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# AFM characterization of ss-DNA probes immobilization: a sequence effect on surface organization

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**Abstract**. The biological sensitivity of a DNA chip depends on the molecular organization of the immobilized probe molecules, single stranded DNA (ss-DNA), on the substrate in terms of accessibility and non specific interactions between probes and substrate. In this article, Amplitude Modulation - Atomic Force Microscopy (AM-AFM) was used to characterize at a molecular scale, the morphological organization of different immobilized probes. In our system, three different ss-DNA were covalently grafted on a silicon substrate with the same deposit process. We studied the influence of probe length (25 bases, 12 bases) and sequence arrangement (two different 25 base oligoprobes) on the morphological organization. We showed that immobilized probes organize themselves in different structures depending on their sequence.

## 1. Introduction

Deoxyribonucleic acid (DNA) chips are major tools in the biomedical research field for DNA diagnosis, gene monitoring and genome sequencing [1,2]. They are structured in an array of single-stranded DNA chains called probes, covalently bonded to a solid support, which are able to specifically recognize complementary single-stranded DNA called targets, by hybridization. Among the required properties, sensitivity and specificity of the chip are crucial and hinge on the surface probes' organization. In most cases, sensitivity is evaluated after biological recognition by measuring the amount of hybridized targets with fluorescent spectroscopy or radioactivity analysis. The reliability and reproducibility of measurements could be strongly influenced by the manufacturing process. Then, it appears necessary to characterize the chip at a molecular scale before hybridization.

Atomic Force Microscopy (AFM) [3] has become one of the most widely used techniques in biological sciences by contributing to major progress in the understanding of the structure and functionalities of biological samples [4,5]. Its success firstly lies in its ability to directly image biomolecules in environmental conditions [6,7] and secondly in the possibility to determine interaction forces between biological molecules [8,9,10]. Development of dynamic modes of operation adapted for the imaging of soft samples has significantly contributed to overcome the usual issue of damage done to these biological objects by the scanning tip [11,12].

 

In this article, we show that AFM is well-adapted to study the molecular organization of ss-DNA chips. Using the amplitude modulation mode in the mostly attractive regime [13,14], molecules could be observed in high resolution images. Studying a model chip with three different ss-DNA probes immobilized, we demonstrate the influence of the sequence arrangement in the molecular layer structuring.

## 2. Experiment

### 2.1. Model chip preparation

To analyze the influence of the probes length and the arrangement of the sequence, chips were designed with three separate areas containing the three different probes:
the 12A region corresponds to 12-base strands (5'-AGC CCG GAG GCA-3'),
the 25A region contains 25-base strands (5'-GCT AAT CCA ACG CGG GCC AAT CCT T-3')
the 25B region correspond to 25 base strands (5'-CTC CTA CCA TGC GGA CGA TCC ATA G-3').
These synthetic oligonucleotides were modified by an aminolinker ($H_2N$-$(CH_2)_6$) at the 3' end (purchased from Eurogentec, Belgium).

These oligonucleotide probes (oligo-probes) were immobilized on N-hydroxysuccinimide (NHS)-ester activated supports. Polished Silicon wafers were preferred to glass substrate because of its lower roughness. The Si (100) with 120 nm of thermally oxide (Tronic's microsystem) was functionalized following protocols previously described [15]. Briefly, wafers were cleaned in piranha solution (H2SO4/H2O2, 3:1), thoroughly rinsed in ultra pure water and dried at 140°C under N2 atmosphere for 2 hours. Silanisation was performed with a monofunctional silane tert-butyl 11-(dimethyl dimethyl aminosilyl) undecanoate (C10) by impregnation process. Subsequently, the terminal ester function was hydrolyzed to the corresponding acid under mild conditions with iodotrimethylsilane. The generated carboxylic acid terminal groups were activated with an equimolar mixture of NHS and di(isopropyl)carbodiimide (0,1 M) in dry tetrahydrofurane to obtain the NHS- activated ester. Amino-modified oligo-probes react with the NHS- activated ester to form oriented stable bonds.
The silanisation step was assessed by contact angle measurements (Digidrop GBX). Nine repetitions with water droplet deposition were used to determine the experimental standard deviation.

The oligo-probes were supplied under lyophilized conditions. They were resuspended in a Phosphate Buffer Saline (PBS) solution at pH = 8.5 to a similar final concentration of 25 µM. 2.5 µL of each solution were deposited on the functionalized substrate. The immobilization proceeds through complete droplet evaporation under air. The reactive surface sites empty of probes were "blocked" by a last capping step with dimethylamine in vapor phase. Finally, the chips were washed in ultrapure water at 80°C for 1 hour to remove unbound oligo-probes.

### 2.2. AFM – setup

AFM topographical imaging was carried out in air with a Stand Alone SMENA (NT-MDT) microscope operating in Amplitude Modulation - AFM (AM-AFM).

Amplitude ranges were chosen between 10-15 nm with a set point value $A_{setpoint}$ maintained at 95% of the free amplitude $A_{free}$. These conditions were chosen in order to realize images in the mostly attractive regime, limiting tip contact on the surface [13,14]. AFM tips were supported by rectangular cantilevers with spring constant in range of 5-14 N/m oscillating near their resonance frequency of 150-300 kHz. In most cases, the quality factor was greater than 500. Nominal tip curvatures were around 10nm. Images (512 x 512 pixels) were acquired with a scanning frequency around 1.5 Hz. Typical temperature and relative humidity were about 25°C and 40% respectively.

## 3. Results and discussion

12 wafers were prepared as described in the previous section. The DNA chips were all characterized by AFM. They exhibited the similar morphology, demonstrating the high reproducibility of the process.

3.1. Grafting layer characterization

For each chip, the anchoring layer of organosilane was analyzed before ss-DNA immobilization. AFM images revealed a smooth and regular morphology (figure 1a). On high resolution images depicting a square region of 400nm x 400nm, we obtained a typical root mean square (RMS) roughness of 0.25 nm similar to the value measured on the bare substrate of oxidized silicon (figure 1a). Peak to valley heights were measured to 2.3 nm. The average height was 0.5 nm corresponding to the ten carbons containing molecules. The expected silane molecule length, around 1.5 nm, was never observed whereas a water contact angle of 81° (± 0.5) was measured indicating the presence of hydrophobic methylene groups. Therefore, we expected that molecules are lying flat on the substrate.

3.2. ss-DNA immobilisation.

After deposition of the probes, the morphology of the chip evolved, at a molecular scale, the surface appeared more roughen with a peak to valley height reaching 4.6 nm and a RMS roughness of 0.55 nm. Moreover, the surface is covered with granular structures presenting oblong shape for the 25A and 25B regions (figure 1b) and circular shape for the 12A area (figure 1c). Statistical measurements of the island lateral dimensions gave values presented in table 1. They were ranging around 15.2 nm wide and 23 nm long on the 25A area and 13 nm wide and 25 nm long on 25B area. For the 12A region, the island shape is circular with a diameter around 11 nm. On the 25 regions, we showed previously that the density of islands - evaluated by counting them on several images- was in good agreement with the density of probes deposited on the surface [16]. 1.75-2.25 x $10^{11}$ islands $cm^{-2}$ are observed. This is comparable to the surface density measured by radiolabelling measurements ($10^{11}$ molecules $cm^{-2}$) [15]. This means that AFM allows us to quantify surface density with accuracy.

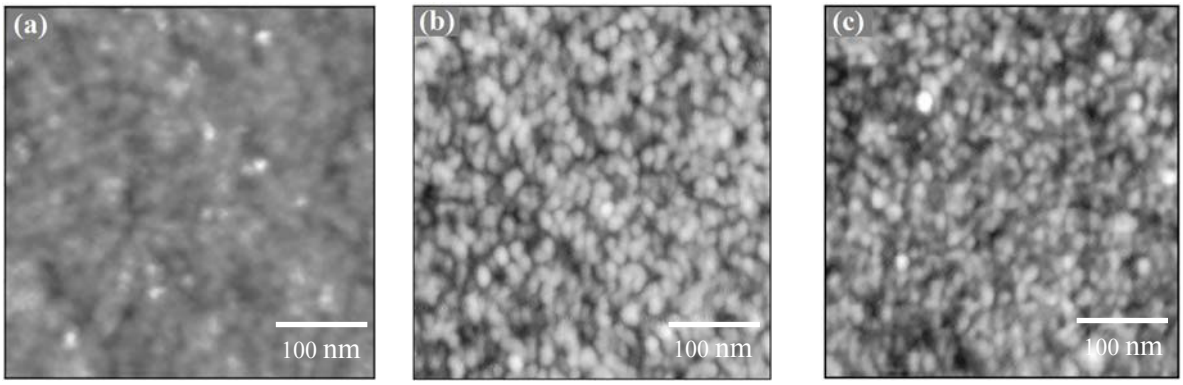


**Figure 1.** High resolution images of the DNA-chip before and after immobilization of the ss-DNA probes. Scanned area is 400nm x 400nm: (a) the silane layer before immobilization, $\Delta z$= 2.3 nm, (b) on the 25A region, $\Delta z$= 4.6 nm and (c) on the 12A region, $\Delta z$= 3.7 nm. Probes show up as isolated islands. Notice that they appear smaller in size on the 12A region, despite the tip enlargement.

**Table 1.** Statistical measurements of island dimensions

| | Width (nm) | $SD_{width}$ [a] (nm) | Height (nm) | $SD_{height}$ (nm) |
|---|---|---|---|---|
| 25A | 15.2 | 2.6 | 23.4 | 5.6 |
| 25B | 13.3 | 3 | 24.9 | 5.8 |
| 12A | 11 | 2.5 | 11 | 2.5 |

[a] Standard Deviation

This means that each island should correspond to one or two probes (i. e. DNA molecules). The scale difference between island and molecule could be attributed to the dilatation effect induced by the tip radius [17]. Despite this tip enhancement, probes could be identified and different island sizes were measured as a function of the probe length. On the 12A region, the tip apex was too large to attain the molecular resolution but islands appear smaller than the 25A or 25B one.

These high resolution images showed that probe molecules could be observed and identified by AFM. Larger scale images gave us interesting information concerning the molecular organization of the ss-DNA on the surface. Figure 2 presents a series of images of a square region of 1.4µm x 1.4µm. A homogeneous distribution of ss-DNA is observed on figure 2a and figure 2c representative of the 25A and the 12A areas respectively, whereas a different structure appears on the 25B area as shown on figure 2b. 25B probe molecules organize themselves to form a lacy structure with numerous holes, typically of 100 nm in diameter. Some diameters between 50 nm and 400 nm were sometimes observed at some places. Considering no hole was observed on the other regions 25A and 12A, we suggest that this particular molecular organization is induced by different physico-chemical properties probably related to a sequence effect.

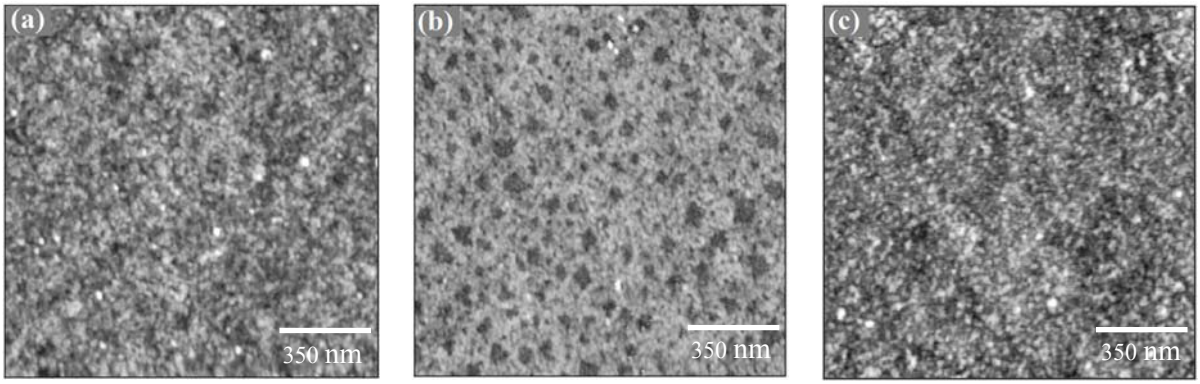


**Figure 2.** Large scale images of the DNA-chip after probes immobilization. Scanned area is 1.4 µm x 1.4 µm: (a) on the 25A region, $\Delta z$= 7.1 nm, (b) on the 25B region, $\Delta z$= 8.3 nm, (c) on the 12A region, $\Delta z$= 5.7 nm. Ss-DNA molecules organize themselves in different structures depending on their sequence. Homogeneous layers for 25A and 12A, and presence of holes for the 25B.

The role played by the sequence arrangement is not obvious and is not yet understood. The selected sequences should not self-hybridize or make hairpin structure [18]. Mechanical and physico-chemical properties of each DNA strand are driven by the base sequence. Thus the observed differences may be related to some macroscopic phenomenon like differences in air/liquid and liquid/solid interactions. For instance, as reported in [19] the spreading of droplets containing the oligoprobes, and subsequently the repartition of immobilized probes on the surface were shown strongly depending on the nature of the sequences. One suggested explanation concerns the difference of superficial surface tension at the air/liquid interface.

The different surface organization observed can also be related to difference of physico chemical/structural properties of the molecules at the surface or in solution (the sequence 25A and 25B contain the same proportion of A, C, G, and T desoxy-nucleotides but a different sequence, for example for 25A the thymidine are located at the extremities). In order to support this assumption we plan to 1) make molecular mechanics computation in order to better understand molecules' behavior in solution and at the surface 2) perform experimental work to obtain information on sequence influence on the film growth (nucleation, random growth…). We plan to study the influence of thymidine, by modifying the sequence 25A with a central T positioned at distances from the extremity of the strand. We plan also to add an extra T in the middle of the 12A sequence. To study inter-molecular interaction

small complementary oligonucleotides could be hybridized before immobilization and removed before AFM experiments.

However, we can not exclude that the differences observed may be related to other phenomenon (i. e. differences in air/liquid surface tension due to the DNA…) [19]

## 4. Conclusion

This preliminary work shows the ability of AM-AFM to characterize and quantify DNA strands at the surface of DNA chips. The influence of the length and nature of the sequences (base arrangement) on the surface organization of the probes on a solid surface is demonstrated. Subsequently, chip's surface morphology is sequence dependent and may influence the following hybridization step (level of detection of complementary strands in biochips experiments).

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