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Biosensor based on glutamate dehydrogenase immobilized in chitosan for the determination of ammonium in water samples

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ABSTRACT

An optical biosensor based on glutamate dehydrogenase (GLDH) immobilized in a chitosan film for the determination of ammonium in water samples is described. The biosensor film was deposited on a glass slide via a spin-coating method. The ammonium was measured based on β-nicotinamide adenine dinucleotide (NADH) oxidation in the presence of α-ketoglutaric acid at a wavelength of 340 nm. The biosensor showed optimum activity at pH 8. The optimum chitosan concentrations and enzyme loading were found to be at 2% (w/v) and 0.08 mg, respectively. Optimum concentrations of NADH and α-ketoglutaric acid both were obtained at 0.15 mM. A linear response of the biosensor was obtained in the ammonium concentration range of 0.005 to 0.5 mM with a detection limit of 0.005 mM. The reproducibility of the biosensor was good, with an observed relative standard deviation of 5.9% ($n = 8$). The biosensor was found to be stable for at least 1 month when stored dry at 4 °C.

Ammonia is a colorless gas at atmospheric temperature with a characteristic pungent odor. It can easily dissolve in water and become liquid ammonia or ammonium [1]. Ammonium is widely used in farming and in the chemical and automotive industries [2]. It is known to be toxic to various organisms. Even at low concentrations, ammonium can adversely affect aquatic life, causing injury to gill tissue, liver, and kidneys [3]. In excess, it can also be harmful to humans by causing coma and even death [4]. Therefore, ammonium is an important parameter in the assessment of water quality used for drinking and industrial processes. In Malaysia, the maximum concentration limit of ammonium in water recommended by the Department of Environment for the protection of public health is in the range of 0.1 to 2.7 mg/L [5].

Numerous methods have been used for the determination of ammonium at a lower concentration, typically requiring a chemical reaction to transform the analyte into a derivative amenable for colorimetric, fluorimetric, or chemiluminescent detection [6]. The reaction with indophenol-based reagents is still the most widely used technique for ammonium determination. However, this technique is time-consuming and involves a complicated sample pretreatment. Recently, many reports on biosensors employing a glutamate dehydrogenase GLDH)[1] enzyme for the determination of ammonium in water samples based on electrochemical methods have been reported [1,4,7]. However, not many studies have reported the application of an optical biosensor for ammonium determination [8]. An optical biosensor offers some advantages: (i) the electrode fouling problem is avoided, (ii) there is no interference from electroactive species, and (iii) there is no requirement of a reference electrode [9].

This article describes the use of GLDH derived on chitosan immobilization for the determination of ammonium in water samples using an optical method. The application of chitosan as an immobilization matrix for several types of biocatalysts in biosensor development has been revealed by several researchers [10–13]. Chitosan is a natural biopolymer product found in the exoskeleton of crustaceans, in fungal cell wall, and in other biological materials. It is formed by the deacetylation process of chitin [11]. Properties of chitosan such as biodegradability, nontoxicity, biocompatibility, antibacterial nature, high mechanical strength, good adhesion, and the presence of many amino and hydroxyl groups make it a promising matrix for enzyme immobilization [12,13]. Furthermore, chitosan is safe, abundant, and inexpensive.

Therefore, this work describes the development of an optical biosensor based on immobilization of GLDH in chitosan for the determination of ammonium. The GLDH–chitosan film was prepared by depositing a chitosan solution containing GLDH onto a microscope glass slide via a spin-coating technique. It was the aim of this study to prepare a simple, easily prepared, inexpensive disposable biosensor for the determination of ammonium in a water sample.

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[1] *Abbreviations used:* GLDH, glutamate dehydrogenase; NADH, β-nicotinamide adenine dinucleotide; UV, ultraviolet; HRP, horseradish peroxidase; RSD, relative standard deviation.

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Materials and methods

Reagents

GLDH, β-nicotinamide adenine dinucleotide (NADH), copper chloride, ferrous sulfate, zinc chloride, silver nitrate, mercury chloride, calcium chloride, potassium chloride, and sodium nitrate were purchased from Sigma. α-Ketoglutaric acid was obtained from Fluka. Chitosan was supplied by Chito-Chem (M) Sdn Bhd. Sodium nitroprusside was obtained from Merck. Thymol was acquired from BDH Chemicals. Sodium hypochlorite 10% was acquired from Systerm (ChemPur), and ammonium chloride was purchased from R & M Marketing. All chemicals were of analytical grade and used without further purification.

Preparation of stock solution and biosensor development

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 (1 mM) and α-ketoglutaric acid solution (2 mM) were prepared by dissolving 0.0035 g of NADH in 5 ml of phosphate buffer solution (pH 8, 50 mM) and 0.0029 g of α-ketoglutaric acid in 10 ml of phosphate buffer (pH 8, 50 mM), respectively. Then GLDH stock solution (40 mg/ml) was prepared by dissolving 0.012 g of GLDH powder in 300 μl of 50 mM phosphate buffer solution (pH 8). This solution was then divided into 20-μl aliquots and kept at −20 °C for later use. A homogeneous stock solution of GLDH/chitosan mixture was prepared by mixing GLDH solution (40 mg/ml) and chitosan solution (2%) at a volume ratio of 0.25:1.0 (v/v).

For enzyme immobilization, 10 μl of the GLDH/chitosan mixture was deposited onto a glass slide in an area of 9 × 10 mm. Then it was spun at 2000 rpm for 3 s. The biosensor was kept at 4 °C for drying.

Evaluation of biosensor response

All absorption measurements were conducted by using an ultraviolet (UV)–visible spectrophotometer (Varian Cary 50). The biosensor was soaked in 50 mM phosphate buffer solution (pH 8) for 5 min to remove unbound enzyme, and then it was washed with distilled water. It was then exposed to 1 ml of 50 mM phosphate buffer solution (pH 8) consisting of ammonium (1 mM), α-ketoglutaric acid (0.15 mM), and NADH (0.15 mM) for 10 min of reaction time. The response of the biosensor was studied at a fixed wavelength of 340 nm as described in Eq. (1):

$$\Delta\mathrm{Abs}(340\ \mathrm{nm}) = I_{0\ \mathrm{min}} - I_{10\ \mathrm{min}}, \quad (1)$$

where $I_{0\ \mathrm{min}}$ and $I_{10\ \mathrm{min}}$ are the absorbance intensities of the NADH when the biosensor was immersed in the solution at 0 and 10 min of reaction time, respectively.

Comparison of biosensor response and analysis of spiked real samples

The responses of the developed biosensor to various concentrations of ammonium ion were compared via a colorimetric (indothymol) method, which is based on the previous work reported by Moliner Martinez and coworkers [14]. For indothymol formation, standard ammonium ion solution was mixed with 0.017 M nitroprusside, 0.013% sodium hypochlorite, and 0.027 mM thymol. The absorption was measured after 5 min at a wavelength of 690 nm. The concentration of ammonium ion used in this study was in the range of 0.00 to 0.45 mM.

For the evaluation of the biosensor performance with real samples, five water samples were used for the analysis. The water samples were collected from the agricultural area in Labis, Johor, Malaysia. The water samples were adjusted to pH 8 with 50 mM phosphate buffer solution prior to the evaluation. The recovery tests for ammonium were conducted after the addition of a known concentration of ammonium to the water samples.

Results and discussion

Evaluation of biosensor response

The determination of ammonia in an aqueous solution employing immobilized GLDH has been reported previously [4,7]. As shown in Fig. 1, the immobilized GLDH requires the cofactor NADH and ammonium in the enzymatic conversion of α-ketoglutaric acid to L-glutamate. During the reaction, NADH is oxidized to NAD^+, thereby making possible the indirect monitoring of ammonium by measuring the consumption of NADH at a wavelength of 340 nm. Thus, the wavelength of 340 nm was used for monitoring the response of an optical biosensor.

The influence of pH on the biosensor response was investigated using 50 mM acetate, phosphate, and Tris–HCl buffer solutions at pH 5, pH 6 to 8, and pH 9, respectively (Fig. 2). The biosensor exhibited an almost bell-shaped pH profile within the pH range of 7 to 9. As can be seen in Fig. 2, the optimum biosensor response was obtained at pH 8. A similar finding was also observed by Quiles and coworkers [8], who immobilized GLDH on a controlled-pore glass for the determination of ammonium detection in plasma using a fluorimetric method.

The effect of chitosan concentration on the biosensor response was also determined by employing various concentrations of 0.25% to 3.00% (w/v). As shown in Fig. 3, the biosensor gave the optimum response at a chitosan concentration of 2% (w/v). Similar results were observed in our previous work [15], where optimum responses of the horseradish peroxidase (HRP) biosensor and the tyrosinase biosensor were obtained at a chitosan concentration of 2% (w/v). As reported, chitosan showed a strong affinity toward protein, which may permit high adsorption of the enzyme [16]. This suggests that the GLDH can be entrapped within the interstitial space of the water-insoluble chitosan biopolymer, with the biosensor exhibiting a high response. Therefore, in this study, a chitosan concentration of 2% (w/v) was sufficient for the enzyme immobilization.

Fig. 4 shows the effect of enzyme loading in a chitosan film on the biosensor response. On increasing the enzyme loading to 0.08 mg, the biosensor response increased steeply to reach a maximum value, indicating a saturation of enzyme in the film. Thus, 0.08 mg of GLDH was used for the preparation of the biosensing film.

The effect of NADH concentration on the biosensor response was also examined by varying the concentrations between 0.05 and 0.25 mM (Fig. 5). It can be observed that the optimum response was found at an NADH concentration of 0.15 mM. Absorption was decreased with concentrations above 0.15 mM. This may be attributed to an excess of NADH concentration, which can competitively inhibit GLDH activity [17].

The biosensor response was further characterized by optimizing the α-ketoglutaric acid concentration (Fig. 6). The α-ketoglutaric acid was used in the concentration range of 0.05 to 0.25 mM. It was found that α-ketoglutaric acid concentration of 0.15 mM was sufficient to obtain a maximum response in this system, and it was used for further experiments. Further increases in the concentration of α-ketoglutaric acid result in decreases in the biosensor response, probably owing to an inhibitory effect [17].

Fig. 7 shows the calibration curve of the biosensor over the ammonium ion concentration range of 0.005 to 1.0 mM. A linear

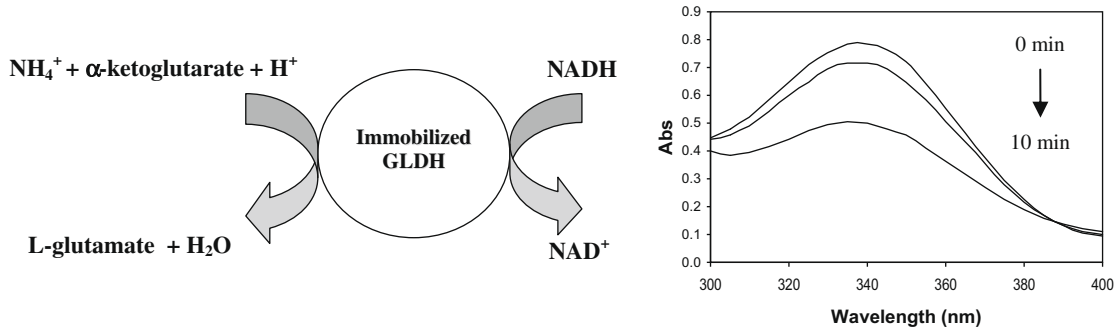

Fig. 1. Mechanism for ammonium ion catalyzed by immobilized GLDH in the presence of α-ketoglutaric acid and reduced NADH.

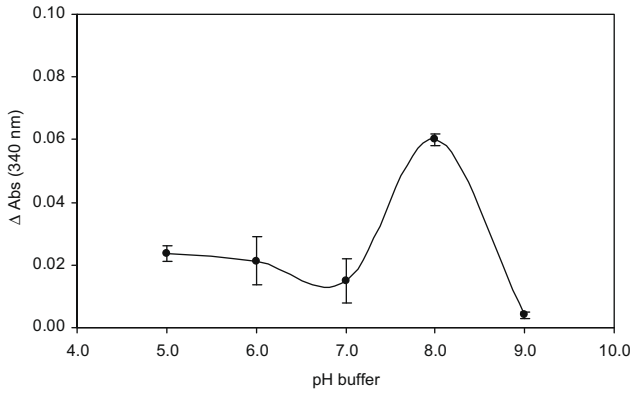

Fig. 2. Effect of pH on biosensor response. The sensor response was determined in the pH range of 5 to 9 using three different buffers (acetate: pH 5; phosphate: pH 6–8; Tris–HCl: pH 9). The concentrations of ammonium ion, α-ketoglutaric acid, and NADH were fixed at 3.0, 0.12, and 0.15 mM, respectively.

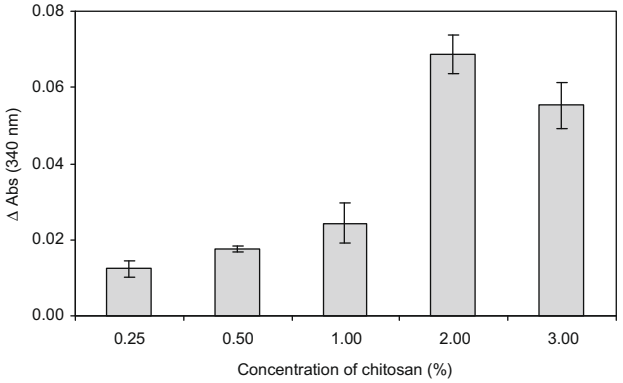

Fig. 3. Effect of chitosan concentration on biosensor response. The experimental conditions used were 3 mM ammonium ion, 0.12 mM α-ketoglutaric acid, and 0.15 mM NADH.

response was obtained at an ammonium concentration range of 0.005 to 0.5 mM (slope = 0.1619, R^2 = 0.9991). The detection limit was calculated based on the method reported by Day and Underwood [18] and Navaz Diaz and coworkers [19], and the value was calculated to be 0.005 mM. The reproducibility in the biosensor fabrication was investigated at ammonium ion, α-ketoglutaric acid, and NADH concentrations of 1.0, 0.15, and 0.15 mM, respectively. The reproducibility of the fabricated biosensor was found to be good with a relative standard deviation (RSD) of 5.9% (n = 8). The stability of the biosensor was also evaluated to be at least 1 month when stored dry at 4 °C. The biosensor retained approximately 70% of its initial activity after 1 month of storage at 4 °C.

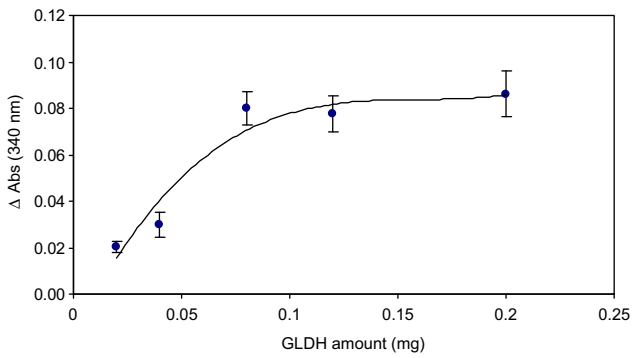

Fig. 4. Enzyme activity of film as a function of concentrations of enzyme immobilized in chitosan film (2%, w/v) at pH 8. The concentrations of ammonium ion, α-ketoglutaric acid, and NADH were fixed at 3.0, 0.12, and 0.15 mM, respectively.

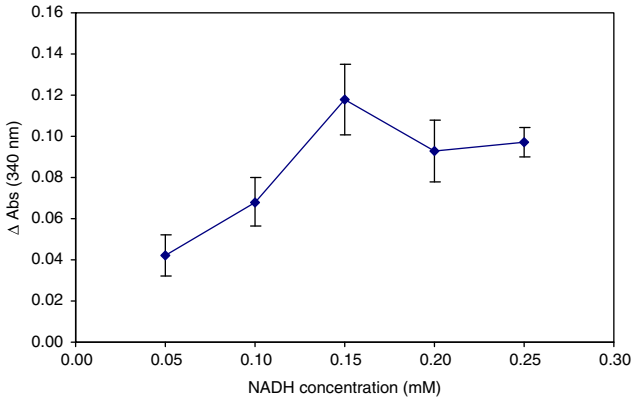

Fig. 5. Biosensor response with respect to various concentrations of NADH. The reaction was conducted at 0.15 mM α-ketoglutaric acid and 1.0 mM ammonium ion.

Interference studies

The effect of potential interferences on the biosensor response was also evaluated in this work. The interference studies were carried out for the following interfering ions that may be present in the environmental samples: Cu(II), Fe(II), Zn(II), Ag(I), Hg(II), Ca(II), K(I), and NO_3^-. These potential interfering ions were studied separately by adding a known concentration to the phosphate buffer solution (50 mM) at pH 8 containing 0.25 mM ammonium ion. The biosensor response was recorded in the presence of interfering ions, and the change of absorbance intensity is shown in Fig. 8. It can be noted that the ions such as Cu(II), Fe(II), Ca(II), K(I), and

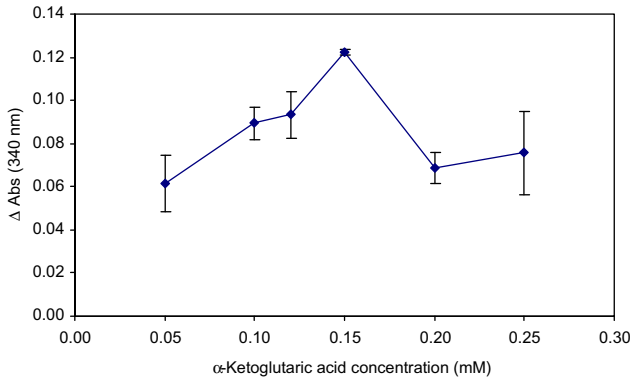

Fig. 6. Effect of α-ketoglutaric acid concentrations on biosensor response at fixed concentrations of NADH (0.15 mM) and ammonium (1.0 mM).

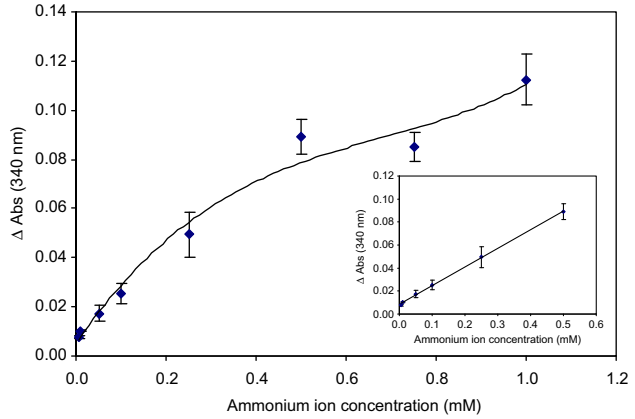

Fig. 7. Dynamic response range of biosensor toward different concentrations of ammonium ion. The inset shows the linearity range of the biosensor toward ammonium ion (0.005–0.5 mM).

NO_3^- did not show any significant interference given that the RSDs of the responses were within 5% of the response for ammonium ion alone at 0.25 mM. As can be seen in Table 1, Zn(II), Ag(I), and Hg(II) appear to cause interference. This could be caused by the inhibition effect of the metals at high concentrations on the enzyme activity. Thus, interference for these metals is not normally expected due to their low concentrations in natural water.

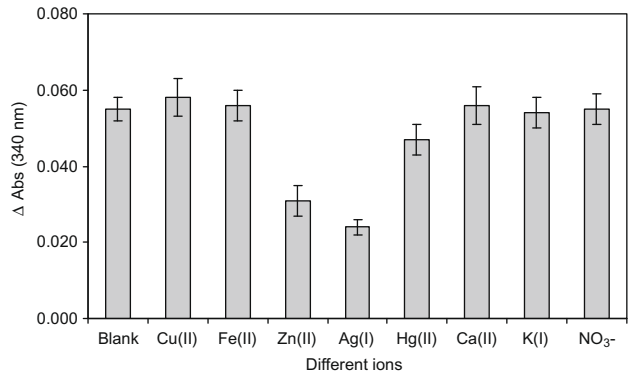

Fig. 8. Effect of different ions on ammonium response of biosensor. The concentrations of ammonium ion, α-ketoglutaric acid, and NADH were 0.25, 0.15, and 0.15 mM, respectively.

Table 1
Results of biosensor response toward different ions at pH 8 of 50 mM phosphate buffer solution.

Possible interferent	Concentration (mg/L)	Change of absorbance intensity (%)
Cu(II)	1.0	−5.0
Fe(II)	1.0	−1.8
Zn(II)	1.0	+43.6
Ag(I)	1.0	+56.4
Hg(II)	1.0	+14.5
Ca(II)	1.0	−1.8
K(I)	1.0	+1.8
NO_3^-	10.0	0.0

Note: The concentrations of ammonium ion, α-ketoglutaric acid, and NADH were fixed at 0.25 mM, 0.15 mM, and 0.15 mM, respectively.

Comparison of biosensor response and analysis of spiked real samples

Fig. 9 presents the results of the developed biosensor compared with the indothymol method for ammonium ion determination. The ammonium ion concentration used in this work was in the range of 0.00 to 0.45 mM. The results show very good agreement between the biosensor and the indothymol method, with slope = 0.9988 and $r = 0.9978$.

The performance of the biosensor for the analysis of water samples spiked with ammonium was tested. The water samples were collected from an agricultural area. For unspiked water samples, no ammonium ion was detected by the developed biosensor or the indothymol method. Therefore, the recovery test was performed by adding known concentrations of ammonium ion to the sample solutions. The results from the water sample recovery are summarized in Table 2. The results were compared with those measured using the indothymol method. The results demonstrate that the biosensor and the indothymol method recovered approximately 97% to 102% and 105% to 111% of the ammonium ion in the water samples, respectively. The relative error between the indothymol method and the biosensor for determination of ammonium ion in the spiked water samples was approximately 6% to 11%.

Statistical analysis for comparing the two means of the developed biosensor and the indothymol method was also evaluated. The statistical analysis was carried out using the method described by Miller and Miller [20]. As shown in Table 2, because the calculated values of $|t|$ are less than the critical value, the difference between the two methods is insignificant at the 5% level and the null hypothesis is retained. This result shows that the two methods used for the determination of ammonium ion in spiked water samples were in good agreement and comparable.

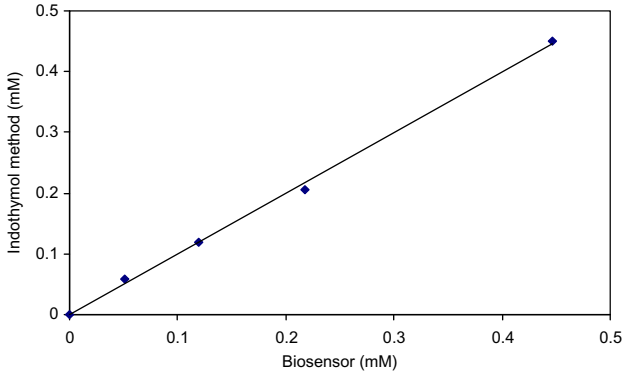

Fig. 9. Comparison between the indothymol method and the biosensor reported in this work.

Table 2
Determination of ammonium ion in spiked water samples using the indothymol method and the biosensor reported in this work.

Water sample	Indothymol method ($n = 3$)			Biosensor ($n = 3$)			Calculated t test
	Added (mM)	Found (mM)	Recovery (%)	Added (mM)	Found (mM)	Recovery (%)	
1	0.222	0.234	105.4	0.222	0.215	96.8	0.29
2	0.222	0.233	105	0.222	0.219	98.6	0.39
3	0.222	0.246	110.8	0.222	0.226	101.8	0.86
4	0.222	0.246	110.8	0.222	0.227	102.3	0.86
5	0.222	0.241	108.6	0.222	0.214	96.3	0.91

Note: The critical value, $t_4 = 2.78$ ($P = 0.05$).

Conclusion

In this study, the fabrication of an optical biosensor by using GLDH immobilized in a chitosan film was successfully developed. The biosensor showed good analytical performance within the dynamic range of 0.005 to 0.5 mM with a detection limit of 0.005 mM. There is no interference from many other ions that coexist with ammonium on the biosensor response except for Zn(II), Ag(I), and Hg(II). As compared with the indothymol method, the developed biosensor demonstrated its suitability for rapid analysis of ammonium in water samples. Other advantages of the biosensor method include simplicity in fabrication, low cost, good sensitivity, and no complicated sample pretreatment.

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