A novel assay for selective determination of polynucleotides using atomic force microscopy in conjunction with the formation of the probe/target/DNA-gold nanoparticle sandwich structure at a gold surface is described. A 17-mer probe was attached to the surface for subsequent hybridization with a polynucleotide target. Due to the flat orientation of the probe-target hybrid with respect to the surface and the spatial obstruction of the unhybridized probes near the hybrids, the AFM images are not clear. The hybridization efficiency was estimated to be about 1.1% since certain surface features could not be resolved. The utilization of 30-mer-capped gold nanoparticles not only provides another dimension of selectivity, but also reorients the previously formed probe-target hybrid in such a way that the strands of the target become tethered with respect to the surface. This reorientation improves the resolution in imaging the hybridized target molecules and provides an accurate determination of the hybridization efficiency (16%).

Key Words: DNA sensing; atomic force microscope; gold nanoparticles; DNA surface orientation.

Sequence-specific DNA assays of target nucleic acids by hybridization with DNA probes at a solid/solution interface (heterogeneous DNA sensors) are an important avenue for detecting DNA samples of biological origins (1, 2). In the past few years, the use of thiolated oligonucleotides immobilized onto gold surfaces has received a considerable amount of attention (3–8), due in part to the control and flexibility in forming self-assembled monolayers (SAMs) of DNA and/or mixed DNA/alkanethiol SAMs. Thiolated DNA probes have also been immobilized onto thin gold films for gravimetric analysis (9, 10), electrochemical detection (11, 12), and AFM imaging of DNA targets (5, 13, 14). More recently, several rapid, sensitive, and selective DNA detection methods using gold nanoparticles covered with oligonucleotides have been developed by Mirkin, Letsinger and their co-workers (15–21). In addition to the fundamental interest and potential applications associated with these systems (3, 4, 6–8, 14, 22–24), the formation of well-ordered DNA films and nanoparticle networks has also enhanced the selectivity and sensitivity of other types of DNA detection schemes. For example, oligonucleotide-capped gold nanoparticles have been shown to improve the sensitivity of techniques such as quartz crystal microbalance (25, 26) and surface plasmon resonance (27) for the detection of DNA hybridization. Recently, a comparison of gold films and nanoparticles for the attachment of thiolated oligonucleotides and for the subsequent DNA hybridization has been made by Demers et al. (15)

We report here the use of a probe/target/DNA-nanoparticle sandwich structure in conjunction with AFM imaging for the detection of hybridization of large polynucleotide targets (M13 phage DNA) across a small and highly localized surface. AFM has been demonstrated to be a powerful and sensitive method for detecting surface-confined DNA molecules at molecular levels (28, 29). The employment of DNA-capped gold nanoparticles in this procedure caused a somewhat unexpected rearrangement of the surface-confined probe-target hybrids and the rearrangement significantly enhanced the quality of AFM imaging. As a consequence, the hybridized target molecules become easily discernable at the microscopic level and the DNA hybridization efficiency can be accurately determined. The oligonucleotide present on the gold nanoparticles provides another dimension of specificity which enabled us to distinguish polynucleotides of very similar sizes and base sequences (M13 mp18(+) vs M13 mp19(+)). The reorientation of the probe-target hybrid by the gold nanoparticle is explained on the basis of steric hindrance and electrostatic interaction with the adjacent probe molecules. We also carried out contact angle measurements in an attempt to under-
stand the surface structures of the various DNA-covered gold surfaces at the macroscopic level.

EXPERIMENTAL

Materials

EDTA and Tris·HCl were both from Sigma (St. Louis, MO). Hydrogen tetrachloroaurat(Iii) hydrate and sodium citrate were obtained from Aldrich Chemicals (Milwaukee, WI). M13 mp18(+) and M13 mp19(+) ss-phage DNA were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and Life Technologies (Rockville, MD), respectively. M13 phage DNA has 7249 bases and the strand length should be around 2.46 μm if 0.34 nm is used as the base spacing. But this value is probably an overestimate since the 0.34 nm spacing is for less flexible double-stranded DNA. The thiolated 17-mer probe, SH-(CH2)6-5′-GTAACGACGGCCAGT-3′ and the thiolated 30-mer 5′-AGAGGATCCCGGTACGGCTCGAATTCC-3′-(CH2)3SH were both acquired from IDT Inc. (Coralville, IA). The 17mer is selective to both targets since the hybridization occurs outside of the multiple-cloning site, but the 30-mer, immobilized onto gold nanoparticles, is only selective to the multiple cloning site of M13 mp18(+). The site on the M13 mp18(+) that is complementary to the 17-mer is 31 bases away from the multiple cloning site. Gold (111) evaporated on mica were used as the substrate for AFM imaging.

Procedures

(a) Solution preparation. The DNA probe stock solutions were prepared in Tris·HCl/EDTA (TE) buffer solutions (10 mM Tris·HCl and 1 mM EDTA) inside a glove box (Pias Labs, Lansing, Mich.). Dilute probe solutions were prepared daily using aliquots pipetted from the stock solution inside the glove box. Target M13 phage DNA solutions were prepared with 10 mM Tris·HCl/0.1 M NaCl (TNE) buffer. The synthesis of gold nanoparticles follows the literature procedure (30, 31), and the size (13 nm in diameter) of these nanoparticles was confirmed with UV–visible spectrometry. The DNA-capped gold nanoparticles were prepared by reacting 5.5 mL of 17 nM gold nanoparticles with 12 mM 30-mer oligonucleotide. After 16 h, the solution was brought TNE solution and allowed to stand for 40 h. This was followed by centrifugation for at least 25 min to remove excess reagents. The red oily precipitate was then washed twice with the Tris·HCl/NaCl solution.

(b) Atomic force microscope experiments. The AFM instrument and operational conditions have been described elsewhere (14). Gold substrates were first soaked in 0.5 μM 17-mer DNA for 4 h at room temperature in the glove box. This was followed by exposing the probe-modified substrate to 6.7 μg/mL target DNA in TNE for 30 min. Prior to AFM imaging, the DNA-covered gold substrate was rinsed with deionized water. The AFM images were then collected in a liquid cell which contained a TNE solution. For experiments that involved the use of the DNA-capped gold nanoparticles, the gold substrate covered with both probe and target DNA molecules was immersed in a 94 mM 30-mer-capped gold nanoparticle solution for 2 h. After thorough rinsing with water, the film was imaged in the TNE solution.

(c) Contact angle measurements. Contact angles at bare gold substrate and gold surfaces covered with DNA molecules and DNA/nanoparticles were measured using a Cam-Plus Microcontact angle meter (KSV Instruments, Monroe, CT). Contact angle measurements and AFM imaging experiments were performed concomitantly at the same substrate in order to provide a one-to-one comparison during data interpretation.

RESULTS AND DISCUSSION

Figure 1 illustrates a simplified schematic of the three-step procedure that improves AFM imaging of polynucleotides. This procedure also provides an opportunity for an additional discrimination of polynucleotide targets of similar size and base sequence. In Step 1, the immobilization of thiolated oligonucleotides produces a DNA film whose coverage and molecular orientations have been well studied by other researchers (3, 4, 7, 8, 22) as well as in our previous AFM work (32). Tarlov and co-workers showed this model to represent the possible arrangements of thiolated DNA at gold surfaces (3, 22). They also demonstrated experimentally that the DNA film is not as compact as that of a regular alkanethiol and the DNA orientation is somewhat random due to the interaction between the DNA bases and the gold surface. In Step 2, the M13 phage DNA target was hybridized by the surface-confined oligonucleotide probes. As revealed in our previous AFM work and confirmed again in the images shown below, the hybrids tend to adopt exclusively an orientation in which the DNA coil(s) are parallel to the underlying substrate (14). We have shown that the
hybridization efficiency between the probe and the polynucleotide is far below that observed between the probe and an oligonucleotide target. Because of this orientation, the hybrids were “buried” underneath the rambling strands of unhybridized probes. Such a configuration renders difficulty to AFM experiments as the imaging relies on the interaction of the AFM tip with surface features without severe obstruction (28, 33, 34). As will be seen below in the description of our AFM images, the use of DNA-capped gold nanoparticles helps resolve this problem. The use of such nanoparticles, with the DNA caps having a sequence complementary to a different portion of the polynucleotide, causes the DNA coil(s) to tilt with respect to the substrate. As a consequence, a major portion of the hybrid DNA strand protrudes above the short unhybridized probe molecules.

Figure 2 shows a series of AFM images obtained upon different DNA immobilization and hybridization reactions. Shown in Fig. 2a is a representative image of a gold substrate modified with the thiolated 17-mer DNA probe. The size and distribution of the oligonucleotides agree well with those of our previous study, with the dots having an average diameter of about 26–40 nm and a surface density of about $1.1 \times 10^{10}$ molecules/cm$^2$. The average height of the probe is about 4–10 nm which is in close agreement with the theoretical value expected from a 17-mer (about 6 nm). The spacing between two neighboring probes is typically around 20–50 nm, which is almost two orders of magnitude wider than that expected from the (\(\sqrt{3} \times \sqrt{3}\))R30$^\circ$ packing of a typical alkanethiol (The nearest spacing is 0.5 nm) (35). A typical AFM image of the surface upon hybridization in a M13 mp18(+) target solution is displayed in Fig. 2b. As can been seen, many circular structures are vaguely discernable. These circles have an average length of 0.7–1.2 \(\mu\)m and a width of about 30–50 nm. The strand width observed by AFM is typically around 13 ± 2 nm from various AFM studies of DNA (13, 28, 29). The somewhat greater strand width found here is mainly ascribed to the uncertainty in obtaining a value from the relatively blurry images of these circular structures. It is therefore apparent that the position of the “buried” polynucleotide relative to the tethered unhybridized probe molecules is not favorable for AFM imaging. It is also worth mentioning that the height of these circles, deduced from the cross-sectional contours, is about 2 nm. Such a value is in close to the theoretical strand width of DNA molecules (36). Owing to the flat orientation and the obstruction arising from the adjacent unhybridized probe molecules, it is difficult to deduce the exact number of the circular DNA molecules with AFM. While Fig. 2b qualitatively proves that hybridization does take place, there is a large uncertainty in the estimation of the hybridization efficiency. When we count only the resolvable circles and treat them as the possible oligonucleotide-polynucleotide complexes, the surface density of the hybrid DNA molecules was found to be about $1.2 \times 10^{8}$ molecules/cm$^2$. Thus the hybridization efficiency appears to be around only 1.1% (calculated by dividing the surface density of the hybrids by that of the probes deduced from Fig. 2a).

Utilizing the same DNA probes immobilized on the gold surface (in Step 1), M13 mp19(+) was also found to be hybridizable and the resultant AFM image is shown in Fig. 2c. A comparison between Figs. 2b and 2c indicates that both surfaces are similar. This is not surprising since the probe has a sequence that is also complementary to M13 mp19(+) and both types of targets are very similar in terms of their structures and base sequences. As expected, the probe binds effectively to both polynucleotide targets.

As mentioned in connection with the description of the procedure, we carried out an additional analysis by using gold nanoparticles covered with a 30-mer whose sequence is complementary only to the multiple cloning site of the M13 mp18(+). While the AFM image of the surface in Fig. 2c remained essentially unchanged after being exposed to the DNA-capped gold nanoparticle solution for 2 h (not shown), the AFM image of the surface represented by Fig. 2b was significantly different. As shown in Fig. 2d, many oblong objects appear upon the implementation of Step 3 in Fig. 1. Close
Hybridization of oligonucleotides immobilized onto gold nanoparticles with the flat-lying polynucleotide molecules present at a gold substrate resulted in a dramatic change in the orientation of the probe-polynucleotide-DNA/nanoparticle sandwich structure. The sandwich structure adopts an orientation in which the polynucleotides become tethered to the surface, thus leading to a significant increase in the contact angle. This change can be attributed to the increased hydrophilicity of the surface due to the hybridization of the probes. The unhybridized probes, as well as the similar repulsive interactions between the oligonucleotides on the gold nanoparticles and the polynucleotide target, contribute to this change.

CONCLUSION

Hybridization of oligonucleotides immobilized onto gold nanoparticles with the flat-lying polynucleotide molecules present at a gold substrate resulted in a dramatic change in the orientation of the probe-polynucleotide-DNA/nanoparticle sandwich structure. This change can be attributed to the increased hydrophilicity of the surface due to the hybridization of the probes, leading to a significant increase in the contact angle. The unhybridized probes, as well as the similar repulsive interactions between the oligonucleotides on the gold nanoparticles and the polynucleotide target, contribute to this change.
With the aid of DNA-capped nanoparticles, the hybridization efficiency was found to be about 16%. This is much greater than 1.1%, an underestimate based on the inclusion of only the resolvable polynucleotides prior to the interaction with the DNA-capped nanoparticles. The reorientation is probably driven by the necessity to reduce steric hindrance and electrostatic repulsion between the polynucleotide-DNA/nanoparticle adducts and the unhybridized probes. Through the second hybridization with the DNA-capped gold nanoparticles, we not only show that the procedure is useful in differentiating targets of similar sizes and base sequences (e.g., M13 mp18(+) vs M13 mp19(+)), but also confirm that the observed reorientation does not result from nonspecific adsorption of the DNA-capped gold nanoparticles. Finally, the agreement between the contact angle measurements and the AFM experiments at the various surfaces suggests that our proposed model is plausible in explaining the processes at such a heterogeneous DNA sensing surface from both macroscopic and microscopic viewpoints.

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REFERENCES