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An Ultrasensitive Electrochemical Enzymatic Urea Biosensor Based on Aniline/N-Butyl Acrylate Conducting Polymer-Modified Screen-Printed Electrode

(Biosensor Urea Elektrokimia Enzimatik Ultrasensitif Berasaskan Elektrod Bercetak Skrin Terubah Suai Polimer Berkonduksi Anilina/N-Butil Akrilat)

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ABSTRACT

An enzymatic electrochemical biosensor for urea detection was developed using the succinimide-modified aniline/n-butyl acrylate (nBA) conducting polymer. This aniline/nBA conducting polymer was synthesized by photopolymerization with the succinimide moiety incorporated during the photocuring procedure. The urease enzyme originating from Jack beans was chemically grafted on the succinimide-functionalized aniline/nBA conducting polymer, which was attached to a screen-printed carbon paste electrode (SPE). The enzymatic hydrolysis of urea by the urease electrode diminished the voltammetric biosensor response as a result of the cascaded chemical reaction between the enzymatically hydrolyzed hydroxide (OH^-) ion and the $K_3Fe(CN)_6$ redox species that generating the side products ($Fe(OH)_3$, CN^- ion, and KCN), which impeded the electron transfer of the redox mediator at electrode-electrolyte interface. In view of the amount of side products produced was proportional to the urea concentration associated in the enzymatic reaction with the immobilized urease enzymes, this has allowed the proposed enzymatic biosensor to demonstrate an inverse sensitivity concept of detecting urea concentration in an ultrasensitive manner. The electron transfer rate constant (k) of the urease electrode based on aniline/nBA hybrid material at the electrode-electrolyte interface was determined at 5.374×10^{-5} cm s^{-1}. The linear response range of the enzymatic urea biosensor was obtained from 1×10^{-10} mM to 1×10^{-1} mM (R^2=0.9834) by differential pulse voltammetry (DPV) with a limit of detection of 4.72×10^{-11} mM at pH 5.0 and enzymatic hydrolysis time of 30 min. The voltammetric urea biosensor response showed good reproducibility with a promising relative standard deviation (RSD) acquired at 5.0% (n=9). The ultra-high sensitivity performance of the developed enzymatic biosensor based on aniline/nBA conducting polymer towards determination of urea concentrations at low levels has demonstrated superior performance across previously reported electrochemical urea biosensors based on various nanostructured conducting materials.

Keywords: Conducting polymer; electrochemical transducer; succinimide-modified polymer; urea biosensor

ABSTRAK

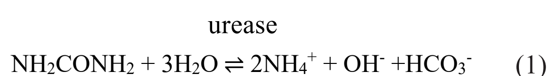
Biosensor elektrokimia enzimatik untuk pengesanan urea telah dibangunkan menggunakan suksinimida terubah suai polimer berkonduksi anilina/n-butil akrilat (nBA). Polimer berkonduksi anilina/nBA ini adalah disintesis melalui pempolimeran foto dengan bahagian suksinimida digabungkan semasa prosedur pempolimeran foto. Enzim urease yang berasal daripada kacang *Jack* telah diikat secara kimia dengan kumpulan berfungsi suksinimida pada polimer berkonduksi anilina/nBA yang dipegun pada elektrod karbon bercetak skrin (SPE). Hidrolisis enzimatik urea oleh

elektrod urease mengurangkan rangsangan biosensor voltammetrik daripada tindak balas kimia melata antara ion hidroksida (OH^-) terhidrolisis secara enzimatik dan spesies redoks $K_3Fe(CN)_6$ yang menghasilkan produk sampingan, ($Fe(OH)_3$, ion CN^- dan KCN), yang menghalang pemindahan elektron pengantara redoks pada antara muka elektrod-elektrolit. Memandangkan jumlah produk sampingan yang dihasilkan adalah berkadaran dengan kepekatan urea yang terlibat dalam tindak balas enzimatik dengan enzim urease pegun, ini telah membenarkan biosensor enzim yang dicadangkan untuk menunjukkan konsep kepekaan songsang untuk mengesan kepekatan urea secara ultrasensitif. Pemalar kadar pemindahan elektron (k) elektrod urease berasaskan bahan hibrid anilina/nBA pada antara muka elektrod-elektrolit diperoleh pada 5.374×10^{-5} cm s^{-1}. Julat rangsangan linear biosensor urea enzimatik diperoleh daripada 1×10^{-10} mM hingga 1×10^{-1} mM (R^2=0.9834) dengan kaedah voltammetri denyutan pembeza (DPV) dengan had pengesanan 4.72×10^{-11} mM pada pH 5.0 dan masa hidrolisis enzimatik selama 30 min. Rangsangan biosensor urea voltammetrik menunjukkan kebolehulangan yang baik dengan sisihan piawai relatif (RSD) yang menggalakkan diperoleh pada 5.0% (n=9). Prestasi kepekaan ultra tinggi biosensor enzimatik yang dibangunkan berasaskan polimer berkonduksi anilina/nBA terhadap penentuan kepekatan urea pada kepekatan rendah telah menunjukkan prestasi unggul merentas biosensor urea elektrokimia yang dilaporkan sebelum ini berasaskan pelbagai bahan berkonduksi berstruktur nano.
Kata kunci: Biosensor urea; polimer berkonduksi; polimer terubah suai suksinimida; transduser elektrokimia

INTRODUCTION

The determination of urea is of great interest in fields such as the pharmaceutical and food industry, environmental protection, and fertilizers, but its most important application is in the biomedical and clinical analysis (Alizar, Lee & Musa 2011; Ertell 2006). Urea is included in the non-toxic category, but it has been noted to cause mild irritation to the skin, lungs, and eyes. Urea is permitted as a component of animal feed to provide a source of nitrogen nutrition and is accepted as a safe food additive (Ertell 2006). Although non-toxicological, analysis of urea in foodstuffs and finished products is essential to prevent the use of unauthorized nitrogen sources in the food and pet food industry. Based on the enzymatic method of analysis, the content of urea in pet food is below 0.1 mg g^{-1} sample (1.67×10^{-3} mmol g^{-1} sample) (Pibarot & Pilard 2012). Besides that, the level of urea in the blood can indicate several diseases related to liver and kidney disorders, whereby a decrease in kidney function can be detected through the content of urea in the blood (Öndeş et al. 2020). The normal urea level in serum is 8–20 mg dl^{-1} (1.3 to 3.5 mM) (Rajesh et al. 2005). Elevated urea concentrations could lead to renal failures, such as acute or chronic urinary tract obstruction with shock, burns, dehydration, and gastrointestinal bleeding (Bozgeyik et al. 2011). Therefore, the diagnosis of kidney function can be made by analyzing the amount of urea contained in the blood (Hao, Das & Yoon 2015; Kuo, Dong & Chen 2021; Rahmanian & Mozaffari 2014).

The analytical methods commonly used to detect urea in pet food, blood, and urine are gas chromatography, colorimetry, fluorimetry, conductometry, and electrochemistry. However, conventional methods such as chromatography and colorimetry analysis sustained from tedious sample preparation procedures, expensive equipment, the need for skilled equipment operators, and lengthy analysis times (Rahmanian & Mozaffari 2014). There are several electrode-based urea biosensors have been developed based on modified membranes or urease-immobilized nanomaterials as the bioreceptors. The immobilized urease bioreceptor catalyzes the enzymatic hydrolysis reaction of urea, which produces ammonium, hydroxide, and bicarbonate ions (Bozgeyik et al. 2011; Dervisevic & Nyangwebah 2017) as shown in the enzymatic equation as follows,



$$NH_2CONH_2 + 3H_2O \xrightleftharpoons{\text{urease}} 2NH_4^+ + OH^- + HCO_3^- \quad (1)$$

The source of urease enzyme which often used as a bioreceptor to design urea biosensors is from *Canavalia enformis* (jackbean) (Alizar, Lee & Musa 2011; Farzaneh et al. 2023; Sankoet al. 2023). The polymers and nanomaterials that have been formulated to function as the immobilization sites for enzyme molecules in the development of urea biosensors are such as poly(2-hydroxyethyl methacrylate-glycidyl methacrylate) nanoparticles (Öndeş et al. 2020); poly(N-3-aminopropyl pyrrole-co-pyrrole) (Rajesh et al. 2005), poly(N-

glycidylpyrrole-co-pyrrole) (Bozgeyik et al. 2011), and poly(o-phenylenediamine) copolymer films; ZnO-polyvinyl alcohol nanostructured hybrid film (Dervisevic & Nyangwebah 2017); functionalized polyaniline thin film (Mello & Mulato 2020); and zirconia-poly(propylene imine) dendrimer nanocomposite (Shukla et al. 2014). These electrode modification materials require elaborate preparation steps in order to immobilize enzyme molecules; besides, the temperature factor plays a role in the preparation of membrane electrodes.

In this study, a facile surface modification material, namely aniline/n-butyl acrylate (aniline/nBA) hybrid material, is introduced that requires a straightforward preparation method simply by undergoing a quick photocuring process within a short period without temperature requirement. The proposed aniline/nBA hybrid matrix is ultilized as both the enzyme-supporting matrix and the conducting element at the carbon-based screen-printed electrode (SPE) surface in the construction of an enzymatic electrochemical biosensor for ultrasensitive detection of urea. The aniline/nBA matrix is a conducting polymer, this can facilitate the transfer of electrons from the electrode to the analyte and can increase the sensitivity of the biosensor. As previously mentioned, the sensitivity of the biosensor can be raised by adding gold nanoparticles to nBA microspheres as a conduction material (Alizar et al. 2014).

Materials and Methods

Electrodes and Apparatus

Electroanalysis based on cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were performed using potentiostat/galvanostat PGSTAT 12 (Autolab, Metrohm). Screen-printed carbon paste electrode (SPE) from Scrint Technology Pvt. Ltd. was used as the working electrode. A rod-shaped glassy carbon and Ag/AgCl electrodes were used as auxiliary and reference electrodes, respectively. The KCl solution at 3.0 M was used as the internal solution of the Ag/AgCl electrode. All the potentials measured in this study were referred to the Ag/AgCl electrode. The homogeneous mixtures of material solutions were prepared using a sonicator bath (Elma S30H).

Chemicals

N-butyl acrylate (nBA), 2-2-dimethoxy-2-phenylacetophenone (DMPP), ethylene glycol dimethacrylate (EGDMA), and 1,6-hexanediol diacrylate (HDDA) were supplied by Aldrich. Urea and urease enzyme (EC 3.5.1.5; 26.1 units mg^{-1} from Jack beans) were provided by Harnstoff and Sigma-Aldrich, respectively. Acrylic acid, N-hydroxy-succinimide (NHS), sodium hydroxide (NaOH) and potassium ferricyanide [$K_3Fe(CN)_6$] electrolyte solution were supplied by Across. All the aqueous solutions were prepared using deionized water.

Conducting Polymer Preparation

The conducting polymer was prepared by photopolymerization as reported by Siti Veren, Alizar dan Syamsi (2021) and Noor Izaanin, Lee and Ling Ling (2022) with slight modifications. Polymer was prepared from aniline, n-butyl acrylate (nBA), ethylene glycol dimethacrylate (EGDMA) and acrylic acid N-hydroxy-succinimide (NHS). The function of N-hydroxy-succinimide (NHS) is to bind the urease enzyme to the matrix via covalent bonds, as has been previously reported for N-acryloxysuccinimide (Alizar, Lee & Musa 2011). These three polymers were analyzed by FTIR to see the bonding between the monomers and the functional groups present.

Electrodynamic Analysis of Aniline/nBA-Modified SPE and the Effect of OH^- Ion on the Enzymatic Biosensor

The electrodynamic characteristics of aniline-/nBA modified SPE has been characterized based on the peak-to-peak potential separation (ΔE_p), electron transfer rate constant (k), and electrical current flow (i_p) by means of cyclic voltammetry in the presence of $K_3Fe(CN)_6$ redox mediator method at pH 7. The k value was determined by the Nicholson equation (Nicholson 1965), whilst both oxidation and reduction diffusion coefficients, i.e., D_O and D_R were calculated with Randles–Sevcik equation (Fanjul-Bolado et al. 2008; Haslinda et al. 2023) as shown herewith,

$$E_p = E^o - (RT/3nF)\ln(4.78\pi^3 D_O/2D_R) - (RT/3nF)\ln(a/kC_o^*) \text{ (Nicholson Equation)} \quad (2)$$

$$i_p = (2.69 \times 10^5)n^{3/2}ACD^{1/2}\,v^{1/2} \text{ (Randles–Sevcik equation)} \quad (3)$$

The analysis of the OH^- ion concentration effect on the electrochemical response of the enzymatic urea biosensor in the presence of $K_3Fe(CN)_6$ redox species was carried out following the procedure recommended

by Kunfeng Chen (Chen, Song & Xue 2014) with some modifications. 2.5 mL of $K_3Fe(CN)_6$ solution at 10 mM was separately mixed with 1 mL of NaOH solution at various concentrations (1×10^{-6}, 1×10^{-5}, 1×10^{-4}, 1×10^{-3}, and 1×10^{-2} M). The electrochemical current response was measured with DPV within the potential range of -1.0-1.0 versus the Ag/AgCl reference electrode.

DETERMINATION OF UREASE ENZYME ACTIVITY

Urease enzyme activity was determined following the previously reported method (Alizar, Lee & Musa 2011) with slight modifications. The aniline/nBA-NHS-modified SPE electrode without the immobilized urease enzyme was used for the control experiments. The urease-immobilized aniline/nBA-NHS SPE was used to check for enzyme activity with 10 mL of 10 mM urea solution for 24 h. The urea solution was then tested with pH indicator strip. The activity of the immobilized urease enzyme is indicated by the colour change of the pH indicator strip due to a change in pH caused by the production of OH^- ions as a result of the hydrolysis of urea by the enzyme electrode.

CONSTRUCTION AND EVALUATION OF UREA BIOSENSOR PERFORMANCE

The proposed electrochemical urea biosensor based on aniline/nBA-NHS conducting polymer was prepared by the photopolymerization method on the working electrode of SPE. In brief, 0.1 g of DMPP was mixed with 1 mL of aniline monomer, 1 mL of nBA monomer, 1 mL of EGDMA and 6 mg of NHS. 100 µL of this mixture was then drop-coated on the SPE and photopolymerized using ultraviolet (UV) light for 10 min under a continuous nitrogen gas flow. Photopolymerization is the process of forming polymers under the UV light to activate free radicals or ions in order to initiate the polymerization reaction under an oxygen-free condition. Urease solution at 2 mg mL^{-1} was prepared in 0.05 M phosphate buffer (pH 7.0). 10 µL of the urease solution was then drop-casted on the aniline/nBA-NHS-SPE and left for 24 h at 4 °C. The resulting urease-modified aniline/nBA-NHS electrode was washed with abundant 0.05 M phosphate buffer (pH 7.0) and kept in the phosphate buffer solution at pH 7.0 until use. Figure 1 demonstrates the construction of an electrochemical biosensor interface for the specific detection of urea. The urea biosensor was rinsed with deionized water before electrochemical signal measurement using electrochemical CV and DPV techniques within the potential range of -1.0-1.0 versus Ag/AgCl reference electrode. To evaluate the electrochemical response of the enzymatic electrode towards assay of urea, the biosensor was tested with several concentrations of urea from 0.001 mM to 1.000 mM range in 10 mM $K_3Fe(CN)_6$ electrolyte solution (Ahmad et al. 2019; Nurashikin et al. 2019). Furthermore, the effect of pH on the urea biosensor response and the catalytic hydrolysis time of urea by the immobilized urease were also carried out.

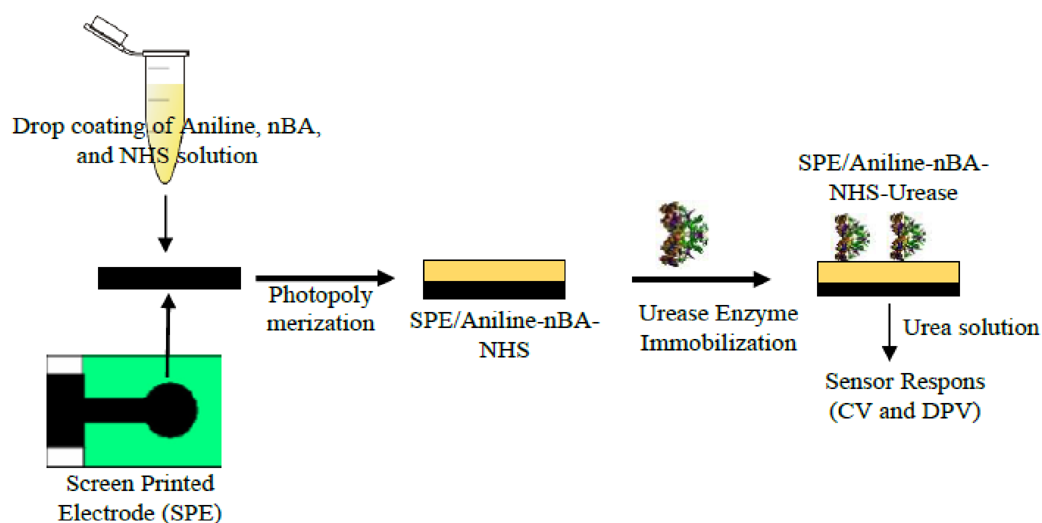


FIGURE 1. The construction of electrochemical biosensor interface based on photopolymerized aniline/nBA-NHS conducting polymer followed urease immobilization for urea detection

Results and Discussion

FTIR

Figure 2(A) presents the appearance of the polymer synthesized by photopolymerization method. The brownish colour of the polymer may be contributed from PANI. Figure 2(B) shows the functional groups obtained from the synthesized of polymer 1-3 by photopolymerization method. Typical peaks shown in the spectra contained polyaniline and stated as N-H stretching (3360-3462 cm^{-1}) (Ansari & Keivani 2006) and C-N stretching vibrations (1146-1171 cm^{-1}). The peaks at 3462 cm^{-1}, 3360 cm^{-1} indicates the presence of primary N-H group which due to $-NH_2$ of aniline. It is suggested that polyaniline formed in this study is Leucoemeraldine from the C-N stretching vibration that can be seen at 1171 cm^{-1}, 1146 cm^{-1} and 1148 cm^{-1}. Furthermore, C=C stretching vibration in aromatic hydrocarbon were shown at around 1602 cm^{-1} (Waware, Hamouda & Majumdar 2020). The C-H functional group was recorded at 3033 cm^{-1} to 2937 cm^{-1} in FTIR spectra for all the synthesized polymers. The $C-H_3$ functional group was also measured at 1450 cm^{-1} (Waware, Hamouda & Majumdar 2020). The peaks around 1716 to 1718 cm^{-1} are referred to the carboxylic group (C=O) (Abi, Cahyo & Suwarno 2018) contained in the chemical structure of ethylene glycol dimethacrylate (EGDMA), n-butyl acrylate (nBA) and acrylic acid *N*-hydroxy-succinimide (NAS) formed in these polymers. Meanwhile, the C=C group was measured in both polymers 1 and 2 (1621 cm^{-1} and 1624 cm^{-1}) (Noviany et al. 2023; Sun et al. 2011). The presence of C=C group indicates that end of polymers' chain that consist of EGDMA and nBA were not completely polymerized. In contrast, no C=C peak appeared in polymer 3. Moreover, all polymers recorded peaks around 1124 cm^{-1} and 1160 cm^{-1}, referred to the ether functional group (C-O) (Sun et al. 2011). The C-O group appear in those spectrums reflected from monomer EGDMA, nBA and NAS contained in the polymers.

Electrodynamic Analysis of SPE Modified with Conducting Polymer

Figure 3(A) shows the cyclic voltammograms of bare SPE and modified SPEs with aniline/nBA conducting polymer and urease, whilst the electrodynamic data of each electrode is summarized in Table 1. The electron transfer rate constant (*k*) of the modified SPEs increased by 3-fold compared to bare SPE, which proves that the presence of aniline as the electron transduction element on the SPE has increased the electron transfer rate of the $K_3Fe(CN)_6$ redox species at the electrode-electrolyte interface. This observation is affirmed by the peak-to-peak potential separation (ΔE_p) result trend of the same electrode series. The SPEs showed a substantial decline in the ΔE_p value after being modified with aniline/nBA, followed by the enzyme, which indicates a faster electron transfer reaction occurred with the conducting polymer-modified electrodes. This corresponds to Nicholson's equation as ΔE_p increases, *k* decreases (Goldstein & Van de Mark 1982). A similar observation was previously reported for the SPE modified with acrylic microspheres and gold nanoparticles (AuNPs) composite (Alizar et al. 2014).

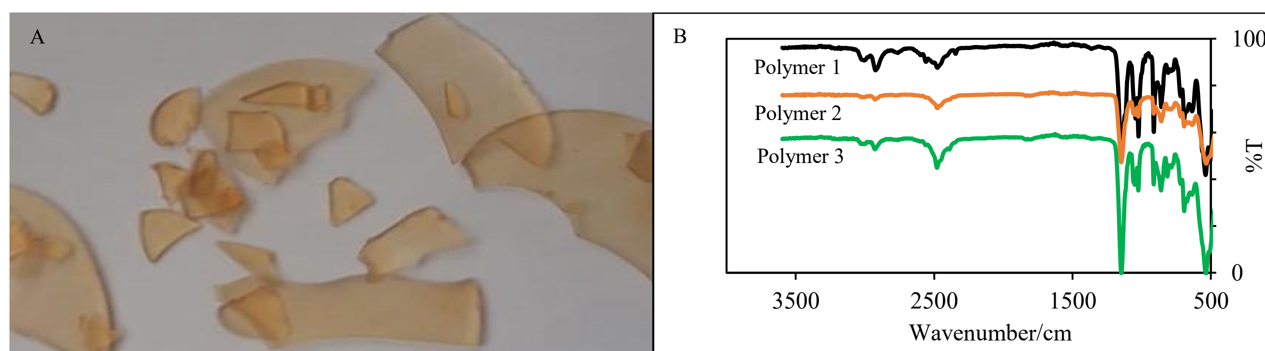


FIGURE 2. Brownish colour conducting polymer (A) and FTIR spectrum of polymer 1-3 (B) by photopolymerization method

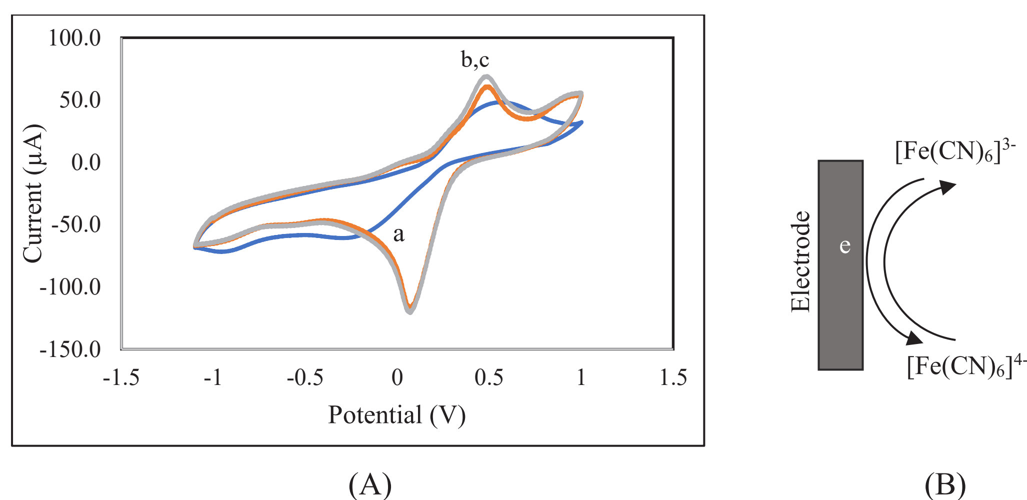


FIGURE 3. Cyclic voltammograms of bare SPE (A): blue line is bare SPE (a), gray line is aniline/nBA-modified SPE (b), red line is urease-modified aniline/nBA-NHS SPE (c) in 10 mM $K_3Fe(CN)_6$ solution and the redox reaction mechanism of the $K_3[Fe(CN)_6]$ electrolyte solution at the electrode-electrolyte interface (B)

TABLE 1. The anodic (E_{pa}) and cathodic (E_{pc}) peaks' potentials of $K_3Fe(CN)_6$; peak-to-peak potential separation (ΔE_p); electron transfer rate constant (k); anodic-to-cathodic peak current ratio (i_{pa}/i_{pc}); and oxidation (D_O) and reduction (D_R) diffusion coefficients of different types of working electrode

Electrode	Peak potential (V)		Peak current (µA)		ΔE_p	k (×10^{-5}) (cm s^{-1})	i_{pa}/i_{pc}	D_O (×10^{-8}) (cm^2 s^{-1})	D_R (×10^{-8}) (cm^2 s^{-1})
	$_{Ep}$a	E_{pc}	i_{pa}	i_{pc}					
Bare SPE	0.548	-0.956	-71.899	48.245	1.504	1.605	1.490	3.824	8.495
Aniline/nBA-NHS SPE	0.489	0.069	-117.830	60.913	0.420	5.522	1.748	7.872	22.813
Urease-aniline/ nBA-NHS SPE	0.499	0.067	-121.00	69.214	0.432	5.374	1.934	6.097	24.059

k, calculated with Equation (1); D_O and D_R calculated with Equation (2)

According to the reversible system, when the i_{pa}/i_{pc} values obtained for both types of the modified and unmodified electrodes are more than 1.0, they indicate that the redox systems are more likely to be oxidized than reduced. When the ferricyanide ($[Fe(CN)_6]^{3-}$) ion in the electrolyte solution receives electrons from the potentiostat through the electrode in the cathodic half-cycle, the anionic $[Fe(CN)_6]^{3-}$ is reduced into ferrocyanide ($[Fe(CN)_6]^{4-}$) ion. The electro-generated $[Fe(CN)_6]^{4-}$ ion will be oxidized in the next anodic half-cycle and transferring the electron to the electrode. In view of the oxidation reaction to form $[Fe(CN)_6]^{3-}$ ion was more significant than the reduction reaction for the formation of $[Fe(CN)_6]^{4-}$ ion in this experiment, this supports the as-obtained i_{pa}/i_{pc} values, which are greater than 1.00 for types of the modified and unmodified electrodes. The i_{pa} and i_{pc} values attained are correspond to the respective oxidation (D_O) and reduction (D_R) diffusion coefficients,

whereby a higher peak current is associated with a lower diffusion coefficient. The redox mechanism of the electrochemical system at the electrode-electrolyte interface solution is illustrated in Figure 3(B) (Chen, Song & Xue 2014).

THE UREASE ENZYME ACTIVITY TEST AND EFFECT OH^- ION CONCENTRATION ON THE VOLTAMMETRIC RESPONSE OF UREA BIOSENSOR

Figure 4(A) shows the activity of both the non-immobilized urease enzyme and immobilized urease on the aniline/nBA-NHS-modified SPE in 0.05 M phosphate buffer at pH 7.0. No colour change with the pH indicator strip was perceived when the enzyme activity test was carried out in the blank solution without urea. A noticeable colour change to the pH indicator strip was discernible upon 10 mM of urea was added into the phosphate buffer (0.05 M, pH 7.0) containing the respective non-immobilized urease and immobilized urease on the aniline/nBA conducting polymer-modified electrode. This was attributed to the catalytic activity of urease in both the non-immobilized and immobilized forms, which have catalyzed the hydrolysis of urea into the corresponding NH_4^+, HCO_3^- and OH^- ions (Alizar, Lee & Musa 2011; Das & Yoon 2015; Dervisevic & Nyangwebah 2017). The resulting OH^- ions generated from the urease enzymatic reaction have changed the pH of the solution from pH 7.0 to pH 10.0 according to the indicator of the pH strip (Alizar, Lee & Musa 2011). This implies that the immobilized urease enzyme possessed the same activity as that of the non-immobilized urease in the solution. The urease enzyme retained its native conformation despite covalent immobilization between the amine group in the urease enzyme and the succinimide moiety from the aniline/nBA-NHS conducting polymer.

In view of the electrochemical response of the proposed enzymatic urea biosensor is tested in the $K_3Fe(CN)_6$ electrolyte solution, and the enzymatic system hydrolyzes urea to produce OH^- ions, which results in the pH change of the medium. Therefore, it is necessary to analyze the effect of OH^- ions on the electrochemical response of the enzymatic electrode in the presence of $K_3Fe(CN)_6$ electron shuttling agent. Figure 4(B) depicts the effect of NaOH concentration from 10^{-6} M to 10^{-2} M on the DPV current of the $K_3[Fe(CN)_6]$ redox mediator on the enzymatic urea biosensor. The voltammetric response of the enzymatic electrode was observed to reduce steadily with the increasing of the NaOH concentration being added to the 10 mM $K_3[Fe(CN)_6]$ redox solution. The increase in the number OH^- ions has impeded the electron transfer of the $K_3[Fe(CN)_6]$ redox species at the electrode-electrolyte interface. Furthermore, the reaction between NaOH and $K_3[Fe(CN)_6]$ could change the $[Fe(CN)_6]^{3-}$ complex to become a strong base and salt according to the following chemical reaction (Luo et al. 2017),

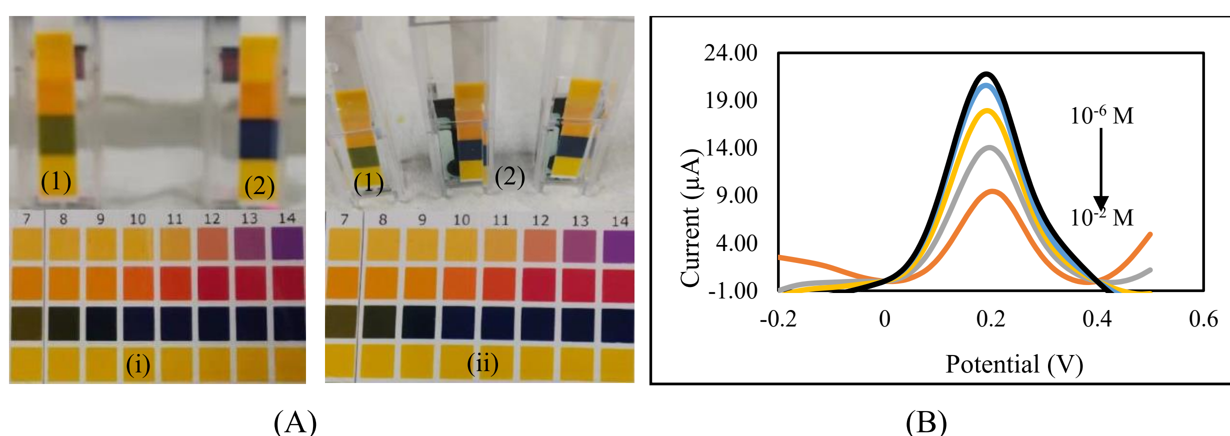


FIGURE 4. The urease enzyme activity test (A) was carried out in 0.05 M phosphate buffer (pH 7.0) containing dissolved urease (i) and immobilized urease on the aniline/nBA-NHS SPE (ii). The enzyme activity tests were carried out in blank solution (1) and 10 mM urea solution (2). Effect of OH^- concentration from 10^{-6} M to 10^{-2} M on the voltammetric response of the urea biosensor based on urease-immobilized aniline/nBA-NHS-modified SPE in the presence of 10 mM $K_3[Fe(CN)_6]$ electrolyte solution (B)

$$3NaOH + K_3[Fe(CN)_6] \rightarrow Fe(OH)_3 + 3NaCN + 3KCN$$

However, in the enzymatic urea hydrolysis system, the urea will be hydrolyzed to produce OH^- ions in the $K_3[Fe(CN)_6]$ electrolyte solution, then the chemical reaction is predicted as follows,

$$3OH^- + K_3[Fe(CN)_6] \rightarrow Fe(OH)_3 + 3CN^- + 3KCN$$

This chemical reaction shows the influence of OH^- ions on the reduction of the $[Fe(CN)_6]^{3-}$ complex, whereby the presence of OH^- ions in the $K_3[Fe(CN)_6]$ electrolyte could change the $[Fe(CN)_6]^{3-}$ ion into a base, CN^- ions, and salts.

THE VOLTAMMETRIC ENZYMATIC UREA BIOSENSOR RESPONSE BASED ON ANILINE/nBA CONDUCTING POLYMER

Figure 5 represents the differential pulse voltammograms of the bare SPE, aniline/nBA conducting polymer-modified SPE, and the enzymatic electrode based on aniline/nBA-NHS conducting polymer before and after reaction with 10 mM urea solution in 0.05 M phosphate buffer (pH 7.0) containing 10 mM $K_3Fe(CN)_6$. As expected, where surface modification of the SPE with aniline/nBA conducting polymer has improved the electrode conductivity, which can be seen by the appreciable increment in the DPV peak current between the bare SPE and the aniline/nBA-NHS conducting polymer-modified SPE by using 10 mM $K_3Fe(CN)_6$ as the electron shuttling agent in the phosphate buffer supporting electrolyte (0.05 M, pH 7.0). Subsequent covalent grafting of the urease enzyme on the aniline/nBA-NHS modified SPE has slightly decreased the urea biosensor response, which can be attributed to the barrier held at the electrode surface that hindered the electron transfer of the redox mediator. However, the enzymatic biosensor exhibited a considerable decline in the voltammetric response after exposure to 10 mM urea in 0.05 M phosphate buffer (pH 7.0) containing 10 mM $K_3Fe(CN)_6$. This phenomenon can be ascribed to the aforementioned chemical reaction involving the enzymatically hydrolyzed OH^- ion with the $K_3Fe(CN)_6$ electron mediator, and producing a base, CN- ions, and salts at the SPE surface, thereby restricting the electron transfer of the redox species at the electrode-electrolyte interface.

OPTIMIZATION UREA BIOSENSOR RESPONSE EFFECTS OF pH AND ENZYMATIC HYDROLYSIS TIME ON THE ELECTROCHEMICAL UREA BIOSENSOR RESPONSE

Each enzyme has an optimum pH range with maximum enzymatic reaction rate. For most enzymes, the optimum

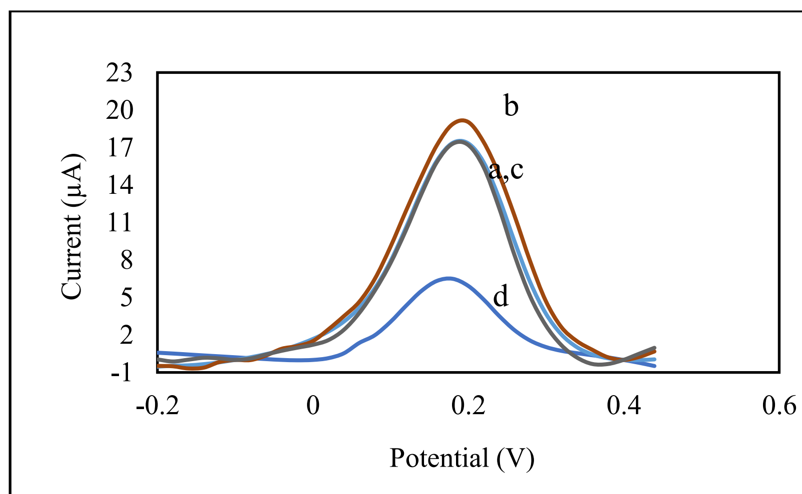


FIGURE 5. Urea biosensor response with differential pulse voltammetry (DPV) method. Bare SPE (a), SPE-Aniline/nBA (b), SPE-Aniline/nBA-NHS-Urease expose in blank solution (c) and SPE-Aniline/nBA-NHS-Urease expose in urea solution 10 mM (d). The experiment was conducted in 0.05 M $K_3Fe(CN)_6$ pH 7.0 with a scan rate of 0.5 V/s versus Ag/AgCl

pH ranges from pH 5 to pH 9. The enzymatic reaction decreases at higher or lower pH beyond the enzyme's typical optimum pH range. This is because changes in pH will change the charge distribution at the enzyme's active sites and consequently changes the enzyme's conformation, thereby affecting the specific binding with its target. The urease enzyme employed in this study, which originated from the Jack beans, has an optimum pH of around pH 7.0 (Qin & Cabral 2002). At this pH, urease's catalytic activity towards urea hydrolysis is the fastest. However, apart from the pH factor, the immobilized enzyme's activity will be influenced by its surrounding immobilized conditions, such as the type of electrode, surface modification materials, and electrolyte solutions (Achmann, Hämmerle & Moos 2008; Štikoniene, Ivanauskas & Laurinavicius 2010). The effect of pH on the voltammetric response of the proposed electrochemical urea biosensor is shown in Figure 6(A). Because the resulting enzymatic product of the proposed electrochemical urea biosensor, i.e., the OH^- ion reacted with the $K_3Fe(CN)_6$ redox mediator and hampering the electron transfer at the electrode surface, therefore, the lowest voltammetric response acquired from the proposed enzymatic biosensor suggests the optimum pH for the enzymatic reaction of urea, which occurred at pH 5.0. Tuning the pH beyond pH 5.0 towards more acidic or basic conditions would not favour the urease enzyme activity for the proposed enzymatic urea biosensor

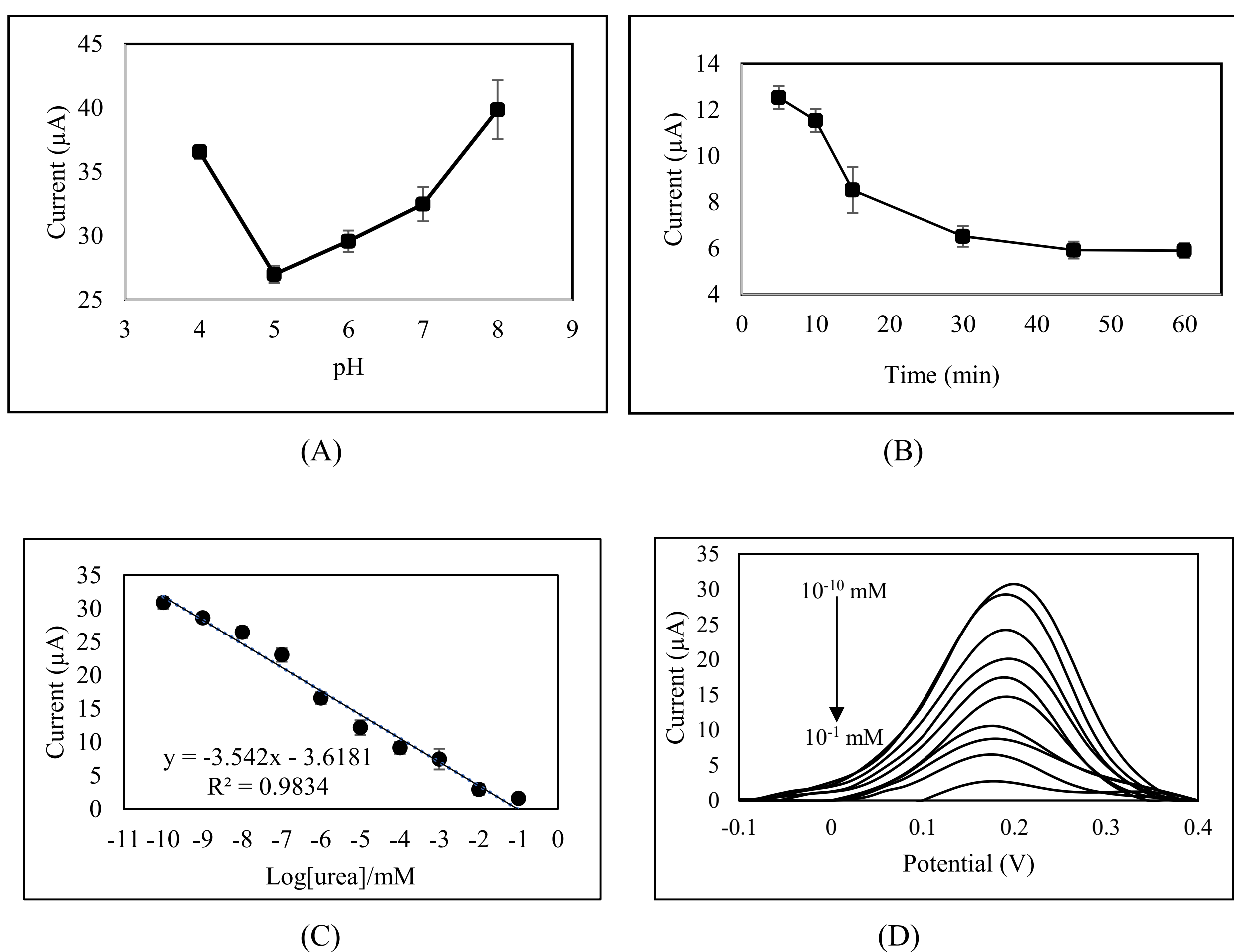


FIGURE 6. Effects of pH (A) and enzymatic hydrolysis time (B) on the voltammetric response of the urea biosensor. The experiment was conducted in 0.05 M phosphate buffer (pH 7.0) containing 10 mM $K_3Fe(CN)_6$ with a scan rate of 0.5 V s^{-1} versus Ag/AgCl. The linear response range of the Aniline/nBA-NHS conducting polymer-based urea biosensor within the urea concentration range of 1×10^{-10} to 1×10^{-1} mM at pH 5.0 and 25 °C with a 30 min of urea hydrolysis time (C). The corresponding differential pulse voltammograms of the enzymatic urea biosensor towards the detection of urea concentration from 1×10^{-10} to 1×10^{-1} mM (D)

based on aniline/nBA conducting polymer. Anyhow, previously reported studies on the optimum pH of the urea biosensor response have been reported at pH 7.5 and pH 8.0 based on poly(2-hydroxyethyl methacrylate-glycidyl methacrylate) nanoparticles (Öndeş et al. 2020) and polyamidoamine grafted multiwalled carbon nanotube (MWCNT-PAMAM) (Dervisevic, Dervisevic & Şenel 2017) electrode surface modification materials, respectively.

Figure 6(B) exhibits the catalytic hydrolysis time of the enzymatic electrode towards 10 mM urea. The enzymatic urea biosensor response decreased with the exposure time to 10 mM urea from 5 min to 30 min. The voltammetric signal remained a rather plateau response thereafter until 60 min enzymatic urea hydrolysis time. The decrement in the voltammetric signal of the enzymatic biosensor signifies the immobilized urease has successfully catalyzed the urea through the cascaded chemical reaction between the product of the enzymatic reaction and the redox indicator in the reaction buffer.

LINEAR RESPONSE RANGE AND REPRODUCIBILITY OF THE ANILINE/nBA CONDUCTING POLYMER-BASED UREA BIOSENSOR

Based on the response curve of the enzymatic urea biosensor portrayed in Figure 6(C), the biosensor demonstrated an inverse sensitivity behaviour with low analyte concentration, yielding a high voltammetric signal. The voltammetric response of the biosensor diminishes as increasing the urea concentration from 1×10^{-10} mM to 1×10^{-1} mM with 30 min urea hydrolysis time at ambient conditions and pH 5.0. This can be explained by the higher the urea concentration was administered into the reaction medium, the higher the cascaded chemical reaction rate was occurred between the enzymatically hydrolyzed OH^- ion and the $K_3Fe(CN)_6$ redox mediator. The generation of the side products, i.e., $Fe(OH)_3$, CN^- ion, and KCN at the enzyme electrode surface, were postulated to be proportional to the urea concentration involved in the enzymatic reaction with the urease enzymes that were conjugated to the aniline/nBA conducting polymer on the SPE. Thus, this limited the electron transfer of the redox species at the electrode-electrolyte interface as increasing the urea concentration to the urea biosensor. The linear concentration range of the proposed electrochemical urea biosensor between 1×10^{-10} mM and 1×10^{-1} mM possessed a linear regression coefficient of R^2=0.9834 with a detection limit of 4.72×10^{-11} mM urea. The detection limit was calculated based on three times the standard deviation of the biosensor response on the response curve close to the detection limit divided by the slope of the linear calibration (Navas Díaz, Ramos Peinado & Torijas Minguez 1998). Figure 6(D) shows the corresponding differential pulse voltammograms of the enzymatic urea biosensor response within the linear urea concentration range of 1×10^{-10}-1×10^{-1} mM.

A study on the reproducibility of the enzymatic biosensor has been carried out by producing several enzymatic biosensors based on aniline/nBA conducting polymer, and were tested with 10 mM urea at optimal conditions, i.e., pH 5.0 and 30 min hydrolysis time. The voltammetric responses of the batch-fabricated enzymatic urea biosensors gave a promising relative standard deviation of 5.0% (n=9), indicating high reproducibility of the developed enzymatic biosensor based on SPE modified with aniline/nBA conducting polymer via the facile photopolymerization method.

COMPARISON OF THE DEVELOPED UREA BIOSENSOR PERFORMANCE WITH PREVIOUSLY REPORTED ELECTROCHEMICAL BIOSENSORS FOR UREA DETECTION

Table 2 compares the developed urea biosensor performance with previously reported biosensors in terms of dynamic linear response range and detection limit. The electrochemical enzymatic biosensor developed using aniline/nBA-NHS conducting polymer-modified carbon SPE in this study showed ultrasensitive detection towards urea with a linear response range targeting urea with a low detection limit at picomolar levels. The higher sensitivity performance of the developed enzymatic biosensor compared to other reported electrochemical biosensors based on various nanostructured conducting materials for urea detection could be mainly attributable by the proposed aniline/nBA-NHS conducting polymer, which functioned as both the enzyme-supporting matrix and the electron conduction pathway of the redox mediator at the electrode-electrolyte interface. This conducting polymer has also assisted the electrons transfer between sample and the electrode interface thus enhanced the biosensor sensitivity performance.

TABLE 2. Comparison of the developed electrochemical urea biosensor with other previously reported electrochemical urea biosensors in terms of dynamic linear range and limit of detection

Reference	Surface modification material and electrode design	Linear range (mM)	Detection limit (mM)
This Work	Aniline/nBA-NHS conducting polymer-modified carbon SPE	1.000×10^{-10}-0.100	4.720×10^{-11}
(Rahmanian & Mozaffari 2014)	ZnO-polyvinyl alcohol nanostructured hybrid film-modified fluorine-doped tin oxide (FTO) conductive glass electrode	0.008-2.100	0.005
(Shukla et al. 2014)	Zirconia-poly(propylene imine) dendrimer nanocomposite-modified carbon SPE	0.010-2.990	0.010
(Dervisevic, Dervisevic & Şenel 2017)	polyamidoamine grafted multiwalled carbon nanotube (MWCNT-PAMAM)-modified Au plate electrode	1.000-20.000	0.400
(Hassan et al. 2018)	Poly(m-toluidine) (PmT) and poly(o-toluidine) (PoT) conducting polymers-modified carbon SPE	0.100-11.000	0.030
(Emami Meibodi & Haghjoo 2014)	single walled carbon nanotube and conducting polymer composite film-modified graphite electrode	0.001-1.000	0.004

CONCLUSIONS

A highly sensitive electrochemical urea biosensor has been fabricated by photopolymerizing aniline/nBA conducting polymer directly on the carbon-based SPE. The urease enzyme was bound to the electrode surface through covalent binding of highly reactive succinimide functional groups from the Aniline/nBA-NHS conducting polymer towards amino groups of the enzymes. The enzyme activity study showed that the catalytic activity of the immobilized urease was retained at its original capacity in spite of undergoing chemical modification. The enzymatic hydrolysis of urea by the developed urease electrode demonstrated that the resulting enzymatically hydrolyzed OH^- ion could diminish the voltammetric biosensor response due to the cascaded chemical reaction with the $K_3Fe(CN)_6$ redox mediator. The inverse sensitivity behaviour of the aniline/nBA conducting polymer-based urea biosensor has made this material an exciting alternative to various nanostructured conducting materials.

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