

3-20-2017

Electrochemical Detection of Neuraminidase Based on Zanamivir Inhibition Reaction at Platinum and Platinum-Modified Boron-Doped Diamond Electrodes

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Ivandini, Tribidasari Anggraningrum; Ariani, Jenny; Jiwanti, Prastika Krisma; Gunlazuardi, Jarnuzi; Saepudin, Endang; and Einaga, Yasuaki (2017) "Electrochemical Detection of Neuraminidase Based on Zanamivir Inhibition Reaction at Platinum and Platinum-Modified Boron-Doped Diamond Electrodes," *Makara Journal of Science*: Vol. 21 : Iss. 1 , Article 6.

DOI: 10.7454/mss.v21i1.7535

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This work was partially supported by a grant from Hibah PUPT BOPTN Universitas Indonesia, Contract No. 0533/UN2.R12/HKP.05.00/2015.

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Electrochemical Detection of Neuraminidase Based on Zanamivir Inhibition Reaction at Platinum and Platinum-Modified Boron-Doped Diamond Electrodes

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Received August 4, 2016 | Accepted February 20, 2017

Abstract

Neuraminidase sensors have been developed using platinum (Pt) and platinum-modified boron-doped diamond (Pt-BDD) electrodes based on the difference of electrochemical responses of zanamivir in the absence and in presence of neuraminidase. Cyclic voltammetry of zanamivir showed that the presence of zanamivir decreases the oxidation-reduction peak currents of Pt. The responses were found to be linear in the zanamivir concentration range of 7.5-150 μM . Comparison between Pt-BDD and Pt bulk electrodes showed that Pt-BDD offers approximately two times higher sensitivity of zanamivir responses than Pt bulk. Further comparison to gold-based electrodes showed that Pt-based electrodes provided about one order higher sensitivity of zanamivir responses. In the presence of neuraminidase, the oxidation-reduction currents of Pt again increase. Observation on the oxidation currents in the system with 2×10^{-5} M zanamivir showed that the responses were linear in the neuraminidase concentration range of 5-20 mU. Good reproducibility with an RSD < 3 ($n = 10$) was achieved even when the measurements were performed in a mucin matrix, suggesting that Pt-based electrodes are suitable to be applied for the detection method of neuraminidase.

Abstrak

Deteksi Neuraminidase secara Elektrokimia berdasarkan Reaksi Inhibisi Zanamivir pada Elektroda Platina dan Boron-Doped Diamond Termodifikasi Platina. Sensor neuraminidase dikembangkan menggunakan elektroda platina (Pt) dan boron-doped diamond termodifikasi platina (Pt-BDD) berdasarkan perbedaan respon elektrokimia zanamivir dengan dan tanpa kehadiran neuraminidase. Voltametri siklik zanamivir menunjukkan bahwa kehadiran zanamivir menurunkan arus puncak oksidasi-reduksi Pt. Respon ini ditemukan linier pada daerah konsentrasi zanamivir 7,5-150 μM . Perbandingan antara elektroda Pt-BDD dan logam Pt menunjukkan bahwa Pt-BDD menghasilkan sensitivitas respon zanamivir sekitar dua kali lebih tinggi daripada logam Pt. Perbandingan menggunakan elektroda berbasis emas menunjukkan bahwa elektroda berbasis Pt menghasilkan sensitivitas satu order lebih baik. Jika neuraminidase dimasukkan ke dalam sistem, arus oksidasi Pt kembali meningkat. Observasi arus oksidasi dalam sistem yang mengandung 2×10^{-5} M zanamivir menunjukkan bahwa respon linier pada daerah konsentrasi neuraminidase 5-20 mU. Kedapatulangan yang baik dengan standar deviasi relatif (RSD) < 3 ($n = 10$) dapat dicapai bahkan jika pengukuran dilakukan dalam matriks lendir, menunjukkan bahwa Pt-BDD sesuai untuk aplikasi dalam metode deteksi neuraminidase.

Keywords: template; instructions; conference; publications; international

Introduction

Neuraminidase is an important enzyme in the life cycle of pathogenic viruses and microbes. It plays an important role in the release process from the host cell. The detection of neuraminidase is important in controlling disease caused by the related virus and microbes. The usual

detection methods rely on polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and enzymatic reactions [1-3], which require various types of chemical reagents, expensive instruments, and highly-skilled operators. Therefore, a simpler, more practical method is urgently required.

Zanamivir{(2R,3R,4S)-4[(diaminomethylidene)amino]-3-acetamido-2-[(1R,2R)-1,2,3-trihydroxypropyl]-3,4-dihydro-2H-pyran-6-carboxylic acid}} is an antiviral. Its chemical structure is shown in Figure 1. Zanamivir inhibits neuraminidase in the influenza A and B viruses through its interaction with the active sites of neuraminidase [3]. Therefore, it is possible to develop a neuraminidase sensor based on the inhibition of zanamivir. However, the existing quantitative analysis methods of zanamivir such as high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) are expensive and less efficient. Also, Zanamivir is unable to cause direct oxidation or reduction at boron-doped diamond (BDD) or even at metal electrodes. Hence, the electrochemical method is inappropriate for the detection of zanamivir. However, the presence of zanamivir increases the electrocatalytic reaction of H⁺ to H₂ at the mercury electrode [4,5]. Since mercury is not desirable because of its poisonous characteristics, the gold electrode was investigated and showed that the presence of zanamivir decreases the oxidation-reduction currents of gold [6,7]. Because of this behavior, electrochemical detection of neuraminidase based on its reaction with zanamivir was developed using gold-based electrodes [6,7].

In this study, a platinum (Pt) electrode was utilized to develop the neuraminidase sensor based on the electrochemical detection of zanamivir. Since the signals were quite small at the bulk platinum, BDD was used as a support of the Pt-based electrode. BDD is established as an electrode with high stability, low background current, and a wide potential window [8-10]. Modification of inert metal nanoparticles on the surface of BDD is reported to produce electrodes with lower background currents. As a result, higher signal-to-background ratios and a better limit of detections can be achieved [11-13]. A comparison performed on gold and gold-modified BDD (Au-BDD) electrodes shows that not only was better sensitivity observed for the detection of zanamivir at Pt-based electrodes [6,7], but better stability was also achieved. The results indicated that Pt-based electrodes are more suitable for the indirect detection of neuraminidase via zanamivir measurements.

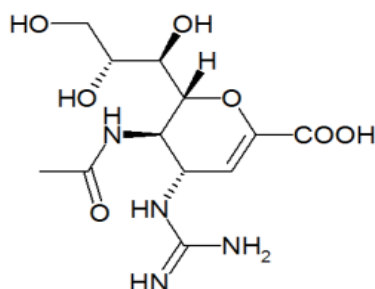


Figure 1. Chemical Structure of Zanamivir

Materials and Methods

Chemicals and Materials. Neuraminidase of *Clostridium perfringens* (1.1 units/mg solid) and mucin bovine submaxillary glands type I-S M3895 were purchased from Sigma Aldrich, while zanamivir, mucin porcine stomach 1% sialic acid bonding, and other chemicals were supplied by Wako Pure Chemical Industries, Ltd. (Nilaco, Japan). Platinum wire (dia 0.2 mm) and silver wire (dia 0.5 mm) were supplied from Nilaco, Japan.

Preparation of Working Electrodes. BDD was grown on a silicon (100) wafer in a microwave-assisted plasma chemical vapor deposition system (Cornes Technologies Ltd., Tokyo, Japan). Microwave power of 5.0 kW was applied for 6 h. Details of the preparation have been described elsewhere with some modifications [15]. Trimethoxy borane was the boron source, while methane was the carbon source (boron-to-carbon ratio was 1:1000). Pure hydrogen was used as the carrier gas. A scanning electron microscope (SEM) image showed a range of diamonds from 3 to 5 μm , while Raman spectra (Renishaw System 2000) showed an intense sp³ carbon peak at 1332 cm⁻¹, which indicated the characteristic of a high degree crystallinity diamond [14,15]. In addition to this peak, two peaks at 500 and 1200 cm⁻¹ were also observed, indicating the presence of boron [14,15]. Platinum particles were deposited on the surface of BDD by the chronoamperometry method in a solution of 1 mM H₂SO₄ containing 1 mM H₂PtCl₆ at the potential -0.2 V (vs. Ag/AgCl) for 30 s. Meanwhile, gold particles were also deposited at the BDD by the same method using 1 mM of H₂SO₄ containing 1 mM HAuCl₄. The morphologies of the BDD and the metal-modified BDD were characterized using SEM, while the surface composition was studied using energy dispersive spectroscopy (EDS).

Electrochemical measurements. A three-electrode electrochemical system was used for electrochemical measurements. Pt, Au, Pt-BDD, and Au-BDD were utilized as the working electrodes with an approximation surface area of 1.3 x10⁻⁵ m². An Ag/AgCl (saturated in KCl) was used as the reference electrode, and a Pt wire was applied as the counter electrode. Prior to use, all electrodes were ultrasonicated in 2-propanol for 5 min and in pure water for 5 min. The zanamivir measurements were mainly conducted in phosphate buffer solution (PBS) pH 6.8, while the measurements in the presence of neuraminidase were performed by the addition of zanamivir into the solution of neuraminidase in 0.1 M PBS pH 6 and 25 mM KCl (1:1). After the incubation process at 37 °C for 30 min, 0.014 N NaOH in 83% ethanol (1:1) was added to stop the neuraminidase activity before the electrochemical measurements were conducted [16].

Results and Discussion

Characterization of the working electrodes. Pt particles were deposited using 0.5 mM H₂SO₄ containing 2 mM of H₂PtCl₆ on the surface of BDD using the chronoamperometry technique. Optimization of the deposition potential, deposition time, and H₂PtCl₆ concentration generates the highest difference between the signal currents and the background current at the deposition potential of -0.3 V for 30 s, which was then fixed as the optimum condition of Pt deposition.

Figure 2 shows the EDS spectrum of BDD electrodes before and after modification with Pt together with their related SEM images in the optimum condition of modification. Pt particles were deposited with an average size of 150 nm. While unmodified BDD shows the existence of carbon (C) (88.67%) and oxygen (O) (11.33 %) on the surface of BDD electrode (Figure 2a), after modification, the EDS spectra shows that some Pt atoms presented on the BDD surface with a 0.77% atom percentage (Figure 2b). Comparison of the atomic percentage extracted from the EDS spectra between both BDD electrodes before and after modification with Pt is displayed in Table 1. The table shows that the presence of Pt changed the oxygen-to-carbon (O/C) ratio from 0.13 to 0.10. It appears that the Pt particles have a tendency to deposit at the oxygen sites rather than at the carbon sites of the BDD surface. This is most likely because the hydrophilic property of oxygen sites provides a better wetting process on the BDD surface and resulted in a better electrochemical deposition process of Pt on the BDD surface. It is well-known that the hydrophilic property of the BDD surface increases after it is oxidized [16,17]. Accordingly, oxidization of

the BDD surface before electrochemical deposition of metal particles was suggested [18,19].

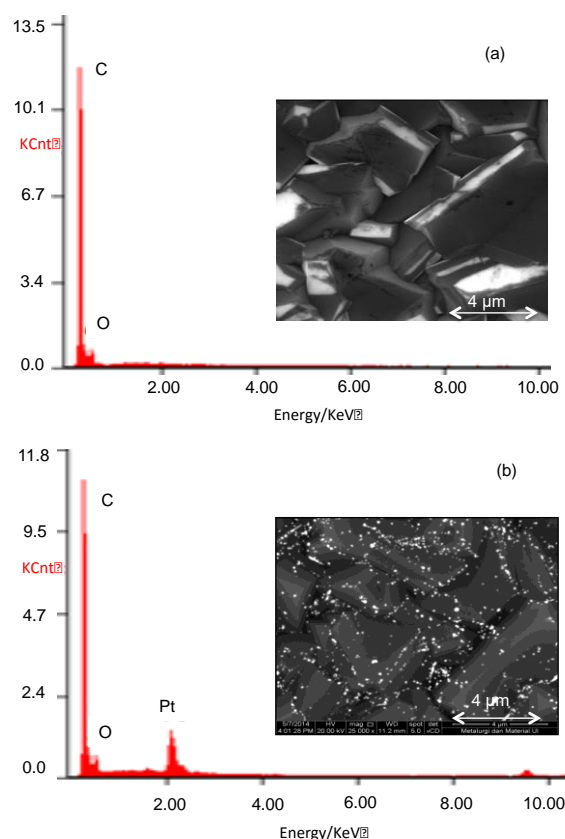


Figure 2. Energy Dispersive Spectrum (EDS) of BDD before (a) and after (b) Modification with Pt. The Insets Show their related SEM Images

Table 1. Comparison of the Atomic Percentage on the Surface of BDD before and after Modification with Pt

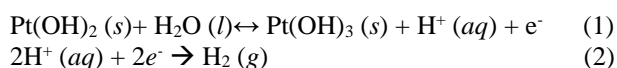
	Unmodified BDD	Modified BDD
Carbon	88.67	89.96
Oxygen	11.33	9.27
Platinum	N.A.	0.77

Table 2. Summary of the Analytical Performance of Zanamivir Detection at Pt-based Electrodes in Comparison with at Au-based Electrodes [6,7]

	Pt		Pt-BDD		Au [6]		Au-BDD [6,7]	
	Oxidation peak at -0.5 V	Reduction peak at -0.9 V	Oxidation peak at -0.4 V	Reduction peak at -1.0 V	Oxidation peak at +1.0 V	Reduction peak at +0.4 V	Oxidation peak at +1.0 V	Reduction peak at +0.4 V
Sensitivity (mA/mM)	0.32	NA	0.68	0.65	0.36	1.57	0.04	0.03
S/B ratio of 5 x 10 ⁻⁵ M	1.14	1.05	1.03	1.07	0.20	0.25	0.29	0.24
Concentration Range (M)	7.5 x 10 ⁻⁶ – 1.5 x 10 ⁻⁴	N.A.	7.5 x 10 ⁻⁶ – 3.0 x 10 ⁻⁴	7.5 x 10 ⁻⁶ – 1.5 x 10 ⁻⁴	1.0 x 10 ⁻⁵ – 1.0 x 10 ⁻⁴	1.0 x 10 ⁻⁵ – 1.0 x 10 ⁻⁴	5.0 x 10 ⁻⁶ – 1.0 x 10 ⁻⁴	5.0 x 10 ⁻⁶ – 1.0 x 10 ⁻⁴
LOD (mol/L)	5.36 x 10 ⁻⁵	N.A.	6.40 x 10 ⁻⁶	5.40 x 10 ⁻⁵	1.25 x 10 ⁻⁵	1.19 x 10 ⁻⁵	1.53 x 10 ⁻⁶	1.49 x 10 ⁻⁶
RSD (%)	1.70 (n = 20)	N.A.	1.64 (n = 20)	1.75 (n = 20)	2.55 (n = 9)	0.33 (n = 9)	1.14 (n = 9)	0.50 (n = 9)

Electrochemical behavior of Zanamivir at Pt-Based electrodes.

The electrochemical behavior of zanamivir was studied using cyclic voltammetry. Figure 3 shows the cyclic voltammograms (CVs) of 0.1 M PBS in the absence and in the presence of 1.5×10^{-4} M zanamivir. A typical CV of PBS pH 7.0 at the Pt electrode in Figure 3a clearly shows one oxidation peak at -0.5 V (vs. Ag/AgCl) and two reduction peaks at +0.0 V and -0.9 V. General oxidation-reduction reactions of the Pt electrode in a neutral pH is presented below [20]:



The oxidation-reduction peaks at -0.5 V and +0.0 V, respectively, are considered to represent the reversible reactions in Eq.1, while the peak at -0.9 V is related to the reduction of H^+ to H_2 as shown in Eq. 2.

At the same time, the CV of 0.1 M PBS at the Pt-BDD electrode (Figure 3(b)) showed similar types oxidation-reduction peaks as those of the Pt electrodes. However, the sharpness of the peaks was different. Whereas well-defined peaks were generally formed at the Pt electrodes, poor peaks were observed when the Pt-BDD electrode was applied. Moreover, higher currents were generated at the Pt electrodes. The probable reason is the limited number of Pt particles at the modified electrodes. In addition, the separation of the oxidation and reduction peaks was wider at the Pt-BDD as shown by the oxidation and reduction peaks at -0.4 V and -1.0 V, respectively; this is reasonable due to the increased resistance on the surface of electrode.

In the presence of zanamivir, neither new oxidation nor reduction peaks appeared in the potential range of -1.2 to +1.5 V at both Pt and Pt-BDD; however, it is obvious in the CVs of Figure 3 that the presence of zanamivir led to a decrease of the currents of the three Pt peaks. Since the reduction peaks at +0.0 V were too small to be analyzed, the oxidation and reduction peaks at -0.5 V and -0.9 V, respectively, were investigated. Both peaks showed the linear decrease of the peak currents ($R^2 = 0.99$) from 7.5×10^{-6} M to 1.5×10^{-4} M of zanamivir at the Pt electrodes (Figure 4a), while the measurements at the Pt-BDD electrode gave linear current responses at a wider concentration range from 7.5×10^{-6} M to 3×10^{-4} M (Figure 4b). About two times higher sensitivity was shown by the Pt-BDD as is also clearly indicated by the decrease of the peak currents in Figure 4b. The estimated limit of detections (LODs) extracted from the oxidation peaks was found to be 5.36×10^{-5} M and 6.40×10^{-6} M at the Pt and Pt-BDD electrodes, respectively, while from the reduction peak it was found to be 5.40×10^{-6} M at Pt-BDD. However, the reduction peak at the Pt electrode showed a low correlation of the peak currents to zanamivir concentration. Therefore, for the next observation, the oxidation peak at -0.4 V was

applied. Furthermore, the lower LOD at the Pt-BDD electrode suggested that the electrode was more suitable for application than the Pt electrode.

Typical behavior of zanamivir at Pt-based electrodes was also reported to occur at the Au-based electrodes [6,7]. Zanamivir is known to have a guanidine group, which actively forms hydrogen bonds to inhibit the activity of neuraminidase. This group is also predicted to have a main role in the adsorbing activity on the surface of the metal electrodes [4-7]. It was reported that guanidine compounds serve as a donor ligand [21], in which the electron pair at $=\text{N}-\text{H}$ is predicted to contribute in the adsorption of zanamivir on the metal surfaces such as those of mercury (Hg) and Au electrodes [6]. However, whereas zanamivir adsorption at Hg electrodes catalyzes the reduction reactions of H^+ to H_2 as shown in Eq. 2, its adsorption at the Au blocks the active surface of Au and reduces the oxidation-reduction reaction of Au. The same behavior appears to occur at the Pt electrodes, which show the decrease of the voltammetry currents in the presence of zanamivir. It was previously reported that the binding of an organic layer to the surface of an electrode can induce the slowing down of electron transfer on the electrode surface [22]. In this case, the adsorbed zanamivir covered the electrode surface and disrupted the oxidation-reduction reactions of the Pt surface. Therefore, the reduction of H^+ to H_2 was also blocked.

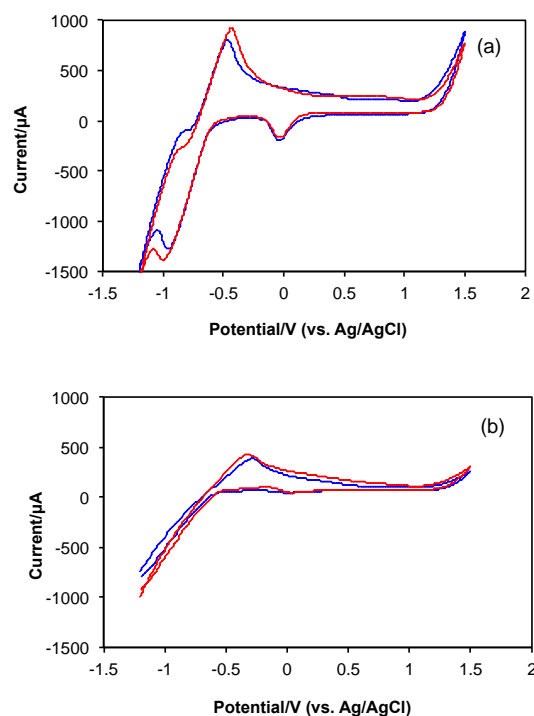


Figure 3. Cyclic Voltammograms of 0.1 M PBS in the Absence (red line) and in the Presence (blue line) of 1.5×10^{-4} M Zanamivir at (a) Pt and (b) Pt-BDD Electrodes. Scan Rate was 100 mV/s

Comparison of the analytical performance of zanamivir with gold (Au)-based electrodes is displayed in Table 2. While the responses of zanamivir using the oxidation peaks at the Pt and Au bulk electrodes showed comparable sensitivities, the Pt-BD showed an approximately 15 times higher sensitivity of zanamivir than Au-BDD [6]. The stability of the peak potentials was also compared. The oxidation-reduction peak potentials at -0.5 V and -0.1 V, respectively, at the Pt electrodes are relatively stable (Figure 4). However, the shift of the oxidation-reduction peaks to the more positive potentials was observed at the Au-based electrodes [6,7]. This is possibly due to the better stability of the adsorption of zanamivir at the Au electrodes, which causes slow recovery of the electrodes.

Excellent reproducibility of 20 consecutive measurements of cyclic voltammetry for 0.1 M of PBS pH 7 containing 1.5×10^{-4} M zanamivir at both the Pt and Pt-BDD electrodes can be achieved with relative standard deviations (RSDs) of 1.70% and 1.64%, respectively. Reproducibility of each electrode on the intermittent days was also investigated by measuring the oxidation current responses for five consecutive days using the cyclic voltammetry method. The better stability of Pt electrode compared to the Pt-BDD electrode was indicated by the decrease of 3.55% and 4.68% after five days at the Pt and the Pt-BDD electrodes, respectively. The physical interaction and the size of Pt particles on the surface of BDD might be the main problem of the stability of Pt-BDD [23,24].

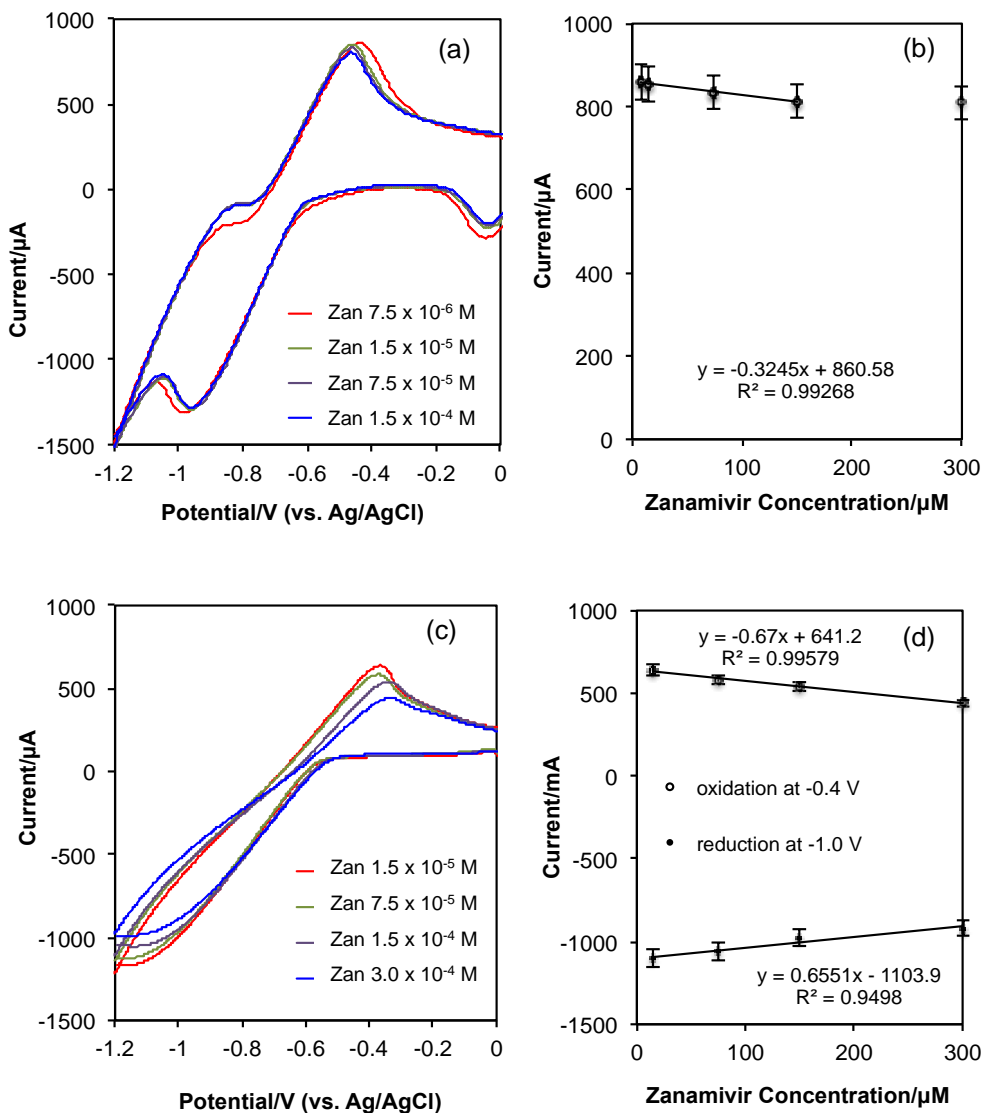


Figure 4. Cyclic Voltammograms of Various Concentrations of Zanamivir in 0.1 M PBS pH 7.0 at (a) Pt and (c) Pt-BDD Electrodes. Plots of the Peak Current at -0.5 V are shown in (b) and (d) for Pt and Pt-BDD, Respectively. Other Conditions were Similar to those Shown in Figure 3

Electrochemical behavior of zanamivir in the presence of neuraminidase. The influence of neuraminidase on the electrochemical behavior of zanamivir was investigated by using cyclic voltammetry at the Pt-BDD electrode. Figure 5a shows that the addition of neuraminidase into the solution containing zanamivir reincreases the oxidation-reduction peaks of the Pt. The dependence of the reduction current at -0.4 V in the form of signal-to-background (S/B) ratio on pH shows that the S/B increased from pH 6.0 to pH 6.8 and then decreased (Figure 5b). Moreover, investigation of the contact time with neuraminidase shows the increase of S/B with the contact time from five to 30 min, then reach saturation after 30 min (Figure 5c). Accordingly, the optimum condition for the inhibition of neuraminidase by zanamivir was fixed at pH 6.8 with an incubation time of 30 min at 37 °C.

It is reported that zanamivir causes the inhibition reaction of neuraminidase [3]. The active site of zanamivir, which can interact with the electrode surface, is predicted to bind with neuraminidase [6,7]. Therefore, the concentration of adsorbed zanamivir on the surface of the Pt-BDD decreased, and the decreases of the oxidation-reduction currents of Pt were reduced when the measurement was performed and then reached saturation. The behavior of zanamivir in the absence and in the presence of neuraminidase is illustrated in Figure 6.

Figure 7 shows that the presence of neuraminidase in the optimum condition of inhibition reincreases the peak currents of Pt linearly in the concentration range of 2-20 mU neuraminidase and then reaches saturation, suggesting that the maximum concentration of neuraminidase that could be inhibited by 2×10^{-5} M zanamivir was 20 mU with an LOD of 0.515 mU. Furthermore, excellent reproducibility of neuraminidase measurement with an RSD of 0.468 was observed for 10 times measurement using the cyclic voltammetry method.

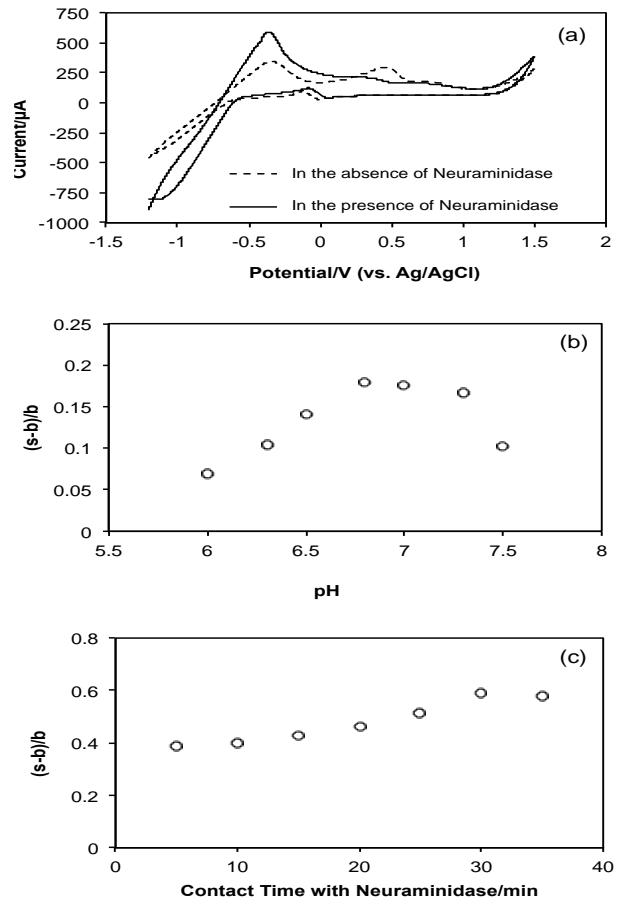


Figure 5. The Dependence of signal-to-background Ratios of the Peak Currents of 2×10^{-5} M Zanamivir in 0.1 M PBS in the Presence of 20 mU Neuraminidase (a) at Different pHs with a Contact Time of 20 min and (b) at pH 6.8 at Different Contact Times. The Working Electrode was the Pt-BDD Electrode. Other Conditions were Similar to those shown in Figure 3

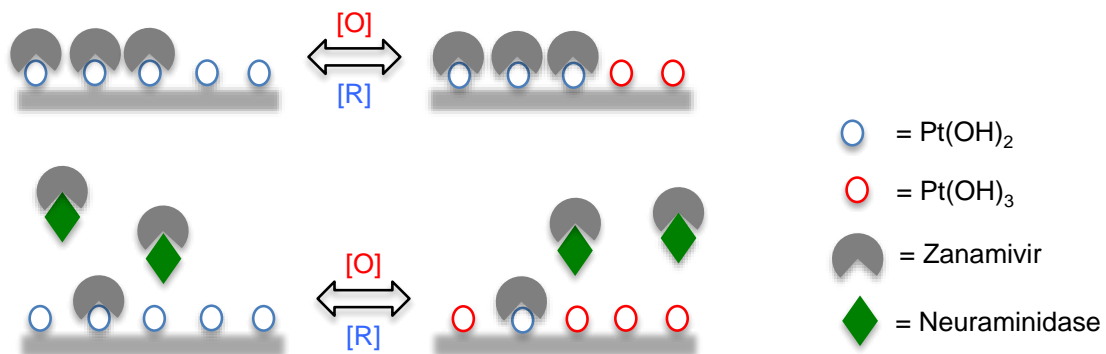


Figure 6. Illustration of Zanamivir Behavior in the Presence of Neuraminidase

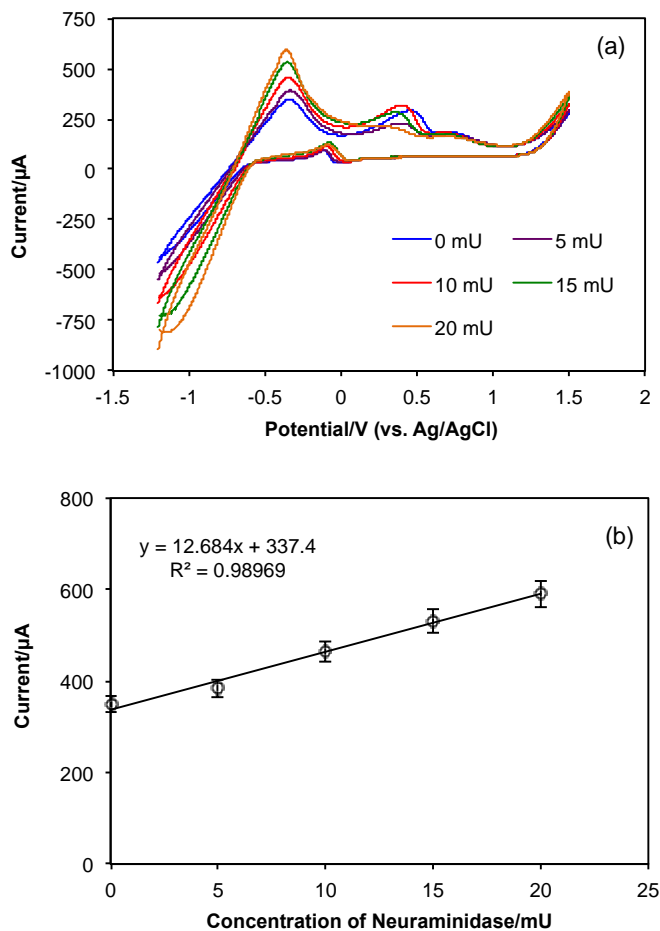


Figure 7. (a) Cyclic Voltammograms of 1.5×10^{-5} M Zanamivir in 0.1 M PBS pH 6.8 in the Presence of Various Concentrations of Neuraminidase at the Pt-BDD Electrode. Contact Time with Neuraminidase was 30 min. The Correlation between the Oxidation Peak Currents at -0.5 V and the Concentrations of Neuraminidase is shown in (b). Other Conditions were Similar to those shown in Figure 3

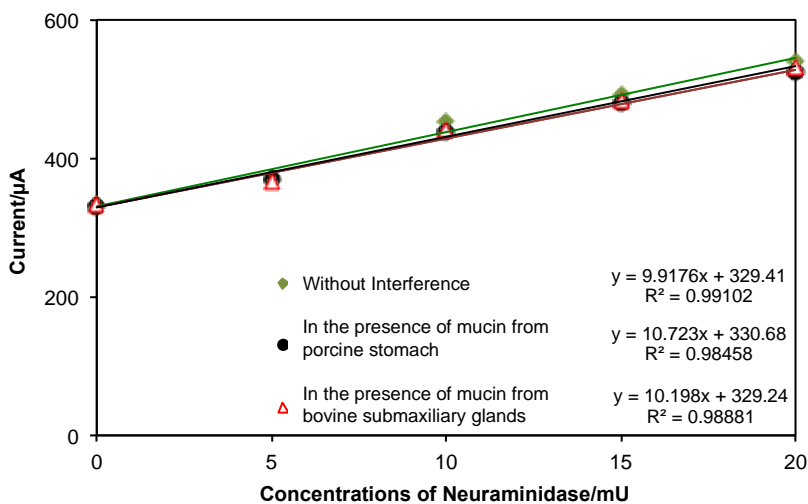


Figure 8. Plots of the Peak Currents of Cyclic Voltammograms of 0.1 M PBS pH 6.8 Containing 1.5×10^{-5} M Zanamivir and 20 mU Neuraminidase in the Absence and in the Presence of Mucin from Porcine Stomach and from Bovine Submaxillary Glands. The Currents were Extracted at the Potential of -0.4 V. The Contact Time of the Solution with Neuraminidase was 30 min. Other Conditions were Similar to those shown in Figure 3

Selectivity of the sensor. Selectivity was examined using the cyclic voltammetry method in the presence of mucin porcine stomach and mucin bovine submaxillary glands as the interferences. Mucin was selected as the matrix for a real sample model since neuraminidase is generally found in an influenza virus. It is well known that a common symptom of a person infected with influenza is the production of nasal mucus, a type of mucin. Based on the observation of oxidation peak currents, the responses were decreased by 0.856% with the addition of 0.011 mg/mL mucin from porcine stomach, and decreased by 0.577% with the addition of 0.011 mg/mL mucin from bovine submaxillary glands. However, the plots of current responses versus neuraminidase concentrations were linear under the presence of 0.011 mg/mL of the interferences (Figure 8), suggesting that the sensor shows promise for being applied in a real sample.

Conclusion

Zanamivir is not directly electroactive on the surface of the Pt and Pt-PDD electrodes. However, similar to that at the Au-based electrodes, zanamivir can be adsorbed on the surface of Pt particles and affect the oxidation reaction of Pt. This behavior was successfully applied for the detection of neuraminidase due to the inhibition reaction of neuraminidase by zanamivir. Furthermore, the measurement could be also performed in the presence of interferences.

Acknowledgments

This work was partially supported by a grant from Hibah PUPT BOPTN Universitas Indonesia, Contract No. 0533/UN2.R12/HKP.05.00/2015.

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