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Mediated Amperometric Biosensor for the Determination of Ammonium

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Abstract- A mediated amperometric biosensor based on bi-layer glutamate dehydrogenase (GLDH)/diaphorase immobilized on screen printed carbon paste electrode (SPCE) for the determination of ammonium has been described. In this work the electrochemical oxidation of NADH was used as indicator reaction for the quantification of ammonium concentration. The use of ferrocene carboxaldehyde as an electron transfer mediator lowered the oxidation potential of NADH to +0.4 V *vs.* Ag/AgCl. Parameter optimization improves the analytical performance of the biosensor within the linearity range of 2.5 µM to 500 µM with the detection limit of 2.5 µM. The reproducibility of the fabricated biosensor was also evaluated and it was found to be good with a relative standard deviation (RSD) of 4.17% (n=5). A good agreement with the Indothymol method was obtained for the measurement of ammonium in water samples.

Keywords- Ammonium Biosensor, Glutamate Dehydrogenase, Diaphorase, Ferrocene Carboxaldehyde, Chitosan

1. INTRODUCTION

Ammonia is widely used in farming, chemical and automotive industries and also occurs naturally as human and animal excrements, industrial waste and wastewater treatment plants

[1]. It is recognized as a highly toxic compound where in excess it can alter the acidity of the soil and this will disrupt the nutrition cycle and the ecological balance [2]. In addition, it can also be harmful to humans by causing coma and death [3]. Even at low concentrations, it can adversely affect aquatic life, such as reduction in growth rate, injury to gill tissue, liver, and penetrate the cell membrane and kidneys [4]. For humans, ingestion of food contaminated by ammonia can cause corrosion of mouth lining, esophagus and stomach. Thus, the assessment of ammonia in the potable water and industrial process is very important .

The permissible level of ammonia in surface water for drinking purpose is about 0.5 mg/L in United Kingdom [5], whereas the maximum level allowed in aquaculture according to the U.S Environmental Protection Agency (USEPA) is about 2.1 mg/L [6]. In Malaysia, Department of Environment, the recommended maximum concentration limit in water is in the range of 0.1 to 2.7 mg/L [7].

Various methods have been developed for quantification of ammonia concentration in different samples. Ammonia has been determined by a flow injection analysis (FIA) system constructed with a sequential immobilized enzyme reactor and chemiluminescence detector [8], HPLC coupled with fluorescent detector [9,10], and colorimetric methods (Indophenol-based reaction [11,12] and Berthelot reaction [13]). However, all these conventional methods for ammonia measurement are time-consuming and tedious. Several methods involved highly toxic reagents and interference by other photoactive substances, resulting in quite large biases of the analysis. Pre-column sample treatment is required for chromatographic method. In addition, they are not suitable for real-time measurements.

The importance of enzyme based amperometric biosensor has increased during the past decade due to high selectivity of the bio-recognition element and the sensitivity of the electrochemical signal transduction [3]. Biosensors for ammonium determination have previously been reported employing GLDH [14], bi-enzymes urease and GLDH [15] and GLDH and glutamate oxidase (GOX) [3] using NADH as a cofactor. Amperometric quantifications with biosensor or bioassays using enzymes NAD^+/NADH dependent are generally based on the electrochemical oxidation of NADH produced during the enzymatic reaction [16]. In theory, the direct oxidation of NADH at the electrode surface can be used as an indicative reaction and high over potential is required. However, this method will lead to electrode fouling as well as interferences from electroactive species [17-20]. To avoid such undesirable phenomena and to increase the biosensor selectivity, electron transfer mediators have been used. Electron transfer mediator for the NADH oxidation can act as one electron oxidant, e.g. ferrocene and its derivatives [21-25]. Enzymes that are frequently employed for catalyzing electron donation from NADH to several kinds of mediators including artificial dyes are diaphorase or lipoamide dehydrogenase [26].

The mono-layer and bi-layer technique provide biosensors with high sensitivity per functional molecule [26]. The functional efficiency of each enzyme molecule is influenced by

the efficiency of the substrate supply, the efficiency of the enzymatic process and the efficiency of the enzyme/electrode electron transfer [26]. Enzyme bi-layer modified electrodes are advantageous with regard to the efficiency of the substrate supply.

In the present work, bi-layer GLDH/diaphorase modified SPCE was constructed for ammonium determination by monitoring the consumption of NADH. The performance of the developed biosensor, its analytical features and application in a real matrix are described.

2. EXPERIMENTAL

2.1. Materials

Glutamate dehydrogenase (GLDH), diaphorase, ferrocene, ferrocene carboxylic acid, ferrocene carboxaldehyde and β-nicotinamide adenine dinucleotide (NADH) were purchased from Sigma. α-ketoglutaric acid was obtained from Fluka. Chitosan was supplied by Chito-Chem (M) Sdn Bhd. Ammonium chloride was purchased from R&M Marketing. Sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from BDH Chemicals. All chemicals were of analytical grade and used without further purification.

2.2. Preparation of stock solution

Chitosan solution (2%) was prepared by dissolving 0.4 g of chitosan powder in 20 ml of acetic acid (1%, v/v). The viscous solution was stirred overnight at room temperature. NADH solution (5 mM) and α-ketoglutaric acid solution (50 mM) were prepared by dissolving 0.035 g of NADH and 0.0725 g of α-ketoglutaric acid in 10 ml of phosphate buffer (pH 8, 50 mM), respectively. Ferrocene and its derivatives were dissolved in absolute ethanol. GLDH (20 mg/ml) and diaphorase (20 mg/ml) stock solutions were prepared by dissolving 8 mg of each powder in 200 µl of 50 mM phosphate buffer pH 8, respectively. Aliquots were kept in Eppendorf tubes at -20 °C for further use.

2.3. Fabrication of biosensor

Screen printed carbon paste electrode (SPCE) was supplied by King Mongkut University of Technology Thonburi, Bangkok, Thailand. The electrode is composed of a two channel membrane, where one channel with carbon surface acted as working electrode for enzyme immobilization and another channel with Ag/AgCl served as the reference/counter electrode. The procedure for preparation of bi-layer GLDH/diaphorase was described as follows: Initially, 2.5 µl of the diaphorase/chitosan mixture (ratio 1:1, v/v) was pipetted and smeared onto carbon surface of working electrode followed by a short spin at 2000 rpm for 2 s. After drying at 4 °C for 1 h, 2.5 µl of GLDH/chitosan mixture (ratio 1:1, v/v) was applied and

smeared on the dried film of diaphorase/chitosan. It was spun again at 2000 rpm for 2 s and kept at 4 °C for drying process. The biosensor was stored at 4 °C prior to use.

2.4. Experimental procedures

Cyclic voltammetric (cv) and amperometric measurement was performed using PalmSensPC version 1.7.3 (Netherlands). All measurements were conducted in a 10 ml electrochemical cell containing 5 ml of 50 mM phosphate buffer, ferrocene and its derivatives (0.03 mM), α-ketoglutaric acid (2.0 mM) and ammonium chloride (0.0-0.5 mM), respectively. A three-electrode system consisting of enzyme modified SPCE as working electrode, a Ag/AgCl as reference electrode and a platinum wire as counter electrode were employed for cyclic voltammetry experiments. The redox couple of the mediator was monitored at scan rates of 0.05 Vs^{-1} between potential of -0.5 to +1.0 V. For amperometric experiments, the enzyme modified SPCE was polarized at a potential of +0.4 V *vs*. Ag/AgCl. After the baseline was stable, 150 µl of NADH (0.16 mM) was added and steady-state current response was measured. The solution was disposed after recording the amperometric response of single addition. Sensitivity value was obtained from the calibration graph of current response versus ammonium chloride concentrations.

2.5. Comparison of the biosensor performance and analysis in spiked samples

The performance of the developed biosensor at various concentrations of ammonium chloride was compared with a colorimetric method (Indothymol), which is based on the reaction of ammonium with 0.017 M nitroprusside, 0.013% sodium hypochlorite, and 0.027 mM thymol at pH 10.0 to form a green color compound. The absorption was measured after 5 min at a wavelength of 690 nm. The concentration of ammonium chloride used in this study was in the range of 0.0047-0.047 mM.

For real sample analysis, five samples were taken from a local fish pond. The samples were adjusted to pH 8.0 using phosphate buffer prior to evaluation. The recovery tests were conducted by addition of known concentration of ammonium chloride to the real samples.

3. RESULTS AND DISCUSSION

3.1. Parameter optimization study on the biosensor sensitivity

The application of NADH-dependant dehydrogenase based biosensor for ammonium detection using bi-layer GLDH/diaphorase was evaluated. In this work the electrochemical oxidation of NADH was used as indicator reaction for quantification of ammonium concentration. The bi-layer GLDH/diaphorase biosensor works on the basis of the following sequence reaction:

$$NH_4^+ + \text{NADH} + \alpha\text{-ketoglutaric acid} \xrightarrow{\text{GLDH}} NAD^+ + \text{NADH}_{(\text{unreacted})} + \text{L-glutamate} + H_2O \quad (1)$$

$$\text{Diaphorase}_{ox} + \text{NADH}_{(unreacted)} \longrightarrow \text{Diaphorase}_{red} + NAD^+ + H^+ \quad (2)$$

$$\text{Diaphorase}_{red} + Fc^+ \longrightarrow \text{Diaphorase}_{ox} + FcH \quad (3)$$

$$FcH \longrightarrow Fc^+ + 2e^- + H^+ \quad (4)$$

In the presence of ammonium, GLDH catalyzed the conversion of α-ketoglutaric acid to L-glutamate. During the enzymatic reaction, NADH is oxidized to NAD^+. The consumption of NADH depends on the ammonium concentration present, which may result in excess of unreacted NADH. This excess of unreacted NADH is then oxidized to NAD^+ in the presence of diaphorase and mediator. Subsequently, the mediator is re-oxidized at the surface of the electrode [27] and the current produced corresponds to NADH concentration.

Electrocatalytic oxidation of NADH on bare electrode surface usually requires a high overpotential of about +0.70 V and this condition leads to electrode fouling due to adsorption of $(NAD)_2$ dimers on the electrode surface especially when high concentration of NADH was employed [28,29]. This problem can be minimized by using redox mediator that can act as a catalyst at a lower potential and favored simultaneous oxidation of NADH [16]. In order to improve NADH-electrooxidation, three types of mediators such as ferrocene, ferrocene carboxylic acid and ferrocene carboxaldehyde were tested. Fig. 1 illustrates cyclic voltammograms of 0.05 mM of respective ferrocene, ferrocene carboxylic acid and ferrocene carboxaldehyde at scan rate of 0.05 Vs^{-1}. The CV was recorded from potential of -0.5 V to +1.0 V at different NADH concentrations. As can be seen, a remarkable oxidation peak was recorded at +0.08 V, +0.2 V and +0.4 V *vs*. Ag/AgCl for ferrocene (a), ferrocene carboxylic acid (b) and ferrocene carboxaldehyde (c), respectively. At various concentrations of NADH studied it was observed that ferrocene carboxylic acid (b) and ferrocene carboxaldehyde (c) gave high current response indicating success of the electro-oxidation of NADH at respective applied potential. The highest current response towards NADH was obtained with ferrocene carboxaldehyde and it was used for further studies.

In order to confirm the optimum working potential of the biosensor, hydrodynamic voltammetry was performed with various concentrations of ammonium chloride (0.0-0.5 mM) in 50 mM phosphate buffer pH 8 containing ferrocene carboxaldehyde (0.05 mM), α-ketoglutaric acid (2 mM) and NADH (0.16 mM), respectively. The working potential ranged from +0.1 V to +1.0 V *vs*. Ag/AgCl. As shown in Fig. 2, maximum sensitivity was obtained at potential of +0.4 V. Therefore, applied potential of +0.4 V was employed for further studies.

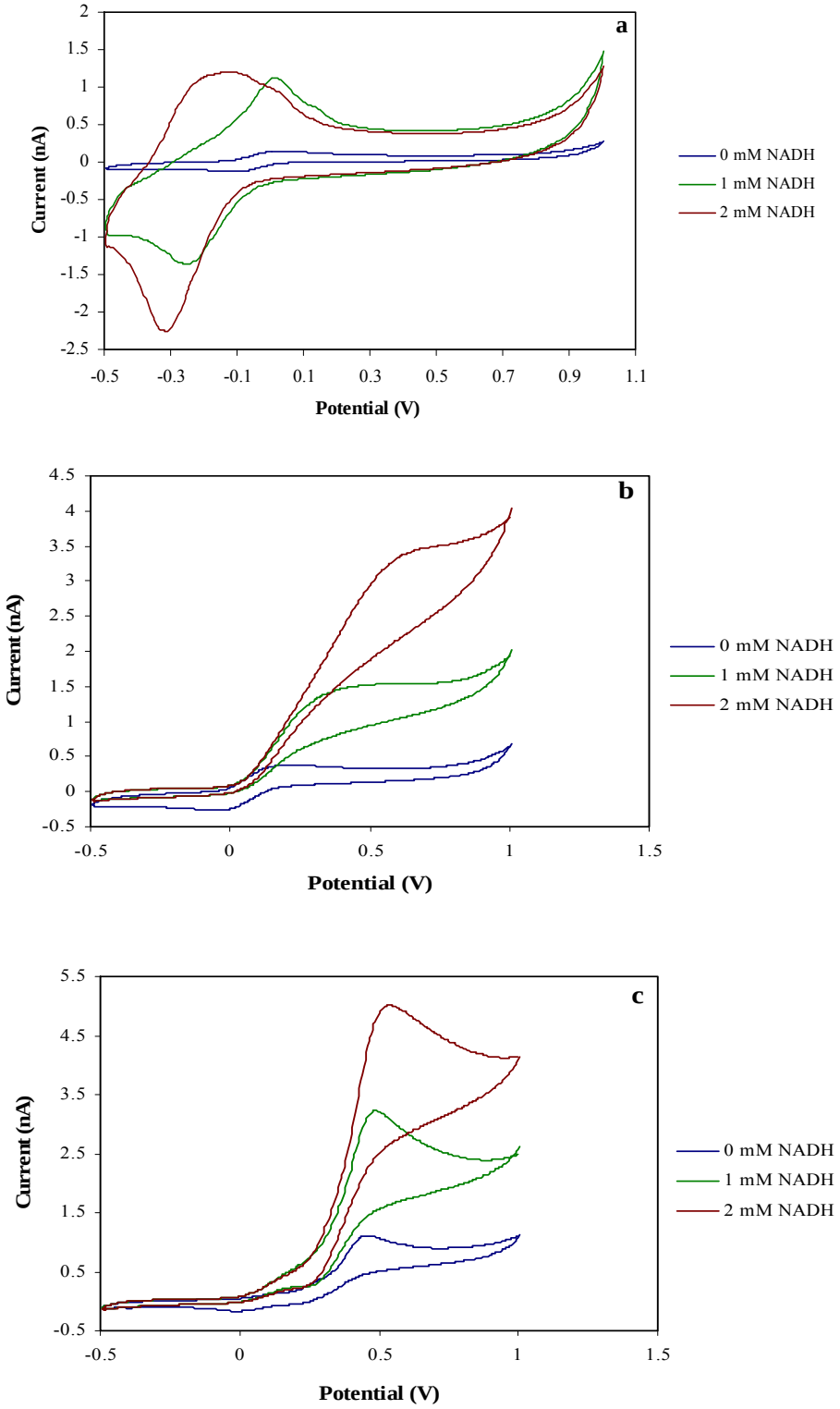

Fig. 1. Cyclic voltammogram of three types of ferrocene and its derivatives (a) 0.05 mM ferrocene, (b) 0.05 mM ferrocene carboxylic acid (c) 0.05 mM ferrocene carboxaldehyde in various concentrations of NADH. Scanning potential in the range of -0.5 V to +1.0 V with scan rate of 0.05 Vs^{-1} *vs*. Ag/AgCl

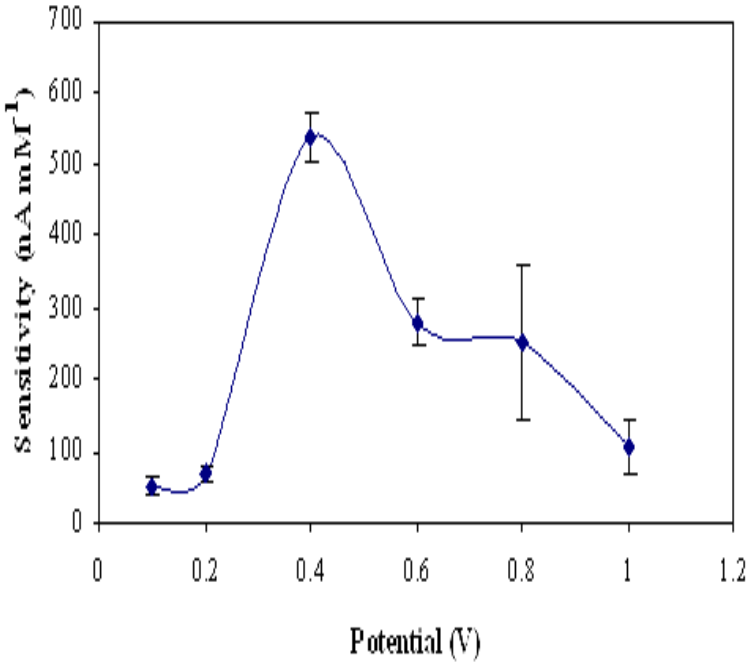

Fig. 2. Hydrodynamic voltammogram recorded by chronoamperometry based on 50 mM phosphate buffer pH 8 containing 0.05 mM ferrocene carboxaldehyde, 0.16 mM NADH, 2 mM α-ketoglutaric acid and NH_4Cl (0 to 0.5 mM), respectively

The effect of ferrocene carboxaldehyde concentrations on the sensitivity of the biosensor was also investigated in the concentration range of 0.02 mM to 0.15 mM. As illustrated in Fig. 3, the highest sensitivity of the biosensor was observed at ferrocene carboxaldehyde concentration of 0.03 mM. Further increase in the concentration of ferrocene carboxaldehyde results in saturation of enzyme-substrate kinetic behavior [30]. Thus, ferrocene carboxaldehyde concentration of 0.03 mM was selected for all subsequent experiments.

The influence of pH on the amperometric response of the biosensor was studied in the pH range between pH 5 to 9. The pH profile of the bi-layer GLDH/diaphorase modified SPCE showed that the maximum sensitivity was obtained at pH 8 (Fig. 4). Therefore, pH 8 was chosen in subsequent studies.

The effect of chitosan concentration on sensitivity of the biosensor for ammonium determination was evaluated in the concentration range of 0.1% to 3.0% (w/v). Fig. 5 shows the biosensor gave maximum sensitivity at chitosan concentration of 2% (w/v). High adsorption of enzyme was obtained due to strong affinity of chitosan towards protein [31]. Thus, chitosan concentration of 2% (w/v) was chosen for further studies.

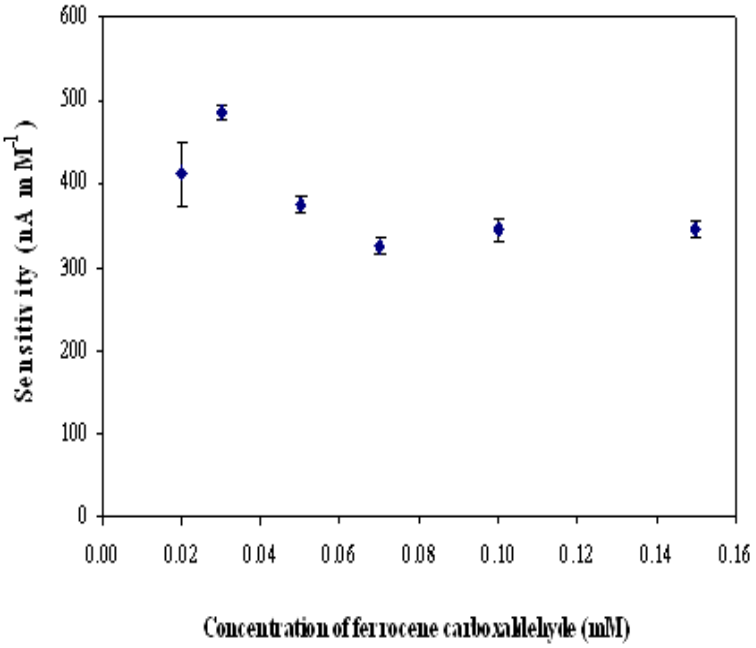

Fig. 3. Effect of ferrocene carboxaldehyde concentrations (0.02 mM to 0.15 mM) on the biosensor sensitivity. The experimental condition: 50 mM phosphate buffer pH 8 containing 2 mM α-ketoglutaric acid, 0.16 mM NADH and ammonium ranging from 0 to 0.5 mM. Potential was set at +0.4 V *vs*. Ag/AgCl

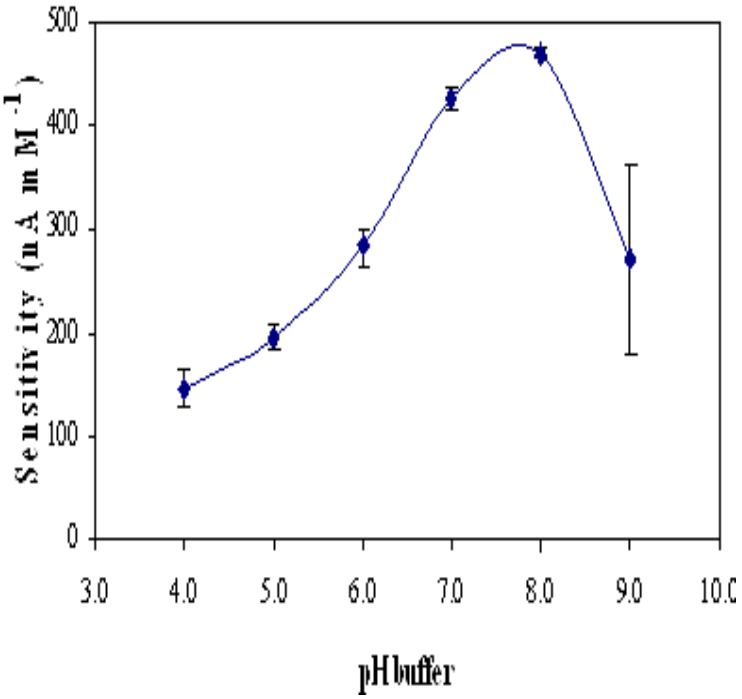

Fig. 4. Influence of pH on the biosensor sensitivity. Experimental condition: 50 mM phosphate buffer containing ferrocene carboxaldehyde (0.03 mM), NADH (0.16 mM), α-ketoglutaric acid (2 mM) and ammonium (0 mM to 0.5 mM), respectively. Potential was set at +0.4 V *vs*. Ag/AgCl

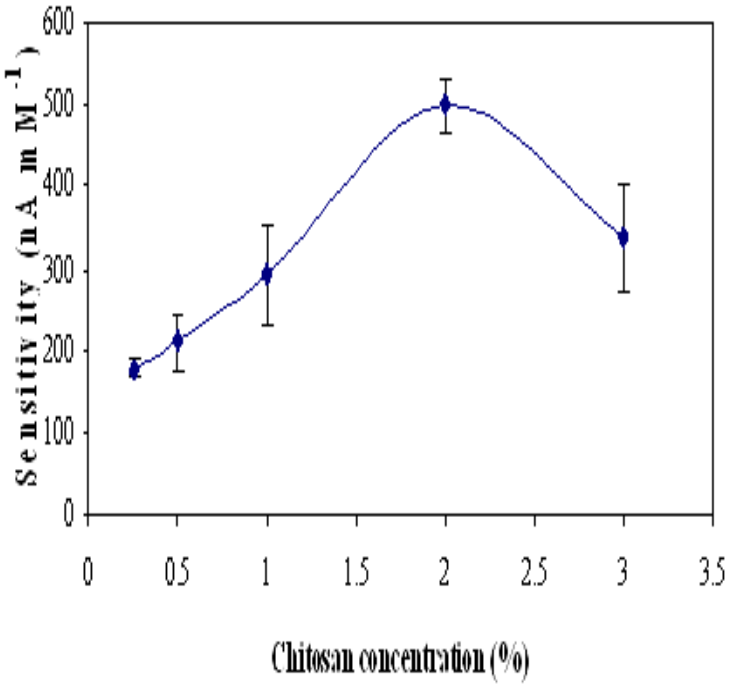

Fig. 5. Effect of chitosan concentration on the biosensor sensitivity. The reaction was conducted at 0.03 mM ferrocene carboxaldehyde, 0.16 mM NADH, 0.15 mM α-ketoglutaric acid and NH_4Cl (0 mM to 0.5 mM), respectively. Potential was set at +0.4 V *vs*. Ag/AgCl

The amount of enzymes loading employed can affect the sensitivity of the developed biosensor. In this work, bi-layer immobilization of GLDH and diaphorase in chitosan film was used. Fig. 6a and b shows the sensitivity of the modified biosensor with different enzymes (GLDH and diaphorase) loading. Upon increasing the enzymes loading, the sensitivity of the biosensor increased sharply to reach a maximum value at 0.05 mg for both enzymes studied. Thus, the enzyme loading of 0.05 mg for both GLDH and diaphorase were used for the preparation of the biosensing films.

To further improve the performance of the developed biosensor, the effect of different NADH and α-ketoglutaric acid concentrations were also evaluated. The influence of the biosensor sensitivity at various NADH concentrations is shown in Fig. 7. The sensitivity increased with increasing NADH concentration and reaches an optimum value at a concentration of 0.16 mM. Above the concentration of 0.16 mM, a decrease in sensitivity was observed. This behaviour may be due to an excess of NADH concentration which can competitively inhibit enzyme activity [32].

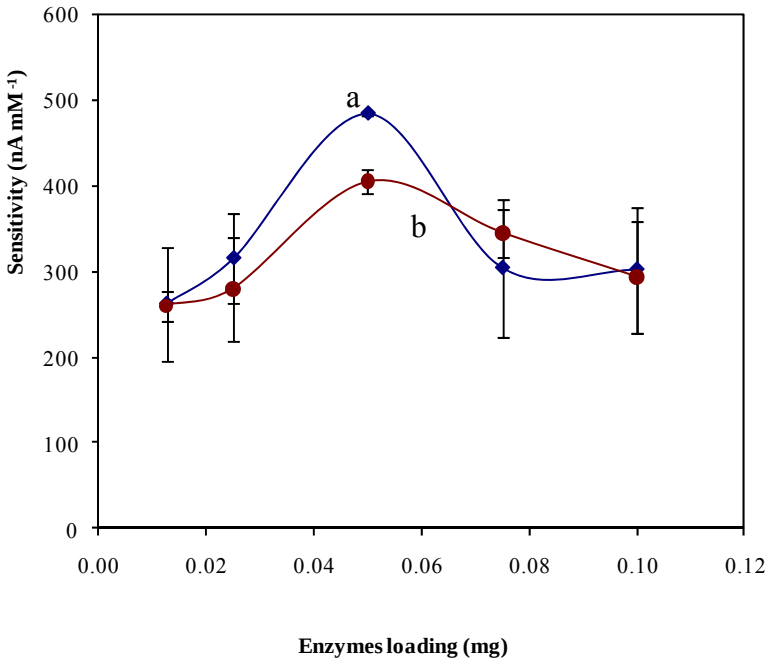

Fig. 6. Influence of GLDH (a) and diaphorase (b) concentrations. Sensitivity obtained from anodic current of 50 mM phosphate buffer pH 8 containing 0.16 mM NADH, 0.15 mM α-ketoglutaric acid, 0.03 mM ferrocene carboxaldehyde and NH_4Cl (0 mM to 0.5 mM) at potential of +0.4 V

The effect of α-ketoglutaric acid concentration on biosensor performance was further evaluated at various concentrations ranging from 0.1–3.0 mM. As shown in Fig. 8, the optimum concentration of α-ketoglutaric acid was obtained at 2.0 mM. Further increase in the concentration of α-ketoglutaric acid results in reduction of the biosensor sensitivity, which may attribute to an inhibitory effect of α-ketoglutaric acid on the enzyme activity [32].

3.2. Analytical performance

Under the optimized experimental conditions, calibration curve for ammonium chloride determination was studied at concentrations ranging from 2.5 µM to 1000 µM. A linearity response was obtained in the concentration range 2.5 µM to 500 µM (slope=0.5314, r=0.9974) with the detection limit of 2.5 µM (Fig. 9). The reproducibility of the fabricated biosensor was also evaluated and it was found to be good with a relative standard deviation (RSD) of 4.17 % (n=5).

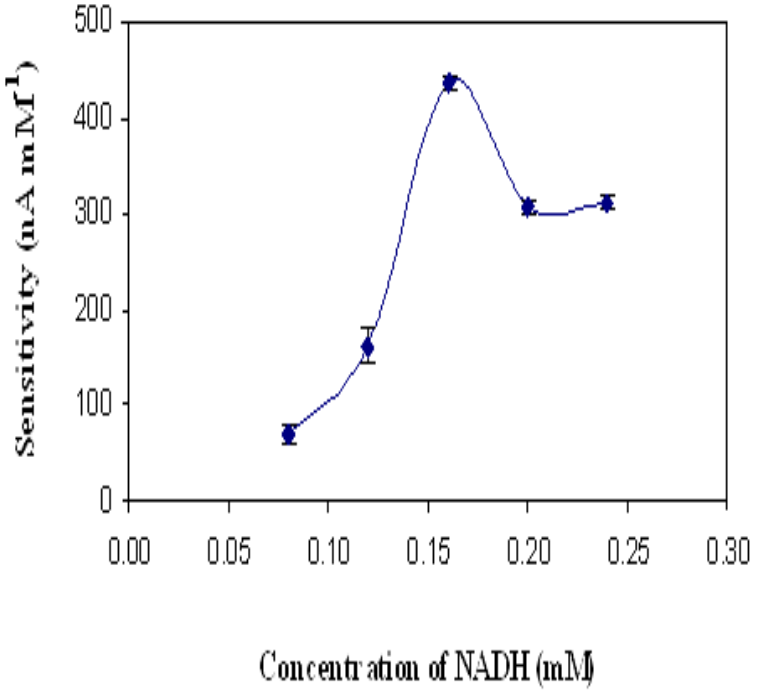

Fig. 7. Effect of NADH concentrations on the biosensor sensitivity. The reaction was carried out at 0.03 mM ferrocene carboxaldehyde, 0.15 mM α-ketoglutaric acid and NH_4Cl ranging from 0 mM to 0.5 mM. Potential was set at +0.4 V *vs*. Ag/AgCl

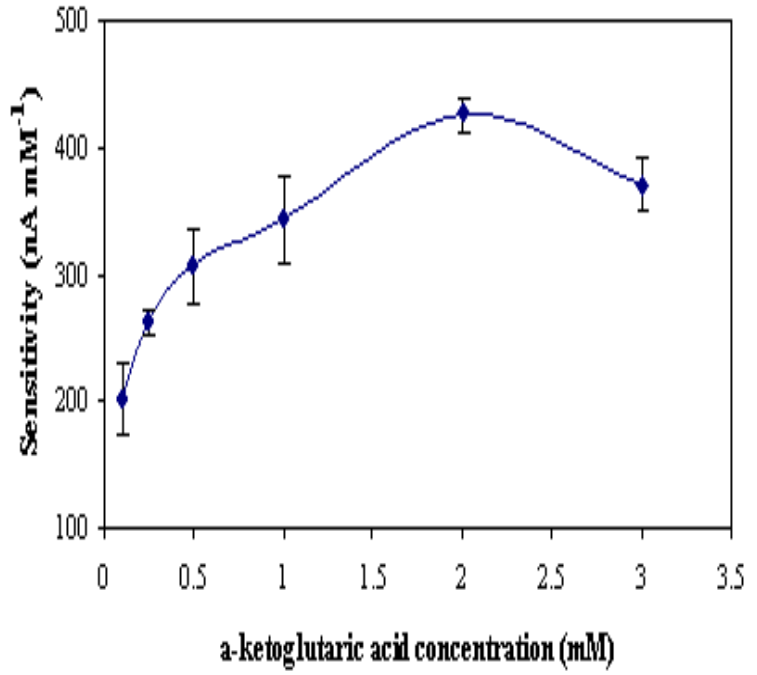

Fig. 8. Effect of α-ketoglutaric acid concentrations on the biosensor sensitivity. The concentration of ferrocene carboxaldehyde and NADH were fixed at 0.03 mM and 0.16 mM, respectively. Concentration of NH_4Cl ranging from 0 to 0.5 mM

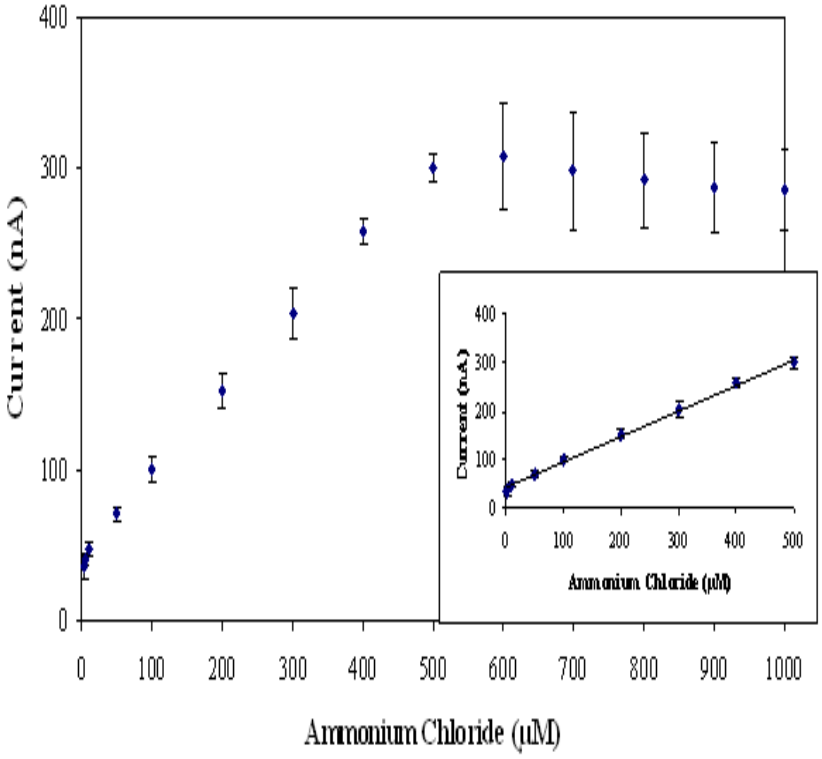

Fig. 9. Dynamic range of the biosensor towards different concentrations of NH_4Cl (0-1000 µM). Inset is the linearity of the biosensor towards NH_4Cl (2.5–500 µM)

A comparison study between the developed biosensor with the Indothymol method was conducted for the determination of ammonium chloride in the concentration range of 0.0047-0.047 mM. The results showed a very good agreement between these two methods with slope=0.9993 and r=0.9991 (Fig. 10).

In order to evaluate the application of the developed biosensor for ammonium determination, real sample from pond water was analyzed. The amperometric assay was performed on the sample solutions before and after spiking with known concentration of ammonium chloride. Table 1 summarized the results of unspiked and spiked water samples. It shows that the biosensor recovered about 86-111% of the ammonium chloride from the water samples. The recovery study of the biosensor was also compared with Indothymol method. The accuracy of the obtained results was confirmed using a t-test. Experimental values of t were always less than the critical value at 5% level, which means that there were not significant differences between the obtained values for both methods.

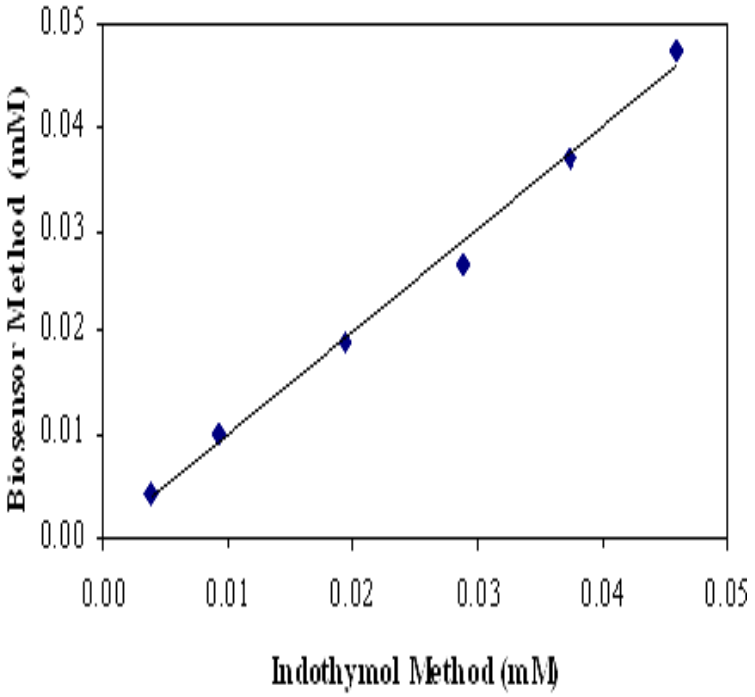

Fig. 10. Comparison between the biosensor method and the Indothymol method

Table 1. Determination of ammonium chloride in unspiked and spiked water samples employing Indothymol method and the biosensor

Water sample	Indothymol method (n=3)			Biosensor (n=3)			Calculated t-test
	Original (mM)	Added (mM)	Found (mM)	Original (mM)	Added (mM)	Found (mM)	
1	ND	0.028	0.038	0.009	0.028	0.035	0.0004
2	ND	0.028	0.042	0.012	0.028	0.043	0.0002
3	ND	0.028	0.047	0.015	0.028	0.039	0.0033
4	ND	0.028	0.042	0.014	0.028	0.043	0.0003

ND – none detected; n = 3; the critical value, t4 = 2.78 (p = 0.05)

4. CONCLUSIONS

The mediated amperometric biosensor based on bi-layer GLDH/diaphorase immobilized in chitosan film for the determination of ammonium was successfully developed. In the assay system, ferrocene carboxaldehyde was used as an electron transfer mediator to lower the potential of NADH to +0.4 V *vs*. Ag/AgCl. The biosensor displays good analytical performance with a simple examination procedure, low detection limit, good sensitivity and

reproducibility. As compared to the conventional method, the developed biosensor demonstrated its suitability for rapid analysis of ammonium in aqueous samples.

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