Int. J. Electrochem. Sci., 7 **(2012)** 4702 - 4715

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

Screen-printed Histamine Biosensors Fabricated from the Entrapment of Diamine Oxidase in a Photocured Poly(HEMA) Film

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Received: 17 February 2012 / *Accepted:* 5 April 2012 / *Published:* 1 May 2012

A three-electrode system amperometric biosensor that consisted of screen-printed carbon paste working electrode (SPE), a commercial platinum rod counter electrode *vs* Ag/AgCl reference electrode was developed for rapid determination of histamine in prawns. The biosensor was designed from entrapping diamine oxidase (DAO) enzyme in a poly(2-hydroxyethyl methacrylate) (photoHEMA) film prepared *via* a simple and one-step direct photocuring process on a carbon paste screen-printed electrode (SPE). The photoHEMA film exhibited water absorption of 34.14% after four hours exposure to water and no leaching of DAO was observed from the hydrogel film. The histamine biosensor showed response time of < 50 s with a linear response range from 0 to 60 ppm histamine (R^2 of 0.9946). The sensitivity of the biosensor was of 5.56 $nAppm^{-1}$, with a limit detection of 0.65 ppm histamine. The performance of the fabricated biosensor in the analysis of histamine in tiger prawn (*Penaeus monodon)* samples was comparable to high performance liquid chromatography (HPLC). The three-electrode system was then converted to an all-screen-printed histamine biosensor by printing onto a polyester substrate with carbon paste to form the working and counter electrodes and Ag/AgCl paste as the reference electrode. The performance of all-screen-printed histamine biosensor was evaluated using potassium hexacyanoferrate (III) as a mediator deposited electrochemically on the carbon-paste SPE. The presence of this mediator demonstrated improvement to the response of the all-screen- printed histamine biosensor.

Keywords: Screen-printed amperometric biosensor; diamine oxidase; histamine; tiger prawn, photocurable methacrylate film.

1. INTRODUCTION

Seafood products are important for nutrition and also as an item of international trade and foreign exchange earner for a number of countries in the world. Unlike other animal products, the quality of seafood products is more difficult to control due to the variations in species, sex, age, habitats and action of autolytic enzymes [1]. Therefore, simple and rapid techniques for the estimation of seafood freshness are important in the seafood industry. During storage, histamine was observed to accumulate in tissues of fish and other seafoods when spoilage by bacteria commenced [2]. However, this does not alter the seafood normal appearance and odor initially [3]. The *in vitro* determination of histamine level in seafood products is normally by chromatography analysis, which sometimes requires toxic reagents, expensive instrumentation and not practical for *in situ* analysis due to complex sample treatment and the requirement of trained personnel. Amperometric sensors based on screen-printed electrodes allow the production of simple, inexpensive and portable devices for rapid determination of freshness and spoilage in seafood products in the field. The oxidase enzyme that produces hydrogen peroxide is commonly used in enzyme electrodes or amperometric sensors [4]. Amperometric biosensors can be used in turbid media, has comparable instrumental sensitivity and is more amenable to miniaturization [5].

A miniaturized biosensor offers several advantages, including the requirement of only a small amount of enzyme for biosensor fabrication. Mass production of miniaturized biosensor with several enzyme electrodes integrated in one biosensor is possible and thus disposable-type of biosensors may be produced [6]. Thick and thin film technologies play an important role in the construction of miniaturized biosensors [6]. In the application of simple, rapid, inexpensive and disposable biosensors in clinical, environmental or industrial analyses, thick film technology permits the construction of solid-state, mechanically robust and planar sensors. This is achieved by the sequential deposition of thick films on a substrate, commonly by screen printing process. A thick film biosensor is flexible in terms of design and the choice of the material. The configuration is normally considered to be one which comprises layers of special ink (or paste) deposited sequentially onto an insulating support or substrate [7].

In this study, an amperometric histamine biosensor was developed based on immobilizing the enzyme diamine oxidase in a photocured poly(2-hydroxyl ethyl methacrylate) film directly deposited on a screen-printed carbon paste electrode. The use of direct photocuring enable a simple fabrication procedure for constructing a histamine biosensor to be used in rapid analysis of histamine, a substance related to the freshness and spoilage in seafood products. The study also involved in the evaluation of the enzyme immobilization characteristics and the electrochemical behaviour of the screen-printed histamine biosensors. Two types of screen-printed histamine biosensors were evaluated, i.e. the screen-printed working electrode alone and a biosensor consisted of screen-printed working, counter and reference electrodes (all-screen-printed biosensor). The use of potassium hexacyanoferrate (III) as a

possible mediator to improve the response of the all-screen-printed histamine biosensor was also examined.

2. MATERIALS AND METHODS

2.1. Reagents

Diamine oxidase (DAO, 0.16 U/mg), histamine dihydrochloride and 2,-2, dimethoxyphenylacetophenone (DMPP) and monomer 2-hydroxyethyl methacrylate (HEMA) and potassium hexacyanoferrate (III) were purchased from Sigma. Other chemicals were of analytical grade and used as received without further purification. All aqueous solutions were prepared with de-ionized water.

2.2. Fabrication of screen-printed electrodes

Miniaturized three-electrode system was manufactured in-house by a multi-stage screen-printing process using a semi-automated DEK-J202RS thick film printer. High modulus mesh of monofilament polyester (SEFAR® PET 1000) stencil (specification of 90-48 W) was designed as with a straight, short and wide conductor of parallel square pads. This design provided better conductivity compared to those designs with curved, narrow conductor and circular pads. The stainless steel screen mesh (78 μm fabric thickness) was mounted at 45^{o} to the print stroke with an emulsion thickness of 12 ± 2 μm for the printed pastes. All three electrodes were screen-printed onto a polyester substrate (50 x 60 mm). Prior to the printing process, the polyester sheets was baked in the oven for 5 hours at 130 ^{0}C to avoid shrinking of the foil during the subsequent heating steps [8]. Each printing cycle produced three miniaturized screen-printed electrodes (SPE). Carbon paste (Screen Technology, BBI 440) was used for the working and counter electrodes and AgCl paste (Dupont, B166) for the reference electrode. A silver layer (Dupont, B111) was printed as basal track layer to increase the conductivity and adhesion of the paste on the substrate. The paste was dried in an oven at 110 ^{0}C for 10 minutes after which each printed layer to remove the solvents.

2.3. Enzyme immobilization

Diamine oxidase (DAO) (5 mg/ml) was immobilized in poly (2-hydroxyethyl methacrylate) (photoHEMA) film via photocuring process on the carbon paste screen-printed working electrode using a procedure before reported by Bean et al. [9]. Before immobilization, an appropriate amount of enzyme was dissolved in 0.1 M phosphate buffer (pH 7.4) prior to mix with the monomer HEMA (100% w/v) and photoinitiator DMPP (1.6% w/w). A volume of 1.2 μl of DAO solution was mixed with 4.8 μl of HEMA mixture and the resulting solution was drop-coated onto the SPE and photocured for 300 s under an inert nitrogen atmosphere.

2.4 Leaching Test (Bradford Assay)

Colorimetric protein determination as reported by Bradford [10] was adopted in this study to examine the leaching level of entrapped DAO from both of the methacrylate membrances. DAO was mixed using both PhotoHEMA and pHEMA in the ratio (v/v) of 1:1, 1:2, 1:3, 1:4, 1:5 and 0:5 (as a negative control). The mixtures were immobilized onto a membrane and immersed in 2 ml of phosphate buffer. A 5 µl of this solution was mixed up with 200 µl of Bradford reagent in the microtitre wells (Immunomini 96-well plate) following by 15 µl of PBS to give a final volume of 220 µl. The absorbance reading at 595 nm was taken after 6 minutes of incubation using a microreader (Model 550; BioRad, USA). DAO entrapped by photoHEMA and pHEMA were tested for 24 and 3 hours, respectively. A BSA (1mg/ml) standard curve was obtained by measuring the absorbance for BSA of 2,4,6,8 and 10 µg at wave length 595 nm. The enzyme concentrations were determined by comparing with a BSA protein standard curve.

2.5. Water absorption study

A known weight of the photoHEMA matrix without DAO was deposited onto a supporting material under UV irradiation. The net weight of the dry film was recorded as W_d before the film was exposed to distilled water. After exposure, the change in weight was recorded for every 10 minutes. And the water uptake was calculated based on the weight of the swollen film, Ws and the weight of the dry film, W_d according to Arica and Bayramoğlu et al. [11] formula:

$$\text{Water content (w/w \%)} = [(Ws - W_d) / W_d] \times 100$$

2.6. Enzyme loading

DAO was prepared in a stock solution of 500 mg/ml and then diluted to 50, 5, 0.5 and 0.05 mg/ml (8×10^{-2}, 8×10^{-3}, 8×10^{-4}, 8×10^{-5} and 8×10^{-6} units). Different concentration of DAO (1.2 µl) was mixed with photoHEMA (4.8 µl) in a ratio of DAO:photoHEMA equals to 1:4. A 5 µl of the mixture was dropped coated onto the SPE and photocured under UV. Different DAO loading working electrodes were used for study for histamine determination. For every single concentration, triplicate of the experiments were performed and all current changes were measured.

2.7. Electrochemical evaluation of histamine biosensor

Initial experiments were performed using Autolab PGSTAT 12 Potentiostat/Galvanostat with GPES software with a three-electrode system consisted of a carbon paste screen-printed electrode as working electrode, platinum rod as counter electrode and Ag/AgCl as reference electrode. These three-electrode systems were then miniaturized by screen-printing technique to from a screen-printed three-electrode sensor (as described in 2.2). For this miniaturized SPE, the working electrode was modified

further by electrodepositing a layer of potassium hexacyanoferrate (III) using a cyclic voltammetric method described by Jaffari and Pickup [12]. The SPE was cycled 15 times at 0.2 Vs^{-1} in a solution of 0.1 M potassium hexacyanoferrate (III) under stirring. The modified SPEs were washed, rinsed with deionized water and stored dry at room temperature until use. All the samples were tested in phosphate buffer pH 7.4, 0.1 M using a potential of 0.35 volt.

2.8. Prawn sample analysis

Tiger prawn (*Penaeus monodon)* samples at 30 ^{0}C from 0 to 5 hours were analyzed using the electrochemical assay developed to determine the freshness and spoilage level of those samples. The prawn's shell, head and tail were removed. Ten grams of prawns body region was blended together with 100 ml of 0.1 M phosphate buffer, pH 7.4. The samples were collected within five hours with every hour interval and kept below -20^{O}C until used.

2.9. Histamine Analysis by High performance liquid chromatography

Histamine was extracted from tiger prawn samples [13] and derivatized as proposed by Hauschild [13]. Separation of derivatized benzoylated histamine was carried out by isocratic reversed-phase HPLC (Waters 1500 Series) equipped with a C_{18} column of 4.6 mm x 250 mm I.D. and column packing size of 5 μm. The derived histamine was detected spectrophotometrically at 254 nm with a Water Model 2487 Dual λ Absorbance Detector.

3. RESULTS AND DISCUSSIONS

3.1 Water absorption of photocured poly(HEMA) film and enzyme immobilization

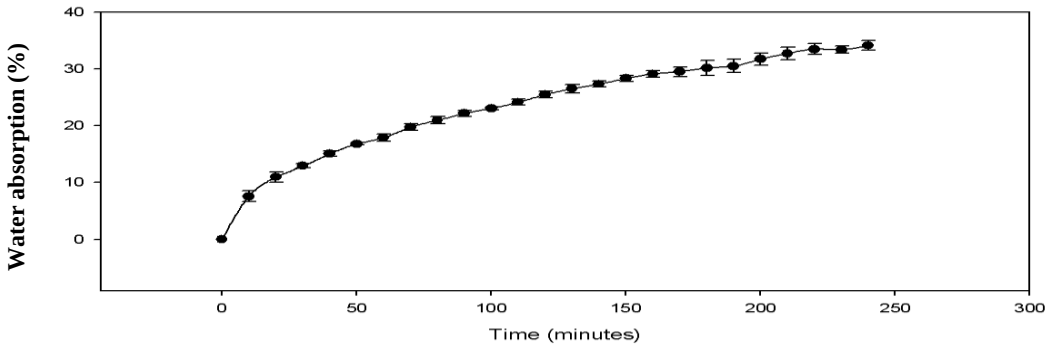

Figure 1. Profile of water absorption of a photoHEMA film.

The rate of water absorption of photoHEMA was rapid at the first hour with 17.90% and increased to 25.48% at the second hour (Figure 1). The rate of water absorption started to decrease at

the fourth hour to 34.14%. The hydroxyl functional groups of the photoHEMA film can facilitate the water absorption and penetration of water into the polymer film is an important because it allows the analytic to diffuse into the membrane and hence for the enzyme reaction to take place.

3.2. Reaction mechanism of histamine for biosensor

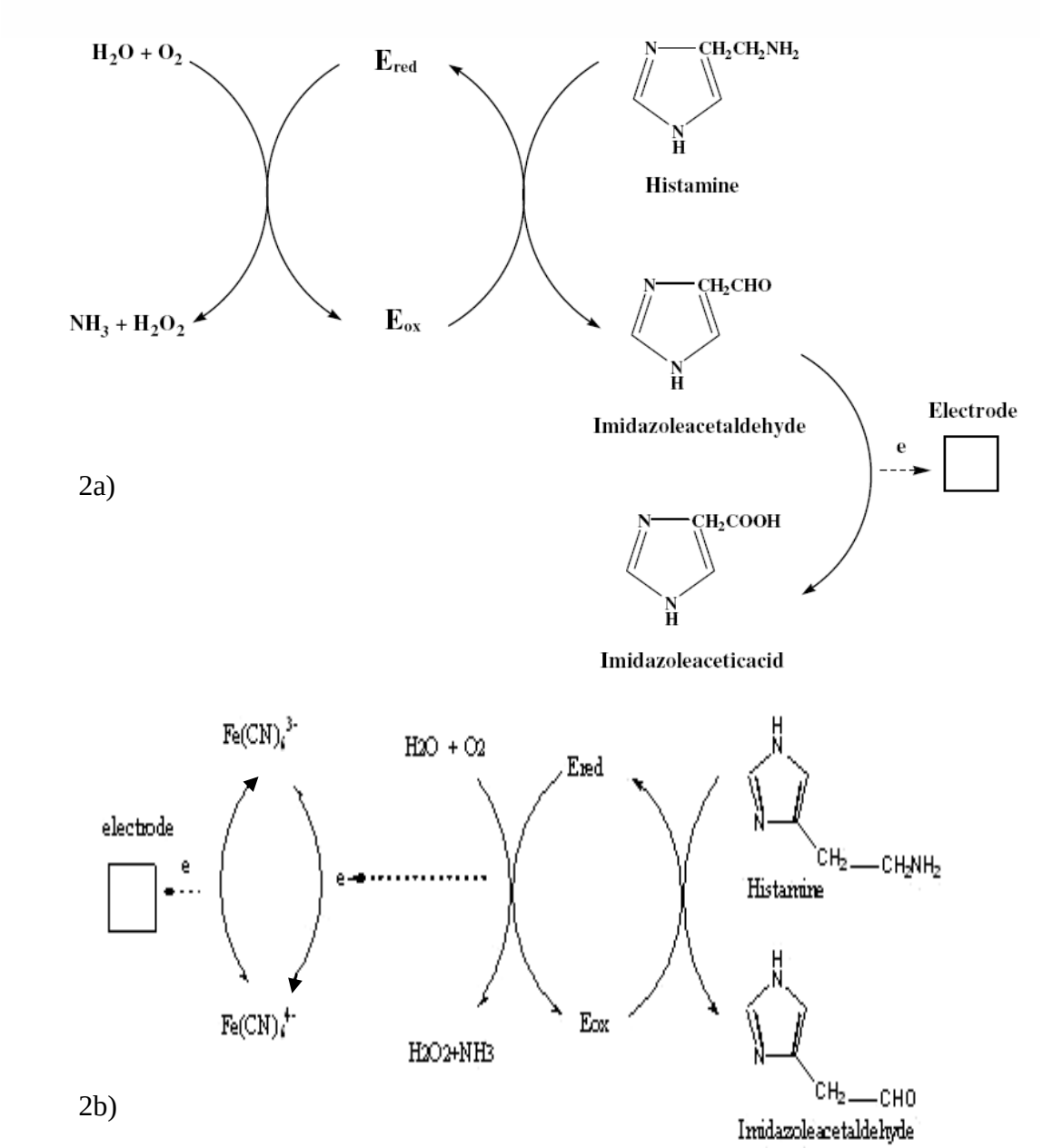

Figure 2. The possible mechanisms of the histamine biosensor; (a) Mechanism without mediator – Electron transfer from the electro-oxidation of the aldehyde product, (b) Mechanism with mediator – Direct electron transfer from DAO catalytic reaction.

The mechanism of reaction of histamine with DAO is shown in (Figure 2). The enzyme DAO causes the oxidation-deamination of histamine to form imidazole acetaldehydes and subsequently

imidazole acetic acid. The electro-oxidation of the imidazole acetaldehyde at 0.35 V results in electron transfer to the electrode and the product imidazole acetic acid is finally obtained. By electrodepositing a layer of potassium hexacyanoferrate (III) on top of the working SPE, the potassium hexacyanoferrate (III) is expected to act as a mediator for the direct transfer of electron from the oxidation of histamine to imidazole acetaldehyde by DAO.

The above mechanism is supported by the hydrodynamic studies performed on both non-immobilized and immobilized DAO. The highest current values were obtained at oxidation peaks of 0.35 and 0.60 V (Figure 3) and thus enzyme reaction between histamine and DAO could be detected amperometrically.

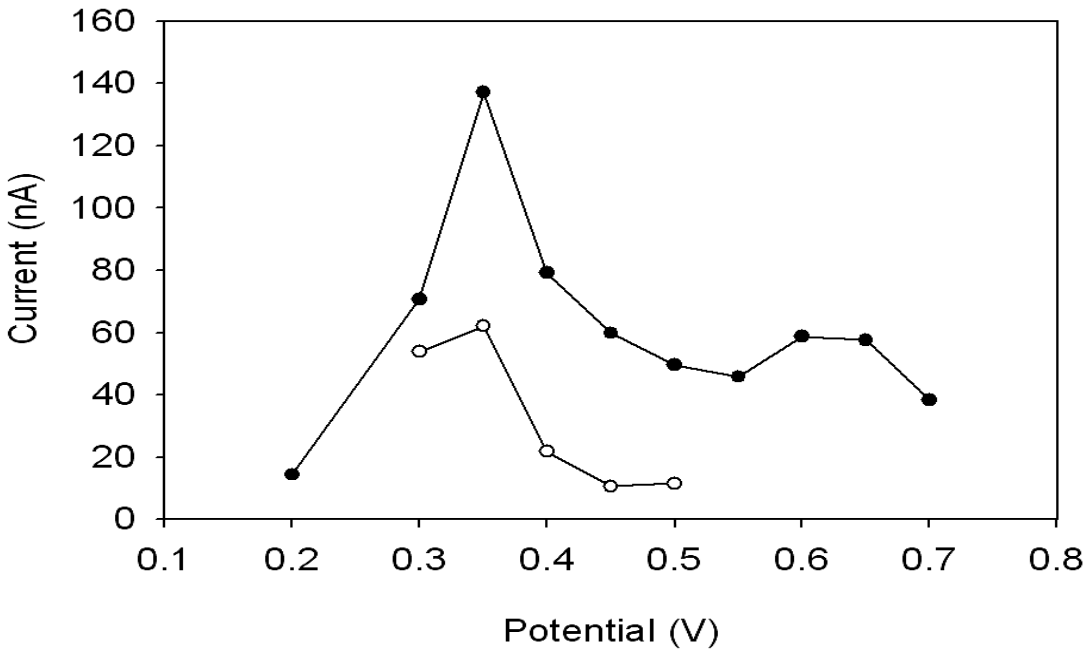

Figure 3. The hydrodynamic voltammograms of non-immobilized (●) and immobilized (○) DAO for histamine at concentration of 1 mg/ml (0.1M Phosphate buffer, pH 7.4).

At 0.60 V, the current is due to the production of hydrogen peroxide as has been reported for most oxidases [4]. In most oxidase enzyme based biosensors, the working potential for the oxidation of H_2O_2 always at a range of 0.60 to 0.70 V [5, 15]. The drawback of utilization of electrochemical oxidation of H_2O_2 as the detection system is the possible interferences from other easily oxidizable substances such as uric acid, ascorbic acid, glutathione and acetaminophen when the system is at such a high positive potentials for detection [15-17]. This interference may be eliminated by decreasing the electrode potential [18].

The current observed at 0.35 volt may be attributed to the oxidation of the reaction product imidazoleacetaldehyde as has been proposed by Kapeller-Adler and Fletcher [19]. This lower potential will be used as a detection potential for the histamine biosensor developed in this work.

3.3 Optimization of DAO loading in biosensor

The ratio 4: 1 was the optimum ratio because it yielded the highest current change as shown in Figure 4. This ratio caused a current change of 283.83 nA ± 4.47% at 50 ppm of histamine. The ratios of 1:1, 2:1, and 3:1 demonstrated a lower response to histamine. Too low of HEMA composition (e.g. ratio 1:1) has been observed to cause the film to be non-homogeneous and brittle and too little enzyme (e.g. ratio of 5:1) will cause a reduction in enzyme activity [20].

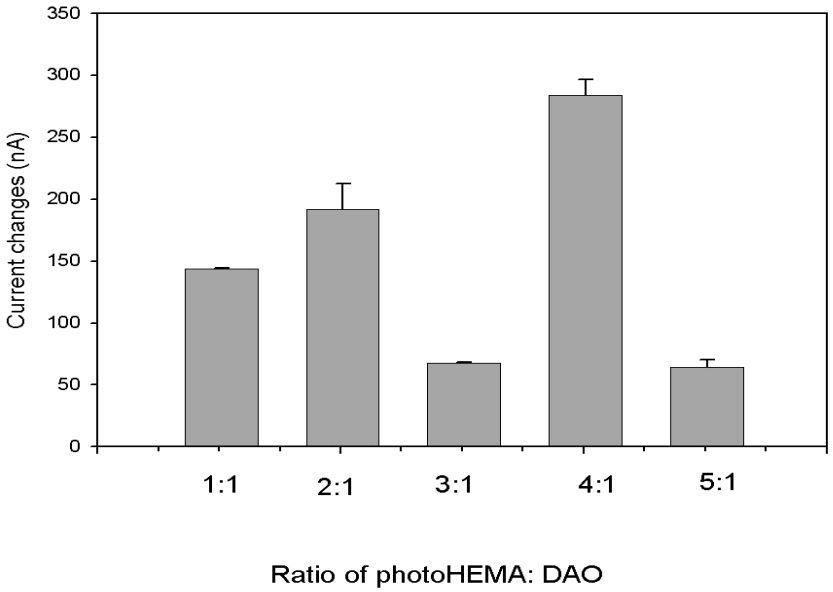

Figure 4. Optimum ratio of photoHEMA: DAO for histamine (50 ppm) determination.

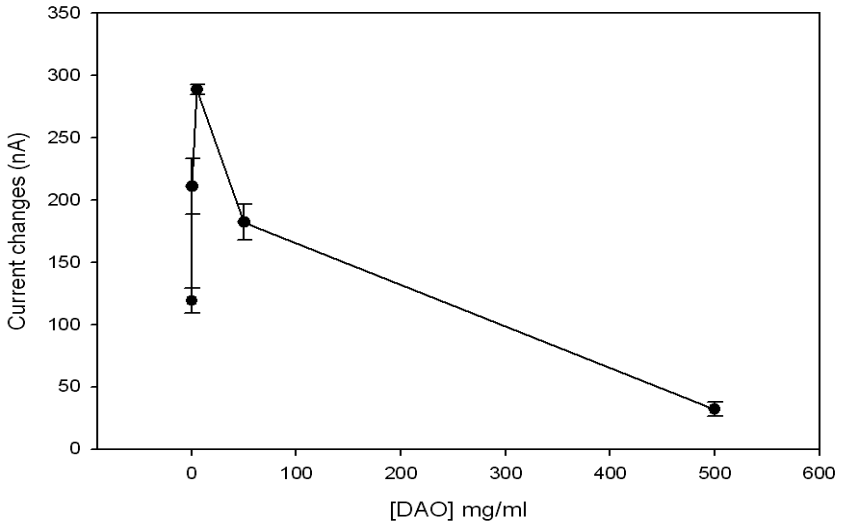

Figure 5. Enzyme loading of immobilized DAO in the photocured membrane of the histamine biosensor. (Response of 50 ppm histamine).

A 1.2 µl of DAO (5 mg/ml) was obtained as the optimum enzyme loading for the histamine biosensor (Figure 5). The current changes were increased by approximately 2.5 times when the DAO concentrations were increased from 8 $x10^{-2}$ to 8 $x10^{-6}$ units. But excessive immobilized enzyme (give unit of enzyme) caused a decrease in enzyme activity and this was also reported by Male and coworkers [2] when porous preactivated nylon membrane was used in the immobilization of DAO with glutaldehyde for biogenic amine determination. The loss of DAO response at high enzyme loading is, likely due to mass transfer diffusion limitation through the membrane as the protein loading on the membrane increases, a consequence of the over saturation of the membrane pore space where the diffusion of substrate and product are restricted [2, 21].

3.4. The analytical performance of the screen-printed working electrode for histamine biosensor

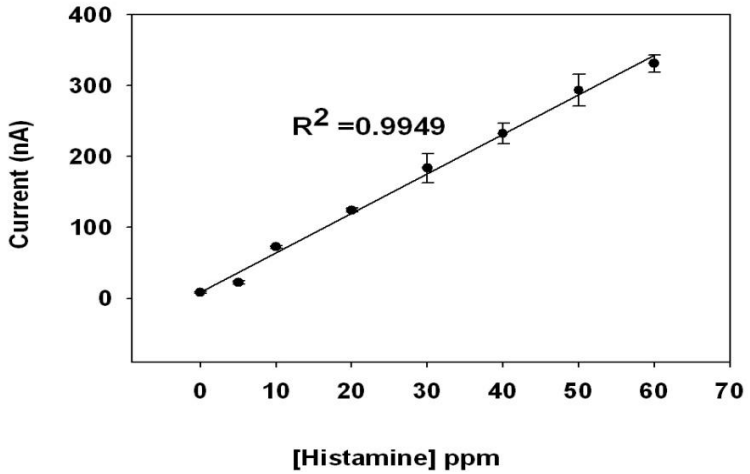

Figure 6. The linear response range of the screen-printed histamine biosensor to histamine concentrations (Detection potential at 0.35 V, phosphate buffer pH 7.4, 0.1 M).

The screen-printed histamine biosensor showed a linear response to histamine for up to 60 ppm of histamine (Figure 6), and this range included the 50 ppm level for the indication of spoilage of fish and fishery by FDA, USA. The limit of detection, calculated based on three times the standard deviation of the blank responses was 0.65 ppm of histamine. The sensitivity of the biosensor was 5.56 $nAppm^{-1}$, with satisfactory stability even after several analyses were performed using the same biosensor, no significantly decrease in the current measured (data not shown). The relative standard deviation of six measurements is less than 2.3%.

The screen-printed biosensor was found to be pH dependent with optimum response at pH 7.4 (n =3, RSD = 5.5%), in the following range 6.4 to 8.4. This pH response of the immobilized DAO was similar to that of the non-immobilized enzyme [22]. Similar pH response was also reported for immobilized monoamine oxidase [23]. The response time of the biosensor was about 50 s (n=3; RSD = 7.6%) at a histamine level of 50 ppm.

To show that the screen-printed histamine biosensor has performance similar to conventional method for histamine analysis such as HPLC, histamine level in prawn samples during aging processes

at room temperature were analysed by the screen-printed histamine biosensor and also by the HPLC method. The results of the study demonstrated that there was good correlation between both the methods where the R^2= 0.9612, indicating that the results obtained by the biosensor were close to that obtained from HPLC (Figure 7).

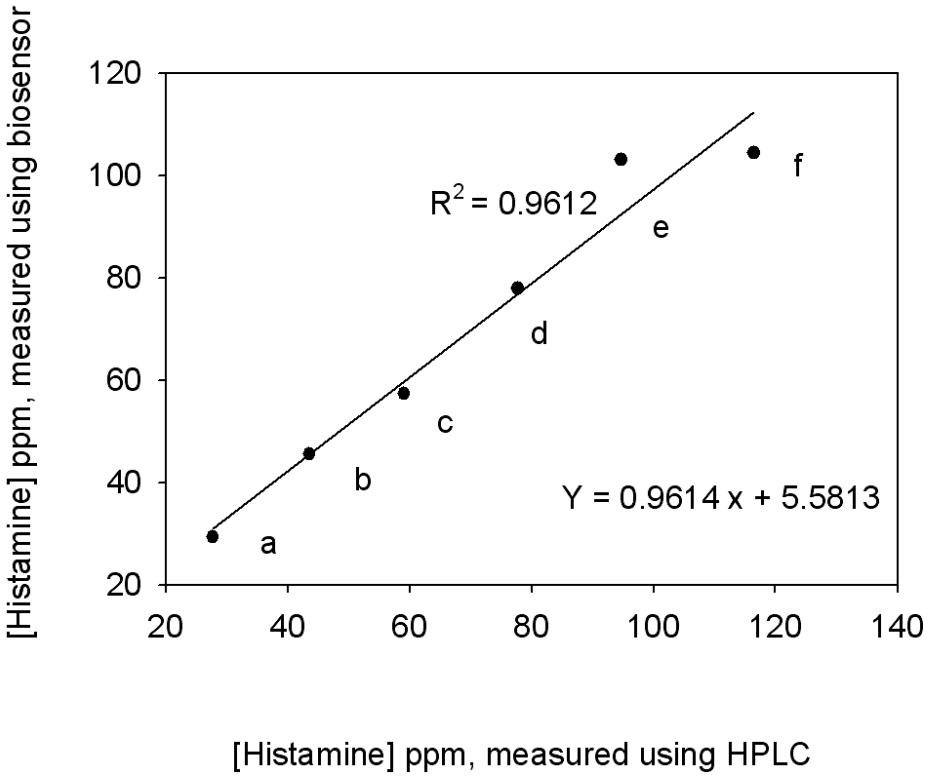

Figure 7. Correlation of histamine levels determined in prawn samples with both histamine biosensor and HPLC method Duration of exposure at 30 ± 20C: a = 0 h, b = 1 h, c = 2 h, d = 3 h, e = 4 h, f = 5 h).

3.5. The analytical performance of the histamine biosensor with screen-printed working, counter and reference electrodes (all-screen-printed histamine biosensor)

An all-screen-printed histamine biosensor was constructed by screen-printed the reference, counter and working electrode on the same substrate. The printing processes, different ink compositions such as type size or loading of graphite particles, the printing and curing conditions strongly affect the electron transfer reactivity and overall analytical performance of the resulting SPE [24]. Thus, electrochemical studies such as cyclic voltammogram (CV) were examined in the behaviour of the SPE constructed with three screen-printed electrodes, i.e. working, counter and reference. The electrochemical behaviour of the three-electrode SPE was scanned using 0.1 M potassium hexacyanoferrate and the CV was compared to that of an SPE working and counter electrodes coupled with a commercial (non-screen-printed) reference electrode (Figure 8).

The oxidation peak of the three-electrode SPE was 0.583V (n = 5; RSD = 1.53%) whilst that of the working and counter SPE with conventional Ag/AgCl reference electrode was shifted to 0.695V (n

= 5; RSD = 1.69%). The potential difference of the oxidation peaks was 0.112 V. The negative shift of the oxidation potential for the three-electrode SPE may be differentiated in the behaviour of the screen-printed Ag/AgCl reference electrode. Furthermore, a three-electrode screen-printed histamine biosensor with the reference electrode printed with Ag/AgCl also demonstrated non-reproducible characteristic when used repeatedly compared to a screen-printed biosensor with conventional Ag/AgCl as a reference electrode (data not shown).

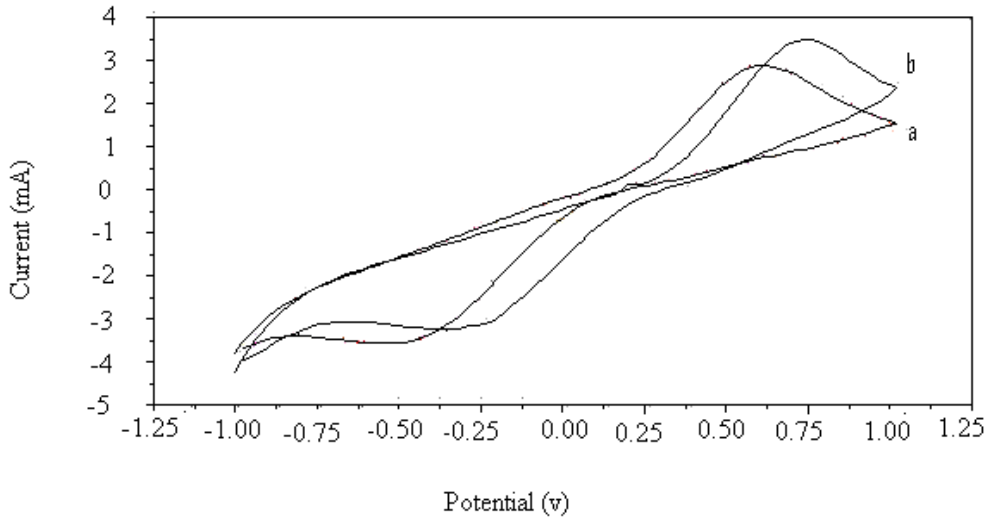

Figure 8. The cyclic voltammogram of (a) Three-electrode SPE; (b) SPE working and counter electrodes versus conventional Ag/AgCl electrode (in 3 M KCl internal electrolyte); in 0.10 M potassium hexacyanoferrate (III)/0.10 M KCl.

The poor stability of the all-screen-printed histamine biosensor may be attributed to the instability of the screen-printed reference electrode. For a conventional Ag/AgCl reference electrode, the stability is attributed to the ability of interaction between the Ag/AgCl electrode with the concentrated and confined KCl electrolyte, which allows the charge and electron transfer at the interface of the Ag/AgCl electrode to occur [25,26]. For the screen-printed reference, owing to the lack of direct contact with a constant concentration of KCl, the charge-electron transfer process is hindered, thus, the stability of this screen-printed reference cannot be maintained.

The instability of screen-printed thick film Ag/AgCl reference electrodes has been reported before by Simonis et al., [27]. Such instability will lead to very limited lifespan of a biosensor. The printed AgCl layer even when exposed to 3M KCl as inner electrolyte would dissolve after a short time caused by the formation of the AgCl complexes [27]. Many miniaturised biosensors incorporating screen-printed reference electrode suffered from the disadvantages of instability and high cross-sensitivity towards anions [28,29].

Under these circumstances, the all-screen-printed histamine biosensor could be operated at 0.25V, much lower than the potential of 0.35V used for the screen-printed biosensor with a conventional reference. Such a difference in operating potential is probably arises from the use of screen-printed reference and conventional reference electrodes. This all-screen-printed biosensor

showed a broader linear response range to histamine from 0 to 100 ppm (Fig 9) in comparing to screen-printed histamine biosensor coupled with a conventional Ag/AgCl reference electrode. However, the all-screen-printed biosensor demonstrated a much lower sensitivity i.e., 0.03 $nAppm^{-1}$ due to the lower conductivity of the carbon paste after that newly design the screen-printed electrodes [30]. This sensitivity was 185 folds lower than that exhibited by screen-printed histamine biosensor with a conventional reference electrode. The limit of detection of all-screen-printed histamine biosensor was 2.46 ppm of histamine.

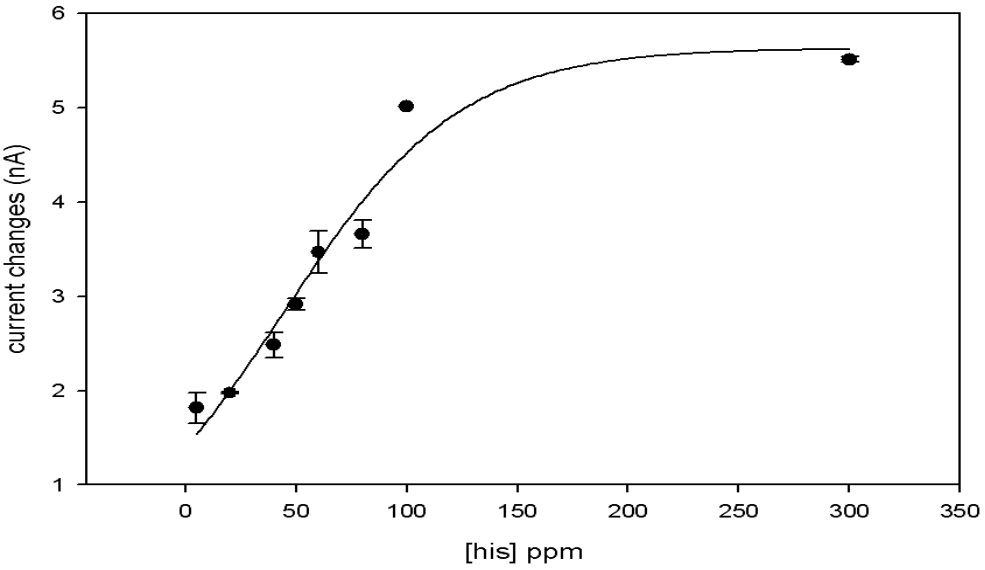

Figure 9. The response range of all-screen-printed histamine biosensor towards histamine at 0.25 V.

Potassium hexacyanoferrate (III) has been successfully employed as a mediator for many biosensors due to its excellent bioelectrochemical properties, where $[Fe(CN)_6]^{3-}$ is easily reduced to $[Fe(CN)_6]^{4-}$. It has been used in commercial biosensors such as Glucocard (Kyoto Daiichi, Japan) and Accucheck Advantage (Boehringer Mannheim, USA) [31]. To increase the sensitivity of the all-screen-printed histamine biosensor, potassium hexacyanoferrate (III) was deposited on the surface of the screen-printed electrode. For the all-screen-printed biosensor modified with potassium hexacyanoferrate (III), the reaction mechanism may follow that of Equations 1 to 3.

$$\text{Histamine} + O_2 + H_2O \longrightarrow \text{Imidazoleacetaldehyde} + NH_3 + H_2O_2 \quad (1)$$

$$H_2O_2 + 2\,Fe(CN)_6^{4-} + 2\,H^+ \longrightarrow 2\,H_2O + 2\,Fe(CN)_6^{3-} \quad (2)$$

$$Fe(CN)_6^{3-} + e \xrightarrow{DAO} Fe(CN)_6^{4-} \quad (3)$$

Hydrogen peroxide produced by the reaction between immobilized DAO reacted and histamine [31] (Equation 1). The following process (2) occurred same as reported by Marcinkeviciene and Kulys [18] and Yao and coworkers [33] when the same mechanism was used to modify the SPE for glucose

biosensor and flow injection enzyme reactors together with peroxidase electrode use for biogenic amines and hypoxanthine determination. This characteristic also is agreeable with reported by Tombelli and Mascini [34] and Bartlett and Cooper [34] that in the presence of mediators such as electron acceptors could catalyze the reduction of oxygen to H_2O_2. The $Fe(CN)_6^{3-}$ was then reduced to $Fe(CN)_6^{4-}$ as in equation 3 for reuse.

Calibration curve of histamine for the modified SPE histamine biosensor was able to detect histamine concentrations from 10 to 200 ppm histamine. The linear range of modified SPE histamine biosensor with the R^2 of 0.9931 showed a broader range of histamine detection from 0 to 80 ppm (results not shown) and similar sensitivity (5.31 $nAppm^{-1}$) compared to screen-printed biosensor with conventional reference electrode.

4. CONCLUSIONS

In this paper, we demonstrated a simple preparation of photo film histamine biosensor *via* the incorporation of potassium hexacyanoferrate and enzyme diamine oxidase (DAO) into the poly (2-hydroxyethyl methacrylate)-based polymer. From the cyclic voltammetry and amperometric measurements, the entrapped ferrocene compounds were found to function as electron shuttling agent between DAO and screen-printed electrode. The histamine sensitive membrane with immobilized DAO, after this device used for determination histamine in tiger prawns. The histamine biosensor showed a good reproducibility and repeatability. Due to the simplicity in preparation of these ferrocene-containing films, photolithography technique can thus be applied to the fabrication of various reagentless biosensors.

ACKNOWLEDGEMENTS
The authors would like to acknowledge the Ministry of Science, Technology and Innovation of Malaysia for the NSF scholarship awarded to Ching Mai Keow and the National Biotechnology Directorate of Malaysia for the financial support through research grant IRPA 09-03-03-006 NBD.

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