Site-Specific Bio-Functionalization of Surfaces by Means of Metal Nanostructures

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Abstract— We present an approach to control the immobilization process of biomolecules by functionalization of SiO₂ substrates decorated with nanostructures. The creation of specific protein permissive and protein resistant areas leads to a directed and site-specific immobilization of proteins. We make use of molecular beam epitaxy and laser assisted cluster deposition to create Au nanostructures on PEG-silanized SiO₂ substrates. The chemical contrast provided by the two materials, namely Au and PEG on the substrate surface supports the specificity of thiol-based Au functionalization for biomolecule attachment.

I. INTRODUCTION

The immobilization of biomolecules on solid substrates plays an important role in fundamental as well as application-driven research for biosensing, catalysis, tissue engineering, bioseparation and bioelectronics. The immobilization approach strongly influences the structure of the molecule and finally its functionality. Since biomolecules are in general attached to surfaces different from their native environment, it remains challenging to preserve their biological activity. Nanotechnology offers the possibility to control the site-specific attachment of biomolecules with minimized contact between adjacent molecules. Surface-deposited metallic nanostructures with sizes comparable to those of biomolecules are promising for achieving nanoscale control of protein attachment [1]. By providing specific binding sites for immobilization, biomolecules can be attached either by direct chemisorption or via linker-chemistry and bioaffinity immobilization. Inter-molecular interaction between bound molecules can be reduced by tuning the inter-particle spacing. However, the control of the immobilization at a low-density level requires a reduction of non-specific attachment of biomolecules on the substrate material. This is of high importance as silicon-based materials, e.g. silicon, silicon dioxide, silica and quartz, are frequently used as substrate material for the manufacturing of biosensors but show a substantial tendency for protein

adsorption and hence bio-fouling [2]. The limitation of non-specific protein adsorption on the substrate material is therefore crucial [3]. A promising way to circumvent this problem is the grafting of a polyethylene(glycol) (PEG) layer onto the substrate. A silane-modified PEG (PEG-silane) layer was shown to reduce the amount of non-specifically adsorbed protein on SiO₂ [4]. However, for a nano-structured system it needs to be taken into account, that a PEG_{6/9} molecule is 2 - 3 nm long [5] and hence might be in the same size range as the deposited nanostructures. As the polymer chains are highly flexible, one would expect that PEG-silane molecules cover or shield neighboring nanostructures and limit or hinder their bio-functionalization. The increase of the nanostructures size to values much higher than the polymer chain is one solution [8]. In this paper, we describe an approach to deposit the metallic nanostructures directly on the protein-repellent layer, thus reducing the amount of protein non-specifically attached to the background while presenting specific binding sites. Such a two-material system exploits the chemical contrast between the substrate and the cluster material. To investigate these nanometer-scale systems we have used atomic force microscopy (AFM), which, in combination with contact angle measurements (CA) and quartz crystal microbalance (QCM), yields direct information on the surface morphology and chemistry.

II. MATERIALS AND METHODS

We used thermally oxidized (300 nm) p-doped Si wafers (SiO₂), cleaned in Piranha solution (3:1 H₂SO₄:H₂O₂ at 100°C, 15 min) and APM (Standard clean 1, 4:1:1 DIW:NH₄OH:H₂O₂ at 70°C, 15 min). QCM crystals (QSX301 and QSX303, Q-Sense, Sweden) were cleaned in acetone, isopropyl alcohol (each 5 min) and UV/O₃ (15 min). Immediately after the cleaning, the samples were transferred to their respective incubation solution. For the protein-repellent layer, PEG-silane (10 mM PEG_{6/9} ((CH₃)(PEG)_n(CH₂)₃OSi(OCH₃)₃, n = 6-9)) was grafted to cleaned SiO₂ in water free toluene for 18 h, followed by rinsing with toluene, acetone, DIW and methanol and a short annealing under inert atmosphere at 110°C. The thiol-linker molecules (HS(CH₂)₁₁(PEG)₆-biotin) were attached in ethanol for 3h, followed by rinsing with ethanol. MBE Au nanostructures (high and low coverage) were deposited *in situ* by evaporation from a Knudsen cell at a

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deposition rate of 0.64 and 0.01 Å/s, respectively.

III. RESULTS AND DISCUSSION

A. Grafting of a protein-repellent layer onto SiO₂ substrates

After grafting PEG-silane onto clean Si/SiO₂ substrates, the static contact angle of water (CA) increased from $11\pm3^\circ$ to $38\pm2^\circ$, comparable to previously reported values [6]. This decrease in hydrophilicity can be attributed to the composition of the PEG-silane polymer, namely a combination of a hydrophobic terminal group (R-CH₃) and the hydrophilic backbone ((CH₂CH₂O)_n). The incorporation of water into the backbone plays an important role in the protein-repellent properties of the film [10]. The rms roughness, determined by analysis [7] of AFM topography images, slightly increased and the grafted film appeared to a large extent flat and uniform (Fig. 1a and b). XPS analysis (data not shown) of the C 1s spectra

Fig. 1. AFM topography images of a) clean SiO₂ and b) of a SiO₂/PEG-silane substrate. The insets show the line profile of the corresponding lines. A vertical scale bar (1 nm) is included next to the cross section in a) and is the same for both cross sections.

showed the C-O and the C-C/C-H peaks at 283.5 eV and 285 eV, respectively, characteristic for PEG moieties [2]. Force spectroscopy (data not shown) in air reveals an increase in adhesion between the SiO₂-tip and the SiO₂ sample after PEG-silanization by over 200%. This increase in adhesion can be attributed to the highly hydrated PEG-silane layer attached to surface. To evaluate the protein-repellent properties of the PEG-silane layer we quantified the molecule adsorption on the PEG-silane. A quartz crystal microbalance (QCM) relates the change in the resonance frequency of a piezoelectric crystal to the mass adsorbed on the crystal surface, i.e. the amount of adsorbed molecules, by the Sauerbrey equation [9]. The non-specific adsorption of human IgG to SiO₂/PEG-silane covered quartz crystals was evaluated. QCM data revealed a reduction of non-specific adsorption of IgG on a PEG-silane surface was by approximately 95% (Tab. I), resulting in a protein coverage of less than 10% of the crystal surface. Non-specific attachment of IgG on clean SiO₂ substrates led to a multi-layer coverage of IgG, calculated based on the mass of one streptavidin molecule and its projected area (2.49E-10 ng and 58.5 nm², respectively) and assuming the molecules forms a dense, flat layer on the crystal.

TABLE I

ADSORBED MASS AND CORRESPONDING PROTEIN COVERAGE ON THE QCM CRYSTALS AFTER INCUBATION WITH HUMAN IgG. ACCURACY OF THE DETERMINATION OF THE FREQUENCY IS 1 Hz, RESULTING IN AN UNCERTAINTY OF THE ADSORBED MASS OF ± 3 NG/CM².

Crystal surface	Adsorbed mass of IgG (ng/cm ²)	Number of IgG molecules (1/cm ²)	Coverage IgG (monolayer)
SiO ₂	860	3.5E12	2.0
SiO ₂ /PEG-silane	38	1.5E11	0.1
Au	814	3.3E12	1.9
Au/PEG-silane	422	1.7E12	1.0

The attachment of PEG-silanes on polycrystalline Au surfaces was investigated by AFM and CA analysis. The CA decreased from $83\pm3^\circ$ to $64\pm3^\circ$ for Au and Au/PEG-silane surfaces, respectively. The increase in hydrophilicity suggests the attachment of the hydrophilic PEG-silane molecules on the substrate surface. Analysis of the morphology of the Au surface after PEG-silane attachment confirms, that PEG-silanes are attached to Au and reveals that those molecules agglomerate on the surface. As a result, the rms roughness increased stronger compared to PEG-silane on SiO₂ from 1.0 ± 0.2 nm (scan size $1\times1\ \mu\text{m}^2$) to 1.4 ± 0.2 nm, respectively. The non-specific adsorption of IgG molecules on silanized Au surfaces (Au/PEG-silane) was further investigated by QCM analysis (Tab. I) and shows a decrease in non-specific attachment of IgG on the Au/PEG-silane crystal surface compared to a clean Au surface. This implies that the PEG-silane layer is not effectively removed from the Au surface and hence prohibits the adsorption of IgG to the same extent as for a clean Au surface. Based on those considerations we conclude that the very flexible PEG-silane polymers might also cover deposited nanostructures or attach non-specifically to them, rendering them inaccessible for further functionalization. This is especially important for nanostructures in the size range of only a few nm as the length of a PEG-silane molecule is of a comparable size and the final thickness of the PEG-silane layer might even be larger depending on the grafting time [2].

B. Functionalization of Au nanostructures on a PEG-silane layer

Based on the above mentioned considerations, we have firstly grafted the protein-repellent PEG-silane onto the SiO₂ surface, followed by the deposition of the Au nanostructures. We have deposited Au nanoclusters, preformed in a laser vaporization cluster source [11], *in situ* on PEG-silane covered samples. An abundance spectrum of the Au nanoclusters recorded before the deposition by a time-of-flight (TOF) mass spectrometer is shown in Fig. 2a. The size distribution of the detected clusters obtained from the TOF mass spectrum assumes a spherical shape for the clusters. Please note that neutral clusters are not detected

in the mass spectrum but are deposited on the sample. The AFM topography image of nanoclusters deposited on

Fig. 2. a) Mass spectrum of non-mass selected, charged Au nanoclusters produced by the cluster deposition apparatus [11]. b) AFM topography image of non-mass selected Au nanoclusters, deposited on a $\text{SiO}_2/\text{PEG-silane}$ sample, corresponding to the abundance spectrum shown in a). A vertical scale bar (1 nm) is included for the cross section in b).

a $\text{SiO}_2/\text{PEG-silane}$ sample is shown in Fig. 2b. Next we deposited Au nanoclusters with a density of approximately $1680 \text{ clusters}/\mu\text{m}^2$ on a $\text{SiO}_2/\text{PEG-silane}$ QCM crystal directly after the deposition on a SiO_2 reference sample. We used the reference sample to exactly determine the coverage of nanoclusters on the sample surface. We used biotinylated thiols as linker molecule for the bioaffinity immobilization of streptavidin (see insets in Fig. 3). The streptavidin was successfully immobilized on this $\text{SiO}_2/\text{PEG-silane}/\text{Au}/\text{biotin-complex}$ as can be seen in Fig. 3. After the introduction of streptavidin-buffer solution, an irreversible shift in frequency occurs, which indicates the attachment of streptavidin onto the crystal. The amount of adsorbed protein calculated based on the frequency shift was $129 \text{ ng}/\text{cm}^2$, corresponding to a protein-coverage of the sensor surface of 0.46 ± 0.02 monolayer. We used molecular beam epitaxy (MBE) to deposit Au nanostructures with different surface coverage onto $\text{SiO}_2/\text{PEG-silane}$ surfaces. The topography of $\text{SiO}_2/\text{PEG-silane}$ samples with 1.0, 0.5, 0.4 and 0.2 monolayer Au coverage is shown in Figs. 4a-d, respectively. It can be inferred from these images that the density of the coverage increases from low to high densities. However, at very high coverage (≥ 0.5 monolayer), the nanostructure-density is too high in view of the framework of laterally distance-controlled biomolecule attachment. Using lower Au coverage, namely 0.2 and 0.4 monolayer (Figs. 4c, d), respectively, the density of the nanostructures is sufficiently low. We used a biotinylated thiol as linker molecule to immobilize streptavidin to the MBE-Au

Fig. 3. QCM analysis of streptavidin adsorption. QCM crystal covered with a $\text{SiO}_2/\text{PEG-silane}/\text{Au}/\text{biotin-complex}$. The shift of the resonance frequency of the QCM crystal indicates the adsorption of proteins to the complex.

nanostructures. The linker molecule was attached via thiol-binding to the Au. The immobilization of the linker-biotin-streptavidin-complex increased the rms roughness from 0.5 to 1.2 nm (based on $1 \times 1 \mu\text{m}^2$ topography images) and the size of the topographical features on the sample, as can be observed in Fig. 4e and f, for 0.2 and 0.4 monolayer, respectively. The height of these features observed in the AFM images cannot be correlated with the expected size of a streptavidin molecule (approximated size $5 \times 4.5 \times 4.5 \text{ nm}^3$, [12]). This might be attributed to the fact that the streptavidin molecule appears much smaller than its actual size in topographical images due to compressive force applied by the AFM tip [12]. The AFM imaging conditions during our experiment, namely intermittent contact mode in ambient air, leads to a dehydration of the molecule and might lead subsequently to a conformational change. However, supported by the results of the QCM experiments we can conclude that streptavidin is immobilized on the Au nanostructures. The large increase in lateral feature size can be rather attributed to a bending of the flexible linker backbone (as it is not supported by other molecules as for example in a self assembled monolayer of thiols) with a large, rather heavy streptavidin molecule attached to it. However, the results indicate a local attachment of streptavidin onto the nanostructures deposited on the surface.

Fig. 4. AFM topography images of Au nanostructures, deposited on SiO_2/PEG -silane by MBE in different densities, namely a) 1.0, b) 0.5, c) 0.4 and d) 0.2 monolayer. Images e) and f) show the respective sample c) and d) after the attachment of the biotin-linker molecule and its functionalization with streptavidin. A vertical scale bar is included next to the respective cross section.

IV. SUMMARY AND CONCLUSIONS

We have shown an alternative method to create nanometer sized structures for a chemical contrast with controlled density by sub-monolayer deposition of Au onto SiO_2/PEG -silane surfaces. The PEG-silanes grafted to SiO_2 substrates, form an effective protein-repellent layer on which we deposited Au nanostructures by vacuum techniques, namely by MBE and by preformed clusters from a laser vaporization cluster source. QCM analysis of streptavidin immobilization on SiO_2/PEG -silane/Au-biotin-complexes shows the successful attachment of the streptavidin to the Au

nanostructures. AFM topography images reveal an increase in lateral dimensions after the streptavidin immobilization. In conclusion, we have created metallic nanostructures on a protein-repellent background which allows for the site-specific functionalization of the nanostructures.

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