Synthesis of DNA oligonucleotides in mesoporous silicon

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1 Introduction The development of versatile 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 this purpose over the last decade. The porous structure provides significant advantages in its ability to filter out impurities by size and its substantial increase in reactive surface area. There have been a number of label-free porous silicon devices proposed, such as porous silicon single layer interferometers [1], rugate filters [2], microcavities [3], and waveguides [4, 5]. In order to take advantage of the large internal surface area of porous silicon devices, molecules need to be effectively immobilized within the confined nanoscale pores. Consequently, a major challenge facing biosensors that utilize porous materials is the effective infiltration of molecules into the pores [6].

In this work, we demonstrate a method for improved probe molecule coverage in nanoscale 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 glass pores, the DNA is cleaved from the template and collected. There have been very limited reports in the literature on DNA synthesis in porous silicon templates [7–9]. We present a first report of in-situ DNA synthesis within a mesoporous silicon optical waveguide structure for label-free sensing. In this case, the pore size is only about fifteen times larger than the synthesized DNA. After DNA synthesis, the DNA probes are not cleaved; they remain as part of an active, functionalized sensing platform. The method of in-situ DNA synthesis in the porous silicon not only increases the probe DNA density on the pore walls compared to traditional methods of direct attachment of presynthesized DNA oligonucleotides, 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 thymine is possible. It is estimated that 50% of the available internal pore surface is occupied by the 8-base oligonucleotides, which is a substantially greater coverage than obtained by using traditional methods of direct attachment of presynthesized DNA oligonucleotides. The greater surface coverage by DNA probes will enable more effective biosensing devices.

2 Experimental

2.1 Porous silicon waveguide preparation In order to evaluate the effectiveness of the DNA synthesis in the porous silicon nanoscale pores, two-layer porous sili-
Table 1 Etch parameters for porous silicon waveguide fabrication. Resulting refractive index and thickness values (measured by SEM) are included for each layer.

<table>
<thead>
<tr>
<th>layer</th>
<th>current density (mA/cm²)</th>
<th>etch time (sec)</th>
<th>refractive index</th>
<th>thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>waveguide</td>
<td>5</td>
<td>42</td>
<td>1.98</td>
<td>240</td>
</tr>
<tr>
<td>cladding</td>
<td>48</td>
<td>60</td>
<td>1.41</td>
<td>1500</td>
</tr>
</tbody>
</table>

Porous silicon waveguides were utilized. As will be explained in Section 2.2, monitoring the effective refractive index of the waveguides enables highly sensitive detection of the synthesized oligonucleotides. The porous silicon waveguides were fabricated by traditional electrochemical etching techniques [10], using 15% hydrofluoric acid in ethanol as the electrolyte. P-type silicon wafers (~0.01 Ω cm) were chosen to enable mesopore formation. Before anodization, the wafers were first oxidized at 800 °C for 30 minutes under 20% oxygen. The oxide layer was next 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. Anodization then proceeded as detailed in Table 1. Resulting average pore diameters were approximately 30 nm.

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 (Fig. 1). Light couples 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. The presence and quantity of DNA inside the waveguide is determined by monitoring the resonant angle. 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 porous silicon layer. The porous silicon waveguide is a resonant structure for which the most sensitive sensing region is the layer that is most easily accessible to infiltrates. Given the fast response time and 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. Hence, 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 DNA synthesis

To prepare the porous silicon waveguides for DNA synthesis, the waveguides were thermally oxidized and surfaces were functionalized with N-(3-triethoxysilylpropyl)-4-hydroxy-butyramide. A 4% 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.

Figure 2 Synthesis scheme for DNA oligonucleotide. Cycle starts with addition of the first (protected) nucleotide phosphoramidite onto the silane functionalized porous silicon. The generated phosphate linkage is oxidized, and then the DMT protecting group is cleaved. A second (protected) nucleotide phosphoramidite is introduced, and attaches to the deprotected 5'-hydroxyl group of the growing chain. The cycle continues as desired to generate the oligonucleotide sequence. During the synthesis, a capping step is used to quench the reactivity of any 3'-OH groups that fail to couple to the next added nucleotide phosphoramidite reagent in order to prevent synthesis of incorrect nucleotide sequences. The capping step is performed in each cycle after the nucleotide coupling reaction.
tion for 16 hours. The samples were then rinsed with ethanol and placed in an oven for 1 hour at 100 °C (in air).

The oligonucleotide bases were attached directly to the functionalized surface through use of an Applied Biosystems Model 392 DNA Synthesizer that was modified to direct reagent flow onto the waveguide surface. The phosphoramidite method [11] was used, and is outlined in Fig. 2. The waveguide is attached to the 3′-hydroxyl end of the oligonucleotide, 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. After the synthesis, the waveguide was removed from the synthesizer and rinsed sequentially with ethanol and water.

3 Results

3.1 Single nucleotide sensing During the synthesis of a series of eight thymine (T) oligonucleotides on porous silicon, the waveguide resonance angle was measured after each T addition. As shown in Fig. 3, a consistently increasing resonance angle was measured after the addition of each base. The first attached thymine showed a shift in resonance angle approximately twice that of the subsequent steps. The larger shift for the addition of the first base is believed to be a result of the protecting group on the first thymine, which is approximately the same size as a single base. The average shift for the first base was 0.233 degrees, and each subsequent base resulted in a shift of approximately 0.091 degrees. The resolution of the prism coupler rotation stage is 0.002 degrees; the measured waveguide resonance shifts are well within the detection limit of the instrument. It should be noted that the thymine additions begin to deviate slightly from the linear fit as the oligonucleotide probe increases in length. Experiments have indicated that this deviation is a result of slight deg-

oradation of the silane linker layer, and not a result of inefficient in situ chain growth.

From Fig. 3, it is clear that the sensitivity of the porous silicon waveguide is sufficient to detect the addition of a single nucleotide at a time. The large surface area of the porous silicon waveguide and resonant sensing mechanism enable the detection of such small molecules. This measurement would not be possible using a traditional surface plasmon resonance sensor. Furthermore, the demonstration of in-situ DNA synthesis and measurement in porous silicon waveguides is noteworthy due to the size range of the pores in which the DNA was synthesized. Previous reports of DNA synthesis on glass [6, 12] or silicon [13, 14] supports have been on a much larger pore scale, often in the micron range.

3.2 In-situ synthesis advantages One of the primary advantages of using in-situ synthesis of DNA, compared to the addition of prepared eight-base DNA strands to a silanized surface, is a substantially increased DNA density in the porous silicon. As shown in Fig. 4, a total shift in the waveguide resonance of only 0.23° was observed when porous silicon waveguides functionalized with 3-aminopropyltriethoxysilane and sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate were incubated in 100 µM of eight-base oligonucleotide solution (MWG Biotech) for one hour [6]. The phosphoramidite synthesis method of eight-base oligonucleotides on the porous silicon surface resulted in an average overall resonance angle shift of 0.956° as shown in Fig. 3. This significant difference in the magnitude of the resonance angle shift is evidence of greater DNA coverage by the stepwise phosphoramidite method. We believe the effects of size exclusion and repulsive forces between charged oligonucleotides are magnified when pre-synthesized eight-base oligonucleotides were added to the waveguide surface.
oligonucleotides are infiltrated into the pores instead of only single oligonucleotides. Each eight-base oligonucleotide has a length of approximately 1.8 nm, while the porous silicon pore size is 30 nm. The advantage of the phosphoramidite method over direct addition will likely be even more significant for the immobilization of larger oligonucleotides in the 30 nm pores. Tunability of porous silicon materials parameters (e.g., pore size) will also allow the fabrication of biosensors for molecules covering a wide range of sizes [15, 16].

### 3.3 Predicting pore surface coverage

In order to estimate the degree of pore surface coverage by the synthesized probe DNA oligonucleotides, calculations of the expected resonance shift for different numbers of DNA bases and different percentages of surface coverage were performed [5]. The calculations assumed that each thymine nucleotide can be modeled as a 2.2 Å molecule [17] with a refractive index of 1.5 [18]. The magnitude of the resonance shift then scales with the magnitude of the effective refractive index change induced by the oligonucleotides, which scales with the number of thymine bases attached and the percent of the porous silicon internal surface area covered by the DNA. As shown in Fig. 5, the experimental data are fit well by the theoretical calculations assuming 50% pore coverage by probe DNA. The slope of the linear theoretical curve is a direct indication of the magnitude of the refractive index change induced by the addition of single oligonucleotides covering 50% of the available surface area. We note that the theoretical curve takes into account the addition of the DMT protecting group added along with the first thymine by adjusting the initial resonance shift position.

The estimate of 50% pore coverage for this synthesis method is highly favorable for sensing applications. The implication is that 50% of the pore surface is covered with probe oligonucleotides, leaving the remaining 50% for the hybridization of complementary target oligonucleotides.

Preliminary hybridization experiments suggest that the porous silicon waveguide sensor response is significantly larger when in-situ synthesized eight-base oligonucleotide probes are utilized.

### 4 Summary

In-situ DNA oligonucleotide synthesis inside mesoporous silicon waveguides using the phosphoramidite method was demonstrated. The attachment of each base was confirmed by monitoring the effective refractive index of the porous silicon waveguide. In-situ synthesis of the oligonucleotides resulted in a higher internal surface coverage in the waveguide than direct attachment of DNA probes. Calculations suggest that the synthesized oligonucleotides achieve 50% pore coverage. The probe DNA functionalized porous silicon waveguides have promising applications in the area of label-free biosensing.

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### References


