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Microstructured bioreactive surfaces: covalent immobilization of proteins on Au(1 1 1)/silicon via aminoreactive alkanethiolate self-assembled monolayers

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Micrometer-scale patterns of a defined surface chemistry and structure were produced on both ultraflat Au(1 1 1) and on gold-coated monocrystalline silicon surfaces by a method combining microcontact printing, wet chemical etching and the replacement of etch-resist self-assembled monolayers (SAMs) by functionalized or reactive SAMs. Key steps in this methodology were characterized by X-ray photoelectron spectroscopy (XPS), ellipsometry and contact angle measurements. The covalent immobilization of (functional) biological systems on these surfaces was tested using an *N*-hydroxysuccinimide ester ω-functionalized disulphide (DSU), which covalently binds primary amines without the need for further activation steps. Atomic force microscope images of native collagen V single molecules immobilized on these patterned surfaces revealed both high spatial resolution and strong attachment to the monolayer/gold surface. Microcontact printing of DSU is shown to be feasible on specially prepared, ultraflat Au(1 1 1) surfaces providing a valuable tool for scanning probe experiments with biomolecules. The retention of enzymatic activity upon immobilization of protein was demonstrated for the case of horseradish peroxidase. The described approach can thus be used to confine biological activity to predetermined sites on microstructured gold/silicon devices – an important capability in biomedical and biomolecular research.

1. Introduction

The covalent immobilization of functional, biological molecules onto a defined and conductive surface provides the basis for sophisticated biomolecular architectures with numerous applications for *in vitro* studies on the behavior of biological structures such as proteins and cells, for implant ''surface tailoring'' or for biosensor devices. In recent years, much work has been devoted to the development of new techniques for protein immobilization on different substrates [1]. These

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developments have taken place in parallel with rapid progress in protein engineering technology. The immobilization of small, protein-containing structures such as cells or bilayer membranes can be facilitated by patterned surface chemistries or even topographically structured surfaces. Our ultimate goal is to build such structures mimicking parts of biological organelles, which are amenable to scanning probe microscopy. The goal of the present paper is to describe the foundations of such structures, based on functionalized alkanethiols or disulphides chemisorbed on gold surfaces.

The structure and formation of self-assembled monolayers (SAMs) of alkanethiols and dialkyldisulphides on gold surfaces has been intensively studied since 1983 [2–5] (for recent reviews see: [6, 7]). A broad range of applications of such systems has arisen from the synthesis of alkanethiols having functional groups in the ω-position. These make it possible to impart virtually any chemical property to a gold surface, and thus, parameters such as wettability [8, 9], and reactivity [10–14] can be controlled on such a surface. Monolayers on gold have been used to selectively and/or covalently bind cells [15–18], proteins [11, 13, 19–21], membranes [22], and other subcellular structures [23] (for a review see: [24]).

Micro- and nano-meter patterning of such alkanethiolate SAMs on gold can be achieved by the recently developed microcontact printing (μCP) method [25–27], providing monolayer surfaces with spatially resolved head-group functionalities. This method was favored in the present study over photolithographic techniques [28], because of its simplicity and wide applicability. In addition, this technique can be directly combined with wet-etching procedures to achieve topographical patterns [29].

We previously developed a procedure for producing Au(111) substrates for these monolayers, having extreme flatness over very large areas [30, 31]. More recently, we have shown that an amino-reactive, ω-substituted alkanethiolate self-assembled monolayer could be used to immobilize and study proteins in their native state [13, 23].

In this paper we report the application of the μCP technique to a functionalized alkanedisulphide – 11,11′-dithio-bis(succinimidyl-undecanoate) (DSU). We also describe a strategy for rendering etched silicon/gold structures accessible to this reactive crosslinker SAM. We subsequently present the site-specific immobilization of proteins onto such surfaces (Fig. 1) demonstrating the retained functionality of the immobilized protein. The atomic force microscope (AFM) was used to image the immobilized proteins and to determine their amount and spatial distribution on the patterned, template-stripped gold (TSG) surface.

2. Materials and methods

All chemicals were of the highest available purity. Ultrapure water was prepared by passage through a Barnstead purification system. 11-Hexadecanethiol (95%) and *N*-succinimidyl palmitate (X-ray photo electron spectroscopy; XPS reference) were purchased from Fluka (Buchs, Switzerland), silicon wafers

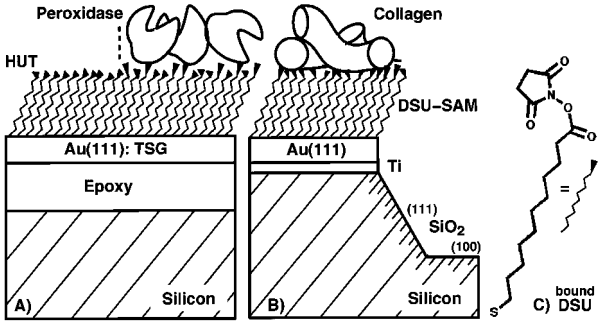

Figure 1 (A) Schematic section through a chemically microstructured TSG sample after μCP with reactive DSU, formation of a HUT SAM and immobilization of proteins. Primary amines of proteins react with the end groups of the DSU crosslinker (11, 11′-dithio-bis(succinimidylundecanoate), Fig. 1C: bound DSU (thiolate form) with the formation of an amide bond. (B) Schematic section through a microstructured silicon/gold structure after μCP, etching the gold, titanium and silicon, removal of the etch-resistant monolayer, formation of a reactive monolayer and finally reaction with protein.

(Si(100), *n*-type, 2–50 Ω cm) from Faselec AG (Zürich, Switzerland) and gold (99.99%) from Cendres et Metaux SA (Biel, Switzerland). Lyophilized bovine uterus collagen V (800 μg ml^{-1} in 0.1 M acetic acid, purified according to a slightly modified protocol from Miller and Rhodes [32] was a gift from Dr Beat Trüeb, Maurice E. Mueller Institute for Biomechanics, Berne. Horseradish peroxidase (POD, type II, EC 1.11.1.7) was purchased from Sigma (Buchs, Switzerland). The synthesis of DSU was carried out as previously described [13]. 11-Hydroxyundecanethiol (HUT) was synthesized according to Bain *et al*. [2].

2.1. Preparation of gold surfaces

Two different gold surfaces have been used in this study:

(a) A 200-nm Au layer was deposited by thermal evaporation using a BAE 370 vacuum coating system from Bal-Tec (Liechtenstein) onto silicon wafers (Faselec AG, Zürich, Switzerland) coated with an adhesion layer consisting of 8 nm of Ti (e-beam). These substrates were placed under an argon atmosphere immediately after the preparation in order to avoid contamination of the gold from the ambient. In the present work we always refer to this type of gold, if not otherwise stated.

(b) Ultraflat, template-stripped gold (TSG) with a mean roughness of 0.2–0.5 nm over 25 μm^2 was prepared as described previously [14, 31]. These surfaces are protected from contamination by the mica template itself, which can easily be lifted off mechanically with the help of tweezers immediately before use. One can reproducibly obtain bare TSG samples without remaining mica pieces by cutting the edges of the glued sample with a sharp knife prior to the mica template removal.

2.2. Chemically patterned surfaces

Monolayer patterns of different alkanethiolates (dodecanethiol, hexadecanethiol (HDT), HUT, DSU) were prepared according the ‘‘microcontact printing’’

method [25, 33]. Briefly, an elastomeric stamp with the desired pattern was made by pouring poly(dimethylsiloxane) (PDMS) onto a silicon chip master, with subsequent curing at 80 °C for 5 h. For route A, the stamp was exposed to a solution of DSU (1 mM in dioxane). After drying with nitrogen and one minute in air, the stamp was lightly pressed onto the gold sample: alkanethiol was thereby transferred to the gold, forming an adsorbed layer. The DSU-pattern was then rinsed with HUT (5 mM in ethanol) for 30 s in order to cover the residual bare gold surface with a defined chemical functionality. For route B, the gold was patterned with a monolayer of hexadecanethiolate (1 mM in ethanol) by μCP and then subjected to chemical etching to create topographically patterned surfaces.

2.3. Physically patterned surfaces

Gold areas not covered by monolayers were selectively etched by immersion in an aqueous solution of 2 mM $K_3Fe(CN)_6$, 20 mM KCN and 1 M KOH at 3 °C for 10 min under stirring. The titanium layer and the underlying thin silicon oxide layer were then removed by etching with 1% HF. Subsequent boiling in 4 M KOH in 15% v/v isopropanol for 4 min at 60 °C resulted in V-shaped, anisotropic etching of the silicon. These substrates were then washed with 1 M KOH and water, dried under nitrogen and exposed to ''piranha'' solution consisting of 30% H_2O_2/conc. H_2SO_4 (3 : 7), at 60 °C for 2 min, to remove the organic monolayer etch-resist from the gold surface. Finally, after washing with water and drying, these substrates were incubated in a solution of 1 mM DSU in dioxane for 2 h, washed with dioxane and were then ready for immobilization of protein.

2.4. Immobilization of collagen

The acidic collagen V solution (800 μg/ml) was diluted 1 : 4000 with high-salt buffer (phosphate-buffered saline; PBS, pH 7.9, supplemented with 500 mM NaCl) and then applied to a 1 cm^2 HUT/DSU patterned surface for 2 h at room temperature. After washing with 15 ml of the buffer, the surface was again incubated in high-salt buffer for 2 h under gentle shaking. In some cases, non-specifically adsorbed protein had to be removed by brief sonication in NaCl-supplemented buffer with 0.05% Tween-20, followed by rinsing with phosphate buffered saline (PBS). The collagen pattern was examined by atomic force microscopy AFM either in PBS or after air-drying. This strategy was also used for the immobilization of collagen V on the 3D-patterned surfaces.

2.5. Peroxidase activity test

One milliliter of a solution of horseradish peroxidase (POD type II, 200 U mg^{-1}) in PBS (pH 7.5, activity 30 mU ml^{-1}) was applied to different DSU- and/or HUT-monolayer substrates and kept for 4 h at room temperature under gentle shaking. These surfaces were washed three times with a stream of PBS containing 0.1% (v/v) Tween-20 and further soaked for 20 min in the same buffer. The enzymic activity of the chemisorbed peroxidase was quantified as follows: after washing (as described) the samples were incubated individually at 24 °C in 1 ml of a 0.42 mM solution of 3,3′-5,5′-tetramethylbenzidine (TMB) in PBS (pH 6, containing 1% DMSO and 0.004% H_2O_2). After 45 min the blue supernatants were transferred into disposable cuvettes, the reaction stopped by adding 100 μl of 2 M H_2SO_4 and the absorption measured at 450 nm (Jasco 7800 UV/Vis Spectrophotometer). For the high-resolution AFM samples, POD (12.5 U ml^{-1}, 10 h at 4 °C) was used and the sample dried under nitrogen after rinsing with water.

2.6. Surface analysis

The AFM images were obtained either on a Bioscope or a NanoScope III equipped with a fluid cell and a ''J-type'' piezoscanner with a maximum scan range of 140 μm (Digital Instruments, Santa Barbara, CA). Monocrystalline silicon cantilevers (Lot, Darmstadt, Germany) were used with force constants ranging from 0.06 to 0.17 N m^{-1} (13–100 N m^{-1} for tapping-mode). In contact mode, the z-feedback loop was frequently adjusted to guarantee minimal forces (the force, just before the tip jumps out of contact if further retracted) between tip and substrate.

Prior to scanning electron microscopy (Philips SEM 505, 30 kV), an 8-nm thick gold layer was deposited on the etched sample in a sputtering device (Balzers Union, Balzers, Liechtenstein) to avoid electrostatic charging of the surface.

The XPS spectra were recorded using an ESCA 5400 instrument (Physical Electronics, Eden Prairie, MN) with MgK_α radiation at 300 W (15 kV). Measurements were taken at a take-off angle of 45° with respect to the sample surface. The analyzed area was 3.5 mm^2. Survey scans were taken for each sample with a constant detector pass energy range of 50 eV, followed by high-resolution XPS measurements (pass energy 17.9 eV) between 528 and 738 eV. Electron binding energies were calibrated to the Au 4f (84.0 eV) line.

The thickness of the stamped DSU layers was mesured using a Plasmos SD 2300 ellipsometer at 623.8 nm. The angle of incidence was set to 70° and the layer thickness measured relative to non-modified areas of bare gold on the same sample, assuming a refractive index of 1.44 for organic thin films. DSU (20 mM) in dioxane was used for stamping.

Advancing contact angle measurements were carried out on a G-I contact angle meter (Krüss, Hamburg, Germany) by applying a 3-μl drop of ultrapure water to a freshly prepared surface. A second drop was centered on the first, and the advancing contact angle measured within 30 s. A set of six locations was averaged per sample.

3. Results and discussion

Many aspects have to be taken into account when developing interfacial devices involving native biological molecules and inorganic, microstructured surfaces: biological objects, because of their sensitivity and complexity, need a well-defined, mild ''physiological'' environment for their structural integrity and activity. On

the other hand, these systems have to be rendered convenient, making them accessible to experimental research or applications in artificial devices. In this regard, an important tool is the spatial direction of the site of immobilization of biomolecules on microstructured surfaces. However, care has to be taken not to denature the biological systems upon immobilization and to make sure that the attachment is strong enough to resist changes in the chemical and physical composition of the liquid environment, which can substantially impair the strength of immobilization of, for example, physisorbed proteins. Structured and chemically defined surfaces based on the alkanethiolate-SAM chemistry offer several advantages for the purposes mentioned above: alkanethiolate monolayers on gold are stable under aqueous conditions and they are ideal crosslinkers between biomolecules and the inorganic substrate. Moreover, the densely packed arrangement of the hydrocarbon chains with a defined spacer length provides a mechanical as well as a chemical barrier protecting the underlying substrate from direct contact with the environment. There are also no chemical limitations with respect to the synthesis of alkanethiols having complex end-group functionalities.

We have produced (by μCP) and then tested two different sets of structured surfaces for the covalent immobilization of biomolecules via SAMs: on ultraflat gold (i.e. on TSG) we prepared patterned DSU/HUT monolayers and tested them using proteins such as collagen V and POD (Fig. 1a). In a second strategy, we prepared structured silicon/gold surfaces with immobilized collagen (Fig. 1b).

3.1. Immobilization of biomolecules on chemically patterned TSG surfaces

While hexadecanethiol has been used in innumerable μCP studies and has been shown to be readily printable as a complete monolayer, it is not suited to the applications described in this paper, as its hydrophobic character is likely to induce protein adsorption with subsequent denaturation. Stamping the reactive SAM first, appropriate monolayer end-group chemistries can be chosen at will for the remaining, free gold areas, and the reactivity of the surface can either be tuned towards the immobilization of a specific ligand or to minimize non-specific adsorption of contaminants. We have concentrated on functionalization by stamping the reactive DSU disulphide. The higher polarity and increased size of this molecule compared to the *n*-alkanethiols used in standard μCP techniques also changes the stamping performance; ellipsometric measurements show that after stamping, a mean layer thickness of 0.48 ± 0.19 nm is obtained, compared to 1.7 nm for a fully formed DSU SAM after incubation [13]. Thus, only 1/4 of a monolayer is transferred to the substrate, on average. Subsequent rinsing with HUT, which adsorbs between the DSU molecules, gives rise to a SAM composed of mixed DSU and HUT molecules. The smaller amount of reactive sites available in the resulting mixed SAM may be counterbalanced by an increased reactivity of the DSU molecules – because of a reduced steric hindrance by neighboring DSU molecules towards nucleophilic attack [13, 34]. Additionally, it is beneficial to embed the reactive species into a matrix with non-adsorbing surface chemistry, leading to reduced non-specific adsorption on the reactive areas.

3.1.1. Collagen V immobilization

Collagen V is a typical extracellular matrix protein with a triple helical, 300-nm long and 1.4-nm wide structure consisting of 3042 amino acids. One hundred and fifty-two lysine residues are fairly homogeneously spread over its surface, enabling the protein to develop multiple bonds with the NHS-ester groups of a DSU monolayer.

Individual (i.e. three sub-units) molecules of collagen V were applied to a pattern of DSU and HUT monolayers. Fig. 2 shows an AFM image at high magnification of collagen V molecules immobilized only on those areas covered by DSU – resulting in a network of single collagen monomers – while no protein is observed on ultraflat-gold areas covered by HUT molecules. The amount of covalently immobilized collagen monomers on this system can be controlled by using different pH values and collagen concentrations in the buffer solution [13]. Densities ranging from a few molecules per square micron to dense networks of collagen can be obtained. The very characteristic filamentous shape of these proteins makes them clearly distinguishable from the template-stripped gold morphology in the background of the AFM images. They are visibly flexible and often follow the boundaries of HUT/DSU patterns. Interestingly, despite its size and shape, collagen V forms very sharp boundaries between patterned DSU and the protein-resistant, OH-terminated

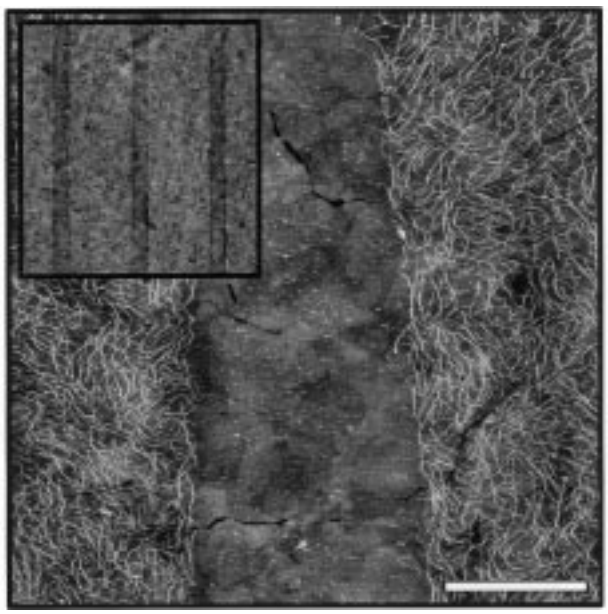

Figure 2 Immobilization of proteins on ultraflat Au(111): a patterned network of collagen V single molecules covalently immobilized on a DSU monolayer. Due to the very low roughness of the ultraflat, template-stripped gold surface, the molecular structure of the immobilized proteins can be resolved over large areas. Inset: the same surface at 18 × lower magnification. Bar = 1/μm.

monolayer. Conceivably, once a collagen molecule has bound with one lysine to a monolayer-site close to the DSU/HUT-SAM border, the non-bound triple-helical strand may be induced to zip-like binding also. In other words, being unable to create covalent bonds with HUT-SAM regions, the protein is thermodynamically forced to find its final location upon reaction with the NHS headgroups of the DSU monolayer. Contact-mode AFM revealed the very strong binding of the covalent (amide) bond between the proteins and the monolayer: an increased loading force on the scanning tip did not sweep aside the collagen until the protein chain itself was ruptured by the strong lateral forces applied. Physisorption of collagen on a HUT monolayer is also fairly stable, but can be overcome by special washing procedures, as described in Materials and Methods. Other ω-terminated alkanethiols (i.e. amino-, carboxyl-, or methyl-terminated) were also tried as fill-in monolayers for DSU patterns, but none proved superior to HUT. We are currently exploring the use of a polyethylene-glycol-terminated SAM, which was shown to resist physical adsorption of many proteins and even of cells [19, 35, 36], as an alternative protein-resistant monolayer coating.

3.1.2. Immobilization of peroxidase

In order to investigate the influence of the monolayer immobilization method on the activity of a model-protein, we adsorbed horseradish peroxidase on a similar pattern as described above and detected its enzymatic activity. Survival of the enzymatic activity at the solid–liquid interface cannot be taken for granted, since proteins often suffer large conformational changes – with partial or complete loss of their activity – when adsorbed at surfaces [37]. Non-covalently adsorbed peroxidase could be very efficiently removed by washing with a buffer solution containing 0.1% (v/v) Tween-20. This mild, non-polar detergent is known not to interfere with the activity of most proteins. The AFM image in Fig. 3a demonstrates that peroxidase molecules were exclusively adsorbed on those areas containing DSU in the monolayer. The height of these regions determined by contact mode AFM was 3.3 ± 0.6 nm, which is a realistic size if compared with X-ray structural data of the enzyme [38], taking into account some degree of compression and convolution caused by the interaction of the tip with the sample. A quantification of bound peroxidase can be attempted on the basis of tapping mode AFM images, on which individual immobilized peroxidase molecules can be distinguished as globular structures which can be counted and thence an average surface-coverage determined. Fig. 3b shows a typical TSG surface with individual POD molecules. A peroxidase surface density of 0.13 pmol cm^{-2} peroxidase could be calculated for an immobilization time of 10 h in PBS pH 7.5 at 4 °C, on ultraflat gold. X-ray data show [38] that the size of the enzyme is approximately $4 \times 4 \times 6$ nm. A maximal density of 3 pmol cm^{-2} can thus be expected for a tightly packed protein monolayer on ultraflat gold, assuming an exclusion radius of half the diameter around each protein.

The enzymatic activity of the covalently bound

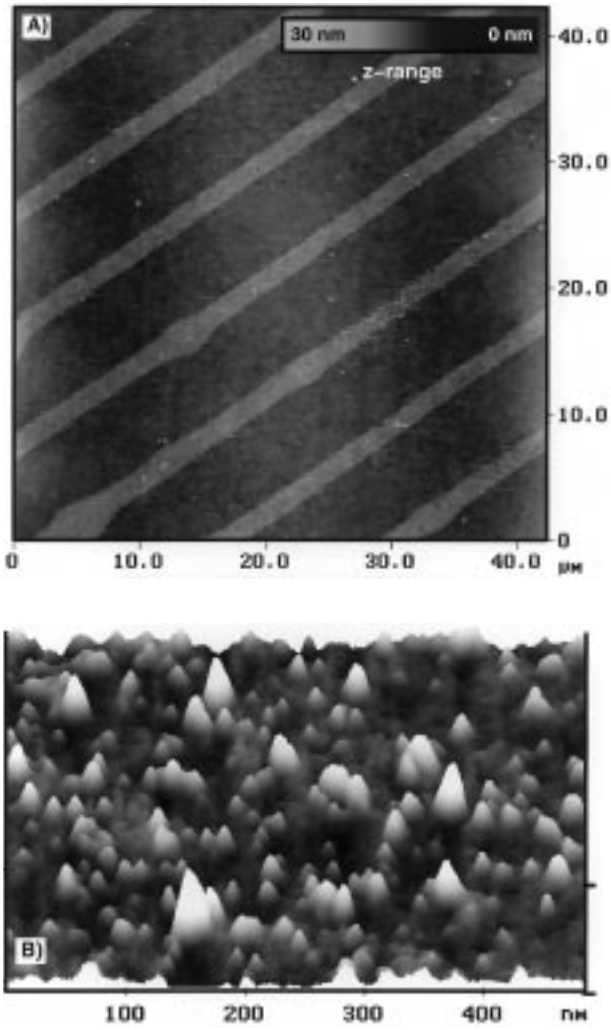

Figure 3 AFM image of bands of covalently immobilized horseradish peroxidase (A, light, higher areas) on ultraflat Au(111). Prior to the attachment of peroxidase, the gold was microcontact printed with a solution containing DSU and then rinsed with a solution of HUT, which covers the areas that appear dark in the image. (B) Individual POD molecules can be distinguished -and counted- at higher magnification on the tapping-mode AFM image. z-Bar = 5 nm.

peroxidase was estimated using 3,3′-5,5′-tetramethylbenzidine (TMB) (see Materials and Methods and Fig. 4). Three different surfaces have been examined for activity of the attached peroxidase after removing physisorbed enzyme by washing with PBS containing detergent (Tween): (i) surfaces totally covered by DSU binding the enzyme covalently; (ii) surfaces covered by a HUT monolayer; (iii) surfaces with DSU/HUT monolayer patterns. In three control experiments, the peroxidase activity was always the highest in the first case (i) 6.2 mU cm^{-2} activity on TSG and the lowest in the second case (ii) 1.4 mU cm^{-2} activity on TSG, while the patterned samples (iii) 3.3 mU cm^{-2} activity on TSG displayed intermediate activities. As expected, the enzyme activity correlates at least roughly with the amount of DSU present on the respective gold surface and, thus, with the number of bound enzyme molecules.

The influence of roughness of the gold surface on the amount of bound enzyme was investigated by comparing two types of surfaces: (i) a substrate prepared by thermal

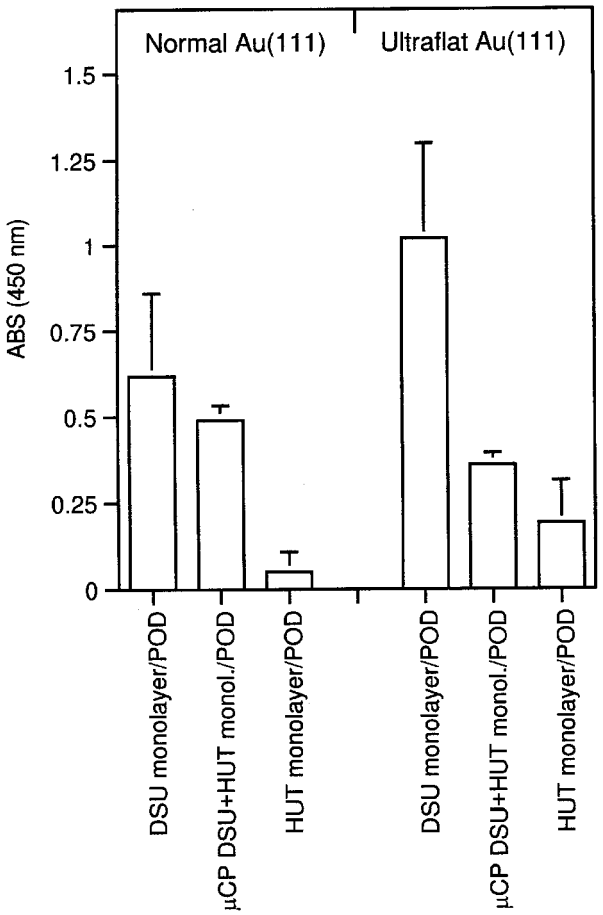

Figure 4 The relative activity of covalently immobilized peroxidase on two different Au(111) substrates precoated with three different SAM compositions: Au(111) normal = gold thermally evaporated on a Ti-primed Si(1 0 0) wafer; ultraflat Au(111) = ultraflat, TSG gold (see Materials and Methods) with a mean roughness of 0.2–0.5 nm/25 μm^2; DSU monolayer = the gold was incubated with the aminoreactive DSU-SAM prior to the immobilization of peroxidase (POD); HUT monolayer = the gold was incubated with 11-hydroxyundecanethiol prior to the immobilization of POD; µCP DSU + HUT monol. = the gold was first microcontact printed with DSU followed by rinsing with a solution of HUT, prior to POD incubation.

evaporation of gold on silicon wafers having a roughness of approx. 2–6 nm and (ii) ultraflat gold surfaces having mean roughness values one order of magnitude lower. Bound POD proved to retain enzymatic activity in both cases (Fig. 4), but only half as much activity was detected on the rather rough surface of the first substrate compared to the second type consisting only of monoatomic steps over cm^2 areas. Increased peroxidase activity on smooth surfaces was observed for the DSU-, HUT- and DSU/HUT-monolayer samples mentioned above. This may be explained by preferential adsorption of the protein in the (more reactive) grooves of the rougher substrate, which might lead to a different protein conformation caused by multiple binding with concomitant loss of activity and/or to unstirred layers during testing, which are known to result in an apparent increase of K_m and decrease in V_{max}. An equivalent activity of 0.08 pmol dissolved POD was found immobilized on 1 cm^2 of a TSG surface covered with DSU.

3.2. Generation of etch-patterned surfaces with immobilized biomolecules

The creation of etch-structured gold/silicon surfaces was achieved by a multistep procedure. First, a hexadecanethiolate monolayer pattern resistant to a cyanide-based gold etch solution was prepared on a 200-nm thick film of gold (deposited on a silicon substrate with a 8-nm titanium layer as an adhesion mediator) by microcontact printing. Non-protected, i.e. monolayer-free, gold areas were etched by subsequent treatment with a cyanide solution. A second etching step with 1% hydrofluoric acid was carried out to remove the titanium and the underlying thin native silicon oxide layer, followed by a third bath to create several-micron-deep, V-shaped grooves (cavities) in the monocrystalline Si(100) substrate by anisotropic etching. This resulted in flat, elevated micrometer-sized gold islands on a silicon support. No changes in the Au(111) morphology could be observed as a result of the etching. The hexadecanethiolate monolayer still left on these gold areas was finally removed by exposing the surface to hot ''piranha'' solution (30% H_2O_2/conc. H_2SO_4 = 3 : 7; 60 °C) for 5 min (*caution: this solution can explode in contact with organic matter*). Contact angle measurements on non-etched test Au samples (Fig. 5) confirm the removal of the hydrophobic etch-resist SAM upon 2 min exposure to piranha. This treatment leads to a bare gold surface which is now again accessible to the adsorption

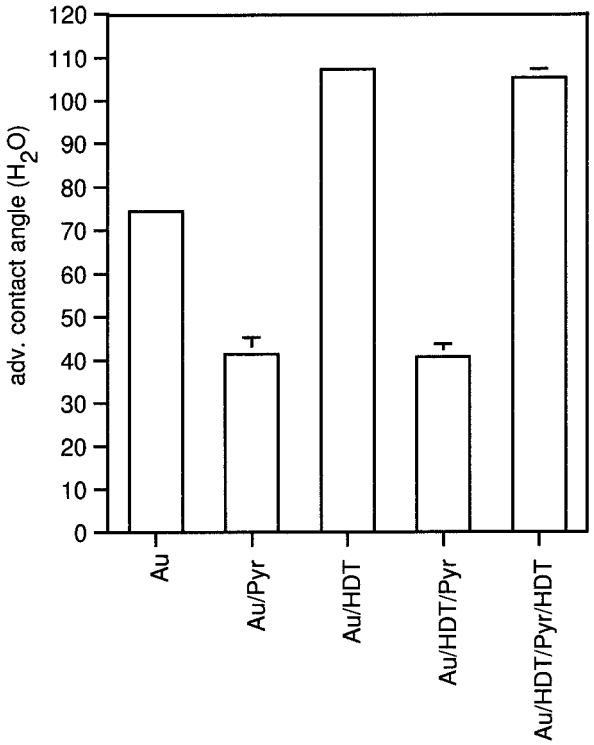

Figure 5 Changes in contact angle related to the process of oxidative SAM removal with piranha solution. Legend: Au: pure Au(111) with natural contamination layer; Au/Pyr: Au treated with hot Piranha solution for 2 min. Au/HDT: Au(111) after 2 h exposure to hexadecanethiol (5 mM in ethanol); Au/HDT/Pyr: Au after the removal of the hexadecanethiolate-SAM (hot piranha for 2 min); Au/HDT/Pyr/HDT: new formation of a hexadecanethiolate-SAM on Au(111) after piranha removal of a previous SAM (5 mM in ethanol).

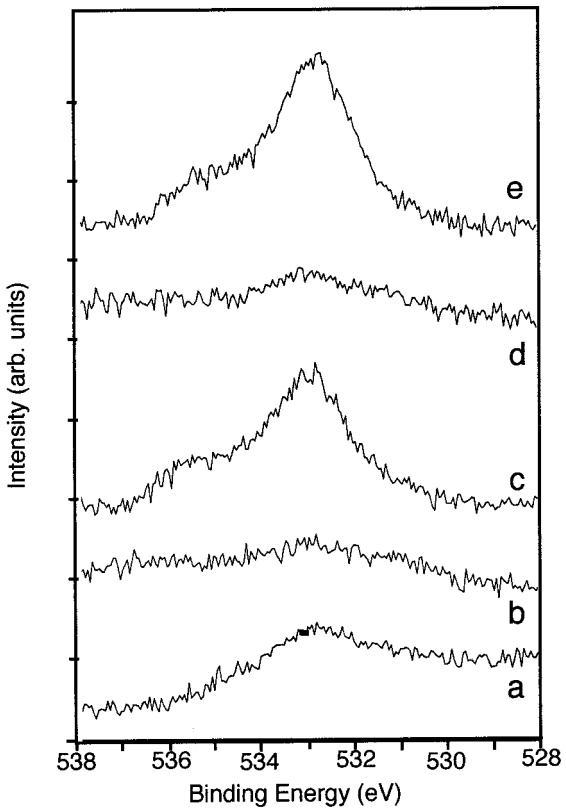

Figure 6 O(1s) X-ray photoelectron spectrum progression of a gold sample at different stages of functionalized-SAM replacement. (a) Fresh, naturally contaminated gold; (b) gold after exposure to 2 min piranha (60 °C); (c) DSU-SAM on gold (0.5 mM in dioxane for 18 h); (d) sample c exposed to 2 min piranha (60 °C), (e) sample d upon repeated exposure to DSU.

of a new SAM, as can be seen from the recovery of hydrophobicity upon exposure to a 5 mM hexadecanethiol solution. The same behavior can also be demonstrated for functionalized SAMs, such as DSU: Fig. 6 shows the progression of the O1s XPS spectra after the different reaction steps: only a very weak oxygen contamination peak can be detected on freshly deposited gold, which then disappears after inmersion in hot piranha solution. After formation of a DSU SAM, the O1s envelope of the succinimidyl-ester functionality appears as a strong band showing two shoulders at 532.2 ± 0.5 eV (C = O) and 534.3 ± 0.13 eV (C – O – C). The peak shape is similar for DSU chemisorbed on Au(111) as for a powder-spectrum of *N*-succinimidyl palmitate, a commercial compound containing the same succinimidyl-ester group (data not shown). Nevertheless, in no case could a good 1/1/2 curve-fitting for the oxygen peak be obtained, probably due to partial hydrolysis of the succinimidylester, which leads to the appearance of carboxylate and hydroxy peaks within the same binding-energy region. Exposure of the DSU-covered substrate to piranha solution leads to a complete disappearance of the oxygen peaks, whereas upon repeated formation of a DSU SAM, these peaks reappear in the original shape, demonstrating the reversability of the SAM formation. After removal of the hexadecanethiolate etch-resist and formation of a DSU SAM, the quality and specificity of this amino-reactive DSU pattern was again examined by immobilization of collagen V molecules. Fig. 7 shows a SEM image of such a topographically and chemically patterned sample. AFM imaging of the monolayer-free silicon oxide areas reveals no physisorbed collagen, while a network of single, covalently bound collagen monomers can be distinguished on the Au(111) islets. Collagen immobilized on the gold tracks of etch-patterned surfaces shows the same characteristics as that on chemically patterned surfaces. However, the apparent edge definition of the collagen on this kind of surface (Fig. 7) is much lower than on TSG (Fig. 2), because AFM imaging is negatively affected by the rough substrate morphology of Au(111) surfaces prepared by standard thermal evaporation of gold on titanium-primed silicon.

Many materials are processable by the sequence described above, since gold layers of well-defined thickness and topography can easily be deposited on a large number of substrates by vacuum-evaporation techniques. Subsequent structuring steps in the micrometer range can be performed by applying traditional wet-etching procedures. The possibility of removing a SAM from gold either by means of an electrical field, or heating above 80 °C in a suitable solvent [2], or by oxidation [39] allows the specific replacement of one monolayer by another. Interesting applications of this include the described possibility of using inert alkanethiols to pattern and etch a surface by μCP and then – through SAM exchange – to impart a desired chemical reactivity to it. Additionally, the process allows monolayer-immobilized proteins that have become non-functional to be replaced by fresh ones: silicon devices containing enzymes on small gold areas could be regenerated through a simple three-step procedure: removing the damaged monolayer/protein layer by photo-oxidation; formation of a new monolayer by rinsing with reactive compounds, and finally binding native enzyme. This kind of surface recycling can be of use in biophysical research applications where complex devices can only be produced in small numbers.

4. Conclusions and outlook

In this study, we have presented a mild procedure for covalent immobilization of proteins on microstructured gold surfaces. The use of ultraflat, TSG as a substrate opens the possibility of making patterned biomolecule structures accessible to scanning probe methods on a background with defined surface chemistry. On microstructured $Si/SiO_2/Ti/Au$ surfaces produced by μCP and etching, the immobilization site of proteins could also be spatially controlled using an amino-reactive SAM. The production of these micropatterned substrates including SAM exchange and protein immobilization, can all be performed in one day, thus providing a high degree of flexibility in experimental work. Although most biological samples have amino groups on their surfaces and can thus be immobilized via DSU, the need may arise for other ω-substituted alkanethiols with chemical reactivity directed towards other functional groups. For example, aryldiazonium-terminated alkanethiols [14] selectively

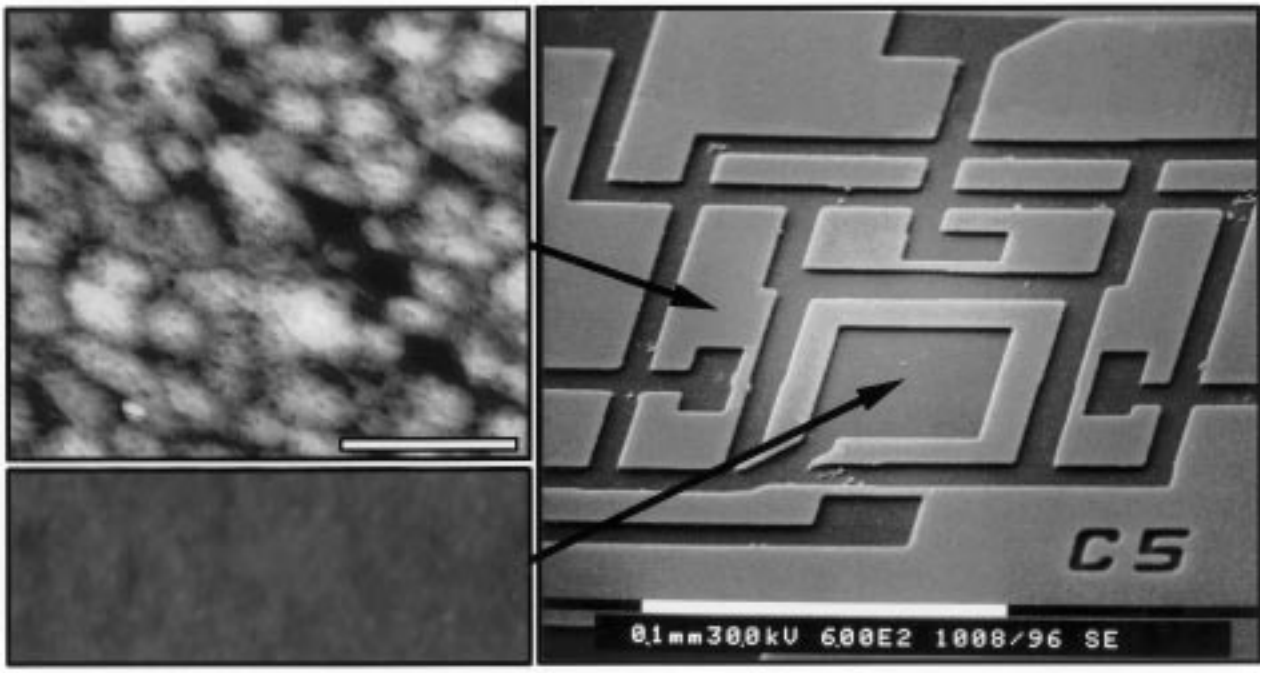

Figure 7 Covalently immobilized proteins on microstructured Au/Si surfaces: (right), SEM image of a structured Au/Si surface produced by μCP, etching, SAM replacement and covalent collagen immobilization (top, left; bar = 200 nm; z-range = 30 nm): An AFM (tapping mode in air) zoom of the Au/DSU-SAM areas reveals the presence of a two-dimensional network of covalently bound collagen V molecules. The resolution is lower than in Fig. 2 because the round-shaped (rough) morphology of this Au(111) impairs AFM measurements. (bottom, left): no collagen is visible on the silicon areas on the surface (same scales as top, left).

bind activated aromatic hydrogens of, e.g. histidine and tyrosine residues; Ni^{2+}-chelating monolayer-headgroups bind histidine-tagged fusion proteins [12]; (Wagner *et al*., manuscript in preparation). We are currently exploring the use of this technology for the immobilization of cells and biological bilayer membranes.

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