**MICROMACHINED SILICON SENSOR FOR DNA SEQUENCING BY
HYBRIDIZATION**

PHASE I FINAL REPORT

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Larry J. Kepley, Ph.D.

Principal Investigator

**TPL, Inc.
3921 Academy Parkway North, NE
Albuquerque, NM 87109-4416**

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1. EXECUTIVE SUMMARY OF PHASE I RESEARCH

The development of the porous, flow-through FPW sensor for genomic information is a major undertaking with several fabrication, engineering, and measurement challenges. While many components of the proposed device and feasibility tests were demonstrated during Phase I, including fabrication of porous, permeable, free-standing membranes, initial characterization of their acoustical properties, immobilization of oligonucleotide probes, and amplifier circuit design; the integration of all the tasks to provide definitive feasibility data was not completed by the end of the Phase I effort. Although electrochemical growth of porous silicon was achieved early on, producing linear micron-sized pores normal to the wafer surface, processing steps to remove the remaining nonporous silicon and produce flow-through membranes remained a difficult challenge until late in the program. Wet-etchant methods were pursued in conjunction with reactive-ion etching (RIE, i.e., gas-phase plasma etching) to implement successfully the alternate method proposed for membrane fabrication.

The scope of the proposed effort was indeed ambitious for the time and resources available in a Phase I program. In retrospect, the situation was made more challenging by the proposed approach of testing the acoustical properties of thick membranes by reflection of large diameter acoustic beams, which required producing thick (100-200 μm), large area samples, prior to producing devices from thin membranes (2-10 μm). The need for both thick and thin membranes, combined with limited thin-film deposition equipment available in house and the anticipated fragility of 2-10 μm membranes containing 1-10 μm pores, prompted the discovery of a more general procedure capable of producing membranes of arbitrary thickness from commercially available silicon wafers. The method involved a combination of wafer thinning by wet etching from the back side and electrochemical dissolution and/or RIE removal of the last few microns of nonporous Si to open the pore bottoms and give flow-through membranes. The new method allows formation of membranes from a wide range of silicon conductivity (i.e., doping levels) consistent with the desired range of pore diameters. The new method overcame some deficiencies of the proposed mask, a thermally grown oxide layer, for membrane area definition. The porous silicon layer was destroyed at the high temperatures needed for oxide growth and the oxide mask did not stand up well to the proposed caustic etching solution for membrane formation. Furthermore, the combined electrochemical-RIE approach provided faster etch rates than RIE alone and avoided the heavy doping required for etch-stop to leave a thin membrane, which creates stress in the membrane that complicates device production. Observed violent reaction of etch solution with porous silicon and gas evolution that lead to membrane fracture, so an alternate mask/etchant system was developed.

The overall purpose of the research is to determine the ability and sensitivity of a new, yet unrealized design of a microgravimetric device, a porous flow-through Flexural Plate-Wave (FPW) device, for direct transduction (i.e., label-free) of the hybridization of complementary (i.e., target) DNA to immobilized gene probes into an electrical signal. The critical physical phenomenon investigated to test the feasibility of detecting the hybridization event by the approach was the effect of the adsorption of DNA upon the velocity (i.e., RF frequency) of an

ultrasonic wave traveling in the porous membrane of the FPW device. As the ultimate demonstration of feasibility, analysis of the sensitivity and selectivity of device response to target oligonucleotide containing a complimentary sequence, relative to that for negative controls, was proposed to show detection of hybridization without false positives or negatives.

Porous silicon (por-Si) membranes containing linear macropores (i.e., channels) normal to and spanning between the membrane surfaces were created by photoelectrochemical etching of polished, n-type silicon wafers. By a process similar to that of Lehmann and Foll,ⁱ silicon wafers in contact with dilute HF electrolyte were etched under anodic bias while illuminated on their opposite (i.e., back) side to produce photo-oxidative dissolution at the electrolyte interface. Pore initiation occurs at microscopic surface depressions or pits due to the higher field gradients generated there by the applied potential. The surface morphology produced, ranging from linear pores of identical diameter to an electropolished surface, depended on photocurrent density, which was controlled mainly by the illumination intensity. Below a critical current density, the dissolution reaction is rate-limited by flux of holes from the bulk, rather than the diffusion of electrolyte to the surface, and pore formation occurs. Stable, sustained pore growth at constant diameter was achieved under conditions where photo-generated, conduction-band holes (i.e., minority charge carriers, h^+) formed in the bulk of the semiconductor migrate to the pore bottoms under the field of the space-charge region surrounding the pore bottoms and are consumed by the dissolution reaction. Pore walls remain passivated against dissolution by the electrical isolation caused by lack of charge carriers in the space-charge region at the base of the walls and by complete consumption of h^+ at the pore bottoms.

The thickness of the space-charge at the solution interface, which is a function of semiconductor's charge-carrier density (i.e., doping density or resistivity) and the applied voltage, controls the pore diameter and the spacing between pores. However, pore diameter and spacing can be varied over a small range for a given doping density by manipulation of the photocurrent density, applied voltage, and the density of initiation sites, that is the surface roughness. Conditions for two doping densities were established to provide por-Si membranes with one of two different sized pores. High pore density and porosity, $>10^6$ pore/cm² and $>50\%$ porosity, were achieved using polished, moderately doped wafers. Pores with diameters as small as 1 μm on center-to-center spacings of about 2 μm were grown into phosphorus-doped (10^{16} cm⁻³) silicon wafers with 0.3 to 1.2 ohm-cm resistivity (Figure 1). Pore diameters as small as about 0.3 μm have been reported and even smaller diameters have been predicted.ⁱⁱ Larger diameter pores with larger spacing between pores were formed in more resistive (3 to 6 ohm-cm) wafers (Figure 2). Pore diameters beyond an upper limit of about 20 μm appears to be constrained by formation of bubbles in the pores.

It was initially thought that an array of pore initiation pits would have to be pre-etched into the surface by high resolution photolithography using an alkaline-resistant photoresist, but our results show that pore density exceeding that possible by photolithography, that is higher porosity, can be achieved from the random surface irregularities on polished wafers. Pore initiation sites present after polishing are more densely and evenly distributed than the feature resolution possible by present lithographic methods. This work shows that the time and cost of high-resolution lithography can be avoided. Furthermore, experiments showed that pore growth could be limited easily to a particular region by masking the area exposed to illumination on the back of the wafer. The pattern of a wire screen mask placed against the dry, illuminated side of the wafer created isolated squares of por-Si visible on the front of the wafer. This means that masking both for selective deposition of por-Si and etching to form FPW membranes can be

conveniently defined by application of one mask to the back of the wafer, avoiding the complicated process of aligning masks on the top and bottom of the wafer.

Figs. 1&2 {check scale before adding caption with two doping densities and pore diameters here}

After application of reactive-ion etch to remove any nonporous silicon blocking the bottoms of the pores, micrographs showed intact pore open on both ends with little damage other than surface roughening. In fact, the density of linear, transmembrane channels was sufficient enough to see light transmission at normal incidence only through the membranes with the naked eye. The acoustic properties of large area samples with uniform thickness to within a few microns were measured to ascertain the size of the effects of the binding of DNA to the membrane. In these experiments, the acoustic wave was generated by a transducer under water and the wave incident on the plate caused phase-matched transfer of energy from the acoustic wave to a leaky guided plate mode. The guided wave sampled the acoustic properties of the plate, including its density (mass/cm^3) and stiffness. Presumably, the adsorbed DNA will not alter the plate stiffness appreciably, but the mass loading did strongly alter the frequency of the guided plate wave. Data (reflected wave intensity in arbitrary units plotted vs. frequency) are shown in Figures 3-4 for the same membrane before and after adsorption of synthetic 18-mer DNA, coagulation factor V: 5' TGGACAGGCAAGGAATAC 3', (incubation 5-10 min in 44 μM solution and thorough flushing of pores with water). Figure 5 shows data for the membrane after removal of the adsorbed DNA by dissolution in strong base. These plots contain the sum of the intensities of the reflected and guided waves, where the plate-wave energy interferes destructively with the nonresonant, reflected energy, so the resonance for plate waves appears as negative peaks in the plots. The position of the resonance vs. angle of incidence (α) was used to assign the identity of the plate mode, symmetric (S_0) or anti-symmetric (A_0) 1st-order modes. The S_0 mode for por-Si occurred at much higher α and lower frequency than that for nonporous silicon, because the porous plate has a much lower stiffness and density than the solid plate. The adsorption of DNA into the plate caused a significant shift in the position of the resonances, particularly that near 3 MHz. Additional washing of the pores with water had little effect on the spectrum, but DNA removal by highly basic solution regenerated the original spectrum for the most part (Fig. 3 vs. 5). In the words of Professor Chimenti, "In my judgement after 20 years of these kinds of acoustic measurements, these data confirm within the unknowns that still exist, the return of the sample to its pre-DNA condition, clearly demonstrating the feasibility of this genosensor technology."

Much more work needs to be done, but the preliminary work reported here demonstrates qualitative proof of FPW-device resonance as a rapid and sensitive probe of specific nucleotide

binding. Quantitative studies of DNA binding and optimization of devices and protocols for highly specific binding that is discriminate to a point mutation need to be carried out. By applying finer and finer surface polishing, the density of initiation sites can be increased to achieve the highest porosity and smallest pore size possible, that still allows solution flow and DNA binding. The optimum pore diameter for the highest FPW device sensitivity will be the smallest diameter achievable that permits pore wall access by probe and target without undesirable perturbation of chemical and electrostatic environments crucial to selective hybridization. We predict that this diameter will be made very small, say 100 nm or even less; however, the practical limit for small pore size will depend upon the size of the DNA fragments under investigation. Smaller pores will be impractical when the membrane becomes a nonselective filter and pressure differentials from solution flow through the membrane exceed its strength.

2. Phase I Accomplishments

The primary objective of the Phase I effort was to design, fabricate, and test prototype sensors, electronics, and software required to test the feasibility of using porous FPW devices as commercial sensors for genetic information. The specific Phase I research objectives were to:

1. Fabricate and characterize porous-silicon FPW devices.
2. Test their compatibility and characteristics while in contact with aqueous solutions.
3. Design and test supporting electronics.
4. Establish protocols for attachment of probe DNA strands to porous-silicon sensor elements.
5. Measure ability of attached probe DNA to specifically bind target DNA.
6. Determine response characteristics (sensitivity, response time, temperature dependence) of porous-Si FPW sensors to hybridized target DNA.

TPL believes the primary objective was accomplished in that all of the specific research objectives for Phase I were attained to a degree to answer in a positive fashion all questions that affected the overall feasibility of the sensor concept. While obviously, much more work is required to optimize the design of the FPW sensor and to fully characterize its performance response, sufficient information was acquired on all aspects of the sensor design to establish the feasibility of the concept. More importantly, TPL believes the experimental data acquired in Phase I indicates that the porous-Si FPW sensor is capable of both the sensitivity and selectivity quantitative measurements that represents a breakthrough in genosensor and, more broadly, biosensor capabilities for a variety of molecular diagnostic applications.

In summary, TPL, in the critical first issue, Objective 1, developed an etching process whereby as small as 1 μ m wells could be created in thin silicon substrates. This porous substrate/membrane dramatically increases the surface area available for attachment of probe molecules as well as increases the sensitivity of the device response by lowering the effective density of the sensor element. The design of an existing electronics system for surface acoustic wave (SAW)-based sensors was modified for

application to a FPW-based sensor (Objective 3) based on relevant characteristics of the thin, porous membrane as deduced from preliminary laboratory characterization (Objective 2). The engineering development of the electronics system can proceed with maximum confidence in the Phase II effort. Using protocols developed by the Biological Technology Division of the Oak Ridge National Laboratory, Dr. Tom Williams, University of New Mexico and TPL's collaborator on this project, demonstrated the ability, through the use of tagged molecules, to attach DNA probe molecules to the well walls of the silicon membrane and to further demonstrate that target DNA molecules would attach (hybridization) to the probe molecules (Objectives 4 & 5). While Phase II will address the issues of flushing out from the wells non-specific molecules and the minimum amount of target DNA that can be detected with a complete instrumentation system, Phase I clearly established that probe and target molecules could be effectively utilized. Finally, at the laboratory of Dr. Dale Chimenti, Iowa State, it was demonstrated that the response characteristics of "bare" porous Si membranes, membranes with only probe DNA molecules attached, and membranes with target DNA molecules attached to the probe molecules, showed significant difference in the response characteristics when subjected to acoustic excitation and that the capability to measure the incremental influence of attached target molecules was established (Objective 6). This accomplishment was obviously the second critical objective of the Phase I effort.

2.1 Task 1 Fabrication of Porous FPW Device Structures

Porous substrates were made from n-type (100) silicon wafers with resistivity of 0.7 or 5 Ωcm . Silicon wafers were either used as received or with a thermally grown oxide mask or a spin coated silica sol-gel coating.

In all pore-growth experiments, a platinum wire was used as a counter/pseudo-reference electrode and a dilute hydrofluoric acid solution as electrolyte. Backside illumination was provided by both filtered light and a chilled water filter. The photocurrent was maintained at the desired current density by controlling the power to the lightsource with a variac. Ohmic contact was made to the wafer by either a stainless steel foil mask, GaIn eutectic and gold wire, copper foil, or sputter coating gold directly onto the backside of the wafer.

Two experimental configurations were used for pore growth. In the first configuration wafers were quartered and mounted with a teflon seal and grease between a teflon bottomless cup and a metal plate with a hole (aligned to allow for backside illumination). The solution was stirred throughout the experiment to remove hydrogen bubbles from the surface. The second configuration consisted of a plexiglass electrochemical flow cell, which allowed for mounting of whole wafers.

Following growth of the pores, the wafers were either electrochemically etched in a dilute hydrofluoric acid solution or chemically etched with hot alkaline materials in their respective pore-growth configurations to a thickness appropriate for the opening of the pores via reactive ion etching.

2.2 Task 2 – Measurement of Acoustical and Electrical Properties of Porous FPW Devices

2.2.1 Acoustic Properties

Acoustic properties were measured using techniques developed by Prof. Dale Chimenti at Iowa State University. The ultrasonic approach uses a combination of swept frequency and variable angle of incidence to determine the ultrasonic velocity as a function of frequency for plate modes within the target sample. Inversion of these data can then be used to determine the elastic moduli of the test material.

The dispersion curves for the FPW membranes were measured in both a bare state and after application of probe oligonucleotides. These data, coupled with measurement of membrane bonding efficiency, were then used to determine the realizable sensitivity limits of these membranes when used as DNA sensors in a mass sensing mode. The results of these measurements are included in section 5.5.

2.2.2 Electrical Properties

The electrical properties of the membranes are critical for integrated device applications such as in the case of the conventional FPW or SAW device where metalization and electric fields are applied directly to the, typically piezoelectric, substrate. Evaluation of measurement methods and economic considerations have steered TPL away from this type of approach. Instead TPL will seek to generate and detect the flexural wave using permanent external transducers. This results in a more cost-effective sensor (no lithography or metalization of IDTs) and improved ultrasonic capability. Another positive consequence of this configuration is that there is no direct application of voltages or electric fields to the membrane. Consequently the largest motivation for a detailed understanding of membrane electrical properties is eliminated.

There are, however, other reasons to consider membrane electrical properties. The most notable is the potential for using applied electrostatic potential to preferentially accelerate the attachment of particular molecules; this is a technique used by some of the "DNA on a chip" manufacturers. Spatial control over these potentials could improve both measurement time and specificity. If awarded, the Phase II effort will investigate the potential to both use applied potential to accelerate binding and inhibit binding of unwanted molecules.

2.3 Task 3 – Design and Fabrication of Supporting Electronics

Although selection of a fundamental operating frequency for the porous FPW membrane will depend upon the final dimensional and coating parameters determined during Phase II, a baseline oscillator circuit was designed and tested during the Phase I project. This design, based on a modification of the TPL SA-1000 SAW driver used in

2.4 Task 4 – Attachment of Probe DNA to Pore Walls and Measurement of Hybridization Activity

During Phase I Dr. Tom Williams developed a measurement method for quantifying the efficiency of Probe DNA attachment using ^{32}P tagged oligonucleotides. Known dilutions of the labelled oligo nucleotides are introduced to porous membrane samples and the radiation levels measured. In order to account for attenuation of radiation by the silicon, measurements of radiation levels through a solid piece of the silicon wafer were made. These measurements show a maximum reduction of radiation levels of approximately 65%. In practice the attenuation will be lower owing to the distribution of the ^{32}P throughout the length of the pores. This deviation can be corrected for by performing similar measurements on porous samples without the oligo nucleotides; this will be done during the Phase II project.

2.5 Task 5 – Measure Ability of Attached Probe DNA

Initial measurements indicate a high binding efficiency of the oligo nucleotides to the porous silicon membrane. The initial measurements, however, were dominated by the presence of a blue dye in the ^{32}P solution. This dye is a fairly large organic compound that had dramatically altered the surface tension properties of the fluid. As a consequence, the initial measurements are not quantitative. During Phase II a dye-free tagging solution will be used.

2.6 Task 6 – Ultrasonic Characterization of Sensor Membranes

The porous membrane was characterized using ultrasonic spectroscopic methods by Prof. Dale Chimenti at Iowa State University. This technique uses interference between the front-side specularly reflected wave and the plate mode to measure plate wave velocities that are then used to ascertain elastic properties. The preliminary nature of the Phase I work prevented full inversion of data sets, but the measurements demonstrate both extremely high contrast between bare and DNA bonded membranes and reversibility of the sensor binding. Figures 4 through 6 show spectra for the bare membrane, the membrane after attachment of DNA, and the membrane after removal of the DNA using a base wash.

Figure 4 Ultrasonic spectrum for membrane prior to application of DNA

Figure 5. Ultrasonic spectrum for the bare membrane.

Figure 6. Ultrasonic spectrum after removal of the DNA. Note the high degree of reversibility as this spectrum is nearly identical to the initial spectrum.

These data can also be used to make an estimate of membrane sensitivity. The first minima in the spectra shifts off-scale after application of the DNA. The full-scale bandwidth of the system is approximately 14 MHz. This means that the *minimum* frequency shift caused by mass addition was approximately 11 MHz based on the first minima original position at 3 MHz. The system has an estimated noise level of approximately 10 Hz. Based on the estimated DNA addition of $25 \mu\text{g}/\text{cm}^2$ we can estimate the functional dependence of the frequency upon mass loading as:

$$25 \mu\text{g}/\text{cm}^2 / 11 \text{ MHz} = 2.3 \text{ pg}/\text{cm}^2 \text{ Hz}^{-1}.$$

Since the noise level is approximately 10 Hz, this means that the minimum detectable mass concentration (based on setting the signal-to-noise ratio = 1) 23 pg/cm^2 . This can then be converted to the more standard molar concentration as 4 fmole/cm^2 . Assuming an reasonable sensor area of 0.03 cm^2 , this means that the minimum detectable DNA amount = 0.12 fmoles. Clearly this approach can provide DNA sensing without the need for amplification.

3. CONCLUSION

TPL believes the primary objective was accomplished in that all of the specific research objectives for Phase I were attained to a degree to answer in a positive fashion all questions that affected the overall feasibility of the sensor concept. While obviously, much more work is required to optimize the design of the FPW sensor and to fully characterize its performance response, sufficient information was acquired on all aspects of the sensor design to establish the feasibility of the concept. More importantly, TPL believes the experimental data acquired in Phase I indicates that the porous-Si FPW sensor is capable of both the sensitivity and selectivity quantitative measurements that represents a breakthrough in genosensor and, more broadly, biosensor capabilities for a variety of molecular diagnostic applications

ⁱ V. Lehmann and H. Foll, J. Electrochem. Soc., 137, 653 (1990)

ⁱⁱ V. Lehmann and U. Gruning, Thin Solid Films, 297, 13-17 (1997)