Chapter V:

Improvement of biosensor stability and study of biosensor output

5.1. Introduction and objectives

The stability of the enzyme cytochrome P450 is one of the major problems faced by the researchers for its practical application [1]. The enzyme system shows poor in vitro stability because of its multicomponent nature either as integral protein or loosely bound to the plasma membrane. In the previous chapter it has also been reported that the fabricated sensor functioned stably only for two days. However, proper immobilization of the bio-catalyst using lyotropic support matrix may provide suitable environment to retain its native conformation and increase stability for continuous and long term use. The use of hydrophobic support matrix for immobilization may provide the isolated preparation of cytochrome P450 monooxygenase with a non-aqueous stationary phase close to the native cellular environment and thus can enhance the stability. The use of colloidal liquid aphrons (polyaphrons) for the entrapment of cytochrome P450 has already been reported to enhance its stability [2,3].

Polyaphrons are oil in water micro emulsions in which spherical droplets of solvent encapsulated within an aqueous shell, stabilised by an ionic and non-ionic surfactant mixture [4]. In general, the size of the polyaphrons ranges from 1-50 microns. Their size depends on the nature of the surfactant in the non-polar phase and the concentration of the surfactant (higher the concentration of the surfactant, smaller the size of the aphrons [5]. For the polyaphrons stability, the organic phase should be barely miscible or immiscible in water. In general, this is obtained by adding two

surfactants: a non-ionic surfactant in the organic phase and another in the aqueous phase, which may be either ionic or non-ionic [6].

For immobilisation of the enzyme the polyaphrons were prepared using bacterial rhamnolipid derived from *Achromobacter sp. TMB1 in* methanol as the polar phase and tween20 in olive oil as the non-polar phase. Rhamnolipids are a type of glycolipid that produced mainly by the *Pseudomonas aeruginosa* and were initially formed as exoproducts of the species and can act as a surfactant [7]. They consist of a rhamnose moiety and a lipid moiety linked via an O-glycosidic linkage [8,9]. They are capable of attracting both polar and non-polar molecules, allowing the mixing of two immiscible liquids such as oil and water. The rhamnose end of the molecule draws polar molecules including water whereas the fatty acid will attract non-polar solvents including fats. They can reduce surface and interfacial tension significantly which promotes foaming to ultimately form microemulsions [10].

These chapter reports, immobilization of a partially purified cytochrome P450 component isolated from extremophile *Bacillus stratosphericus Sp.* via entrapment in a novel polyaphrons matrix, prepared from bacterial rhamnolopid and olive oil and its subsequent use as biological receptor in an Enzyme Field Effect Transistor (ENFET) for detection n-hexadecane. The enzyme-polyaphron layer was immobilized over a Si_3N_4 gate for its response in presence of substrate n-hexadecane and co-factor nicotinamide adenine dinucleotide phosphate (NADPH). The principle of operation of

the reaction and the operating principle of the device is same as described in chapter 4. The variation of potential difference across gate to source with respect to concentration of the substrate n-hexadecane was recorded for the effects of pH, temperature, storage, hysteresis and concentration of the co-factor at constant currents. The device output showed a good repeatability with a sensitivity of 67 mV/molar and stability up to a week.

5.2. Objectives

1. Screening of materials for polyaphron synthesis.
2. Synthesis of stable polyaphrons
3. Immobilisation of CYP450 on the fabricated ISFET using polyaphrons.
4. Characterisation of the device for different parameters.

5.3. Materials and Methodologies

5.3.1. *Materials used*

5.3.1.1. 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, Polyethylene glycol (PEG) Oil of Olive (Himedia), glycerol and Agarose (Sigma), Calcium chloride dehydrate AR (Qualigen), Glycine, Lysozyme (SRL), N-hexadecane (Merck).

5.3.1.2. 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, Micropippette (Thermo scientific), Mini centrifuge (Tarsons Spinwin), pH meter, Plasma-enhanced chemical vapor deposition (PECVD) machine, Ultracentrifuge, Vortex (Tarsons Spinot).

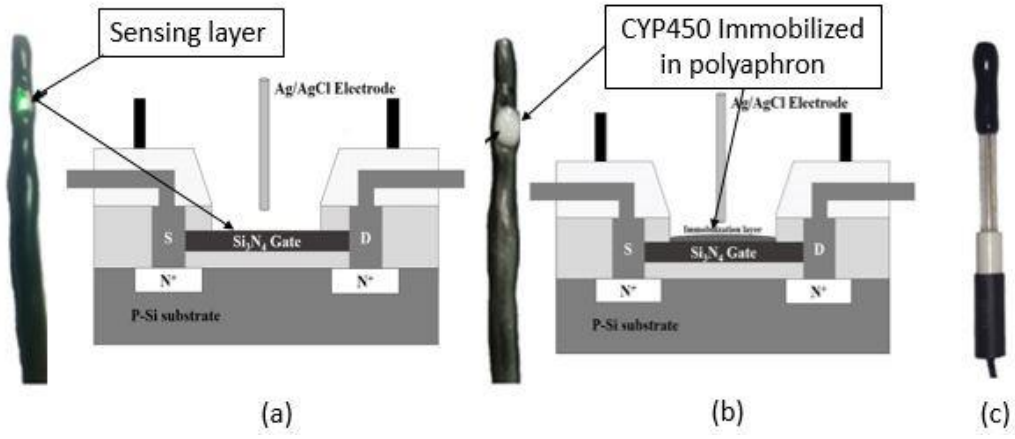


Fig. 5.1: Representation of the actual ISFET and ENFET along with its schematics. (a) WINSENCE ISFTE (WIPSK-S) with Silicon Nitride gate dielectric (b) Enzyme immobilised on the WINSENCE ISFET using polyaphron (c) Ag-AgCl reference electrode. (Purchased from Winsense Co., LTD, Bangkok, Thailand.)

5.3.1.3. The bioreceptor

Crude and partially purified enzyme Cytochrome P450 monooxygenase from the bacterial strain of *Bacillus stratosphericus.*

5.3.2.4. ISFET, Reference electrode and the circuit

A pH-sensitive Silicon nitride gate (Si_3N_4) ISFET (Fig 1. (a)) was used as the basic structure of the biosensor for immobilization. The reference electrode (Fig 1. (c)) used in the experiments is an Ag/AgCl electrode submerged in an electrolyte (KCl) enclosed in a plastic casing of length 4 cm and diameter 5mm. The end of the casing is fitted with a porous Alumina ceramic membrane with a drift less than 0.05mV/hr. The ISFET along with the reference electrode comprises three terminals, the Gate (G), Source (S) and drain terminals (D). The ISFET has a sensitivity of 50 mV pH^{-1}. The ISFET (WIPSK-S) and the Ag/AgCl reference electrode (WIPSK-RE) were purchased from Winsense Co., LTD, Bangkok, Thailand.

The measurement circuit used in this experiment for all measurements as well as the measurement set up necessary for that have already been described in chapter 4 [11]. Measurements were taken in two different modes, viz. constant current and constant voltage mode. A high accuracy quad operational amplifier (op-amp) "OPA4277" along with a voltage follower was used to maintain a constant voltage across drain to source. Another op-amp has been used for amplification (with a gain of 1600) of the low magnitude output voltage. A 10 KΩ potentiometer has been used

to maintain the required gate to source voltage. Also, the voltage applied to the reference electrode was kept constant using a voltage follower circuit using OP07. For measurements, the drain current was recorded for the corresponding variable drain to source voltage at different $V_{GS.}$

5.3.2. Methodologies

5.3.2.1 Preparation of polyaphrons and Enzyme immobilisation

500µl of bacterial rhamnolipid solution derived from *Achromobacter sp. TMB1* was mixed in 86% methanol (0.80 mg/ml) and agitated to form a gas foam. To the gas foam, 10 ml olive oil (non-polar phase) containing 4% Tween20 and 100ul of the crude enzyme (conc.: 558 µg/µl) were added progressively at certain intervals(the non-polar phase was added in volumes of 125µl, 250µl, 500µl, followed by 1 ml volumes for eight consecutive times). After each addition, the polyaphron phase was agitated to continue the foam preparation. 500µl of 10% PEG was added to the polyaphron to enhance its stability

5.4. **Results**

5.4.1. The polyaphron characterisation

The polyaphrons prepared from the aqueous phase (Rhamnolipid in methanol) and a non-polar phase (olive oil with 4% tween 20) were found to be stable for more than two months. The prepared polyaphrons were spread in a glass slide and observed under the microscope in different magnifications. They were found to have a central hydrophobic core (non-

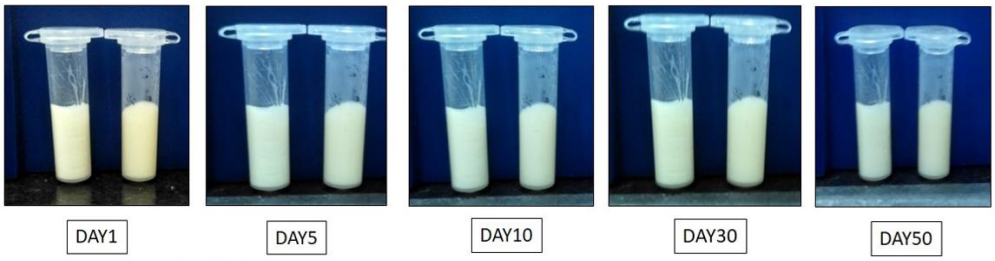
polar) that was surrounded by an aqueous double layer as described by Cao [4]

Spreading coefficient is a measure of the ability of a liquid to spread on the surface of another liquid or solid [5,6]. It is defined as the "difference between the work of adhesion between the two phases and the work of cohesion of the spreading liquid" [12,13]. For formation of stable polyaphrons, the surfactant-containing hydrogen-bonded liquid and the non-polar liquid are selected in such a manner that the spreading coefficient is more than or equal to 0, thus ensuring the spreading of non-polar liquid on the Hydrogen-bonded liquid to form the aphrons. A positive value is assigned corresponding to spreading and negative to non-spreading [5,6] The spreading coefficient values of the prepared polyaphron as presented in Table 5.1 were calculated from the surface tension values measured by a tensiometer (Ludwig Wilhelmy) using ring test method as per the Antonoff's rule [14] which was found to be 0. This confirmed that these two phases were efficient enpugh to allow formation of stable polyaphrons for enzyme immobilization.

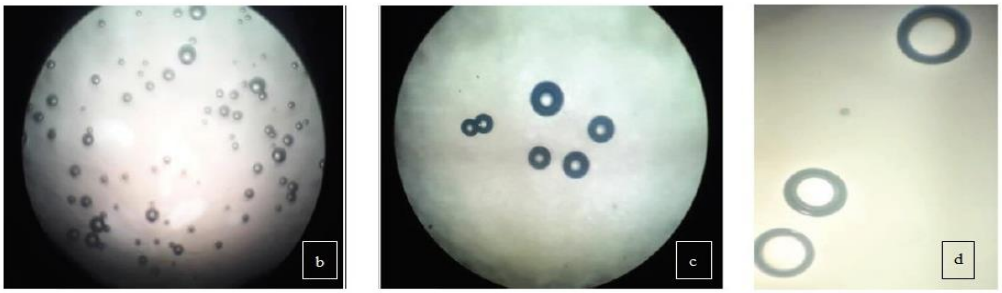
Further, the stability of the polyaphrons can also be determined employing the phase volume ratio (PVR). The total PVR can be up to 50 [5,15,16]. With the increase in PVR the viscosity increases and the stability of the polyaphron also increases. This is due to the decrease in the size of the aphrons [16]. The calculated phase volume ratio was found to be 20, confirming the higher stability of polyaphrons (table 5.1)

Table 5.1: Calculation of spreading coefficient based on surface tension values (in Dynes/cm) Calculation of phase volume ratio (PVR)

Calculation of Spreading Coefficient		
Surface Tension (Rhamnolipid in water) : γa	Surface Tension (Olive oil) : γb	Interfacial Surface Tension : γab
34.2	32	2.2
Spreading coefficient = γa-γb-γab= 0		
Calculation of phase volume ratio (PVR):		
V_{org} (ml)	V_{aq} (ml)	PVR= V_{org} / V_{aq}
.5	10	20



(a)



(b) (c) (d)

Fig. 5.2.a: Polyaphron shows stability for more than two months in room temperature. (b), (c), (d), Polyaphrons prepared were observed under the microscope under 4X and 10 X lenses and the typical double layer was observed.

5.4.2. Sensitivity

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:

$$S_{ENFET} = \left.\frac{\Delta V_{GS}}{\Delta C}\right|_{I_D = Constant} \qquad (2)$$

It can be easily illustrated form the plot (fig. 3.a) that the variation of V_{GS} increases along with the change in substrate concentration in a range of 0.1 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-

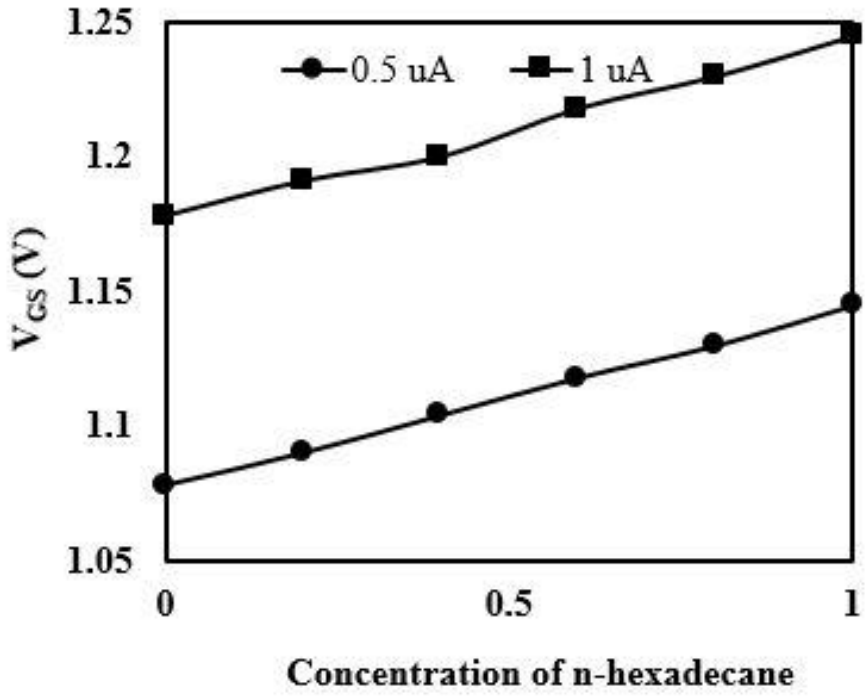
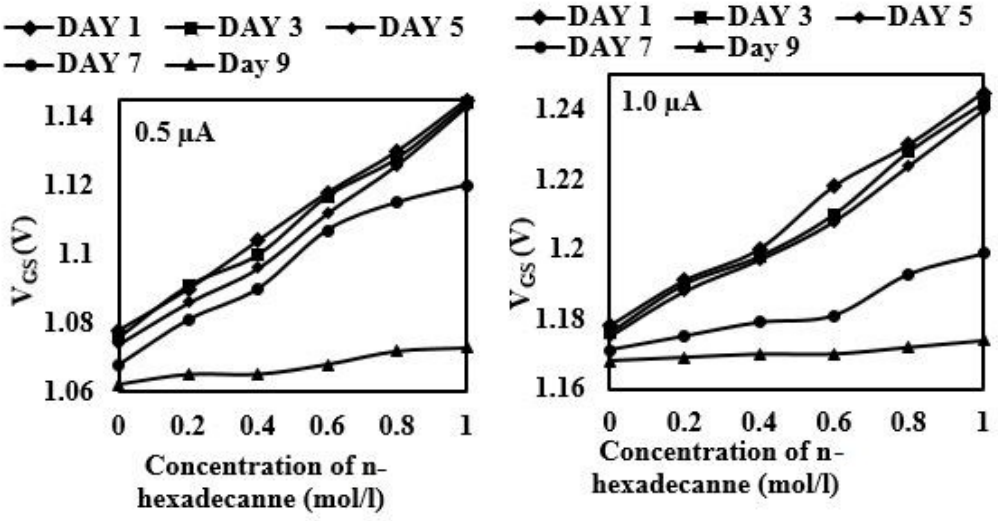


Fig. 5.3: Variation of V_{GS} with respect to change in concentration of n-hexadecane at constant currents of 0.5μA and 1μA.

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 67 mV/molar using equation 2.



(a) (b)

Fig. 5.4: Variation of V_{GS} with respect to concentration of n-hexadecane for constant current of 0.5 µA (fig a) 1 µA (fig b)

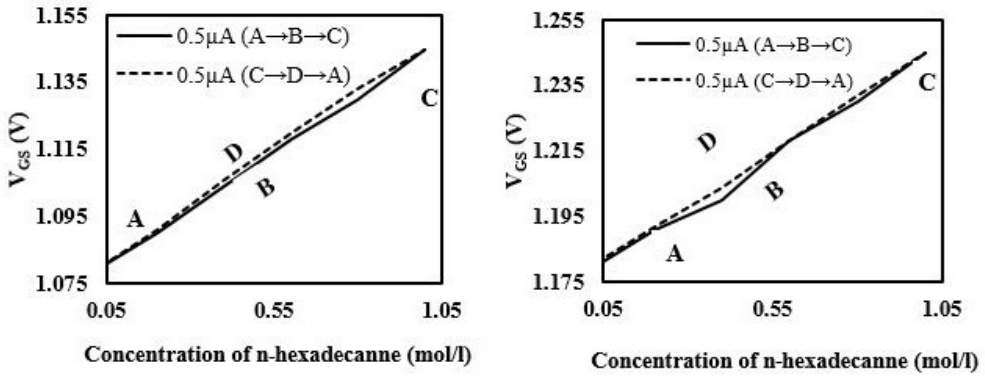
5.4.3. Reproducibility and stability of the sensor system

Reproducibility of a biosensor is the closeness of agreement while taking the measurements. For checking the reproducibility of the device, the measurements were taken at room temperature (27◦C) in every 12 hours for ten successive days. While for storage the ENFET was kept at 4 ◦C. The device reported here possess a high reproducibility of with 99% accuracy for seven days. It is evident from plot fig.4 that the variation in the sensitivity for the first week was negligible. Beyond the seven days, the stability gradually decreases and eventually after the 10th day it drastically

changes to nil. The gradual loss of stability of the device may be attributable to the unstable nature of the enzyme outside the live microorganism or the cellular environment.

5.4.4. Hysteresis

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 a and b. 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%.



(a) (b)

Fig. 5.5: Hysteresis observed in the ENFET device for current .5 (fig. a) and 1 μA (fig. b).

5.4.5. Variation of the device V_{GS} with respect to the change in pH

The voltage across gate to source (V_{GS}) was checked for a pH range from 6.2-9.2 changing the pH of the buffer solutions. It has been observed that

there is an increase in the V_{GS} with the increase in pH up to pH 8.2. But at pH 9.2 there was not any significant change in V_{GS} for change in concentrations implicating the inability of the device to work in the alkali conditions.

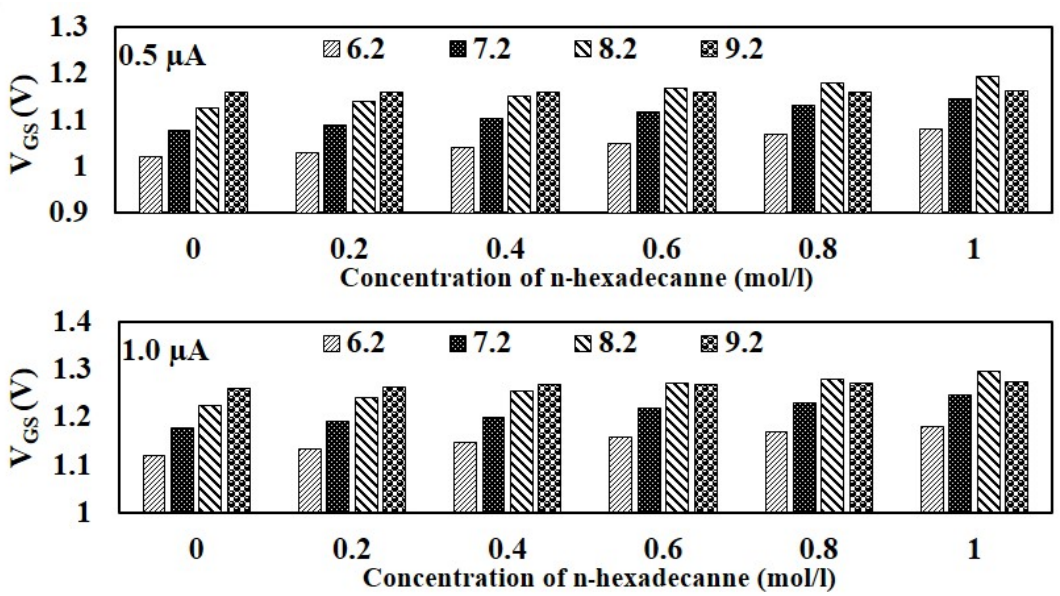


Fig. 5.6: (a) Variation of V_{GS} with respect to concentration of n-hexadecane measured for constant current of 0.5 µA (fig a) 1 µA (fig b) showing its change in different pH.

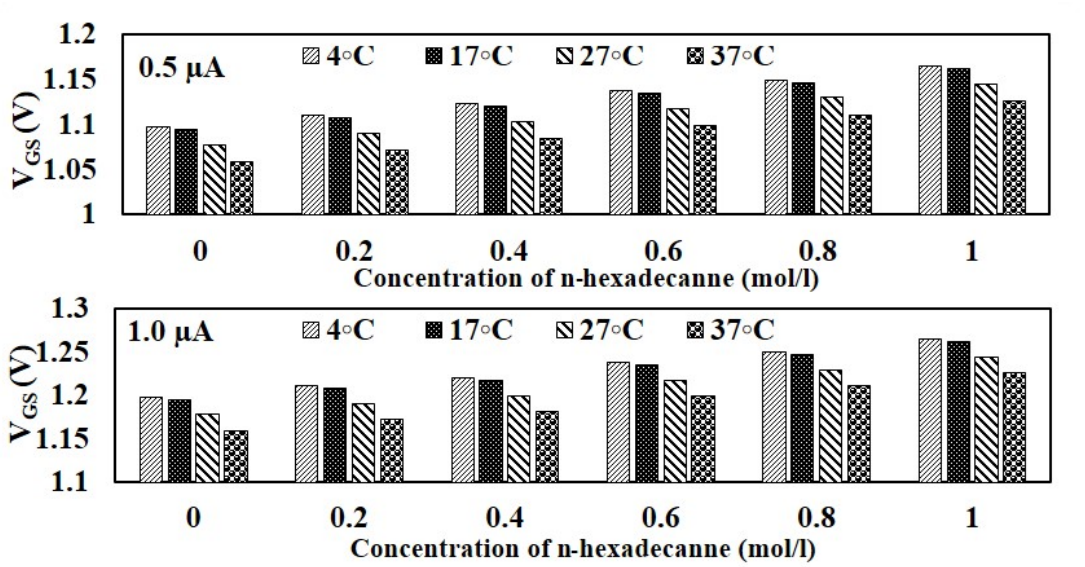


Fig. 5.7: (a) Variation of V_{GS} with respect to concentration of n-hexadecane measured for constant current of 0.5 µA (fig a) 1 µA (fig b) showing its change in different temperature

5.4.6. Variation of the device V_{GS} with respect to the change in Temperature

The voltage across the gate to source (V_{GS}) was measured for a temperature range from 4◦C to 37◦C. It has been observed that with the increase in temperature there was a decrease in output V_{GS}. The reason behind could be associated with possible presence of some metal impurities on the ISFET sensing layer.

5.4.7. Variation of the V_{GS} with respect to the change in time.

The reaction time for the ISFET biosensor was calculated by measuring the change in V_{GS} with the change in time in every 30 seconds up to 600 seconds. It has been observed that it takes about 180-200 seconds for completion of the reaction.

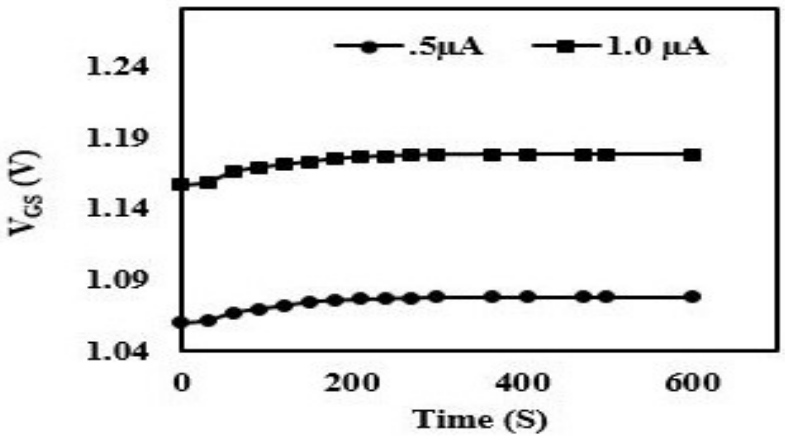


Fig. 5.8: Variation of V_{GS} with respect to time (S) measured for constant current of 0.5 and 1 µA

5.4.8. Effect of NADPH

In the hydroxylation reaction from RH to ROH (reaction 1) the electrons as hydride ion flow from NADPH to facilitate the reduction of the enzyme-

-substrate complex and eventually forms ROH and H_2O molecule. From the fig. 9 it can be very easily illustrated that without the NADPH the reaction does not take place. Further, this also shows the variation in outputs for different concentrations of the NADPH, and it was found that at a concentration of 1 µM/ml it gives optimum sensitivity, beyond which the NADPH didn't have any impact on the sensitivity implying the saturation at the particular concentration.

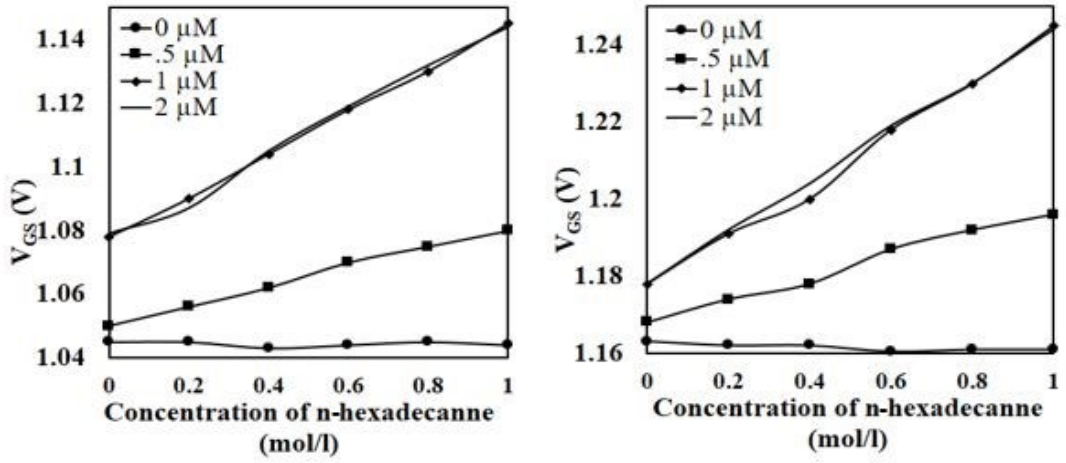


Fig. 5.9: Variation of V_{GS} with respect to concentration of n-hexadecane measured for constant current of 0.5 and 1 µA at different concentrations of NADPH.

5.4.9. 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. 3, where correlation coefficient was 0.9992 and, the regression equation was y = 0.067x + 1.0773 (X-values assays the concentration of n-hexadecane depending on the Y-values)

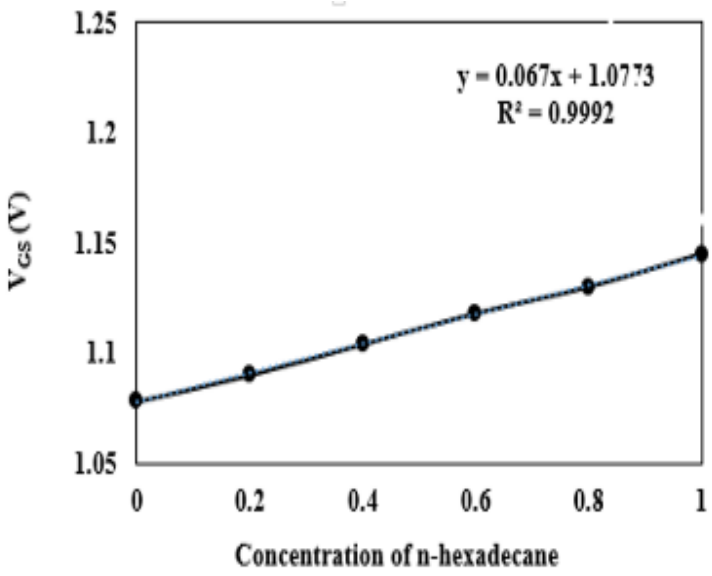


Fig 5.10: Standard curve for determining the unknown n-hexadecane concentration for a particular current 0.5 μA.

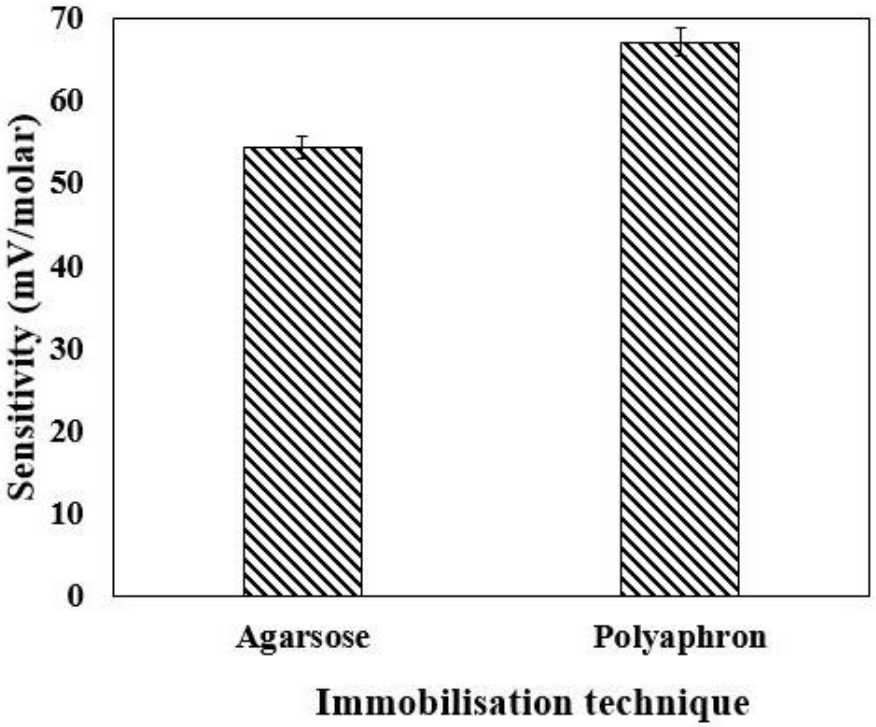


Fig 5.11: Comparison of sensitivity of the agarose based and polyaphron based immoblisation

5.5. Discussions

Studies carried out on the immobilization of the purified enzyme preparation revealed that the use of polyaphron could be considered for

using as suitable supprt matrix for better and stable functioning of the protein. Since the enzyme is membrane bound hence, it is relatively nonpolar and would be attracted to oil in water interphase. Enzyme immobilization using this macroemulsion technique has been attempted earlier, and 85% efficiency was reported [15,17]. This study reports a highly stable novel polyaphron matrix, mimicking somewhat the native cellular environment for a functional cytochrome P450 monooxygenase which can be used for various purposes apart from its use as recgnition element in biosensors, for example biotrnaformation, bioremediation etc.

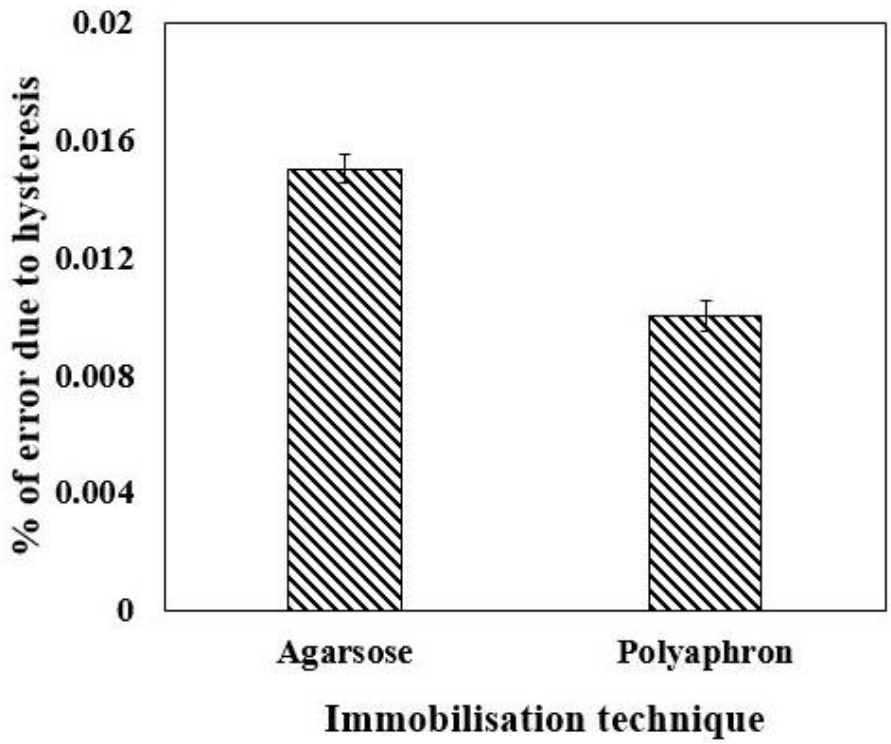


Fig 5.12: Comparison of hysteresis of the agarose based and polyaphron based immobilisation

In the previous chapter (chapter 4) the reported sensitivity was 54.34 mV/molar, which has been improved here by about 23% to 67 mV/molar (Fig.: 5.11). Similarly, the stability of the device has been improved by 250%. Further the hysteresis has also been lowered by about 34%. This may be because enzymes such as cytochrome P450 monooxygenases are usually (partially) membrane bound and need hydrophobic anchoring and interaction for maitaining stability in isolated form. The hydrophobic environment used for the immobilization of cytochrome P450 monooxygenase may provide a non-aqueous mimic environment of in-vivo counterpart to enhance the stability of the enzyme considerabily to be used for biosensor and other similar applications.

5.6. Conclusions

1. Polyaphrons produced have stability of more than two months in room temperature and pressure (NTP) with a spreading coefficient of 0 and PVR value of 20.
2. The average sensitivity of the ENFET device was calculated to be 67 mV/molar, with 35% corresponding increase in sensitivity.
3. The device stability has been extended to two and half times (250%).
4. The reproducibility parameters showed 99% accuracy of the device in daily operations for a period of seven days.
5. The average error in sensitivity due to hysteresis was calculated to be ±0.01%.

6. With the increase in temperature there was a decrease in output V_{GS}.

7. With the increase in pH the there is an increase in V_{GS} up to pH 8.2. However, at pH 9.2 there was not any significant change in V_{GS} for change in concentrations.

8. The response time was found to be 180-200 seconds.

9. Without NADPH there is no reaction, and hence there is no change in output V_{GS}. The variation of output with respect to different concentrations of the NADPH showed that 1 μm/ml was optimum for sensitivity.

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