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Preparation of flat gold terraces for protein chip developments

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Abstract: A simple method to prepare flat gold terraces on mica for atomic force microscopy biomolecular characterisation is described. The procedure includes preheating of the substrate, metal deposition and an annealing step. All of these steps are at elevated temperatures (300–420°C). This approach allows one to prepare large flat gold terraces (200–500 nm), which constitute ideal substrates for visualisation and characterisation of a self-assembly monolayer of biomolecules at the nanoscale. The authors illustrated this potential of characterisation with the reconstitution of a protein monolayer.

1 Introduction

Repartition and orientation of immobilised proteins on biodevices are very important to ensure their high performance. There have been a few methods to evaluate surface coverage, molecule repartition and even tridimensional structure and orientation of immobilised proteins. One of them is time-of-flight secondary ion mass spectrometry (TOF-SIMS), which is able to analyse the upper surface of one layer of molecules [1, 2]. Atomic force microscopy (AFM) is a technique which enables us to assess changes in surface topography, biofilm homogeneity, protein surface coverage and protein 3D structure without any need of biological sample labelling [3].

Gold surfaces provide convenient supports for a number of reasons. Gold is chemically inert to biomacromolecules; on the other hand, it is accessible to chemisorption via the formation of stable gold-thiol bonds. Based on this, a protein with a unique cystein, that is, a unique sulphhydryl residue at the periphery of the three-dimensional structure, will naturally self-assemble on gold substrate. Bearing this sulphhydryl moiety, cytochrome b5 is able to perform direct self-assembly onto a gold surface by the chemisorption process, leading to a packed and oriented self-assembled monolayer (SAM) [2].

The classical way of preparing thin gold films is vacuum evaporation of gold onto glass, silicon or mica, at low pressure. This deposition method induces metal films presenting globular gold grains measuring 25–30 nm in diameter [4]. Such rough surfaces present limitations in resolution when gold-immobilised biomolecules have to be imaged and characterised. Atomically flat gold substrates seem ideal to better characterise surface-bound biomolecules using scanning microscopy methods. Mica substrate is often preferred as it provides a clean and highly flat surface. When gold is deposited on such an atomically flat surface, the metal film can be stripped from mica and used on this 'mica-like face' [5]. The other possibility is to thermally anneal the gold-covered mica since this method allows improvement of the flatness of the gold film. Nevertheless, the first 'stripping' method often needs the deposition of another layer on the gold, in order to propose a new 'support' to the gold-stripped film. This method is, moreover, delicate to realise owing to the stripping step of the metal film in the case of a mechanical pealing. Stripping can also be obtained through chemical treatment using tetrahydrofuran (THF). This last possibility presents limitations, especially in terms of surface contamination by mica flasks [6].

We propose here a new method to prepare gold films presenting large flat terraces on mica. Our method is rapid

and highly controlled in terms of pressure, temperature and limitation of surface contamination, because the whole process occurs under non-ruptured vacuum (substrate pre-heating, metal deposition and annealing). We present the relevance of such a flat gold surface in the characterisation of a protein monolayer specifically self-assembled on it.

2 Materials and methods

2.1 Materials

Pure 99.99% gold metal and mica sheets (G250-1, from Agar Scientific) were used. Engineered cytochrome b5 was prepared as described in the reconstitution procedure. Octylglucopyranoside (OG) and dithiothreitol (DTT) were purchased from Sigma-Aldrich.

2.2 Metal deposition and annealing run

Gold thin films were deposited on mica substrates to prepare flat terraces by electron beam evaporation in an Alliance Concept EVA450 vacuum system at a pressure of 4×10^{-6} mbar. Indeed, we wanted to obtain a very thin grain size in order to mimic the flat state of mica's surface while realising flat gold terraces. Before gold deposition, substrates were first heated at a stabilised temperature of 300°C for half an hour and then etched with an argon ion beam powered by a hollow cathode of 3 cm diameter at a pressure of 3.6×10^{-4} mbar. Positive argon ions were neutralised by flow rates of negative species before impact of the substrates. The argon flow rate was 5 sccm in the cathode and neutraliser, and the pressure in the chamber was obtained by a high-speed turbo molecular pump, Leybold, of 1200 l/min coupled with a primary rotative pump. Finally, evaporation was obtained by heating the pure 99.99% gold metal in a liner of graphite. For the first time, deposition was made on the shadow between the liner and substrates until the right and regular speed rate control was reached by an Inficon XTC2 quartz crystal.

Gold was deposited in either one or two steps. For sample I, deposition of the gold film was made in two steps: first, 150 nm was deposited at a rate of 5 nm/s, which will be reduced to 0.05 nm/s to achieve a thickness of 200 nm. For samples II, III and IV, gold films were obtained in one step at a low gold deposition rate.

For all samples, heating, etching and gold layer depositions were all realised at 300°C. Samples I and II stayed in the same chamber for a run of 30 min and 8 h of annealing at 300°C. Samples III, IV and V were annealed in another vacuum system at 2.5×10^{-6} mbar for 3, 20 and 32 h at 420°C, respectively. Sample VI was obtained after a classical gold deposition of 150 nm (without heating) followed by an annealing step of 20 h at 420°C.

2.3 Reconstitution of cytochrome b5 monolayer

Engineered cytochrome b5 was derived from human microsomal cytochrome b5 by genetic engineering resulting in the substitution of (i) the 26 C-terminal amino-acid residues by the –NGHHHH–COOH sequence and (ii) the serine 23 in $Hb5(His)_4$ by cystein as previously described [7]. Modified human cytochrome b5 bears a unique cystein, that is, a unique sulphydryl residue, and is processed in a saline phosphate buffer (PBS, 100 mM at pH 7.4 with NaCl 50 mM). Protein is reduced by a reducing agent in excess (DTT) (20/1 by moles) during 10 min. Reduced cytochrome b5 (20 μl at 1 μM) is able to react directly by chemisorption onto gold substrate. During 15 min incubation, the reconstitution of a packed and oriented protein monolayer by direct self-assembly is obtained. A washing step by buffer first (20 μl 3 times) and then by a detergent (OG, 20 μl at 40 mM) was realised after protein immobilisation in order to remove not covalently bound proteins.

2.4 AFM characterisation

The AFM used was a Nanoscope III (Veeco, Santa Barbara, CA, USA). Imaging was performed in contact mode using NPS-oxide sharpened silicon nitride probes (Veeco). For the feedback controls, typical values of set-point for imaging were between 0.5 and 1.5 V, depending on scan size.

Table 1 Various conditions of gold deposition and annealing runs

Sample number	Heating parameters	Etching at 300°C (min)	Gold thickness and deposition rate at 300°C	Annealing parameters
I	30 min at 300°C	1 min at 3.6×10^{-4} mbar	150 nm at 5 nm/s^{-1} 50 nm at 0.05 nm/s^{-1}	30 min at 300°C
II			50 nm at 0.1 nm/s^{-1}	8 h at 300°C
III			150 nm at 0.1 nm/s^{-1}	3 h at 420°C
IV				20 h at 420°C
V				32 h at 420°C
VI	–	–		20 h at 420°C

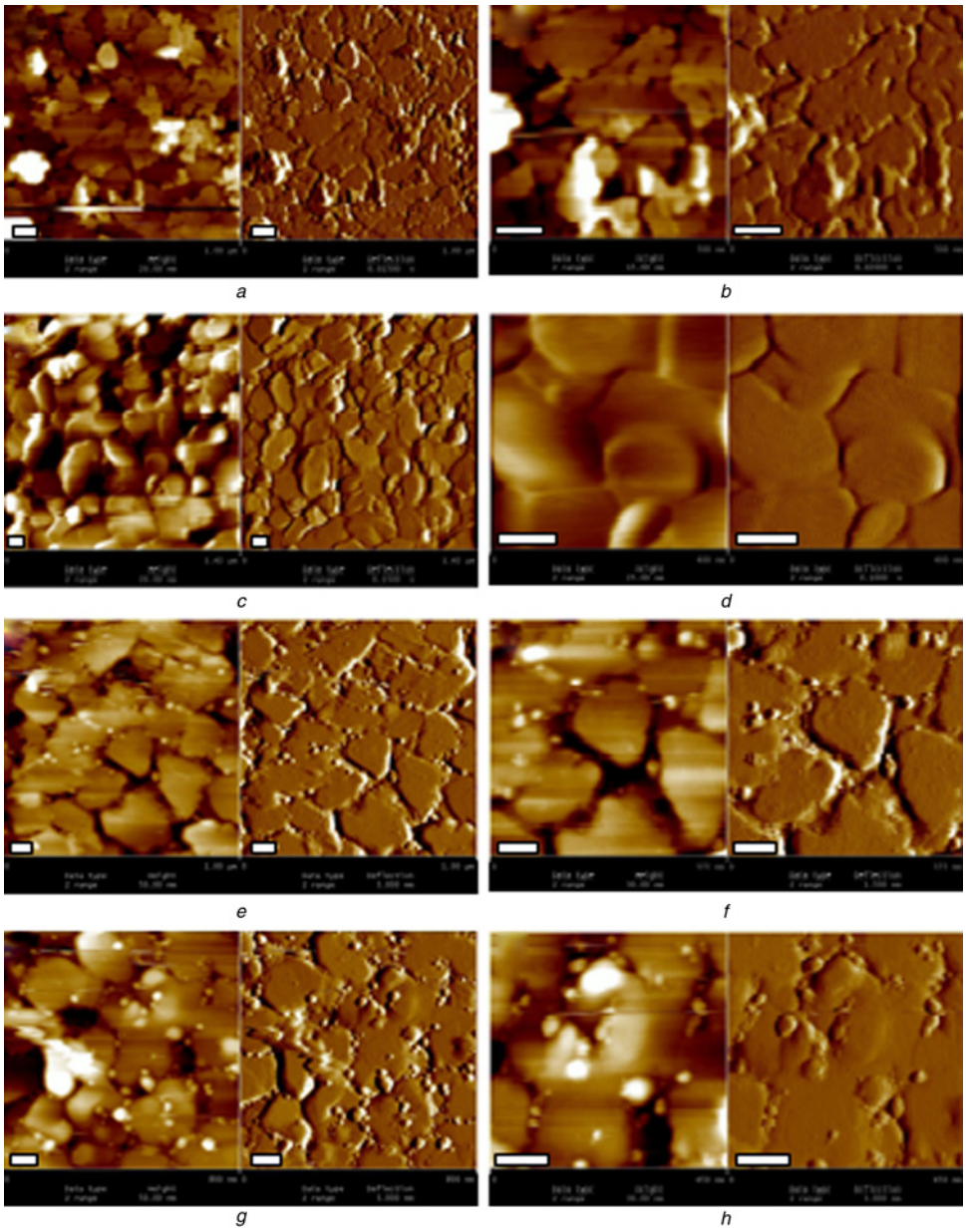

Figure 1 *Gold terraces on mica obtained with different conditions of metal deposition rate, thickness and temperature.*

For each sample, a large scan (around 1 μm) and one zoom (around 500 nm) are presented
a,b Sample I obtained with 200 nm gold deposited in two steps: 150 nm at 5 nm/s and then 50 nm at 0.05 nm/s, followed by 30 min 300°C annealing
c,d Sample II obtained with 50 nm gold at 0.1 nm/s followed by 8 h 300°C annealing
e,f Sample III obtained with 150 nm gold deposited at 0.1 nm/s followed by 3 h annealing at 420°C
g,h Sample IV obtained with 150 nm gold deposited at 0.1 nm/s followed by 20 h at 420°C
Scale bar: 100 nm

2.5 Image J software

We used Image J software to determine the area of gold terraces obtained on different substrates.

3 Results and discussion

We chose the electron beam evaporation method for deposition of gold, since it offers the possibility to work under elevated temperatures until 300°C before, during and after the gold deposition. This temperature of 300°C corresponds to a transition structure of densely packed fibrous grains in the Thornton diagram [8], enabling one to keep the state of mica's surface and to obtain flat gold terraces. From this diagram it is also possible to understand that evaporation with a lower pressure and little energies of particles – that is, less than 1 eV – corresponds to a better solution than sputtering at a pressure of 5×10^{-3} mbar of argon and particle energies from 5 to 10 eV. According to the Thornton diagram, it

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is important to choose evaporation conditions with a lower pressure and a temperature T/Tm in the range of 0.2–0.3. T corresponds to the temperature of the condensation vapour, that is, the temperature of the substrate, and Tm is the gold liquid temperature, that is, 1063°C.

According to the same Thornton diagram, this also means that with sputtering conditions, the gold layer grew in a columnar structure. Evaporation with a low deposition rate is the best way to form gold flat terraces. In the same field, the evaporation of gold with high speed was not chosen because the roughness of the coating increased with thickness and the flat terraces disappeared. We found that the only way to better mimic the flatness of the mica substrate and obtain flat gold terraces was an evaporation beam with a temperature of 300°C and a pressure less than 5×10^{-5} mbar.

It appears that long bake-outs of mica help to outgas contamination, which prevents direct stacking of Au atoms onto the mica surface [9]. We decided to use etching with a neutral argon ion gun to clean the surface without high particle energies and to avoid disturbance of the mica substrate.

3.1 Morphology and size of terraces

We have evaporated gold on a pre-heated mica substrate and have tested different conditions of metal deposition and temperature. Different gold deposition rates, thickness and temperature conditions were experimented with (Table 1). Indeed, the rate of metal deposition influences the roughness of the gold film. A classical evaporation without heating induces a rough gold film consisting of grains with a diameter around 30 nm[4]. While decreasing the gold deposition rate, the surface topography gets smoother [5]. Thermal annealing of the evaporated films dramatically improves the flatness of the gold substrates, producing atomically flat terraces [10, 11].

For sample I, the gold thickness is 200 nm and only a short annealing time at 300°C (30 min) is applied. Small terraces with irregular shapes were obtained (Figs. 1*a* and *b*).

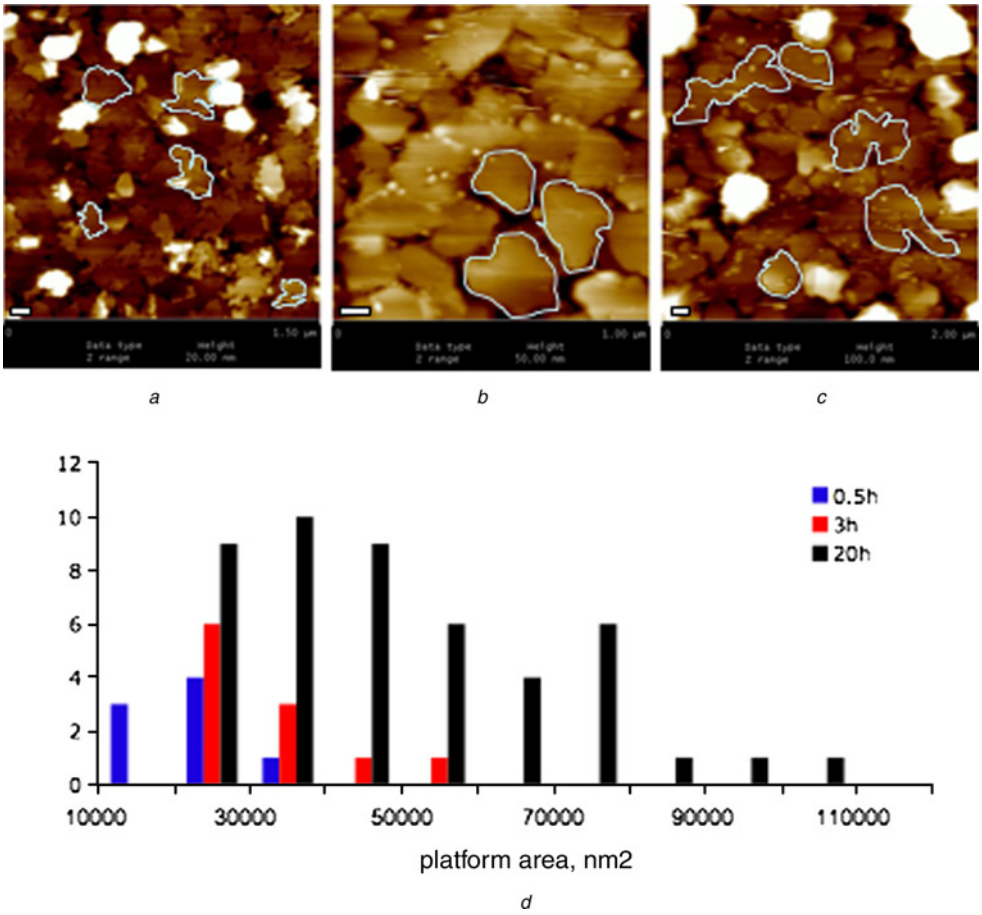

Figure 2 *Gold terrace areas*

a Sample I
b Sample III
c Sample IV
d Area of terraces function of annealing time
Scale bar: 100 nm

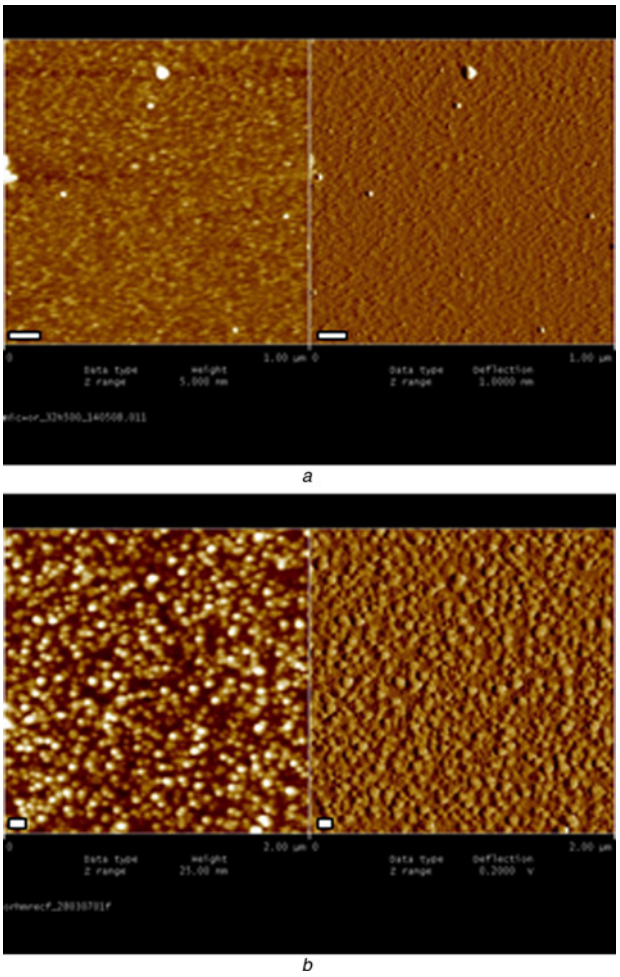

Figure 3 *Effect of heating during gold deposition and annealing time*

a Gold surface after an annealing time of 32 h at 420°C (pre-heating of substrate at 300°C, 150 nm gold thickness at 300°C)
b Gold deposition without heating the substrate (150 nm gold thickness followed by annealing at 420°C for 20 h).
Scale bar: 100 nm

Sample II was obtained after 50 nm gold deposition followed by a long annealing time at 300°C (8 h). In this case, terraces present round-shaped morphology (Figs. 1*c* and *d*). In the case of samples III and IV, 150 nm of gold was deposited but the annealing time was 3 or 20 h at 420°C. In this last case, we obtained better results. Indeed, after 3 h annealing, terraces are larger than sample I and present a homogeneous shape (Figs. 1*e* and *f*). After 20 h annealing, the surface still presents these individual terraces, but several terraces seemed to have fused together to give very large ones (Figs. 1*g* and *h*).

Concerning the size, we managed to prepare terraces from 22 500 nm^2 for sample I (Figs. 2*a* and *d*) to 55 000 nm^2 for sample III (Figs. 2*b* and *d*) and more than 100 000 nm^2 (Figs. 2*c* and *d*) for sample IV. Thus, the tendency for the terraces to grow in size with annealing time appeared clearly (Fig. 2*d*).

Nevertheless, there is a maximum heating duration beyond which the gold surface is completely disorganised like 'melt' on/with mica (sample V and Fig. 3*a*). AFM images do not present gold terraces anymore and the surface topography is a sort of intermediary between classical rough gold film and highly flat mica. Nogues and Wanunu [12] also reported this phenomenon, saying that overexposure of gold films to high temperatures had to be avoided. Indeed, when mica was heated to temperatures above 500°C, Derose *et al.* [9] remarked that the surface deteriorated, becoming quite rough. It appears that the mica disintegrated at these temperatures, causing poor epitaxy.

We also made two observations: First, there is a minimum gold thickness allowing the preparation of relatively large terraces. Indeed, when only a deposition of 50 nm of gold is realised at 300°C (sample II), followed by an annealing time of 8 h at 300°C, small round and 'donut' shaped terraces were obtained (Figs. 1*c* and *d*). Second, if gold deposition is realised without heating the substrate, but followed by an annealing time at 420°C during several hours (sample VI), this does not succeed in gold terrace realisation. The gold substrate presents in this case a rough surface presenting gold grains of around 30 nm diameter (Fig. 3*b*), which corresponds to the roughness of 'classical' gold films.

Our work then presents an easy way of preparing flat gold surfaces under highly controlled conditions of pressure and temperature. Moreover, this approach, using equipment in a 'clean room', presents the advantage of protecting the gold interface against contamination by polluting species in air. This last point is of great importance when the substrate has to be efficiently and homogeneously functionalised by proteins for biosensor development.

3.2 Terrace flatness

Higher resolution images revealed nicely individual gold terraces. These terraces are smooth on 100 (for the smallest) to 200 nm length or more and present a maximum height variation of only around 5 Å (Fig. 4). The morphology exhibited by such gold surfaces is therefore ideal for clear visualisation, distinction and characterisation of immobilised molecules and proteins.

3.3 Visualisation and counting of immobilised protein

We developed flat surfaces in order to better characterise the protein monolayer immobilised on a gold surface. Indeed, classical evaporated gold films present a high roughness, with a surface composed of small grains in the same size range as proteins. The topography of such a rough surface prevents the clear visualisation and characterisation of a protein layer grafted on it. In our aim to characterise the reconstituted biomolecular film at the interface of the sensor surface, the development of a flat surface could allow one to count individual molecules and determine their surface

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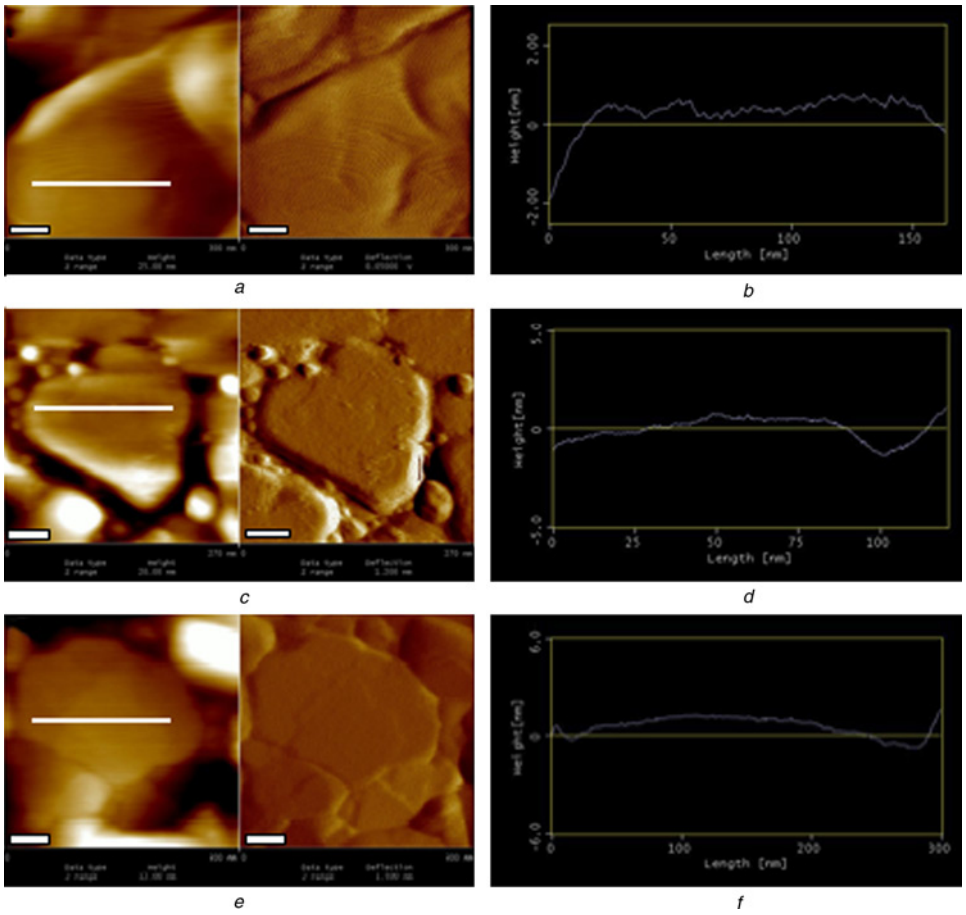

Figure 4 *Flatness of individual gold terraces*

a Gold terraces prepared with 50 nm gold evaporated at 0.1 nm/s followed by 8 h at 300°C annealing (sample II)
b Cross-section along the white line in A
c Gold terraces prepared with 150 nm gold evaporated at 0.1 nm/s followed by 3 h annealing (sample III)
d Cross-section along the white line in B
e Gold terraces prepared with 150 nm gold at 0.1 nm/s followed by 20 h at 420°C (sample IV)
f Cross-section along the white line in (e)
Scale bar: 50 nm

coverage, and eventually their tridimensional structure and orientation. As a model, we worked with a protein containing a single cystein residue allowing its binding to gold. In Fig. 5 we show clearly the difference between before (Fig. 5*a*) and after (Fig. 5*b*) incubation of proteins on the gold terraces. Binding of proteins induced surface changes and the appearance of a number of motifs on the gold surface. As a comparison, a classical evaporated gold film, without annealing, is represented before (Fig. 5*c*) and after (Fig. 5*d*) protein adsorption on the surface. Proteins are indistinguishable, showing the difficulty of characterising the protein layer on this kind of rough surface. In contrast, on surfaces presenting terraces, proteins are visible and distinguishable, and it is possible to control the density and homogeneity to count proteins and determine their surface coverage. We counted around 1200 proteins per μm^2, meaning 1.2×10^9 molecules/mm^2 and thus 2 fmol/mm^2. Aoyagi *et al.* [2] mentioned that it is possible to perform SAMs with various densities in a window of 1–200 fmol/mm^2. Our AFM results then fit in this range. Images also revealed that the protein layer is homogeneous on a gold surface, with proteins regularly distributed on the substrate. This AFM investigation revealed that neither particular concentration of molecules on the sides of terraces nor aggregation of proteins appeared on the surface.

4 Conclusion

A new rapid, reproducible and highly controlled procedure for the preparation of clean flat gold terraces has been presented. We demonstrated the usefulness of such flat surfaces for reconstitution of a protein monolayer and its easy characterisation by AFM in terms of surface coverage, density and repartition of immobilised proteins. In order to use such flat gold surfaces in a biosensor, we are currently working on the gold terraces formation on glass. With collaborators working on the theoretical modelling of surface behaviour, we are studying the ability of these flat surfaces to generate plasmons. This last development would pave the way for this innovative atomically gold substrate

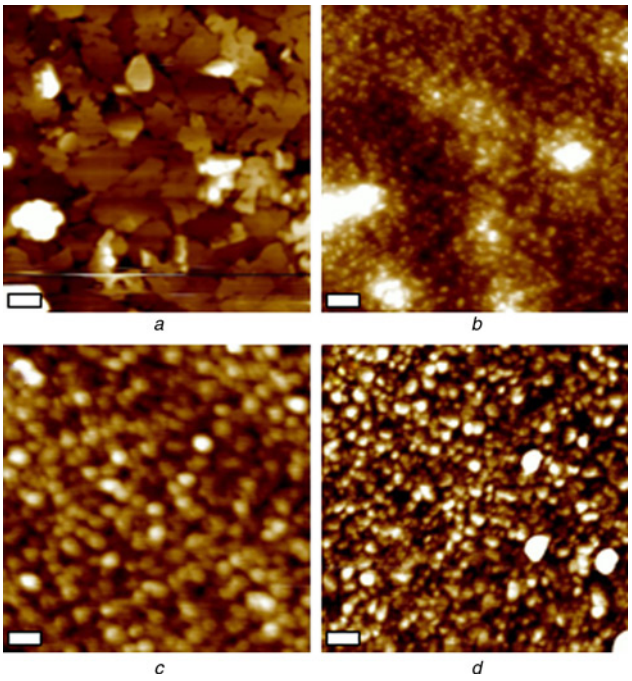

Figure 5 *Visualisation of immobilised protein on gold surfaces*

a Gold terraces before cytochrome b5 immobilisation
b Gold terraces after cytochrome b5 immobilisation
c Comparison with a classical rough gold surface before protein immobilisation
d Comparison with a classical rough gold surface after protein immobilisation
Contact mode images in air, *z* scale 20 nm (*a, b*) and 3 nm (*c, d*)
Scale bar: 100 nm

investigation, giving information on kinetics and mass loading. Thus, these flat gold surfaces could represent a really interesting flat and 'active' substrate for biochip development.

5 Acknowledgment

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