Electronic Supplementary Material (ESI) for Analytical Methods

Electronic Supplementary Information (ESI): Miniaturized silicon biosensor for the detection of Triglyceride in blood serum

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I. Sensor fabrication

A 0.8 µm thick thermal oxide is grown on a p-type 1-10 Ω-cm (100) silicon wafer after cleaning it with standard RCA cleaning procedure. An array of 1500 μm X 1500 μm windows is opened in the oxide layer with 2 cm spacing between the windows. The exposed silicon is bulk micro machined using semiconductor grade KOH (44.44 wt %) to a depth of 100 μm for a period of 2 hours at 80°C. The wafer is cleaned again using standard RCA cleaning procedure to eliminate the K^+ ion contamination and the remaining oxide mask is completely removed from the wafer using semiconductor grade BHF solution. A field oxide layer of 0.8 μm thickness is grown thermally which plays the dual role of isolating one sensor from the other, as well as reducing the surface roughness resulting from the KOH etching. After removing the field oxide from the etched pits, a 10 nm gate oxide is grown in a rapid thermal oxidation (RTO) system at 1000 °C with an oxygen flow of 50 sccm for 1 minute followed by annealing in nitrogen flow of 50 sccm for 3 minutes. The RTO system is ramped down to 100 °C and the wafer is immediately loaded in a plasma enhanced chemical vapor deposition (PECVD) system to grow a nitride layer of 30 nm thickness followed by annealing at 800 °C in nitrogen ambient for 10 minutes. The stack of nitride and oxide on silicon is the gate insulating layer of the electrolyte insulator semiconductor (EIS) device under study.

The RTO grown oxide ensures a good Si-SiO2 interface and the PECVD deposited nitride is the active pH sensitive layer which is resistant to ion penetration from the electrolyte into the silicon dioxide. The rear side of the silicon wafer is scratched using a diamond tip metal rod for better ohmic contact and Aluminum is thermally evaporated. The chips are diced out using an Ultra slice dicing machine. The micro machined reactor in each silicon die acts as a miniaturized electrolyte insulator semiconductor capacitor (EISCAP) pH sensor. The fabrication process steps are shown in Fig. 1.

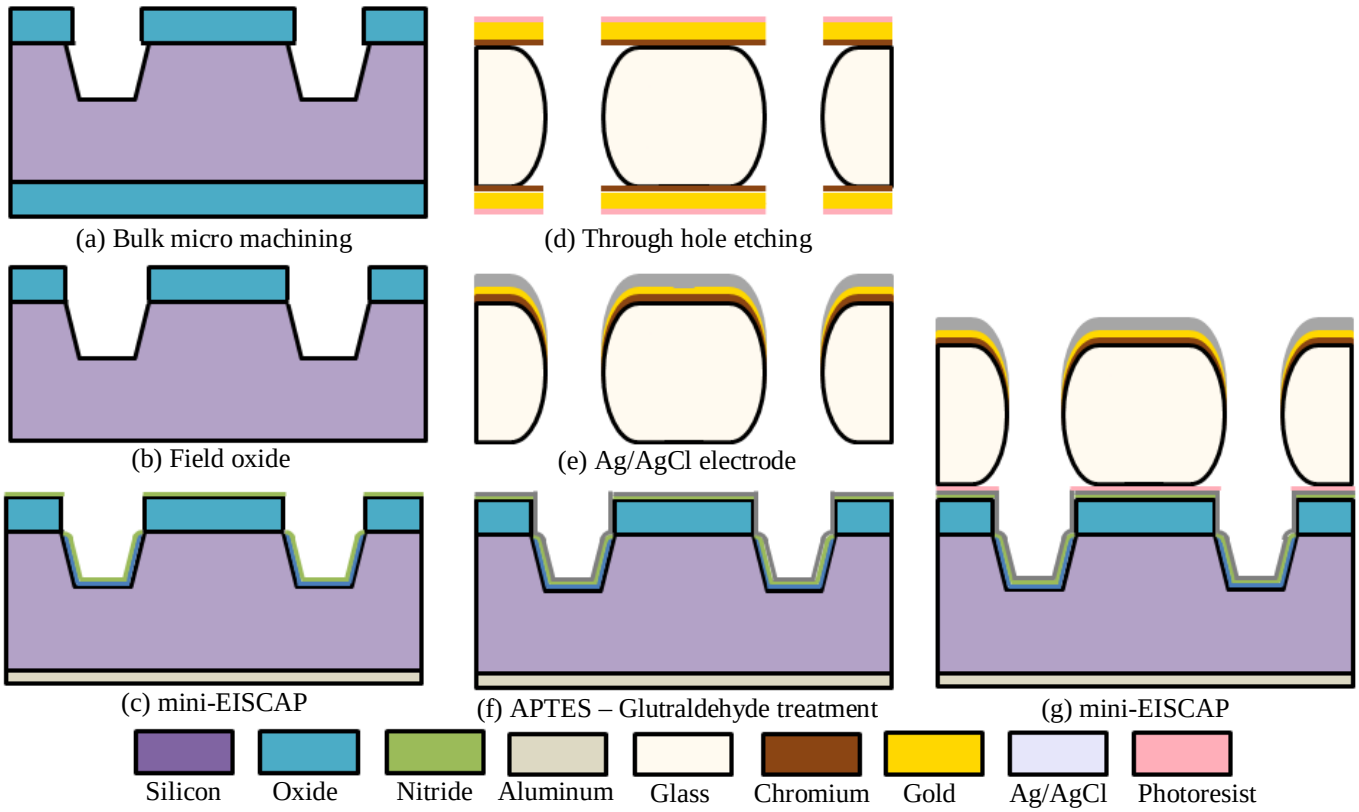

Fig.1: Schematic of the process steps of the enzyme immobilized and electrode integrated mini-EISCAP structure (not to scale)

Silver-silver chloride electrode deposited on a boro-float glass wafer is used for the solid state electrode fabrication. A through hole is initially formed in the glass wafer by bulk micro machining using e-beam deposited chrome-gold as the masking layer on both sides. The Au layer thickness is further increased to 1µm by electroplating, using gold sulfite (TSG-250) solution at 60 °C. A thick layer of PPR (SPR-220) is spin coated on both sides of the glass substrate and circular windows of 1 mm diameter are opened to etch out the Au and Cr layers.

The glass wafer is immersed in concentrated HF for about 60 minutes to etch through holes of 3 mm diameter in the glass wafer. The remaining Cr-Au-PPR layer is removed and now silver is deposited on one side of the micro machined glass wafer. Since the adhesion of silver is poor on glass, a stack of Cr-Au is initially deposited on the glass wafer at 120 °C followed by a thick layer of 99.9% pure silver. Silver chloride is formed by placing the silver deposited glass wafer in an electrochemical cell. A standard Ag/AgCl reference electrode acts as the cathode and the Ag deposited glass wafer acts as the anode. A resistor of 1 kΩ is connected in series with the 1.5 V voltage source to limit the current flow in milli-amperes range and an ammeter monitors this current flow. Once the current approaches a steady state of about 50 µA, the electrolytic process is terminated. Solid state Ag/AgCl electrode embedded to the through hole etched glass wafer is shown in Fig. 1.

After the formation of the Ag-AgCl layer on the glass electrode, the glass wafer and the silicon wafer are bonded using photoresist as an adhesive as shown in Fig. 1. The Ag-AgCl layer deposited on the glass wafer acts as the top plate and Aluminum layer deposited on the rear side of the silicon wafer acts as the bottom plate of the EISCAP. The silicon microreactor, after bonding with the glass wafer, can hold a sample volume of 10 µl.

II. Biochemical Characterization and Calibration

A. Free Enzyme Assay

Crude *Pseudomonas Cepacia* lipase [100 mg] is dissolved in 100 ml of buffer (200 mM, Tris-HCl, pH 7.2), and centrifuged at 12857×g in 4 °C using Eppendorf® cooling centrifuge (model 5810R). The supernatant is dialysed overnight in 18.2 MΩ-cm resistivity de-ionized water to remove any contaminants. The enzyme used in the experiment is lipase from *P.cepacia* prepared in Tris-HCl (pH 7.2, 200 mM) buffer. The free enzyme activity is determined by *p*-Nitro Phenol Butyrate (*p*NPB) assay and used as a calibration data for the determination of immobilized enzyme activity later. The enzyme assay is discussed briefly as follows. A stock solution of *p*NPB is prepared by dissolving 20.92 mg in 1 ml of acetonitrile. The reaction mixtures contain the determined concentration of *p*NPB (viz. 0, 3, 6, 9, 12, 18, 25, 50, 100 mM *p*NPB), lipase (1mg/ml) in a final volume of 1.0 ml of 0.2 M Tris-HCl buffer, pH 7.2, containing 0.9% NaCl and the final concentrations of acetonitrile in all reaction mixtures is 1% (v/v). The hydrolysis of *p*NPB is determined by monitoring the increase in absorbance at 410 nm continuously using no-enzyme incubation mixture as a blank in UV- spectrophotometer (Jasco, Model V 530).

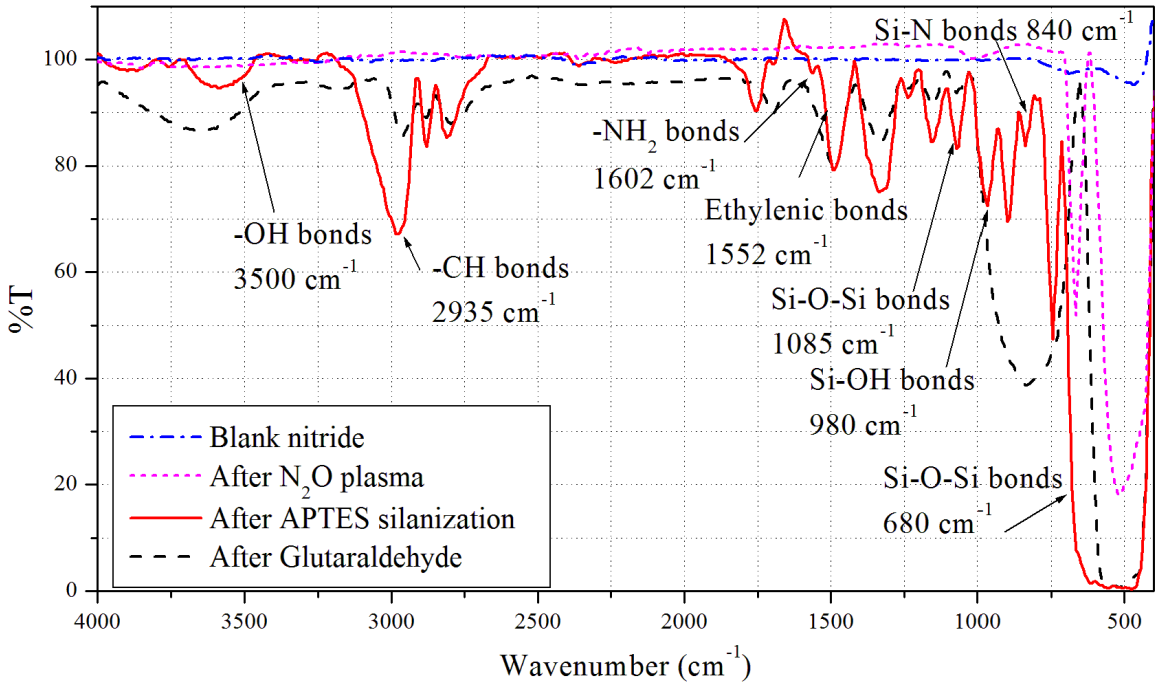

Fig. 2: FTIR spectrum taken after each of the immobilization process steps.

B. *Immobilized Enzyme Assay*

The immobilized enzyme activity is also measured by *p*NPB assay. Briefly, the enzyme immobilized sensor is dropped into the cuvette instead of free enzyme and the final volume is made upto 5 ml using 0.2 M Tris-HCl buffer, pH 7.2, containing 0.9% NaCl. The concentration of acetonitrile in all reaction mixtures is 1% (v/v). The hydrolysis of *p*NPB is determined by monitoring the increase in absorbance at 415 nm using a no-enzyme microreactor in the cuvette of the UV-Spectrophotometer.

C. *Fourier Transform Infrared (FTIR) Spectra*

The FTIR spectra (shown in Fig. 2) are measured on a JASCO 550 operating in transmission mode. Spectra are recorded at 2 cm^{-1} resolution, averaged using 32 scans. The samples are dried in nitrogen ambient, transported using vacuum desiccators. The bands at 2935 and 2885 cm^{-1} are attributed to the C-H bonds which are present in the silanized, glutaraldehyde activated and enzyme immobilized samples. The bands at 3300 and 3380 cm^{-1} are assigned to NH_2 bonds, which are present mainly in silanized samples. APTES treated samples show a broad peak around 3500 cm^{-1} due to the presence of Si-OH bonds. In the silanized samples a peak at 1602 cm^{-1} is evident which is attributed to NH_2 bonding. In glutaraldehyde activated and enzyme immobilized samples the peak at 1602 cm^{-1} is missing and a new peak, around 1552-1582 cm^{-1} is found, due to the ethylenic bond which is a sign of proper glutaraldehyde activation and subsequent immobilization. The spectrum exhibits bands characteristic of surface-OH, SiO-H. The Si-O-Si asymmetric bands are visible around 1085 cm^{-1} and Si-OH hydroxyl groups stretching bands are located around 980 cm^{-1}. The partially masked SiN band is visible around 840 cm-1.

III. Principle and Design of pH Readout

A. *Principle*

The principle of measuring pH is explained using the C-V curves of the EISCAP sensor shown in Fig. 3. The C-V curve $C(V_{ref})$ corresponds to the reference electrolyte of known pH value (pH_{ref}) and $C(V_x)$ corresponds to the electrolyte of unknown pH value (pH_x). The capacitance of the EISCAP varies with the bias voltage applied across its terminals. The non-linear capacitive device if biased at a particular voltage can be made to charge from initial voltage V_L to final voltage V_H and discharge back to V_L using a constant current in theory. The frequency of charging and discharging can be measured if the EISCAP is embedded into a relaxation oscillator loop.

The schematic of the EISCAP relaxation oscillator is shown in Fig. 4. The comparator and the positive feedback resistance R_a and R_b form an inverting Schmitt trigger. The voltage (υ_c) at the top plate of the EISCAP is detected by the comparator. The bottom plate of the EISCAP is controlled by V_{bias} that fixes the operating point of the sensor in the region of interest. The EISCAP is charged and discharged between the threshold voltages V_H and V_L set by

$$V_H = \frac{V_{ref}R_a + V_{dd}R_b}{R_a + R_b} \quad (1)$$

$$V_L = \frac{V_{ref}R_a}{R_a + R_b} \quad (2)$$

When the electrolyte with pH_{ref} is used in the sensor, the relaxation oscillator of Fig. 4 produces a frequency of oscillation f_{ref} and when the electrolyte is replaced with pH_x, the C-V curve shifts in the positive direction with ΔV voltage change and produces a frequency of oscillation f_x. Assume that $pH_x > pH_{ref}$ and voltages (V_H, V_L, V_{bias}) are initially set appropriately as shown in Fig. 3 with respect to the $C(V_{ref})$ curve.

$$f_{ref} = \frac{I}{2}\left[\int_{V_L}^{V_H} C(\upsilon_{ref} - V_{bias})d\upsilon_c\right]^{-1} \quad (3)$$

$$f_x = \frac{I}{2}\left[\int_{V_L}^{V_H} C(\upsilon_x - V_{bias})d\upsilon_c\right]^{-1} \quad (4)$$

$$C(\upsilon_x) = C(\upsilon_{ref} - \Delta V) \quad (5)$$

$$\Delta V = 0.0592(pH_x - pH_{ref}) \text{ at } 25\,^{\circ}C \quad (6)$$

ΔV of (6) is derived from ideal Nernst equation. From Fig. 3, in case of pH_x, because of the ΔV shift in voltage, the capacitance that lies between V_H and V_L becomes larger than what it was when the electrolyte was pH_{ref} and this makes the frequency of oscillation f_x smaller than f_{ref}. If the bottom plate voltage V_{bias} of the sensor is modified as V_{bias} - ΔV, then the frequency f_x can be made equal to f_{ref}. The change in the bottom plate voltage (ΔV) of the sensor is used in (6) to calculate the unknown pH (pH_x). However, the actual voltage sensitivity (ΔV) to pH changes in the EISCAP sensors varies from device to device. The measurement of voltage sensitivity (ΔV) to pH changes is explained in the sub-section B.

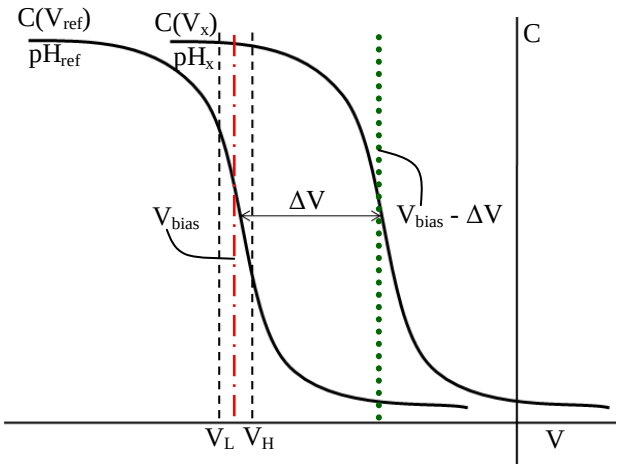

Fig. 3: C-V curves with different electrolyte of pH_{ref} and pH_x

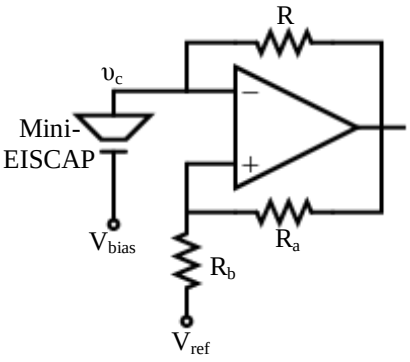

Fig. 4: Schematic of EISCAP relaxation oscillator

Due to the quality of nitride layer of the EISCAP sensor, it is not possible to achieve in practice the theoretical pH sensitivity limit of 59.2 mV/pH. The pH sensitivities that we measured with EISCAP vary between 50 mV/pH and 58 mV/pH from device to device that are batch processed. Clearly, using (6) to measure pH_x would result in gross measurement errors and hence, we need to calibrate the EISCAP to make the pH measurements independent of sensitivity. The actual pH sensitivity is

$$\Delta V = 0.0592\alpha(pH_x - pH_{ref}) \text{ at } 25\,℃ \qquad (7)$$

The parameter α in (7) is dimensionless and it depends upon the intrinsic buffer capacity and the differential capacitance of the nitride layer. It varies between 0 and 1. Intrinsic buffer capacity is the measure of the capability of the nitride layer to absorb H^+ ions at the electrolyte nitride sensing interface layer and the differential capacitance is the measure of the capability of the electrolyte to adjust the amount of absorbed H^+ charges to change in electrolyte nitride interface electrostatic potential.

There are two unknown variables, pH_x and α in (7), hence we need two equations to solve it. The second equation can be obtained by using one more reference pH. The pH value of 4 (pH_4) and pH value of 8 (pH_8) are used as reference pH values in calibrating the sensor. The unknown pH (pH_x) in (10) is calculated by solving ΔV_{ref} and ΔV_{test} using (8) and (9) respectively. ΔV_{ref} is the voltage sensitivity to change in pH from pH_4 to pH_8 ($\Delta pH = 4$). ΔV_{test} is the voltage sensitivity to change in pH from 8 to pH under test (pH_x).

$$\Delta V_{ref} = 0.0592\alpha(pH_8 - pH_4) \qquad (8)$$

$$\Delta V_{test} = 0.0592\alpha(pH_8 - pH_x) \qquad (9)$$

$$pH_x = pH_8 - \Delta pH_{ref}\left(\frac{\Delta V_{test}}{\Delta V_{ref}}\right) \qquad (10)$$

B. Calibration and measurement

The sensor is calibrated as follows:

- The sensor is biased in accumulation and inversion regions after placing pH_4 in the sensor. The EISCAP relaxation oscillator produces frequencies of oscillation f_L and f_H when biased in these constant capacitance regions of the C-V characteristics. To achieve this, fine DAC input code is reset to all 0's and the coarse DAC input code is set to all 1's followed by all 0's so that the senor is biased in the extreme ends of the C-V curve. To bias the EISCAP with the help of the coarse DAC in the mid of the depletion region independent of the flatband voltage, the centre frequency f_C is calculated as an arithmetic mean of f_L and f_H. The coarse DAC input code is now adjusted using successive approximation algorithm driven by the firmware till it achieves f_C frequency of oscillation. This is the first step in calibration.
- In second calibration step pH_8 is placed in the sensor. A reduced frequency of oscillation is noticed. Now, the fine DAC input code is adjusted with the help of successive approximation algorithm to bring back the frequency of oscillation to f_C. The fine DAC input code that results in oscillation of f_C corresponds to the voltage shift (ΔV_{ref}) in the EISCAP C-V characteristics for a change in pH of 4.

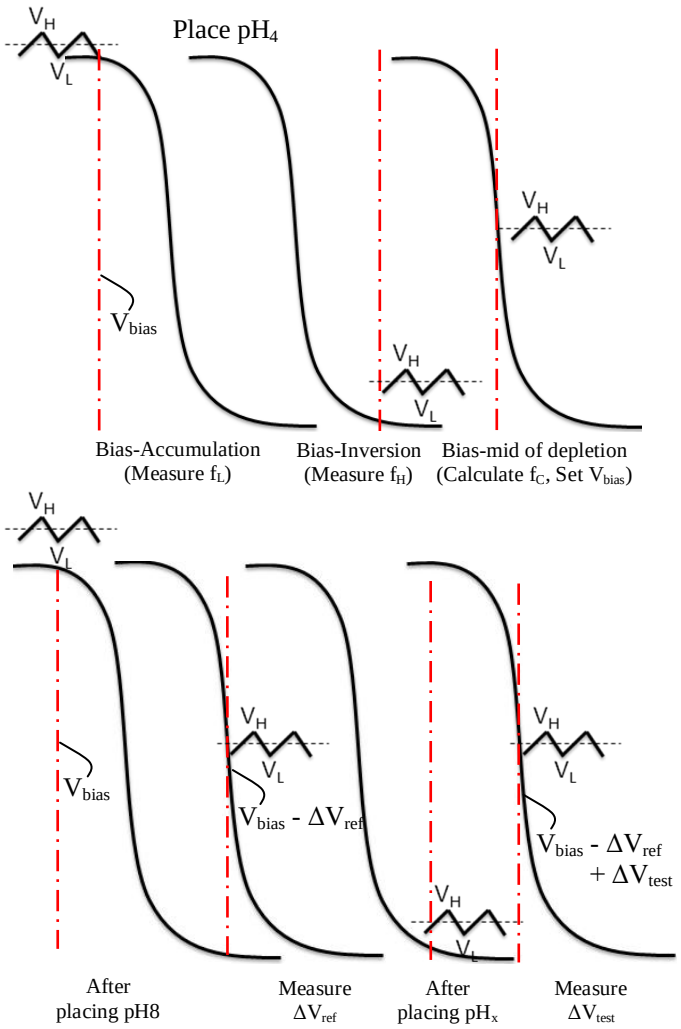

Fig. 5: Calibration and measurement modes using C-V curves

The pH under test is measured as follows:

- The electrolyte, whose pH (= pH_x) to be determined is now placed in the sensor. Successive approximation algorithm adjusts again the fine DAC code required to bring back the

frequency of oscillation to f_C. The new input code corresponds to the voltage shift (ΔV_{test}) in the EISCAP C-V characteristics.

- The unknown pH_x is now determined using (10). The result of the test pH_x is displayed on the LCD screen. The calibration and measurement modes are shown in Fig. 5 using EISCAP C-V characteristics.

The successive approximation algorithm works as follows:

- After calibrating the sensor for the centre frequency (f_C), the MSB of the DAC is set to 1 and the rest all set to 0. The measured frequency of oscillation ($f_{measure}$) is compared to f_C. If $f_{measure} < f_C$, the next significant bit is set to 1 else if $f_{measure} > f_C$, the MSB is set to 0 and the next significant bit is set to 1.
- The above process is repeated until it covers all the bits of the DAC input code.