Optimization of a Hydrogel-Based Bienzyme Amperometric Sensing Device for L-Glutamate and β-N-Oxalyl-α,β-Diaminopropionic Acid (β-ODAP) Using Glutamate Oxidase and Horseradish Peroxidase

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Improvements in a previously reported hydrogel-based amperometric biosensor using glutamate oxidase and horseradish peroxidase have been achieved in terms of dynamic linearity range, sensitivity and detection limits. The bilayer approach in the construction of the enzyme modified electrode in the present work, together with high enzyme loading, have enabled detection limits of 0.7 μM of the primary substrate for the enzyme system, L-glutamate and 2 μM for the secondary substrate β-N-oxalyl-α,β-diaminopropionic acid (β-ODAP), a neurotoxin found in Lathyrus sativus *(grass pea) seeds. The sensor response was found to be linear up to 250 μM for both substrates. Good reproducibility between equally prepared electrodes was found and the electrodes showed an operational stability of more than 50 h.*

Enzyme electrodes with amperometric transduction have recently attracted much attention in the biomedical and food research areas (1-3). The range of oxidase enzymes available for constructing enzyme electrodes has provided a convenient basis for constructing enzyme electrodes based on the detection of the electroactive species (O_2 or H_2O_2) involved in the catalytic reaction (1). The glucose oxidase containing Clark (O_2) electrode is one of the most studied. Measurements based on oxygen detection have, however, practical inconveniences and limitations. The response is low, and the dependence on oxygen can reduce the accuracy and reproducibility of the device. The detection of H_2O_2 through its electrochemical oxidation is the most commonly used alternative to overcome these drawbacks. However, the high potential employed to oxidize hydrogen peroxide (• > +600 mV *vs.* Ag|AgCl) poses a problem of electrochemical interference, resulting from a range of electro-oxidizable species in real sample matrices, such as ascorbate and urate (1,4). Furthermore, low ambient oxygen concentrations may prove rate limiting for oxidase enzymes (5).

In recent years, enzyme electrodes have been developed which use a non-physiological small redox mediator to shuttle electrons between the reduced enzyme and the electrode (6-8). These constitute a second generation of biosensors. In order to prevent mediators from diffusing out of the device, it is possible to covalently attach them to a polymer backbone (9,10) and additionally further covalently bind the mediator-polymer to the enzyme (11). In this way, electrical communication between the redox center(s) of the enzyme and an electrode is attained. Mediators have most commonly been used with hydrogen peroxide-producing oxidases but the concept is applicable to other classes of redox enzymes in biosensor research as well. In conjunction with oxidases, the function of the artificial electron acceptor is to replace oxygen as the natural electron acceptor and to ensure the current response can be obtained at a more modest potential than that of direct electrochemical oxidation of hydrogen peroxide. One has to keep in mind that any suitable mediator has to compete with the enzyme's natural electron acceptor, molecular oxygen, and during the catalytic cycle it has to oxidize the reduced oxidase in

a very fast step. Another requirement would be that it should be regenerated at potentials where oxidation of interfering compounds does not occur, or is at least significantly reduced.

Previously it was shown that Os-based polymeric mediators do not work optimally with glutamate oxidase (GlOx) (12). Another approach to circumvent this problem is the use of a bienzyme electrode construction. Such a method was introduced in 1981 (13) using glucose oxidase in combination with horseradish peroxidase (HRP) catalyzing the electroreduction of hydrogen peroxide at a low applied potential (0-0.2 V) and capable of working in the presence of soluble oxygen. Since then many other publications reported on amperometric bienzyme electrodes with oxidases and HRP (or other peroxidases) using a milder applied working potential of 150 mV (14-16). Gorton et al. (17) recently described a bienzyme (GlOx and HRP) carbon paste electrode operated at an "optimal" working potential of -50 mV whereby most of the electrochemical interfering components could be eliminated. That work describes the use of direct electron transfer between the electrode and HRP. The lower limit of detection and sensitivities did not, however, meet the demands for medical purposes. A mediated approach has been used to make the electron transfer between the electrode and HRP very much faster, thereby increasing the sensitivity and lowering the detection limit (12). In that study an Os-containing polymer was used to speed up the electron transfer between the electrode and the immobilized HRP.

GlOx has a high activity primarily for L-glutamate but also for β-ODAP (18). The second substrate, β-ODAP is a potent neurotoxin principle present in the seeds of *Lathyrus sativus* (grass pea), a staple crop in countries like Bangladesh, India and Ethiopia. A sustained consumption of the crop causes an irreversible neurodegenerative crippling disease called *Lathyrism* (19). Further studies in the bienzyme hydrogel based biosensor to improve the sensitivity, extend the linear response range, and improve the lower detection limit for both L-glutamate and β-ODAP compared to what was reported previously from this laboratory (12) is very desirable. There is also a considerable growing interest in having a convenient analytical method for rapid determination of glutamate in a variety of foods and biological materials (20,21). In foodstuffs, glutamic acid is involved in flavor enhancement of the food. An on-line monitoring and process-controlling set-up for glutamic acid production in fermentors is also highly demanded (22). On the other hand, glutamic acid is a potent neuroexcitatory amino acid associated with certain behavioral patterns. Its accurate and reliable quantification below the μM level is therefore useful in brain dialysates to obtain further knowledge of the function of both non-pathogenic and pathogenic disorders of the brain as well as in clinical diagnosis of some myocardial and hepatic diseases (12).

In the present investigation further optimization of a previously reported bienzyme biosensor prototype (23) is described. Three issues were of particular interest: A) the effect of a single layer vs. a bilayer in the enzyme-based electrode construction, B) the effect of further addition of polyethylenimine (PEI) to the sensing layer and C) the effect of loading oxidase enzyme on the analytical parameters of the sensor is considered.

Experimental

Chemicals and Reagents

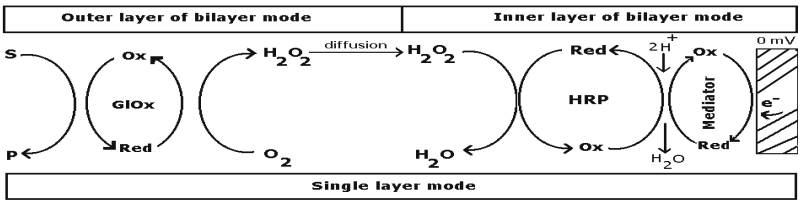
The enzyme electrode was prepared using spectroscopic graphite (SGL Carbon, Werke Ringsdorff, Bonn, Germany, type RW001, 3.05 mm diameter). Tris (hydroxymethyl)-aminomethane and potassium chloride were from Merck (Darmstadt, Germany). L-glutamate oxidase from *Streptomyces* sp. was obtained from Yamasa Corp. (Tokyo, Japan). Horseradish peroxidase (HRP, type VI, EC 1.11.1.7, cat. no. P-8375), polyethylenimine (PEI), L-glutamic acid, β-N-oxalyl-α,β-diaminopropionic acid (β-ODAP) and *o*-phthalaldehyde (OPT) were purchased from Sigma (St. Louis, Missouri, USA). Poly(1-vinylimidazole){osmium(4,4'-dimethylbpy) 2Cl}$^{2+/+}$ denoted PVI_{12}-dmeOs (24) was a gift from TheraSense Inc. (Alameda, California). Poly(ethylene glycol) (400) diglycidylether (PEGDGE) was purchased from Polysciences, Inc. (Warrington, Pennsylvania, USA Cat. No. 08210).

Preparation of Electrode

For the construction of the enzyme-based biosensor, graphite electrodes were used as the conducting solid support. They were first polished on wet emery paper (P1200) to obtain a smooth surface. The electrodes next were carefully rinsed with MilliQ water and allowed to dry at room temperature. Two different constructions of bienzyme electrodes were investigated; a single layer and a bilayer approach. In the single layer approach, a premixed solution composed of 1 μL of PVI_{12}-dmeOs (10 mg mL^{-1} in water), 1.5 μL of HRP (10 mg mL^{-1} in 10/10 mM Tris/Cl^{-1} buffer pH 7.5), 0.5 μL of a freshly prepared PEGDGE solution (5 mg mL^{-1} in water), and 3 μL of 10 and/or 20 mg mL^{-1} GlOx was placed on top of the polished end of the electrode and spread evenly using the micro syringe tip. In some experiments, additionally 1.5 μL of PEI (0.1 %, w/w in water) was added before applying the premixed solution onto the electrode surface. The electrode was allowed to stand a couple of hours at room temperature before mounting in the flow-through electrochemical cell. For the preparation of the bilayered bienzyme biosensor, the premix for the inner layer was composed of all the components as above except GlOx. This was placed on top of the polished

F1

Sequential reactions occurring on bilayer and single-layer electrodes. Single-layer electrodes have an electrically wired HRP, and H_2O_2 diffuses within the film. Bilayer electrodes have an electrically wired HRP inner layer and a non-wired GlOx outer layer. H_2O_2 diffuses between the layers.



graphite electrode. After 10 minutes waiting time, the second layer composed of 3 µL of GlOx (20 mg mL^{-1} in 10/10 mM Tris/Cl^{-1} buffer pH 7.5) was evenly spread on top of the first layer of the enzyme electrode and allowed to stand two hours hours at room temperature before it was mounted to the electrochemical cell.

Flow Injection Analysis

The enzyme-modified working electrodes were mounted in a three-electrode flow-through amperometric wall jet cell (25). It contained a platinum wire auxiliary electrode and Ag | AgCl (0.1 M KCl) reference electrode. The cell was connected to a potentiostat (Zäta Electronik, Lund, Sweden) and the readout response was registered with a recorder (Kipp and Zonen, The Netherlands, model BD 112). All measurements were carried out at an applied potential of -50 mV vs. Ag | AgCl. Tris/KCl buffer (10/10 mM, pH 7.5) was the carrier buffer delivered by a pump (2150, LKB Bromma) at a flow rate of 0.8 mL min^{-1}. Samples (50 µL volume) were injected using an electrically operated injection valve (Rheodyne, 7125, California, USA). All connections between parts of the set-up were made with PEEK tubing (0.3 mm i.d.).

Results and Discussion

Mediation of the bienzyme electrode

There are no reports on direct electron transfer between GlOx and electrodes. Previous reports on glutamate sensors are based on three different principles. Immobilization of GlOx on platinum or platinized electrodes allows the hydrogen peroxide produced to be electrochemically oxidized at around +600 mV *vs.* Ag | AgCl (26-28). GlOx has also been immobilized on Prussian Blue-modified glassy carbon where hydrogen peroxide can be electrocatalytically reduced at around 0 mV *vs.* Ag | AgCl, thus decreasing the influence of easily oxidizable interfering species (29). Finally, co-immobilizing GlOx with HRP has also been reported both in carbon paste and on solid graphite (4). As with Prussian Blue-modified electrodes, peroxidase-modified electrodes allow electrocatalytic reduction of hydrogen peroxide at low applied potentials. In a previous report from this laboratory (23), the working potential of the electrode was reduced significantly (-50 mV *vs.* Ag|AgCl) by coupling glutamate oxidase with HRP and by the use of Os-based polymer as an electron transfer mediator between HRP and the graphite electrode. Much work with GlOx-based sensors has focused on finding either an efficient mediator for GlOx or a way to immobilize GlOx on an electrode further modified with a catalyst enabling the electrochemical conversion of hydrogen peroxide at a low over potential (29,30). It seems as though the reaction rate between reduced GlOx and artificial mediators is not very high with the most commonly used mediators. When hydrogen peroxide-producing oxidases are co-immobilized with HRP, there are two possibilities for electron transfer between the electrode and HRP, either through a direct electron transfer mechanism or through a mediated electron transfer with the aid of an added artificial electron transfer mediator. The direct electron transfer will in this case take place between HRP and the electrode. The direct electron transfer between electrodes and HRP is, however, characterized by sluggish kinetics (31). In general, the bilayer electrode construction would be a better approach for hydrogen peroxide-generating enzymes that show electrical wiring with the mediating polyelectrolyte polymer (32). Otherwise the reduced cofactor of the oxidase is directly and efficiently oxidized by the electrical wiring material, resulting in short circuiting of the system with less production of H_2O_2 in the presence of oxygen (24). The reaction sequence involved in the signal generation process for the bienzyme electrode is depicted in ***F1***.

Most commonly, either glassy carbon or gold has been used as the electrode material for $Os^{2+/3+}$-wired enzymes (11,24,32,33). However, in this work graphite was used. Compared with carefully polished glassy carbon or gold, polished graphite offers a higher loading of immobilized enzyme and polymer mediator due to its surface porosity. Thus, even if the background current exceeds that of glassy carbon or gold, the signal-to-noise ratio is higher for graphite (12,23).

Single layer vs. Bilayer

Two approaches of electrode construction have been considered in optimizing the L-Glutamate/β-ODAP sensor—a single layer and a bilayer approach. In the single layer bienzyme electrode preparation, both HRP and the hydrogen peroxide-generating enzyme, GlOx, were mixed together before being immobilized within the 3-D PVI_{12}-PEG-DGE redox epoxy network. Previous comparison of H_2O_2 electrodes based on direct, diffusionally mediated, and redox polymer-relayed electroreduction of HRP showed that the wiring of HRP to an elec-

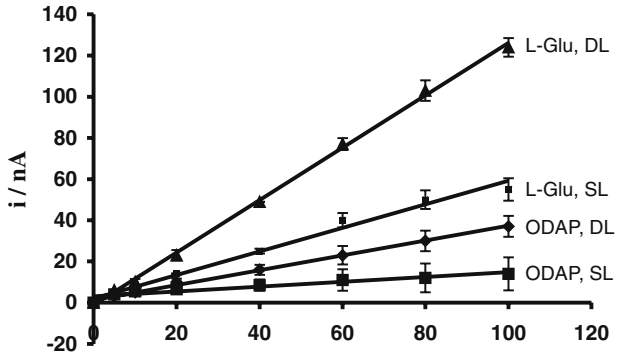
trode through a covalent binding of HRP to a hydrophilic Os-based 3-D electron-relaying redox network has increased sensitivity compared with the other two (33). Such electrode construction could be appropriate when any anodic currents resulting from mediated electron transfer from the active site of GlOx to the redox polymer is nil. However, when the redox centers of the H_2O_2-generating enzyme are directly wired by the mediating redox polymer, the measured cathodic response currents are reduced through partial electro-oxidation of the reduced active site of GlOx caused by the wiring to the redox polymer. This will be particularly true when the substrate concentration is high (24). Under this condition, the competition between electro-oxidation of $FADH_2$ centers of GlOx by the directly wired redox polymer and the reaction with O_2 favors the former. The reason is that the system will be O_2 depleted at high concentrations of the substrate. The single layer electrode construction also tested in the present work has shown that the response of the electrode was low and the linearity was not good at high substrate concentration. Similar results were obtained previously (23). A calibration curve for an electrode based on the single layer mode using L-glutamate and β-ODAP substrates is shown in ***F2***.

In the bilayer electrode construction mode, prevention of close contact between the redox polymer in the inner layer and GlOx in the outer layer is of essence (32). One way to prevent this is to avoid the interdiffusion of the two layers as much as possible so the redox centers of the GlOx are prevented from direct wiring with the mediating redox polymer. High temperature (50 °C) curing of the electrode, as was used in (23), was abandoned in the construction of both single and bilayer bienzyme modified electrode in the present work as this may facilitate the interdiffusion of the two layers. Instead, room temperature and a long curing time (two hours) were employed. The bilayer electrode preparation gave significantly higher electrode response in a reproducible manner for the same ranges of concentration tested as in the single layer mode. ***F2*** also shows calibration curves for L-glutamate and β-ODAP for an electrode based on the bilayer approach and with the same total amount of enzymes as the single layer approach. What is clearly seen is that for both substrates, the bilayer approach yields much higher currents. Therefore, it is highly probable that in the single layer electrode construction of GlOx, the Os-based redox polymer electrically communicates with the reduced GlOx centers and brings about reduction of the measurable cathodic current through "short circuiting" the electron transfer pathway.

In the previous work (23) where a single layer bienzyme electrode was used, the linearity did not extend beyond 50 µM for β-ODAP. The bilayer electrode preparation employed in the present study was far superior in extending the linear range of the sensor, particularly for the second substrate, β-ODAP. In addition to the short-circuiting problem described above in the single layer electrode design, the high weight fraction of electrically insulating protein could also render poor electron transport in the single layer approach. In this case, the electroreduction of H_2O_2 is limited both by the flux of electrons from the electrode via the redox hydrogel to HRP, as well as for H_2O_2 produced by GlOx in the outer layer to diffuse and reach immobilized HRP in the inner layer. The bilayer electrode prepared with GlOx as an outer layer and redox epoxy incorporated HRP as an inner layer was clearly superior to the single layer mode for bioelectroreduction of H_2O_2.

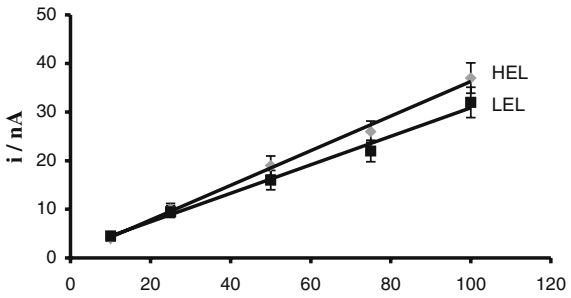
F2

Current vs. concentration of L-glutamate/β-ODAP plot for single layer electrode (SL) and double layer (DL) electrode construction. (In both cases 0.3 U GlOx per electrode.)



F3

Effect of enzyme loading. Upper high enzyme loading (HEL, 0.3 U GlOx per electrode) and low enzyme loading (LEL, 0.15 U GlOx per electrode).

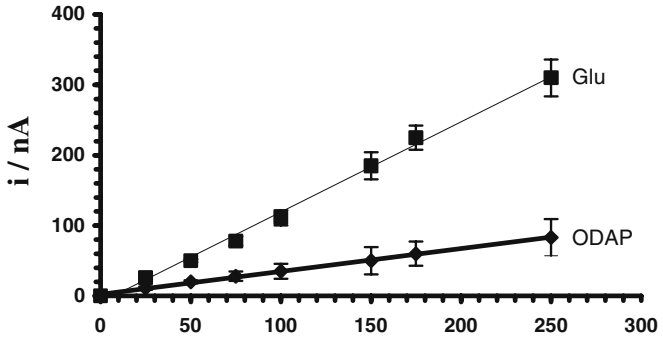


Enzyme Loading

By having a high loading of GlOx in the second layer, still better current densities are expected for β-ODAP because GlOx has a comparatively low activity for this substrate. This is indeed the case in the present investigation, which helped in improving the lower detection limit for the secondary substrate, β-ODAP. Two sets of electrodes (three in each case) were prepared, all modified with PEI (1.5 µL, 0.1% w/w) but containing different amounts of GlOx, with one set of electrodes containing 0.15 U GlOx per electrode

F4

Calibration curve of enzyme-modified electrode. Conditions: enzyme loading 0.3 U per electrode, substrate concentration 1 - 250 µM, flow rate 0.8 mL min.$^{-1}$, injection volume 50 µL.



and the other set 0.3 U GlOx per electrode. The average response curves of these electrodes in FIA were compared and are presented in ***F3***. An increase in response was observed with increase in the enzyme (GlOx) loading and the linearity of response was slightly improved, R^2 values being 0.995 and 0.991 for high GlOx loading and low GlOx loading, respectively. The enzyme loading of 0.3 U per electrode was then chosen for all the works in this study. For economy reasons, higher enzyme loadings could not be tried.

Effect of Polyethylenimine (PEI)

In a number of previous publications, mainly describing enzyme-modified carbon paste electrodes, the addition of a polyelectrolyte such as PEI proved very beneficial in terms of both electrode stability and sensitivity (17,34-38). To investigate the effect of further modification of the bilayer enzyme electrode preparation in the present study, polyethylenimine (PEI) additive was used in the hydrogel premix as a modifier. For this purpose a 0.1% w/w PEI solution was prepared from a 50% w/v stock solution of PEI. Although addition of PEI has not exhibited an enhancement of electrode response in a statistically verifiable way, operational stability and reproducibility of the electrode preparation were assured. A similar stability enhancing effect was observed for a carbon paste electrode containing co-immobilized alcohol oxidase and HRP (38). In another study (39) however, a 45% increase in enzyme activity was obtained by the presence of 0.1% PEI in fructose dehydrogenase electrode preparation using carbon paste. In our study an increase in enzyme activity or in general electrode response was not consistently observed. Two different amounts of PEI added to the hydrogel premix, 1.5 µL and 3.0 µL, were tried and both gave similar responses. Other amounts were not tried. The mechanism behind stability enhancement in our electrode preparation is not understood. Electrostatic interaction between the polycationic PEI and enzyme or substitution of hydrogen bonding with bound water could change or stabilize the enzyme conformation. Interaction of the polycationic polymer with both enzyme and the net negatively charged graphite electrode, which can alter the orientation of the enzyme on the electrode surface, cannot be ruled out. Although the effects of the PEI modifier have not been evaluated with cyclic voltammetry in our study, comparison of the electrode response obtained in flow injection analysis, both in the presence and absence of PEI, adequately showed the beneficial effects of PEI addition.

Linearity Range, Sensitivity and Detection Limit

The electrode preparations were tested for linear response by putting them in the FIA system. A typical calibration curve showed a linear relation between electrode response and concentrations of L-glutamate and β-ODAP over the range 1-250 µM (R^2 0.995 and 0.998, respectively) (***F4***). As would be expected, the enzyme-modified electrode (0.3 U GlOx) was more sensitive to its main substrate, L-glutamate. It was also found to be sufficiently sensitive to the second substrate, β-ODAP. This was, of course, another attraction to the biosensor development as this substance is found in a food/feed crop and is a potent neurotoxin for both humans and animals (40). The lower detection limit was calculated based on a signal-to-noise ratio of 3, and was found to be 0.7 and 2 µM for L-glutamate and β-ODAP, respectively. The electrode responses above 250 µM concentrations have not been tested for any of the substrates. The lower detection limit with the single layer enzyme-modified electrode was 5 µM for the substrate β-ODAP, and about 1 µM for L-glutamate.

In order for an analytical method/sensor to find practical application in the measurement of L-glutamate (as a neurotransmitter) in brain extracellular fluid, its linear range should be at least 2-100 µM. This is the most likely concentration range of glutamate in brain extracellular fluids (28). The present bienzyme sensor in the bilayer mode therefore meets the demands of a practical sensor for extracellular fluid. For food analysis, however, a practical sensor should have upper detection limits in mM range concentrations (20).

Operational Stability and Reproducibility of Electrode Preparation

Operational stability of the electrode has been studied by continually injecting 85 µM β-ODAP standard samples for more than 60 hours at an interval of three minutes. Although some unexplainable increase in electrode response is observed for some duration at the beginning, about 98% of the electrode response remained intact for 50 hours of operation. Even after 58 h of operation, the residual response was still high (86%). The operational stability of the electrode is graphically presented in ***F5***. Each point is an average of 45 responses (RSD, 1.4%) and the first point is taken as reference (relative response of 1) for estimating the relative responses

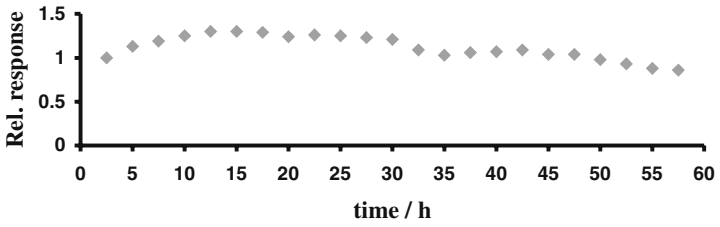
over the studied time. It is logical to take the first point as reference when the sensor was fresh and compare all other responses obtained when operating the sensor relative to this one. Fluctuation in temperature when the system was run day and night and variation in oxygen concentration in the carrier buffer could account for the differences of electrode response up to 50 hours of operation. The consistent decrease in response after 50 hours of operation, however, could be fairly ascribed to the instability of the immobilized enzymes on the electrode surface, and quoting the sensor stability as "at least 50 hours" is quite reasonable. For immobilized enzyme systems it may be expected that the change in enzyme stability could be abrupt. This kind of empirical observation is quite common.

The storage stability of the electrode preparation procedure used in the present study was also studied for four days by keeping the electrodes dry in a desiccator at room temperature. The electrode response was not altered. A longer storage time was not studied, but clearly would be required for commercial applications.

In order to assess the reproducibility of the system, three identically prepared electrodes were mounted to the flow injection analysis system one at a time and run under identical conditions the same day. Their responses were tested in the range between 2 and 50 µM β-ODAP. The results as shown in ***F6*** where the average response is plotted vs. concentration with error bars fitted, are remarkably reproducible. ***T1*** shows the individual responses for the three electrodes prepared to test reproducibility at different concentrations of β-ODAP.

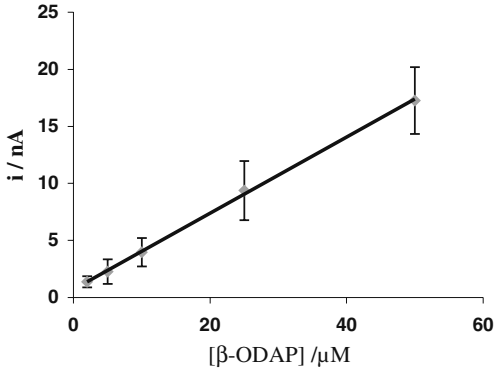
F5

Operational stability of the electrode. Conditions: 85 µM β-ODAP, standard solution, injection volume 50 µL, and flow rate, 0.8 mL min.$^{-1}$and enzyme loading 0.3 U per electrode.



F6

Reproducibility of electrode preparation. Conditions: enzyme loading 0.3 U per electrode, substrate concentration 2-50 µM, flow rate 0.8 mL min.$^{-1}$, injection volume 50 µL.



T1

Reproducibility of electrode preparation.

Conc. β-ODAP, µM	Electrode responses, nA.		
	Electrode 1	Electrode 2	Electrode 3
2	1.5	1.3	1.3
5	2.5	2.3	2.0
10	4.3	3.8	3.8
25	10	9.3	8.8
50	17.8	17.5	16.5

Conclusion

An operationally stable, sensitive and reproducible biosensor for glutamate and β-ODAP is reported in the present work. The achievement of sufficient response with respect to the substrate β-ODAP is of paramount importance in efforts to detoxify the crop *Lathyrus sativus*. The presence of analytical potential to determine this toxin is central in such efforts. Modification of the sensor with PEI, which provides long operational stability, is another beneficial advantage for cost-effective analysis of many samples. Both the reproducibility and stability of the biosensor preparation of the hydrogel-based enzyme electrode have been found to be suitable for using the biosensor as a detection device in real sample analysis after chromatographic separation of L-glutamate and the neurotoxin, β-ODAP as is reported separately (41).

Acknowledgement

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