

Surface plasmon resonance imaging sensors based on mucin – antimucin interaction

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Surface Plasmon Resonance imaging (SPRi) is a label-free sensitive method, designed for the study of ultrathin layers of biomolecules and biomolecular interactions. It can detect the presence of biomolecules on modified metallic surfaces by monitoring the change of the local reflectivity, without using molecular labels. This work reports our efforts in designing a biosensor based on the antibody-antigen bioaffinity, as possible tool for non-invasive early tumour diagnose. Mucin is a good candidate as cancer marker protein, since it could be correlated with different type of tumours. In this work, antimucin antibodies are successfully used for the development of SPRi sensors for identifying mucin overexpression. Antibody immobilisation was performed after self assembled thiol monolayers were created onto gold SPRi chips. Atomic Force Microscopy, X-ray Photoelectron Spectroscopy, SPR and SPRi were used as analysis methods.

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1. Introduction

SPR imaging (SPRi) is a label-free sensitive method, specially designed both for the study of ultrathin layers of biomolecules as well as for the evaluation of biomolecular interactions. It can detect the presence of biopolymers on modified metallic surface by monitoring the change of the local reflectivity, without using molecular labels. Nevertheless, the imaging technique has high potential since it allows the simultaneous analysis of the surface of a multiaarray sensor. Reference literature in this field has mainly arisen from two well-known groups of Knoll [1-2] and Corn [3-6]. In this work we report on the immobilization of antimucin (AM) antibody on the gold surface of a SPRi sensor, as sensing phase for the further detection of mucin overexpression in saliva samples. Mucin was identified among others, as a promising cancer marker protein [7-13]. Monitoring its concentration in saliva could represent an easy way of early cancer diagnose, based on a non-invasive and very sensitive label-free method, with a negative control consisting in the normal level of the protein in healthy people saliva. The potential advantages of this biosensor are very important and they were detailed elsewhere [14].

The immobilization of the antibody on the sensor surface was performed in a two steps procedure. First, a thiol monolayer was created with the aim to improve the orientation of the antibody molecules, to have the specific active sites available for mucin detection. Then, the antibody was bound on the thiol after the activation of the carboxylic groups of the latter. AM immobilization was

successful and its ability to specifically detect mucin was preliminary tested.

2. Experimental

2.1. Materials

The biosensor substrate consisted in commercially SPR gold slides (sSens, Netherlands), based on borosilicate glass ($n = 1.51$ and 0.3 mm thickness). These gold slides (10×10 mm $\times$ mm) correspond to commercial SPR instruments but also to our modular device. The good adherence of the gold on the glass was improved through an intermediate very thin titanium layer [15]. A second substrate was used in this study, consisting in gold directly evaporated on the observation prism (BK7). The gold surface was obtained as hexagonal patterned mesh, with the diagonal of hexagons $70 \mu\text{m}$. This was very helpful in the further image analysis of the obtained SPRi maps.

Gold surface was used immediately after rinsing with piranha solution (75% concentrated H_2SO_4 and 25% H_2O_2), for 1 minute, followed by distilled water cleaning and drying under nitrogen stream). The thiol 16-Mercaptohexadecanoic acid was used without further purification, as 1mM solution in ethanol, freshly prepared.

For the activation of the carboxylic groups of the thiol, N,N -(3-dimethylaminopropyl)- N' -ethyl-carbodiimide hydrochloride (EDC) and N -Hydroxysuccinimide

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(NHS) were used, as 20 mM and 5 mM in phosphate buffer, pH 7.4 [14].

As sensing phase we have selected monoclonal anti-human gastric mucin, clone 45M1, used as solution in phosphate buffer (pH 7.4).

Mucin was from porcine stomach (Type III partially purified powder).

The phosphate buffer was prepared as presented in a recent report [14], using monosodium phosphate monohydrate and disodium phosphate heptahydrate in distilled water.

2.2. Methods

Antibody immobilization on gold

Fig. 1 schematically presents the procedure of antibody immobilization. Self assembled monolayers (SAMs) were created on neat gold through the direct exposure of the metallic surface (immediately after rinsing with piranha) at 1 mM thiol fresh ethanolic solution. Thiol spontaneously adsorbing and self-organizing (step 1 in Fig. 1) on gold was performed in 24 hours, at room temperature, in Teflon dishes (to avoid retention of mercaptans on glass). The SAMs containing gold slides were cleaned with a low flow of ethanol and then they were dried under a nitrogen stream. SPRi and atomic force microscopy were used to visually map the modified surface versus neat gold. The surface modification was proved by X-ray Photoelectron Spectroscopy.

The antibody immobilization (schematically shown as step 2 in Figure 1) was performed as previously described [14]. AM molecules were bound stoichiometrically on the activated thiol SAMs through direct exposure of the slides to the protein solution (via protein spotting or via exposure in a flow cell). Several concentrations of antibody were used: 5, 10, 25, 50, 100, 250 ppm in phosphate buffer, pH 7.4. The activation of the carboxylic groups of the thiols was realized through the treatment with EDC/NHS (30 minutes, room temperature, in the dark). At the end of the immobilization time, the slides were rinsed with buffer to remove any antibody excess.

SPR imaging

The SPRi principle was already detailed presented in a recent work [14], with special emphasis on the label-free character of the technique as well as on the potential of investigating a high number of biological samples at once, in a simple and very sensitive procedure.

The device used in this work was a modular device build-up in the group. The optical scheme is shown in Fig. 2.

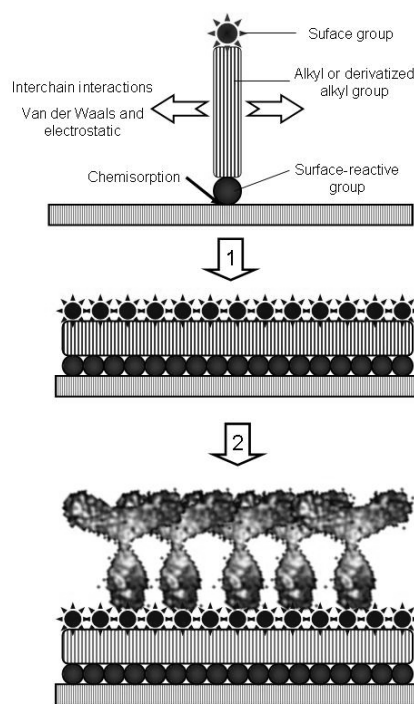


Fig. 1. Antibody immobilization on gold. 1 – thiol; 2 – thiol SAM formation; 3 – Antibody specifically oriented binding on the thiol SAM.

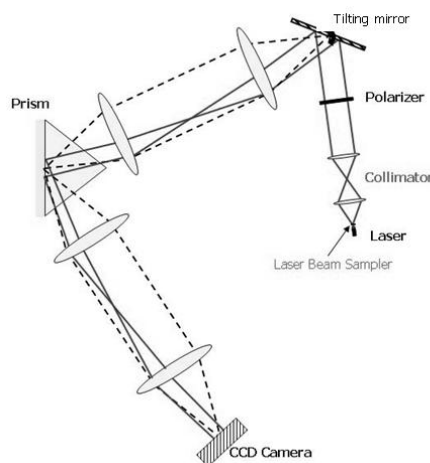


Fig. 2. Optical set-up for the SPRi modular device.

Monochromatic light (632.8 nm) is provided by a He-Ne laser LGK 7653-8 (LASOS GmbH, Ebersberg, Germany). The laser beam is p-polarized through a polarizer and then 10 times expanded through a collimator. 1 tilting mirror is responsible for adapting beam direction to different incidence angles on the surface of the prism, corresponding to SPR occurrence. The prism used is made in BK7 glass ($n = 1.51$), 90°. The sensing phase was immobilized on the gold chip that is fixed on the surface of the prism through a drop of oil with the same refractive index as the glass (1.51). The reflected light is magnified

by 2 lenses and brought to the CCD camera (Retiga 1300 Qimaging, Burnaby, British Columbia, Canada).

Further, the functioning principle of the SPRi method is schematically shown. Briefly, the material immobilized on the surface of the sensor, at the interface gold-observation medium, induces a mass gain corresponding in a change of the local reflectivity of the surface. The SPR angle is the incidence angle at which the condition of minimum reflectivity is respected for a certain material, so the SPR phenomenon occurs. Fig. 3 presents how this angle increases when several species are added on the sensor's surface. It appears as a grayscale map with brighter spots (corresponding to higher refractive indexes) easy to identify on a dark background (corresponding to the immobilization substrate). Image analysis might provide a relationship between AM concentration and the values of the SPR angle.

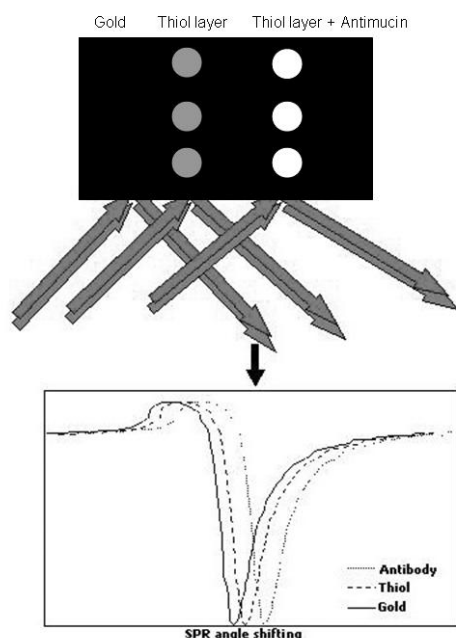


Fig. 3. Schematic view of how the SPRi works.

SPR spectroscopy

Classical angular interrogation experiments were performed on a BIOSUPPLAR spectrometer built by Analytical micro-Systems (Regensburg, Germany).

Atomic force microscopy (AFM)

AFM is a powerful imaging technique that was used to explore the change of the topography of the surface when AM was immobilised on the sensor. AFM was performed using an Autoprobe CP-Research microscope (Thermomicroscopes, Sunnyvale, California), in tapping mode with ULCT-AUTM-AB ultralevers (Veeco, Dourdan, France). The samples were imaged in air. 3D

topography images of the surfaces were rebuilt automatically from the computer, after storing the distance the scanner has vertically moved (Z) at each data point (X,Y).

X-ray Photoelectron Spectroscopy (XPS)

XPS was selected as a reliable method to characterise the self assembling of thiol molecules on gold. XPS was performed using AXIS ULTRA spectrometer (Kratos Analytical, England), with X-ray source Mono-Al $K\alpha_{1,2}$, power of the X-ray source 300 W at 20 mA, analyser pass energy 160 eV (survey spectra) and 20 eV (high-resolved spectra), with charge compensation switched on. Different take-off angles were used: 0° corresponding to an information depth ID ~ 8 nm [16, 17], 60° corresponding to ID ~ 4 nm and 75° corresponding to ID ~ 2 nm. All spectra were charge compensated using the Au 4f7/2 peak as reference (BE = 84.00 eV). Negative values of charge compensation indicate an overexpression guaranteeing unadulterated peak shapes. To determine elemental ratios, normalized peak areas were calculated from peak areas (Raw area [CPS]) of survey spectra according to the equation (1), respecting sensitivity factors (RSF) experimentally determined for Kratos Axis Ultra and spectrometer's transmission function (Tx. Function).

$$\text{Norm. Area} = \text{Raw Area} / (\text{RSF} * \text{Tx. function}) \quad (1)$$

(CPS = counts per second; BE = binding energy [eV])

3. Results and discussion

3.1. Thiol assembling on gold

SPRi evaluation

SPRi has indicated a very low reflectivity change associated with thiol immobilisation on the gold. Fig. 4 shows the SPRi map and the SPR angle shifting obtained from the analysis of the SPRi image. The brighter spot corresponds to the light increase in the refractive index due to the presence of thiol on gold.

This analysis visually confirms the presence of the 16-Mercaptohexadecanoic acid on the gold sensor, but it can not provide informations about the regular assembling of the thiol as a monolayer or multiple layers. However, the low reflectivity variation proves that a very small amount of this small molecule (compared with AM and mucin which are proteins with big molecular weights) was immobilized on gold, probably as monolayer assembly.

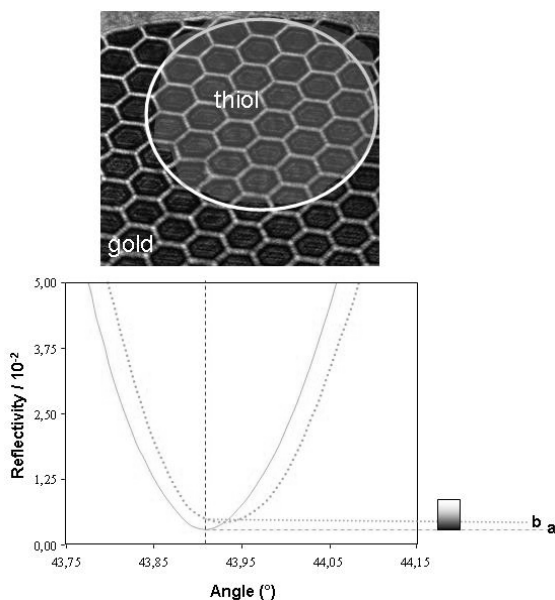


Fig. 4. SPRi result for thiol spotted on gold, measured in air.

XPS analysis

The monolayer formation was confirmed by XPS analysis.

The survey spectra of the sample surface (Table 1) showed the following elements:

carbon (6): as C 1s peak,
 oxygen (8): as O 1s, O 2s, O KL1 = O KL₂₃L₂₃ and O KL2 = O KL₁L₂₃ peaks (the intensity of the corresponding O KL₁L₁ peak was too low to be seen in the spectra), sulphur (16): as S 2p_{3/2}, S 2p_{1/2} and S 2s peaks, zinc (30): as Zn 2p_{1/2}, Zn 2p_{3/2} and Zn LMM = Zn L₂M₄₅M₄₅ peaks (the intensities of the corresponding peaks Zn 3s, Zn 3p_{1/2}, Zn 3p_{3/2}, Zn LM2 = Zn L₃M₄₅M₄₅, Zn LM3 = Zn L₂M₂₃M₄₅(¹P), Zn LM4 = Zn L₃M₂₃M₄₅(³P), Zn LM5 = Zn L₃M₂₃M₄₅(¹P), Zn LM6 = Zn L₂M₂₃M₂₃, Zn LM7 = Zn L₃M₂₃M₂₃ were too low to be seen in the spectra), gold (79): as Au 5p_{3/2}, Au 5p_{1/2}, Au 4f_{7/2}, Au 4f_{5/2}, Au 4d_{5/2}, Au 4d_{3/2}, Au 4p_{3/2}, Au 4p_{1/2} and Au 4s peaks.

Table 1. Survey spectrum.

	0°	60°	75°
survey spectrum	-3.35 eV	-3.65 eV	-3.35 eV
S 2s spectrum (E _{pass} = 160 eV)	-3.35 eV	-3.65 eV	-3.35 eV
C 1s spectrum	-3.52 eV	-3.88 eV	-3.58 eV
Au 4f spectrum	-3.52 eV	-3.88 eV	-3.58 eV

As previously explained in Section 2.2 Methods, elemental ratios were estimated using equation (1).

Table 2. Elemental ratios obtained from XPS results of the thiol covered gold slide.

	0°	60°	75°
[C]:[Au] _{sp}	0.832	1.916	3.283
[O]:[Au] _{sp}	0.384	0.724	1.051
[S]:[Au] _{sp}	0.072	0.119	0.146
[Zn]:[Au] _{sp}	0.021	0.023	0.03
[O]:[C] _{sp} ^{a)}	0.461	0.378	0.32
[S]:[C] _{sp} ^{b)}	0.087	0.062	0.044

The stoichiometric ratios for 16-Mercaptohexadecanoic acid (HS-C15H30-COOH) are: [O]:[C]_{stoi} = 0.125 (^{a)}); [S]:[C]_{stoi} = 0.0625 (^{b)}). The ratios obtained through the XPS analysis, as shown in Table 2, are higher than the stoichiometric values. It can be said that not all the sulphur on the gold is bound to carbon atoms. Indeed, some saturated hydrocarbons as well as sulphur are due to inherent contamination of the surface during the analysis. However, it can be assumed that all organically bound sulphur (S-C), detected as maximum amount in the proximity of gold (0°, corresponding to ID ~8 nm) was introduced through adsorption of the thiol on gold. When the information depth is lowered (through increasing the take-off angle), the whole amount of carbon strongly increases. This clearly demonstrates that the top layer of the covered slide is formed by a very dense carbon layer, also confirming the SAM formation.

AFM mapping

AFM mapping failed to indicate a significant difference between the topography of the surfaces of unmodified freshly cleaned gold and thiol-modified gold. However, this is not a negative result and it could be easily explained through the low dimension of 16-Mercaptohexadecanoic acid molecules, that probably coincides with the roughness parameters of neat gold sensor. Since the tip of the cantilever follows the surface through the adsorbed thiol small molecules, it is likely that the resulting roughness is similar with the one from neat gold surfaces.

Fig. 5 presents the AFM maps for gold (panels 1 – a,b,c) and thiol (panels 2 – a,b,c). Panels a show 10 nm × 10 nm AFM representative topographies of non-modified gold (a – 1) and thiol-treated gold (a – 2). The white lines show the distance and the direction used to obtain the topography profile scans presented in panels b. These profiles show homogeneous surfaces with a mean roughness depth in the range of 0.6 nm both for bare gold and thiol-coated slide. The roughness horizontal pathway is estimated at approximately 0.25 nm for neat gold and 0.3 for thiolated surface. Panels c display the 3D topographic images of the surfaces.

3.2. AM immobilisation

SPRi analysis

SPRi has successfully proved the AM binding at the surface of the thiol SAMs assembled on gold sensors.

The shifting in the local reflectivity was clearly depending on the concentration of the antibody solution used in the immobilisation step, as shown in Fig. 6. All the images were recorded at gold SPR angle (43.9° in air).

The increase of the concentration was followed by an increase of the amount of antibody bound on the thiol and finally it lead to higher SPRi signals (brighter hexagons - b,c,d in Fig. 6 - compared with the dark non-modified gold background - a in Fig. 6). Panel c shows even both the non-modified gold (left darker hexagons) and the AM - modified area (right brighter hexagons).

The panel below the SPRi images shows the angle shifting as estimated through grey level analysis of the recorded responses.

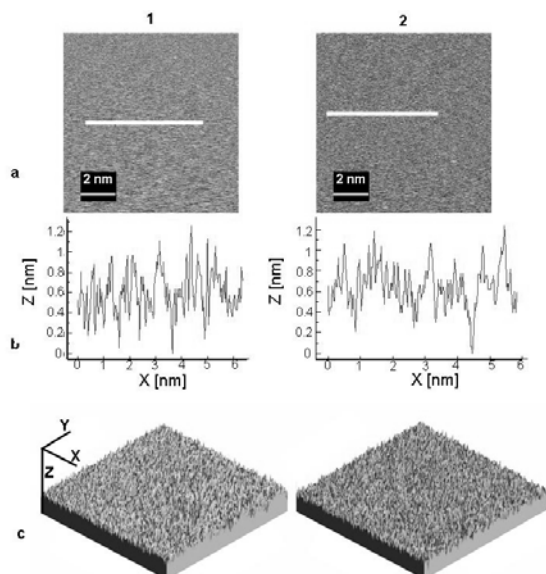


Fig. 5. AFM micrographs for 1 – neat gold and for 2 – 16-mercaptohexadecanoic acid coated gold. a – $10\text{ nm} \times 10\text{ nm}$ AFM topographies; b – topographic profiles obtained when scanning the white line in panels a; c – 3D images (scan size $10\text{ nm} \times 10\text{ nm}$, Z range 2 nm).

AFM mapping

AFM results have confirmed the AM immobilisation on the thiol-covered gold slides. Fig. 7 presents the appearance of AM-layer obtained when using 25 ppm antibody solution in buffer. Different magnifications are shown. The left panels (1) display the representative topographies of AM bound on the surface, while the right panels (2) show the corresponding 3D micrographs. Globular features homogeneously spread can easily be distinguished in both left and right panels, at all magnifications. These are associated with AM molecules assembled on the thiol SAM. The AM layer formed on top of the thiol layer is homogeneous and it is likely that the slide is completely covered by protein molecules with diameters in the range of 20-35 nm. These results are well

matching the expectations, since AM has large macromolecules.

Besides these observations, a second confirmation of the successful AM modification of the surface comes from the comparison of the surface roughness recorded for non-modified gold (or for thiol-coated slides) with the surface topography of AM treated surfaces. In Fig. 5 panels c, the 3D scans of gold and thiol-coated gold are shown, for a scan size of $10\text{ nm} \times 10\text{ nm}$.

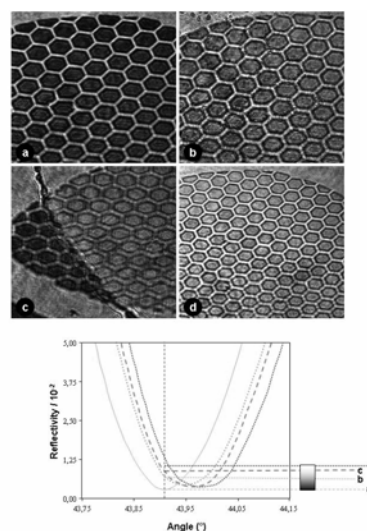


Fig. 6. SPRi maps for a – gold, b – gold + 5 ppm AM, c – gold + 25 ppm AM, d – gold + 150 ppm AM, measured in air.

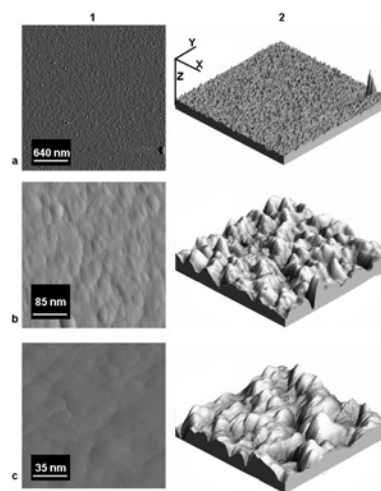


Fig. 7. AFM images of AM-coated slides (25 ppm) 1a – $3.2\text{ }\mu\text{m} \times 3.2\text{ }\mu\text{m}$; 2a – scan size $3.2\text{ }\mu\text{m} \times 3.2\text{ }\mu\text{m}$, Z range 36.6 nm; 1b – $425\text{ nm} \times 425\text{ nm}$; 2b – scan size $424.9\text{ nm} \times 424.9\text{ nm}$, Z range 8.2 nm; 1c – $175\text{ nm} \times 175\text{ nm}$; 2c – scan size $176.3\text{ nm} \times 176.3\text{ nm}$, Z range 7 nm.

In the panel 2c from Fig. 7, the same 3D topography is displayed for AM-coated surface, with a scan size of $176.3\text{ nm} \times 176.3\text{ nm}$. It was previously shown that the features detected on neat gold have horizontal dimensions (pathways) of 0.25 μm , on the thiol-treated surfaces they

have values of 0.3 nm and on the AM-covered surfaces the features are 20-35 nm large. These results are consistent with the affirmation that an homogeneous AM layer is formed on top of the thiol-coated gold slide.

3.3. Mucin capture affinity of the sensor

The AM sensor was preliminary tested with respect to its capacity of specifically interacting with mucin. In order to be sure that the sensitivity of the sensor is high enough to detect high level of the antigene in the test solution, the sensor obtained from the immobilization with 25 ppm AM was selected. It was exposed directly to a flow of mucin (100 ppm) in buffer, pH 7.4. The non-reacted species were removed through rinsing with buffer.

SPRi analysis

The SPRi showed a new shifting in the local reflectivity, corresponding to the increase of the refractive index due to the binding of mucin (data not shown).

SPR angular interrogation

In order to analyse the angle shifting due to the specific interaction antibody-antigene, a complete experiment starting with thiol adsorption on gold and finishing with mucin-AM affinity was performed. The result is presented in Fig. 8, where the angular shifting is shown in time. First, the thiol was immobilized on the gold surface. Then, for about 1 hour, the surface was exposed alternatively to AM (25 ppm in buffer pH 7.4) and buffer. The graph shows a clear modification of the SPR angle, due to AM stable immobilization. The angular shifting is of about 0.15° , value that is consistent with the shifting obtained from the treatment of the SPRi images (c in Fig. 6). The last step of the experiment has successfully monitored the increase of the angle due to the reaction with mucin. These results are consistent with the SPRi response, proving the efficiency/potential of the AM sensor in mucin over-expression detection.

AFM analysis

AFM measurements have revealed large globular features on the surface of the sensor, following the treatment with the mucin solution (Fig. 9). The dimensions of these features (38 - 45 nm) are superior to those detected for AM layer (20 - 35 nm). This analysis confirms the presence of the mucin protein on the surface.

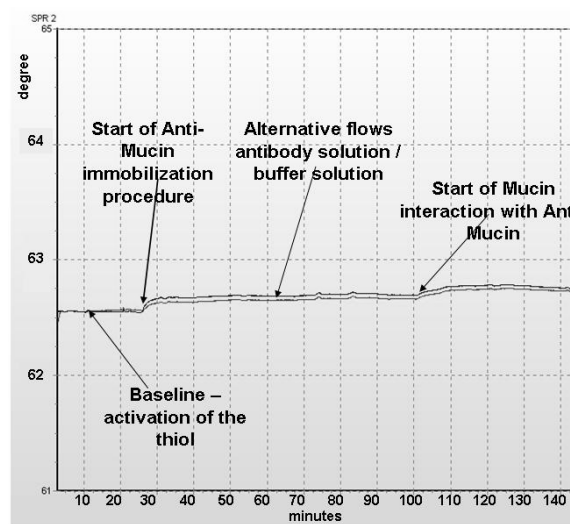


Fig. 8. SPR angular shifting.

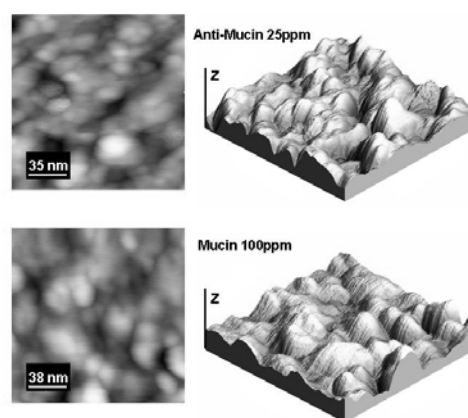


Fig. 9. AFM height images of AM and mucin covered surfaces.

4. Conclusions

This work reports on the successful immobilisation of AM antibody on gold-SPR slides, using an intermediate 16-mercaptohexadecanoic monolayer. The antibody can be bound using different concentrations of AM solution. However, to be sure that the sensor will be further sensitive enough to detect mucin overexpression, we have selected as optimal concentration 25 ppm AM in buffer pH 7.4. The results recorded by SPRi, SPR and AFM were successfully consistent. In order to prove the further functionality of this sensor, mucin solution was used for a specific affinity reaction. As expected, the interaction occurred successfully emphasising the potential of the sensor in specifically and label-free detecting mucin as tumour marker. Further experiments will be performed to optimise the procedure and to fix the detection limits of this sensor.

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