Hydrogen Peroxide (H$_2$O$_2$) Biosensor Based on the Conducting Polymer Using Self- Assembly Technique

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Abstract: Biosensors are in principle fabricated by immobilized biomaterials on a detector membrane and combining them with electrochemical equipment. The applications of enzyme-based biosensors can be explored such as in the process of gas detection, medicine, pathogen detection and detection of toxic levels of substances before and after bioremediation. In this study, H$_2$O$_2$ detection was performed using HRP/PANI, HRP/PPY, HRP/PT and HRP/PT/PPY/PANI layers. The HRP/PANI layer from Variable Pressure Scanning Electron Microscopy (VPSEM) image exhibited a dry surface. The HRP/PPY layer exhibited a surface with agglomerate molecules. The HRP/PT layer, on the hand, exhibited a layer surface with almost the same molecular size. This is confirmed by the higher surface roughness value for HRP/PPY compared to other layers obtained via characterization with Atomic Force Microscopy (AFM). The increasing current response for all three layers was arranged in HRP/PANI> HRP/PT> HRP/PPY. VPSEM and AFM images exhibited surfaces with molecules being in an agglomeration state after the H$_2$O$_2$ detection process. In terms of current response, the response rate of H$_2$O$_2$ on the surface of the HRP/PT/PPY/PANI electrode caused the current response obtained to be fast. The roughness value increased with time due to the reaction that took place between the surface of the HRP/PT/PPY/PANI layer with H$_2$O$_2$. The day-based current response showed that day 1 to day 14 exhibited a uniform graph pattern but from day 21 to day 30 there was a change in the graph pattern due to the HRP/PT/PPY/PANI layer undergoing degradation. The activity of the HRP enzyme was studied by looking at its absorption effect for 30 days. From day 1 to day 14, there was a difference in the overall rate of absorption. However, from day 21 to day 30, the rate of absorption remained constant which explains the slowing down of HRP activity.

Keywords: Hydrogen peroxide, biosensor, conducting polymer, self-assembly, polyaniline

1. Introduction

Interests in research and developments of biosensors have surged in recent years because of their benefits such as the generation of rapid, scalable and portable responses. Cosnier and Gondran reported that the design concept of a biosensor is the specific combination of biological systems, the physical sensitivity of the transducer and the processing power of microcomputers [1]. The polymer matrix can be used either in the detector mechanism or in the stabilization process to detect the material. In general, surface modification with polymers depends on several factors such as identification of functional groups, cross-linking on nearby functional groups, degradation of polymer molecules and absorption on the surface. These factors affect only a few layers of the molecule (up to 100 nm) and produce a surface charge suitable for the storage process.

Foulds and Lowe explained that the unique characteristics of conduction polymers with their ability to trap enzymes via electropolymerization method have been used for the fabrication of biosensors [2]. Various conduction polymers including polypyrrole (PPY), polyaniline (PANI), polyethylene (PE), polyindole (Pln), polythiophene (PT) are used because they have the ability to transfer electrical charge. The conduction properties of polymers offer good electrocatalytic properties and fast electron transfer ability. Enzyme trapped in the conduction polymer causes the active site of the enzyme to be close to the back chain group of the conduction polymer. Nigel explained that Horseradish Peroxidase (HRP) is widely used in molecular biology applications because of its ability to enhance weak signals and improve the detection of target molecules [3]. HRP produces color which allows itself to be detected and quantified. HRP is typically used as a conjugate (a genetically or chemically reacted molecule) to study the presence of target molecules. HRP is said to be superior because it is used in many fields and has its own benefits such as its small size, good stability and low cost. Moreover, it has a good signal acquisition rate that allows the generation of strong signals in a short period of time.

Pandey et al. explained that conduction polymers have a booster state resulting from the addition of doping ions at the molecular level [4]. It can be driven between the conductor and insulator levels via an electrochemical reversing process in a solid polymer electrolyte solution. PPY is used in industrial applications due to its shrinkage strength [5], tensile rate, ease of synthesis, good reaction time [6] and so on. Mello et al. explained that the degree of deposition affects the surface capacity of a monolayer when the positive contribution of the double layer increases with increasing protonation of the polymer molecule [7]. The stability of the monolayer and its transfer can be improved when subphases with low pH are used. The properties of the monolayer depend on the type of acid used in the subphase. The surface energy of doped PANI is different when compared to non-doped polymers. The degree of deposition then affects the surface capacity and is expected to increase with increasing acidic subphase.

The Langmuir-Blodgett (LB) technique is used to control the molecular orientation and material structure [8]. Through this technique, organic matter is fabricated into an assembled molecular form where the film thickness is controlled at the molecular level. When amphiphilic molecules are dissolved in a solvent, the solution is allowed to evaporate. Then it is spread onto the water-air surface to form a monolayer. By applying pressure to the surface of a monolayer, a solid monolayer can be obtained when the surface pressure point has increased. The layer by layer (LBL) method is also used to produce a sensitive and stable glucose biosensor by stagnation of glucose oxide on a glass indium oxide (ITO) substrate. Marystela et al. explained that the biosensor was stable for 20 days and exhibited a constant amperometric response [9]. Biosensors that combine enzyme and transducer components have been applied in chemistry and biology due to their high sensitivity and selectivity [10], their low cost and their scalable size. This research looks into the effect of HRP/PT/PPY/PANI deposited on the ITO glass for fabrication of H_2O_2 biosensor. The layer will be characterized using UV-Visible Spectroscopy (UV-Vis.), Impedance analyzer, Variable Pressure Scanning Electron Microscopy (VPSEM) and Atomic Force Microscopy (AFM).

2. Materials

PANI emeraldine base (EB) (Mw: 10,000), PPY (Conductivity: 10- 40 Scm^{-1}), PT (kept in 2-4 °C in freeze), HRP and *p*-toluene sulfonic acid (PTSA) was bought from Sigma-Aldrich. Methanol (Merck) act as solvent for PANI, PPY and PT for dilution process. Hydrogen peroxide (H_2O_2) from R & M chemical used in biosensor process. While PTSA will used for doping process of PANI, PPY and PT. Silver conductive paint (Electrolube) with surface resistivity 0.03 ohms/sq will used together with Indium Tin Oxide glass. The phosphate buffer is prepared from Disodium Hydrogen ortophosphate salt (Na_2HPO_4) and Potassium Dihyrogen Phosphate (KH_2PO_4).

3. Methodology

3.1 Formation of PANI Monolayer Using LB Technique

PANI was dissolved in 0.1 M methanol and put in ultrasonic bath for 1 hour and then filtered. The resultant solution was injected on the subphase surface (pH 1) that contained the mixing solution of PTSA in LB trough. The barrier was moved in and out to control the surface pressure. Next, the ITO glass (1.5cm x 1.0cm) was dipped in the subphase solution to deposit the PANI monolayer.

3.2 LBL Process of PPY and PT onto PANI

PPY and PT were dissolved in 0.1M methanol. Both solutions were kept in ultrasonic bath for 1 hour and then filtered. PTSA was added to both solutions. The previously deposited PANI monolayer was dipped into the PPY solution using a dipper probe at a speed of 10 mms^{-1}. After the PPY/PANI was dried, the layer was dipped in the PT solution.

3.3 Electrodeposition of HRP on PT/PPY/PANI Using Electrodeposition Technique

Before the electrodeposition process, 0.1 mM HRP was injected into 0.1M phosphate buffer. The current used was 750A with 15V and the process was run for 5 minutes.

3.4 H_2O_2 Biosensor

0.1M H_2O_2 was injected in 0.1M phosphate solution before the potentiostatic experiment. The sensor process was done for 30 seconds. Three electrodes were used in this system: the auxiliary electrode (Ag/ AgCl), the working electrode (HRP/ PT/ PPY/ PANI) and the reference electrode (Platinum).

4.   Results and Discussion
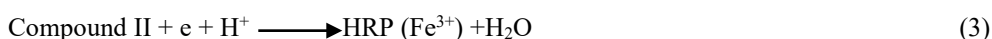
4.1 H_2O_2 Concentration

The H_2O_2 concentration was varied to study the suitability of the concentration to be used in the sensor process. Concentration variations ranged from 0.1 M up to 1.0 M. The UV-Vis. spectrum in figure 1 shows that at a concentration of 0.1 M, the absorption reading is high. Absorption is related to the species that performs the absorption. High H_2O_2 concentrations cause an increase in molecular size. At a certain time, the molecules will agglomerate together and become larger [11]. Compared to systems with low concentrations, when molecular agglomeration is slower, the resulting molecules become smaller. The relaxation and deformation of the chain resulting from the addition of electrons or gaps to the chain causes a variety of particles, solitons, polarons and bipolarons with distinctive carrier properties to be produced. When electrons are added to a chain, they cause a change in the shape of the chain. Molecular shape change is described as a tendency to change from a single bond to a double bond. The change in the shape of the chain causes a change in the structure of the energy level [12].
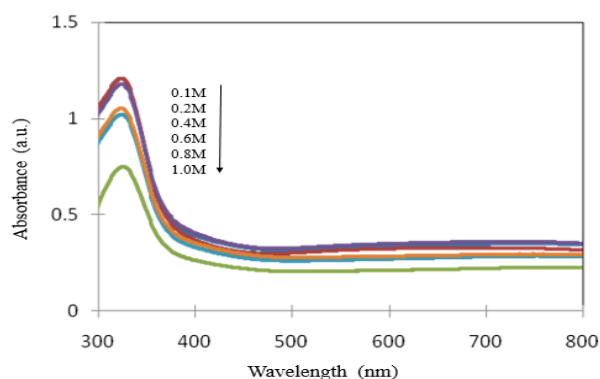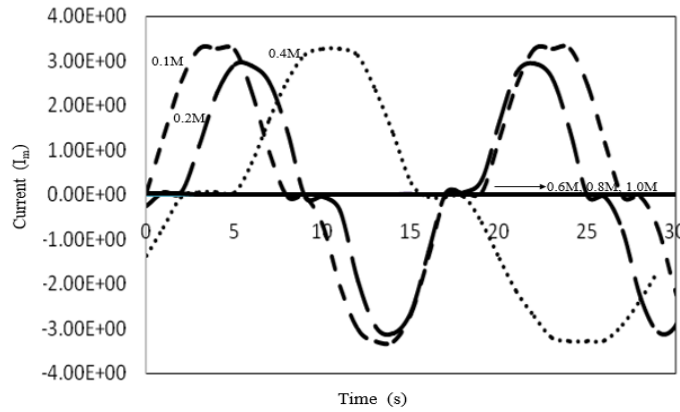

Fig. 1 - UV-Vis. Spectrum of H_2O_2 with different concentration of H_2O_2 (a) 0.1 M; (b) 0.2 M; (c) 0.4 M; (d) 0.6 M; (e) 0.8 M; (f) 1.0 M

Figure 2 shows the fluctuating current due to the electron transfer process. The immobilization of HRP on polymers provides a favorable micro-environment for maintaining HRP bioactivity and aids electron transfer between protein redox and electrode surfaces [13]. When 0.1M of H_2O_2 solution was used, the current response shown was fast which explained why the H_2O_2 molecules performed the response process on and in the electrode surface easily. The current response was detected as early as 0.01 seconds when compared to other H_2O_2 concentrations which produced a slower current response within 2.03 or 5.02 seconds. The lines for the current responses of 0.6M, 0.8M and 1.0M H_2O_2 solutions are horizontal on the x-axis. This occurred because the larger the concentration value that is used, the slower the response rate [14]. Thus, the concentration value indirectly affects the current, and more time is required to allow the response process to take place.

The mechanism of H_2O_2 biosensors occurs based on the following reactions:

$$HRP\ (Fe^{3+}) + H_2O_2 \longrightarrow Compound\ I\ (Fe^{4+} = 0) + H_2O_2 \qquad (1)$$

$$Compound\ I\ (Fe^{4+} = 0) + e + H^+ \longrightarrow Compound\ II \qquad (2)$$

$$Compound\ II + e + H^+ \longrightarrow HRP\ (Fe^{3+}) + H_2O \qquad (3)$$

Fig. 2 - The current rate obtained when the different molarity of H_2O_2 injected into the buffer solution

The H_2O_2 rate of response on the electrode surface caused a process of H_2O_2 reduction in the presence of HRP in the reaction. Compound I was produced as a result of the reduction process by HRP, where Fe^{3+} and Fe^{4+} represented forms of oxidation and reduction by HRP. The oxidation and reduction processes caused a decrease in current that was directly proportional to the concentration of H_2O_2 in the buffer solution [15].

4.2 Surface Layer of HRP/PANI, HRP/PPY and HRP/PT Due to H_2O_2 Detection Process

Figure 3 shows the response of H_2O_2 current to the three types of electrodes HRP/PANI, HRP/PPY and HRP/PT. It was found that the current response given by the HRP/PANI electrode was faster by giving a response within 6.01 seconds. This was due to the HRP/PANI surface which further promoted the electron exchange rate on the HRP/PANI surface. The initial rate of current response indicated by the HRP/PPY electrode was slow with its current detection time starting at 22.05 seconds. This phenomenon can be explained by the molecules on the surface of the HRP/PPY electrode that agglomerated and in turn inhibited and slowed down the electron exchange process on the surface. Thuc et al. reported that agglomeration formed on the surface of the layer has low conductivity and leads to low current flow as well as slow electron movement [16]. This surface also causes the response time obtained to be long because the penetration rate of the target molecule on the surface of the conduction polymer is slow [17]. Meanwhile, the HRP/PT electrode showed a slightly slower current response compared to the HRP/PANI electrode at 13.01 seconds. Virgi et al. reported that the current flow rate is related to the surface and molecular size to further promote the electron exchange process on the surface [18]. It is explained here that the H_2O_2 current response rate for the three electrodes was expressed as HRP/PANI> HRP/PT> HRP/PPY.
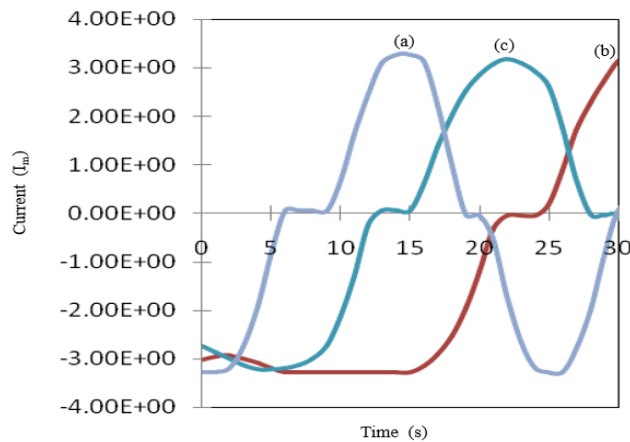
Fig. 3 - H_2O_2 current response to electrode (a) HRP/PANI; (b) HRP/PPY; (c) HRP/PT

Figure 4 shows the VPSEM images for HRP/PANI, HRP/PPY and HRP/PT when the detection process for H_2O_2 was performed. For Figure 4 (a) the surface of the HRP/PANI layer was flat but underwent flake-shaped exfoliation. Exfoliation occurs due to the process of degradation of the deposition process. Factors influencing degradation include moisture, light and more [19]. In this case, the probability of degradation was due to dampness. This was because the HRP/PANI electrodes were stored in the refrigerator at 4°C when not in use. Conduction layer degradation involves the

process of either disrupting conjugation patterns that limit the length of the conduction chain or altering the molecular dopping effect[13]. Figure 4 (b) shows the larger molecular size structure of HRP/PPY. This was because the process of detection of H_2O_2 on the HRP/PPY layer has changed the redox properties of the layer. The reduction and oxidation processes of the HRP/PPY layer caused disturbances in the charge between the surface of the layer and the H_2O_2. The changes that took place in the structure of these molecules affected the electroactive properties and even the conductivity of the polymer. This is particularly important because the redox stage is involved in most applications of PPY technology [20]. On the other hand, Figure 4 (c) shows a more uniform and flatter image with the size of the molecules formed being almost the same. The detection of H_2O_2 on the surface of HRP/PT has transformed the agglomeration of HRP/PT particles into smaller particles. This occurred because the reaction between H_2O_2 and the HRP/PT layer has changed the density of HRP/PT chain bond to elongate. Chain elongation allows more reactions to take place with the production of cations and anions that act as charge carriers to produce electrical conductivity [21].
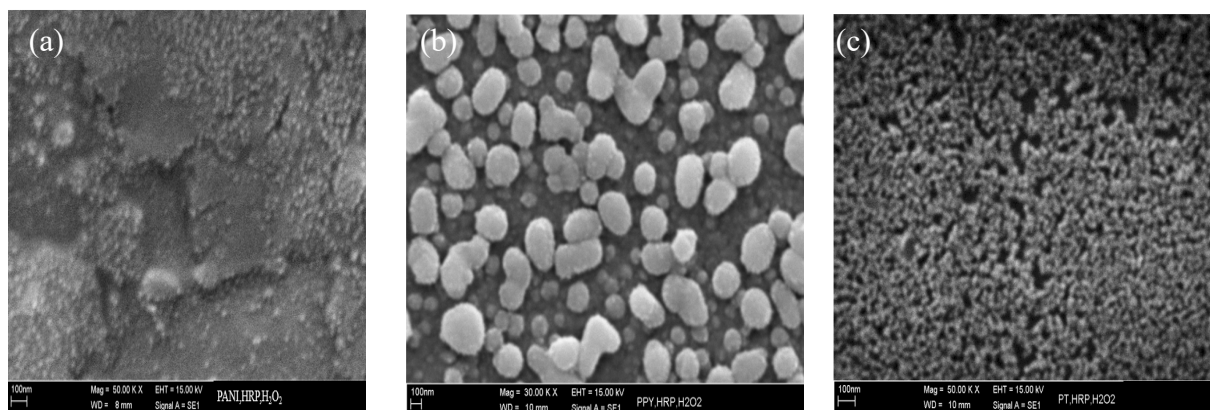

Fig. 4 - VPSEM image (a) H_2O_2/HRP/PANI; (b) H_2O_2/HRP/PPY; (c) H_2O_2/HRP/PT

Figure 5 shows the AFM images for HRP/PANI, HRP/PPY and HRP/PT when the detection process for H_2O_2 was performed. The film roughness values obtained support the film surface conditions described by VPSEM. Figure 5(a) shows a flat HRP/PANI surface with average surface roughness, R_a at 6.93 nm. Figure 5(b) exhibits an uneven and bumpy HRP/PPY surface due to the molecular agglomeration process [22] that occurred with an average surface roughness value, R_a obtained was 7.41 nm. Figure 5(c) shows a flat surface of PT/HRP and a molecular size that is almost uniform with the average surface roughness, R_a obtained was 6.80 nm. The average surface roughness, R_a for the three surfaces is shown in Table 1.
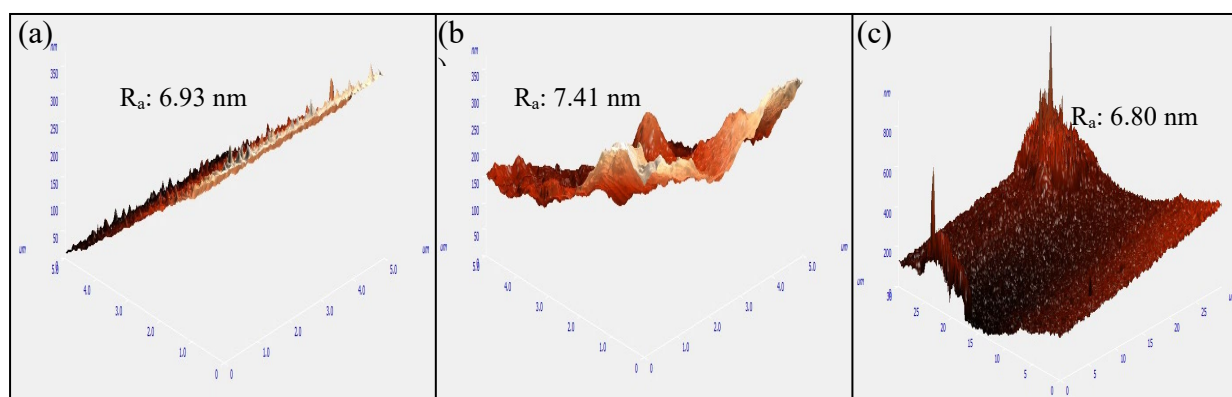

Fig. 5 - Image AFM of (a) H_2O_2/HRP/PANI; (b) H_2O_2/HRP/PPY; (c) H_2O_2/HRP/PT

Table 1 - The average surface roughness value of the film, R_a

| Film	R_a
H_2O_2/HRP/PANI	6.93 nm
H_2O_2/HRP/PPY	7.41 nm
H_2O_2/HRP/PT	6.80 nm

4.3 Surface of HRP/PT/PPY/PANI layer Due to H_2O_2 Detection Process

Figure 6(b) shows the VPSEM image of HRP/PT/PPY/PANI after the detection process for H_2O_2 was carried out. The resulting surface after the H_2O_2 detection process was almost the same as before (Fig. 6(a)). The surface has a uniform molecular size. This condition proved that the detection process that took place did not have an extreme effect on the surface of the HRP/PT/PPY/PANI electrode and the current response generated was also stable. The correct orientation of the protein on the electrode surface is very important because an electrostatic reaction exists and takes place in that process. The high enzyme content will interfere with the capacity of electrons reaching the film. The polymer/enzyme concentration needs to be maintained at a high rate to allow all enzyme molecules to carry the same current rate in electron transmission [19].
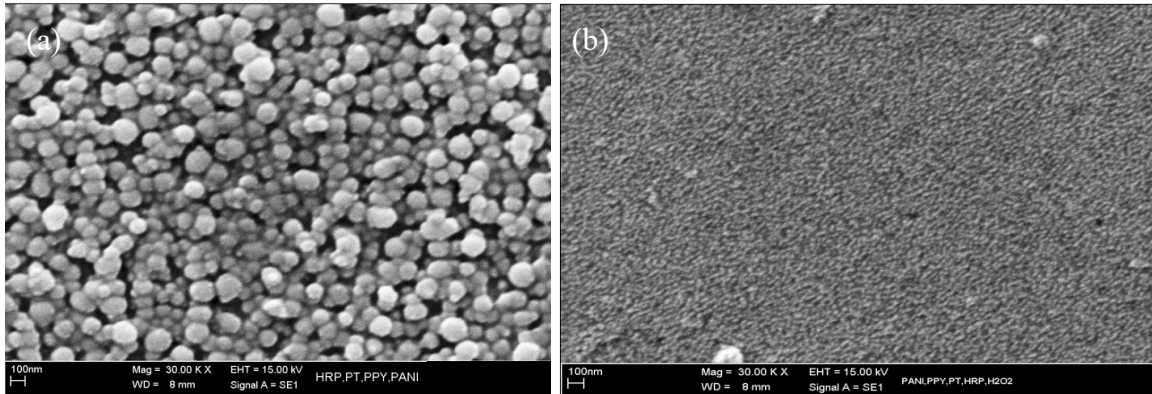
**Fig. 6 - Different image of VPSEM (a) HRP/PT/PPY/PANI; (b) H_2O_2/ HRP/PT/PPY/PANI**

Figure 7 shows the resulting AFM image when HRP/PT/PPY/PANI was tested with H_2O_2. The mixing process by H_2O_2 caused HRP to react and indirectly reduced the average surface roughness, R_a. The average surface roughness obtained was 7.670 nm. There was a reduction in the roughness value compared to before the H_2O_2 detection process. This occurred due to the surface of the HRP/PT/PPY/PANI electrode layer undergoing degradation (peeling) [19].
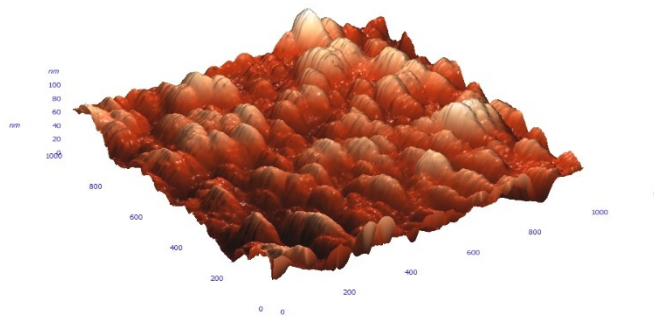
Fig. 7 - Image of AFM H_2O_2/ HRP / PT/ PPY/ PANI with average surface roughness, R_a: 7.670nm

4.4 Current Response of HRP/PT/PPY/PANI Electrode By-Day

A study of the number of days was also performed to see the stability and suitability of HRP/PT/PPY/PANI electrodes to H_2O_2 (Figure 8). Findings from day 1 to day 14 exhibited a similar pattern due to the uniform response rate and absence of obstruction on the electrode surface that could impede the process of electron entry and exit. From the day 21 to the 30, there was a change in the pattern due to the effect of the change on the non-uniform electrode surface (degradation). This indirectly limited the transfer of H_2O_2 electrons directly to the electrode surface. Thus, the time taken by an electron to complete its cycle is longer and it can be seen at the peak width obtained [23].
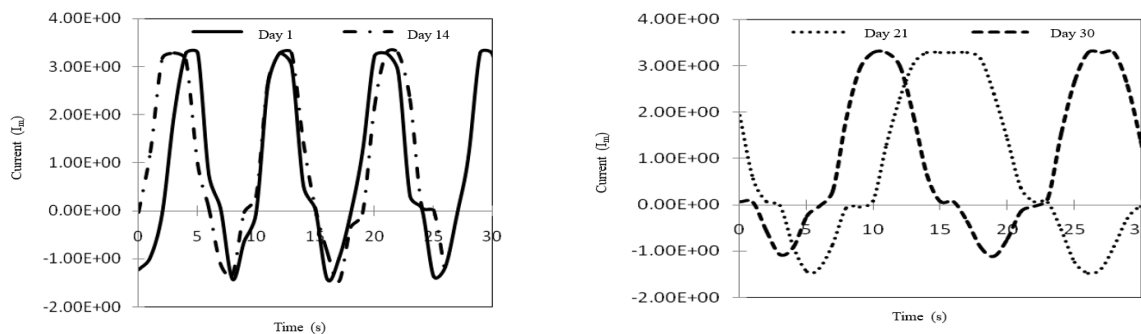
Fig. 8 - H₂O₂ current response to HRP/PT/PPY/PANI electrodes on the different day

Figure 9 shows the AFM image for the surface of the HRP/PT/PPY/PANI layer after a 30 day H_2O_2 detection process taken on days 1, 14, 21 and 30. The surface roughness increased due to the presence of agglomeration as a result of the reaction on the polymer chain which began to dissolve. Large white spots on day 30 (Fig. 9(d)) indicated the presence of blisters. There are many external factors that cause degradation of polymeric materials such as heat, light, mechanical stress, oxygen, ozone, humidity, atmospheric pollution, processing time, presence of reactive sites and so on [13]. Roughness values based on 1, 14, 21 and 30 days are shown in Table 2. Increasing surface roughness will affect the response of the detected H_2O_2 current.
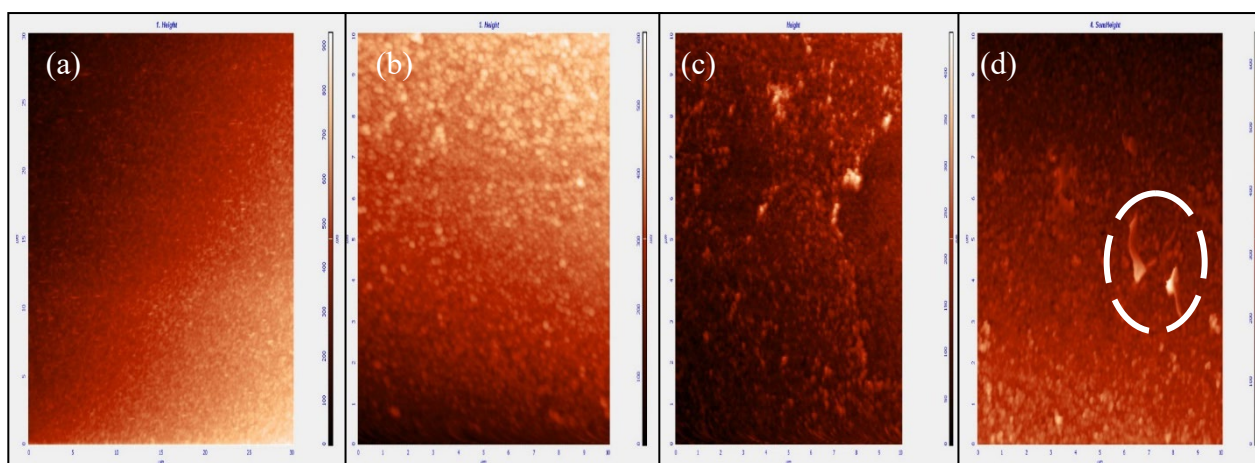
Fig. 9 - AFM film images of HRP/PT/PPY/PANI on the day (a) 1; (b) 14; (c) 21; (d) 30

Table 2 - Surface roughness value, R_a for H₂O₂/HRP/PT/PPY/PANI

Day	R_a
1	7.67 nm
14	8.87 nm
21	11.76 nm
30	13.01 nm

4.5 HRP Enzyme Activity

The HRP/PT/PPY/PANI electrodes were studied to see the effect of activity based on the peaks obtained up to the 30 days (Figure 10). Enzyme activity is related to the quantity of active enzyme present [24]. It was found that absorption increased with an increase in the number of days. The activity of HRP enzyme was studied by looking at its absorption effect based from day 1 until day 30. It was found that the absorption rate increased with time. As explained previously, the rate of absorption is related to the species that performs the absorption. From day 1 to day 14, there was a difference in the rate of absorption. Whereas from day 21 to day 30, there was a similarity in the rate of absorption which explained the nature of the slowing HRP activity. This condition occurred due to an increase in system charge. This indirectly affected the structure and surface obtained. With increasing charge, the combination of polymer structure and HRP will become a dense structure by producing a high repulsive force to expand the spiral structure [20].
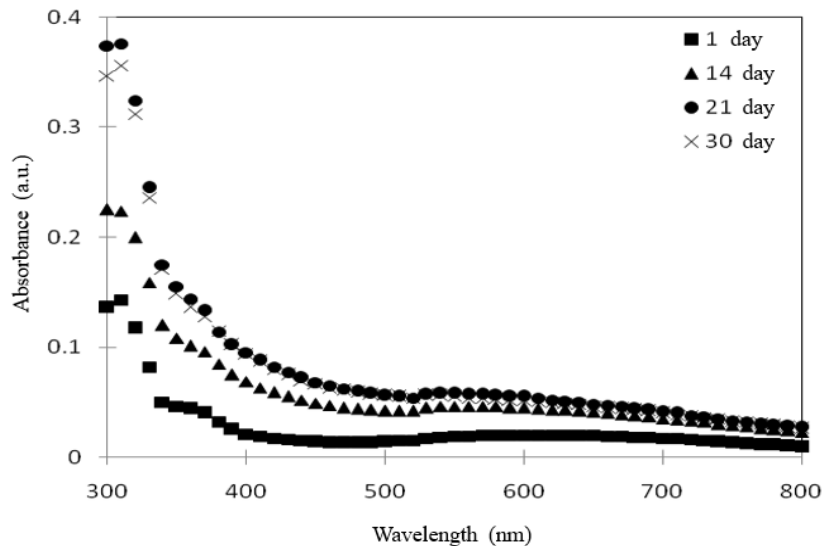
Fig. 10 - UV-Vis spectrum of HRP/PT/PPY/PANI against H_2O_2 on different day

5. Conclusion

This study found that 0.1M H_2O_2 concentration is suitable for use in the H_2O_2 detection process. It was confirmed by the UV-Vis spectrum which showed that at 0.1M, the absorption rate was high compared to other concentration values. The change occurred due to the process of H_2O_2 detection on all three types of layers. The combination of polymer support consisting of PT/PPY/PANI provides good polymer support to HRP to enable it to be applied to detect H_2O_2. It was continued by determining the effect of the HRP/PT/PPY/PANI layer on H_2O_2 detection. The study found that the reaction process between layers affected the surface roughness value obtained. This indirectly caused the response rate to become slower and in turn limited the electron transfer rate. It was also found that the absorption rate increased over time. As explained previously, the rate of absorption is related to the species that perform the absorption.

Acknowledgement

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