Thin Film Biosensor for Rapid Visual Detection of Nucleic Acid Targets

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Background: We have developed a silicon-based biosensor that generates a visual signal in response to nucleic acid targets.

Methods: In this system, capture oligonucleotide probes are immobilized on the surface of the biosensor. Interaction of the capture probes with a complementary target and a biotinylated detector oligonucleotide allows initiation of formation of an organic thin film on the biosensor. Thin film formation is completed by enzymatic activity of peroxidase conjugated to an anti-biotin antibody. Peroxidase catalyzes deposition of an insoluble product onto the silicon surface, generating a uniform thin film. The increased thickness on the surface alters the perceived color of the biosensor through changes in the interference patterns of reflected light from the surface, causing a color change from gold to purple.

Results: The biosensor results may be evaluated by direct visual inspection or quantified by ellipsometry. Results are obtained in 25 min with a detection limit of 5 pmol/L (150 amol/sample). Selectivity of the biosensor is demonstrated by discrimination of single nucleotide mismatches. Multitarget arrays are also analyzed with the thin film biosensor, and the system is capable of detecting targets from human serum and urine.

Conclusions: The biosensor surface is inexpensive to produce, and the assay format is simple and rapid. The thin film biosensor is adaptable to a wide variety of nucleic acid detection applications, including rapid diagnostic testing for infectious disease panels, antibiotic resistance panels, or allelic discrimination of specific genetic markers.

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duce a visible color change from gold to purple. Surfaces are constructed such that angstrom (Å) level changes in thickness produce color transitions to which the human eye is maximally sensitive. The method is sensitive to optical thickness changes as small as 10–20 Å.

Qualitative interpretation of results is determined visually. Quantitative analysis of the results can be performed with the fixed polarizer ellipsometer (FPE) (7, 8). The FPE detects changes in the polarization state of linearly polarized light caused by reflection from thin films on the silicon surface. The polarization change is proportional to the thin film thickness and therefore relates to the concentration of analyte bound to the biosensor surface.

We report the application of thin film detection to construct a simple, highly sensitive and selective biosensor for detection of nucleic acid targets. The system is compatible with typical clinical matrices, and results are obtained in less than 30 min.

**Materials and Methods**

**SURFACE CONSTRUCTION AND ANALYSIS**

Optical coatings were applied by an ion beam deposition tool (Commonwealth Scientific) onto a porous polycarbonate membrane with 8-μm channels (Osmonics). A channel size of 8 μm is small enough to avoid interference with light reflection yet large enough to allow for free flow of liquids. All surface characterization was performed at Rocky Mountain Laboratories (Golden, CO) by one of the authors (W.B.). The x-ray photoelectron spectroscopy (XPS) analysis was performed using a Fisons SSX-100 x-ray system on an elliptical area of 150 × 300 μm. The depth analysis varied from 10 to 100 Å with a sensitivity of 0.01–1 atom%. Atom% is a measurement of the percentage of composition of each element in the surface being characterized. The Auger analysis was conducted using a PHI 610 spectrometer on a 1-mm diameter spot. The depth analysis was between 50 and 100 Å with a sensitivity of 0.1–2 atom%. The secondary ion mass spectroscopy analysis was performed on a SIMS 100 Å with a sensitivity of 0.1–2 atom%.

The depth analysis varied from 10 to 100 Å with a sensitivity of 1 atom%.

**OLIGONUCLEOTIDE SEQUENCES**

All oligonucleotides were synthesized by IDT and purified by HPLC. The sequences used in this study are summarized in Table 1.

**ASSAY FORMAT**

The mecA capture probe (1 μmol/L) was spotted on the surface in 0.1 mol/L carbonate buffer, pH 10.0, and dried overnight at room temperature. To begin the assay, the surface was rinsed with distilled H2O to remove unbound capture probe. mecA target (30 μL) in hybridization buffer (5× standard saline citrate (0.75 mol/L NaCl, 0.075 mol/L sodium citrate) containing 1 g/L sodium dodecyl sulfate and 5 g/L casein) was incubated on the surface for 10 min. The surface was rinsed with a stream of distilled H2O. mecA biotinylated detection probe (30 μL of a 1 μmol/L solution) in hybridization buffer was then added to the surface. After 5 min, the surface was rinsed with distilled H2O, and 0.9 mg/L anti-biotin antibody labeled with HRP (Jackson Immunoresearch) was added to the surface for 5 min. After the surface was rinsed, 35 μL of precipitating tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry) was added and incubated for 5 min. After a final rinse, the assay was complete. All incubations occurred at room temperature (19–25 °C). Total incubation time was 25 min.

**CAPTURE PROBE QUANTIFICATION**

The mecA capture probe was 5’ labeled with γ-32P-labeled ATP (ICN) using T4 kinase (Promega) by standard methods (9). The thin film assay was carried out, and the counts remaining on the surface were quantified by Cerenkov counting after each wash step in a Wallac scintillation counter.

**SINGLE NUCLEOTIDE MISMATCH DISCRIMINATION**

Six mecA target molecules (T1–T6) were synthesized with single-base mismatches in either the capture or detector hybridization region (Table 1). The thin film assay was performed at room temperature. However, the wash solution was heated to 37 °C. After each assay incubation step, the 37 °C wash solution was used to rinse the surfaces. The relative signal intensity of each mismatch target was compared to control assays with 100% homologous target or no added target and quantified with the FPE.

**ARRAY HYBRIDIZATION**

The array experiment was performed by hybridization of a biotinylated oligonucleotide to its surface-bound complement. All steps in the array hybridization experiment were performed at room temperature on a 14-mm square thin film surface. The capture oligonucleotides A, B, or C (100 μmol/L) were adsorbed to the surface in a 2-μL volume, with 2-mm spacing between each spot. The entire surface was covered with the biotinylated probes A, C, or A plus C (100 μL of 1 μmol/L) and incubated for 10 min. After the surface was washed, the anti-biotin HRP-antibody conjugate was added for 10 min, followed by washing and incubation with TMB substrate for 3 min. Total assay time was 23 min.

**DETECTION OF TARGET FROM SERUM AND URINE**

The mecA target (1 nmol/L) was equilibrated in human serum (Sigma), human urine, or water for 1 h at room temperature. These samples were then diluted 1:1 in 2× hybridization buffer, and the standard assay protocol was followed. The results were evaluated both visually and by ellipsometry.
ASSAYS INCORPORATING COMPETITOR DNA
Sheared salmon sperm DNA (10 and 100 mg/L; 5 Prime-3 Prime) was added to 1 nmol/L target DNA, and the standard assay protocol was followed.

**Results**

The optical surfaces are composed of discrete thin films of amorphous silicon and diamond-like carbon (DLC) deposited on a porous polycarbonate membrane, creating a gold-colored reflective test surface coated with capture probe (Fig. 1A). The sample is applied to the center of the surface, and target detection is enhanced through deposition of an HRP-catalyzed organic thin film (Fig. 1B). The additional thickness alters the antireflective properties of the surface such that the red and green components of visible light (500–700 nm) are attenuated, whereas wavelengths corresponding to purple and blue (400–500 nm) are reflected, producing a color change from gold to purple.

**Surface Construction**

The optical surfaces are prepared using a dual ion beam deposition system to deposit three discrete layers. To produce the proper intensity of reflected light, antireflective surfaces are designed such that the refractive index of each layer is approximately the square root of the one below. Thus, the choice of materials is essential for proper surface response. In our biosensor, the first layer is amorphous silicon of 2000 Å with a refractive index of 4.0. Above the silicon layer, silicon nitride is deposited. Both silicon and silicon nitride are deposited using ion source bombardment of a pure single crystal silicon target in an argon or nitrogen atmosphere. Silicon nitride is chemically inert and has an index of refraction of 2.0 and a nominal thickness of 150 Å. In the same apparatus, a DLC layer of 200–250 Å, with a refractive index of 1.9–2.1, is deposited using the second ion source, which provides radio frequency-assisted ion beam decomposition of methane gas. The final DLC coating functions both as a component of the antireflective layer and to mediate capture probe attachment. The DLC layer is hydrophobic and serves as a suitable substrate for passive adsorption of nucleic acid or protein capture molecules. The final hybridization dependent thin film has a refractive index of 1.4–1.45.

The DLC layer was analyzed for chemical composition by three methods. Auger electron spectroscopy uses a

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\begin{table}
\centering
\caption{Oligonucleotide sequences.\textsuperscript{a}}
\begin{tabular}{ll}
\textbf{Name} & \textbf{Sequence, 5' to 3'} \\
\hline
mecA capture & CTTTGTGACGCGAGGGGAAC-C3 amine \\
mecA target & AAAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
mecA detector & Biotin-CGACCGAGCTGACCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Mismatch T1 & AAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Mismatch T2 & AAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Mismatch T3 & AAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Mismatch T4 & AAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Mismatch T5 & AAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Mismatch T6 & AAAAAGACCTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
Array oligonucleotides & \\
A capture & ATGCGACTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
A probe & Biotin-ATGCGACTCTGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
B capture & ATAGCCTCGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
B probe & Biotin-ATAGCCTCGCTCAACAAAGGTCCAGATTACAACCTCACCATCCTCAACAAA \\
C capture & GCATCAGACTGATGATC \\
C probe & Biotin-GCATCAGACTGATGATC \\
\end{tabular}
\textsuperscript{a} Bold underline, single-base mismatch.
\end{table}

Panel A shows an unreacted surface with optical and capture probe layers. The optical pathlength of the light is demonstrated in the lower half of the panel. Panel B shows a reacted surface and demonstrates the deposition of the target-dependent organic thin film. The lower half of the panel shows the increased optical pathlength and resulting color change from gold to purple.

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focused electron beam to create secondary electrons near the surface of the sample that have energies characteristic of the element from which they are released. All elements can be detected except hydrogen and helium. We found that the DLC layer was composed of pure carbon, free of contaminants. To determine how the carbon was bound to hydrogen, the DLC was analyzed by secondary ion mass spectroscopy analysis, which bombards the surface with a narrow beam of ions. Ejected ionized species provide a mass spectrum composed of fragment ions of the various functional groups of the surface. The DLC surface composition was mainly ionic fragments of \( \text{C}^+, \text{CH}^+, \text{CH}_2^+, \text{CH}_3^+ \), and \( \text{CH}_3^+ \).

Further characterization of the DLC surface was provided by XPS. In XPS, monoenergetic soft x-rays bombard the DLC surface and eject electrons, and the presence of the elements on the surface can be directly distinguished by analysis of the kinetic energy of the ejected photoelectrons. XPS revealed the presence of carbon in the DLC coating and confirmed that no contaminants other than a small amount of oxygen were incorporated in the outermost layer of the surface. XPS also allowed examination of the hybridization state of carbon-to-carbon bonds. The ion beam-deposited DLC in this study was composed of about 80% sp\(^3\) plus 20% sp\(^2\) carbon, indicating that the carbon structure was diamond-like rather than graphite-like.

**CAPTURE PROBE CHARACTERIZATION**

The target-specific surfaces were prepared by applying the 20-nucleotide (nt) capture probe solution to the optically coated membranes and allowing the spot to dry. No further manipulation was required to permanently adhere capture probe to the DLC membrane. Various modifications of the 3′ end of the capture probe were tested for optimal attachment to the optical surface, including fluorescein, \( \text{C}_3 \), \( \text{C}_6 \) amine, and \( \text{C}_{18} \). These modifications either enhance hydrophobicity or provide a spacer between the surface and the capture probe sequence to alleviate potential steric hindrance to hybridization. The optimal capture probe was a 3′-modified \( \text{C}_3 \) amine. The amount of capture probe retained on the surface was quantified by radiolabeling the 5′ end with \( \text{P}^{32} \) and measuring the counts retained on the surface at each step in the assay. The application of 10 pmol of capture probe to the membrane in a 4-mm diameter spot produced retention of 38 fmol, or 0.4% of the starting material. Therefore, the density of capture probe was 1825 molecules/\( \mu \text{m}^2 \) (3 zmol/\( \mu \text{m}^2 \)).

**LIMIT OF DETECTION**

The experimental hybridization assay target was a 60-nt synthetic sequence from the *mecA* gene of *Staphylococcus aureus* encoding methicillin resistance. The capture probe contained a 3′-terminal amine, a 3-carbon spacer, 5 nonhomologous nt, and a 15-nt sequence homologous to the target. The detector probe contained 15 nt of homologous sequence and 5 nonhomologous nt terminated with a 5′ biotin. The nonhomologous nucleotides served as a spacer between either the surface and the hybridizing sequences or the hybrid and biotin. The calculated melting temperature for target interacting with immobilized capture or detection probe under our standard assay conditions was 44 and 43 °C, respectively (9).

Target concentrations of 0.5–1000 pmol/L were tested in the thin film biosensor by incubating the target oligonucleotide for 10 min on the capture probe-coated surface. After hybridization of biotinylated detection probe, the organic thin film was enhanced by binding the HRP-labeled anti-biotin antibody and subsequent precipitation of TMB substrate. Total assay time was 25 min, and the visual limit of detection was 5 pmol/L or 150 amol/sample.

**SINGLE NUCLEOTIDE MISMATCH DISCRIMINATION**

To assess the ability of the silicon biosensor to discriminate single-nucleotide polymorphisms, six target molecules were synthesized with single transversion point mutations (Table 1). The mismatch was either in the center or near the terminus of the probe hybridization region. Hybridization efficiency of these targets was compared to that of the 100% complementary target. All hybridization steps were carried out at room temperature. Stringency was mediated by rinsing the surface with 37 °C wash solution after each hybridization step. Assay results were quantified with the FPE (Fig. 2). Both the visual appearance of the membranes and the ellipsometric values demonstrated that this simple selection procedure allowed discrimination of single-base mismatches between the target sequence and the capture or detection probe. The intensity values from the internally located mismatches (T1, T2, and T5) were indistinguishable from the negative control. The intensity values from the mismatches located one nucleotide from the end of the hybrid...
(T3, T4, and T6) were higher but still below that of the perfectly matched hybrid. There was no difference in hybridization efficiency if the mismatch was located in the capture or detection sequence.

ARRAY HYBRIDIZATION
To investigate the adaptability of the thin film biosensor to multitarget analysis, we constructed a 3 × 3 array of capture probes. Capture probes were either 100% complementary (probe A), 80% complementary (probe B, 12 of 15 nt), or noncomplementary (probe C, 100% mismatch) to a biotinylated target sequence (Fig. 3). Target detection was enhanced by HRP-catalyzed thin film formation. All hybridizations and washes were performed at room temperature. The arrays were probed with multiple target combinations. In each case, the expected hybridization pattern was generated. There was no hybridization to capture probe B, the 80% match. The results were visually interpreted, and only hybridization to perfect complements was seen.

TARGET DETECTION FROM SERUM AND URINE
To test the ability of the thin film biosensor to detect target nucleic acid from simulated clinical samples, the mecA target was added to control solution, or human serum or urine. The samples were stored at room temperature for 1 h. The thin film assay was then performed and interpreted visually. Target was detected in both serum- and urine-treated samples with signal strength equal to the control. No signal interference or false-positive results were observed. Quantification by ellipsometry demonstrated <5% signal intensity difference in the results from serum or urine compared with those of the control. The effect of competitor DNA on the thin film assay was analyzed by addition of a 5000-fold excess of salmon sperm DNA to the target. No signal difference was observed compared with samples without competitor DNA.

Discussion
We have described a non-instrument silicon-based biosensor that permits rapid visual detection of nucleic acid target sequences through their interaction with immobilized complementary capture probes. Detection of hybrid formation is enhanced with a labeled probe that triggers enzymatic formation of a thin film. The thin film alters the interference pattern of visible light on the silicon surface and produces a perceived color change. The color change depends on the unique composition and thicknesses of the surface layers of the biosensor. The composition of the optical layers is designed such that small increases in thickness occur in a color range where the human eye is most sensitive, i.e., a gold-to-purple transition.

The relationship between color change and molecular thickness can be simulated theoretically and used to predict nonreacted surface color and the increased thicknesses required to produce various colors. For example, with the present layer composition and thickness, we would expect the addition of 10 Å of uniform thin film to alter the surface color from the initial gold to a light purple color. The color change is a function of the alteration in the destructive interference pattern from antireflective to blue (producing a gold appearance) to a higher percentage of blue reflection as the thickness increases. Alternative materials may be modeled and constructed to customize the surface response to a desired thickness and refractive index change.

A key feature of the silicon surface is that it is relatively two-dimensional compared with typical surfaces used for hybridization assays, such as nitrocellulose or polystyrene. The flatness of the biosensor surface may improve on-rates of hybridization and decrease nonspecific binding. Low nonspecific binding creates an assay format with a high signal-to-noise ratio, thus allowing for detection of very low concentrations of analyte (10). The thin film assay is compatible with typical clinical matrices, and assay performance is not affected by competitor DNA. To our knowledge, detection of target from a true clinical matrix has not been reported for other biosensor technologies (3–6). Biosensor methods based on refractive index changes, such as evanescent field or reflectometric interrogation, cannot discriminate nonspecific adsorption from specific effects (11) and thus may have difficulty with direct examination of clinical specimens.

At present, detection of nucleic acid targets is primarily based on fluorescent or chemiluminescent methods (12). Although these methods are sensitive, they require sophisticated instruments and specialized reagents and are not suitable for field use or for poorly equipped laboratories. The silicon-based biosensors described here can be manufactured at low cost, and the reagents used to generate the thin films are commonly available and relatively inexpensive. Thin films can be generated in a variety of ways, using substrates that produce insoluble enzymatic products or with film-forming particles.

Although the assay described here is qualitative, the thickness of the film generated by the hybridization event can be accurately quantified by ellipsometry. Ellipsometric quantification allowed the discrimination of targets that differed by a single nucleotide. The FPE is capable of detecting changes in thickness of <10 Å, yet the instrument is compact and inexpensive (7, 8). Thus, the thin
film biosensor can be easily interpreted by either visual or instrumented detection.

Other direct visual detection methods for nucleic acid targets have reported detection limits of 0.1–10 nmol/L (13, 14). The thin film biosensor described here achieved a visual detection limit of 5 pmol/L (150 amol/sample) in under 30 min without target amplification. The format is simple, and the material cost is consistent with current commercial diagnostic formats. The assay lends itself to automation for increased sample throughput and multi-target analysis for such applications as infectious disease panels, antibiotic resistance panels, or allelic discrimination of specific genetic markers.

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