Characterization of DNA chips on the molecular scale before and after hybridization with an atomic force microscope

Marie Hélène Rouillat\textsuperscript{a}, Vincent Dugas\textsuperscript{b}, Jean René Martin\textsuperscript{a}, Magali Phaner-Goutorbe\textsuperscript{a,*}

\textsuperscript{a}Laboratoire d’Electronique d’Optoélectronique et Microsystèmes, Ecole Centrale de Lyon, 69134 Ecully, France
\textsuperscript{b}Rosatech Society, Ecole Centrale de Lyon, 69134 Ecully, France

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Abstract

Using two different 25-mer oligonucleotide probes covalently grafted on a silicon substrate, we demonstrate how efficient atomic force microscopy (AFM) can be for monitoring each step of DNA chip preparation: from probe immobilization to hybridization on the molecular scale. We observed the probe-molecule organization on the chip after immobilization, and the target molecules, which hybridized with probes could be individually identified. This article presents a method of straightforwardly identifying not only single and double DNA strands, but also, and more significantly, the hybridized part on them. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

DNA chips are necessary to keep up with the demands of biomedical goals such as DNA sequencing, clinical diagnostics and gene expression monitoring \cite{1,2}. They consist of an array of single-stranded DNA (ssDNA) chains of different sequences called probes that are tethered to a solid substrate. These DNA chips are exposed to DNA strands called targets that are complementary, so that they may be captured by hybridization. During the fabrication of the chip, the density and the distribution of the immobilized probes on the substrate should be optimized to improve the biological selectivity of the chip. Moreover, after hybridization, the identity and the abundance of the captured targets should be determined by sensitive readout methods. When they are few DNA targets, the commonly used detection techniques (such as the radiochemical method \cite{3} and fluorescent microscopy \cite{4}) fail, so high-resolution methods like atomic force microscopy (AFM), for example, should be used.

* Corresponding author. Tel.: +33 4 72 18 62 32; fax: +33 4 72 18 62 32.
E-mail address: Magali.Phaner@ec-lyon.fr (M. Phaner-Goutorbe).
Since its invention [5], AFM has become one of the most widely used techniques for the probing of soft materials [6–10]. It has demonstrated its unique ability to directly observe single molecules in air or in solutions, opening up exciting opportunities in biology. DNA is the most commonly studied molecule and numerous papers relate experiments concerning its adsorption on various substrates observed in different media such as air, inert atmospheres and liquids [11–16]. Few articles are concerned with AFM studies on DNA microarrays [17–22]. Some papers have reported analyses, which allowed the complementary regions (where the hybridization occurred) to be identified by comparison with the non-complementary regions. This difference could be measured by force changes between the tip and the scanned surface. For example, Mazzola et al. [18] used an ssDNA-modified tip to analysis an ssDNA array. They showed that oligonucleotide regions, which are complementary to the tip exhibited a stronger friction force than non-complementary regions. Wang and Bard [19] measured the electrostatic force between the surface and a tip modified by the attachment of a spherical silica bead. They observed the surface charge evolution between regions with single and double strand DNA arrays. Other, morphological, studies mentioned a roughening of the surface induced by immobilization [20] and also by hybridization with a broadening of the structures [21,22]. All these experiments have demonstrated the capabilities of AFM for DNA chip analysis, but a topographical description on the molecular scale has never been mentioned.

In this article, we show that AFM can be a useful tool to monitor each step of the DNA chip preparation from immobilization to hybridization. Using the amplitude modulation mode in the mostly attractive regime [23,24], most of the main features can be straightforwardly extracted from the images, even in air. Our approach was a step-by-step comparison, first of all, of the thermally oxidized silicon after silanization (taken as a reference), then of two 25-mer oligonucleotide probe areas before and after testing by hybridization with our selected DNA target. One of the probes presents a sequence, which perfectly matches one part of our selected target, and the other probe does not. In this way, we can individually identify the target molecules, which have hybridized with probes. Furthermore, single and double DNA strands on targets, on the molecular scale, can be distinguished.

2. Experiment

2.1. Chip preparation

Several chips were fabricated and analyzed. In this study, we consider a model chip presenting two separate areas (Fig. 1). Each is composed of a 25-mer oligonucleotide probe: the 5'-TTC CTA ACC GGG CGC AAC CTA ATCG-3' chain and the 5'-GAT ACC TAG CAG GCG TAC CAT CCTC-3' chain (purchased from Eurogentec Seraing, Belgium). Each probe was bonded to the substrate by an aminohexyl moiety linked to its 5' extremity. The first area was made up of an oligonucleotide presenting a sequence, which perfectly matched one part of the selected target. For this reason, it was called 25-C (C for complementary). The second area was formed of a sequence, which was totally different to the 25-C sequence. Since no hybridization could occur with the target, it was called 25-NC (NC for non-complementary).

2.2. Targets for hybridization

The target is a long strand of 1500 base-paired DNA obtained by polymerase chain reaction (PCR) amplification of the 16S-rDNA gene of Agrobacterium tumefaciens with 25 bases matching the 25-C probe (from the 185th to the 209th base, situated at around 1500-base target)

![Fig. 1](https://example.com/fig1.png) Schematic drawing of the model DNA chip, circles delimit areas where probes are grafted: 25-C (C for complementary) and 25-NC (NC for non-complementary). Only the 25-C probes can hybridize with the selected targets (a 1500-mer fragment terminated with a fluorescent dye: Cy3).
80 nm from the 3’ extremity) [25,26]. A fluorescent dye (indocarbocyanine, Cy3) was added at one extremity to perform fluorescent microscopy.

2.3. Amplitude modulation atomic force microscopy

The topography of the chip was investigated by a stand-alone SMENA (NT-MDT) AFM operating in tip scanning mode. The images were taken in air using the amplitude modulation mode. The AFM probes were silicon tips with spring constants of 5–14 N m$^{-1}$ driven near their resonant frequency of 150–300 kHz. The set-point amplitude was maintained at 95% of the free amplitude, this typically corresponds to 10–15 nm. These conditions were chosen in order to realize images in a mostly attractive regime, limiting tip contact on the surface [23,24]. Nominal tip curvatures were around 10 nm. Scan rates were about 1 μm s$^{-1}$. Images were processed using a first-order plane fit to remove sample tilt.

3. Results and discussion

In this process, oligonucleotides were tethered onto a thermally oxidized silicon surface by means of an organosilane anchoring layer. Silanol groups constitute anchoring sites for the organosilane molecules, and so for oligonucleotides. Moreover, thermally oxidized silicon exhibits a smooth and featureless surface favorable to AFM characterization. Small silane molecules of tertiobutyl-[(dimethylamino)dimethylsilyl] undecanoate (C10) were grafted on the substrate by an impregnation process [27]. Each molecule was covalently bonded to the substrate. This process produces a smooth and homogeneous silane monolayer. Fig. 2a presents an AFM image of the substrate surface after silanization. Several cross-sections were performed on different images to obtain a statistical value of the roughness height. We measured an average height of 0.5 nm, whereas the root mean square (RMS) value typically reached 0.25 nm. The silane molecule length is expected to be

![AFM images of the chip. A (400 nm × 400 nm) scanned area before immobilization: (a) the silane layer, after immobilization, Δz = 2.3 nm, (b) on the 25-C area, Δz = 4.6 nm and (c) on the 25-NC area, Δz = 4.6 nm. Oligoprobes show up as homogeneous islands lying on the surface.](image-url)
around 1.5 nm, but no such height was measured on AFM images. Moreover, contact angle measurements (digidrop GBX) gave an angle $\theta = 90^\circ$, representative of the presence of hydrophobic groups (the methylene groups $-(\text{CH}_2)-$) on the surface. These observations lead us to believe that the molecules are lying flat on the substrate [28].

The two probe areas were formed by deposition of 2.5 $\mu$L drops containing, respectively, the 25-C and 25-NC single strands at a concentration of 25 $\mu$M in a phosphate buffer saline solution (pH 8.5). The grafting was carried out by evaporation under ambient conditions ($T = 25^\circ C$, humidity rate = 40%). The chip was then washed in a 1% sodium dodecyl sulfate (SDS) solution in ultra-pure water at 80 $^\circ C$ for 1 h and thoroughly rinsed with ultra-pure water. This left a density of $(3.8 \pm 1) \times 10^{11}$ probes cm$^{-2}$ on each zone (measured by a $^{32}$P-radiolabeling experiment [26]). This oligoprobe immobilization step roughens the surface with a RMS of 0.55 nm. The surface is covered with granular structures 1.5–2 nm in height (Fig. 2b for the 25-C region and Fig. 2c for the 25-NC region). A statistical analysis of the lateral dimensions of these islands was realized on different images and reported in Fig. 3. Islands are oblong in shape with an average width of 15.2 nm (with a standard deviation (S.D.) of 2.61 nm) and an average length of 23.37 nm (S.D. of 5.59 nm) for the 25-C region; slightly thinner, 13.27 nm (S.D. of 2.98 nm) and 24.9 nm (S.D. of 5.77 nm), for the 25-NC region. These values are mostly higher than the expected molecule dimensions. Although we have no idea what the real size or the conformation of the molecule-probe is, we could estimate a molecule length of 12.3 nm for a stretched conformation in accordance with the Tinland et al. [29] calculations. Moreover, a width ranging between 1 and 1.5 nm for a single strand was found in the

![AFM images after hybridization](image)

Fig. 3. Statistical measurement histograms of the island dimensions on the 25-C and 25-NC regions. These values were obtained by directly measuring the length (a) and width (b) of the islands at 80 different points on several images.

Fig. 4. AFM images after hybridization. Large-scale image (2500 nm × 2500 nm): (a) on the 25-NC area—as expected no evidence of fragments was observed and (b) on the 25-C area—DNA fragments are lying flat on the chip. Different average heights of 1 and 2 nm were measured on strands labeled X and Y, respectively.
literature [30]. The size difference between island and molecule could be attributed to the well-known dilatation effect induced by the tip radius [6]. We estimated the density of islands by counting them on several images. We obtained a value of around \( (1.75–2.25) \times 10^{11} \) islands cm\(^{-2}\), in good agreement with the density of probes deposited on the surface, evaluated by radiolabeling measurements [26]. This means that each island observed on the image should correspond to one or two probes.

The hybridization step was realized with double-stranded DNA (dsDNA) targets resuspended at 0.1 \( \mu \)M, in 5\( \times \) saline sodium citrate (5\( \times \) SSC) buffer. The solution was first heated to 95 \(^\circ\)C for 10 min to denature the dsDNA by thermal effect. This leads to two complementary single strands present in the solution. Only one single strand matches the 25-C probe. A drop (50 \( \mu \)l) of this solution was immediately deposited on the chip surface. The hybridization was then carried out for 2 h at 47 \(^\circ\)C in a humid chamber. The chip was washed for 2 min at room temperature in 2\( \times \) SSC, 0.1% SDS and 2\( \times \) SSC. Before drying with compressed nitrogen, the chip was rapidly rinsed with ultra-pure water. These washes were performed to remove all adsorbed targets and non-specific hybridization, despite the risk that such heavy rinsing could also produce partial denaturation on the complementary area. The objective of the rinsing was to ensure that the DNA targets present on the surface were really hybridized. The washing was followed by fluorescent microscopy [31] and the chip was deliberately rinsed until the fluorescent signal measured on the complementary region fell below the detection limit.

As expected, there was no evidence of targets in the non-complementary region (Fig. 4a), and some were observed to be lying flat on the surface in the complementary region (Fig. 4b). This confirms that fragments imaged on the 25-C region were actually hybridized and that non-specific adsorption was totally eliminated by washing. Targets mostly adopt a stretched configuration, and are present as individual entities that are sometimes (not always) oriented in the same direction as in Fig. 4b. In some places, they join like in the upper right part of the image. A DNA length of around 650 nm was measured on the images. This value is in good agreement with the expected value of 643 nm [32] corresponding to the 1500-mer single strand length. On this chip, we estimated a target density of \( 5 \times 10^8 \) molecules cm\(^{-2}\), which could not be evaluated by fluorescent microscopy.

Two different strand heights are observed in Fig. 4b: one (denoted X-strand) globally reached a height of 1 nm and the other one (denoted Y-strand) attained 2 nm. This was evidenced on several images and on different investigated chips. A high-resolution characterization of some X-strands was carried out (Fig. 5). It showed that one individual hybridization target could be identified by AFM even with the probe topography visible on the substrate. A precise measurement of the height along the fragment gave

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**Fig. 5.** High-resolution image on the 25-C region representative of one individual hybridization event (X-strand). Cross-sections show that two different heights ((A) 1 nm and (B) 2 nm) are obtained along the single strand. The 2 nm height is attributed to the hybridized region.
a value of around 1 nm (cross-section ‘A’ in Fig. 5) globally, but reached 2 nm in a small region (cross-section ‘B’ in Fig. 5). This region covers the measured 25-C probe length and is observed at 80 nm from one extremity. As we measured it on several strands and always in the same place, we consider that this 2 nm mound corresponds to a double strand height attributed to the 25 bases of the target, which have hybridized with the 25 bases of the probe. This indicates that the hybridized region could be identified along the target. Moreover, the two different X- and Y-strands could thus be interpreted as two kinds of hybridization process. The X-strand corresponds to the expected single strand target hybridized with a 25-C probe. The Y-strand mainly forms a duplex as a result of two consecutive hybridizations: one with the 25-C probe and the other with the complementary fragment of the target. The hybridization solution is obtained by denaturing the ds DNA target, and the two 1500-base single strands are present. One is the fitting target, which hybridizes with the 25-C probe, and the other one is its complementary sequence with which partial re-hybridization could occur. The formation of duplexes and also triplexes has been mentioned in the literature [33]. It is not so obvious to make quantitative measurements of biological structure height by AFM. The great interaction between the tip and a soft surface induces some flattening of the biological molecule, and lower heights than expected were often measured [11–16]. In our experiment, we could distinguish between a single strand and a double strand DNA molecule lying on a surface, for two different reasons. Firstly, we deliberately used AFM conditions under an attractive regime, thus limiting direct contact with the surface [23,24]. Secondly, in our system, DNA targets lie on a DNA “carpet”, a surface of the same nature, unlike other studies where DNA fragments lie on different stiff substrates [11–16]. In this way, the interactions between the AFM tip and the probe-covered surface on one hand, and between the tip and the targets on the other, are similar. Minor interaction changes occur when scanning from probes to targets [34,35].

4. Conclusion

In the present work, each preparation step of a DNA chip was followed by AFM on a molecular scale. We show that AFM allows probes and targets to be identified and the hybridized region along the target to be recognized. This demonstrates that AFM is an appropriate technique to monitor the molecular organization of probes and targets on DNA chip. It could also replace conventional techniques for the readout of DNA chips when only a small number of probes and targets are available. In addition, with appropriate experimental conditions and depending on the interaction between DNA targets and the chip, single and double strand heights can be distinguished.

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