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A New Biosensor Based on Nanogold Doping in P-HEMA Alcohol Oxidase Detects Formaldehyde in Fresh Food

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ABSTRACT

Formaldehyde is a known carcinogen which may cause cancer when accumulated in the body. This study showed the results of a formaldehyde biosensor which was fabricated by nanogold doping in a poly-2-hydroxy ethyl methacrylate (p-HEMA) membrane. The biocatalysts used for the biosensor were 1.0% ferrocene mediator and alcohol oxidase which was then deposited on a carbon screen-printed electrode. 2,2-Dimethoxy-2-phenyl-acetophenone (DMPP) was applied to the membrane as a polymerization agent. The amperometric method was employed with a phosphate buffer solution (pH = 7.2). The optimum potential was selected to be 0.3 V which obtained good linear calibration ($R^2 = 0.99$) for a range of 0.02-0.16 mM formaldehyde (n = 4). The RSD (Relative Standard Deviation) and LOD (Limit of Detection) were found to be 5.62% and 0.007 mM formaldehyde, respectively. The fabricated biosensor successfully detected formaldehyde in selected fresh foodstuffs (tauhu, meatballs, shrimp and dried and wet fish) and the results were well correlated with the NASH standard method.

Key words: Formaldehyde, alcohol oxidase, p-HEMA membrane, nanogold

INTRODUCTION

Industrial revolution has generated various hazardous contaminants including food preservatives, pharmaceutical products, cosmetics, disinfectants, paints and pesticides. Accumulated levels of food preservatives, such as formaldehyde, may have negative influence on biological cells, tissues and body fluids, as well as on the food itself, even though formaldehyde is a natural metabolite found in living organisms (Dzyadevych *et al*., 2001; Ali *et al*., 2006). For example, in special cases, frozen fish can generate up to 200 mg of formaldehyde per kilogram wet weight due to enzymatic reactions (Ali *et al*., 2006).

Nevertheless, many industries depend on formaldehyde as a low-cost solvent. It is used in industrial processes such as wood processing, dry cleaning, petroleum refining, pulp manufacturing and textile production. In addition, formaldehyde is used as a preservative in food, paints, cosmetics and pharmaceutical items in order to protect products from microbial attack (Vianello *et al*., 2007). Finally, formaldehyde is also often applied to food in order to maintain its appearance and make

it appealing for consumers (Cui *et al*., 2007). Despite the wide use of formaldehyde, high exposure may cause central nervous disorders and damage to the immune system, as well as respiratory tract irritation and blindness (Ali *et al*., 2006; Vianello *et al*., 2007). Previous reports have revealed that formaldehyde is a mutagenic agent, a human carcinogen and a chemical mediator in cell death in cancer (Tyihak *et al*., 1998). Previous reports have shown that although formaldehyde plays a major role in many industries, in practice, it is a very harmful substance. Therefore, a formaldehyde sensor is urgently needed. Current formaldehyde assays involve applying combined High Performance Liquid Chromatography (HPLC) and mass spectrometry analyses which is an elaborate, time consuming method and relies on skilled operators. To our knowledge, only a few published reports about formaldehyde assays related to foodstuffs exist in the current literature. Ngamchana and Surareungchai (2004) used an amperometric method to detect formaldehyde in water on rinsed fruit and vegetables. Cui *et al*. (2007) detected trace amount of formaldehyde in food samples by using formaldehyde as a catalyst for the oxidation of rhodamine B. Furthermore, Wang *et al*. (2007) detected formaldehyde in food and Chinese herbs based on a color chart reaction.

Applications of the biosensor method have been increasing in the last decade. A biosensor was designed for estimating the concentration of heavy metal pollutants in natural wastes using cyanobacteria (Shing *et al*., 2008; Chay *et al*., 2009; Sundaram and Soumya, 2011). Similarly, Shyuan *et al*. (2008) used an electrochemical biosensor based on alkaline phosphatase to screen pesticides and heavy metal toxicants. Furthermore, Sharma *et al*. (2011) reviewed the use of lipase biosensors in the qualitative determination of triacylglycerols and Rahaie and Kazemi (2010) studied the promising lectin-based biosensors for the quantitative determination of pathogens. A literature review by Chen *et al*. (2005) suggests that the biosensor technology offers promising prospects in the development of bioengineering processes for fermentation products in China. Lawal and Adeloju (2012) recently demonstrated interesting comparison of the amperometric and potentiometric techniques on phosphate biosensors, using polypyrrole as the target analyte.

To date, the focus of the biosensor method was primarily on formaldehyde detection in the atmosphere (Korpan *et al*., 2000; Dzyadevych *et al*., 2001; Kataky *et al*., 2002). Dzyadevych *et al*. (2001) proposed the use of a conductometric biosensor for a formaldehyde assay in drinking water using preconcentration method. Therefore, the goal of the present study is to examine a formaldehyde biosensor for foodstuffs.

Because the catalytic reaction of formaldehyde by alcohol oxidase is very slow, ferrocene can act as a mediator by forming a π-π linkage along with enzyme-induced electron transfer (Yang *et al*., 2006b). Because ferrocene is a small molecule, it may snap readily from the platform; hence, a polymer such as poly-2-hydroxy ethyl methacrylate (p-HEMA) is required to form a covalent bond with ferrocene (Kandimalla *et al*., 2006). The polymer molecule possesses poor electrical conductivity; therefore, a carbon Screen Printed Electrode (SPE) is required for the reaction platform to improve electrical conductivity. Although, ferrocene may initiate the electron transfer, the active site of the enzyme is deeply embedded in the molecule; thus, ferrocene is not able to accelerate the electron transfer.

As a result, nanogold particles were introduced in the reaction platform to attract and adsorb all chemical species involved in the enzymatic reaction resulting in good electrochemical performance. Au or Ag nano particles have been broadly used for electrochemical applications because of their outstanding behavior when encountering large surface area-volume ratios, high electron affinity and suitable biocompatibility. Thus far, the application of nanogold particles in

biosensor investigations have involved (1) glucose biosensors using a polytyramine-modified gold electrode (Labib *et al*., 2010), (2) DNA sequence detection in chronic leukemia using a poly (-eriochrome black T) film attached to a thiolated capture probe (Lin *et al*., 2010), (3) an optical immunosensor using a fluorophore mediator to maximize fluorescence response (Hong and Kang, 2006) and (4) a biosensor design using flower-like ZnO crystals (Zhang *et al*., 2009). Nanogold particles therefore provide excellent potential as a platform for binding the active site of alcohol oxidase with ferrocene in biosensor fabrication. To the best of our knowledge, this is the first report of the application of nanogold particles in a p-HEMA membrane deposited onto a carbon SPE for detection of formaldehyde in food samples. The nanogold surface is compatible for both the enzyme and ferrocene and provides good platform for electron transfer; thus, better interactions between electrons and the working carbon SPE are facilitated and this enhances the electrochemical response. Previously, a carbon SPE was used for detecting bacterial tuberculosis by an electrochemical biosensor that was based on the differential pulse voltammetric technique (Issa *et al*., 2010). The present study used the selected fresh food samples (tauhu, shrimp, meatballs and dried and wet fish) for biosensor application which was validated by the NASH standard method.

MATERIALS AND METHODS

Chemicals: Analytical grade chemicals were used in this study: Alcohol Oxidase (AOX) was obtained from *Hansenula* sp., 2,2-dimethoxy-2-phenylacetophenon (DMPP), p-HEMA, ferrocene or iron (II) cyclopentadienyl and commercial nanogold (50-130 nm) were purchased from Aldrich Chemical Company. The formaldehyde stock solution (1.070-1.080 g mL^{-1}) was obtained from AnalaR BDH Ltd. Poole. The phosphate buffer was made by dissolving KH_2PO_4 and KCl in deionized water and then by concentrating in NaOH to obtain final pH of 7.2.

Several chemicals were needed to determine formaldehyde using the NASH method; these included formaldehyde standards, ammonium acetate (Scharlau Chemie), glacial acetic acid (BDH Chemicals Ltd.), acetyl acetone (Riedel de Haen AG) and Trichloro Acetic Acid (TCA) (UNILAB Ajax) for preparing a 5% solution for formaldehyde extraction from food samples.

Membrane fabrication: The carbon SPE (Screen Print Ltd. Malaysia) is suitable for the attachment of p-HEMA membrane cocktails during UV exposure photocuring because it is low cost and easily used. The membrane cocktail consisted of 1.6-% DMPP as the photoinitiator, ferrocene, alcohol oxidase and nanogold particles dissolved in the HEMA solution. The membrane cocktail was then deposited on the 5.0 mm diameter circle of the carbon SPE which was radiated by UV light for polymerization under a constant flow of nitrogen for 500 sec. The membrane electrode was kept dry at 4°C until further use.

Electrochemical transduction: All electrochemical measurements were conducted in a 5 mL cell, containing a phosphate buffer of pH 7.2 and the 3-electrode configuration system: (i) the working electrode, i.e., carbon SPE attached by the p-HEMA membrane; (ii) the glassy carbon-counter electrode; and (iii) the Ag/AgCl/KCl (3 M) reference electrode. A PGSTAT 12 Autolab Potentiostat/Galvanostat (Eco Chemie B.V) was used for all amperometric measurements which was provided by a magnetic stirrer interface connected to a PC running GPES software.

Cyclic voltammetry and differential pulse voltammetry: Cyclic Voltammetry (CV) was used to examine the effect of ferrocene and to optimize the voltage. The working conditions were set as follows: potential range from -0.2 to 0.6 V, potential step for 0.02 V, equilibrium time of 3 sec and a scan rate of 0.06 V sec^{-1}. For Differential Pulse Voltammetry (DPV), the potential range was set from -0.2 to 1.5 V. Sample injections were carried out by aliquots of formaldehyde, with a duration time of 60 sec for each sample.

Chronoamperometry: A potential of 0.3 V was selected for all current responses in the chronoamperometry mode. The calibrations of the formaldehyde standard were prepared by successive injections of 0.02-0.20 mM FA in phosphate buffer (pH 7.2). Aliquots of 0.5 μL were obtained by dissolving 5 g of the food sample in 50 mL of deionized water which were then injected into the phosphate buffer.

NASH reagent preparation: By following the procedure of Kleeberg and Klinger (1982), the NASH reagent was prepared by dissolving 75 g ammonium acetate, 1.5 mL glacial acetic acid and 1.0 mL acetyl acetone in a 500 mL aqueous solution. A serial dilution of 6-30 ppm formaldehyde standard solutions were prepared and 3 mL of the NASH reagent was added to each solution and making the final volume up to 10 mL. The food sample (5 g) was crushed and extracted by 5% TCA in a 50 mL solution and the pH range was adjusted to 6.0-6.5. A UV-Vis spectrophotometer was used for recording the absorbance at 410 nm. A paired sample t-test was used for validation.

RESULTS AND DISCUSSION

Reaction mechanism in p-HEMA membrane: The reactions in the formaldehyde biosensor are expected to be as follows:

$$HCHO + \underset{\text{Enzyme ox}}{AOX} + 2H_2O + O_2 \rightarrow HCOOH + H_2O_2 + \underset{\text{Enzyme red}}{AOXH^+} + OH^- \quad (1)$$

$$\underset{\text{Ferrocirium ion}}{Fe(C_5H_5)_2^{2+}} \rightarrow Fe(C_5H_5)_2^{3+} + e^- \quad (2)$$

$$\underset{\text{Enzyme red}}{AOXH^+} + \underset{\text{Ferricirium ion}}{Fe(C_5H_5)_2^{3+}} + e^- \rightarrow \underset{\text{Enzyme ox}}{AOX} + \underset{\text{Ferrocirium ion}}{Fe(C_5H_5)_2^{2+}} + H^+ \quad (3)$$

The reaction mechanism is based on the previous report (Boujtita *et al.*, 2000). As long as the electrochemical reactions progress smoothly, the [OH^-] produced in reaction (1) and the [H^+] in reaction (3) should be balanced. Once this balance is disturbed by membrane precipitation or by concentration polarization which changes the concentration of [OH^-] or [H^+], the enzyme will be partially inactive.

Although alcohol oxidase acts as a biocatalyst in the formaldehyde reaction, a ferrocene mediator is required to increase the rate of electron transfer in the p-HEMA membrane. The role of ferrocene yielded substantial effects in the current response, as shown by the CV display (Fig. 1). The CV displays the redox reaction of formaldehyde and the enzyme close to the working electrode, or the carbon SPE in both the presence and absence of ferrocene. As the potential approached the reduction potential, ferricinium ions were reduced to ferrocinium ions (reaction 3)

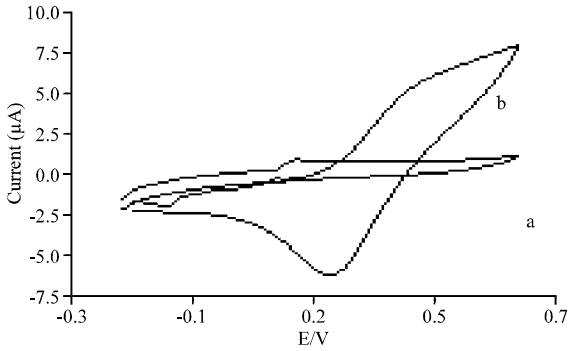

Fig. 1: The CV of carbon SPE-pHEMA-AOX (7.7U)-formaldehyde (2.0 mM) in the a: Absence and b: Presence of 1% ferrocene, Phosphate buffer pH: 7.2, Scan rate, 0.06 V sec^{-1}, 500 sec UV photocuring

and the cathodic current increased until it reached its limiting value, as shown in Fig. 1 (curve b). When all ferricinium ions were completely reduced, a rapid change was observed in the passing current. As the potential approached the oxidation potential, oxidation of both the formaldehyde (reaction 1) and ferrocinium ions (reaction 2) occurred, resulting in a decrease of the anodic current to its limiting value, as shown in Fig. 1 (curve b). When all oxidizable species were completely oxidized, the passing current returned to zero. The above condition occurred when ferrocene was present at a given sweep rate (i.e., 0.06 V sec^{-1}), as shown in Fig. 1 (curve b). On the other hand, it was not possible to generate reduction and oxidation peaks in the CV in the absence of ferrocene at the same sweep rate, because, in this case, the redox reaction progressed slowly, as shown in Fig. 1 (curve a) (Atkins and Paula, 2002). Several trials showed that the most suitable concentration of ferrocene with respect to membrane loading is 1.0%. According to Hall *et al.* (1998), several enzymatic reactions require mediators to wire electrochemical reactions in order to obtain better biosensor performance. Examples of such mediators include Co-phthalocyanine (Boujtita *et al.*, 2000), Co-tris bipyridine (Opallo and Kukulka-Walkiewicz, 2001) and the Os-complex (Castillo *et al.*, 2003; Smutok *et al.*, 2006).

Applied potential: As shown in Fig. 2, the +0.35 V/Ag/AgCl showed the highest sensitivity. It was observed that both the +0.25 V/Ag/AgCl and +0.35 V/Ag/AgCl electrodes yielded non linear amperometric responses. Results obtained using the +0.25 V/Ag/AgCl and +0.35 V/Ag/AgCl electrodes showed higher sensitivities than those obtained with the +0.30 V/Ag/AgCl electrode in the formaldehyde range of interest. Although the explanation for this difference in the sensitivities remains unclear, it has been attributed to the high dynamic movement of the species in the p-HEMA membrane due to enzymatic reactions which results in relatively greater drift response. On the other hand, the +0.30 V/Ag/AgCl electrode yielded more consistent results, leading to a linear amperometric response. According to the results of the study by Boujtita *et al.* (2000) on ethanol biosensor using alcohol oxidase, it appears likely that the electrocatalytic oxidation process controls the biosensor response in the +0.30 V/Ag/AgCl electrode. Owing to its better linear response, the +0.30 V/Ag/AgCl electrode was selected for the study on the whole.

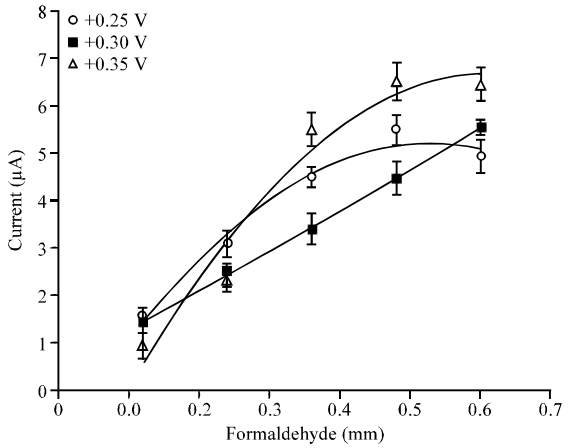

Fig. 2: The sensing response obtained using different working potentials in Ag/AgCl/carbon SPE/pHEMA/AOX/1% ferrocene, Phosphate buffer pH: 7.2, 500 sec UV photocuring

Enzyme loading: Generally, higher enzyme loadings yielded higher responses prior to saturated loading, as has been reported in previous studies (Boujtita *et al*., 2000; Shimomura *et al*., 2008) and this study showed a similar trend with the results found in their studies with respect to enzyme loading. This study used a 7.7 U enzyme in order to increase its sensitivity and sensor performance in the formaldehyde working range used. It is not surprising that higher enzyme loadings yield faster catalytic reactions in the membrane layer, resulting in higher biosensor responses before saturation occurred. The figure is attached in the separated supplemented sheet.

Nanogold entrapment: Nanoparticles play a vital role in the adsorption of biomolecules because of their large surface area and high surface free energy. Many studies have already shown that optical, mechanical, photocatalytic and transport properties can drastically change due to nano scale reactions (Vastarella and Nicastri, 2005). It is therefore expected that the integration of nano materials with biomolecules can result in remarkable bioanalytical chemistry properties. Because many investigators have successfully used nano materials; such as gold, silver and SiO_2 in the fabrication of electrochemical biosensors (Luo *et al*., 2004; Huang *et al*., 2005; Zhang *et al*., 2005; Yang *et al*., 2006a; Li *et al*., 2007), we were inspired to investigate the use of nanogold doping in the p-HEMA membrane for this study.

The entrapment of nanogold particles may induce improvement in the electrochemical response because nanogold particles can act as tiny conduction centers to facilitate electron transfer in the formaldehyde reaction at the electrode surface. The mechanism of nanogold coupling in p-HEMA remains unclear; however, one possibility is that electrons capture by ferricinium ions bound to p-HEMA and the protonated alcohol oxidase (reaction 3). These electrons would then become trapped in the nanogold surface during the p-HEMA membrane fabrication by UV irradiation. Another possibility is because of the fact that strong covalent bonds between ferrocene and the nanogold particles at the sensing interface (Labib *et al*., 2010). It should be noted that the sensing layer provided by nanogold particles possesses a large surface area and good biocompatibility that allows for large quantities of alcohol oxidase and ferrocene to be loaded. Additionally, the three-dimensional structure of nanogold particles is capable of free orientation, thus allowing the

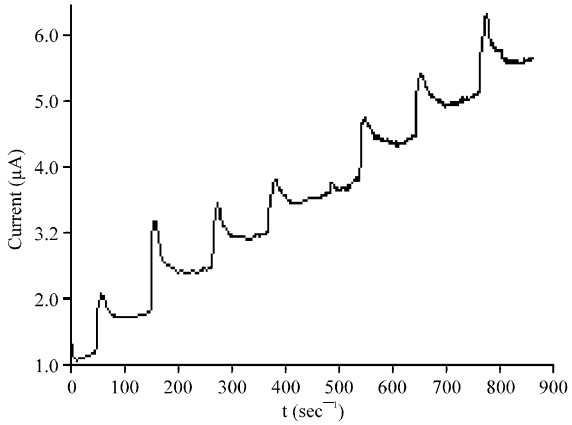

Fig. 3: Typical chronoamperogram by successive formaldehyde additions (0.02-0.16 mM) using 0.2% nanogold particles in stirred solution, 1% ferrocene, 7.7U AOX, 500 sec UV photocuring, Phosphate buffer pH: 7.2, Applied potential: 0.3 V vs. Ag/AgCl, Stirring 100 rev/min

enzyme to retain its active configuration. Therefore, it is not surprising that alcohol oxidase entrapped in nanogold particles could maintain its biological activity. This study has applied several concentrations of commercial nanogold particles (0.05, 0.15 and 0.2%) based on the chronoamperometric method for formaldehyde additions (0.08-0.48 mM). It is reasonable that higher concentrations of nanogold particles results in better sensing response because a higher nanogold content would possess larger surface area and better biocompatibility for enzyme and mediator loading, as was proved by our experimental results. The figure is attached in the separated supplemented sheet.

Formaldehyde calibration: Figure 3 presents the typical chronoamperogram for successive formaldehyde injections (0.02-0.16 mM) in a stirred phosphate buffer of pH 7.2, using 1.0% ferrocene. The chronoamperogram showed sharp peaks which corresponds to the formaldehyde injections during the running time. Figure 4 illustrates the related linear calibration ($R^2 = 0.9990$; n = 4). Shyuan *et al.* (2008) obtained a linear response range with $R^2 = 0.92$ for the detection of environmental toxicants using alkaline phosphatase. On the basis of the formaldehyde calibration, this study found RSD value of 5.62% and an LOD of 0.007 mM of formaldehyde. On the other hand, Shing *et al.* (2008) found an LOD of 8 ppm for the detection of Cd(II) and Chay *et al.* (2009) obtained an LOD of 0.4 ppm for Pb(II) using an electrochemical biosensor with cyanobacteria. The range of formaldehyde calibration applied in this study was determined on the basis of the expected levels of formaldehyde in food samples. This study used the pH 7.2 phosphate buffer because this pH provides optimum catalytic enzyme activity and has been used in previous related reports (Boujtita *et al.*, 2000; Kataky *et al.*, 2002; Khlupova *et al.*, 2007). With respect to the pH of the buffer, Sridevi *et al.* (2008) found a linear response range of 5.10^{-2} to 2.10^{-3} mM for the detection of orange G dyes using a phosphate buffer (pH 5.5) with a carbon paste electrode operating at -0.293 V vs. Ag/AgCl (3 M KCl) reference electrode.

Application of the developed biosensor for food samples: Table 1 lists the formaldehyde content obtained using 5 different types of food samples (tauhu, meatballs, shrimp and dried and wet fish) and the comparison results obtained from the NASH method. The proposed biosensor was

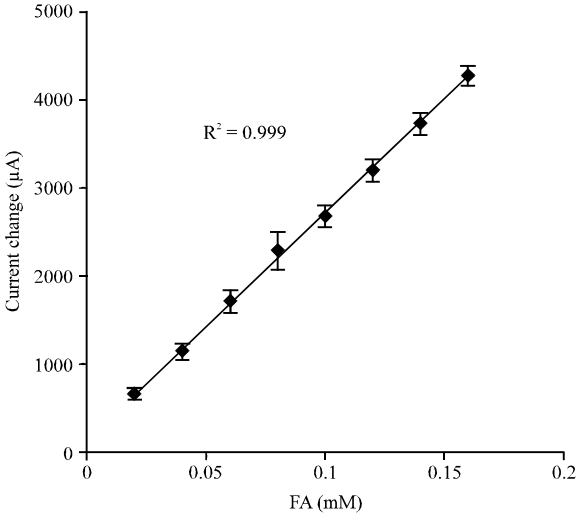

Fig. 4: Linear formaldehyde (FA) calibration (R^2 = 0.9990; n = 4) in the range of 0.02-0.16 mM using 0.2% nanogold particles in stirred solution, 1% ferrocene, 7.7U AOX, 500 sec UV photocuring, Phosphate buffer pH: 7.2, Applied potential 0.3 V vs. Ag/AgCl, Stirring 100 rev/min

Table 1: Developed biosensor for the detection of formaldehyde in food samples

Food samples	Developed biosensor (mg/kg)[a]	NASH standard method (mg/kg)[a]	Wang *et al.* (2007) (µg mL^{-1})	Cui *et al.* (2007) (µg kg^{-1})	Ali *et al.* (2006) (mg kg^{-1})
Tauhu (n = 5)	1.05±0.24	0.96±0.24			
Meatballs (n = 4)	1.13±0.2	1.08±0.08			
Dried fish (n = 4)	0.72±0.04	0.69±0.08			
Wet fish (n = 5)	0.24±0.13	0.27±0.12			
Shrimp (n = 5)	0.51±0.06	0.54±0.04		24.9±0.3	
Sleeve-fish			<0.8	86.0±0.2	
Jelly-fish			<0.8		
Fishes (gadoid species)					up to 200

[a]Values are Mean±SD

validated by the NASH method and the results were well correlated with the paired sample t-test at 95% confidence interval. The method of the statistical analysis is described in the separated supplemented sheet. The results of previous investigation of formaldehyde determination are also shown in Table 1. These include a previous investigation which used reference color cards for absorbance measurements (435 nm) in an acetylacetone reagent Wang *et al.* (2007) and the study by Cui *et al.* (2007) which used a spectrophotometric method based on rhodamine B-potassium bromate in sulfuric acid. Ali *et al.* (2006) reported that in an extreme case, certain frozen fish contained up to 200 mg of formaldehyde per kilogram wet weight and this unusual level is probably due to metabolic changes during cool storage. With respect to the biosensor application for food samples, a polypyrrole-based potentiometric biosensor assay using xanthine oxidase and a ferrocene mediator was carried out for the determination of hypoxanthine in fish samples as an indicator of fish freshness (Lawal and Adeloju, 2008). The observed data can be used as a useful reference for further study regarding the biosensor method, application and the target analyte.

CONCLUSION

The present study shows that biosensor performance is influenced by the successful reactions in the membrane, electrode configuration in the reaction cell and surface smoothness of the membrane. Biosensor performance is further affected by membrane fabrication including enzyme loading, mediator, characteristics of the nanoparticles and number of membrane layers. On the basis of the results of this study, the nanogold doping p-HEMA biosensor can be promoted for broader food analyses.

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