specific template can be easily converted into a specific one by adsorption of a mediator protein with specific binding sites for the secondary target protein.

The EBL allows the generation of patterns over a length scale ranging from centimeters to nanometers,[11,20,23] with no limitations to the pattern shape, including complex, gradient-like assays.[20] Such patterns can become an important tool for mimicking natural biological interfaces which frequently possess gradient character—a typical way of encoding and displaying directional biological information. Such model surfaces can be used as versatile assays to study, for example, cell growth, differentiation, and migration[24] or bacteria adhesion.[25]

Whereas complicated patterns can only be written by a focused electron beam, for example, with a commercially available lithography setup,[20] less complex patterns can be prepared in proximity printing geometry using quite simple equipment.[23] The approach can also be extended to X-ray[26] or deep UV[9,27] lithography, which allows interferometric techniques to be implemented; these techniques are suitable for the fabrication of arbitrary large arrays with a nanometer-scale precision.[9,25]

Experimental Section

EG7 and EG-silane compounds were synthesized in analogy to previously described methods. The SAMs were prepared by immersion procedures, following standard methods. They were either homogeneously irradiated (10 eV electrons) or patterned with either 10 eV (dot patterns) or 1 keV (gradient patterns) electrons. The effect of electron irradiation was monitored by XPS; for which homogeneously irradiated SAM samples were used. The adsorption of proteins on these samples and on SAM patterns was performed according to standard methods. The adsorption was monitored by XPS, using the characteristic N1s emission. The protein patterns were imaged by atomic force microscopy and fluorescence microscopy. For details and further references, see the Supporting Information.

Received: February 18, 2009
Revised: March 31, 2009
Published online: July 6, 2009

Keywords: chemical lithography · gradient patterns · protein patterns · self-assembled monolayers

[1] M. Mrksich, C. S. Chen, Y. Xia, L. E. Dike, D. E. Ingber, G. M. Whitesides, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 10775.
[2] E. Delamarche, A. Bernard, H. Schmid, B. Michel, H. Biebuyck, *Science* **1997**, *276*, 779.
[3] Y. Xia, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 568; *Angew. Chem. Int. Ed.* **1998**, *37*, 550.
[4] A. S. Blawas, W. M. Reichert, *Biomaterials* **1998**, *19*, 595.
[5] G. MacBeath, S. L. Schreiber, *Science* **2000**, *289*, 1760.
[6] J. R. Kenseth, J. A. Harnisch, V. W. Jones, M. D. Porter, *Langmuir* **2001**, *17*, 4105.
[7] K. B. Lee, S. J. Park, C. A. Mirkin, J. C. Smith, M. Mrksich, *Science* **2002**, *295*, 1702.
[8] J. Gu, C. M. Yam, S. Li, C. Cai, *J. Am. Chem. Soc.* **2004**, *126*, 8098.
[9] A. Turchanin, A. Tinazli, M. El-Desawy, H. Großmann, M. Schnietz, H. H. Solak, R. Tampe, A. Gölzhäuser, *Adv. Mater.* **2008**, *20*, 471.
[10] W. Eck, V. Stadler, W. Geyer, M. Zharnikov, A. Gölzhäuser, M. Grunze, *Adv. Mater.* **2000**, *12*, 805.
[11] T. Winkler, N. Ballav, H. Thomas, M. Zharnikov, A. Terfort, *Angew. Chem.* **2008**, *120*, 7348; *Angew. Chem. Int. Ed.* **2008**, *47*, 7238.
[12] N. Ballav, A. Shaporenko, S. Krakert, A. Terfort, M. Zharnikov, *J. Phys. Chem. C* **2007**, *111*, 7772.
[13] M. Zharnikov, M. Grunze, *J. Vac. Sci. Technol. B* **2002**, *20*, 1793.
[14] M. Montague, R. E. Ducker, K. S. L. Chong, R. J. Manning, F. J. M. Rutten, M. C. Davies, G. J. Leggett, *Langmuir* **2007**, *23*, 7328.
[15] J. Rundqvist J. H. Hoh, D. B. Haviland, *Langmuir* **2006**, *22*, 5100.
[16] M. Steenackers, A. Küller, N. Ballav, M. Zharnikov, M. Grunze, R. Jordan, *Small* **2007**, *3*, 1764.
[17] M. Riepl, M. Östblom, I. Lundström, S. C. T. Svensson, A. W. D. Van der Gon, M. Schäferling, B. Liedberg, *Langmuir* **2005**, *21*, 1042.
[18] T. Kraus, T. Kraus, R. Stutz, T. E. Balmer, H. Schmid, L. Malaquin, N. D. Spencer, H. Wolf, *Langmuir* **2005**, *21*, 7796.
[19] J. Genzer, R. R. Bhat, *Langmuir* **2008**, *24*, 2294.
[20] a) N. Ballav, S. Schilp, M. Zharnikov, *Angew. Chem.* **2008**, *120*, 1443; *Angew. Chem. Int. Ed.* **2008**, *47*, 1421; b) S. Schilp, N. Ballav, M. Zharnikov, *Angew. Chem.* **2008**, *120*, 6891; *Angew. Chem. Int. Ed.* **2008**, *47*, 6786.
[21] H. LeVine III, *Anal. Biochem.* **2006**, *356*, 265.
[22] C. M. Yam, J. M. Lopez-Romero, J. Gu, C. Cai, *Chem. Commun.* **2004**, 2510.
[23] N. Ballav, A. Shaporenko, A. Terfort, M. Zharnikov, *Adv. Mater.* **2007**, *19*, 998.
[24] L. Liu, B. D. Ratner, E. H. Sage, S. Jiang, *Langmuir* **2007**, *23*, 11168.
[25] E. A. Burton, K. A. Simon, S. Hou, D. Ren, Y.-Y. Luk, *Langmuir* **2009**, *25*, 1547.
[26] R. Klauser, I.-H. Hong, M.-L. Huang, S.-C. Wang, T. J. Chuang, A. Terfort, M. Zharnikov, *Langmuir* **2004**, *20*, 2050.
[27] S. O. Kim, H. H. Solak, M. P. Stoykovich, N. J. Ferrier, J. J. de Pablo, P. Nealey, *Nature* **2003**, *424*, 411.

