Planar Waveguide Biosensors for Nucleic Acid Hybridization Reactions

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ABSTRACT

Oligonucleotide probes derived from (1) the T3 RNA polymerase promoter sequence (T3) and (2) prostate-specific antigen messenger RNA (PSA) were prepared and labeled with a red-emitting fluorescent dye (Cy5). The complimentary oligonucleotides (anti T3 and anti PSA) were prepared and labeled with biotin. Initially, a feasibility study was performed in which the hybridization rate of the T3/anti T3 oligonucleotide pair was examined. Specifically, biotinylated anti T3 was immobilized to a neutravidin-coated waveguide and solutions containing increasing concentrations of Cy5-labeled T3 were injected into the biosensor. Fluorescence emission was detected with an evanescent wave imaging fluorometer. The hybridization reaction proceeded rapidly with a significant amount of binding occurring during the first 5 minutes. A Michaelis-Menton kinetics model was used to analyze hybridization rate data and gave values of 78 nanomolar for the apparent affinity of the hybridization reaction and 1.4 picomolar for the analytical sensitivity of the hybridization assay. In subsequent studies the hybridization rate of the PSA/anti PSA oligonucleotide pair was examined. Biotinylated anti PSA was immobilized to the waveguide and solutions containing increasing concentrations of Cy5-labeled PSA were injected into the biosensor. The hybridization rate observed for formation of the PSA/anti PSA pair was comparable to the high rates observed for the T3/anti T3 pair. Lastly, the selectivity of the biosensor was examined using an oligonucleotide probe derived from human glandular kallikrein (hGK), which exhibits a high degree of homology to PSA. The two oligonucleotide probes (PSA and hGK) only differed in 7 out of 20 positions. Interestingly, the hybridization rate observed for Cy5-labeled hGK was very low—not statistically different from the non-specific binding rate of the hybridization assay.

Keywords: biosensors, DNA, evanescence, fluorescence, glandular kallikrein, hybridization, MDx, molecular diagnostics, nucleic acid, prostate specific antigen, PSA, waveguides

1. INTRODUCTION

There have been several reports to date of the use of optical biosensors for performing molecular diagnostics (MDx) assays. Although a majority of these were either fluorescent fiber optic sensors[1-3] or label-free surface plasmon resonance (SPR) sensors,[4-8] several other label-free evanescent wave formats have been described including interferometry,[9] diffractometry[10] and evanescent-illuminated light scatter.[11, 12] Two of these articles are particularly relevant because they report the use of evanescent wave biosensors in clinical applications. First, Nilsson *et al.*[5] used surface plasmon resonance to detect the presence of the human tumor suppressor p53 gene in breast tumor biopsy material. Moreover, these authors were able to detect point mutations in clinical DNA specimens because polymerase chain reaction (PCR) products containing mismatched bases gave reduced levels of hybridization relative to the wild type. This result illustrates an important feature of biosensor technology in MDx assays—namely, the ability to monitor the hybridization reaction in real time provides a powerful tool for identifying mismatched bases. Second, Pilevar *et al.*[3] were able to detect 25 pM levels of *Helicobater pylori* RNA using a fluorescent fiber optic sensor. This result is very encouraging because it shows that fluorescent evanescent wave sensors are capable of performing high sensitivity MDx assays. The real question is whether such sensors can be used in high throughput genetic screening applications where hundreds, if not thousands, of hybridization assays would need to be performed in parallel on the same biosensor.

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2. BIOSENSOR FORMAT FOR HYBRIDIZATION ASSAYS

Evanescent wave biosensors can be designed to function either with or without fluorescent labels. Surface plasmon resonance and other label-free optical sensors respond to mass changes in the evanescent wave. In our opinion, mass sensors have at least two limitations over fluorescent sensors. First, a mass sensor will respond to any molecule bound within the evanescent wave—whether it is bound specifically or non-specifically. For this reason, nonspecific binding (NSB) can be a significant problem with these sensors and both Biacore AB and Affinity Sensors have devoted significant effort to developing immobilization chemistries with low NSB. In contrast, detection in a fluorescent biosensor is accomplished by a fluorescently-labeled "tracer" molecule that binds specifically (through an affinity interaction) to the ligand-capture molecule complex; alternatively, a fluorescently-labeled analyte molecule can bind directly to the immobilized capture molecule. This latter case is more appropriate for nucleic acid hybridization assays where the fluorescent label can be directly incorporated into the analyte molecule using PCR. In either case, NSB is only an issue with the fluorescent-labeled molecule, rather than with any molecule that happens to be in the evanescent wave.

The second limitation of mass sensors is that they require a significantly larger sensing area than a fluorescent sensor does to measure a given concentration (because mass detection is less sensitive than fluorescent detection). For this reason, it is difficult to construct medium- and high-density array biosensors using SPR technology. For that matter, fiber optic fluorescence sensors are not particularly well suited for medium- and high-density applications either because a separate sensor fiber would be required for each nucleotide sequence being examined. For these reasons, we believe that planar waveguide sensors are the best choice for rapid, high throughput genetic testing. Furthermore, a fluorescent assay format should afford greater sensitivity than can be achieved with label-free biosensors such as BIACORE® or IAsys®. A planar waveguide fluorescent sensor for detection of nucleic acid hybridization is shown diagrammatically in Figure 1.

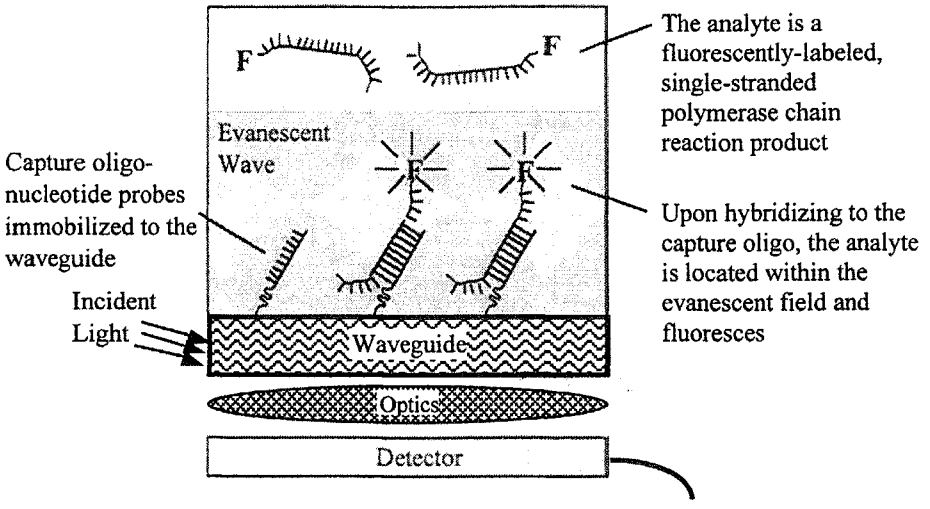

Figure 1. Oligonucleotide hybridization assay using a planar waveguide biosensor. Light is totally reflected within the waveguide, setting up an evanescent wave that decays a few hundred nanometers into the sample. Fluorescently-labeled analyte molecules hybridize to capture oligonucleotides that are attached to the waveguide. Once in the evanescent field, the label fluoresces.

3. EXPERIMENTAL PROCEDURES

3.1. Waveguides

An injection-molded planar waveguide sensor was used in all DNA hybridization studies described herein. These sensors are based on a design patented by two of the authors (Herron and Christensen).[13-15] Sensors were molded from polystyrene by Opkor, Inc. in Rochester, NY and are both disposable and relatively inexpensive, making them ideal for assay development purposes. A schematic illustration of an injection-molded planar waveguide sensor is shown in Figure 2. In addition to the waveguide, the sensor contains two lens—an in-coupling lens used for coupling light into a waveguide and an exit lens used to dump light out of the waveguide. Capture oligonucleotides are immobilized to the top of the planar waveguide and fluorescence emission is collected from the bottom of the waveguide (orthonormal to the plane of the waveguide) using a CCD camera as described in Section 3.3.

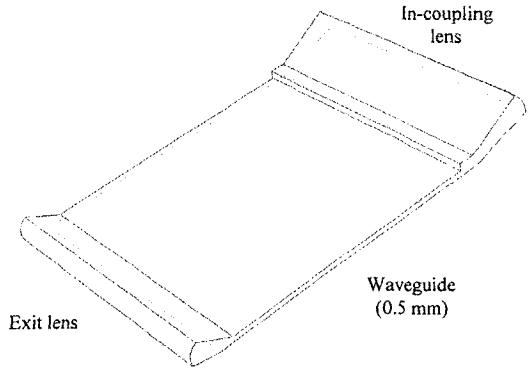

Figure 2. Injection-molded planar waveguide sensor. The waveguide and in-coupling lens are incorporated into the same disposable unit for greater alignment tolerance. This particular version also contains an exit lens for dumping light out of the sensor in a graceful manner. Exciting light (typically generated by a 635 nm diode laser) is collimated into a sheet beam (ca. 20 mm x 2 mm) and steered into the coupling lens. After being coupled, the light propagates down the waveguide setting up the evanescent field. Capture oligonucleotides are immobilized to the top of the planar portion and hybridization assays are performed as described in Figure 1.

3.2. Flowcells

The injection-molded waveguide sensors were used in conjunction with a flowcell developed by Herron, Christensen and Reichert.[16-19] In brief, flowcells were constructed from two separate pieces—a top and a bottom plate—that were fabricated from aluminum. Three different types of top plates were milled that had either one, two or three parallel flow chambers, each with small inlet and outlet ports. The interior of the bottom plate was milled to support the waveguide and provide a clear view of its bottom. The entire flowcell was anodized flat black. The top plate was sealed against the waveguide using a composite gasket with a low-refractive index Teflon™ layer next to the waveguide and a silicon rubber layer next to the top plate. Mechanical pressure to seal the system was produced by tightening four knurled bolts located at the four corners of the flowcell.

A computer-controlled 3-barrel syringe pump (Cavro Scientific Instruments Inc.) was used to inject specimens into the flow chambers. The sample volume of each chamber was 100 μL. The syringe pump was controlled by an instrument control and data acquisition program that was written in the LabView macro language. This software runs on either MacOS or Windows platforms.

The availability of different top plates (1-, 2- or 3-chamber) affords some flexibility in experimental design. For example, the PCR product from a single patient could be screened for a large number of mutations using a single chamber flowcell, or PCR products obtained from three different patients could be screened for a smaller number of mutations using a 3-chamber flowcell. Alternatively, the extra chambers could be used for calibration purposes. For instance, the patient's sample could be injected into one chamber, while positive and negative controls are injected into the other two chambers.

3.3. Optical Configuration

All fluorescence measurements were taken with a Mark 1.5 evanescent wave imaging fluorometer that was constructed at the University of Utah. Waveguides were mounted in the flowcell to form the sensor assembly as described above. The sensor assembly was then locked into a mounting plate on the Mark 1.5 fluorometer that provides tight registration of the waveguide to the exciting light. A diode laser that emits 12 mW of red light at 635 nm was used as the light source. The output of this laser was formed into a sheet beam using a series of collimating lenses and then reflected with a mirror into the coupling lens in the case of the waveguide.

Once trapped inside the waveguide, the light bounced from side to side of the waveguide setting up an evanescent field at each reflection point. This field decayed about 100nm into the solution in the flowcell and excited Cy5-labeled nucleotides that were hybridized to the immobilized capture nucleotides. Fluorescence emission emanated in all directions, but the portion that travels through the waveguide (and through a window in the bottom of the flowcell) was collected and imaged by a CCD camera (Santa Barbara Instrument Group). This camera was equipped with a 55 mm f/2.8 macro lens (Nikon) to focus the light and a 670 nm bandpass interference filter (Orion) to reject scattered light. The CCD image was collected and processed by the aforementioned instrument control and data acquisition program that was written in the LabView macro language. This software "binned" the image into different spatially-resolved sensor zones and also used a non-linear least squares fitting routine to compute the average hybridization rate of a data set over a 5 min. assay period.

4. RESULTS AND DISCUSSION

Our evanescent planar waveguide technology is generic in the sense that it can be used to monitor almost any sort of affinity interaction between biomolecules. Nucleic acid hybridization is an affinity interaction, similar in some respects to antigen-antibody interactions; so it seemed only natural to investigate whether our biosensor system could be used to monitor the hybridization of two complementary oligonucleotides in real time. The T3 RNA polymerase promoter site was chosen as a model system for this feasibility study. The T3 site is a region spanning 20 bases with the following sequence: 5' AATTAACCCTCACTAAAGGG 3'. Oligonucleotide primers for both this sequence (T3) and its complementary sequence (anti T3) are commercially available. They have also been fluorescently labeled and used in nucleic acid sequencing.[20]

We decided to employ the avidin/biotin system for immobilizing the T3 oligonucleotide to the waveguide. This choice was based upon a previous observation that avidin adsorbed strongly to injection-molded waveguides.[21] The T3 20-mer was biotinylated at the 5' end via a six-carbon spacer. It was then immobilized to waveguides (either injection-molded or integrated optical) that had been coated previously with either avidin or neutravidin. The anti T3 20-mer was labeled at the 5' end with Cy5, a red-emitting fluorescent dye. It should be mentioned that this Cy5-labeled analyte is very similar to analytes that are used in clincial MDx assays. Such clinical analytes are prepared by polymerase chain reaction and Cy5-labeled primers can be used to initiate DNA synthesis. Thus, the PCR product can be made to contain Cy5 dye at its 5' end.

The kinetic response of our injection-molded biosensor for the T3/anti T3 hybridization reaction is shown on the next page in Figure 3, along with three controls. The same analyte solution (10 nM Cy5-labeled anti T3) was injected into the flowcell in all four cases, but a different capture molecule (or complex) was examined in each case. Nonspecific binding of Cy5-anti T3 to either avidin or neutravidin is depicted in open circles and open squares, respectively. Interestingly, a significant level of NSB was observed with avidin, but not with neutravidin; presumably because avidin has a high pI value and is positively-charged at pH 7.4, which can lead to electrostatic interactions with the negatively-charged tracer oligo. Because of this observation, neutravidin was used instead of avidin in all subsequent studies. The third control examined whether there was any nonspecific binding between two oligos with the same nucleotide sequence. In this case, biotinylated anti T3 was immobilized to a neutravidin-coated waveguide (closed diamonds). As can be seen from the figure, binding between like oligos was negligible. Finally, hybridization between complementary oligos was examined by immobilizing biotinylated T3 to a neutravidin-coated waveguide (closed circles). In this case, very strong binding was observed, reaching 10^7 sensor units in less than two minutes.

Next, T3/anti T3 hybrization assays were performed at room temperature for a number of different concentrations of Cy5-labeled anti T3, ranging from 10 pM to 100 nM. Hybridization rate was plotted versus tracer oligo concentration in Figure 4 on the next page. A double log plot was used because of the wide dynamic range (4 orders of magnitude) of the assay (the same data is plotted on linear axes in the inset). Once again, the data was found to fit a Michaelis-Menton model with a Michaelis constant of about 78 nM (curve-fitting parameters are also shown in the figure). An analytical sensitivity value of 1.4 pM was computed for these data.

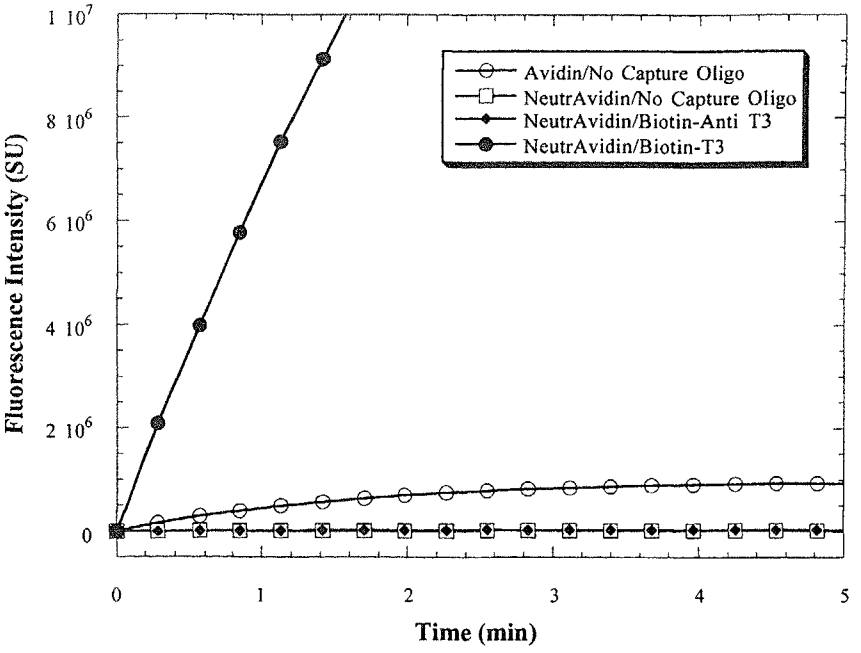

Figure 3. Hybridization of Cy5-labeled anti T3 to several different capture molecules (or complexes). Open circles: nonspecific binding to immobilized avidin. Open squares: nonspecific binding to neutravidin. Closed diamonds: nonspecific binding to immobilized biotin-anti T3/neutravidin complex. Closed circles: hybridization to immobilized biotin-T3/neutravidin complex.

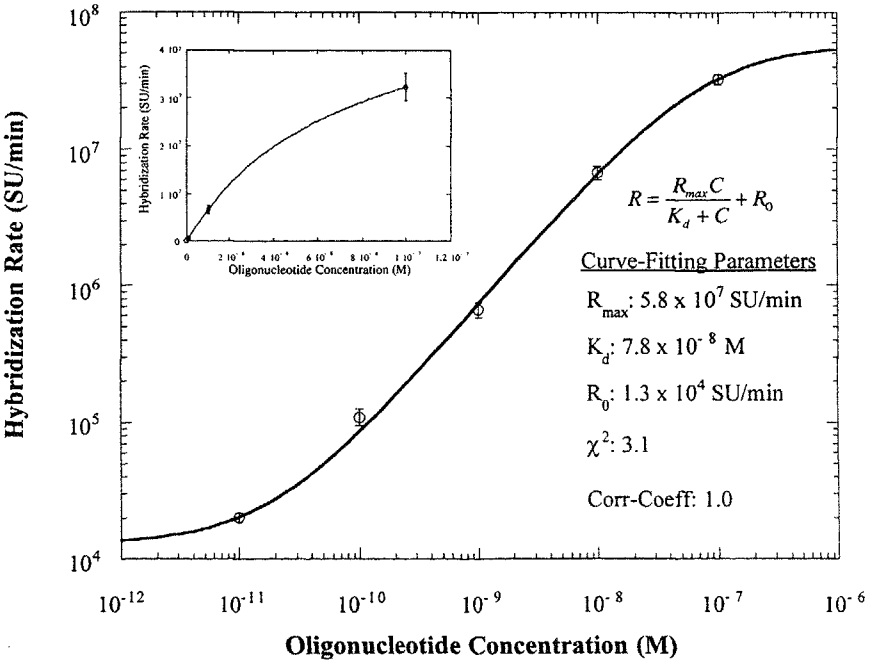

Figure 4. Standard curve for the hybridization of Cy5-anti T3 to immobilized T3. The sensitivity of our T3/anti T3 hybridization assay using injection-molded waveguides was investigated by spiking human Cy5-labeled anti-T3 into PBS. Hybridization rate was plotted versus Cy5-anti T3 concentration to construct the standard curve. An analytical sensitivity (defined as 2σ/slope, where σ is the standard deviation of the zero rate) value of 1.4 pM was determined for the assay.

Having established that our planar waveguide sensors can be used to monitor nucleic acid hybridization reactions, we decided to evaluate our biosensor system in a molecular diagnostics assay for the messenger RNA that encodes for prostate specific antigen (PSA). It turns out that PSA exhibits a high degree of homology to another human protein, glandular kallikrein (hGK2), that is also secreted by the prostate gland.[22-24] For this reason, it is important that the immobilized hybridization probe bind only to cDNA derived from the PSA message and not to that derived from the hGK2 message. We identified a region in exon 4 of the PSA gene where the sequence differs from hGK2 in 7 out of 20 positions. A hybridization probe (5'-GGGGC AAAAGCACCTGCTCG-3', referred to as "anti PSA") that recognizes this sequence was synthesized and biotinylated at the 5' end. Two additional oligonucleotides were synthesized, labeled with Cy5, and then used as model analytes. One of these (5'-CGAGCAGGTGCTTT TGCCCC-3', referred to as "PSA") was derived from the cDNA sequence of PSA, while the other (5'-CCA·CAA GTGTCTTTACCAC -3', referred to as "hGK2") was derived from the cDNA sequence of hGK2. The biotinylated probe was immobilized to a neutravidin-coated, injection-molded waveguide and a 1 nM solution of either Cy5-labeled PSA or Cy5-labeled hGK2 was injected into the flowcell. Hybridization kinetics curves are shown in Figure 5 for both of these reactions. A very high hybridization rate was observed for the homoduplex, while the rate observed for the heteroduplex was not statistically above background.

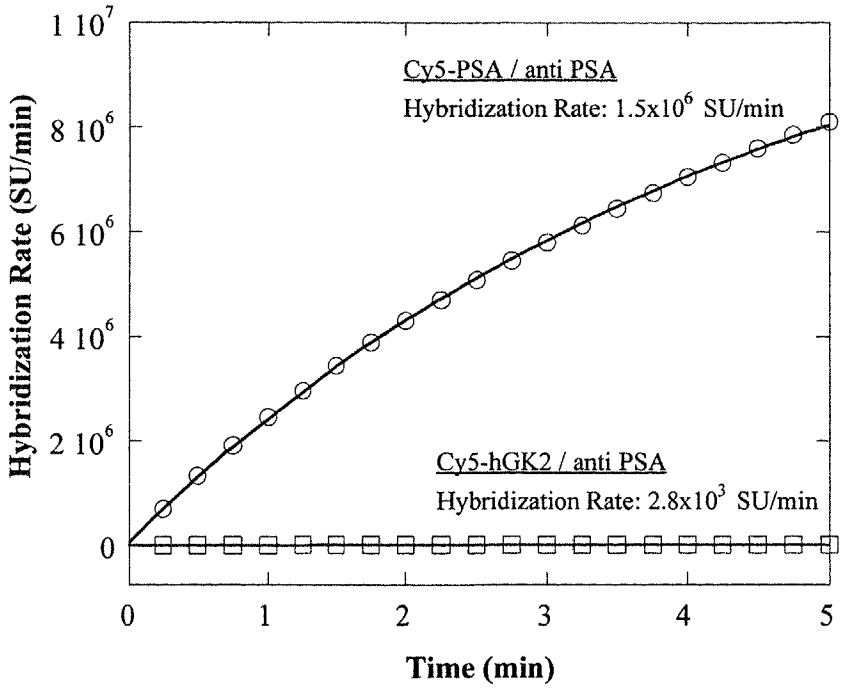

Figure 5. Hybridization kinetics of two Cy5-labeled oligonucleotides (PSA & hGK2) to an immobilized oligonucleotide (anti PSA). An analyte concentration of 1 nM was used for both Cy5-labeled oligonucleotides. PSA and anti PSA are perfectly complementary and exhibited a high hybridization rate ($1.5x10^6$ sensor units per minute). The hGK2 and anti PSA oligonucleotides have mismatched bases in 7 out of 20 positions and exhibited a much lower hybridization rate (2800 SU/min.). This rate was not significantly different from the nonspecific binding rate of this assay (513±1416 SU/min).

5. CONCLUSIONS

We conclude from these studies that our injection-molded planar waveguide sensors are well suited for performing nucleic acid hybridization assays. The 1.4 pM sensitivity observed for the T3/anti T3 system compares very favorably to the 25 pM sensitivity obtained by Pilevar et al.[3] for *Helicobater pylori* RNA using fluorescence fiber optic sensor technology. Our ability to perform rapid (≤5 min.) assays offers the additional advantage of being able to monitor hybridization reactions in real time. Another important feature is the ability to distinguish between two closely-related analytes (PSA and hGK) that would both be present in the same clinical specimen. Taken together, these capabilities make our biosensor system well positioned for performing MDx assays in point-of-care and critical care settings.

ACKNOWLEDGEMENTS

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