

Research Article

Preparation of an Amperometric Glucose Biosensor on Polyaniline-Coated Graphite

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Control of glucose concentration has tremendous significance in medical diagnosis, pharmaceuticals, food, and fermentation industries. Herein, we report on the fabrication of a facile, low-cost, and sensitive enzyme-based amperometric sensor using the electrochemically deposited polyaniline (PANI) film on a graphite electrode. PANI was deposited from an aqueous solution of 0.2 M aniline in 1.0 M hydrochloric acid (HCl) by cyclic voltammetry (CV). Surface morphology and composition characterization of the PANI film were carried out by scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS), and Fourier-transform infrared (FTIR) spectroscopy. Potentiostatic immobilization of glucose oxidase (GO_x) enzyme in the PANI film was carried out at 0.75 V to fabricate an amperometric glucose biosensor (GO_x/PANI/graphite biosensor). The glucose concentration response of the prepared sensor was studied amperometrically by detecting hydrogen peroxide (H₂O₂). The detection of H₂O₂ was optimized by calibrating the effects of pH, reduction potential, and background current. A reduction potential of -0.4 V at pH 6 was the best combination to get a maximum amperometric response of the GO_x/PANI/graphite biosensor. A stable current response was obtained in 4 min with a high reproducibility in linearity within the concentration range of 0.01 M–0.1 M D-glucose. Therefore, the fabricated GO_x/PANI/graphite biosensor could be a promising candidate for glucose sensing.

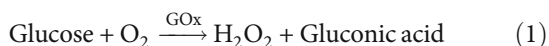
1. Introduction

Glucose concentration measurement is immensely important in food processing, fermentation reactors, pharmaceuticals processes, textile industry, environmental monitoring, and clinical laboratories [1–3]. Rapid determination of blood sugar is essential in the treatment and control of diabetes. There are numerous methods in practice for glucose determination, such as high-performance liquid chromatography (HPLC), polarimetry, capillary electrophoresis, gas chromatography, colorimetry, electrochemiluminescence, and biosensors [4, 5]. The use of glucose biosensors is preferred in the recent past due to its low cost, reliability, fast, and accurate response. The glucose biosensor based on the amperometric method is relatively a better method and easy to fabricate in the laboratory [6, 7].

The response of a glucose biosensor requires enzyme immobilization on the electrode surface. The surface used for immobilization should facilitate a smooth transfer of an electron from a biocomponent to the electrode surface, and therefore, it should impart biocompatibility to the electrode surface [8]. It should also enhance the response properties and be sensitive to perturbation so that a response is correctly recorded [8, 9]. In this regard, intrinsically conducting polymers containing self- π -conjugated system with either a double or a single bond alternating along the polymer chain have promising properties to be used in biosensing. The continuous conjugation in conductive polymers is responsible for remarkable electrochemical properties such as high electrical conductivity, low ionization potential, high electron affinities, and low energy optical transition [10–13]. Among conducting polymers, polyaniline (PANI) has gained keen

interest among researchers due to its dual nature as it creates an immobilization platform for biomolecules and acts as an electron mediator in enzymatic reactions the same time. Such properties make PANI the right candidate for biosensing applications [14–17]. It provides a high surface area and can be quickly deposited on the electrode surface with controlled film thickness [18].

Enzyme immobilization is tempting the researchers as it forms stronger adhesion between the enzyme and the matrix. In combination with a glucose oxidase enzyme (GOx), PANI is a potent combination of glucose sensors [15]. The GO_x adsorbed nicely on PANI by electrostatic interaction due to its negative charge. Such entrapment maintains the accessibility of catalytic sites and prevents the enzyme from leaching [19–22]. GOx is a dimeric protein containing one tightly bound flavin adenine dinucleotide (FAD) per monomer as a cofactor. Therefore, each enzyme has two FAD sites. The FAD can be released from the protein following the protein's partial unfolding since it is not covalently bound. The GOx-based biosensor catalyzes glucose's oxidation in the presence of oxygen to produce gluconolactone, which is hydrolyzed to gluconic acid and hydrogen peroxide (H₂O₂) [23, 24].



The H₂O₂ can further be oxidized or reduced and either way is used for glucose detection. Therefore, surface modification of electrode by a polymeric membrane is an ongoing research approach for improving the sensor's response.

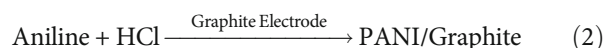
This paper describes a simple glucose biosensor fabricated by electropolymerization of aniline on graphite substrate and potentiostatic immobilization of GOx onto the PANI film on the graphite surface. A quantification of glucose concentration has been achieved by optimizing the response of the biosensor with pH, reduction potential, response time, and background current for the reduction of hydrogen peroxide obtained from enzyme-catalyzed oxidation of glucose.

2. Materials and Methods

2.1. Chemicals and Reagents. Analytical reagent grade aniline (C₆H₅NH₂), glucose oxidase (GOx), sodium monohydrogen orthophosphate (NaH₂PO₄·2H₂O), and D (+) glucose was purchased from Merck, USA. Hydrogen peroxide (H₂O₂), hydrochloric acid (HCl), and potassium nitrate (KNO₃) were acquired from Fischer Scientific, India. Similarly, a graphite rod of 99.99% purity was received from Nilako Corp., Japan. 1.0 M and 2.0 M HCl, 0.2 M C₆H₅NH₂, and 0.1 M KNO₃ solutions were prepared in double-distilled water. Also, buffer solutions of pH 5.0, 5.5, 6.0, 6.5, and 7.0 were adapted with a standard buffer preparation method [25].

2.2. Electrochemical Synthesis of PANI onto the Graphite Electrode. A graphite rod was polished with silicon carbide (SiC) paper starting from #1200 grit size down to #2000 and

ultrasonicated in double-distilled water for 10 min. It was then washed with distilled water and dried with compressed air. The electropolymerization of aniline on the graphite electrode was performed at room temperature (RT) using cyclic voltammetry (CV) in HCl. A saturated calomel electrode (SCE) was used as a reference and a Pt wire as a counter electrode. All the potential mentioned hereafter referred to SCE. The CV was carried out in the potential range of -0.4 V to 1.2 V at a 100 mV/sec scan rate for 10 cycles. Freshly prepared PANI/graphite electrode was washed with a buffer solution, dried under the compressed air, and used for immobilizing the enzyme. The process of PANI formation on the graphite electrode can be represented as



2.3. Characterization of the PANI Film. PANI film on the graphite surface was analyzed by X-ray photoelectron spectroscopy (XPS) (Thermo scientific sigma probe instrument) with a vacuum condition around 2×10^{-8} mbar with Al K α (1486.6 eV) at 400 μm beam size. Pass energy of 50 eV at the resolution of 0.1 eV was used for each scan. The surface was sputtered by Ar⁺ at 1.0 KeV for 2 min before each characterization to clean the surface. The spectra were decomposed with CasaXPS [26]. Similarly, the surface functionality of the prepared PANI was characterized by Fourier-transform infrared spectroscopy (FTIR) (Shimadzu Tracer-100) in the wavelength of 400–4000 cm⁻¹. Alike, the morphology of PANI was observed by scanning electron microscopy (SEM) (Hitachi SU 8000).

2.4. The PANI/Graphite Electrode Response to Hydrogen Peroxide (H₂O₂). The response of the PANI/graphite electrode to H₂O₂ was optimized concerning pH and potential. pH was optimized by recording the reduction current of H₂O₂ at -0.40 V in a phosphate buffer solution of pH 5.0, 5.5, 6.0, 6.5, and 7.0 with 0.1 M KNO₃ as a supporting electrolyte. A steady background current was also measured at -0.4 V without H₂O₂. Then, a reduction current at every addition of H₂O₂ was measured in the concentration range 1.0 mM to 25 mM. The difference in the current value ($\Delta i = i_b - i_a$) against the H₂O₂ concentration was determined, where Δi is the difference of current, i_b is the total current, and i_a is the steady background current.

The electrolyte was stirred uniformly by a PTFE covered magnetic bar during the experiment to get a rapid and reproducible current response. The optimization of potential used for cathodically reducing H₂O₂ was done by performing the experiment mentioned above at a fixed pH (pH 6.0) so that the optimized potential will be used in amperometric determination of glucose concentration.

2.5. Glucose Oxidase (GOx) Immobilization on the PANI/Graphite Electrode. The immobilization of GOx on the PANI/graphite electrode was carried out potentiostatically at 0.75 V for 40 min in a 0.1 M phosphate buffer solution (pH 6.0) containing 200 U/mL GOx and 0.1 M KNO₃ as a supporting electrolyte. Then, the film was carefully rinsed with distilled water to remove unattached enzyme. The

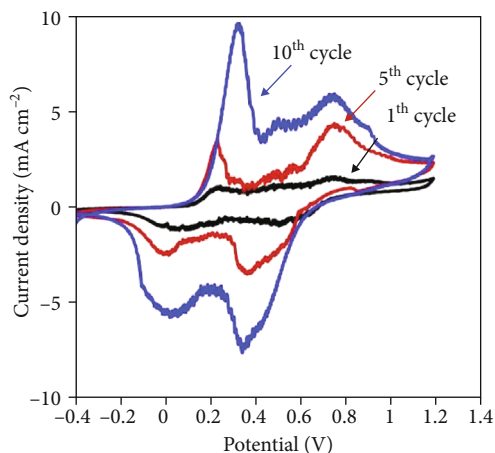
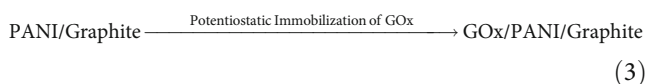


FIGURE 1: Cyclic voltammogram of the PANI/graphite surface. Experimental conditions: 0.2 M aniline monomer in 1.0 M HCl, potential limit -0.4-1.2 V, scan rate: 10 mV/s⁻¹, number of cycles=10.

prepared electrode is named as GOx/PANI/graphite biosensor in this article, and the process can be summarized as



2.6. Amperometric Response Measurements of Glucose Oxidation. The amperometric response of the prepared biosensor was studied by monitoring the concentration of D (+) glucose oxidized by GOx and response measured in terms of cathodic current due to H₂O₂ reduction. The GOx/PANI/graphite electrode was immersed in 0.1 M phosphate buffer of pH 6.0 containing 0.1 M KNO₃ as a supporting electrolyte. A potential of -0.4 V was applied to the electrode, and a stable background current was measured. After the background current reached a stable value, D (+) glucose solution was added dropwise to the buffer solution by using a micropipette. The oxidation of glucose by GOx resulted in the formation of H₂O₂, which was reduced at -0.4 V. The measurements were performed under hydrodynamic amperometric mode to have a faster and reproducible response. The response time of the biosensor was also optimized.

3. Results and Discussion

3.1. Electrochemical Synthesis of PANI onto the Graphite Electrode by CV. Figure 1 shows the cyclic voltammogram of PANI deposition on a graphite electrode. Three subsequent peaks observed in the potential range studied show that the PANI is redox-active. The first peak appeared at a 0.20 V potential, representing the transformation of sufficiently reduced form of leucoemeraldine into a partly oxidized emeraldine state. The peak occurring at approximately 0.80 V corresponds to emeraldine transformation into pernigraniline, the fully oxidized form of polyaniline [27]. The redox peaks at 0.50 V appeared between the two dominant peaks is generally attributed to the transformation of hydroquinone to p-benzoquinone [28]. The leucoemeraldine form has nitro-

gen atoms in the amine state, whereas all nitrogen atoms in pernigraniline are in the imine state. These two forms are not conductive. The conductive form is obtained when the ratio of amine to imine nitrogen is 1 and depending on the pH value.

3.2. Characterization of PANI. Surface composition of the PANI/graphite electrode was characterized by XPS measurement. A thick PANI coating was developed for the XPS analysis in order to avoid the graphite substrate detection, and the electrode was rinsed with water and ethanol to avoid mass protonation. High-resolution spectra of C1s and N1s are generally used to understand the state of PANI. Figure 2(a) shows the high-resolution C1s spectra together with four symmetric deconvoluted peaks. The C1s peaks at 284.75 eV, 285.49 eV, 286.44 eV, and 288.81 eV appeared after fitting. The main peak at 284.75 eV is attributed to the C-C/C-H groups present in the aniline polymer. The peak at 285.49 eV is due to the CN/C=N groups. Similarly, the peak observed at 286.49 eV is related to the CN⁺/C=N⁺ groups. The peak at 288.81 eV might contain C=O/C-O groups due to the contaminations [29, 30]. The two dominated peaks at 284.75 eV (C-C/C-H) and 285.49 eV (CN/C=N) account for approximately 36% and 35% contributions from PANI. The peak at 286.49 eV for CN⁺/C=N⁺ (15%) represents the extensive polymerization of aniline for PANI formation. A relatively high percentage of C-C/C-H and CN/C=N might also indicate for the mixed state, emeraldine, of PANI.

N1s spectra provide more precise information about the PANI structure since amine, imine, and protonated nitrogen can be distinguished (Figure 2(b)). The N1s spectra show three asymmetrical peaks located at 399.1 eV, 399.8 eV, and 401.86 eV, which are assigned for neutral imine, amine, and positively charged nitrogen, respectively [31–33]. The tentative compositional ratio of the three nitrogen compounds imine, amine, and a positively charged nitrogen are 30%, 60%, and 10%, respectively. A large and broad signal at high binding energy is indicative of positively charged nitrogen atom N⁺. The preferential protonation of imine sites, which are more basic, has resulted in a relatively reduced imine component. This result further confirmed the emeraldine as a dominant form in the coated layer.

Figure 2(c) represents a SEM image of the PANI/graphite surface. The deposition of tiny particles of PANI in rod-like shape is apparent. Also, spherical agglomerations of these particles forming coral-like island structure are present. Such a structure is assumed to be a good site for the immobilization of enzymes. Figure 2(d) shows the FTIR spectrum of PANI/graphite. The FTIR peaks at 1600 cm⁻¹ and 1490 cm⁻¹ show C=C stretching vibration of a quinoid structure and a benzene ring, respectively. Similarly, the characteristic peaks at 1302 cm⁻¹, 1150 cm⁻¹, and 810 cm⁻¹ suggest stretching vibration of C-N, C=N, and C-H in-plane. These values are in agreement with the reported values elsewhere [34–36].

3.3. Response of the PANI/Graphite Electrode to H₂O₂. The glucose biosensor works based on detecting H₂O₂. Therefore, optimization of the PANI/graphite electrode response to

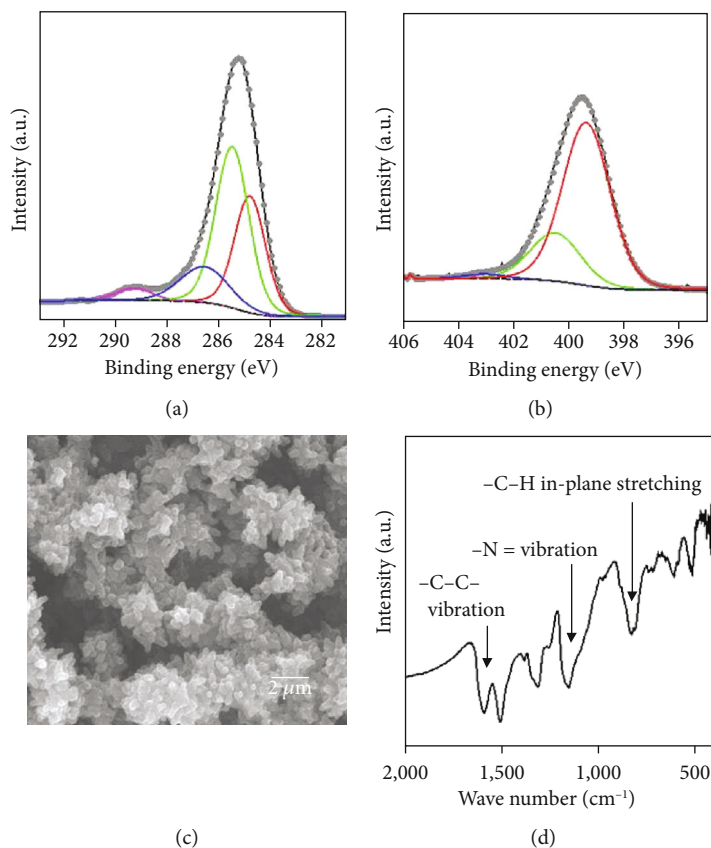


FIGURE 2: (a) High-resolution XPS spectra of C1s, (b) N1s of the PANI/graphite electrode, (c) SEM image of PANI, and (d) FTIR of the PANI/graphite electrode.

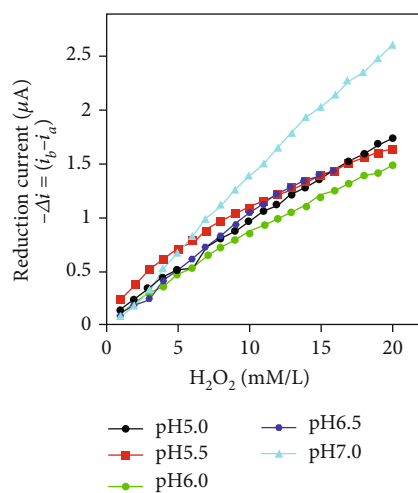


FIGURE 3: Reduction current vs. concentration of H₂O₂ at different pH on the PANI/graphite electrode.

H₂O₂ at different pH was essential. In this regard, the reduction potential for H₂O₂ was set at -0.4, and the resulting current with variable H₂O₂ concentrations at different pH was being measured. The reduction of H₂O₂ takes place as

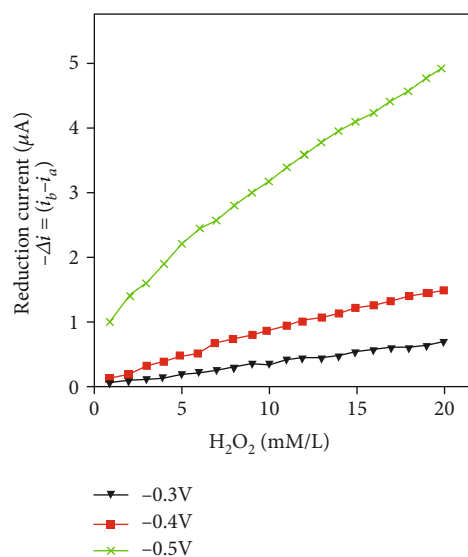
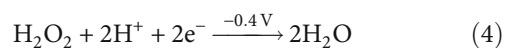


FIGURE 4: Reduction current vs. concentration of H₂O₂ at the PANI/graphite electrode at various applied potentials of -0.3 V, -0.4 V, and -0.5 V at pH 6.0.

A graphical representation of the response current of the PANI/graphite electrode against H₂O₂ concentrations at different pH is shown in Figure 3. A linear current response with H₂O₂ concentration except pH 5 is observed. It is worth mentioning that the optimum pH stability range for GOx

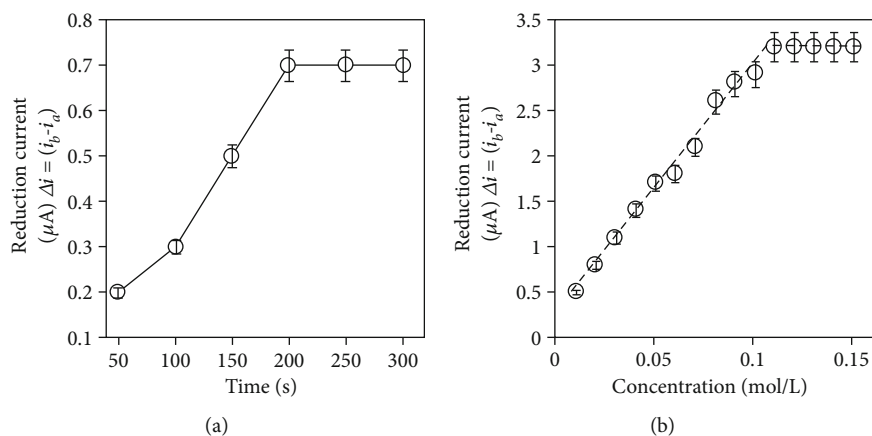


FIGURE 5: Response of biosensor: (a) reduction current vs. time and (b) reduction current vs. concentration of glucose.

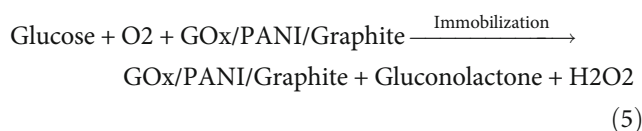
enzyme is pH 5.5 to 7.0. From the current response, the pH 6.0 was selected for biosensor response measurement due to the better conductivity and stability of polyaniline in a slightly acidic medium. The enzyme GOx is also stable in this pH, and H_2O_2 can be reduced.

3.4. Effect of H_2O_2 Reduction Potential on the Response of the PANI/Graphite Electrode. After optimizing the pH for H_2O_2 reduction, the next step was to scrutinize the reduction potential for obtaining an optimum test condition for H_2O_2 reduction. The potential of the PANI/graphite electrode was set at -0.3, -0.4, and -0.5 V, at the fixed pH 6.0 in each measurement. From the plot in Figure 4, the response current is found to increase with potential becoming negative. However, at -0.5 V, a nonlinear current is obtained at a lower concentration of H_2O_2 , which can be detrimental in the lower detection limit of the sensor. Therefore, an optimum response is chosen at -0.4 V with the view that it also reduces the effect of interferences, and that a better sensitivity of the enzyme could be possible.

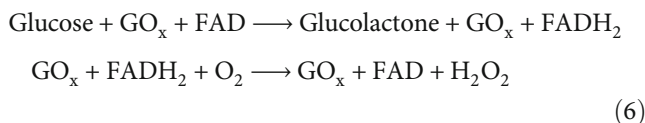
3.5. Response of the GOx/PANI/Graphite Biosensor. After immobilization of GOx in PANI/graphite, the response of the electrode towards glucose oxidation was studied by measuring the reduction current of H_2O_2 produced from glucose oxidation catalyzed by the enzyme GOx. First of all, the time taken for a stable current response in the presence of a fixed amount of D (+) glucose at pH 6.0 buffer solution was studied. Figure 5(a) shows initially the current increased gradually, and a steady current obtained after 4 min. Therefore, the time required to get a total oxidation of glucose by GOx and subsequent reduction of H_2O_2 is 4 min.

After evaluating the response time of the biosensor, its response to glucose concentration was studied. Figure 5(b) represents a response of glucose biosensor against D (+) glucose concentration. A linear response is obtained within the concentration range of 0.01 M to 0.1 M. Above 0.1 M concentration, the current became constant due to saturation of electrode surface, and therefore, the biosensor response could not be used to quantify the glucose concentration. The linearity clearly shows that lower detection limit can be easily brought down by measuring the lower current values.

When the glucose solution is added, it is oxidized by GOx of the GOx/PANI/graphite electrode into the gluconic acid and H_2O_2 . This hydrogen peroxide is detected at -0.4 V due to a cathodic response. The overall response of the sensor toward hydrogen peroxide can be written as



The fundamental concept of the glucose biosensor is that the immobilized GOx catalyses the oxidation of glucose to gluconolactone and hydrogen peroxide by molecular oxygen as an electron acceptor. In this process, gluconolactone is nonenzymatically hydrolysed to gluconic acid. GOx, working as a catalyst, needs a redox cofactor-flavin adenine dinucleotide (FAD). FAD acts as the initial electron acceptor and is reduced to FADH_2 . The FADH_2 is then oxidized by dissolved O_2 producing H_2O_2 and transforming the enzyme to its initial state containing FAD [37–39].



It can be expected that the reversible two-electron redox reaction takes place at the GOx/PANI/graphite electrode that is attributed to FAD, which is tightly bonded to the active site of the GOx, and/or to FAD, which is loosely bonded to the active site of the GOx or is even diffused for the GOx active site [40]. The electrode easily recognizes the number of electron transfers, and this electron flow is proportional to the number of glucose molecules. Alternatively, an electron-transfer mediator, which has a lower oxidation potential, can bridge the electrical communication between glucose oxidase and glucose.

The results show that by correlating with the response current, the concentration of glucose is analyzed. The concentration analyzed by thus fabricated amperometric biosensor is comparable to other reported glucose biosensors [1, 15,

22, 24], and hence, it can be recommended for practical application for glucose determination.

4. Conclusion

This study describes the fabrication of a simple, sensitive, and low-cost glucose sensor ($\text{GO}_x/\text{PANI}/\text{graphite}$) by employing conducting polymer as an entrapment surface for the enzyme. The electrodeposited polyaniline surface has a coral-like island structure, making it a good site for the immobilization of enzymes. The sensor gives an optimum amperometric response to D (+) glucose in the concentration range of 0.01 M–0.1 M at pH 6.0 and the potential of -0.4 V. A stable sensor response is obtained in 4 min, and it gives reproducible results.

Data Availability

The additional data and information of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no competing interests.

Acknowledgments

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