

Highly sensitive optical chip immunoassays in human serum

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Abstract

Over the past decade the ability of refractometric optical sensors to quantitatively measure a wide range of biomolecules has been demonstrated. These include proteins, nucleic acids, microorganisms, and in competitive formats small molecules such as drugs and pesticides. Furthermore, by using high refractive index nanoparticles to amplify the biomolecular binding signal, sensitivities approaching those of well established diagnostic assays have been achieved. However, to date it has not been possible to show rapid detection of analytes in complex bodily fluids such as serum, in a one-step procedure, due to the interference resulting from non-specific binding (NSB) to the sensor surface. We have carried out preliminary work on the control of interference due to NSB using an optical chip based on the Hartman interferometer. This interferometer configuration employs a reference sensing region that can be functionalized separately from the specific sensing region. Optical chips were stored dry after surface functionalization, and rehydrated in serum. The observed level of background drift in serum was reduced by an order of magnitude when an exposed reference was used, compared to a reference which was blind to the sample. An additional 70% reduction in signal drift in serum was achieved by controlling the surface chemistry of the optical chip using a biotin-poly(ethylene glycol) (PEG) blocking agent. This functionalization procedure was combined with a sandwich assay using gold nanoparticles to develop a one-step assay for human chorionic gonadotropin (hCG) in human serum with a detection limit of 0.1 ng/ml for a 35 min assay. © 2000 Elsevier Science S.A. All rights reserved.

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1. Introduction

Direct detection of biomolecules using evanescent field-coupled refractometric optical sensors has become a subject of considerable interest in recent years. This interest derives from the capability of these devices to measure, in real time, both immunological and nucleic acid hybridization reactions, providing a versatile transducer platform for biomolecular diagnostics. Refractometric optical sensors fall generally into one of two categories; (1) interferometers (Heideman, et al., 1993; Schlatter et al., 1993; Brosinger et al., 1997; Schipper et al., 1997; Schneider et al., 1997; Luff et al., 1998; Weisser et al., 1999); and (2) resonance devices, such as surface plasmon resonance (SPR) (Lundström, 1994; Karlsson and Ståhlberg, 1995; Lyon et al., 1998;

O'Meara et al., 1998; Woodbury et al., 1998; Harris et al., 1999), the resonant mirror sensor (Buckle et al., 1993; Watts et al., 1994, 1995) and grating couplers (Tiefenthaler and Lukosz, 1989; Bier and Schmid, 1994; Fattinger et al., 1995; Clerc and Lukosz, 1997). All function by monitoring the effect on optical waves from a coherent light source produced by the refractive index changes associated with biomolecular binding at the sensor/solution interface. In resonance devices the refractive index change brought about as a result of surface binding alters the incident angle at which the optical wave will couple into the waveguide layer or thin gold film in the case of SPR. In interferometers the refractive index change is measured as a phase difference between two propagating optical waves.

Refractometric optical sensors have been shown to be highly sensitive to surface binding. Several groups have demonstrated detection levels of a few pg/mm² (Heideman et al., 1993; Schlatter et al., 1993; Karlsson and Ståhlberg, 1995; Luff et al., 1998). In theory inter-

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ferometers are an order of magnitude more sensitive than resonance devices or grating couplers, with a detection limit of 0.4 pg/mm^2 (Lukosz, 1991; Huber et al., 1992), principally due to the longer interaction path length available to interferometers. Additional sensitivity with refractometric sensors may be gained by using sandwich assay formats, in which secondary antibodies are conjugated to latex or gold nanoparticles (Severs and Schasfoort, 1993; Watts et al., 1994; Kubitschko et al., 1997; Spinke et al., 1997). Even in this approach the real-time, quantitative detection of these sensors is maintained, since it then becomes the direct binding of the antigen-nanoparticle conjugate which is measured. An added benefit of this approach is that the nanoparticle conjugate gives a signal that is independent of the size of the original target analyte, because the signal derived from surface binding of the nanoparticle is orders of magnitude larger than that arising from the antigen itself. The ability of these sensors to measure minute changes in surface binding is also the practical limitation in measurements of clinical specimens, in which non-specific binding (NSB) of sample matrix components introduces a variance and lower limit on analyte detection. It is theoretically possible to null out background signal due to NSB by using a reference surface exposed to the sample. In the Hartman interferometer this is achieved by exposing both arms (sensing regions) of the interferometer to the test sample, but with only the specific sensing region bearing a specific capture antibody while the reference sensing region is coated with an irrelevant antibody layer. In this interferometer design the subtraction of the reference signal from the specific binding signal is carried out optically, before the phase information is converted into an electrical output. This approach yields less noise than first converting the specific binding and reference responses into electrical outputs and then subtracting them, as is necessary for resonance devices and common path interferometers.

Recently, we described the Hartman interferometer, in which a single lightbeam was used to address multiple sensing elements on a microfabricated optical chip (Schneider et al., 1997). The present paper describes further research in our laboratories on the development of this device for rapid, multi-analyte immunoassays in serum, using human chorionic gonadotropin (hCG) as a model system. To obtain rapid measurements in serum a functionalization methodology was required that: (1) allowed the differential immobilization of antibodies onto different sensing regions on the optical chip surface; (2) generated a sufficient level of immunoactivity of the immobilized capture antibody layer for analyte detection at low concentrations; (3) incorporated a blocking scheme to minimize NSB on both the specific and reference sensing regions; and (4) had a stabilizing

treatment to allow full retention of immunoactivity after dry storage. In addition, a method was required that combined a simple, one-step assay format with signal enhancement using nanoparticle conjugates.

2. Experimental

2.1. Materials

hCG was obtained from Scripps Laboratories (San Diego, CA). Monoclonal antibodies (Mabs) against hCG (Mab A94 against the alpha subunit and Mab B56 against the beta subunit), cytomegalovirus (CMV), and 5-dimethyl-aminonaphthalene-1-sulfone (dansyl) were developed by Abbott Laboratories (Abbott Park, IL). NeutrAvidin™ (avidin) was obtained from Pierce Chemical Company (Rockford, IL). Calf serum was obtained from Sigma (St. Louis, MO). Human serum, from males, was supplied by Interstate Blood Bank, Inc. (Memphis, TN). Poly (ethylene glycol) (PEG) derivatives were purchased from Shearwater Chemicals (Huntsville, AL), silane reagents were purchased from United Chemical Technologies (Bristol, PA). Sodium borotrifluoride at a specific activity of 28.0 Ci/mol , NCS Tissue Solubilizer and BCS-NA scintillation fluid were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Nanoparticles (5 nm and 15 nm colloidal gold and 100 nm latex) were obtained from Polysciences (Warrington, PA). Other chemicals used were of ACS grade or equivalent and used as supplied.

Phosphate buffer contained 0.15 M sodium chloride and 0.1% sodium azide in 20 mM phosphate, pH 7.4 (PBS). Additional buffers were PBS containing 0.2% bovine serum albumin (PBS-B) and PBS containing 0.1% Tween-20 (PBS-T).

2.2. Chip design and fabrication

The optical chips used in these studies were fabricated at the Georgia Institute of Technology, and were based on the Hartman interferometer (Hartman, 1997). The basic optical chip structure (Fig. 1) consisted of a silicon nitride (Si_3N_4) waveguiding film (140 nm thick) deposited onto a BK-7 glass substrate using plasma-enhanced chemical vapor deposition. Grating couplers were used to couple light from a 670 nm diode laser into and out of the waveguiding layer. The light was passed through two sensing regions, which were 15 mm long by 1 mm wide and separated by a 1 mm gap, defined using silicon dioxide (SiO_2) overlayers. A 500 nm thick SiO_2 film was coated onto the optical chip surface everywhere except in the two sensing regions, where a thickness of 40 nm was used.

2.3. Biotinylation of monoclonal antibodies

Anti-hCG Mab A94, anti-CMV and anti-dansyl Mabs were biotinylated using NHS-LC-LC-biotin (Pierce) at a 1:15 molar ratio, using a Mab concentration of 2 mg/ml, in 0.1M sodium bicarbonate buffer pH 8.4. NHS-LC-LC-biotin was prepared at 10–20 µg/µl in dimethyl sulfoxide, and 5–7 µl were added. The biotinylation was carried out inside a dialysis cassette of 10 000 molecular weight cut-off (Pierce) with gentle shaking for 2 h at room temperature (RT). The dialysis cassette was placed directly into PBS and free biotin

removed by extensive dialysis at 4°C. The ^3H -labelled Mab was biotinylated essentially by the same procedure, except that the biotinylation buffer was exchanged with PBS, and free biotin removed using Microcon-30 spin columns (Amicon, Beverly, MA).

2.4. Optical chip surface treatments and antibody immobilization

Functionalization protocols were developed to effect the immobilization of different Mabs onto the two parallel sensing regions, and to apply blocking and stabilizing treatments to the immobilized antibody films. Optical chips were cleaned in hot sulfuric–chromic acid cleaning solution (Fisher Scientific, Pittsburgh, PA). Positive photoresist Microposit SC1827 (Microlithography Chemical Corporation, Newton, MA) was spin-coated onto the optical chip surface and soft baked on a hot plate at 114°C for 1 min. The optical chip was then exposed to UV light to develop the photoresist, with a positive chromium mask used to block the sensing regions. After development, the exposed surface was silanized in a 3% solution of (tridecafluoro-1,1,2,2-tetrahydro-octyl)-1-dimethylchlorosilane in hexane at RT. After removing the remaining photoresist, the sensing regions were silanized overnight at 50°C with an aqueous 3% solution of 3-glycidypropyldimethylethoxysilane (GOPS) acidified to pH 2 with 1M sulfuric acid. Oxidation of the GOPS film to aldehyde groups was carried out using 0.5% sodium periodate in 80% glacial acetic acid at RT.

Avidin was immobilized onto both sensing regions via reductive amination coupling carried out for 15 min at RT, using 0.5 mg/ml protein and 10 mg/ml sodium cyanoborohydride in 100 mM citrate buffer pH 5.5. After rinsing with citrate buffer and PBS-T, binding of the biotinylated Mab A94 and control Mabs was carried out at 0.1 mg/ml concentration and RT for 15 min, followed by rinsing with PBS-T.

When blocking treatments using PEG derivatives were used, they followed the immobilization of the biotinylated antibodies. Both sensing regions were covered with the PEG solution and left for 15 min at RT, followed by rinsing with PBS-T. Stabilizer solutions were coated onto the optical chips as a final procedure (see discussion in Results section on Dry Storage). After a 15 min incubation, excess solution was removed and the chips were dried in an incubator for 30 min at 37°C. The dried chips were stored in a desiccator until tested.

2.5. Optical chip test system and assay procedures

Interferometric measurements with dry-stored optical chips were performed using a flow system, as shown schematically in Fig. 2. A flow cell made from clear

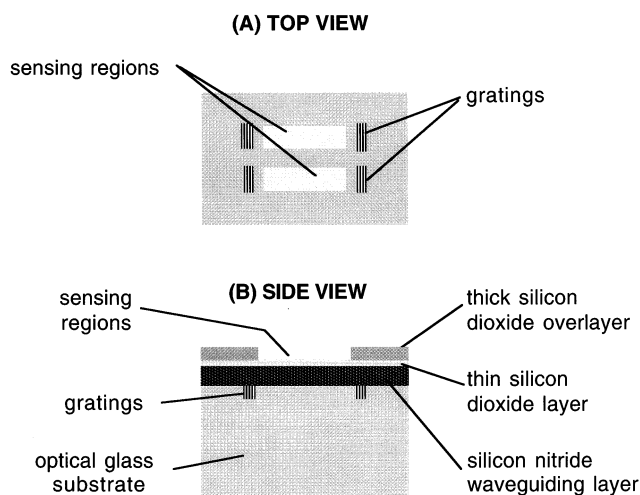


Fig. 1. Schematic view of prototype optical chip: (a) top view; (b) side view. The optical chip consisted of a thin Si_3N_4 waveguiding layer deposited onto an optical glass substrate. A thin SiO_2 layer was deposited on top of the waveguiding layer in the sensing regions to provide the surface for biomolecule attachment. A thick SiO_2 layer was used to bury the evanescent field from the sample outside of the sensing regions.

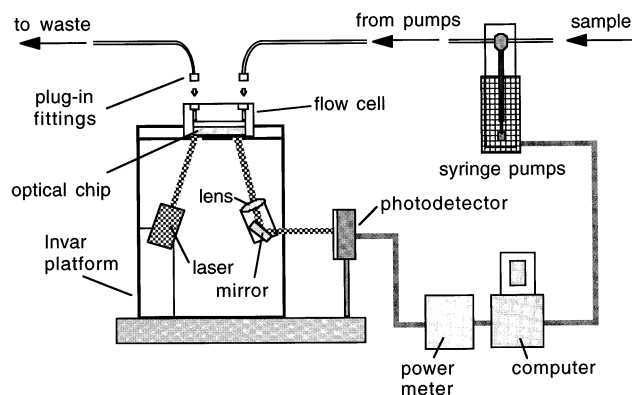


Fig. 2. Schematic view of test system. An optical chip with attached flow cell was mounted in the test platform. A diode laser was coupled into the optical chip from below, and the outcoupled light was focused with a lens onto the photodetector. A computer running LabView software was used to monitor the phase response from the optical chip, while at the same time operating the syringe pumps that controlled sample flow.

methacrylate was sealed against the optical chip sensing surface using a double-sided adhesive gasket with a cut-out center which served as the flow channel across the sensing regions. The thickness of this gap between optical chip surface and flow cell was nominally 100 μm . The flow cell-optical chip assembly was mounted in a slot in the test platform and held in place using a screw-down bracket.

The flow system consisted of syringe pumps fitted with 1 ml glass syringes (Cavro, Sunnyvale, CA) which pushed fluid through the SmartValve (Cavro) and test cell. A PC running LabView™ software (National Instruments, Austin, TX) controlled the pumps and SmartValve, and simultaneously collected data.

Typical experiments comprised the following steps. Test solution was flushed through the cell to rehydrate the dried films and to wet hydrophobic regions of the optical chip surface. Fine adjustments of laser coupling were performed to maximize signal modulation, and typically required less than 2 min to accomplish. After data acquisition was initiated, the sample solution, including added hCG and/or nanoparticle conjugate as described for each experiment, was pumped through the cell at a steady flow rate of approximately 60 $\mu\text{l}/\text{min}$.

2.6. Tritium labelling of proteins for off-line studies

The labeling of glycopeptides by periodate oxidation and subsequent reduction by sodium borohydride has been described previously (Butkowski et al., 1974). Briefly, 1 mg of hCG in 1 ml of PBS pH 7.0 was oxidized by 9 mM sodium periodate for 20 min at RT. The reaction was stopped by incubation with 150 mM glycerol. The solution was run on a Sephadex G-25 desalting column equilibrated with PBS or dialyzed in a dialysis cassette for 2 h at 4°C with rapid stirring. The hCG was reduced for 30 min with 0.29 μmol of sodium borohydride in a solution adjusted to a final concentration of 0.16 M sodium carbonate, pH 9.6. Reduction was completed by incubation in unlabeled sodium borohydride at a final concentration of 6 mM. The tritiated hCG (^3H -hCG) was separated from the unreacted sodium borohydride on a desalting column and dialyzed against 300 volumes of PBS. The hCG was stored at 4°C in PBS-B. The specific radioactivity level of the hCG was 5×10^5 counts per min (cpm) per μg .

The immunoreactivity of the ^3H -hCG was evaluated by measuring its reactivity with nanoparticles conjugated to anti-hCG Mabs. When nanoparticles conjugated to Mab B56 were incubated with the ^3H -hCG and centrifuged, 91% of the counts pelleted with the nanoparticles. In a similar experiment, 84% of the counts pelleted with nanoparticles conjugated to Mab A94.

Mab A94 was labelled with ^3H as described for hCG except that 5.8 mg of oxidized Mab in 2.0 ml of buffer was reduced with 0.36 μmol of sodium borohydride. The specific radioactivity level of the Mab was 3×10^4 cpm per μg .

2.7. Off-line studies

In order to evaluate the antibody immobilization procedure, 10 cm diameter silicon wafers were coated with Si_3N_4 and SiO_2 layers as described above for the BK-7 glass optical chips. These wafers were then diced into 0.16 cm^2 squares, and functionalized with avidin as described above for the sensing regions of the optical chip. Varying concentrations of ^3H -biotinylated Mab A94 in 8 μl of PBS-B were incubated on the surface for 1.5 h at RT in humidified chambers. Chips were washed with PBS, dried, and the amount of ^3H measured. In further experiments, the activity of a non-tritiated, immobilized Mab A94 surface was determined by incubating an excess of ^3H -hCG in 50 μl of PBS-B on 1 cm^2 silicon wafers. Anti-dansyl Mab was immobilized on control silicon wafer chips.

The ^3H bound to the chips was measured as dried samples solubilized with a NCS: H_2O (5:1) mixture for 30 min with shaking. BCS-NA scintillation fluid was added to give a final ratio of scintillation fluid to solubilizer of 17. A correction factor, which was determined by comparing counts in the presence and absence of a chip, was used when chips were counted.

Experiments correlating the interferometric response with ^3H -hCG bound to optical chip surfaces were performed in the open well test system described previously (Schneider et al., 1997). In order to obtain a maximum radiolabel signal, biotinylated Mab A94 was immobilized to one half of the optical chip surface and the biotinylated anti-dansyl control Mab was immobilized onto the other half. Each sensing region remained 15 mm^2 . ^3H -hCG at 100 ng/ml in PBS-B was incubated on the functionalized surface until a response $\geq 1.9 \pi$ radians was recorded. The surface was washed three times with PBS-B. The ^3H -hCG was solubilized with NCS: H_2O (5:1) and counted.

2.8. Antibody conjugation to latex and colloidal gold

Mab B56 was conjugated to 15 nm diameter colloidal gold by passive adsorption. The gold colloid was processed at approximately 1 OD and pH 7. The gold particles were coated with a final Mab concentration of 10 $\mu\text{g}/\text{ml}$, which was enough to nearly saturate the particles, and mixed gently. Then, the particles were blocked by the addition of 0.6% BSA with continued gentle mixing. The conjugate preparation was centrifuged at 12 000 rpm for 10 min. Approximately 90% of the supernatant was removed and the conjugate was

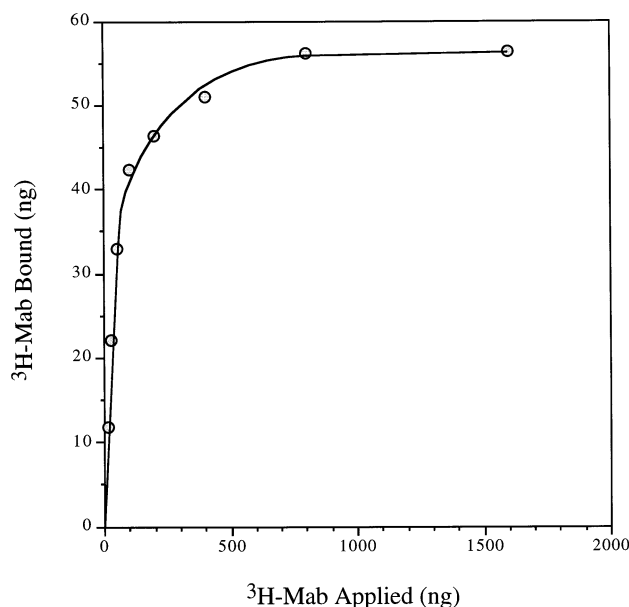


Fig. 3. Off-line determination of the amount of biotinylated ^3H -Mab A94 bound to avidin-coated SiO_2 surfaces measured as a function of the Mab concentration applied.

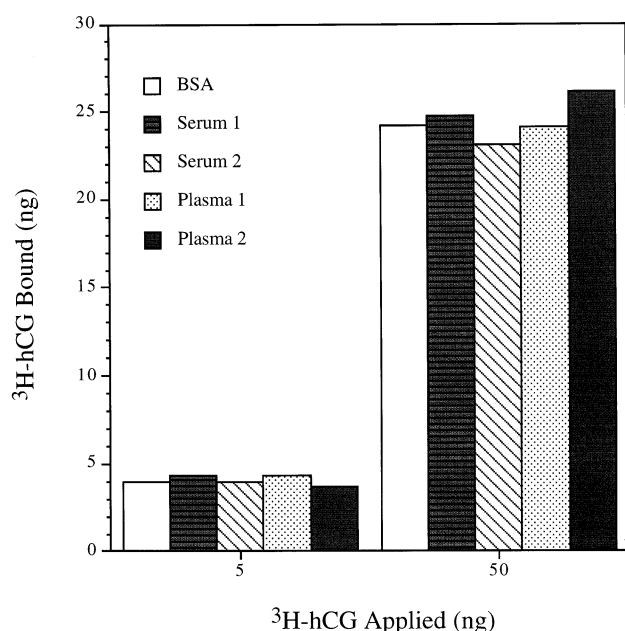


Fig. 4. Binding of ^3H -hCG to Mab A94-functionalized SiO_2 surfaces. The amount of ^3H -hCG bound at equilibrium from PBS-B, serum and plasma was measured at 5 and 50 ng of ^3H -hCG applied, which were sub-saturating and saturating levels, respectively.

resuspended and stored at 4°C. Conjugation to 5 nm diameter gold followed the same procedure, except the centrifugation step was replaced by diafiltration using 100 K Microgon cartridges to remove free antibody.

Mab B56 was conjugated to 100 nm diameter latex nanoparticles by passive adsorption at pH 7.0, using a protein concentration of 300 $\mu\text{g/ml}$ and a latex concen-

tration of 0.26%. After incubation at RT for 1 h with shaking, the latex surface was blocked with 1% BSA. The latex was washed twice with 1% BSA using centrifugation. The washed nanoparticles were resuspended in 1% BSA to approximately 2.6% solids.

3. Results and discussion

3.1. Evaluation of optical chip functionalization

Off-line measurements were used to evaluate the immobilization of Mab A94 and the subsequent binding of hCG antigen. The amount of Mab A94 immobilized as a function of the concentration added is shown in Fig. 3. A saturation level of 3.52 ng/mm² (a total of 56.3 ng bound) was determined, which corresponds to a surface coverage of 2.33×10^{-14} mol/mm², consistent with a randomly orientated IgG monolayer (Buckle et al., 1993; Spinke et al., 1997). Silicon wafer chips functionalized with saturating levels of Mab were tested for their binding activity for hCG in PBS-B, serum and plasma using 50 µl of ³H-hCG solution per chip. At 1 µg/ml ³H-hCG (50 ng applied), which was above the concentration necessary to saturate the binding capacity of the Mab monolayer, there was little difference in hCG bound when the three solutions were compared (Fig. 4). Likewise, only minor differences were observed when the subsaturating level of 100 ng/ml (5 ng applied) was tested. The observation that 2.33×10^{-14} mol/mm² of Mab bound 6.5×10^{-15} mol/mm² of hCG (0.26 ng/mm²) indicated that approximately 28% of the immobilized Mab bound antigen.

A separate set of studies using functionalized optical chips were carried out to correlate directly phase response with the amount of hCG bound. A value of 53.3 pg /mm² of hCG bound correlated with a 1 π radian phase response (a total of 800 pg hCG bound onto a 15 mm² sensing area). This corresponds to a coverage of 8×10^8 molecules/mm², which is 2% of a theoretical monolayer (4×10^{10} molecules/mm²). Hartman (1998) performed an analysis of hCG binding to an anti-hCG Mab coated optical chip surface using a multilayer waveguide model. Assuming a protein refractive index of 1.48, the model estimated a 2.7% surface coverage resulting from a 1 π radian phase response, which agrees well with the experimental results reported here.

3.2. Dry storage

Before evaluating surface blocking methods for controlling NSB it was a practical necessity to develop a method for stabilizing functionalized optical chips during dry storage, so that retained activity was independent of storage time. The primary objective of this stabilizing treatment was to prevent irreversible confor-

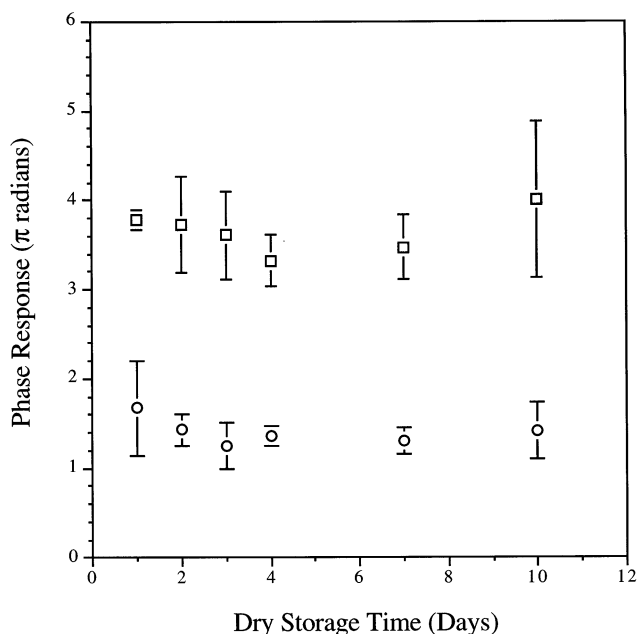


Fig. 5. Activity of optical chips as a function of dry storage time. Functionalized optical chips were coated with GIC stabilizing treatment and dried in an incubator. Triplicate measurements to a test solution of 50 ng/ml hCG in PBS-B were obtained after 1–10 days of dry storage in a desiccator. Error bars represent 1 SD from mean values. (○) Analyzed response after 10 min; (□) analyzed equilibrium response after 40 min.

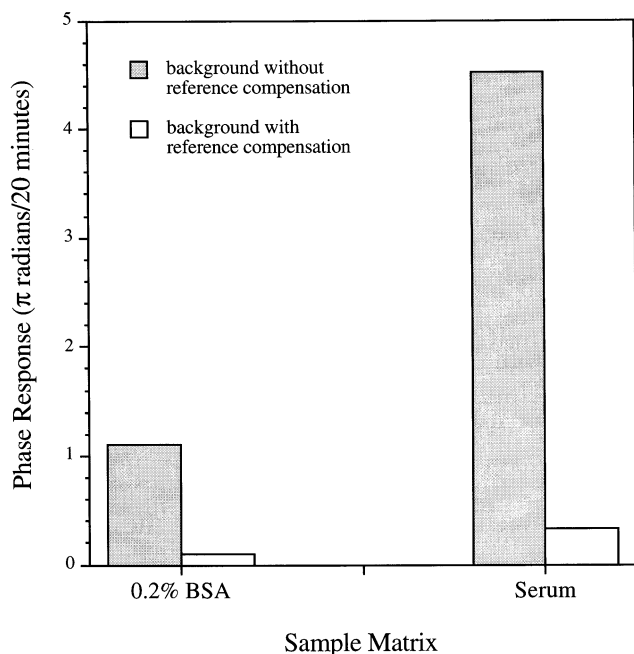


Fig. 6. Background phase response measured over a 20 min period for optical chips with background compensation (exposed reference) and without background compensation (buried reference). Experiments in calf serum used optical chips treated with the biotin-PEG-5000 blocker.

mation changes from occurring during dehydration, which could lead to loss of activity. Polyhydroxylated compounds, such as poly-alcohols and sugars have been used to stabilize proteins (Larreta-Garde et al., 1984; Monsan and Combes, 1984; Gibson and Woodward, 1992; Flounders et al., 1997). We found that a mixture of glucose, inositol and casein (GIC coating) worked effectively to stabilize the immobilized antibodies. Functionalized optical chips that had been treated with the GIC coating, then dried and stored in a desiccator, were tested with 50 ng/ml hCG. While a slight drop in immunoactivity was observed during the first 2–4 days of dry storage, there appeared to be little subsequent drop in activity, either in the initial 10 min dynamic response or the 40 min equilibrium response (Fig. 5). The GIC coating was used in all further experiments.

3.3. Measurement of NSB in serum: exposed versus buried reference

In standard sandwich immunoassays, where a secondary labeled antibody provides the measurement signal, NSB of this antibody can be a major source of false-positive signals, which affects assay performance. In the case of refractometric sensors, including the Hartman interferometer, indiscriminate NSB caused by sample matrix components can also give rise to false-positive signals. However, the self-referencing capability of the interferometer provides a mechanism whereby all sources of NSB can be, at least in principle, cancelled out. A major objective of this work was to measure the degree to which this cancellation may be effected in practice.

The phase signals for optical chips with and without an exposed reference sensing region were compared using either PBS-B or serum as the test solution (Fig. 6). Optical chips with only a single exposed sensing region were fabricated by coating the reference sensing region with a SiO_2 film sufficiently thick to place the sensor/solution interface beyond the evanescent field. Mab A94 was bound to the exposed sensing region. Optical chips with both sensing regions exposed had Mab A94 on the specific sensing region and anti-CMV Mab on the reference sensing region. The average level of phase signal in PBS-B for experiments with an exposed reference sensing region was 0.106π radians in 20 min. In most experiments this level of drift decreased as the chip continued to equilibrate. When the reference sensing region was buried, the measured phase signal in PBS-B was 1.102π radians, which was an order of magnitude greater. This reflects the larger imbalance between the exposed specific sensing region and the buried reference sensing region. Some of the possible sources of this imbalance are surface phenomena such as adsorption/desorption, surface wetting and thermal-mechanical differences between the two sensing regions.

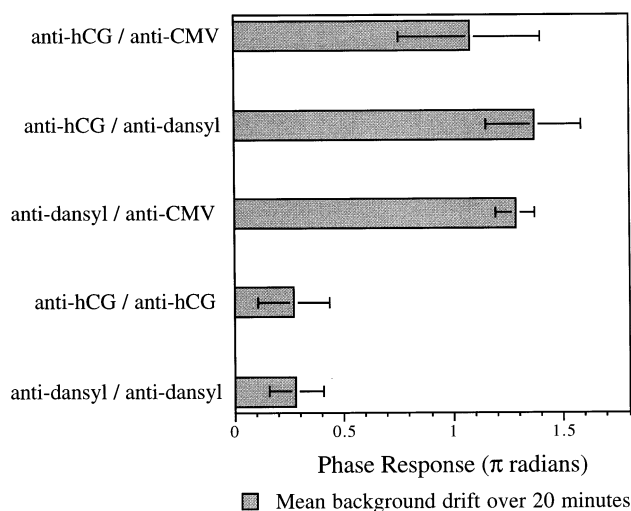


Fig. 7. Measurement of background phase response for different combinations of Mabs on the specific and reference sensing regions (exposed reference). Error bars represent 1 SD from mean values, which were based on triplicate measurements.

The main component, however, may have been residual porosity of the thin (40 nm) SiO_2 layer coating the exposed sensing regions, resulting in slow water penetration. This problem could be solved by using the denser films obtainable from electron-beam deposition, which was used to deposit the thick (500 nm) SiO_2 films.

In further experiments, in which this background drift was measured in serum, the optical chips were blocked with the biotin-PEG-5,000 surface blocking treatment (see following section). The signal with the reference region buried (4.515π radians) was again significantly greater than with the reference region exposed (0.323π radians). This background signal was greater in serum than in PBS-B whether the reference region was buried or exposed. If it is assumed that this difference in background drift for measurements in serum compared to PBS-B represents NSB, then the differential signal measured with the exposed reference sensing region was only 6% of the actual NSB signal, which was represented by the measurements with the buried reference sensing region. Thus, the reference sensing region was able to compensate for about 94% of the signal due to NSB occurring on the specific sensing region. Since the optical chip was exposed to serum for about 1–2 min prior to data acquisition, these measurements were not able to provide absolute values for the total amount of NSB that had occurred. They do represent, however, some measure of the continuing adsorption to the surface and the interferometric referencing provided by the exposed reference sensing region. Based on off-line measurements, this increased signal in serum relative to PBS-B for optical chips with a buried reference sensing region corre-

sponds to 182 pg/mm^2 of protein, in the range of 3–7% of a monolayer growth (depending whether this adsorption is primarily due to albumin, immunoglobulins, or other biomolecules) over the 20 min measurement period.

3.4. Interferometric control of NSB

The results in serum shown in Fig. 6 represent the total background phase signal measured over a 20 min period. The phase signals recorded during this measurement were not linear, but decreased as a function of time for optical chips with either exposed or buried reference sensing regions (results not shown). With the exposed reference region, phase drifts $< 0.005 \pi/\text{min}$ in serum were observed if the optical chips were left to equilibrate for longer periods of time, suggesting that the rate of NSB to each individual sensing region decreases with exposure time to serum. Thus, after long exposure the difference between the rates of NSB on the specific and reference sensing regions becomes extremely low, resulting in low background drift. Kubitschko et al. (1997) stabilized the signal of a bidiffractive grating coupler by applying a blocking solution immediately before introducing the serum sample. This device, which has a common path reference that does not allow discrimination of NSB through a separately functionalized reference surface, was capable of detecting thyroid stimulating hormone at the clinically-relevant level of 1 pg/ml using signal enhancement resulting from a latex nanoparticle-based sandwich assay. Although demonstrating high sensitivity, this test format is not applicable to the simple, rapid assays required for point-of-care testing.

The signal measured with the exposed reference sensing region was a differential signal derived from NSB to both the specific and reference sensing regions. This point was demonstrated effectively by measuring the total background phase signal during a 20 min exposure to serum on optical chips with different combinations of Mabs on the two sensing regions (Fig. 7). When the same Mab was immobilized on both sensing regions the resulting signal was reduced by 78% (4.5 times lower) compared to the signal measured when different Mabs were immobilized on the two sensing regions. Furthermore, more signal drift was observed when one of the antibodies was the anti-dansyl Mab. Thus, the anti-CMV Mab was a better match than the anti-dansyl Mab as a reference for the anti-hCG capture Mab.

A complementary approach to lowering NSB was surface blocking treatments. Commercially available blocking buffers, for example SuperBlock and SeaBlock buffers from Pierce Chemical Company, were tested but were not effective in reducing the initial background phase signal in serum (results not shown). Surface-at-

tached PEG films have been found to be particularly good at reducing protein adsorption (Gombotz et al., 1992; Bergström et al., 1992; Österberg et al., 1993; Van Alstine and Malmsten, 1997; Seigel et al., 1997). Therefore we tested several different PEG derivatives as surface blockers by measuring the background signal in serum. The results of these experiments are shown in Fig. 8. Compared to the unblocked control, PEGylating the surface with PEG-SPA-20 000, an *N*-hydroxy-succinimidyl ester derivative expected to couple to amine groups on both the Mab and avidin layers, resulted in a small reduction in the background signal. The biotinylated PEG derivatives, which should fill free biotin-binding sites on the avidin layer, resulted in a more stable signal. Overall, the biotin-PEG-5000 derivative reduced the background signal by 70% as compared to the control, which approximated the re-

duction achieved by using the same Mab on both sensing regions.

3.5. Signal amplification using nanoparticle conjugates

The clinically relevant range for hCG detection is 5–25 mIU/ml, which corresponds to 0.3–1.5 ng/ml of the hCG used in these studies. Our goal was detection of 0.1 ng/ml hCG in human serum using a rapid, one-step assay without wash steps. The level of background drift observed in direct binding studies necessitated the use of signal amplification by high refractive index nanoparticles in a sandwich format as a means of achieving the desired detection limit.

Both colloidal gold and latex polystyrene nano- and microparticles have been widely used in immunodiagnostic assays, and both are capable of providing signal enhancement with refractometric optical sensors (Severs and Schasfoort, 1993; Watts et al., 1994; Kubitschko et al., 1997; Spinke et al., 1997). We tested 5 and 15 nm diameter colloidal gold and 100 nm latex particles that were conjugated to Mab B56. The results of hCG measurements in PBS-B with and without signal enhancement are shown in Table 1. These results were obtained after first allowing the background signal to fully stabilize. Compared to unamplified assays, the use of 5 nm gold resulted in an enhancement of 22-fold, while 15 nm gold and 100 nm latex gave an enhancement of 220- and 250-fold, respectively.

3.6. Dose-response measurements for hCG in human serum

A series of experiments were performed to evaluate the dose response, variation, and limits of detection of the hCG assay in human serum. The optical chips were functionalized with Mab A94 and anti-CMV Mabs on the specific and reference sensing regions respectively, followed by application of the biotin-PEG-5000 blocking treatment and the GIC stabilizer coating. The 15 nm gold colloid, conjugated to Mab B56, was used for signal amplification in a one-step, sandwich assay format. The following assay procedure was employed: (1) a dry-stored, functionalized optical chip was rehydrated with unadulterated serum and fine adjustments were made to achieve optimal light coupling; (2) the gold conjugate and hCG were added to the serum sample to be tested, and a syringe pump was used to pump this sample through the flow cell and measure the binding response in real time. Because the optical chips used in these studies were manufactured in a research setting there were slight variations in dicing that required each optical chip to be individually positioned with respect to the laser in order to achieve optimal light coupling into the waveguiding film.

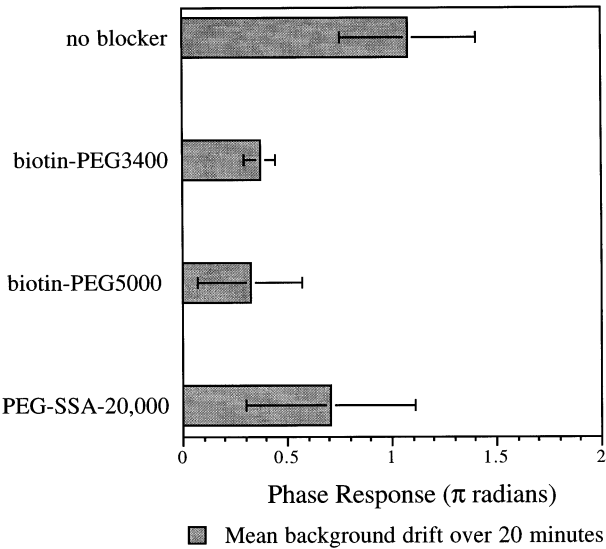


Fig. 8. Measurement of background phase response after different surface blocking treatments (exposed reference). Optical chips were functionalized with A94 and anti-CMV Mabs bound to the specific and reference sensing regions, respectively. Error bars represent 1 SD from mean values, which were based on triplicate measurements.

Table 1
Comparison of hCG binding in PBS-B with and without signal enhancement from nanoparticle conjugates^a

Nanoparticle type	hCG Concentration (ng/ml)	Phase response ^b (π radians)
100 nm latex	0.5	4.28
15 nm gold	0.5	3.89
15 nm gold	0.1	0.97
5 nm gold	5	3.67
None	50	1.58
None	5	0.17

^a Measurements were made after first allowing the background signal to equilibrate.

^b Analyzed response (15 min).

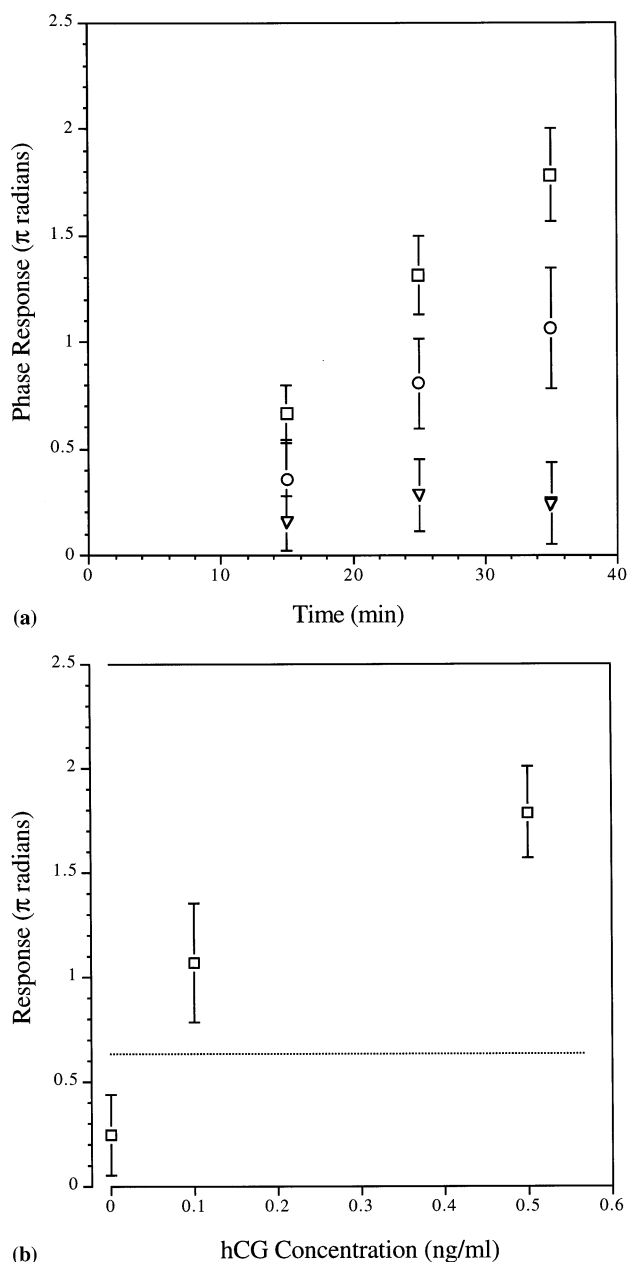


Fig. 9. (a) One-step sandwich immunoassay for hCG in human serum using gold nanoparticles for signal amplification. The response to hCG as a function of assay time is shown for 0 (∇), 0.1 ($\circ$) and 0.5 ng/ml ($\square$) of hCG. Error bars indicate 1 SD from mean values. A 15 nm gold conjugate of Mab B56 was added to the serum sample prior to testing with dry-stored optical chips. The x -axis reflects assay time after beginning sample flow. The first 5 min after sample introduction was not included in the data calculation. (b) Data presented in Fig. 9a is plotted to show the dose-response curve for the 35 min assay of hCG. Error bars indicate 1 SD from mean values. The dotted line indicates the response level 2 SD above the mean control value.

A minimum of nine replicates of each solution containing 0, 0.1, and 0.5 ng/ml of hCG were tested using 18 different optical chips. A large degree of signal variability was observed in the first few minutes follow-

ing sample introduction, which could be due to variations in surface rehydration. Therefore, the first 5 min of data collection were not used in determining the sensor response. The control signal reached a fairly stable level after 15 min exposure to serum, while the response to hCG binding continued to rise in a monotonic fashion (Fig. 9a). At longer assay times, more discrimination was achieved between the control and the hCG concentrations tested as well between the two concentrations of hCG. At 35 min the standard deviation (SD) of the mean response to 0.1 ng/ml hCG did not overlap with 2 SD of the mean of the control (Fig. 9b). Resolution of 0.1 and 0.5 ng/ml of hCG was observed after 25 min of data collection. After subtraction of the mean control signal, the net response at 15 min exposure (10 min analyzed data) for 0.1 and 0.5 ng/ml were 0.39 π radians and 0.77 π radians, respectively. This two-fold difference in response was significantly lower than the 5-fold difference that would be expected since these hCG concentrations were not saturating. These measurements in serum were 60% and 30% of the equivalent signals in PBS-B at 0.1 ng/ml and 0.5 ng/ml respectively. Since the off-line results showed no difference in hCG binding in serum compared to PBS-B, the cause of the lower signals in serum was not apparent, and is currently under investigation.

4. Conclusions

The promise of refractometric optical sensors — real-time, quantitative detection and a simple assay procedure — was demonstrated for the first time in a clinical sample using an optical chip based on the Hartman interferometer. Immunoassay measurements for hCG in human serum were obtained using a one-step assay procedure, without separation or wash steps, and with a detection limit of 0.1 ng/ml for a 35 min assay. The ability of the Hartman interferometer to compensate for NSB, one of the major hurdles to more widespread use of refractometric optical sensors, was shown by using a reference sensing region exposed to the test sample and by controlling surface chemistry. Further improvements are needed to lower both the detection limit and assay time, and to demonstrate that the optical chip can quantitate analytes in whole blood.

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