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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 AFM biomolecular characterization is described. The procedure includes preheating of the substrate, a metal deposition followed by an annealing step, all of these steps lead at elevated temperature (300 to 420°C). This approach allowed to prepare large flat gold terraces (200 to 500 nm), that constituted ideal substrates for visualization and characterization of self-assembly monolayer of biomolecules at the nanoscale. We illustrated this potential of characterization with the reconstitution of a protein monolayer.

1. Introduction

Repartition and orientation of immobilized proteins on bio-devices are very important to assure their high performance. There have been few methods to evaluate surface coverage, molecules repartition and even tridimensional structure and orientation of immobilized proteins. One of them is time-of-flight secondary ion mass spectrometry (TOF-SIMS) that is able to analyze upper surface of one layer of molecules^{1,2}. Atomic force microscopy is one of the methods of choice to achieve the protein surface coverage, the biofilm homogeneity with a molecular resolution until protein structure, without any “denaturing” sample preparation or labeling³.

Gold surfaces provide convenient supports for a number of reasons. Gold is rather chemically inert to biomacromolecules; but, on the other hand, it is accessible to chemisorption via the formation of stable gold-thiol bonds. Based on that, a protein having a unique cysteine, i.e. a unique sulfhydryl residue at the periphery of the 3D structure, will naturally self-assemble on gold substrate. Bearing this sulfhydryl moiety, cytochrome b5 is able to perform direct self-assembly onto gold surface by chemisorption process leading to a packed and oriented self assembled monolayer (SAM)².

The classical way to prepare 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 to 30 nm in diameter⁴. Such rough surfaces present limitations in resolution when gold immobilized biomolecules have to be imaged and characterized. Atomically flat gold substrates seem ideal to better characterize 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 **improving** the flatness of the gold film. Nevertheless the first “stripping” method often needs the deposition of another layer on gold, in order to propose a new “support” to the gold stripped film. This method is moreover delicate to realize due to the stripping step of metal film in the case of a mechanical peeling. Stripping can be also obtained through a chemical treatment using THF. This last possibility presents also limitations especially in terms of surface contamination by mica flasks⁶.

We propose here a new method to prepare gold films presenting large flat terraces on mica. Our method is rapid, highly controlled in terms of pressure and temperature and limitation of surface contamination, since the whole process happens under a non-ruptured vacuum (substrate preheating, metal deposition and annealing). We present the relevancy of such a flat gold surface in the characterization of a protein monolayer specifically **self-assembled on it.**

2. Material 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 very thin grain size, in order to mimic the flat state of the mica's surface while realizing flat gold terraces. Before gold deposition run, the substrates were first heated at a stabilized temperature of 300°C for half an hour and then etched with argon ion beam powered by a hollow cathode of 3cm diameter at a pressure of $3,6 \times 10^{-4}$ mbar. Positive argon ions were neutralized by flow rates of negative species before impact of the substrates. An Argon flow rate was 5 sccm in cathode and neutralizer, and the pressure in the chamber was obtained by a high-speed turbo molecular pump Leybold of $1200 \text{ l}\cdot\text{min}^{-1}$ coupled with a primary rotative pump. Finally, evaporation was obtained by heating the pure 99,99% gold metal in a liner of graphite. In a first time, deposition was made on the shadow between liner and substrates until the right and regular speed rate control by an Inficon XTC2 quartz crystal was reached.

Gold was deposited either in one or two steps. For sample I, deposition of gold film has been made in two steps: first, 150 nm was deposited at a rate of $5 \text{ nm}\cdot\text{sec}^{-1}$ which will be reduced to $0.05 \text{ nm}\cdot\text{sec}^{-1}$ to achieve the thickness of 200 nm. For samples II, III and IV, the gold films were obtained in one step at low gold deposition rate.

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

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 $3.6 \cdot 10^{-4}$ mbar	150 nm at 5 nmsec^{-1}	30 min at 300°C
II			50 nm at 0.05 nmsec^{-1}	8h at 300°C
III			150 nm at 0.1 nmsec^{-1}	3h at 420°C
IV				20h at 420°C
V				32h at 420°C
VI				20h 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 described⁷. Modified human cytochrome b5 bears a unique cystein, i.e. a unique sulfhydryl residue, and is processed in saline phosphate buffer (PBS, 100 mM at pH 7.4 with NaCl 50 mM). Protein is reduced by a reducing agent in excess (dithiothreitol, 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 secondly by a detergent

(Octylglucopyranoside (OG), 20 μ l at 40 mM) was realized after protein immobilization in order to remove not covalently bond proteins.

2.4. AFM characterization

The AFM used was a Nanoscope III (Veeco, Santa Barbara, CA). 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 to 1.5 V, depending on scan size.

2.5. Image J software

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

3. Results & discussion

We chose deposition of gold using electron beam evaporation method, since it offers possibility to work under elevated temperature 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 Thornton diagram⁸ enabling to keep the state of mica's surface and to obtain the flat gold terraces. On this diagram, it is also possible to understand that evaporation with lower pressure and little energies of particles – i.e. less than 1eV- corresponds to a better solution than sputtering with a pressure of 5×10^{-3} mbar of argon and particles energies from 5 to 10 eV. According to Thornton diagram, it is important to choose evaporation conditions with lower pressure and a temperature T/T_m in the range of 0,2 to 0,3. T corresponds to the temperature of the condensation vapor, i.e. the temperature of the substrate, and T_m is the gold liquid temperature, i.e. 1063°C.

According to the same Thornton diagram, it also means that with sputtering conditions the gold layer grew in columnar structure. The evaporation way with low deposition rate is the best one 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 the thickness and the flat terraces disappeared. The only way we found to better mimic the flatness of the mica substrate and obtain flat gold terraces, was evaporation beam with a temperature of 300°C and a pressure less than 5×10^{-5} mbar.

It appears that long bake-outs of the mica helped to outgas contamination which prevents direct stacking of Au atoms onto the mica surface⁹. We decided to use etching with neutral argon ion gun, in order to clean the surface without high particles energies and to avoid disturbance of the mica substrate.

3.1. Morphology and size of terraces

We have evaporated gold on pre-heated mica substrate and have tested different conditions of metal deposition and temperature. Different gold deposition rate, thickness and temperature conditions were

experimented (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 nm⁴. While decreasing the gold deposition rate the surface topography gets smoother⁵. 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 shape were obtained (Fig1A,B). The sample II was obtained after 50 nm gold deposition followed by a long annealing time at 300°C (8h). In this case, terraces present round shaped morphology (Fig1C,D). In the case of sample III and IV, 150 nm of gold were deposited but annealing time was 3h or 20h at 420°C. In this last case, we obtained better results. Indeed, after 3h annealing, terraces are larger than sample I and present an homogeneous shape, (Fig1E,F). After 20h annealing, the surface still presents these individual terraces, but several terraces seemed have fused together to give very large ones (Fig1G,H).

Concerning the size, we managed to prepare terraces from 22 500 nm² for sample I (Fig2A,D), to 55 000 nm² for sample III (Fig2B,D) and until more than 100 000 nm² (Fig2C,D) for sample IV. Thus, the tendency for the terraces to grow in size with annealing time appeared clearly (Fig2D).

Nevertheless, there is a maximum heating duration beyond which the gold surface is completely disorganized like “melt” on/with mica (sample V and Fig3A). 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 (2004)¹² also reported this phenomena, saying that overexposure (long time) of gold films to high temperatures had to be avoided. Indeed, when mica was heated to temperatures above 500 °C, Derose et al⁹ 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 preparation of relatively large terraces. Indeed, when only a deposition of 50 nm of gold is realized at 300°C (sample

II), even followed by an annealing time of 8h at 300°C, small round and “donuts” shape terraces were obtained (Fig1C,D). Second, if the gold deposition is realized without heating the substrate, but followed by an annealing time at 420°C during several hours (sample VI), we did not succeed in gold terrace realization. The gold substrate presents in this case a rough surface presenting gold grains of around 30 nm in diameter (fig3B), that corresponds to the roughness of “classical” gold films.

Our work present then an easy way to prepare flat gold surfaces under highly controlled conditions of pressure and temperature. Moreover, this approach, using equipments in a “clean room”, presents the advantage to protect the gold interface against contamination by polluting species in air. This last point is of deep importance when the substrate has to be efficiently and homogeneously functionalized by proteins for a biosensor development.

3.2. Terraces flatness

Higher resolution images revealed nicely individual gold terraces. These terraces are smooth on 100 (for the smallest) to 200 nm **in length** or more and present a maximum height variation of only around 5Å (Fig4). The morphology exhibited by such gold surfaces is therefore ideal for clear visualization, distinction and characterization of immobilized molecules and proteins.

3.3. Visualization and counting of immobilized protein

We developed flat surfaces in order to better characterize the protein monolayer immobilized at a gold surface. Indeed, classical evaporated gold films present a high roughness, with a surface composed of small grains in the same size range than proteins. The topography of such a rough surface prevents a clear visualization and characterization of a protein layer grafted on it. In our aim to characterize the reconstituted biomolecular film at the interface **of sensor surface**, the development of flat surface could allow to count individual molecules and determine their surface coverage, 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 Fig5 we show clearly the difference between before (Fig5A) and after (Fig5B) incubation of proteins on the gold terraces. Binding of proteins induced surface changes and appearance of lot of motifs on the gold surface. As a comparison, a classical evaporated gold film, without annealing, is represented before (Fig5C) and after (Fig5D) protein adsorption on the surface. Proteins are undistinguishable showing the difficulty to characterize the protein layer on this kind of rough surface. In opposite, on surface presenting terraces, proteins are visible, distinguishable and it is possible to control the density, homogeneity, to count proteins and determine their surface coverage. We counted around 1200 proteins per μm^2 , meaning $1.2 \cdot 10^9$ molecules/ mm^2 thus 2 fmol/ mm^2 . Aoyagi S. et al (2008)² mentioned that it is possible to perform SAMs with various density in a window of 1 to 200 fmol/ mm^2 . Our AFM results then fit in this range. Images also revealed that the protein layer is homogeneous on gold surface, with proteins regularly distributed on the substrate. This AFM investigation revealed that neither particular concentration of molecules on 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 was presented. We demonstrated the usefulness of such flat surfaces for reconstitution of a protein monolayer and its easily characterization by AFM in terms of surface coverage, density and repartition of immobilized proteins. In order to use such flat gold surfaces in a biosensor, we are currently working on gold terraces formation on glass. With collaborators working on theoretical modeling of surface behavior, we are studying the ability of these flat surfaces to generate plasmons. This last development would open the way of these innovative atomically gold substrate investigation through their plasmon interrogation, giving informations on kinetics and mass loading. Thus, these flat gold surfaces could represent a really interesting flat and “active” substrate for biochip developments.

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Figure legends

Fig1: 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 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 8h 300°C annealing. Sample III (E,F) obtained with 150 nm gold deposited at 0,1nm/s followed by 3h annealing at 420°C. Sample IV (G,H) obtained with 150 nm gold deposited at 0,1nm/s followed by 20h at 420°C. **Scale bar: 100 nm.**

Fig2: Gold terrace areas of (A) sample I, (B) sample III and (C) sample IV. (D) Area of terraces function of annealing time. **Scale bar: 100 nm.**

Fig3: Effect of heating during gold deposition and annealing time. (A) Gold surface after an annealing time of 32h at 420°C (preheating 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 20h). **Scale bar: 100 nm.**

Fig4: Flatness of individual gold terraces. (A) Gold terraces prepared with 50 nm gold evaporated at 0,1nm/s, followed by 8h 300°C annealing (sample II). (B) Cross-section along the white line in A. (C) Gold terraces prepared with 150 nm gold evaporated at 0,1nm/s followed by 3h annealing (sample III). (D) Cross-section along the white line in B. (E) Gold terraces prepared with 150 nm gold at 0,1nm/s followed by 20h at 420°C (sample IV). (F) Cross-section along the white line in E. **Scale bar: 50 nm.**

Fig5: Gold terraces before (A) and after (B) cytochrome b5 immobilization. Comparison with a classical rough gold surface, before (C) and after (D) protein immobilization. Contact mode images in air, z scale 20 nm (A,B) and 3 nm (C,D). **Scale bar: 100 nm.**