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ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · MAY 2003

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Hybridization-Based Unquenching of DNA Hairpins on Au Surfaces: Prototypical "Molecular Beacon" Biosensors

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Recent intense interest in the use of rapid genetic analysis as a tool for understanding biological processes,[1] in unlocking the underlying molecular causes of disease, and in the development of biosensors has led to a need for new sensitive and arrayable chip-based analytical tools. Of high importance is the need for techniques that do not require labeling of the target sample,[2] because that increases the time, cost, and potential for error inherent in the analysis. In the context of solution-phase assays, the molecular beacon concept has proven itself to be both sensitive and reliable.[3,4] Molecular beacons consist of a DNA hairpin functionalized at one end with a fluorophore and at the other with a quenching agent.[5] In the absence of the target DNA sequence, the quencher is brought in close proximity to the fluorophore, and no signal is generated. Addition of the target sequence leads to hairpin unfolding, concomitant duplex formation, and signal generation.

Although a few reports of surface-immobilized molecular beacons have appeared in the literature,[6] to our knowledge all of these employ an attached single molecule as quencher, while the material on (or in) which the hairpin is immobilized serves only a passive role. As part of a general program aimed at developing "label-free" optical biosensors,[7] we decided to investigate whether the substrate material itself could be used as a quenching agent, by immobilizing a fluorophore-functionalized hairpin on a gold film. We report herein the first implementation of such a scheme.

We designed two DNA hairpins **H1** and **H2** (Table 1) incorporating portions of the *Staphlococcus aureus FemA*[8] and *mecR*[9] methicillin-resistance genes, and bearing a 5′ end-linked disulfide and a 3′ end-linked rhodamine. Both **H1** and **H2**, and their respective complementary strands **T1** and **T2**, were obtained from a commercial supplier. Computational predictions[10] of the hairpin secondary structure for **H1** and **H2** were confirmed through thermal melting experiments.

Careful preparation of the substrates for oligonucleotide immobilization was critical to obtaining a high quenching efficiency. Briefly, Au films on quartz substrates were annealed at 200 °C, and then cleaned using acid. DNA hairpins were assembled on the surface by immersing the substrate in a DNA hairpin:mercaptopropanol (MP) solution at a ratio of 1:10, respectively. After 2 h, the substrate was thoroughly rinsed with hot water (90 °C or higher) to remove unbound DNA. Optimization of the DNA to MP ratio and the immersion time was critical. Longer incubation times and lower relative concentrations of MP would be expected to result in Au surfaces with larger amounts of bound DNA. However, these conditions should also lead to complications resulting from nonspecific adhesion of DNA to the surface,[11] or by a lack of sufficient interstitial space for high hybridization efficiency.[12] Indeed, we found that deviation from the conditions described above resulted in significant background fluorescence intensity.

Table 1. DNA Sequences Used in This Study

entry	sequence
H1	5′-(C6Thiol)-ACACGCTCATCATAACCTTCAGCAAGC TTTAACTCATAGTGAGCGTGT-Rhodamine-3′
T1	5′-ACGCTCACTATGAGTTAAAGCTTGCTGAAGGTTA TGA-3′
H2	5′-(C6Thiol)-AATGATGATAACACCTTCTACACCTCCA TAATCATCATT- Rhodamine-3′
T2	5′-TATGGAGGTGTAGAAGGTGTTATCATCATT-3′

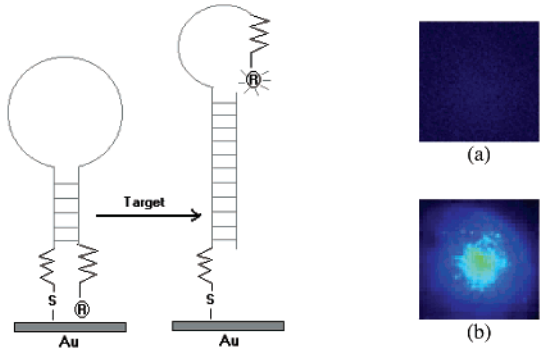

Figure 1. Operation of Au-immobilized molecular beacon. Right: (a) CCD image prehybridization; (b) CCD image posthybridization.

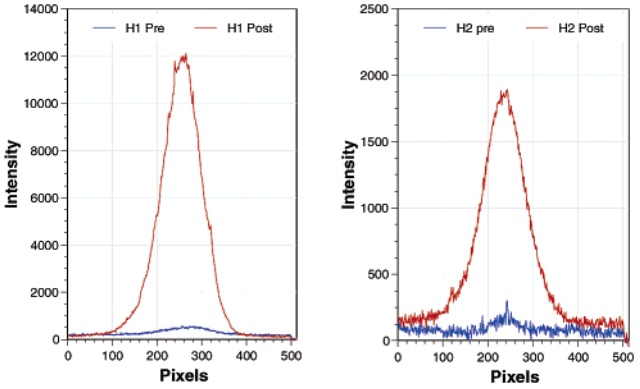

Figure 2. Hybridization-dependent fluorescence efficiency: CCD images with dark counts subtracted, and all pixels binned in the vertical direction, for both sequences before and after hybridization. The integration time was 10 s.

Using epi-fluorescence confocal microscopy, we examined the fluorescence of **H1**- and **H2**-functionalized Au films in the presence and absence of **T1** and **T2**, respectively (Figures 1 and 2). Films were excited at 514 nm. Strong reflected laser scatter was removed using a dichroic beam splitter and a laser-line notch filter. Sample emission was collected by a CCD attached to an imaging spectrograph and passed through a band-pass filter (585 ± 5 nm) to ensure that only rhodamine fluorescence was being observed. As

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10.1021/ja0290781 CCC: $25.00

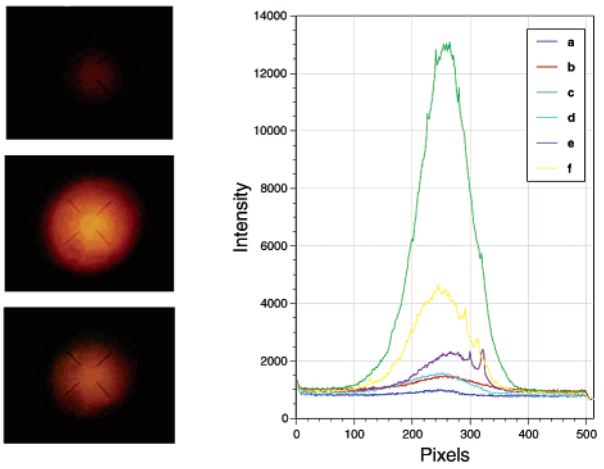

Figure 3. True-color digital photos (left) and binned CCD intensity images (right) demonstrating assay selectivity. Top: Postimmobilization of H1. Center: Posttreatment with 1.38 μM **T1**. Bottom: Posttreatment with 48.1 μM (nucleotide concentration) salmon sperm DNA. (a and d) Preimmobilization of **H1**. (b and e) Postimmobilization of **H1**. (c) Posttreatment with 1.38 μM **T1**. (f) Posttreatment with 48.1 μM (nucleotide concentration) salmon sperm DNA.

shown in Figure 3, our sensitivity was limited by a small background signal at 585 nm. This signal had a spectrum similar to that arising from just a pure Au film or a quartz coverslip, indicating that it was due to autofluorescence from the optical system.

Fluorescence quenching of the hairpins prior to addition of T1 or T2 was found to be 96 ± 3% for **H1** and 95 ± 4% for **H2** (Figure 2). This is similar to quenching efficiencies obtained in solution-phase assays.[4] Viewed another way, this corresponds to a 26-fold fluorescence enhancement for **H1** in the presence of 1.38 μM **T1** or to a 20-fold enhancement in the presence of 2.29 μM **T2**. Preliminary experiments designed to test the sensitivity of this technique indicated that we could detect complementary DNA concentrations as low as 10 nM. However, this is by no means an optimized value. On the basis of recent measurements of the coverage of oligonucleotides on Au surfaces,[13] we anticipate that the optimization of the probe, site size, site density, and instrument design will improve detection to the femtomolar level. We have observed that fluorescence unquenching of the chip is reversible, as washing the hybridized ("on") surface with unbuffered water restores it to a quenched ("off") state. Cycles of hybridization/washing result in a monotonic decrease in fluorescence intensity, presumably due to the loss of probe hairpin from the Au surface.

Binding specificity (sequence selectivity) is obviously a crucial measure of the utility of a diagnostic device or biosensor. To evaluate the extent to which the Au-immobilized probes retained their hybridization selectivity, we compared the ability of equivalent concentrations of **T1** and salmon sperm DNA to produce a signal when incubated with a **H1**-functionalized gold substrate. As shown in Figure 3, we measured an approximately 26-fold increase in intensity (over background) for the sample corresponding to the appropriate complementary DNA. In contrast, a similar concentration of salmon sperm DNA (USB) produces only a 4-fold increase of fluorescence intensity. This result suggests that DNA hairpins immobilized on a gold surface retain their ability to bind complementary DNA sequences selectively. That the salmon sperm DNA produces a net increase in intensity is not surprising, as a standard database search[14] of the sequences T1 and T2 indicates that sequences homologous to portions of these are present in a variety of organisms.

While under laser illumination, the fluorescence intensity was observed to irreversibly decay with time, likely due to photobleaching of the dye molecule. For an excitation intensity of 600 W/cm^2, the signal intensity was reduced by a factor of 2 in 1 s. However, the rate of decay was linearly proportional to the excitation intensity for intensities in the range from 6 to 600 W/cm^2. Thus, to avoid any ambiguities caused by the permanent photobleaching, all measurements were taken at intensities less than 20 W/cm^2. This is a lower intensity than is commonly employed by commercial microarray scanners; however, direct comparisons are difficult given the differences in scan times.

In conclusion, we have demonstrated that fluorophore-tagged DNA hairpins attached to gold films can function as highly sensitive and selective sensors for oligonucleotides. For two distinct DNA hairpin sequences, binding by the complement caused an increase in signal by over a factor of 20, while nonspecific sequences resulted in a minimal response. Efforts to implement this design in a microarray format, to optimize sensitivity through surface enhancement provided by roughened metal substrates,[15] to replace dye molecules with semiconductor quantum dots affording orders of magnitude more photostability, and to examine the utility of this new technique in biosensor development are in progress.

Acknowledgment. The authors thank Professor Doug Turner and Jessica Childs for assistance with melting studies of DNA hairpins, and Dr. Jonathan Hibbs (Evident Technologies, Inc.) for helpful discussions. Financial support was provided by Evident Technologies, Inc., and by the Center for Future Health.

Supporting Information Available: Secondary structure predictions, thermal melting curves for **H1/T1** and **H2/T2**, descriptions of hybridization conditions and fluorescence measurements, and the definition of quenching efficiency (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0290781