DNA Oligonucleotide Synthesis in Mesoporous Silicon for Biosensing Applications

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ABSTRACT

We report a method for improving the sensitivity of label-free optical biosensors based on in-situ synthesis of DNA probes within porous silicon structures. The stepwise attachment of up to 15mer probes inside 30 nm mesopores was accomplished through a series of phosphoramidite reactions. In this work, a porous silicon waveguide was utilized as the sensor structure. Synthesis of DNA probe, as well as sensing of target DNA, was verified by monitoring the change in effective refractive index of the porous silicon waveguide through angle-resolved attenuated total reflectance measurements. The average resonance shift per oligo of 0.091° during stepwise synthesis corresponds to surface coverage slightly less than 50%, according to theoretical models. When compared with the traditional method of direct attachment of pre-synthesized oligonucleotide probes, the sequential phosphoramidite method resulted in an approximately four-fold increase in DNA probe attachment. This increased surface coverage by DNA probes increases the likelihood of target molecule binding, leading to improved sensitivity for bio-molecule detection. Exposure to a 50 μM solution of target 8-base DNA in deionized water produced a 0.4236° change in the waveguide resonance angle. Nanomolar detection limits for small molecule sensing are realizable with this sensor scheme.

Keywords: Porous silicon, Waveguides, DNA Hybridization, Label-free optical sensor, DNA synthesis

1. INTRODUCTION

The development of versatile, real-time detectors for biomolecules is an active area of current research, specifically for applications in medical diagnostics and biotoxin detection. Porous silicon has become a widely studied material for these applications over the last decade. The porous structure provides significant advantages over planar surface substrates due to its substantial increase in reactive surface area and the inherent size-exclusion properties of the pores, preventing contamination by large molecules. A number of innovative porous silicon structures have been proposed and studied, such as single layer interferometers, rugate filters, microcavities, and waveguides.

1.1 Previous work on in-situ DNA synthesis

In previous work, we demonstrated a method for improved probe molecule coverage in mesoscale pores based on in-situ DNA synthesis. Synthesis of oligonucleotides in a porous template, for which the template pore size is substantially larger than the synthesized oligonucleotides, is the traditional method of producing DNA strands. After synthesis, for example in micron-sized controlled pore glass (CPG), the DNA is cleaved from the template and collected. While there have been limited reports in the literature on DNA synthesis in porous silicon templates, we presented a first report of in-situ DNA synthesis within a mesoporous silicon optical waveguide structure for label-free sensing. After synthesis, the porous silicon template was not removed like a CPG substrate; it remains as an active, functionalized sensing platform. This method of in-situ DNA synthesis in the porous silicon not only increases the probe DNA density on the pore walls by a factor of four compared to traditional immobilization techniques, but it also allows for flexibility in defining the probe sequence. The detection sensitivity of the porous silicon waveguide is sufficient to detect oligonucleotides containing only a single base. Hence, combining the sensitivity of the waveguide platform with the high
probe coverage obtained using the in-situ DNA synthesis method, the functionalized porous silicon waveguides serve as the basis for a highly efficient sensing device.

2. EXPERIMENTAL DETAILS

2.1 Porous silicon waveguide formation
In order to evaluate the effectiveness of the probe DNA synthesis in porous silicon nanoscale pores, two-layer porous silicon waveguides were utilized. The porous silicon waveguides were fabricated by traditional electrochemical etching techniques\textsuperscript{16}, using 15\% hydrofluoric acid in ethanol as the electrolyte. P-type silicon wafers (~0.01 Ω-cm) were chosen for mesopore formation. The native oxide layer was first removed by soaking for one minute in the 15\% HF solution, followed by ethanol rinsing. This procedure was used to facilitate the pore initiation process. A two-step anodization procedure followed, and has been reported previously\textsuperscript{17}. Resulting average pore diameters were approximately 20 nm. In order to expand the pores to approximately 30 nm, the waveguides were then etched in 9 mM solution of KOH in ethanol for 30 min\textsuperscript{18}, followed by rinsing with ethanol and DI water. The resulting waveguide has a thickness of approximately 240 nm and a refractive index of approximately 1.98. The lower, cladding layer has a thickness of approximately 1500 nm and refractive index of approximately 1.41.

2.2 Porous silicon waveguide measurements
A Metricon 2010 prism coupler was used to evanescently couple 1550 nm light from a diode laser into the porous silicon waveguide, as illustrated in figure 1. A coupling head presses the porous silicon waveguide up against the Metricon prism, leaving a small air gap whose size may be controlled by adjustment of the coupling pressure. Light couples across the air gap and into the waveguide at a resonant angle that corresponds to a wavevector match between the incident light and the propagating waveguide mode. This resonant angle directly correlates to the effective index of the porous silicon waveguide. Monitoring the resonant angle makes it possible to detect the presence and quantity of DNA inside the waveguide. DNA attachment inside the pores causes the refractive index of the porous silicon to change, leading to a shift of the resonant angle. The magnitude of the resonance shift increases as a function of the amount of DNA immobilized in the pores. The waveguide allows particularly sensitive detection of the DNA since the electric field is primarily confined in the top layer of porous silicon waveguide where the majority of the DNA molecules are attached. A major advantage of this sensing platform over multilayer microcavities and rugate filters is that the porous silicon waveguide is a resonant structure for which the most sensitive sensing region is the layer that is most easily accessible for infiltration. Given the strong field confinement observed in porous silicon waveguides, even small concentrations of DNA attached in the pores result in a measurable shift of the coupling angle. For this investigation, the coupling angle of the porous silicon waveguide was monitored at each step in the fabrication of oligonucleotides to ensure that the synthesis was proceeding as desired.

2.3 Porous silicon surface treatment
To prepare the porous silicon waveguides for DNA synthesis, the waveguides were thermally oxidized in a furnace under atmosphere at 800°C for 30 minutes. Surfaces were functionalized with N-(3-triethoxysilylpropyl)-4-hydroxybutyramide (TEOS-HBA). A 4\% TEOS-HBA silane solution was prepared, combining 1900 μL ethanol, 100 μL DI water, and 83 μL of the silane. The porous silicon waveguide samples were incubated in the silane solution for 16 hours. The samples were then rinsed with ethanol and placed in an oven for annealing under atmosphere for one hour at 100°C.

As compared with similar silanes for silicon functionalization, the TEOS-HBA silane was found to be highly sensitive to annealing and hydrolyzing conditions\textsuperscript{19}. Due to its alcohol functional group, the silane is likely to form weakly bound multilayers on the silicon surface, which must be removed before the DNA synthesis begins\textsuperscript{20}. If they remain on the surface, these weakly bound multilayers may detach during the DNA synthesis and hybridization, compromising the later measurements. Experiments showed that the TEOS-HBA silane could be stabilized on the porous silicon surface through annealing at 200°C for 16 hrs, followed by hydrolyzing the surface by soaking in DI water for 4 hrs. This extended annealing and hydrolyzing procedure produced a functionalized porous silicon surface for which stable and repeatable resonance angle measurements were obtained after waveguide exposure to a variety of aqueous and organic...
reagents. This extended functionalization procedure, used for the chain capping experiments described below, produces a waveguide with long-term stability, observed as measurements were taken over the course of several weeks.

![Prism coupling measurement configuration](image)

**Figure 1:** Prism coupling measurement configuration. Etched into a silicon substrate is a high refractive index waveguide layer on top, and a low refractive index cladding layer below. Biomolecules infiltrate primarily into the top porous layer, in the region of strongest field confinement. To achieve optimal coupling, the width of the air gap separating the prism and waveguide can be controlled with the pressure applied by the coupling head onto the substrate. Light from the laser source couples into the waveguide at a given incident angle, at which point the intensity of light reaching the detector is a minimum.

### 2.4 In-situ DNA synthesis

The oligonucleotide bases were attached directly to the functionalized surface using an Applied Biosystems Model 392 DNA Synthesizer. This system was modified to direct reagent flow across the waveguide surface. The phosphoramidite method\(^2\) was used, a standard DNA synthesis technique often used on CPG supports. The waveguide is attached to the 3'-hydroxyl end of the first nucleotide, and subsequent nucleotide additions attach onto the 5'-hydroxyl end. A dimethoxytrityl (DMT) protecting group ensures that only one end of the adding nucleotide is reactive, thus controlling the stepwise linear growth of the chain. Oligonucleotide probe sequences were synthesized on the porous silicon waveguides using the phosphoramidite method, demonstrating up to 15 bases in length. After the synthesis, the waveguide was removed from the synthesizer and rinsed sequentially with ethanol and water before measurement.

### 2.5 DNA hybridization and sensing

Once the oligonucleotide probe is attached to the porous silicon surface, it may be used for detection of the complementary oligonucleotide sequence. The probe DNA is prepared for sensing by deprotection in a 50:50 v/v solution of ethylenediamine and ethanol for 1 hr. Once the probe is deprotected, the porous silicon waveguide is exposed to a DNA target in buffer solution. For these experiments, a DNA target concentration of 50μM is used. Porous silicon waveguides with DNA probes are exposed to both complementary DNA target sequences as well as the non-complementary sequences in order to assess the selective binding ability of the oligonucleotide probe. DNA hybridization on the porous silicon surface is monitored by prism coupling measurements as described in Section 2.2.
3. EXPERIMENTAL RESULTS

3.1 Infiltration of probe oligos into porous silicon waveguide

In working with a porous silicon waveguide structure, the significant advantage of increased total surface area is dependent upon efficient infiltration of reagents and molecules into the porous structure. In order to demonstrate the presence of the TEOS-HBA silane linker and oligonucleotide probe following in-situ DNA synthesis, secondary ion mass spectrometry (SIMS) analysis was performed on waveguide samples, as shown in figure 2. While the SIMS data below cannot be interpreted quantitatively, the presence of both carbon and nitrogen atoms in the waveguide are a strong indication that silane and/or oligos are able to infiltrate deep into the top and bottom layers of the waveguide. Also valuable is a comparison of the SIMS data from a DNA probe synthesized inside a porous silicon waveguide using the in-situ method compared with traditional whole probe DNA attachment in a porous silicon waveguide. Note that the presence of the carbon and nitrogen in the in-situ sample appears greater. This agrees well with previous results indicating superior surface coverage with the in-situ DNA synthesis.

Figure 2: SIMS measurement results for a 16-mer oligo attached to the porous silicon waveguide in the traditional method (bottom), as compared to an in-situ synthesis of an 8-mer oligo (top). Both the carbon and nitrogen lines can be seen as indicators of DNA. For the traditional method, note that the amount of carbon and nitrogen in the waveguide and cladding layers is only slightly more than the background. Compare this to the relative quantities in the in-situ sample, in which a considerable difference can be noted between the presence of carbon and nitrogen in the pores compared with elsewhere. The SIMS data suggests that in-situ synthesis significantly increases the quantity of DNA probes attached in the porous silicon.
3.2 In-situ oligonucleotide chain growth

The ability of DNA solutions during phosphoramidite method synthesis to infiltrate into the porous structure has a considerable impact on the viability of the sensing platform, affecting not only the sensitivity, but also the selectivity of the oligonucleotide probe. In traditional DNA probe surface attachment, oligonucleotide solutions are pre-screened for incorrect DNA probe sequences after separation from the CPG substrate and before immobilization on the sensor. While the in-situ method greatly increases probe density on the porous surface, it does eliminate the possibility for filtering out failed DNA sequences. As a result, the efficiency of the DNA synthesizer, including the frequency of failed sequence production and effectiveness of the phosphoramidite capping step, is critically important. In order to determine capping efficiency, a forced DNA failure sequence was performed. The DNA synthesizer was utilized to begin an oligo probe on a porous silicon waveguide but, for the last base in the sequence, the synthesizer does not introduce the nucleotide. Thus at this step, all growing DNA probes should be failed sequences and capped, preventing any further chain growth. On average, there is zero shift of the waveguide resonance angle following a 4-base addition procedure in the DNA synthesizer on a fully capped probe sequence.

For sensing of more complex molecules, longer oligonucleotide sequences must eventually be achieved within this sensing platform. In previous work, 8-mer oligonucleotide probes were demonstrated. Herein, we report in-situ synthesis of DNA probes up to 15-mer in length in the 30 nm diameter porous silicon pores. Through the addition of 15 bases, there is a distinct shift noted in the resonance angle upon each base addition, as shown in figure 3. The nonlinearity of the resonance shift may be due, in part, to less efficient attachment of the last few bases, possibly from the narrowed pore opening or an increased chance of having some failed chains.

![Figure 3: Shift noted per base addition for in-situ synthesized DNA probe, increasing from 8 to 15 bases. The large magnitude of the shift from base 8 to base 9 is attributed to the addition of a protecting group onto the sequence. While small, the shift upon addition of base #15 produces a distinctly measureable shift of 0.02486°.](image)

3.3 DNA hybridization and sensing capabilities

In order to demonstrate selective detection of DNA target molecules, a porous silicon waveguide functionalized with TEOS-HBA and an in-situ 8-mer oligonucleotide probe was exposed to 50μM solution of complementary and non-complementary 8-mer sequences in DI water solution. The target DNA sequence (5’-CCC CCC CC-3’) is 100% complementary to the synthesized probe. The non-complementary DNA (5’-AAA AAA AA-3’) is a 100% mismatch towards the DNA probe. As shown in figure 4, upon exposure to the complementary target DNA solution, a significant change, 0.4236°, in the resonance angle was observed by attenuated total reflectance measurements. Exposure to non-complementary oligo solution also produced a shift in the resonance angle, although approximately one order of
magnitude smaller. This suggests some residual non-complementary binding occurred under current experimental conditions. The changes in resonance width are due in part to the coupling conditions, including air gap thickness, which were not changed during the course of the experiment. This resulted in different levels of coupling efficiency for different waveguide effective indices.

Given angular resolution of the Metricon instrument of 0.002°, the waveguide has a detection limit of ~250 nM of complementary DNA. Theoretical calculations for this particular waveguide configuration however, indicate a detection limit that is approximately one order of magnitude lower. Likely, an even more commercially competitive detection limit can be achieved through experimental optimization of parameters such as solution dwell time, surface preparations, and buffer solution composition.

![Figure 4](image)

*Figure 4:* Hybridization with a complementary DNA target (left) is compared with exposure to a non-complementary (right) target in a porous silicon waveguide. The complementary target produces a shift that is about an order of magnitude larger than the shift due to non-specific binding of the non-complementary target, demonstrating selective binding of the complementary strand.

4. **CONCLUSIONS**

Selective 8-mer DNA detection was demonstrated using in-situ probe DNA synthesis by phosphoramidite chemistry. The in-situ DNA synthesis produced a considerably higher concentration of oligonucleotide probe molecules on the active sensor surface compared to traditional probe molecule infiltration techniques. The increased probe coverage was quantified by attenuated total reflectance measurements, and was verified by SIMS elemental analysis. The increased probe density allowed a detection limit of ~250nM for DNA target molecules in DI water. Sensor selectivity was also demonstrated. The resonance shift of the porous silicon waveguide upon exposure to complementary DNA was an order of magnitude larger than that after exposure to non-complementary sequences. This work is a promising initial demonstration of the sensitivity and selectivity of synthesized DNA probes in porous silicon waveguides. Future work will be directed towards lowering the detection limit and reducing non-specific binding.

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REFERENCES