Hartman interferometer: versatile integrated optic sensor for label-free, real-time quantification of nucleic acids, proteins, and pathogens

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The Hartman interferometer, a proprietary integrated optic sensor, provides a basis for a broad range of biomedical diagnostics, including antibody-based and gene probe-based assays. As with other evanescent-wave optical sensors, the interferometer measures the refractive index change resulting from biomolecular binding on a waveguide surface. The exciting promise of evanescent-wave sensors lies, in general, in their potential to be used as label-free, real-time transducers that can operate in a true mix-and-read fashion and provide fast, quantitative results. One of the major issues facing their development, however, is creating a simple, low-cost configuration for multianalyte testing. The Hartman interferometer addresses this challenge by relying on linearly polarized light and a planar waveguide format, thereby avoiding the problems associated with circular polarization and channel waveguides. We report preliminary experiments that demonstrate the applicability of this sensor configuration to detection of a wide range of protein, nucleic acid, and pathogen analytes.

Considerable interest has been shown over the past few years in the field of evanescent-wave optical sensors for the direct measurement of bioaffinity reactions [1–11]. These devices operate on the basic principle that light confined or resonating within a film <1 μm thick extends on the order of one wavelength into the outside environment (the evanescent-wave region). As shown in a simplified scheme in Fig. 1, the binding of analyte in solution to receptors confined on the surface of a waveguiding film is detected by the resulting change in refractive index of this surface layer (displacement of water by analyte), which affects the phase of a propagating lightwave. This effect confers these optical devices with several highly desirable aspects from the point of view of clinical diagnostics, including the real-time direct detection of an analyte without the prerequisite for labeling of either receptor or analyte. As a result, the response is not only rapid but also inherently quantitative.

Of the various technologies developed, several have been brought to market in the form of research instruments, most noticeably surface plasmon resonance [12], the resonant mirror [13, 14], and the grating coupler [15]. Devices based on optical interferometric techniques [10, 11, 16], while not yet commercialized, are inherently more sensitive than other types of evanescent-wave optical sensors [17]. In particular, the increased interaction pathlength available to interferometers results in at least an order of magnitude improvement in detection limits. This translates to an estimated detection capability of 1 pg/mm² for bound analyte [18].

Optical interferometers function by using two distinct light paths that are combined to form an interference signal. This signal is therefore a differential measurement of the phase propagation in these two light paths, one acting as a reference to the other. By exposing both light...
paths to the sample, and by coating only one with a specific recognition element (e.g., antibody, nucleic acid probe), the differential signal measured becomes referenced to nonspecific binding, which affects both light paths equally.

Taking evanescent-wave interferometers beyond research instrument status and into use as a broad platform for medical diagnostics requires the development of a simple, low-cost format with simultaneous, multianalyte detection capability. Here we discuss the Hartman interferometer, a proprietary integrated optic interferometer [19] that uses a single planar wave of linearly polarized light, thereby greatly simplifying optical coupling and signal processing. Fig. 2 shows both side and top views of the Hartman configuration. Light from a diode laser source is coupled into the waveguiding film as a single broad beam by means of an input grating fabricated into the optical chip. The light passes through multiple, parallel sensing regions on the surface of the chip, which are defined by conventional photolithographic processes. An array of integrated optic elements (e.g., mirrors and beam splitters) is used to combine light passing through adjacent regions, which have been functionalized with specific or nonspecific receptors. Each pairing creates an interferometer, the number of interferometers being limited by the resolution of thin-film manufacturing processes (currently ~1 μm). The interference signals from this array of interferometers are outcoupled through another grating into a photodiode array. The use of a single planar wave of linearly polarized light to operate an array of interferometers simplifies optical coupling and signal processing, and semiconductor manufacturing technology provides for easy fabrication.

Here we present recent results we obtained with a simplified version of this device (a single interferometer with generation of the interference signal external to the chip), thereby demonstrating the general applicability of the device to biomedical diagnostics. As examples of the assays that can be performed with the Hartman interferometer, we show direct immunoassays for human chorionic gonadotropin (hCG)\(^3\), influenza A virus, and Salmonella typhimurium and direct nucleic acid hybridization measurements preliminary to the development of an assay for the differentiation of Mycobacterium species. The current detection capability and the future optimization of these assays are discussed.

**Materials and Methods**

*Reagents.* Anti-influenza A nucleocapsid protein monoclonal antibody (anti-NP) was provided by the Influenza Branch of the Centers for Disease Control and Prevention (CDC). Synthetic oligonucleotides were provided by the Pulmonary and Molecular Biology Core Laboratories of the Emory University Atlanta VA Medical Center. Polyclonal antibody to Salmonella common serum antigen (anti-Salmonella) and Salmonella typhimurium-positive control samples were from KPL Labs. (Gaithersburg, MD). hCG was from Scripps Labs., and monoclonal antibody to hCG (anti-hCG) was from Abbott Labs. All other chemicals were from Pierce Chemical Co., Sigma Chemical Co., or Fisher Scientific, except where indicated.

**Influenza A cell culture samples.** Influenza A virus-positive and negative (control) samples were also provided by the Influenza Branch of the CDC. These were prepared from MDCK (dog kidney) cell cultures, with the positive (virus-containing) culture being infected with influenza virus A/Beijing/333/89 (H3N2 subtype). The supernatants from both control and infected cultures were centrifuged at low speed to remove cell fragments. Viral inactivation and rupture was carried out by using heat treatment (10 min at 56 °C). A rapid immunoperoxidase-staining cell culture assay used to determine the infectivity of the viral preparation before heat shock gave the result as 6.25 × 10\(^{11}\) PFU (plaque-forming units)/L.

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\(^3\) Nonstandard abbreviations: hCG, human chorionic gonadotropin; NP, influenza A nucleocapsid protein; GOPS, 3-glycidoxypropyldimethyl-ethoxysilane; PFU, plaque-forming units; NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido) hexanoate; SSC, standard saline citrate; PBS, phosphate-buffered saline; and BSA, bovine serum albumin.
Synthetic oligonucleotide probe and targets. A biotinylated capture probe, TB-Probe (37 bases), was synthesized with a biotin on the 5' end and a 19-base recognition sequence complementary to a section of the M. tuberculosis 16S rRNA. Two synthetic target oligonucleotides were prepared, one containing this 19-base sequence from the MTB (TB-Target), and the other the same 16S rRNA sequence but from M. avium (MAV). The mismatch between the MTB and MAV sequences was 4 bases.

Hybridization buffers. Hybridization measurements for both complementary and noncomplementary synthetic oligonucleotides were carried out in standard saline citrate (SSC) buffers, either at 5× SSC or 2× SSC concentration (1× SSC = 0.15 mol/L NaCl and 0.015 mol/L trisodium citrate), with 5× Denhardt’s solution [1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, and 1 g/L bovine serum albumin (BSA) in water], 5 mmol/L phosphate buffer, pH 6.8, and 1 g/L Tween-20. All measurements were carried out at ambient room temperature without temperature control. The capture probe was regenerated with a 50 mmol/L solution of sodium hydroxide.

Biotinylation of anti-hCG monoclonal antibody. Biotinylation with sulfo succinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) was carried out for 2 h at room temperature with sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) in hexane for 2 h at room temperature. After a rinsing with citrate buffer and PBS containing 1 g/L Tween-20 (PBS-Tween), the biotinylated receptor (antibody or oligonucleotide probe) was coupled to the avidin-sensing region at room temperature for 40 min and again rinsed with PBS-Tween.

Laboratory test system. Measurements were performed with optical chips that had a single sensing region coated with a specific receptor and a single reference region coated with BSA. An open 2-mL well was affixed to the waveguide with ultraviolet-curing optical adhesive (Norland Products, New Brunswick, NJ). The cell was filled with 1 mL of buffer (PBS containing 2 g/L BSA for immunoassay measurements, hybridization buffers for nucleic acid detection) and stirred by means of a paddle attached to a small dc motor controlled by a rheostat. The background drift was established before sample addition, which was done in small aliquots (typically 5–10 μL) of high concentration, to give the desired final concentration in the cell when diluted into the cell volume. Dose–response curves were compiled by removing the sample solution from the cell and replacing it with new buffer, then adding an aliquot of a higher concentration of analyte.

Results and Discussion

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hCG immunoassay. Measurement of hCG was used as a model for generalized immunoassay detection of protein antigens. Fig. 3 (top) shows the dynamic binding of hCG to the capture anti-hCG antibody, plotted with phase response as a function of time, for concentrations from 10 to 500 μg/L. Each binding curve provides a real-time measure of the immunogenic reaction. As a result, both the initial rate of phase change and the equilibrium phase response provide a quantitative measure of the amount of hCG bound to the surface. Plotting the initial rate response (in π rad/min) against the hCG concentration at nonsaturating amounts of hCG (5–100 μg/L, or 83.5–1670 IU/L) gives a linear relationship with a slope of 1 (Fig. 3, bottom). Equilibrium responses plotted against hCG concentration also provide a dose–response curve (Fig. 3), but...
the linear range is more limited because of the saturation of antibody-binding sites, which results in the nonlinear portion of the dose–response plot. This behavior for equilibrium measurements is expected from the theory of mass action as applied to solid-phase immunoassay [20], which predicts that the dose–response curve will achieve a slope of 1 (unity) only under conditions of receptor excess (i.e., when the antigen concentration is much less than the concentration of immobilized antibody). In contrast, the initial rate will be proportional to the antigen concentration, with the rate controlled by diffusion or binding kinetics. Thus, the upper limit to initial rate detection will be the instrumental resolution of the rate of phase change, which will start to fall off rapidly at very high antigen concentrations.

A direct detection limit of ~2 μg/L has already been demonstrated, whether measured in terms of the initial rate (~<5 min) or at quasi-equilibrium (30–60 min), a phase response of 0.05 rad (a 10% change in signal intensity) being used as the detection limit. Optical improvements and active signal processing can reduce this detection limit at least 10-fold. However, nonspecific binding effects will likely restrain the overall improvement that can be achieved.

Although the Hartman interferometer can be used as a label-free sensor platform, detection limits can be enhanced by using a sandwich format with secondary antibody-coated nanoparticles (100 nm in diameter). As demonstrated elsewhere, up to three orders of magnitude improvement in the detection limit can be achieved by using either gold [9] or latex [21] nanoparticles. By premixing target analyte with secondary antibody-coated nanoparticles, the sensor can still be used for a direct measurement of the surface-binding of the labeled antigen without necessitating a wash step.

**Direct detection of influenza A virus.** Dose–response curves for influenza A virus made by using anti-NP antibody adsorbed directly to the cleaned, nonsilanized optical chip surface covered the concentration range of $2.2 \times 10^9$ to $3.5 \times 10^{11}$ PFU/L. The dynamic binding response for increasing concentrations of heat-treated virus specimens (see Fig. 4, top, which shows the phase response plotted on a logarithmic y-axis) was used to quantify viral load, either by measuring the initial rate of phase change or the final equilibrium phase response. The slopes of the dose–response curves (Fig. 3, bottom) were found to be 0.922 ($r = 0.962$) for equilibrium measurements, and 1.125 ($r = 1.0$) for initial rate measurements. This small deviation from the predicted slope of unity for a solid-phase immunoassay may result from experimental error, or may be from incompletely solubilized virus (i.e., aggregate material). No saturation of the immobilized antibody layer was detected at these concentrations of virus. Although heat-treated virus was detectable only down to $2 \times 10^9$ PFU/L (for a 5-min assay), more recent results for detergent-solubilized virus have given an improved detection limit of $1 \times 10^8$ PFU/L (unpublished results). This improvement is primarily attributable to the fact that heat treatment results in aggregation of the target nucleocapsid protein. The use of a sandwich assay as described above would easily achieve the clinically relevant detection limit of $1 \times 10^9$ PFU/L.

**Direct detection of Salmonella typhimurium.** Equilibrium dose–response curves (40 min) were made for the direct detection of whole *Salmonella typhimurium* cells over the concentration range $5 \times 10^8$ to $5 \times 10^{10}$ CFU/L (Fig. 5). At
low bacterial concentrations, the response was diffusion-limited (essentially linear with measurement time). In these experiments cell disruption was not required because the antibody was directed against *Salmonella* surface antigens. The slope of this dose–response curve, 0.75 ($r = 0.989$), deviated considerably from the slope of 1 predicted for immunoassay measurements—an indication that the binding is more complicated than in the other immunoassay results presented here, in which a single protein (hCG or nucleocapsid protein) is being captured by a monoclonal antibody. Although additional measurements (not shown) were found to reproduce the slope observed in Fig. 5, further study is required to understand the binding process.

The direct detection limit of $5 \times 10^8$ CFU/L achieved thus far reflects an extremely low antigenic capture efficiency. Even with a sample containing $1 \times 10^{10}$ CFU/L, the bound cells (viewed under an optical microscope) appear to cover only $\sim 1\%$ of the surface. In comparison, $\sim 10\%$ surface coverage was reported with a sample containing $1 \times 10^{10}$ cells/L Staphylococcus aureus and an IgG-functionalized resonant mirror sensor [9]. We therefore believe that significant room for improvement remains in this direct detection assay. Even without improved capture efficiency, a nanoparticle-based sandwich assay should improve the detection limit of the sensor to $\sim 5 \times 10^5$ CFU/L.

**DIRECT HYBRIDIZATION ASSAY FOR NUCLEIC ACID DETECTION**

The real-time hybridization of target nucleic acid in solution to a complementary single-stranded probe immobilized on the interferometer surface was measured as easily as antibody–antigen binding. Fig. 6 (left) shows the binding of the complementary target oligonucleotide (TB-Target) for concentrations ranging from 4 to 2000 $\mu$g/L. The initial rate of response correlates linearly with concentration (not shown). Specific hybridization detection for complementary (TB-Target) vs noncomplementary (GQLF) oligonucleotides was confirmed (Fig. 6, right). Even at a saturating concentration of the noncomplementary oligonucleotide, the phase response was essentially indistinguishable from background. Differentiation of the
closely related MTB and MAV sequences was more difficult, because out of 19 bases (in the target region) there are present a continuous, identical 14-base sequence and only a 4-base mismatch. However, by using a more stringent hybridization buffer prepared in 500 mL/L formamide, we reduced the signal response for MAV (4 base mismatch) to ~10% of that for MTB (complete match) (Fig. 7). However, the response rate for hybridization of the MTB target in the formamide buffer was greater than that for buffer without formamide, a result that requires further investigation. We expect that resolution of single-base mismatches will simply require the application of current DNA hybridization protocols, as opposed to current room temperature procedures. The current nucleic acid detection capability is ~4 μg/L, which corresponds to ~10^11 copies of target nucleic acid per 1-mL sample volume (assuming a molecular mass of 15 kDa for a 44-base oligonucleotide). A nanoparticle-enhanced sandwich assay format should improve this detection limit to 10^8 copies, or about the number of copies of Mycobacterial rRNA expected at a clinically meaningful concentration of 10^7 cells/L (~10 000 copies per cell).

In conclusion, these preliminary experiments demonstrate that the Hartman interferometer can be used for a broad range of biomedical measurements. Direct detection of analytes at 2 μg/L has already been demonstrated, with an order of magnitude or more improvement likely. If required, particle-labeled sandwich assay formats offer the potential of 1000-fold amplification. Real-time detection allows assays to be completed in at most 5 min. As with other integrated optic configurations, the chip format of the Hartman interferometer allows simultaneous measurements of several analytes in a single assay and built-in reference channels to monitor nonspecific binding. However, the unique use of linearly polarized planar waves greatly reduces both the practical difficulty and the production costs.

![Fig. 6. Real-time detection of nucleic acid: (left) dynamic phase response for the hybridization of complementary target nucleic acid to a capture probe immobilized on the sensor surface plotted as a function of time for various concentrations of target; (right) differentiation of complementary (TB-Target) and noncomplementary (GQLF) nucleic acids determined through direct hybridization measurements to a capture probe immobilized on the sensor surface.](image)

![Fig. 7. Differentiation of closely related nucleic acid sequences determined under various hybridization conditions. The selective detection of MTB nucleic acid (fully complementary sequence) from MAV nucleic acid (4-base mismatch) is observed in a hybridization buffer containing 500 mL/L formamide, but not in hybridization buffer without formamide.](image)
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