Silicon-based Biosensors for Rapid Detection of Protein or Nucleic Acid Targets

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Background: We developed a silicon-based biosensor that generates visual, qualitative results or quantitative results for the detection of protein or nucleic acid targets in a multiplex format.

Methods: Capture probes were immobilized either passively or covalently on the optically coated surface of the biosensor. Intermolecular interactions of the immobilized capture probe with specific target molecules were transduced into a molecular thin film. Thin films were generated by enzyme-catalyzed deposition in the vicinity of the surface-bound target. The increased thickness on the surface changed the apparent color of the biosensor by altering the interference pattern of reflected light.

Results: Cytokine detection was achieved in a 40-min multiplex assay. Detection limits were 4 ng/L for interleukin (IL)-6, 31 ng/L for IL1-β, and 437 ng/L for interferon-γ. In multianalyte experiments, cytokines were specifically detected with signal-to-noise ratios ranging from 15 to 80. With a modified optical surface, specificity was also demonstrated in a nucleic acid array with unambiguous discrimination of single-base changes in a 15-min assay. For homozygous wild-type and homozygous mutant samples, signal-to-noise ratios of ~100 were observed. Heterozygous samples yielded approximately equivalent signals for wild-type and mutant capture probes.

Conclusions: The thin-film biosensor allows rapid, sensitive, and specific detection of protein or nucleic acid targets in an array format with results read visually or quantified with a charge-coupled device camera. This biosensor is suited for multianalyte detection in clinical diagnostic assays.

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Simultaneous detection of multiple protein or nucleic acid targets is necessary for the diagnosis of many diseases. An ideal detection platform would be inexpensive, recognize targets rapidly with high sensitivity and specificity, possess high stability, and in many cases, create an interpretable signal without instrumented intervention. We describe here an optically coated, highly reflective, silicon-based surface designed to permit visual detection of biological interactions in white light (1, 2). Specific capture molecules are immobilized on the coated surface. After interaction with specific targets, the addition of an antibody conjugated to horseradish peroxidase (HRP) catalyzes formation of an organic thin film in the presence of a precipitating substrate formulation. The thin film increases the thickness of the surface, producing a visible color change attributable to destructive interference. Film thicknesses on the order of 5 nm are readily detected visually by untrained users. The optical layer composition and thickness are selected on the basis of refractive indices, biomolecule compatibility, and the known sensitivity of the human eye to various color transitions (1, 2).

We previously described rapid, single-analyte biosensors that use thin-film technology for protein assays. Diagnostic targets have included conserved nonstructural proteins in rhinovirus (3) and influenza virus (4), and the brain trauma marker S-100B (5). These assays take 6–20 min to complete with analytical sensitivities equal to a similarly configured ELISA format.

Recently, thin-film technology has been applied to the detection of nucleic acid targets. The nucleic acid biosensor demonstrated a detection limit of 60 000 copies, or 10 fmol/L, for a single-stranded DNA target (6). Using this format, our laboratory developed a test to rapidly and specifically detect the mecA gene in methicillin-resistant Staphylococcus aureus (6, 7). This biosensor also provides a rapid and simple replacement for gel electrophoresis for the detection of multiplex reverse transcription-PCR.
products from RNA viruses present in respiratory infections (8).

We describe here further applications of the biosensors for simultaneous detection of multiple cytokines and for discrimination of single nucleotide polymorphisms in the human cystic fibrosis transmembrane conductance regulator (CFTR) gene.

ASSAY PRINCIPLE

The base substrate of the thin-film biosensor is a crystalline silicon wafer that provides a highly reflective, chemically inert, flat surface. The surface is coated with silicon nitride (Si$_3$N$_4$), which serves as an antireflective optical layer. Si$_3$N$_4$ was chosen as the optical coating because of its ability to be easily applied to silicon by chemical vapor deposition techniques and because it has an appropriate refractive index. A Si$_3$N$_4$ coating of 47.5 nm was necessary to obtain a gold surface color (Fig. 1A). Although the Si$_3$N$_4$ layer is sufficient for the optical coating, a more biologically compatible layer is required to obtain efficient immobilization of capture moieties. Optimal performance of this attachment layer was obtained with the polymer aminoalkyl functional T-structure polydimethylsiloxane (TSPS), which was applied by spin coating to a thickness of 13.5 nm. The polypeptide poly(phenylalanine-lysine) (PPL) was passively adsorbed onto the TSPS layer. PPL served to provide amino functionality to the surface, allowing the covalent attachment of activated N-hydroxysuccinimide esters of N-terminal-modified oligonucleotides or to improve the efficiency for passive adsorption of proteins.

The thin-film biosensor surface can be coated with capture moieties that recognize and bind target molecules, including nucleic acids and proteins. Capture moieties applied to the thin-film biosensor surface retain functional stability for more than 1 year at 45 °C. Surface-immobilized targets can be detected by binding of a labeled detector molecule, such as a biotin-labeled anti-human detector antibodies; and streptavidin-HRP were from the DUOSET ELISA development reagent set obtained from R&D Systems. Rabbit anti-goat IgG was obtained from Pierce. Genomic DNA samples were obtained from Coriell Cell Repositories.

MATERIALS AND METHODS

REAGENTS

The cytokines interleukin-6 (IL-6), IL1-β, and interferon-γ (INF); capture antibodies; biotin-labeled goat anti-human detector antibodies; and streptavidin-HRP were from the DUOSET ELISA development reagent set obtained from R&D Systems. Rabbit anti-goat IgG was obtained from Pierce. Genomic DNA samples were obtained from Coriell Cell Repositories.

SURFACES

Si$_3$N$_4$ was applied to crystalline silicon wafers in a vapor deposition chamber (Nordiko). The polymer aminoalkyl functional TSPS (UCT) was applied, using a spin coater (Machine Technologies, Inc.), and cured at 150 °C for 24 h to produce the thin-film biosensor surface. These surfaces are available under the trade name SILAS at www.thermobiostar.com. The amino-functionalized biosensor was prepared by soaking a SILAS wafer in a 50 mg/L
solution of PPL (Sigma) in 1× phosphate-buffered saline (PBS; pH 6) containing 2 mol/L NaCl overnight at room temperature (15). The surface was then washed extensively with H2O and 1× PBS, dried under a stream of nitrogen, and stored dry at 4 °C.

**OLIGONUCLEOTIDE SYNTHESIS**

All DNA oligonucleotides were synthesized by IDT (Corvalle, IO) by standard phosphoramidite chemistry. To facilitate detection of the PCR product, the BP1 primer for each of the mutations was terminated with a C18 biotin phosphoramidite (Glen Research), designated X in the sequence. Primers were as follows:

For exon 10, BP1 was 5'-X TCT GTT CTC AGT TTT CCT GG-3', and P2 was 5'-TTG GCA TGC TTT GAT GAC GC-3'.

For exon 11, BP1 was 5'-X TAG GAC ATC TCC AAG TTT GC-3', and P2 was 5'-CAA TAA TTA GTT ATT CAC CTT GC-3'.

For exon 21, BP1 was 5'-X TGC TAT AGA AAG TAT TTA TTT TTT CTG G-3', and P2 was 5'-AGC CTT ACC TCA TCT GCA AC-3'.

To facilitate covalent attachment to the surface, all capture sequences were terminated with an amino modifier C18 phosphoramidite, designated Y in the sequence. Capture sequences were as follows:

For ΔF508, the wild type was 5'-YAC ACC AAA GAT GAT ATT TT-3', and the mutant was 5'-YCC GAA ACA CCA ATG ATA TTT TC-3'.

For N1303K, the wild type was 5'-YAC GGA TCC AAG TTT TTT CTA A-3', and the mutant was 5'-YAC GGA TCC AAC TTT TTT CTA A-3'.

For G551D, the wild type was 5'-YAA CTC GTT GAC CTC CAC TC-3', and the mutant was 5'-YAA CTC GTT GAC CAC TC-3'.

For G542X, the wild type was 5'-YAA CAC CTT CTC CAA GAA CTA TA-3', and the mutant was 5'-XAA CAC CTT CTC AAA GAA CTA TA-3'.

**ANTIBODY IMMOBILIZATION**

Capture antibodies were diluted to 2 mg/L in 0.1 mol/L sodium bicarbonate buffer, pH 9.3, and 200 nL was spotted using a Hamilton MP2200 pipetting robot equipped with a modified dispense head. The antibodies were incubated in a humid chamber for 1 h at room temperature and then allowed to dry. Surfaces were washed with H2O and dried under nitrogen. Surfaces were then dip-coated with a preservative solution (ThermoBioStar) and dried.

**CAPTURE PROBE IMMOBILIZATION**

Capture probes were dried and resuspended in 5 µL of 0.1 mol/L triethylammonium acetate, pH 7.8. Disuccinimidyl suberate (Pierce) was dissolved to 28 g/L in dimethyl formamide (Aldrich), and 22 µL was added to the capture probe and incubated at room temperature for 10 min. Ice-cold H2O (200 µL) was added, followed by extraction with 600 µL of ethyl acetate (Aldrich) three times. Ice-cold H2O (200 µL) was added, and the sample was extracted twice with 400 µL of iso-butyl alcohol (Aldrich). The activated DNA was brought to 500 µL with H2O and placed on ice. To spot on the surface, activated capture probe was diluted with 0.1 mol/L phosphate buffer, pH 7.8, and then 200 nL was spotted using a Hamilton MP2200 pipetting robot equipped with a modified dispense head. Surfaces were covered to prevent drying and incubated at room temperature for 3 h. Surfaces were then washed with 1× PBS and H2O, and any remaining loosely associated capture probe was removed by treating the surface with 1 g/L sodium dodecyl sulfate (SDS) at 53 °C for at least 2 h. Surfaces were washed with H2O, dried under nitrogen, and stored at 4 °C until further use. Input concentrations for the probe sets was as follows: ΔF508, 150 nmoL/mL for both the wild type and mutant; G542X, 75 nmoL/mL for the wild type and 750 nmoL/mL for the mutant; N1303K, 300 nmoL/mL for both the wild type and mutant; G551D, 150 nmoL/mL for both the wild type and mutant.

**CYTOKINE ASSAY**

Recombinant cytokines (50 µL) were incubated on the surface for 10 min at room temperature in binding buffer (20 mmol/L Tris, pH 7.3; 1 g/L bovine serum albumin; 0.5 mL/L Tween 20), and surfaces were washed with wash buffer (1× PBS, pH 7.3; 0.1 mL/L Tween 20). The surface was then incubated with 50 µL of detector antibody mixture (18 mg/L biotin-labeled goat anti-human IL-1β, 18 mg/L biotin-labeled goat anti-human INF-γ, and 36 mg/L goat anti-human IL-6) in binding buffer for 10 min at room temperature and washed with wash buffer. Surfaces were then incubated with 50 µL of 10 mg/L streptavidin-HRP in binding buffer for 10 min at room temperature and washed with wash buffer. Finally, 50 µL of Fast Substrate (BioFX) was added and incubated at room temperature for 10 min. The surface was washed with wash buffer and dried under nitrogen. CD values were determined as described previously (6).

**MULTIPLEX REVERSE TRANSCRIPTION-PCR**

Genomic DNA targets were amplified in a 50-µL multiplex PCR reaction using Qiagen HotStarTaq Master Mix (Qiagen). DNA (100 ng) was added to 1× Qiagen PCR buffer; 200 µM each of dATP, dCTP, dGTP, and dTTP; 1 µM exon 11 and 0.2 µM each of exon 10 and exon 21 primers; 1.5 mM MgCl2 and 2.5 U of HotStarTaq. Reactions were incubated at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 50 °C for 60 s, and 72 °C for 60 s. Final extension was at 72 °C for 7 min.

**SINGLE-NUCLEOTIDE POLYMORPHISM (SNP) ASSAY**

Aliquots (1 µL) were taken from the PCR reaction, diluted with 9 µL of H2O, and boiled at 95 °C for 3 min. The denatured PCR product was then diluted into 190 µL of 1× hybridization buffer [3 mol/L tetramethylammonium
chloride (Aldrich), 50 mmol/L Tris-HCl, pH 8.0; 1 g/L SDS; and 4 mmol/L EDTA] prewarmed to 57 °C in a 96-well microtiter plate containing the biosensors. The PCR product was hybridized at 57 °C for 5 min, and the biosensor was then washed with wash A (0.1× SSC containing 1 g/L SDS) followed by wash B (0.1× SSC). Anti-biotin antibody-HRP conjugate (200 µL of an 1 mg/L solution; Jackson ImmunoResearch Laboratories, Inc.) diluted in conjugate buffer [5× SSC, 1 g/L SDS, 5 g/L BlockAid (ThermoBioStar)] was added to the surface, incubated at 23 °C for 3 min, and washed with wash B. Finally, 200 µL of Fast Substrate was added, incubated at 23 °C for 4 min, washed with H2O, and dried, and the biosensor was read. Washes consisted of a stream of buffer sufficient to remove unbound reactants (total time, 5–10 s). CD values were determined as described previously (6).

**Results**

**CYTOKINE DETECTION ARRAY**

Because many cytokines have overlapping biological functions, are modulated depending on cell status, and regulate each other’s production, it is often of interest to simultaneously detect multiple cytokines. To analyze cytokines in a multiplex assay, we created an array of monoclonal antibodies that recognize IL-6, IL-1β, and INF-γ on the biosensor surface. Each antibody was printed in triplicate 600-µm (diameter) spots to create a 3 × 4 array on a 5-mm² biosensor chip. The biosensor was incubated with cytokine mixtures at room temperature for 10 min. Surface-bound cytokines were detected by binding a mixture of biotin-labeled, goat antibodies specific for each of the cytokines in the panel, followed by addition of a streptavidin-HRP conjugate. In the presence of a precipitating substrate formulation containing 3,3′,5,5′-tetramethylbenzidine, an HRP-catalyzed precipitate was deposited to create a thin-film signal. Total assay time was 40 min. An anti-goat antibody control was included to serve as a positive control for the detection reagents (i.e., the goat anti-cytokine antibodies) and to establish orientation of the array.

To test for nonspecific binding of capture antibodies with the inappropriate cytokine or with detector antibodies, we applied individual cytokines to the array. Binding of individual cytokines showed no cross-reactivity; signal was observed only for the specific binding of a cytokine-monoclonal antibody pair (Fig. 2, A and B). Upon simultaneous binding of multiple cytokines, specific binding was maintained; no cross-reactivity was observed. Nonspecific binding of cytokines was limited; INF-γ binding was slightly reduced in the presence of IL1-β (Fig. 2, C–E).

CD values were used to establish that the signal-to-noise ratio in this experiment ranged from 15 to 80 (Fig. 3). The signal-to-noise ratio was defined as the CD value of a reacted spot divided by a background, or unreacted, spot. Variability in this value was related to differences in the extent of specific binding because no nonspecific binding was observed.

![Fig. 2. Multiplex detection of cytokines.](https://academic.oup.com/clinchem/article-abstract/47/10/1894/5639396)
To assess sensitivity, we tested dilution series of cytokines individually. To maintain a multiplex format, surface-bound cytokines were detected with the mixture of three antibodies specific for each of the cytokines. The data showed specific, saturable binding with a dynamic linear range of two orders of magnitude (data not shown). Lower limits of detection were 4 ng/L for IL-6, 31 ng/L for IL-1β, and 437 ng/L for INF-γ as defined with reference to the DUOSET reagent set.

**SNP discrimination panel**

To provide a stringent test for the sequence-specific detection of nucleic acid targets in an array format, we developed a panel for the detection of four mutations in the human CFTR gene. One mutation, ΔF508, is a 3-bp deletion, whereas the other three, G542X, G551D, and N1303K, are SNPs. Wild-type sequences were printed in the top row, and mutant sequences were printed in a second row (Fig. 4). Individual chips were fixed into the bottoms of wells in a 96-well microtiter plate. Multiplex PCR was carried out using human cell line DNA of known genotype. To facilitate detection, amplicons were prepared with one biotin-labeled primer pair. One micro-liter of the PCR product was heat-denatured for 3 min, diluted into 200 μL of hybridization buffer, and hybridized to the biosensor array for 5 min. The immobilized complex was then treated with an anti-biotin antibody-HRP conjugate. Addition of a precipitating substrate for HRP caused deposition of a thin film on the surface. Total assay time was 15 min.

Optimization of the input concentrations of capture probes was essential to obtain unambiguous genotype discrimination. The optimized input concentration of each capture probe was determined empirically with known genotype targets. For homozygous targets, specific hybridization ratios were varied to provide maximum signal in the absence of detectable visual noise; probe concentrations were varied to give 1:1 signals for heterozygous samples. Final capture probe relative input concentrations varied from 1:1 to 1:10. With the optimized assay, hybridization of PCR-amplified wild-type DNA to the array produced unambiguous and highly specific signals. No cross-reactivity was observed with the mutant capture probes (Fig. 4A). Hybridization of homozygous mutant DNA PCR products produced signals only on the appropriate mutant capture probes (Fig. 4, B, D, and F). Hybridization of PCR product from heterozygous samples produced equivalent signal intensities for both the wild-type and mutant capture probe sets (Fig. 4, C and E). The signal-to-noise performance based on CD values for both homozygous wild-type and mutant samples was ~100 for all four mutations tested (Fig. 5). This ratio was the practical upper limit for the biosensor because saturating CD values were 1.0 and background CD values were 0.01.

**Discussion**

We have described a sensitive silicon-based platform for specific detection of nucleic acid or protein targets in a
Multiplex array format. In this system, binding of target analytes to surface-immobilized capture probes triggers enzyme-catalyzed deposition of a localized thin film that increases the surface thickness. The increased thickness is detected by a change in the surface color from gold to purple. Assay results can be read visually to generate qualitative results or by a CCD camera or ellipsometer to give quantitative results. The biosensor can be formatted in microscope slide and microtiter well dimensions and is compatible with widely used automated devices, including pipetting robots. Optically coated flow-through membranes have also been configured.

Application of multiple capture molecules to each surface requires robotic printing of capture moieties. The biosensor surface is quite hydrophobic (contact angle of 105°), making it compatible with the application of capture probes in high densities. Previous work with commercial printing devices has shown that arrays can be produced with a spot diameter of 150 μm and a center-to-center spacing of 200 μm (data not shown), suggesting that immobilization of capture molecules in densities at least as high as glass substrates is possible.

For multiplex cytokine detection, we demonstrated, in a 40-min assay, limits of detection that are similar to those reported for a single analyte/well ELISA format that takes a 40-min assay, limits of detection that are similar to those of microscopy has indicated surface roughness. Analysis of the surface by atomic force microscopy may be attributable to its chemical simplicity: the surface has limited chemical reactivity and low surface roughness. Analysis of the surface by atomic force microscopy has indicated <1 nm variance in thickness (data not shown). Application of target nucleic acids or proteins to the biosensor surface in the presence of clinical samples, including whole blood, serum, sputum, and feces, has shown no adverse effect on assay performance.

The ability to distinguish between single-nucleotide base changes without instrumentation may have applications in various settings where relatively few markers are assessed and/or resources are limited. At present, ~100,000 copies of target are required for an easily detectable signal. Target amplification allows for very short hybridization times and a rapid overall assay. Further improvements in detection may obviate the need for amplification of human genomic DNA targets.

The detection system described here is suitable for rapid and inexpensive multitarget detection in clinical samples. The material costs of the silicon substrate and the detector (ellipsometer or CCD camera) are low. Furthermore, the detection of multiple targets on the same surface reduces sample reagent usage per test. The format can potentially increase clinical diagnostic throughput in multitarget analysis for such applications as infectious disease panels, antibiotic resistance panels, cancer marker panels, or allelic discrimination of disease-associated markers.

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**References**