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Talanta 67 (2005) 479–485
Talanta
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Towards single-spot multianalyte molecular beacon biosensors

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Available online 2 August 2005

Abstract

The separate developments of microarray patterning of DNA oligonucleotides, and of DNA hairpins as sensitive probes for oligonucleotide identification in solution, have had a tremendous impact on basic biological research and clinical applications. Herein, we will discuss several successful efforts to develop oligonucleotide sensors based on the surface immobilization of functionalized DNA hairpins. We also will discuss the development of prototypical single-spot multianalyte "Molecular Beacon" biosensors. Importantly, we show that organic fluorophores will likely be inadequate in moving this technology forward and new approaches, such as the use of nanotechnology, will be needed.
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Keywords: Biosensor; Molecular beacon; Nanocrystals; Single-base mismatch; Microarray

1. Introduction

Currently, the ability to cure most viral and bacterial infections depends critically on an accurate diagnosis [1]. This ever-increasing need for more sensitive diagnostics coupled with the increasing availability of genetic information for a broad range of infectious organisms has led to a surge in the development of new DNA-based diagnostic aids. With respect to high levels of sensitivity and selectivity, few techniques can match the performance that has been exhibited by solution-phase molecular beacons [2]. Molecular beacons, as first described by Tyagi and Kramer [3], consist of short (∼30–40) oligonucleotide strands that are modified at one terminus with a fluorescent dye, and at the other with a quenching moiety, which can either be organic or metallic in nature. The molecular beacon will naturally adopt a hairpin (or stem-loop) structure which forces the dye and quencher into close proximity causing the fluorescence to be quenched via nonradiative energy transfer. Upon introduction of a complementary oligonucleotide, the beacon undergoes a thermodynamically driven hybridization to form double-stranded DNA. The result of DNA duplex formation is that the quencher and fluorophore are separated to such an extent that energy transfer is no longer possible between the two, resulting in a large and easily observable fluorescent signal [3,4].

Due to the inherent specificity that encompasses DNA duplex formation, along with the strong energetic driving force for DNA duplex formation to occur, molecular beacons have become a leading tool in genetic analysis and species identification [5]. However, in addition to high accuracy there exists a pressing need for expediency in diagnosis, which also critically determines the probability that most viral and bacterial infections can be cured. Thus, development of arrayable sensing technologies, allowing one to perform thousands of experiments in the identical time it takes for a single one, has received significant recent attention [6]. For example, several successful attempts have been reported that involve adaptations of the standard solution-phase molecular beacon methodology to one that is performed immobilized on a surface [7–10]. The goals of these attempts are clear: to leverage the sensing capabilities of molecular beacons in an arrayable format, providing highly parallel data processing in a single experiment.

Herein, we will discuss several successful efforts to develop oligonucleotide sensors based on the surface

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doi:10.1016/j.talanta.2005.06.040

immobilization of functionalized DNA hairpins, followed by a more in depth discussion of work performed by our group towards the development of prototypical single-spot multianalyte "Molecular Beacon" biosensors. Importantly, we believe that nanotechnology (specifically, in the form of semiconductor nanocrystals) will be instrumental in moving this technology forward.

2. Immobilized molecular beacons

In the majority of studies on immobilized molecular beacons, the substrate serves only a passive role in sensing. For example, in work by Malayer and coworkers [11] a traditional molecular beacon, (consisting of an 18 base loop, a 4 base-pair stem, and incorporating a black hole quencher on the 3′ end, a Cy3 fluorescent dye on the 5′ end, and terminated with a C6 amine modified thymine), was immobilized on a commercially manufactured aldehyde glass slide via reversible imine formation. This surface immobilization technique was then employed to create arrays of beacons capable of detecting synthetic mimics of the 16S rRNA from the bacterium *Francisella tularensis*, a feared bioweapon. A modest 4–6-fold increase in fluorescence intensity upon DNA duplex formation was observed, and the authors were capable of discriminating between totally matched and totally mismatched targets, demonstrating a *general* specificity for their intended target. An alternative immobilization strategy [8] used a traditional (but not biologically relevant) molecular beacon (3′ DABCYL, 5′ Rhodamine) containing a biotin linker covalently attached to the hairpin's stem to bind avidin immobilized on a silica surface. Fluorescence enhancements of 10-fold were observed after incubation with a target. Similar surface immobilization strategies based on avidin–biotin interactions have been used to detect hairpin invasion and subsequent DNA hybridization by a target oligonucleotide on the single molecule level [12,13]. Recently, surface immobilized molecular beacons have been combined with microfluidics to enable rapid surface hybridization reactions [14], and attached to microspheres for high throughput multiplexing [15].

3. Gold film DNA sensors

An alternative to a passive surface is to involve the substrate itself as a participant in the signal transduction process. Heeger and coworkers have recently reported on an immobilized molecular beacon-based sensor that provides an electrochemical readout [10]. Creation of these sensors is accomplished via the immobilization of DNA hairpins, modified at one terminus with a thiol and at the opposite terminus with a ferrocene derivative, onto a Au film. Once immobilized, the ferrocene tag is brought into close proximity with the Au surface via hairpin formation, which allows for efficient electrochemical oxidation/reduction (redox) of the ferrocene. Upon hybridization with complementary DNA, the ferrocene moiety is separated from the Au surface, causing a cessation of the ferrocene redox. Using this technique, Heeger and coworkers demonstrated remarkable speed and sensitivity: a significant signal can be achieved in the presence of as little as 5 fmol target DNA within 30 min. However, this type of sensor employs an "off-signal" mechanism for determining the presence of its designed target (that is, signal strength *decreases* in the presence of the target, rather than increases), which is not ideal. For example, use of this type of mechanism allows for the possibility that contamination of a sample by a DNase could result in the improper destruction of the redox signals.

The use of Au films as functional components of biosensors are not limited to electrochemical processes. Noting the ability of metals to quench fluorescence, we have developed a label-free optical assay that merges the sensitivity and selectivity of molecular beacons with the quenching efficiency of gold [9]. Using an Au film as both the immobilizing substrate and quenching agent has the advantage of simplicity in design, as it limits the number of components that could potentially fail in assembly of the sensor (Fig. 1). The level at which an Au film is capable of quenching fluorescence is critical; efficient fluorescence quenching implies small background and thus potentially a high signal-to-noise ratio. Large signal-to-noise ratios become increasingly critical as the similarity between two potential targets increases, and in the case of single-base mismatches, they are essential.

To test our theory that Au films would provide an ideal functional substrate, rhodamine-labeled molecular beacons specific for genes known to confer methicillin resistance to the bacterium *Staphylococcus aureus* (SA) were immobilized onto thin gold films. With these prototypical molecular beacon biosensors, we have obtained quenching efficiencies in excess of 95% (Fig. 2). Put another way, we routinely

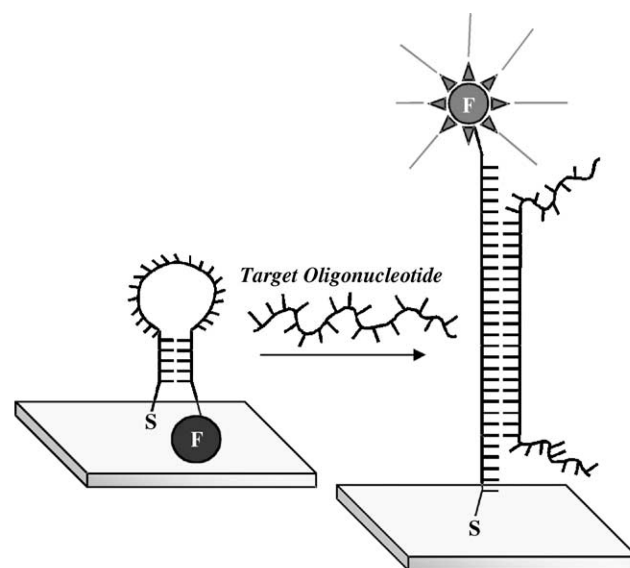

Fig. 1. Working principle of the DNA sensor.

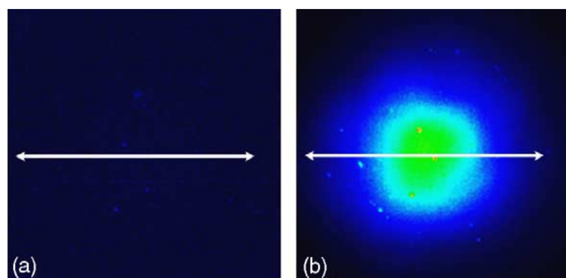

Fig. 2. Typical CCD fluorescence images of before (a) and after (b) hybridization. Excitation was typically at 514 nm.

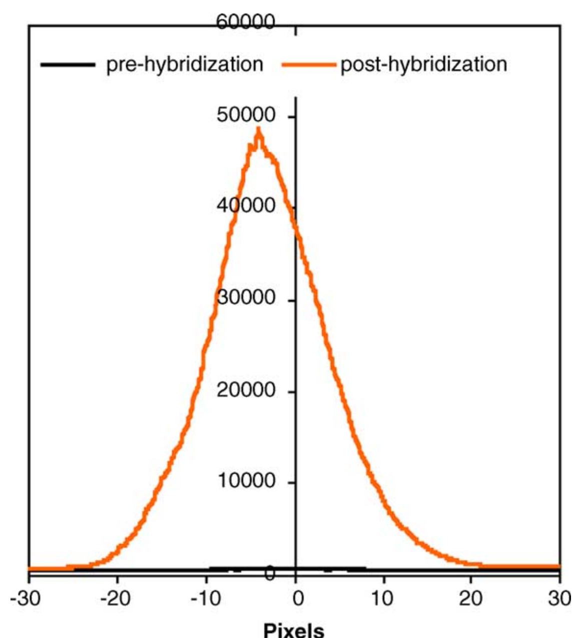

Fig. 3. Fluorescence intensity of the chip obtained by binning the CCD fluorescence images along the lines in Fig. 2. The difference between pre and post-hybridization signal intensities is over a factor of 100. The hairpin probe used is given by H1 in Table 1, and the target is given by T1.

observed signals greater than 20 times baseline, and in some cases signals over 100 times greater (Fig. 3). Importantly, these sensors have also demonstrated the proper ability to discriminate against not only completely mismatched DNA, but also against a large amount of genetic material, as illustrated by the limited response elicited by the treatment of the sensors with a high concentration of salmon sperm DNA (Fig. 4). This result is important because any future use in microarray technology for the purpose of genomic screening will be done in the presence of a large amount of non-specific oligonucleotides.

Fig. 4. True color fluorescence image of a Rhodamine-labeled DNA array on Au exposed to buffer (left), salmon sperm, which approximates a random DNA assortment (middle) and the complimentary sequence (right). The substrates were excited at 514 nm and the images were obtained on an inverted optical microscope. The intensity ratio of the fluorescent spots is 1:3:30.

4. Immobilized beacons for single-base mismatch detection

The ability to distinguish single-base mismatches demonstrates the ultimate sensitivity of DNA-targeted detection. Single-base mismatches are the most common form of genetic polymorphisms and can often be used to diagnose particular genetic predispositions toward disease and drug-response. Thus, the ability to accurately and rapidly discriminate them has direct implications for medical diagnostics. Solution-phase molecular beacons have traditionally excelled at discriminating single-base mismatches [3]. For example, single mismatch discrimination with DNA probes immobilized on gold nanoparticles exhibits a 4–25-fold difference in signal between the complements and mismatch [4,16,17]. However, in these experiments the reaction conditions (temperature or ionic strength) were biased in order to energetically destabilize the mismatched duplex relative to the fully complementary duplex. Using unfavorable reaction conditions for the mismatch is commonly referred to as changing the stringency of the assay, or a “stringency test”. Single mismatch discrimination without such “stringency tests” has been demonstrated for DNA hairpins immobilized on fiber-optic surfaces [18] and agarose coated glass [19]. However, the differences in signal between a perfect DNA match and the mismatch were small, approximately a factor of two in both cases.

We have investigated the ability of Au-surface immobilized molecular beacons to also distinguish single-base mismatches [20]. Two identical Au surfaces containing identical surface immobilized hairpin probes were prepared at the same time and then were respectively incubated with perfectly matched target and single mismatched target solutions of equal concentration. Typically, a significantly weaker fluorescence by an order of magnitude occurs for the

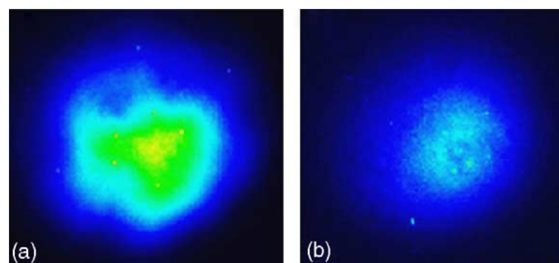

Fig. 5. Single mismatch discrimination: (a) CCD fluorescence intensity image of the perfect match; (b) CCD fluorescence image of the single-base mismatch. The probe is given by H2 (Table 1) and the target and mismatch are given by T2 and T2M1, respectively. The concentration of both the perfect match and mismatch is 2 μM.

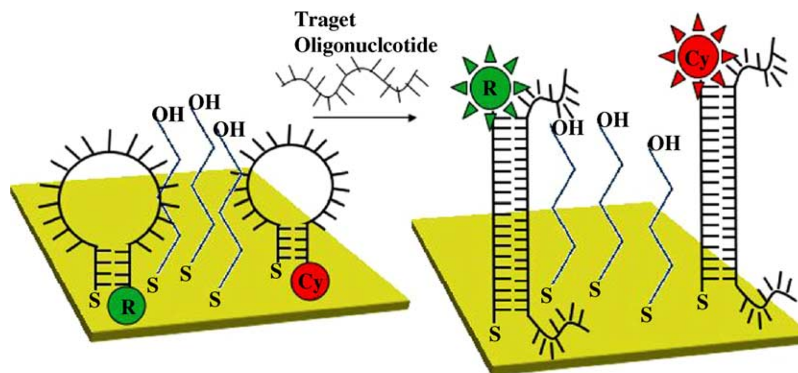

Fig. 6. Working principle of the two-color DNA sensor. Each probe spot consists of two different hairpins, one labeled with rhodamine and one with Cy5, and mercaptoethanol as a spacing molecule to prevent all the hairpins in the probe spot from interacting.

single mismatched target compared with the complement (see Fig. 5), although we did find that the mismatch discrimination varies with target concentration [20]. Significantly, for our studies hybridization takes place under identical reaction conditions for both the perfect match and the mismatched target (as opposed to including high stringency), which allows for a more direct comparison between responses. The high selectivity observed suggests that the molecular beacon itself only partially provides for the highly selective assay. Currently, efforts are underway in our laboratories to determine why we observe a significantly higher selectivity than that observed by researchers working with alternative immobilized molecular beacon formats.

5. Single-spot arrays

The high throughput afforded by microarray technology has revolutionized the field of molecular biology and is playing a significant role in both basic research and clinical genomic studies [6]. Their success has created a desire for improved microarrays to solve more complex problems. Multi-probe single-spot chips are an attractive strategy for increasing the number of probe types that can be immobilized per unit area without increasing fabrication complexity. Because of the inherently high specificity of molecular beacons, and gold's ability to quench fluorescence over a wide range of wavelengths, we have begun to examine the feasibility of immobilizing multiple molecular beacons on Au surfaces in the hopes of creating a functional, single-spot array.

As an initial test of the concept, we have examined the possibility of a single-spot, two-color "array" using two different organic dyes for fluorophores, as shown in Fig. 6. In both cases, we used two SA probes AH2 and BH2, derivatized initially with rhodamine and Cy5, respectively (Table 1). Organic dyes are generally ill suited to such schemes in which a single wavelength light source is used, even in a simple two-color implementation, since very few dyes have the requisite overlapping excitation spectra and well-separated emission spectra. Nonetheless, we believed that the spectral overlap between rhodamine and Cy5 would be sufficient to at least give some indication as to the effectiveness of molecular beacons when immobilized in a heterogeneous monolayer.

Solution-phase absorption and emission spectra for the AH2-Rhodamine and BH2-Cy5 probes are shown in Figs. 7 and 8, respectively. Results from two chips prepared from a 1:1 mixture of AH2-Rhodamine and BH2-Cy5 in mercaptopropanol/water [21] and excited at 532 nm are shown in Fig. 9. As expected, both fluorophores are efficiently quenched by the gold surface in the absence of added complementary DNA. Addition of 1.0 μM AH2-complement yields a chip with significant fluorescence around 585 nm, while addition of 1.0 μM BH2-complement produces only weak Cy5 fluorescence (675 nm).

The weak fluorescence of the Cy5 post-hybridization is due to many contributing factors. First, although there is some overlap in the absorption spectra, the absorption maxima are

Table 1
DNA hairpin probes and their targets

Name	Sequence
H1 (probe)	5′-(-C6Thiol) ACACGCTCATCATAACCTTCAGCAAGCTTTAACTCATAGTGAGCGTGT-(-3′-Amino C7) (TMR)-3′
H2 (probe)	5′-(-C6Thiol) ACACGCTCATCAAGCTTTAACTCATAGTGAGCGTGT-(-3′-Amino C7) (TMR)-3′
T1 (H1complement)	5′-ACGCTCACTATGAGTTAAAGCTTGCTGAAGGTTATGA-3′
T2 (H2complement)	5′-ACGCTCACTATGAGTTAAAGCTTG-3′
T2M1 (single mismatch of H2)	5′-ACGCTGACTATGAGTTAAAGCTTG-3′
AH2 (probe)	5′-CGATAATATGATGCCTAGGCAGAAATATTATCG-(-3′-Amino C7) (TMR)-3′
BH2 (probe)	5′-TATCAATAATAAACGAATAGGGGTGTTAATATTGATA-(-3′-Amino C7) (Cy5)-3′

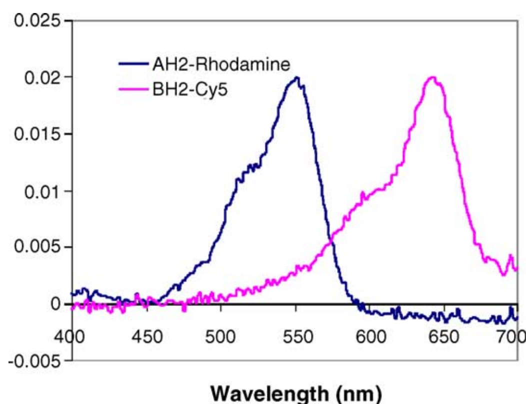

Fig. 7. Absorption spectra for AH2-Rhodamine and BH2-Cy5. Vertical axis has units of absorbance.

separated by approximately 100 nm. Thus, resonant excitation of rhodamine will not efficiently excite the Cy5, due to the low oscillator strength of Cy5 at ∼550 nm. Indeed, exciting the chip containing BH2-Cy5 at 633 nm, which is closer to the absorption maximum of Cy5, increases the signal by factors of 2–3. Second, the fluorescence quantum yield of Cy5 (∼25%) is over a factor of three smaller than that of rhodamine (∼90%), which further adds to a reduction in the Cy5 signal.

The high signal to noise required for the use of two molecular beacons immobilized in a single probe spot is further complicated by possible optical and hybridization interference between the two probes and their respective targets. For example, when using AH2-Rhodamine and BH2-Cy5, the Cy5 fluorescence is so weak relative to the rhodamine that it is difficult to differentiate the tail of the rhodamine emission from Cy5 emission, implying that unequivocal identification of unknowns containing mixtures of oligonucleotides would be problematic. In other words, a large positive response for AH2 would also show up as a (smaller) response for BH2 even with no target for BH2 present. Further, care must be taken in probe design to ensure that the target of one probe has minimal sequence complementarity with the other probe, since any significant base-pairing between one probe and its mismatched target will also result in a false-positive signal.

We have also examined the performance of a BH2-Oregon Green conjugate in combination with the AH2-Rhodamine probe, and since Oregon Green is a more robust emitter we expected some improvement in performance. However, the overall performance was basically unchanged, which demonstrates that new approaches are needed for single-spot, multianalyte assays. Of course, one could potentially employ multiple excitation sources in order to circumvent the problem of low spectral overlap. However, this increase in instrument complexity would at least partially negate the advantages of the single-spot format. Although recent innovative approaches to the development of fluorescent oligonucleotides may provide one solution to this problem [22], we have opted to initially pursue a nanotechnology-based approach.

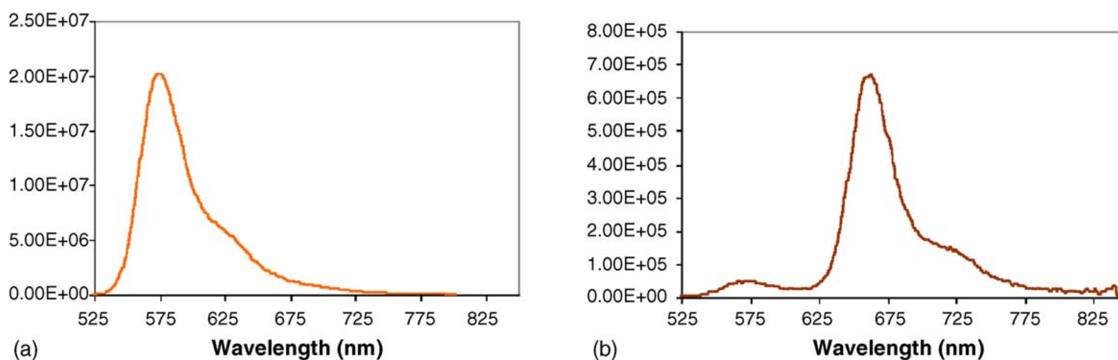

Fig. 8. (a) Fluorescence spectrum for AH2-Rhodamine; (b) fluorescence spectrum for BH2-Cy5.

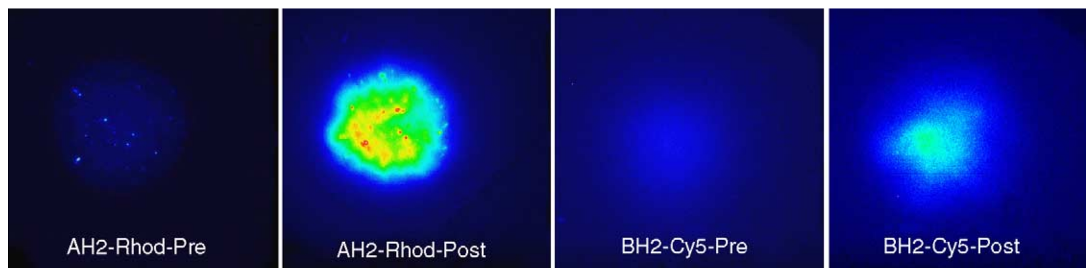

Fig. 9. CCD images of fluorescence from AH2-Rhodamine and BH2-Cy5 chips prior to (pre) and following (post) addition of their respective complementary DNA sequences.

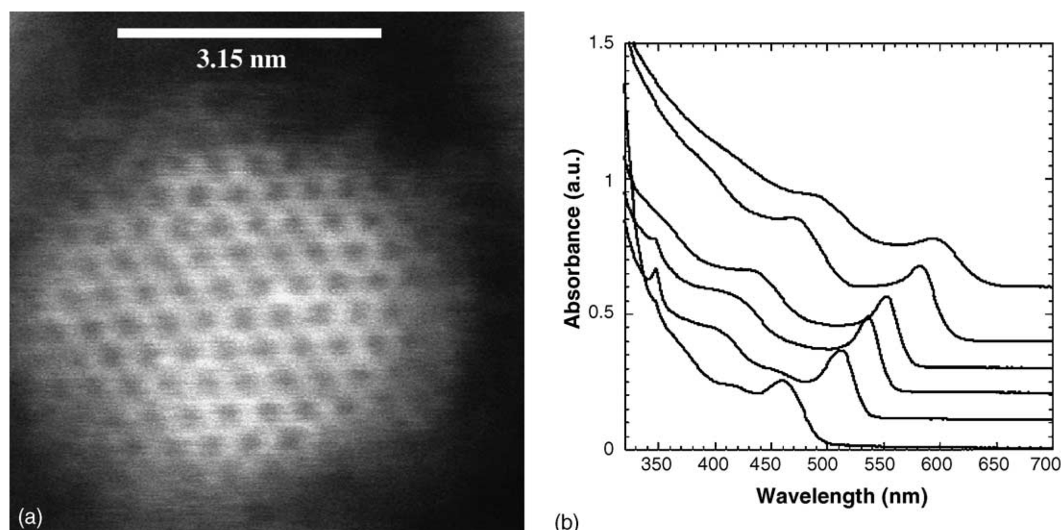

Fig. 10. (a) High resolution annular darkfield scanning transmission electron microscope image of a CdSe nanocrystal showing the individual atoms of the nanocrystal lattice. The image was acquired in collaboration with Mick Thomas and John Silcox, Cornell University; (b) absorption spectra of a series of CdSe nanocrystals with varying diameter. The diameter increases from ∼2.2 to ∼6.0 nm for the lowest to highest absorption spectra. Spectra are offset for clarity.

6. Towards nanocrystal-based single-spot arrays

Semiconductor nanocrystals are inorganic particles (typically spherical) containing a few thousand atoms, with sizes commonly in the range of 2–20 nm (Fig. 10(a)) [23–25]. Significantly, the electronic structure of these materials can be easily tuned by varying the size of the nanocrystal (Fig. 10(b)). Semiconductor NCs are intensely fluorescent, with quantum yields the same order as those of dye molecules [26,27]. In addition, several other properties make semiconductor NCs attractive as possible alternatives for organic dyes with respect to surface immobilized beacons. First, fluorescence from an ensemble of NCs is much brighter than for dye molecules over a short integrated period, since NCs typically last well over an order of magnitude longer than dyes before they irreversibly photobleach [28,29]. Second, NCs fluoresce with the same color regardless of where they are excited, permitting many nanocrystal sizes (i.e. many colors) to be excited with one excitation source. Third, the emission spectrum of nanocrystals is typically much narrower than that of dyes. Altogether, the advantages of semiconductor nanocrystals are particularly exciting, since they provide an alternative pathway for achieving parallel detection through single-spot multi-analyte arrays.

Considerable effort by a number of laboratories has gone into the development of efficient strategies for the covalent attachment of CdSe nanocrystals to synthetic oligonucleotides [28,30–33]. We have recently succeeded in adapting these methods for the production of Au-immobilized, nanocrystal-functionalized DNA hairpins. Although these efforts have not yet led to the production of a functioning sensor, they provide an initial indication that such a device is feasible. The construction of a CdSe NC-DNA microarray first begins with the chemical attachment of an oligonucleotide to the NC. We have successfully synthesized water-soluble CdSe NCs by exchanging the hydrophobic surface coating with dihydrolipoic acid (DHLA) [34] thus giving the NC surface carboxylic acid functionality. We activated the acids on the NC with EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide) and reacted the activated acids with an amine on the 3′ end of a DNA hairpin to form an amide bond, which is similar to coupling schemes already reported for CdSe nanocrystal bioconjugation [35]. The 5′ end of the DNA strand contained a free thiol, which was used to bind the DNA to a gold surface. Attachment of nanocrystal-DNA conjugates was verified by atomic force microscopy (AFM). As seen in Fig. 11, NCs bound through the thiolated DNA show up clearly on an atomically flat gold surface. NCs treated with EDC but without DNA did not bind to the gold surface and were washed away with a light rinsing of the surface with water. These initial results clearly show that we have at least one viable route to attach CdSe NCs to DNA strands and subsequently attach DNA-NC bioconjugates to gold surfaces.

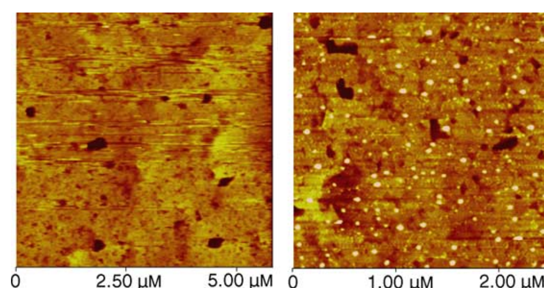

Fig. 11. Tapping mode AFM image of (left) an atomically flat gold thin film and (right) a gold thin film with covalently bound CdSe-DNA nanocrystal conjugates.

7. Conclusion

Molecular beacons have proven to be an exceptionally useful tool for genetic analysis. In prior work, we have demonstrated that the Au-immobilized molecular beacon format produces sensors that are highly selective and sensitive. Initial attempts described herein to produce single-spot/mutli-analyte sensors indicate that this approach will be successful; however, they also highlight the deficiencies of currently available organic dyes. A successful demonstration of the immobilization of semiconductor nanocrystal-functionalized oligonucleotides on Au films suggests that this will be a promising line of future research. Further efforts in our laboratories will focus on the application of these sensors to a broad range of biomedically relevant targets, as the first step towards the production of new, rapid, and highly selective diagnostic aids.

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