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Zinc oxide/Vancomycin-based Electrochemical Chiral Sensor for the Recognition of Penicillamine Enantiomers

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In this study, an electrochemical chiral sensor based on Zinc oxide/vancomycin-modified indium tin oxide glass substrate (ZnO/Van/ITO) was successfully fabricated and used in the chiral recognition of penicillamine (Pen) enantiomers. The preparation of the modified electrode was a two-step procedure in which electrodeposition of ZnO was conducted first on the ITO glass substrate. The deposition parameters were conducted at –0.9 V, in a buffer solution of pH 7 at 60 °C for 900 s. The second part of the procedure involved drop casting Van on the surface of the ZnO/ITO modified electrode. Different oxidation peak current, I_p of L- and D- Pen were observed in the differential pulse voltammograms (DPV). Under optimal condition, the chiral sensor exhibited good linear response to Pen enantiomers in a linear range of 5 mM to 30 mM with a detection limit of 23.56 mM and 14.73 mM (S/N=3) for the D-Pen and L-Pen, respectively. The modified electrode fabricated possessed exceptional performance of having low detention limit and good enantioselectivity for Pen enantiomers. The association constant from the experiment, K_D and K_L obtained were 2.314×10^3 L/mol and 1.076×10^3 L/mol for the D and L-Pen, respectively. The value of K_D was larger compared to K_L indicating that the binding effect is higher between ZnO/Van with D-Pen. The findings suggested that this proposed chiral sensor has the ability to discriminate Pen enantiomers which can bring good use in the pharmaceutical industries.

Keywords: chiral sensor, vancomycin, ZnO, enantioselectivity, penicillamine

1. INTRODUCTION

Chiral recognition and separation of racemic drugs has been an interesting research subject since the introduction of the racemic switch strategy adapted by pharmaceutical industries [1,2]. The medical and pharmaceutical industries would require pure enantiomer over the racemate in the production of medicines and effective treatments. The development of a reliable detection system in the discrimination of chiral drugs is necessary as chirality affects the drug's biological and pharmacological properties [3,4]. There are several stereoselective techniques utilized in chiral analysis such as spectroscopy methods, high-performance liquid chromatography, and electrochemical methods. The electrochemical approach brings a plethora of benefits of high sensitivity, rapid detection and low-cost as compared to the conventional chromatography methods [5–8]. Hence, enantioselective sensor is a reliable tool in the monitoring of different interactions of enantiomers, determining purity in chirally pure marketed products and studying toxicological effects of enantiomer.

Vancomycin (Van) is a macrocyclic glycopeptide used to discriminate enantiomers in many techniques such as high-performance liquid chromatography (HPLC) and electrochemical methods. The interesting property of vancomycin containing an array of chiral center possessing functional groups that interacts with the analyte is worth to be explored. Van has shown to be an efficient chiral selector in the enantio-recognition of chiral molecules in electrochemical chiral sensor. Previously, vancomycin based electrochemical sensor have been developed on various chiral compounds such as carnitine [9], perindopril [10], butaclamol [11] and penicillamine [12]. Penicillamine (Pen), is a commonly known drug among the medical fraternity marketed under the brand names Cuprimine and Depen. D-Pen is utilized in Wilson disease and rheumatic disorders treatment. The application of D-Pen is a non-naturally occurring chiral compound exhibiting racemic structures which is an important thiol compound as a chelating agent to decrease copper stores in Wilson disease patients [13,14]. Besides that, the application of D-Pen is also extended to the treatment of a condition known as cystinuria due to the accumulation of stones in the kidney and bladder [15]. On the other hand, L-Pen exhibited no antibiotic properties and is toxic.

Previously, several chiral recognition sensors have been developed that shown to have high sensitivity, selectivity, and compatibility in biological assay. Wang et al., [16] have reported on the chiral recognition of Pen enantiomers using bovine serum albumin (BSA) embedded in Al_2O_3 sol gel coated on glassy carbon electrode (CGE). The results showed that larger current response was obtained from D-Pen due to the difference of hydrogen bonds, hydrophobic interactions, and the stereospecific site between BSA and Pen enantiomers. On the other hand, Lin et. al., [17] developed a chiral recognition of penicillamine via electrochemiluminescence using hemoglobin and gold nanoparticles functionalized graphite-like carbon nitride nanosheets.

The inclusion of ZnO as part of the modifier is mainly attributed to the ability of ZnO for the immobilization of enzymes while retaining their biological activity [18–20]. Since, ZnO is known to exhibit various desirable traits for biosensing such as high catalytic efficiency, strong adsorption capability, and high isoelectric point (IEP; ~9.5) which are suitable for adsorption of certain proteins (e.g. enzymes and antibodies with low IEPs) via electrostatic interaction [21]. Although ZnO has shown to exhibit poor electrochemical behavior [22], we hope to understand the capability of ZnO in biosensing capability upon reacting with a macrocyclic glycopeptide for the discrimination of chiral molecules. Combining the unique properties of Van with ZnO as a hybrid couple is expected to enhance the chiral detection of the enantiomers.

To date, there are no published reports on the usage of ZnO/Van modified ITO in the chiral analysis of Pen enantiomers. The simple experimental design of the nanohybrid electrode is hoped to be a supplementary reference for the development of Pen chiral sensor. This will thus give important implications for biosensing, asymmetric syntheses and separation, and understanding the nature of chiral interactions in biological systems. The outcome could hold good application in the fields of electroanalytical chemistry and biosensors. Answers to these puzzling questions will bring significant impact on the understanding of the processes involving chiral molecules in living organism.

2. EXPERIMENT

2.1 Chemicals

Vancomycin hydrochloride (Van) was purchased from European Pharmacoeia Reference Standard. Hygroscopic L-Pen and D-Pen were obtained from Toronto Research Chemicals Inc. Zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) was obtained from Friendemann Schmidt. Ethanol (C_2H_5OH) and acetone (CH_3COCH_3) were purchased from HmbG Chemicals. Potassium chloride (KCl) and hydrochloric acid (HCl) were obtained from Merck and R&M Chemicals, respectively. Ultrapure 18.2 M **Ω**-cm deionized water obtained from Barnstead Nanopure Ultrapure Water Systems (Thermo Scientific) was used to prepare the supporting electrolyte. All other chemicals were of analytical grade and were used without further purification.

2.2 Apparatus and measurements

The electrochemical analyzer Nova 1.1 Autolab potentiostat (PGSTAT204, Metrohm Autolab, Netherlands) connected to an external computer was utilized to carry out electrochemical measurement and electrochemical impedance spectroscopy (EIS). The three-electrode system consisted of a conductive indium-tin oxide (ITO) thin film electrodes (7 cm x 1 cm) as the working electrode, Ag/AgCl/KCl (3M) and platinum as the reference and auxiliary electrode, respectively. The morphology of the modified electrode was characterized by using the FESEM microscope (JEOL JSM-7600F) operated at 5 kV.

2.3 Fabrication of ZnO/ITO modified electrode

ITO was washed and cleaned under ultra-sonification for 30 minutes thoroughly with acetone, ethanol and distilled water, respectively. The glass substrate was then dried in a desiccator under ambient temperature to obtain a working electrode with refurbished electrode surface. A mixture of zinc nitrate hexahydrate $Zn(NO_3)_2.6H_2O$ (0.05M) and 5 ml of 0.1 M of KCl (0.1M) acting as the bath solution were placed in a borosilicate glass container and bubbled with stream of N_2 gas. The ZnO electrodeposition was carried out using on an electrochemical potentiostat applying the chronoamperometry (CA) technique. The parameters employed in the electrodeposition procedure was based on our previously reported work [23]. In short, the ZnO deposition applied a deposition temperature of 60^{o}C in a pH buffer of 7, with a deposition potential of -0.9V for 900 s.

2.4 Fabrication of ZnO/Van/ITO modified electrode

Prior to the inclusion of Van, the ZnO modified electrode is washed with distilled water and dried under a stream of nitrogen gas for 15 minutes. Van was dispersed well in dimethylformamide (DMF) followed by sonication for 2 hours. Then, 4 µL of the Van was drop-casted onto the ZnO modified electrode and dried under ambient temperature.

3. RESULTS AND DISCUSSION

3.1. Morphology of bare ITO, ZnO/ITO, Van/ITO and ZnO/Van/ITO electrode

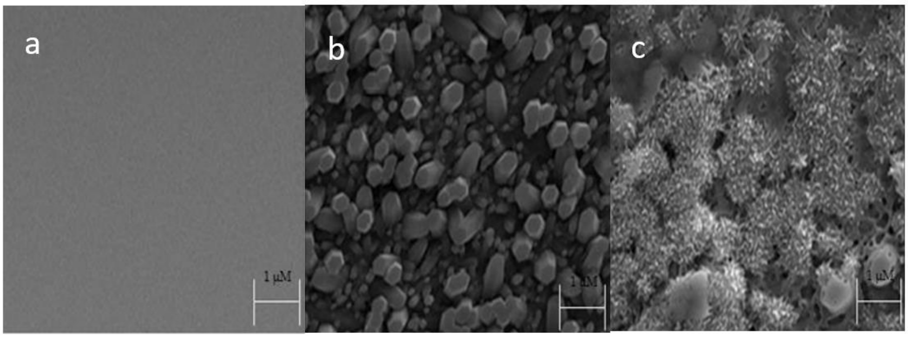


Figure 1. Field emission scanning electron microscopy images of (a) bare ITO, (b) ZnO/ITO and (c) ZnO/Van/ITO

Figure 1 depicts the morphology surface of bare ITO, ZnO/ITO and ZnO/Van/ITO modified electrode, respectively. From the SEM images, significant morphological distinctions can be easily identified. The bare ITO exhibited a flat smooth surface as shown in Figure 1a. On the other hand, hexagonal shaped nanorods resembling a branch-like morphology were randomly distributed on the ITO substrate after electrodeposition as depicted in Figure 1b. The morphological findings were in coherent with other previously reported literature on ZnO electrodeposition on a glass substrate [24,25]. The poor size distribution and dimension of the nanorods can be attributed to the lattice mismatch and high surface energy of ZnO [26]. The electrodeposition of ZnO is a flexible growth process in which an array of nanostructure could be obtained via this technique. The different properties of ZnO nanostructure can be achieved by controlling the experimental condition of the electrodeposition process [27–29]. The presence of Van can be confirmed from the spore-like formation coverage on the surface of the ZnO/ITO using a simple drop-casting method as depicted in Figure 1c. It is posited that the Van filled the empty gaps formed between the ZnO nanorods and also formed a coverage on the nanorods. Hence, the film formation between the interaction between ZnO and Van leads a much compact structure.

3.2. Electrochemical behavior of bare ITO, ZnO/ITO, Van/ITO and ZnO/Van/ITO electrode

The electrochemical behavior of the bare ITO, ZnO/ITO, Van/ITO and ZnO/Van/ITO electrodes were investigated by obtaining the current response of 1 mM $K_3[Fe(CN)_6]$ which act as the electroactive probe in 0.1 M PBS electrolyte solution at 100 mV/s scan rate in the potential range from -500 to +1000 mV. As depicted in Figure 2, a standard redox peak was obtained on the bare ITO for the ($K_3[Fe(CN)_6]$) with ΔI_p of 281.56 µA. The modification with ZnO resulted in the decrease of ΔI_p to 122.34 µA. This can be explained by ZnO nanorods blocking the surface of the electrode and hindered the redox reaction from occurring smoothly by introducing additional barrier for electron transfer on the electrode surface [26]. The peak current ΔI_p of Van/ITO recorded at 150.12 µA was higher than that of ZnO/ITO can be explained by the good adsorption ability of Van which enhanced the rate of electron exchange with the surface of the electrode due to their abundant hydrophilic and polarizable functional groups which increased its' conductivity. However, the peak current ΔI_p of ZnO/ITO decreased after immobilization of Van which can be attributed to the blocking of non-binding sites between the medium and the electroactive surface electrode which decelerates the electron transfer [30].

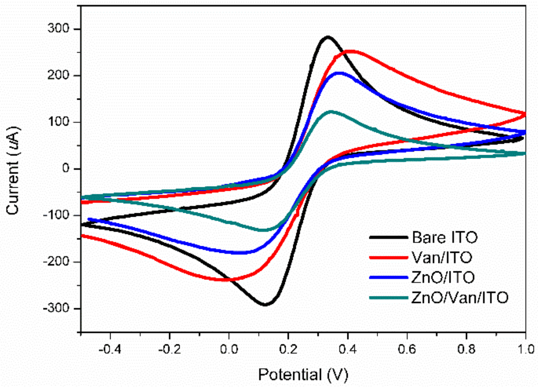


Figure 2. Cyclic voltammogram of 1 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) in 0.1 M PBS electrolyte solution using bare ITO, Van/ITO, ZnO/ITO and ZnO/Van/ITO using scan rate of 100 mV/s at room temperature

3.3. *Electrical Impedance Spectroscopy analysis*

EIS is used to confirm the interface properties of the surface modified electrodes. The impedance spectra depict a linear part at low frequency corresponds to the limited diffusion in transporting redox species to the electrode surface and a semicircle part at a higher frequency corresponds to the limited process of electron transfer.

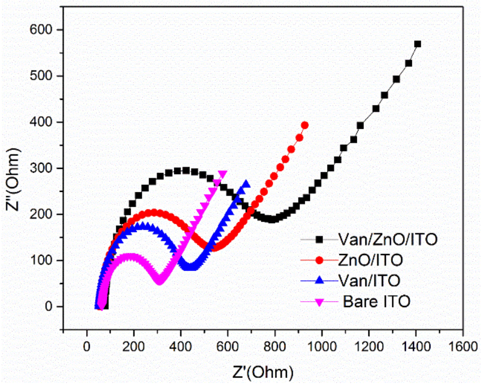


Figure 3. Nyquist plots obtained at a) ZnO/Van/ITO b) ZnO/ITO c) ZnO/ITO and d) bare ITO in 0.1 M PBS containing 5 mM $K_3[Fe(CN)_6]^{-3/-4}$

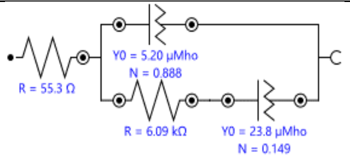
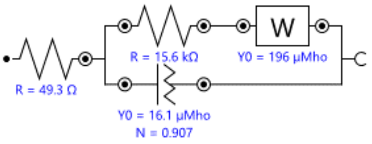
A typical shape of the Faradaic impedance spectrum plotted as Nyquist plot in which the diameter is equal to the interfacial charge transfer resistance (R_{CT}). Figure 3 depicts the impedance retorts for bare ITO, ZnO/ITO, and ZnO/Van/ITO in PBS containing 5 mM $K_3[Fe(CN)_6]^{-3/-4}$ using a frequency range from 100 kHz to 1 mHz, and the AC amplitude was set at 10 mV. Theoretically, the higher the charge transfer resistance (R_{CT}) values, the lower the conductivity of the modified electrode.

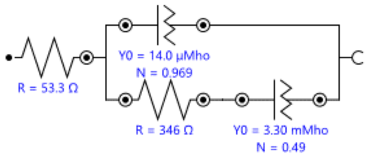
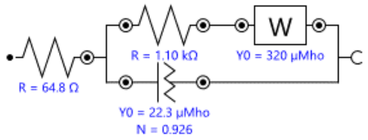
The equivalent circuit in Table 1 is further chosen to fit the impedance data obtained in the experiments. Based on Figure 3, bare ITO showed a semicircle in high frequency region and straight line in low frequency region with charge transfer resistance (R_{CT}) of 1.10 kΩ. The ZnO modified electrode showed an interfacial R_{CT} of 15.6 kΩ which showed an apparent hike upon comparing with R_{CT} value of bare ITO which agrees well with previous report [31]. The inherent conductivity characteristics of ZnO nanorods leads to the increase of R_{CT} [32]. However, the ZnO/Van/ITO showed an interfacial R_{CT} of 6.09 kΩ, which is lower compared to the ZnO/ITO. The decreased value of R_{CT} showed that Van possessed high electrocatalytic efficiency. The diameter of the semi-circle decreased due to the blockage of charge transfer by Van. The decrease in R_{CT} value for Van/ITO can also be explained from the presence of high number of polarizable groups on the Van structure that was polarized at the open circuit potential. This process led to a positive charge on electrode surface which ease the redox reaction and causes the decrease in the R_{CT} [33]. Furthermore, the decrease in the R_{CT} value also confirms the incorporation of the Van of the ZnO/ITO electrode surface [34]. The EIS behavior pattern spectrum matched with the results in the previous section.

3.4. Chiral Discrimination of D- and L- Pen at ZnO/Van/ITO

The recognition of the two enantiomers, D-Pen and L-Pen carried out using DPV were compared using a) ZnO/Van/ITO b) Van/ITO and c) ZnO/ITO in 10 mL of 0.1 M PBS containing 1 mM Pen enantiomers (pH 7.0) at room temperature. Based on the DPV from Figure 4a, the peak current obtained for were 1.14 µA and 0.885 µA for D-Pen and L-Pen respectively with peak current difference ΔI (ΔI = I_D - I_L) at 0.255 µA. A distinct peak current difference, ΔI and peak potential differences, E_p can be observed for the ZnO/Van/ITO. The synergistic effect of ZnO and Van with the Pen enantiomers could be the main factor in the successful discrimination of the Pen enantiomers. Besides, the affinity of the different stereoisomeric configuration that lead to the different formation of H-bonding affinity with the modified electrode is posited to be the underlying for the findings. Based on Figure 4b, a clear ΔI of 0.270 µA is apparent for the Pen enantiomers due to the already known chiral selector characteristics of the Van.

Table 1. Equivalent circuit obtained at a) ZnO/Van/ITO b) ZnO/ITO and c) Van/ITO d) bare ITO

Electrode	**Equivalent Circuit**
ZnO/Van/ITO	
ZnO/ITO	

Van/ITO	
Bare ITO	

Based on Figure 4c, the D-Pen and L-Pen peaks were slightly resolved indicating good property of selectivity. However, the difference in peak current response was not significant. However, it is interesting to note that even though the obtained ΔI is very minimal, but there is small observable change in the E_p showing that the varying symmetry of the ZnO nanorods indeed affects the chirality enhancement of the Pen enantiomers [35].

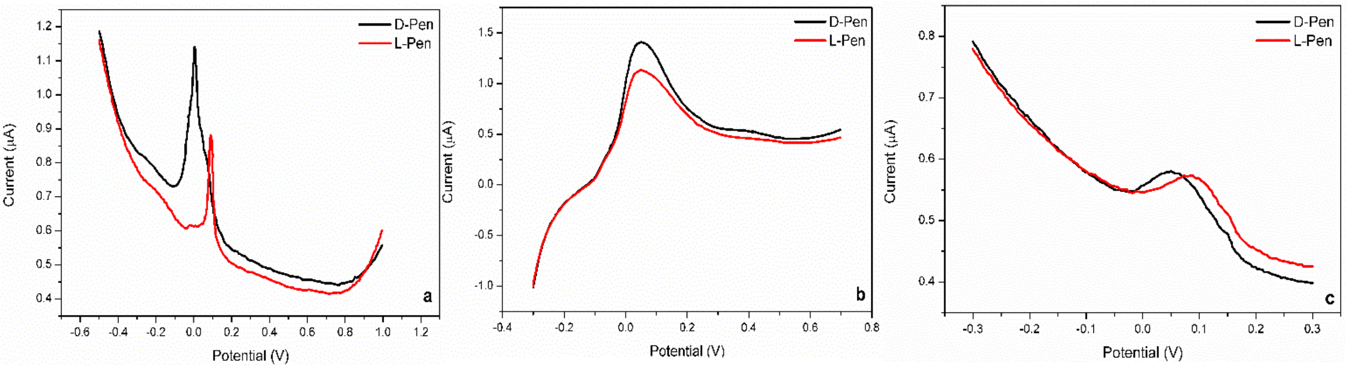


Figure 4. DPVs of 1 mM L-Pen and D-Pen using a) ZnO/Van/ITO modified electrode, b) Van/ITO modified electrode, c) ZnO/ITO modified electrode in 10 mL of 0.1 M phosphate buffer solution (PBS) containing 1 mM Pen enantiomers (pH 7.0)

These findings can be used to meet the purpose of using ZnO/Van/ITO even though the current response is considered lower upon comparing to other previous reports [36,37]. The well-defined E_p for each enantiomers provide qualitative enantio-recognition of enantiomers which can be applied in racemic mixture analysis.

3.5. Optimization of interaction conditions

In order to probe the influence of the amount of Van as the chiral selector on Pen enantiomers chiral discrimination, a study was conducted by drop casting varying amount of Van from the range of 2 µL to 12 µL. It can be observed that the highest current response was attained at 4 µL of Van indicating that saturation point has been achieved from the interaction between ZnO/Van and Pen enantiomers. The presence of excess Van significantly decreases the separation of the two enantiomers. As depicted in Figure 5a, with the amount of Van increased, the peak current obtained for D-Pen increase from 2 µL until 4 µL and then decreased slightly from 4 µL to 8 µL and then rises again until 12 µL. While, for L-Pen, the peak current increased from 2 µL until 4 µL followed by the peak current decrease at 6 µL.

However, the peak current slightly increased at 8 µL and decreases significantly until 12 µL. The continuous decrease of ΔI_L could be due to the increase of Van might be due to physically absorbed L-Pen causing depressed enantioselectivity [16]. The maximum value of $\Delta I = \Delta I_D - \Delta I_L$ was 0.132 µA occurring at 4 µL. Hence, 4 µL was chosen as the optimum amount of Van in the subsequent work.

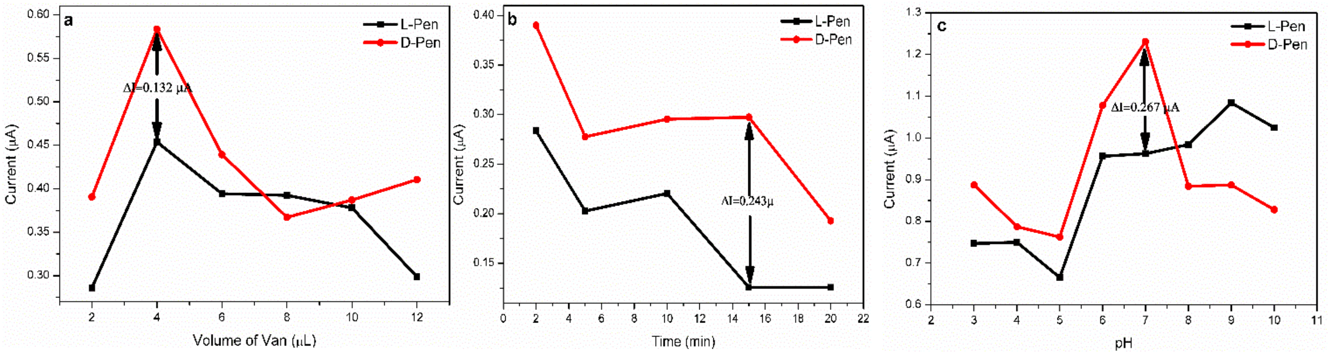


Figure 5. (a) Plot of peak current versus volume of Van casted (b) Plot of peak current versus incubation time (c) Plot of peak current versus pH of electrolyte

The effect of incubation time on the current response at the range of 2 to 20 mins was studied. The change of the peak current with the incubation time was depicted in Figure 5b. The decrease in peak current obtained can be seen from 2nd minute to the 5th minute and a slight increase from the 5th minute to 10th minute could be observed for both Pen enantiomers. However, from the 10th minute to the 15th minute, the peak current increased for the D-Pen and decreased for the L-Pen. The maximum value of ΔI was obtained at 15 minutes which was at 0.243 µA. Hence, 15 minutes was adopted as the best recognition time in this work.

The pH of the supporting electrolyte is pertinent to reveal the Van efficacy to the Pen enantiomers. Based on Figure 5c, it can be seen that the electrocatalytic oxidation of D-Pen at the ZnO/Van/ITO surface was more favoured under neutral, at pH 7 which is also in agreement with previously reported studies [38]. However, the L-Pen gives the highest peak current response at a slightly basic condition at pH 9. The maximum value of ΔI was obtained at pH 7 which was at 0.267 µA which was the largest compared to when the D-Pen and L-Pen were in basic or acidic environment. Hence, pH 7 was used as the best recognition time in this work.

3.6. Effect of concentration of Penicillamine enantiomers

The ZnO/Van/ITO was used to evaluate the current response of varying concentration of Pen enantiomers from 5 mM to 30 mM in order to study the effect of Pen concentration on peak current and also to investigate the optimum testing concentration of Pen enantiomer. It can be seen that the anodic peak current obtained was inversely proportional to the Pen enantiomers in the range of 5 – 30 µM for both the Pen enantiomers at Figure 6. This occurrence can be explained by weaker dissociation of Pen at higher concentrations hence retarding the electron transfer process.

It was found out that the peak current of both Pen enantiomers decreased with the increasing amount of Pen enantiomers. However, it is important to notice that D-Pen showed a more apparent change in peak current than L-Pen. It means that the D-Pen has a stronger affinity to Van. Based on Figure 6, the linear regression of the D-Pen can be expressed as $y= -0.0053x +2.2558$, with a correlation coefficient of 0.96122. The limit of detection was calculated to be 23.56 mM, which was calculated according to the International Union of Pure and Applied Chemistry (IUPAC) definition that is 3 times the standard deviation of the blank value. On the other hand, the linear regression of the L-Pen can be expressed as $y=-0.0071x + 2.0173$, with a correlation coefficient of 0.98458. The limit of detection was 14.73 mM. Hence, the proposed electrode showed excellent performance in terms of wide linearity range, low detection limit and good enantioselectivity for the Pen enantiomers.

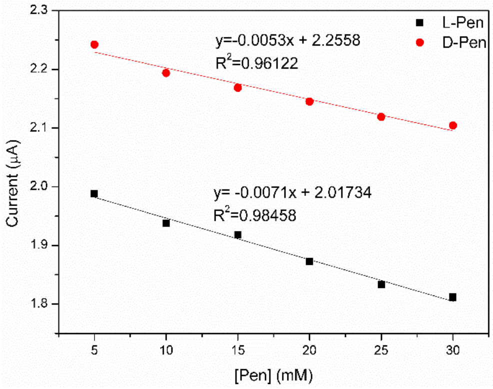


Figure 6. Plot of peak current versus concentration of Pen enantiomers.

The selectivity of the chiral biosensor was quantitatively evaluated using the enantioselectivity coefficient (α) which is defined using the following equation:

$$\alpha = \Delta I_D / \Delta I_L \qquad (1)$$

where α is the enantioselectivity coefficient, a calculable value of the sensor's ability to distinguish one isomer over the other, ΔI_D and ΔI_L are the anodic peak current differences of the modified electrodes after interaction, corresponding to D- and L-Pen, respectively. The enantioselectivity coefficient for the chiral sensor based on the concentration from 5 mM to 30 mM was calculated as shown in Table 2.

Table 2. Enantioselectivity coefficient of ZnO/Van/ITO under different [Pen]

[Pen]	ΔI_D	ΔI_L	α
5	2.285	2.015	1.13
10	2.160	1.887	1.14
15	2.144	1.917	1.12
20	2.143	1.870	1.15
25	2.115	1.832	1.15
30	2.126	1.812	1.17

The determination of association constant between Van and Pen enantiomers is vital in confirming the interaction of Van and Pen enantiomers. Equation 2 is used to obtain the association constant value for the interaction can be determined using Equation 2. The binding number (*m*) and association constant (*K*) of ZnO/Van-mPen were calculated as follow:

$$\log\frac{\Delta I_i}{\Delta I(_{i,max-}\ I_i)} = \log K + m \log [\text{Pen}] \qquad (2)$$

where ΔI_i means peak current change of ZnO/Van and Pen enantiomer. $\Delta I_{i,max}$is the maximum change in peak current and [Pen] is the concentration of the Pen enantiomers.

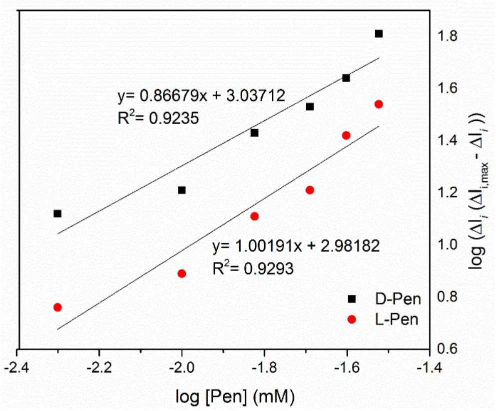


Figure 7. The plot of log $\Delta I/\Delta\, I(_{imax-}\ _i)$ against log [Pen] for D- Pen and L-Pen

According to Equation 2, the plot of $\Delta I/\Delta\, I(_{imax-}\ _i)$ versus log [Pen] was obtained with the mean value of the peak current change by 3 replicate measurements resulted in a linear relationship for both the Pen enantiomers. Based on the intercept of the linear plot from Figure 7 were obtained and from there the K_D and K_L obtained were 2.314 x 10^3 and 1.076 x10^3 L/mol for the D and L-Pen respectively. The value of K_D was larger compared to K_L indicating that the binding effect is higher between ZnO/Van/ITO with D-Pen.

3.7. Effect of enantiomeric ratio of Pen enantiomers

The selectivity is one of the most fundamental concepts in the development of chiral sensor as most enantiomers have almost similar electrochemical behaviors. It is vital to come out with fast, simple, and feasible method to discriminate one enantiomer with the presence of analyte with similar interference peak potential or current. The ZnO/Van/ITO modified electrode has been used for the determination of the mixture of L- and D- Pen.

The ratio of the Pen enantiomers (D-Pen:L-Pen) were studied by differential pulse voltammetry. The ratio concentration employed are 1:0, 4:1, 3:2, 2:3, 1:4 and 0:1 by increasing the L-Pen in the solution containing D-Pen. The peak current gradually decreased from D-Pen to L-Pen along with the increasing amount of L-Pen.

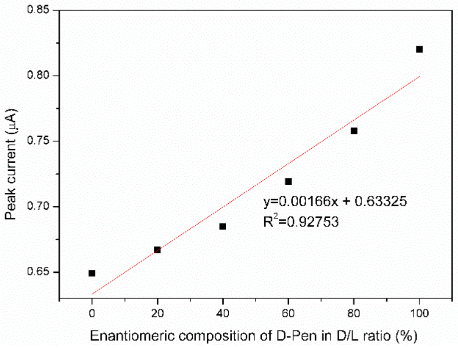


Figure 8. The linear calibration curve for the percentage of D-Pen.

It is important to note that the apparent peak reduction at the 20% addition of L-Pen to the D-Pen. Based on Figure 8, the correlation between Δ*I* and % D-Pen exhibited a satisfactory linear relationship which can be expressed as y=0.00166x + 0.63325 with a correlation coefficient of 0.92753. These results would be beneficial for the analysis of enantiomeric excess of chiral compounds where the unknown % of each enantiomer in the mixture can be determined according to:

$$\%L= [M_L/M_L+M_D] \times 100 \quad (3)$$
$$\%D= [M_D/M_D+M_L] \times 100 \quad (4)$$

where M_D and M_L are the slopes of the linear calibration graphs. From this, the percentage of each enantiomer in the mixture can be calculated using Equation 3 and Equation 4. This kind of application is important in asymmetric synthesis and chiral separation fields.

3.8. Repeatability, Reproducibility and stability studies

Repeatability, reproducibility and stability studies were conducted to access the performance of the modified ZnO/Van/ITO electrode in 0.5 mM of Pen enantiomers using 100 mV/s of CV in a pH of 7.0. The relative standard deviation (RSD) of the modified electrodes was depicted in Table 3. Since the RSD value is significantly small (less than 5 %), the modified electrode is posited to exhibit good repeatability, reproducibility and stability.

Table 3. RSD value obtained for repeatability, reproducibility and stability

Experiment	Relative standard deviation (RSD)	
	D-Pen	L-Pen
Repeatability	0.3012	0.3577
Reproducibility	2.0450	1.4075
Stability	0.2010	1.0077

4. CONCLUSION

A novel chiral sensor based on ZnO/Van/ITO of good repeatability, reproducibility, and stability was successfully fabricated in the enantio-recognition of Pen enantiomers. The differential pulse voltammogram of the ZnO/Van/ITO displayed the expected behavior with a preference for D-Pen than for L-Pen. Optimization of enantio-recognition conditions showed that 4 µL was chosen as the optimum amount of Van suggesting that the interaction between ZnO/Van and Pen enantiomers is best. Pen enantiomers had reached a saturation point. The incubation time effect showed that 15 minutes provides the largest maximum value of ΔI. pH 7 was used as the best recognition time in this work. It was found out that the peak current of both Pen enantiomers decreased with the increasing amount of Pen enantiomers. However, it is important to notice that D-Pen showed a more apparent change in peak current than L-Pen. The limit of detection was calculated to be 23.56 mM and 14.73 mM for both D-Pen and L-Pen, respectively. The proposed electrode exhibited excellent performance in terms of wide linearity range, low detection limit and good enantioselectivity for the Pen enantiomers. From the determination of association constant between ZnO/Van/ITO and Pen enantiomers, the value of K_D was found to be larger compared to K_L indicating that the binding effect is greater between ZnO/Van/ITO with D-Pen.

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