Imaging Gold Nanoparticles for DNA Sequence Recognition in Biomedical Applications

Peter Eaton*, Gonçalo Doria, Eulalia Pereira, Pedro Viana Baptista, and Ricardo Franco

***Abstract*—The hybridization of single-stranded oligonucleotide-derivatized gold nanoparticles (Au nanoprobes) with double stranded complementary DNA was directly observed by atomic force microscopy (AFM). This specific interaction is the basis for an Au nanoprobe-based homogeneous assay for specific DNA sequence detection, based on salt-induced particle aggregation that is prevented when a complementary target is present. For long DNA targets (linearized plasmid DNA) complicated hybridized target DNA–Au–nanoprobes structures were formed, that were interpreted as the basis for stability of the Au nanoprobes against salt-induced aggregation. For shorter DNA targets (PCR amplified fragments) hybridization with the Au nanoprobes occurred, in the majority of cases, in the expected location of the DNA target fragment containing the specific sequence. The formation of the observed DNA hybridized structures provides evidence at the molecular level for specific hybridization to the target sequence as the method of binding of the Au nanoprobes.**

***Index Terms*—Atomic force microscopy, biological materials, biomedical transducers, DNA, nanotechnology.**

I. Introduction

NANODIAGNOSTICS, defined as the use of nanotechnology (materials, devices, or systems) for diagnostics purposes, is a burgeoning field of interest as more and improved techniques become available to meet the demands of clinical diagnostics for increased sensitivity and for analysis of increasingly smaller amounts of biological material. Single analyte molecules can be accessed through one-to-one interactions between the target analyte and signal generating moieties, such as noble metal nanoparticles. These nanoparticle-based detection systems have been widely applied in nucleic acid hybridization and recognition of complementary sequences of interest [1], [2]. Particularly, colloidal gold nanoparticles with ca. 15 nm diameter can be easily synthesized and functionalized with thiolated oligonucleotides, generating DNA-functionalized gold nanoprobes (Au nanoprobes). The use of Au nanoprobes for specific oligonucleotide (DNA or RNA) detection in homogeneous format was first suggested by Mirkin and coworkers [3], who have developed a cross-linking assay using two different functionalized DNA–Au–NPs that hybridize in adjacent positions of the complementary DNA sequence, resulting in the formation of a polymeric network of DNA and Au nanoprobes with a concomitant red-to-blue color change (for reviews see [1], [4]). Conversely, Maeda and coworkers developed a non-cross-linking system with single DNA–Au–NPs in an homogeneous format [5] that was recently adapted into a heterogeneous assay allowing single-nucleotide polymorphism (SNP) detection [6], [7]. Our goal has been the development of a simple, easy-to-use, and inexpensive assay for specific DNA/RNA sequence characterization, with applications for infectious agent detection, mutations/SNP screening, and gene expression. Our assay is based on a non-cross-linking hybridization method, where aggregation of the oligonucleotide-derivatized gold nanoparticles (Au nanoprobes) is induced by an increasing ionic strength of the assay mixture: the presence of a target complementary to the Au nanoprobe born sequence prevents aggregation and the solution remains red, whereas the presence of noncomplementary/mismatched targets does not prevent Au nanoprobe aggregation, resulting in a visible color change from red to blue. This method has been successfully applied to eukaryotic gene expression studies without retro-transcription and polymerase chain reaction (PCR) amplification or any additional signal enhancement steps [8], and in a fast and straightforward assay for detection of *Mycobacterium tuberculosis* DNA in clinical samples [9]. Currently, we are applying this approach for the direct detection of mutations and SNPs in clinical samples [10].

In the present work we used atomic force microscopy (AFM) to study the biological colorimetric detection system in order to elucidate the DNA recognition events that occur at the nanoscale involving hybridization of the target double-stranded DNA and the Au nanoprobes. Due to the ability of AFM to image both metallic and organic particles, in vacuum, air, or even liquid environments, it has been extensively used in studies of nucleic acid interactions with nanoparticles. For example, various authors [4], [11]–[14] have assembled metallic nanoparticles onto stretched DNA segments in order to create nanowires, and

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imaged the resulting complexes by AFM, enabling measurement of the degree of coverage of the DNA template, and thickness of the resulting wire. This "metalized" DNA, however, results from nonspecific interaction between the nanoparticles and the DNA, allowing binding of nanoparticles all along the DNA molecule. Ganguli *et al.* [15] analyzed complex formation between cationic nanoparticles and DNA using AFM, as a model system for biological DNA condensation. The nonspecific interactions led to formation of networks of DNA on the surface, which structures were highly dependent on the relative concentrations of nanoparticles and DNA. Furthermore, as AFM can easily distinguish submolecular features in nucleic acids, it has been used to show binding of anti-dsRNA antibodies to RNA molecules [16], demonstrating preferential binding of antibodies to the ends and certain internal consensus sequences of the RNA molecules. Maeda *et al.* [17] used AFM to follow binding of streptavidin-coated nanoparticles to template DNA previously hybridized with a biotinylated oligonucleotide. AFM images of such complexes were successfully obtained; however, multiple bindings and secondary structure of the single-stranded DNA complicated the interpretation of the images.

In the present work we used AFM imaging in parallel to the colorimetric detection system to elucidate the DNA recognition events occurring at the nanoscale, i.e., hybridization of Au nanoprobes to target double-stranded DNA (plasmid or PCR product) and Au nanoprobes. Complementary and noncomplementary target sequences were used to study the level of specific interaction between the target and the Au nanoprobes. This allowed direct evidence of the mode of action of the Au nanoprobes and in addition allowed assessment of the incidence of nonspecific interactions between the Au nanoprobes and the target.

II. Experimental Section

A. Chemicals

The water used for AFM sample preparation was Water Biochemika for cell biology ultrafiltered and autoclaved (Fluka) or water for molecular biology (Sigma). All other chemicals were of the highest quality available and were used as received.

B. DNA Target Sequences

The β-globin gene locus genomic region was used as a model. Thalassaemia is probably the most common single gene disorder worldwide, and approximately 1 in 14 of the population carries one of the subtypes. The majority of the mutations causing β-thalassaemia have been characterized and a small number of common mutations cause the bulk of disease in each particular population-base [18], [19]. The Au nanoprobe DNA sequence used to prepare the Au nanoprobes (5 *′*-SH-(C_6)-AACCTTGATACCAAC-3 *′*) is complementary to a 15 bp region of the β-globin gene (GenBank accession no. NG_000007) overlapping the region harboring two of the most frequent mutations in the Portuguese population causing β-thalassaemia (β° IVSI,nt1 and β +IVSI,nt6) [20]. As the complementary target, the p158 plasmid (kindly provided by Dr. Wafaa Lemsaddek) containing the full length β-globin gene sequence with ≈8668 bp, or a derived 1559 bp fragment amplified by PCR was used. The noncomplementary target sequence was a 1565 bp PCR amplified fragment from an unrelated genomic sequence (CYP2D6 gene). Plasmid DNA was purified with Sigma's GenElute HP Plasmid Maxiprep kit, according to the manufacturer's instruction. Plasmid p158 was linearized prior to use in the AFM measurement, using BamHI restriction enzyme (Amersham Biosciences). Long PCR amplification of the target sequences was performed in a total volume of 50 μl using 1.25 U Extensor Hi-Fidelity PCR Enzyme Mix (ABgene, U.K.) with 1x Extensor Buffer 1, 200 μM dNTPs, 0.2 μM each upstream and downstream primers, and 200 ng template DNA. The complementary target was amplified with the upstream primer R3B (5' CACTGACCTCCCACATTCCC) and downstream primer HBB103for (5' AACGTGGATGAAGTTGGTGGTGAGG) using the plasmid containing the full length β-globin gene sequence as template. The noncomplementary target was amplified with the upstream primer CYPex4F (5' AGGCGACCCCTTACCCGCATCTCC) and downstream primer CYPex7R (5' TGCTGAGCTGGGGTGAGGAGGGC) using as template a plasmid containing the full-length CYP2D6 gene sequence. Thermocycling conditions for Long PCR were 94 °C for 2 min (initial denaturation), followed by 10 cycles at 95 °C for 20 s, Tm°C for 30 s and 68 °C for 2 min, and further 25 cycles at 95 °C for 20 s, Tm°C for 30 s and 68 °C for 2 min with 1 s increment/cycle, and a final extension at 68 °C for 10 min (Tm temperatures for complementary and noncomplementary target amplification were 68 °C and 57 °C, respectively).

C. Preparation of Au Nanoprobes

Au nanoprobes were prepared as previously described [8], using the 15 bp oligonucleotide described in Section II-B, above. Briefly, gold nanoparticles were synthesized by the citrate reduction method of Lee and Meisel [21], with an average diameter of 17 nm as determined from transmission electron microscopy observations (see inset to Fig. 2 for an example image). These gold nanoparticles (≈8.6 nM) were derivatized with 10 μM of the 15 bp thiolated oligonucleotide (STABVida, Portugal; sequence described in Section II-B, above), and resuspended in 10 mM phosphate buffer (pH 7), 0.1M NaCl, to a final concentration of gold nanoparticles of 9.7 nM as determined by Beer–Lambert law assuming a calculated molar absorptivity for the plasmon resonance band maximum (526 nm) of 2.33×10^8 M^{-1} cm^{-1} [22].

D. Colorimetric Assay

Each colorimetric assay consisted of three mixtures ("positive," "negative," and "blank"). Each mixture had a final volume of 60 μl and contained the Au nanoprobe at a final concentration 2.5 nM, plus: complementary DNA at 36 $\mu g \cdot ml^{-1}$ ("positive"); noncomplementary DNA at 36 $\mu g \cdot ml^{-1}$ ("negative"); 10 mM phosphate buffer (pH 8) ("blank"). After 5 min of denaturation at 95 °C, the mixtures were allowed to slowly cool down to 60 °C for 5 min, 40 °C for 5 min, and 25 °C for 30 min. A 5 M NaCl solution was then added for a final NaCl concentration of 0.5 M. Following 5 min at room temperature for color

development, photographs were taken and UV-visible spectroscopic measurements were carried out.

E. AFM Samples

To prepare the AFM samples of DNA material alone (linearized plasmid or PCR fragments), all buffers were removed from solution and the DNA was resuspended in buffer suitable for deposition for AFM experiments as described below. To prepare the AFM samples of Au nanoprobes alone or with DNA, all the steps of the colorimetric assay, except for NaCl addition were carried out. The mixtures were then centrifuged for 25 min at 30 000 g, followed by removal of supernatant. The red oily precipitate was washed twice with 100 μl of water and finally resuspended in 60 μl of water to discard salt/buffer crystals that would affect AFM measurements and remove nonhybridized nucleic acids.

F. AFM Analysis

For AFM analysis, samples were deposited in one of two ways: from water solution, or from buffer solution (10 mM Tris-HCl, pH 7.5, 1mM $NiCl_2$). All samples were deposited onto freshly cleaved mica sheets, and imaged in air. The samples in pure water were simply pipetted onto freshly cleaved mica followed by drying with Ar and vacuum. DNA samples were deposited from buffer solution incorporating Ni^{2+} ions to increase adhesion to mica [23]. Samples were deposited, left for 2 minutes and then washed with water, followed by drying as above. Imaging was carried out with either a Veeco Multimode AFM with a Nanoscope 4a controller, or a Molecular Imaging PicoLE AFM. No significant difference was found in the images produced by the two machines. In both cases, the AFM was operated in tapping mode, and rectangular silicon cantilevers of resonant frequency about 75 kHz (MikroMasch, Tallinn, Estonia) were used.

III. Results and Discussion

We have been developing a colorimetric assay using Au nanoprobes to detect specific DNA and RNA sequences [8]–[10]. Here we applied the method to the detection of a specific region of the β-globin gene (see Experimental Section for details). Fig. 1 presents the schematics of the method showing the colorimetric detection results. As expected, upon salt addition the "blank" and "negative" samples show extensive Au nanoprobe aggregation detectable by the blue color of the respective solutions; whereas the "positive" sample containing the complementary DNA does not show that effect and the solution remains red. This colorimetric method is thought to be based on the hybridization between the single-stranded DNA sequence present in the Au nanoprobes and the complementary DNA sequence present on a double-stranded DNA target, leading to stabilization of the Au nanoprobes in solution and avoiding its aggregation upon salt addition [8]. Here, we used AFM as an imaging technique in order to elucidate the interactions taking place at the molecular level.

AFM images were first made of the Au nanoprobes and DNA alone (Fig. 2). The three DNA samples were all in the double stranded form and consisted of the linearized p158 plasmid (≈8668 bp) containing the full length β-globin gene sequence; a 1559 bp PCR product including a portion of sequence complementary to the oligonucleotide sequence present in the Au nanoprobes; and a 1565 bp PCR product without any complementary sequence to the oligonucleotide sequence of the Au nanoprobes. Fig. 2 shows representative images of these DNA samples as well as the Au nanoprobes alone. As expected, the Au nanoprobes show a more or less globular morphology and height measurements (diameter) average about 17 nm. Although not shown in Fig. 2(A), some Au nanoprobes were present as small aggregates (2-8 Au nanoprobes); however, the vast majority of the Au nanoprobes were observed in an isolated form. It can be seen from Fig. 2 (B), (C), and (D) that all the visualized DNA molecules were separated and of uniform length and height, as expected. AFM provided a way to verify that no enzymatic digestion had degraded either the plasmid or the PCR products. For the complementary PCR product [Fig 2(B)] the height (diameter) was 0.5 nm, and the length averaged 488 nm. This length is as expected for the DNA fragment used (1559 bp), given the length per base pair and taking into account known errors in AFM measurements of this kind [24], as the theoretical length based on the value of 3.2 å per bp from crystallography would be 499 nm. Similarly, the noncomplementary PCR product, with 1565 bp and a theoretical length of 501 nm, was determined by AFM to measure 477 nm [Fig. 2(C)]. The AFM image of the linearized plasmid shows, as expected, much longer molecules, and the mean length as measured by AFM was 2802 nm, the theoretical value being 2774 nm for the ≈8668 bp. The length of these longer molecules is hard to determine by this technique, as it is not easy to find molecules without "overlaps," which complicate analysis [Fig. 2(D)]. Though typical for AFM measurements, the height of the DNA is rather different to the theoretical value of 2 nm, and is usually attributed to the presence of a thin salt layer on the surface, obscuring some of the DNA, as well as to tip–sample interactions (e.g., squashing of the sample, and electrostatic interactions) [25], [26].

Attempts to visualize hybridization of the Au nanoprobes to specific target sequence within the linearized p158 plasmid were carried out by heat denaturation of the Au nanoprobes and the plasmid at 95 °C for 5 min, followed by slow cooling to room temperature (to allow hybridization to occur), as described in the experimental section. This procedure is standard in DNA–DNA hybridization assays, as the objective is to denature the target DNA and obtain single-stranded DNA. In this way, the exposed DNA sequence and the complementary Au nanoprobe sequence can easily anneal together. The linearized plasmid had been obtained by restriction enzyme digestion so that the complementary sequence to the Au nanoprobes sequence is located at about one third of the way along the length of the linear plasmid. Once cooled, the samples were deposited onto freshly cleaved mica and imaged by AFM. A representative result is shown in Fig. 3, an unambiguous picture of the Au nanoprobes bound to the plasmid–DNA. However, as the DNA appeared to have formed complicated supramolecular structures, it is not clear whether there is a single Au nanoprobe per plasmid, as would be expected if the binding was by hybridization; and it is not possible to ascertain where along the DNA molecule the Au nanoprobe

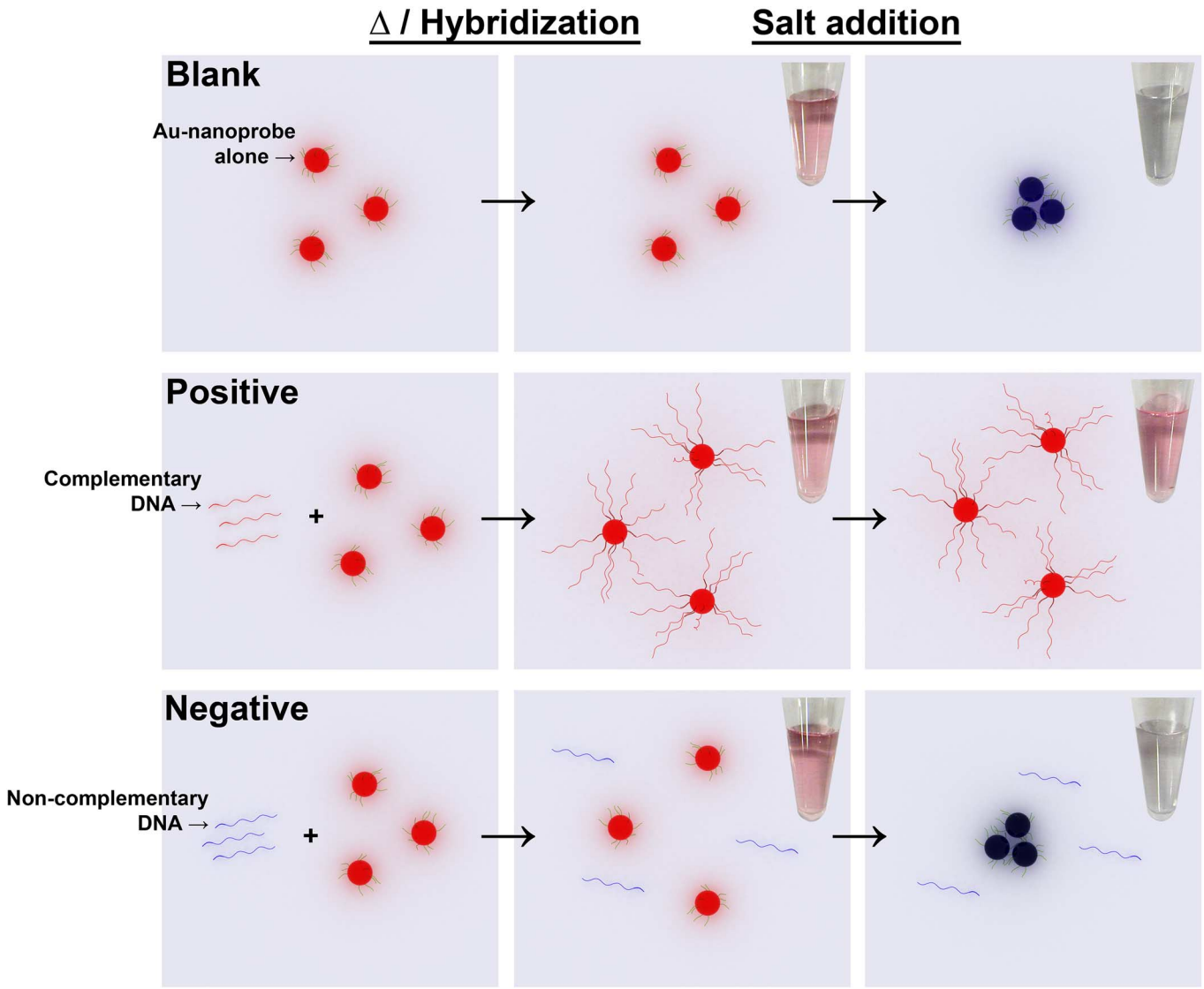

Fig 1. Schematic representation of the colorimetric assay using Au nanoprobes to detect specific DNA sequences. The method consists of visual/spectrophotometric comparison of three test solutions before and after salt induced Au nanoprobe aggregation: Au nanoprobes alone (Blank); Au nanoprobes in the presence of a complementary DNA target (positive); and Au nanoprobes in the presence of a noncomplementary DNA target (negative). After heat treatment to denature the double-stranded target DNA, the Au nanoprobes containing single-stranded DNA are allowed to hybridize at room temperature. After salt addition, both the "blank" and the "negative" control samples turn blue, revealing Au nanoprobe aggregation, while "positive" samples do not present Au nanoprobe aggregation, maintaining its initial red color (see experimental section). Inset pictures in each panel represent results of a detection test of a specific region of the β-globin gene, using the Au nanoprobes and PCR fragments as described for the AFM experiments.

hybridized. Although complicated, these large aggregates involving several Au nanoprobes and several targets are likely to exist in solution when the colorimetric assay is performed, helping to stabilize hybridized Au nanoprobes and avoiding aggregation upon salt addition, as observed for positive samples. This hybridization experiment was repeated several times for AFM imaging, but 1 : 1 Au nanoprobe–target aggregates were very rarely seen. In order to simplify the experiment, a shorter DNA fragment presenting a hybridization site at the end of the molecule was used. A shorter fragment reduces the potential for DNA to wrap up into complex shapes, as seen in Fig. 3, thus simplifying analysis. DNA fragments of ca. 1500 bp, including at one end the complementary region to the sequence carried by the Au nanoprobes, were obtained by PCR amplification, as described in the experimental section. As a negative control (noncomplementary DNA), DNA fragments with a similar size were obtained by PCR amplification of an unrelated DNA sequence. In order to visualize the hybridization of the Au nanoprobe to the target DNA, hybridization with the target PCR fragments was carried out in solution followed by resuspension in pure water, followed by deposition onto mica and imaging by AFM. Fig. 4(A) presents a typical image of the sample obtained in this way, with the complementary PCR fragment: several different species are visible, namely, individual (nonhybridized) Au nanoprobes and targets, individual Au nanoprobe–target aggregates, and more complex structures combining more than one target and more than one Au nanoprobe. Some cases of 1 : 1 probe–target aggregates where the probe is at the end of the DNA molecule (white arrows), and where the probe is in the middle of the DNA molecule (gray arrows) are highlighted. In order to simplify analysis, only cases of aggregates consisting of a single Au nanoprobe and a single target were further ana-

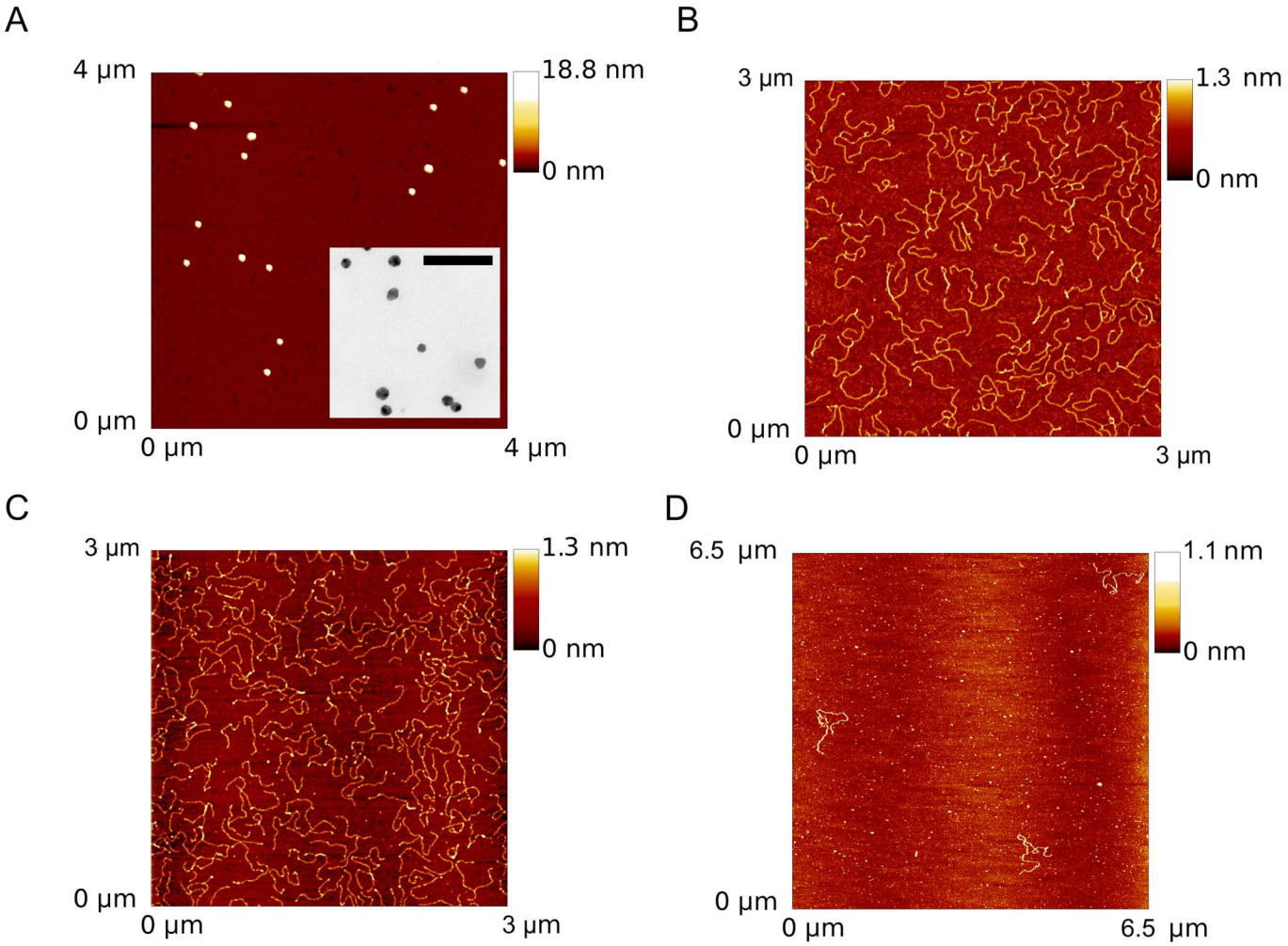

Fig 2. AFM images of: (A) the Au nanoprobes alone—inset shows TEM of bare gold clusters, scale bar = 100 nm); and (B) DNA alone, namely the 1559 bp complementary PCR product; (C) the 1565 bp noncomplementary PCR product; and (D) the ≈8668 bp linearized p158 plasmid.

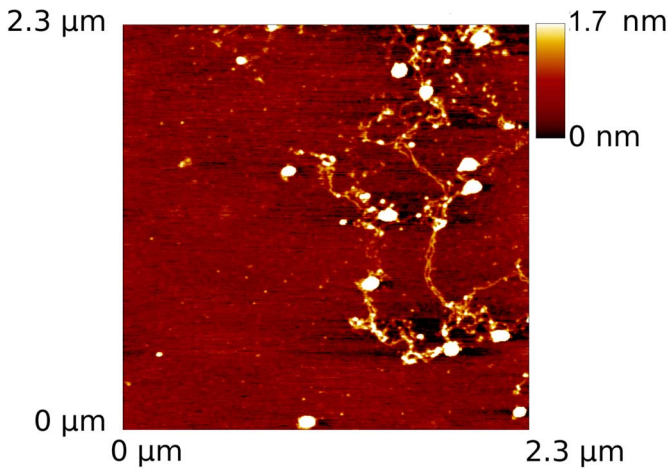

Fig 3. AFM image of Au nanoprobes hybridized to the linearized p158 plasmid.

lyzed. However, it is important to note that Fig. 4(A) shows that the real situation was more complicated than this. To analyze these 1 : 1 Au nanoprobe–target aggregates, the height of the Au nanoprobe was measured firstly to check that it was a single Au nanoprobe. Then the length of the "tail" of the aggregate was measured, in the case where the Au nanoprobe appeared to be hybridized at one end of the PCR fragment. In the case of the Au nanoprobe apparently hybridized in the middle of the PCR fragment (i.e., an aggregate with two "tails"), the distance was measured to the closest end. It was found that in the majority of the analyzed cases (ca. 70%), the Au nanoprobes had bound to the target at the end of the PCR fragment, where the complementary sequence is located. In these cases, the length of the "tail" of DNA extending form the Au nanoprobe varied from 216 to 494 nm, with a mean value of 355 nm. The theoretical length of this complementary PCR fragment (1559 bp; 3.2 å per base pair) was 499 nm. Considering the terminal position of the target sequence on the PCR fragment, the theoretical tail length if the Au nanoprobe was hybridized at the target sequence would be 483 nm. The considerable number of the overhanging PCR fragment sequences smaller than this value can be possible explained if we consider that these overhanging fragments have considerable freedom of movement, and some of them can wind back on themselves, possibly stabilized by nonspecific interactions with the Au nanoprobe. A negative control for this experiment was obtained from a sample prepared with noncomplementary PCR product via an identical procedure. As may be observed in Fig 4(B), there was practically no DNA attached to

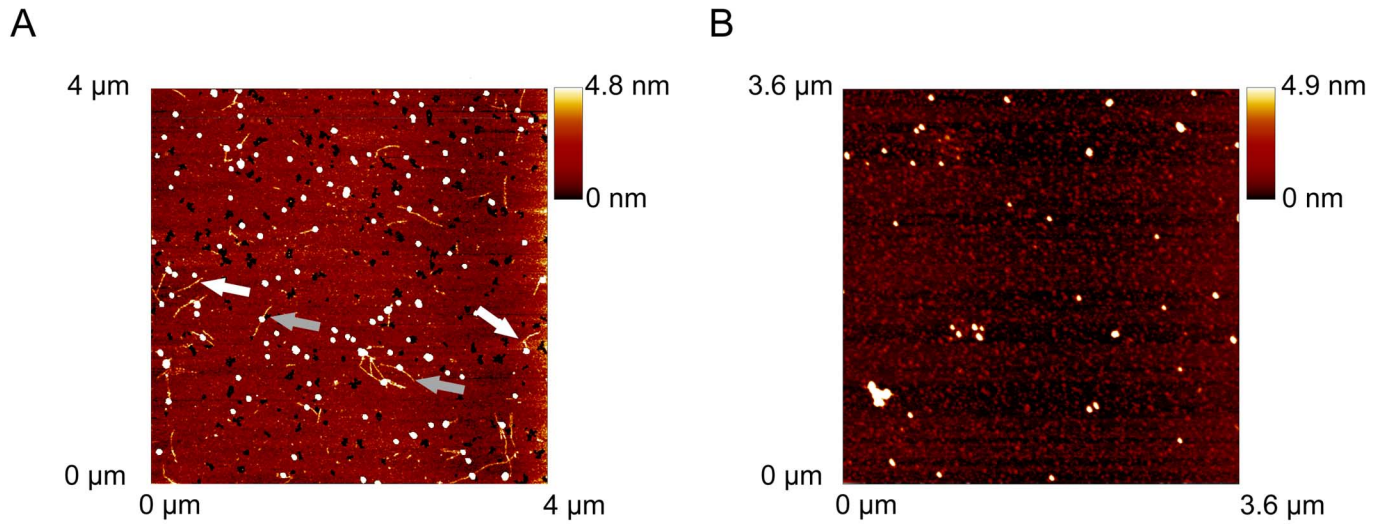

Fig 4. AFM images of: (A) Au nanoprobes hybridised with a complementary PCR product; and (B) with a noncomplementary PCR product. Arrows indicate 1 : 1 probe-target aggregates where the probe is at the end (white arrows), and in the middle (gray arrows) of the DNA molecule.

the Au nanoprobes. This observation of no DNA bound to the Au nanoprobes when submitted to the hybridization protocol with noncomplementary DNA, confirms our theory that specific hybridization to the target sequence is the method of binding in the case of a complementary DNA sequence.

In order to better elucidate the Au nanoprobe–target DNA interaction, future work will attempt to stretch the overhanging plasmid (for example, by methods used to straighten DNA on a surface [27], [28]). With the target DNA extended in one direction, an increase in overall "tail" length is expected, assuming that the majority of the interactions are, indeed, by hybridization to the target sequence. On the other hand, if the interactions are nonspecific, and weak, we may find that attempting to stretch the DNA will produce fewer Au nanoprobes bound to target DNA.

IV. Conclusion

AFM images obtained from the hybridization experiments of Au nanoprobes and PCR fragments containing the complementary DNA sequence, showed that hybridization occurs between those DNA sequences and the sequence born by the Au nanoprobe. Such hybridization is specific for the complementary DNA sequences as can be concluded from the position of the Au nanoprobes along the PCR fragment. In fact, about 70% of the analyzed cases revealed that the Au nanoprobes had bound at the end of the PCR fragment, where the complementary sequence is known to be located. As still about 30% of the interactions apparently occurred away from the end of the PCR fragment, nonspecific interactions cannot be ruled out at this point. It should be noted, however, that the experiments described here do not in any way measure the strength of the interaction, or, indeed, if any is occurring. In conclusion, the fact that the majority of the nanoparticles were found at the ends of the target molecules strongly supports hybridization to the complementary sequence as the primary interaction with the Au nanoprobes. A molecular interaction basis for the colorimetric detection method is thus established, providing further support for a quick and simple detection assay for specific DNA sequences.

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