Chapter IV:

Fabrication and characterisation of the biosensor device.

4.1. Introduction

An ENFET is a modified ISFET device where the enzyme is immobilised over the gate region of the ISFET [1]. This chapter focuses mainly on immobilisation of the purified preparation of Cytochrome P450 enzyme from *Bacillus stratosphericus* recognition element on a silicon ISFET device fabricated in collaboration with Department of Electronics and Communication Engineering, Tezpur University followed by functional characterisation of the device.

4.1.1. Working principle of this device

In an ENFET, the enzyme plays the role of the bioreceptor. As the enzyme comes in contact with the substrate, it specifically recognises the substrate and catalyses the reaction. The device reported here is immobilised with purified preparation of cytochrome P450 monooxygenases and termed conveniently as ENFET device or CYPFET. The reaction by which the enzyme incorporates single oxygen atom into the substrate for hydroxylation is given below.

$$RH + O_2 + 2H^+ \xrightarrow{CYP450} ROH + H_2O \qquad (4.1)$$

In the chemical reaction 1, RH is the hydrocarbon substrate with a hydroxylable site. In every reaction cycle, one molecular oxygen is inserted by the biocatalyst CYP450.

Further, two protons are also consumed in every cycle of conversion, making a local change in the pH in that particular region near the enzyme. Usually, due to the buffer action, this small change gets diluted by the the influx of ions. However, this process of dilution can be slowed down if the enzyme is placed inside a gel matrix thereby restricting ions from normal diffusion [2].

The development of interfacial potential due to the change in electrochemical (H+) gradient with respect to the progression of enzymatic catalysis can be related to the site binding theory [3]. For the formation of active sites, the surface of an ISFET reacts with the H^+ of the electrolyte. The changing electrical property of the active site modifies the threshold voltage of the device. Hence the changes in H^+/OH^- due to the enzymatic reaction further leads to a corresponding variations in the output voltage of the device.

The ENFET reported here has been fabricated with SiO_2 as the sensing layer. Furthermore, since an ENFET has an electrolyte biological membrane insulator semiconductor structure, it is considered to be an extension of EIS structure for the ISFET [4]. Therefore, in conjunction with the site binding theory and electrical double layer theory, an electrolyte biological insulator semiconductor system has to be considered for ENFET modeling.

4.2. Objectives

1. Fabrication of the SiO_2 ISFET
2. Immobilisation of CYP450 on the fabricated ISFET
3. Characterisation of the device for different parameters.

4.3. Materials and Methodologies

4.3.1. Materials used

4.3.1.1. Chemicals used

Ammonium chloride, Ammonium sulphate, Di-Potassium hydrogen phosphate, Magnesium sulphate heptahydrate, Potassium dihydrogen phosphate, Sodium chloride, Acetone, 1% Bromophenol Blue, Ethanol, Formic acid, Hydrochloric acid (about 35% pure), Iron (III) chloride anhydrous, 2-Propanol, Methanol, n-hexane, Manganese (II) sulphate monohydrate, Potassium Chloride, Sodium thiosulfate pentahydrate, Sodium hydroxide pellets GR, Calcium chloride dehydrate (MERCK), Luria Bertani agar, Sodium carbonate anhydrous extra pure, Sulphuric acid, Yeast extract (Himedia), Tris base, Bushnell Haass Agar, Bushnell Haass broth, Ammonium persulphate, bacteriotryptone (Himedia), glycerol and Agarose (Sigma), Calcium chloride dehydrate AR (Qualigen), Glycine, Lysozyme (SRL)., Photoresist Microchem S1818 Microchem CD26 Kapton tape, N-hexadecane (Merck).

4.3.1.2. Instruments used

Autoclave (Labtech), Carbon Monoxide production unit, Electronic balance (Kern), Fraction Collector (Redi frac), Hot water bath, Laminar air flow

hood, Magnetic stirring, heating plate, Metricon 2010M prism coupler Micropippette (Thermo scientific), Mini centrifuge (Tarsons Spinwin), MultiScan NMR, pH meter, Plasma-enhanced chemical vapor deposition (PECVD) machine, Ultracentrifuge, Vortex (Tarsons Spinot).

4.3.2. Methodologies

4.3.2.1. Device Fabrication

A 3″ double sided polished p-type (100) Silicon wafer of resistivity of about 1-10 Ω cm was used as the substrate for EIS pH sensor. The wafer is boron doped and has a thickness of about 380 µm. The wafer was initially degreased to remove wax or any oil contamination. Further, by RCA cleaning process organic and metallic contaminants were removed [5]. After that 450 nm thick SiO_2 layer was deposited by thermal oxidation at a temperature of 1050°C with a gas flow of 2slpm (standard liquid per minute) of oxygen for a duration of 30 minutes /1 hour/ 30 minutes (dry/wet/dry). Lithography was performed by using the first mask (Fig. 4.1.b.i) for outlining the area of 22×7 mm which includes the active area and protective region to prevent metal from coming into direct contact with the active area. The unmasked region was removed by using buffer oxide etchant. The second lithography was done to etch out an active area of dimension 5×5 mm by using a second mask (Fig. 4.1.b.ii).The thickness of the insulating layer at the active region was around ~ 120 nm.

A hard mask was used as the third mask for the metal deposition (The first mask is used again as hard mask). This hard mask avoids the necessity for another level of lithography. This hard mask is a transparent sheet with the imprints of the first mask used with openings at the region where metal is to be deposited. This mask was attached to the wafer with Kapton tape (Polymide film which can sustain high temperature). A 200 nm thick silver layer was deposited for metal contact on the exposed region to form Schottky contact by thermal evaporation at 60-70A current for duration of

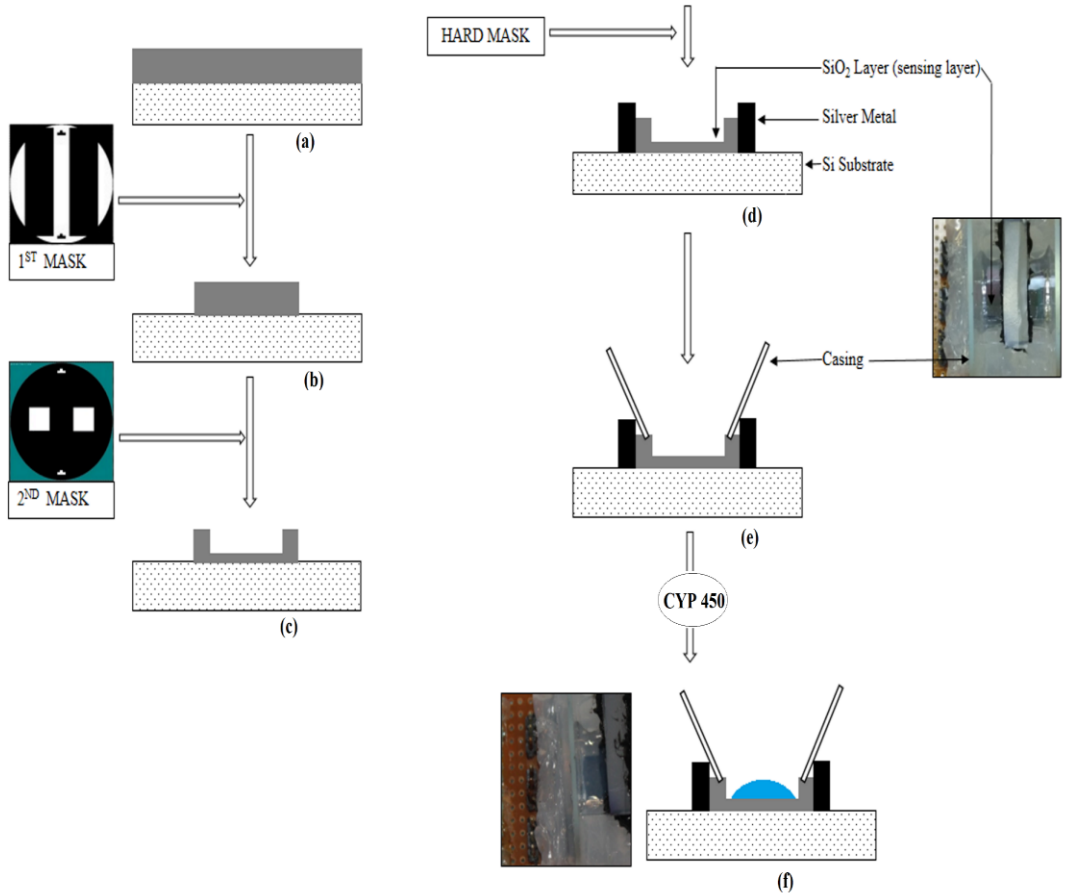


Fig. 4.1: (a) Thermal oxidation of silicon dioxide (b) Photolithography for outlining the region for deposition of the metal layer using 1st mark (inset a) (c) Second lithography defining the active area using 2nd mask (inset b) (d) Metal deposition of silver using hard mask (e) Casing done using cover slip (inset e) (f) immobilization of enzyme on the bare ISFET device to form the ENFET (inset f).

30 seconds. The silicon substrate was then mounted on PCB board with adhesive. Male connectors were soldered on the PCB board for the connections of source, gate and drain. The connectors were bonded to the metal layers using silver paste. A glass chamber using cover slip was constructed for depositing the electrolyte surrounding the active layer. Silicone being almost neutral to pH changes was selected for passivation and sealing purpose. Mask designing was done using Clewin software. The schematics of the process flow showing the fabrication process is depicted in fig. 1.a and the actual device is shown in fig. 4.1.a (inset).

Deposition of metal on the semiconductor forms a Schottky contact when the barrier height is large ($\phi_B > kT$) [29].Since the substrate is p-type, the Schottky barrier height (ϕ_{Bp}) is given by the difference between the valence band edge and the Fermi energy in the metal,

$$\phi_{Bp} = \frac{E_g}{q} + \chi - \phi_m$$

Here E_g is the band gap of silicon semiconductor which is 1.14 eV, q is the electronic charge, χ is the electron affinity which is 4.01 V for silicon, ϕ_m is the work function difference of metal (here silver) which is 4.26V. The barrier height is 0.91V which is much greater than the value of kT thus indicating formation of a Schottky contact [6,7].

Application of a positive bias to the p-type Schottky ISFET, the body region depletes and eventually inverts to form an n-type channel. When the hole

barrier height is large, the inversion mode (electron) barrier height is relatively small [8]. Positive bias when applied to the gate leads toward band bending (downwards) in the region below the oxide. This inversion caused by band bending, consequently reduces the contact potential built up due to metal/semiconductor contact (at source and drain junction), leading to flow of electrons between source and drain through the channel [8].

(a) (b)

Fig. 4.2: (a) the complete measuring Setup (b) enlarged picture of a fabricated device

This type of fabrication process has the advantage over other conventional process such that it mitigates the necessity for source and drain region doping. Thus decreasing the number of fabrication steps. Though work on ISFET has started four and half decades earlier as per authors' knowledge Schottky based ISFET fabricated in this work is first of its kind.

4.3.2.2. Circuit Design

The schematic of the measurement set up for the ISFET and the CYPFET at constant applied potential difference is illustrated in the fig. 4.3a. In this work, the characteristics of the devices are obtained using constant voltage set up by keeping the potential difference between drain and source (V_{DS})

constant at 0.27V and varying the gate to source voltage (V_{GS}). Further, the corresponding drain currents for different pH values were measured. This was repeated for another two different sets of constant drain to source voltage (0.4V and 0.5V) necessary to find the transfer characteristics. The circuit has OPA4277 high precision quad operational amplifier (op-amp) used as voltage follower which keeps the potential difference constant at the drain to source junction. The output potential difference being low in magnitude was amplified by another circuit that uses a JFET based op amp OP07. The same circuit had also been used to measure the CYPFET, i.e., ISFET with immobilised bioreceptor CYP450.

The output characteristics are depicted in the fig. 4.3.b. The gate to source voltage can be varied using the potentiometer of value 10KΩ. Separate power is given to the gate terminal as the Keithley 6517B Electrometer, which is a two terminal device. A stable potential difference was maintained at the reference electrode using a voltage follower (OP07). For the measurement, V_{GS} was kept constant and VDS was varied from 0 V to 9 V using Keithley Electrometer and the corresponding drain current were noted using the same electrometer as it was connected in series with the drain to source terminal.

4.3.2.3. *Preparation of the bioreceptor*

The enzyme Cytochrome P450 monooxygenase was purified from the bacterial strain of *Bacillus stratosphericus* as described in [7,9,10]. Which

involves ultracentrifugation of the bacterial spheroplasts, followed by an ion exchange chromatography and a gel filtration. Finally, the CYP content in the pooled fractions was examined using CO difference spectra [2,11].

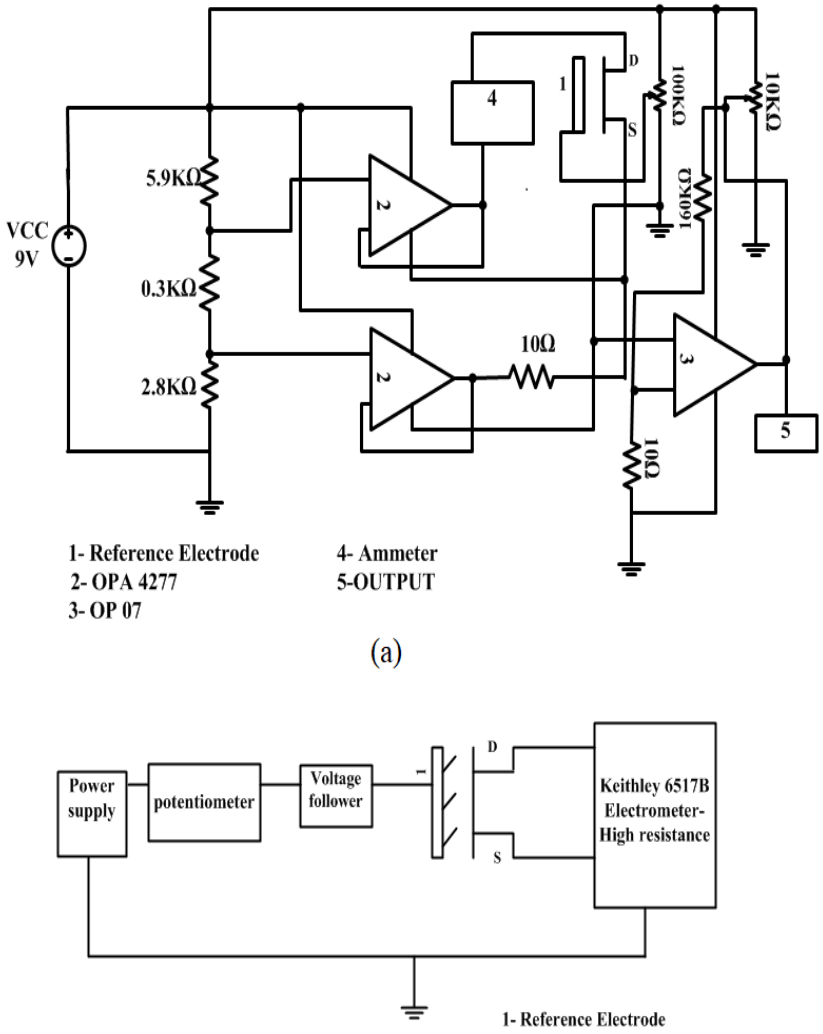


Fig. 4.3: (a) Circuit Diagram of the measurement set up of transfer characteristic, (b) Block diagram for measurement set up for output

4.3.2.4. Immobilisation of the enzyme

For immobilization, the purified fraction of cytochrome P450 enzyme in 50 mM Tris HCl buffer was mixed with equal volume of 5 % (w/v) agarose in

50 mM Tris HCl (pH-7.2) in a vortex and of which 40 µl was laid over the sensing layer of ISFET with the help of a micropipette, and allowed to solidify for around 10 minutes [2].

4.4. Results

4.4.1. Verification of completion of the reaction

Partially purified enzyme CYP450 was immobilised in the gate region of the fabricated device. CYP450 can oxidise the substrate n-hexadecane to n-hexadecanol consuming two protons (H+), as shown in the chemical reaction 2. Further this change in protons also lead to the change in the gate potential.

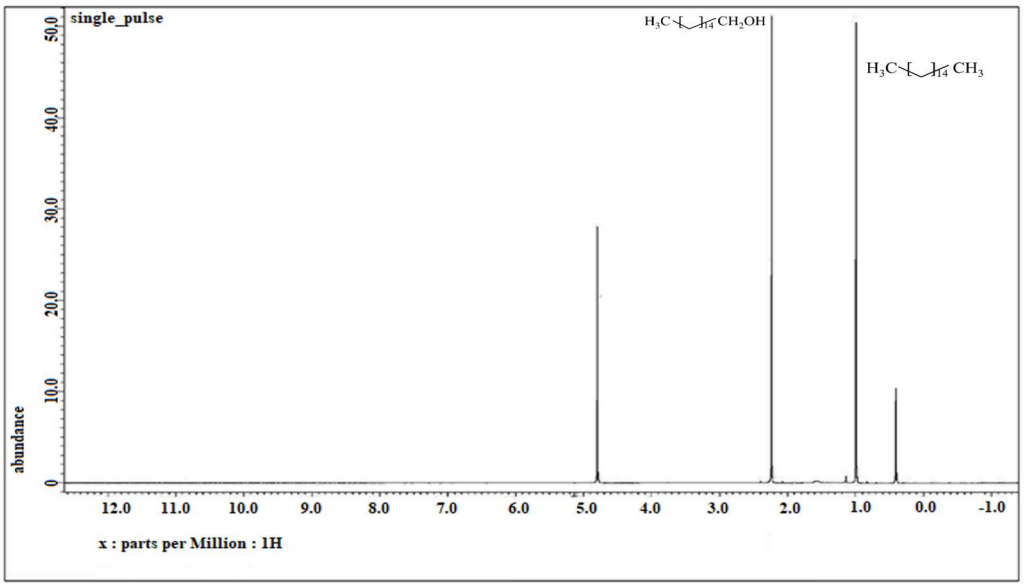


Fig. 4.4. Proton NMR of the reaction mixture after the completion of the reaction.

With variation in the concentration of the n-hexadecane the gate potential also varies making it a functional ENFET for sensing *n*-hexadecane. The production of the *n*-hexadecanol was to be verified by subjecting the reaction mixture to a proton NMR (JEOL, ECS-400) and data analysis was done using software DELTA (Version-4.3.6). The NMR spectroscopy

confirms the presence of both n-hexadecane and n-hexadecanol in the reaction mixture.

$$C_{16}H_{34} + NADPH + O_2 \xrightarrow{CYP450} C_{16}H_{33}O{+}NADP^{+} + H_2O \qquad (2)$$

4.4.2. The sensitivity of the Device

The sensitivity of the device was determined by measuring the difference in potential across gate and source (V_{GS}) against substrate (n-hexadecane) concentration (C) at constant currents (I_D) at a fixed buffer pH (pH 7.2) and operating temperature (27◦C). The sensitivity of the device can be defined as the:

$$S_{ENFET} = \left.\frac{\Delta V_{GS}}{\Delta C}\right|_{I_D = Constant} \qquad (3)$$

It can be easily illustrated from the plot (fig. 4.5) that the variation of V_{GS} increases along with the change in substrate concentration in a range of 0.1

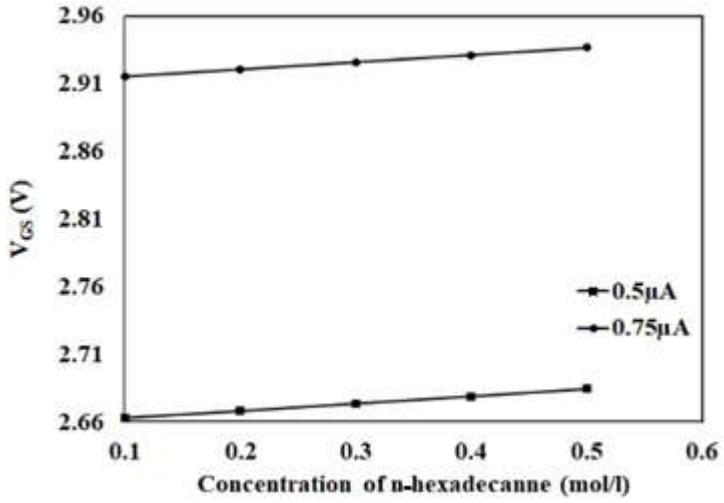
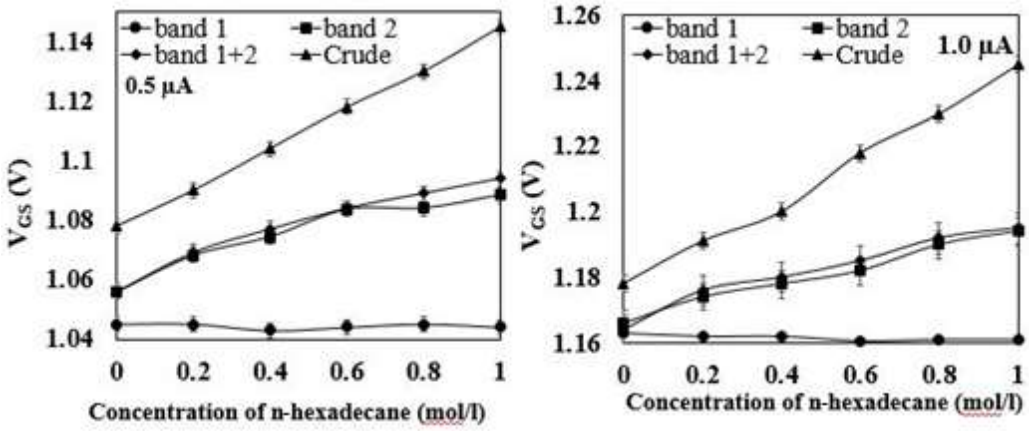


Fig. 4.5: Variation of V_{GS} with respect to change in concentration of n-hexadecane at constant currents of 0.5μA and 0.75μA

to 1 mol/l (step of .2 mol/l) at a constant current of .5 and 1 µA respectively. It is evident from the equation one that with the increase in n-hexadecane concentration, there will be an increase in OH^- ions, leading to a rise in the V_{GS} value. The average sensitivity of the ENFET device was calculated to be 54.34 mV/molar using equation 3.

4.4.3. Efficacy of protein bands

From the previous chapter it was identified that after purification there were two bands a bigger band (68 kDa) and the smaller band (13 kDa). When the bands were compared for the sensitivity it was found that the smaller band alone didn't have any activity. The bigger band have an average sensitivity value of 24.2 mV/molar, which was similar to the value 23.9 mV/molar obtained when the bands are mixed after purification. However the maximum sensitivity was observed in case of the crude



(a) (b)

Fig. 4.6: Variation of V_{GS} with respect to concentration of n-hexadecane measured for constant current of 0.5 µA (fig a) and 1 µA (fig b) showing its change in different protein bands and crude.

enzyme with a value of 54.34 mV/molar. As the maximal sensitivity was observed in case of crude enzyme, in the following experiments crude enzyme was used.

4.4.4. Stability

The fabricated device once immobilised by the enzyme was kept at room temperature (27°C, without the Potassium Phosphate buffer solution) and the results (sensitivity) of the device were monitored after a period of 24 hours continuously for three consecutive days. The process to find the sensitivity of ENFET is similar to the process described in the previous section. It is evident from fig. 4.7(a and b) that the variation in the sensitivity for the first day (day1) and the second day (day 2) was reasonably low indicating its potential as a biosensor for hydrocarbon detection. However,

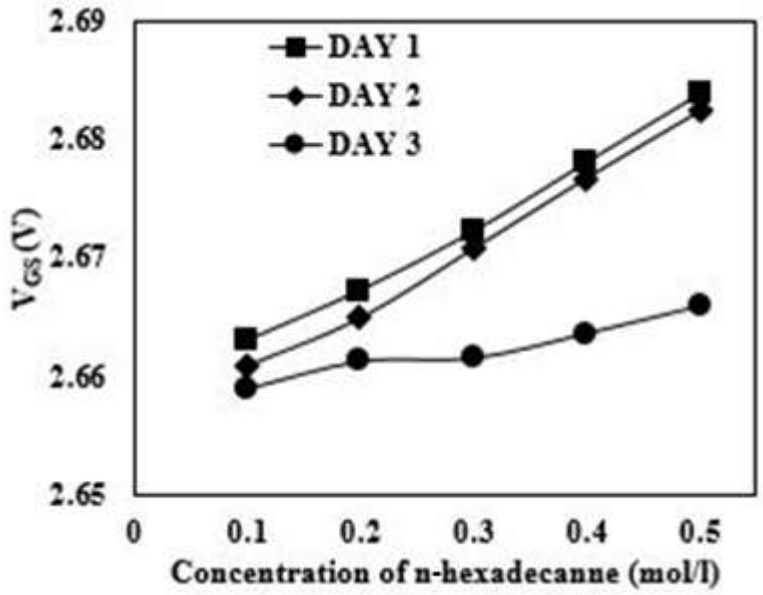


Fig. 4.7: (a) Variation of V_{GS} with respect to concentration of n-hexadecane measured at three consecutive days for constant current of 0.5 µA

from the third day (day 3) onwards a decline in the sensitivity could be observed.

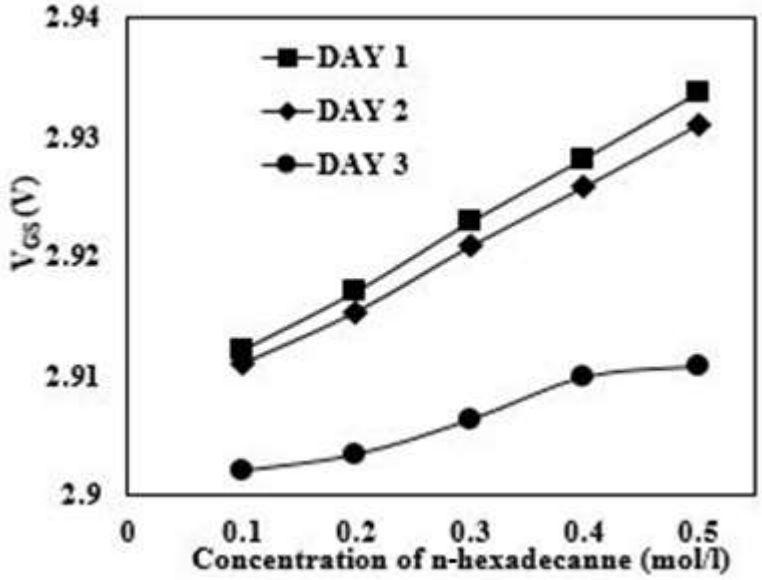


Fig. 4.7: (b) Variation of V_{GS} with respect to concentration of n-hexadecane measured at three consecutive days for constant current of 1.0 μA

4.4.5. Transfer and output characteristics of the fabricated ENFET

The transfer characteristics relate the drain current of a device with that of the applied gate to source voltage. The gate current can be considered to be of zero value as the gate terminal is electrically isolated. The threshold voltage can be derived by extrapolating the curves obtained from $\sqrt{I_D}$ versus V_{GS} plot [3]. Once the threshold voltage is attained, the drain current increases indicating the onset of significant flow of electrons. In the case of an ISFET device, the threshold voltage is a function of pH value. The value increases with decrease in the proton concentration. The immobilised enzyme was maintained at pH of 7.2. Hence the values are similar to pH 7.

Thus, for 0.4M and 0.5M the threshold values were found to be 2.08V and 2.095V respectively.

The output characteristics of the fabricated ENFET are illustrated in the following figure. The drain to source potential was varied from 0 to 5V keeping the gate to source voltage constant at 3V. It was observed that the drain current decreased with the increase in pH value. The buffer solution of the analyte is at pH 7.2. Hence the fabricated ENFET showed similar response trend of sensitivity at two molar concentrations 0.4and 0.5M as that of pH 7 [7].

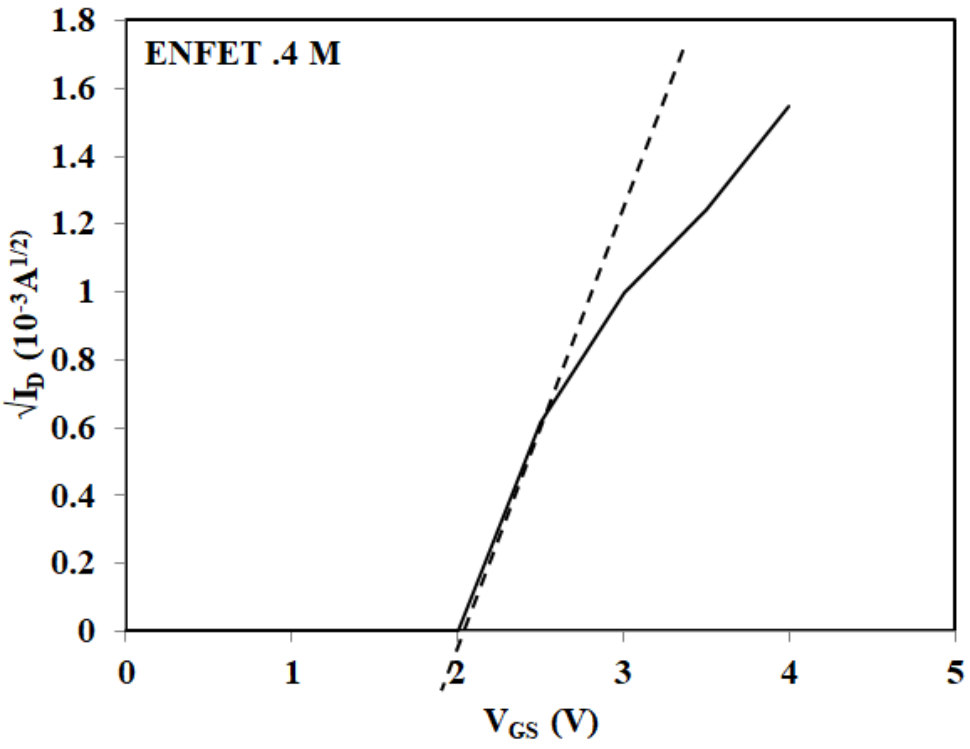


Fig. 4.8: (a) Transfer characteristics of ENFET for 0.4M

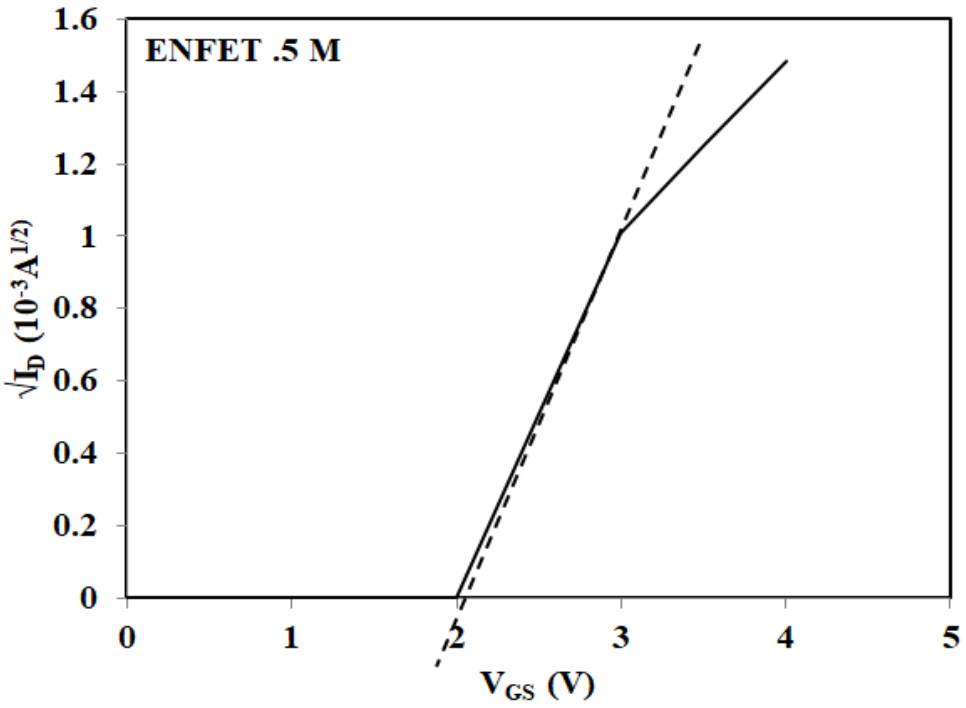


Fig. 4.8: (b) Transfer characteristics of ENFET for 0.5M

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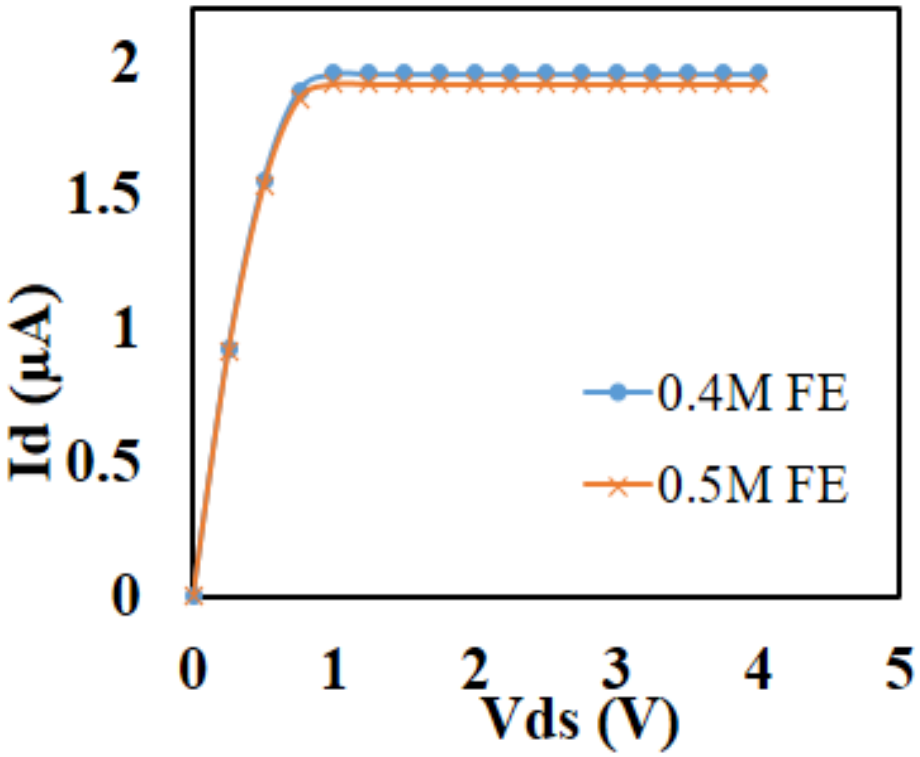

Fig. 4.9: Output characteristics for 0.4molar and 0.5 molar ENFET (FE indicates fabricated ENFET)

Hysteresis is a device characteristic, which corresponds to the inability of a device to produce exact output while running the operation in the opposite direction. It is another parameter showing reproducibility of a particular result in a specific data point. In fig.4.10, the hysteresis path fort the ENFET has been represented as A→B→C→D→A at room temperature (27◦C) and initial pH of 7.2. It was found that the average error in sensitivity was ±0.01%.

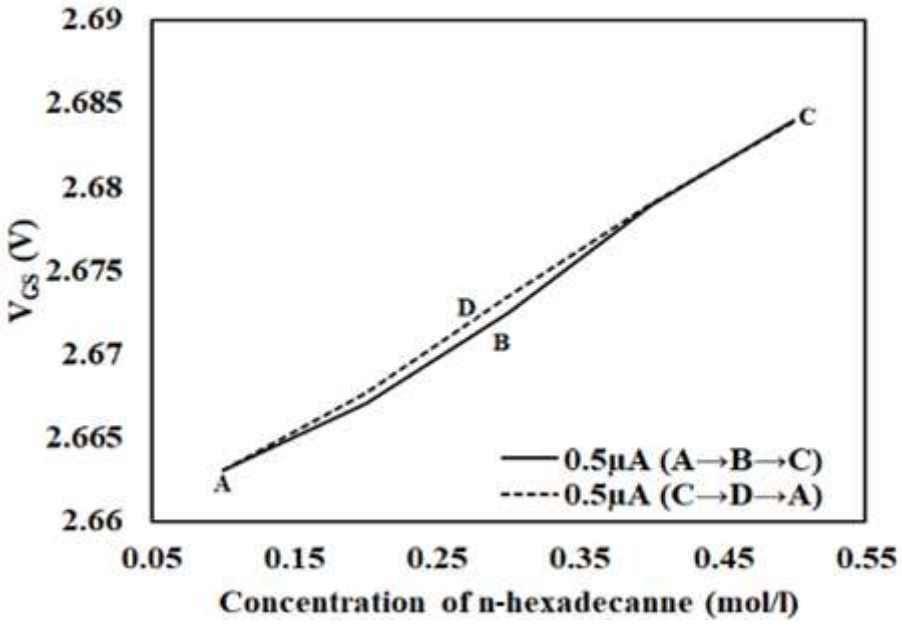

Fig. 4.10: Hysteresis observed in the fabricated ENFET device

4.4.7. Detection limit and variation along with change in pH.

The measurand after the completion of enzymatic reactions was collected to measure their pH values. This was done for perusing the variation in pH value with respect to the concentration of measurand (fig.4.11). The change in pH after reaction was measured using a standard pH meter and it was found that there had been a linear increase in pH with the increase in the concentration of the substrate from .1 molar to .5 molar. However,

negligible change in pH in the concentration range of .001 to .0001 molar was observed (inset fig 4.11), indicating the limit of the sensor to be 0.01 molar.

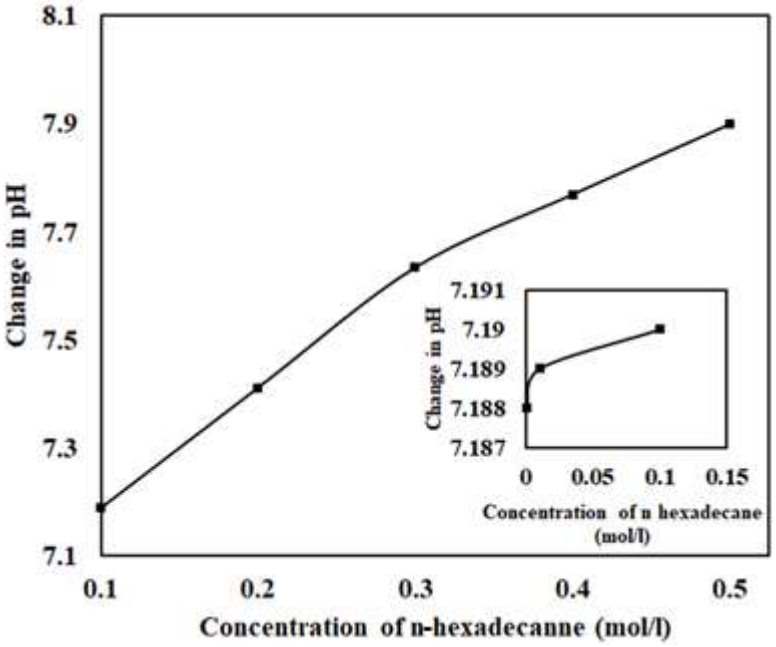

Fig. 4.11: Change in pH with respect to concentration of the n-hexadecane

4.4.8. Procedure for Measurement of n-Hexadecane

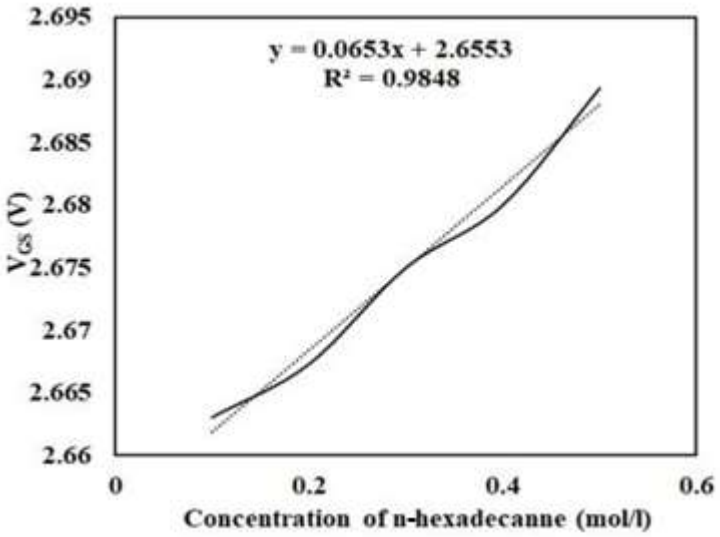

Fig. 4.12: Correlation and regression line for measurement of n-hexadecane

This can be done by considering a particular current say for example 0.5µA, the standard curve for determining the unknown substrate concentration can be obtained from Fig. 4.12, where correlation coefficient was 0.9848 and the regression equation was y = 0.0653x + 2.6553. Where, X-values assays the concentration of n-hexadecane depending on the VGS values in the Y axis.

4.5. Discussions

In the initial trials, when the isolated cytochrome P450 enzyme was used as redox bioreceptor, fabricated ISFET device showed a regular and predictable pattern of variation in potential difference for constant applied current corresponding to changed analyte concentration. The catalytic phenomenon of the analyte *n*-hexadecane being acted upon by the immobilized interface of redox component has been transduced successfully in the used ISFET setup to sense hydrocarbons. However, the device showed inconsistency in producing desired output beyond 3rd day of trial which may be due to limited stability of the protein inside immobilization matrix under given experimental condition. When the experiment was repeated at every 12 hours for three consecutive days , it was observed that the device showed consistent reproducibility only up to four cycles (48 hours) under room temperature and pressure, for a particular current 0.5µA, beyond which the sensor output decreases

significantly as illustrated in Fig. 4.13 This may be due to the degradation of the enzyme protein.

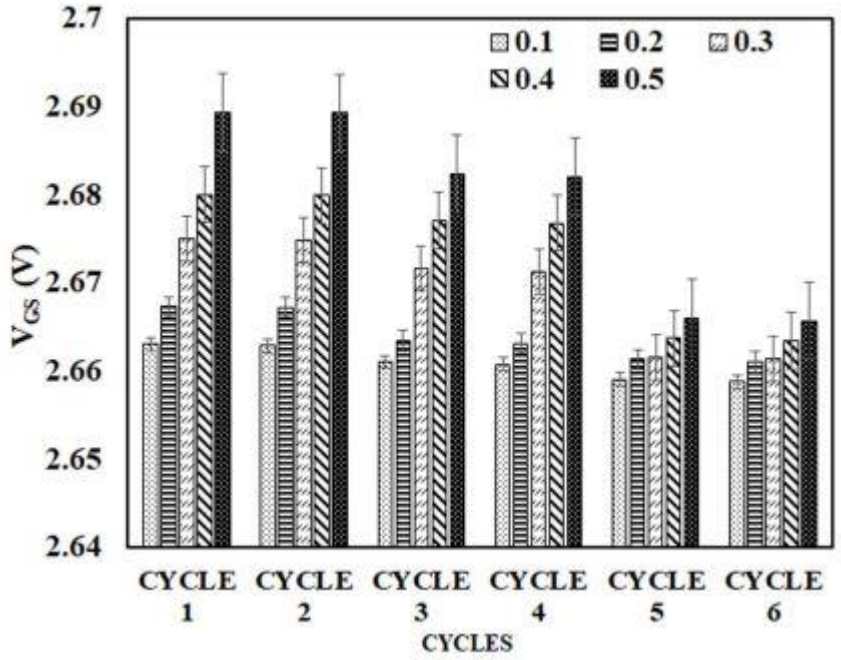

Fig.4.13. Reproducibility of sensor output for a particular current 0.5μA for 6 cycles (72 hours; 12 hours each) for 0.1 to 0.5 mol/l.

The sensing layer of SiO_2 requires a certain amount of hydration of the surface to make it pH sensitive. Nevertheless, the sensing silicon dioxide has been etched enough to form a very thin layer and improve the sensitivity of the device. Further, Schottky contact was formed by directly depositing metal on silicon which has high reverse leakage current. It is known that the reverse leakage current increases with temperature, so the fabricated device might have a thermal stability issue. Since ISFET are low powered devices, this factor can be overlooked. In this device, silicone was used for passivation of the casing. In our experiments of pH changes with respect to time, when a glass slab, coated with silicone is immersed in electrolytes of different pHs, very negligible change in pH was observed for

prolonged duration. The device in its present form with material limitation has shown a sensitivity of about 50.65 mV/pH and 54.34 mV/molar for bare ISFET and ENFET respectively.

4.6. Conclusions

1. The smaller band (band1) of electrophoresed cytochrome P450 protein failed to produce any machine output as bioreceptor in the fabricated ISFET, The larger band (band 2) showed considerable sensitivity which was maximum for the crude preparation.
2. The average sensitivity of the ENFET device was calculated to be 54.34 mV/molar.
3. The device was operated stably for 48 hours with 99% accuracy in reproducibility.
4. The average error in sensitivity due to hysteresis is calculated to be ±0.015%.
5. The detection limit of the sensor is found to be .01 molar.
6. From the transfer and output characteristics, the threshold voltage for ENFET is found to be 2.08 and 2.095 V for respective substrate concentration of 0.4 and 0.5 M.
7. For a current 0.5μA, the standard curve for determining unknown substrate concentration is y = 0.0653x + 2.6553 with correlation coefficient of 0.9848 (X-values assays the concentration of n-hexadecane depending on the Y-values)

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