Chapter 1

A Reagentless, Screen-Printed Amperometric Biosensor for the Determination of Glutamate in Food and Clinical Applications

G. Hughes, R.M. Pemberton, P.R. Fielden, and J.P. Hart

Abstract

A reagentless biosensor has been successfully developed to measure glutamate in food and clinical samples. The enzyme, glutamate dehydrogenase (GLDH) and the cofactor, nicotinamide adenine dinucleotide (NAD^+) are fully integrated onto the surface of a Meldola's Blue screen-printed carbon electrode (MB-SPCE). The biological components are immobilized by utilizing unpurified multi-walled carbon nanotubes (MWCNT's) mixed with the biopolymer chitosan (CHIT), which are drop-coated onto the surface of the MB-SPCE in a layer-by-layer fashion. Meldola's Blue mediator is also incorporated into the biosensor cocktail in order to increase and facilitate electron shuttling between the reaction layers and the surface of the electrode. The loadings of each component are optimized by using amperometry in stirred solution at a low fixed potential of +0.1 V. The optimum temperature and pH are also determined using this technique. Quantification of glutamate in real samples is performed using the method of standard addition. The method of standard addition involves the addition of a sample containing an unknown concentration of glutamate, followed by additions of known concentrations of glutamate to a buffered solution in the cell. The currents generated by each addition are then plotted and the resulting line is extrapolated in order to determine the concentration of glutamate in the sample (Pemberton et al., Biosens Bioelectron 24:1246–1252, 2009). This layer-by-layer approach holds promise as a generic platform for the fabrication of reagentless biosensors.

Key words Glutamate, Reagentless, Carbon-nanotubes, Meldola's Blue, Screen-printed carbon electrode, Glutamate dehydrogenase

1 Introduction

Glutamate is considered to be the primary neurotransmitter in the mammalian brain and facilitates normal brain function [2]. Neurotoxicity, which causes damage to brain tissue, can be induced by glutamate at high concentrations. The accumulation of high concentrations of glutamate leads to the overactivation of NMDA and AMPA receptors [3], which may link it to a number of neurodegenerative disorders such as Parkinson's disease, multiple

Ben Prickril and Avraham Rasooly (eds.), *Biosensors and Biodetection: Methods and Protocols, Volume 2: Electrochemical, Bioelectronic, Piezoelectric, Cellular and Molecular Biosensors*, Methods in Molecular Biology, vol. 1572,
DOI 10.1007/978-1-4939-6911-1_1,

sclerosis [4], and Alzheimer's disease [5]. In cellular metabolism, glutamate also contributes to the urea cycle and tricarboxylic acid cycle (TCA)/Krebs cycle. It plays a vital role in the assimilation of NH_4^+ [6]. Intracellular glutamate levels outside of the brain are typically 2–5 mmol/L, while extracellular concentrations are ~0.05 mmol/L [7] and is present in high concentrations throughout the liver, brain, kidney and skeletal muscle [8]. Glutamate has a significant role in the disposal of ammonia, which is typically produced from the digestion of dietary amino acids, protein and the ammonia produced by intestinal tract bacteria. Many food products contain MSG (monosodium glutamate) as a flavor enhancer, often in unspecified amounts. The determination of glutamate in food products could assist those with a sensitivity to glutamate known as Chinese restaurant syndrome [9].

Electrochemical biosensors for the measurement of glutamate have been based on either oxidase or dehydrogenase enzymes which have been integrated with various transducers using one of several immobilization methods [1, 10]. The main limitation with biosensors systems based on oxidase enzymes is the high cost of the enzyme, whereas dehydrogenase enzymes require that the cofactor NAD^+/NADH be added to the sample undergoing analysis. In the present study we describe a reliable method of incorporating this cofactor with the other biosensor components onto the surface of a screen-printed carbon electrode containing the electrocatalyst Meldola's Blue. This results in a low cost reagentless device which is more convenient to use [11, 12].

The biosensor is utilized in a three electrode configuration consisting of a working (WE), reference (RE) and a counter electrode (CE) which are placed in a cell, as shown in Fig. 1, into which the buffer and analyte of interest are added.

Figure 2 shows a schematic of the overall principle of operation of the biosensor. Glutamate in solution is oxidized to form alpha-ketoglutarate in the presence of the immobilized enzyme GLDH and the cofactor NAD^+. This results in the generation of NADH and NH_4^+. The NADH reduces the mediator Meldola's Blue (MB), which undergoes electrochemical oxidation at the electrode surface resulting in the generation of the analytical response. The mediator subsequently regenerates. The concentration of glutamate determined by the biosensor is proportional to the current generated, when operating within the K_m of the enzyme. The reactions described take place at the surface of the electrode and throughout the layer-by-layer structure [13].

Figure 3 illustrates the structure of the reagentless biosensor produced by the layer-by-layer procedure. The inner (layer 1) and outer layer (layer 3) of the biosensor are composed of multi-walled carbon nanotubes (MWCNTs) mixed with the biopolymer chitosan (CHIT). The enzyme and cofactor are entrapped in layer

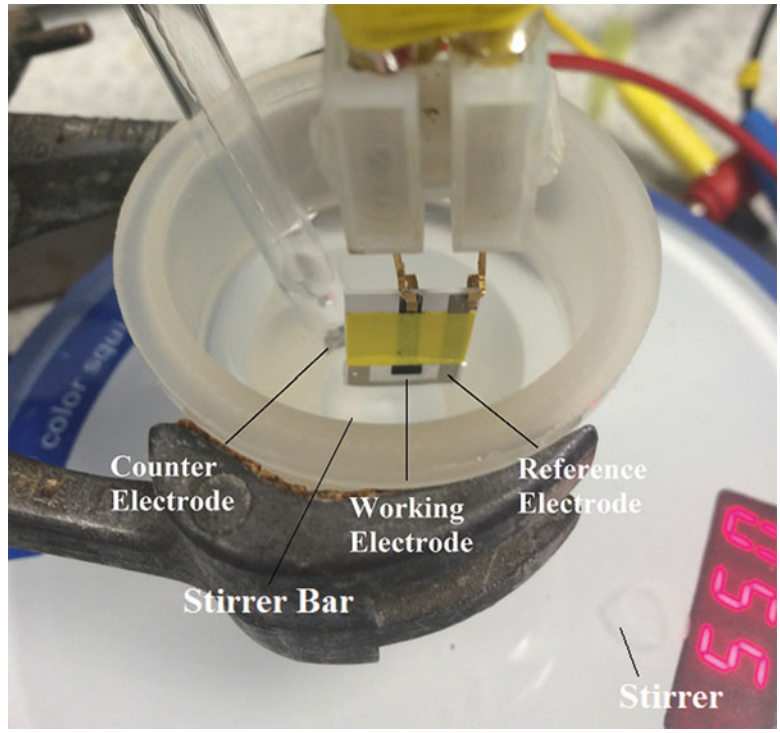

Fig. 1 Photograph of the three-electrode system and the experimental setup

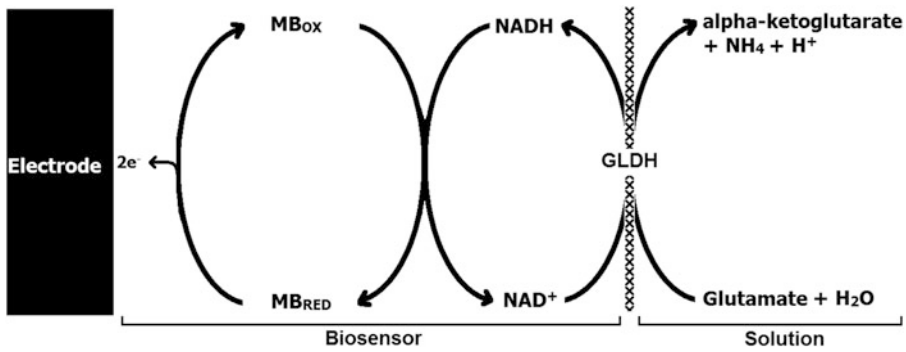

Fig. 2 Schematic displaying the interaction between the immobilized enzyme GLDH and glutamate at the surface of the electrode and the subsequent generation of the analytical response

2 which is retained by layer 3. Additional MB is integrated throughout each layer of the biosensor in order to enhance sensitivity.

Table 1 shows the optimized quantities of each component drop-coated on the MB-SPCE surface. Layers one and two are deposited sequentially then left to dry at 4 °C in a desiccator. Next, the third layer is drop-coated and allowed to completely dry at 4 °C in a desiccator. These are stored under vacuum at 4 °C until required for analysis.

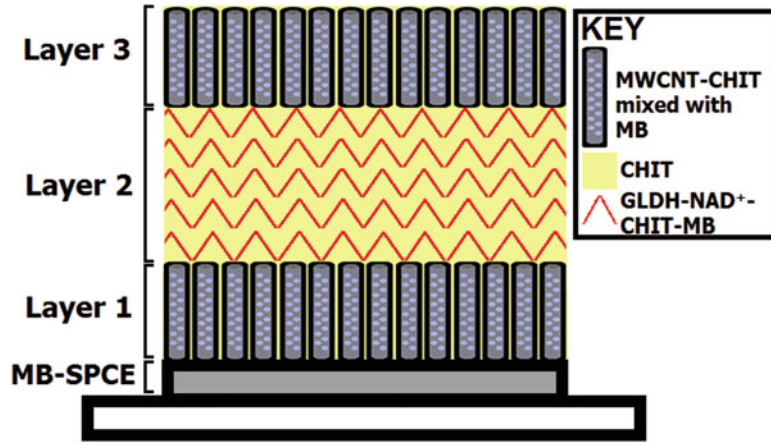

Fig. 3 A schematic diagram displaying the layer-by-layer drop coating fabrication procedure used to construct the reagentless glutamate biosensor, based on a MB-SPCE electrode

Table 1
Total optimized loadings for each biosensor component

GLDH (units)	NAD^+ (μg)	CHIT (μg)
27	13.5	5
27	27	5
27	54	5
27	106	5
27	214	5
27	106	5
27	106	10
27	106	15
27	106	20

2 Materials

2.1 Chemicals

1. All chemicals are of analytical grade, purchased from Sigma Aldrich, UK, except glutamate dehydrogenase (CAT: 10197734001) which is purchased from Roche, UK.
2. The 0.75 M phosphate buffer is prepared by combining appropriate volumes of tri-sodium phosphate dodecahydrate, sodium dihydrogen orthophosphate dihydrate, and disodium hydrogen orthophosphate anhydrous solutions to yield the desired pH.

3. Glutamate is dissolved directly in 0.75 M phosphate buffer. Solutions are prepared fresh per use.
4. $NADH/NAD^+$ is dissolved directly in 0.75 M phosphate buffer. Solutions are prepared fresh per use.
5. An appropriate quantity of glutamate dehydrogenase (GLDH) is dissolved in 600 μL of phosphate buffer (0.75 M) and aliquoted into 60 μL micro cuvettes (3 U/μL). The aliquots are frozen at −4 °C in order to preserve enzyme activity.
6. An appropriate quantity of CHIT is weighed and dissolved in 0.05 M hydrochloric acid (pH < 3.0) to produce a 0.05% solution. The solution is sonicated for up to 10 min in order to fully dissolve the chitosan.
7. The MWCNT–CHIT solution is prepared by mixing 0.6 mg of MWCNT into a 300 μL of 0.05% CHIT solution. The solution is sonicated for 15 min and stirred for 24 h.
8. The 10^{-3} M Meldola's Blue (MB) solution is prepared by dissolving the appropriate weight of MB in distilled water with some mixing to ensure homogeneity.
9. Foetal bovine serum (FBS) (South American Origin, CAT: S1810-500) obtained from Labtech Int. Ltd., is used for serum analysis.
10. Food samples (Beef OXO cubes) are obtained from a local supermarket.

2.2 Equipment

1. All electrochemical experiments are conducted with a three-electrode system consisting of a carbon working electrode containing MB, (MB–SPCE, Gwent Electronic Materials Ltd.; Ink Code: C2030519P5), a Ag/AgCl reference electrode (GEM Product Code C61003P7); both printed onto PVC, and a separate Pt counter electrode.
2. The area of the working electrode is defined using insulating tape, into a 3×3 mm^2 area.
3. The electrodes are then connected to the potentiostat using gold clips. Solutions, when required, are stirred using a circular magnetic stirring disk and stirrer (IKA® C-MAG HS IKAMAG, Germany) at a uniform rate.
4. A μAutolab II electrochemical analyzer with general purpose electrochemical software GPES 4.9 is used to acquire data and experimentally control the voltage applied to the SPCE in the 10 mL electrochemical cell which is used for hydrodynamic voltammetry.
5. An AMEL Model 466 polarographic analyzer combined with a GOULD BS-271 chart recorder is used for all amperometric studies.

6. Measurement and monitoring of the pH is conducted with a Fisherbrand Hydrus 400 pH meter (Orion Research Inc., USA).
7. Sonications are performed with a Devon FS100 sonicator (Ultrasonics, Hove, Sussex, UK).

3 Methods

3.1 Reagentless Biosensor Fabrication

Figure 3 illustrates the structure of the reagentless biosensor produced by the layer-by-layer procedure. The inner (layer 1) and outer layer (layer 3) of the biosensor are composed of multi-walled carbon nanotubes (MWCNTs) mixed with the biopolymer chitosan (CHIT). The enzyme and cofactor are entrapped in layer 2 which is retained by layer 3. Additional MB is integrated throughout each layer of the biosensor in order to enhance sensitivity.

Table 1 shows the optimized quantities of each component drop-coated on the MB-SPCE surface. Layers one and two are deposited sequentially then left to dry at 4 °C in a desiccator. Next, the third layer is drop-coated and allowed to completely dry at 4 °C in a desiccator. These are stored under vacuum at 4 °C until required for analysis

3.1.1 Layer 1

1. When drop coating the biosensor components, utilize the tip of the pipette as if it were a brush. Brush each deposition on the surface of the electrode to ensure full coverage of the working area.
2. Ensure that the drop-coated liquids remain on the working electrode by keeping the screen-printed transducer on a flat surface. Once the initial base of liquid is defined, subsequent drop-coatings become easier.
3. Using a Gilson pipette, 10 μL of MWCNTs solution containing 0.05% CHIT in a 0.05 M HCl solution is drop-coated onto the surface of the working electrode.
4. Following this, an aliquot of 1 μL of 0.01 M MB in H_2O is drop-coated. No premixing is required, the MB will disperse throughout the solution.
5. This layer is allowed to partially dry at 4 °C under vacuum for 10 min.

3.1.2 Layer 2

1. The components for this layer are prepared as follows: 54 μL of GLDH (3 U/μL), 5 μL of 0.1% CHIT, and 6 μL of 160 mM NAD^+ are placed into an eppendorf tube and mixed thoroughly.
2. 9 μL of this mixture is drop-coated onto the surface of the working electrode. It is useful to eject the full 9 μL, holding the liquid as a sphere at the end of the pipette, and then touch the surface of the electrode. The liquid will then distribute itself evenly across the MWCNT–CHIT mixture.

3. This is followed by 1 μL of 0.01 M MB in H_2O.
4. This layer is allowed to partially dry at 4 °C under vacuum for 3 h.

3.1.3 Layer 3

1. 10 μL of MWCNTs suspended in a solution containing 0.05% CHIT in a 0.05 M HCl solution is drop-coated on top of layer 2.
2. Following this, an aliquot of 1 μL of 0.01 M MB in H_2O is drop-coated onto the above layer.
3. The biosensor is allowed to completely dry at 4 °C under vacuum for 2 h.
4. The composition of each layer is shown in Fig. 3.
5. A picture of the final complete biosensor is shown in Fig. 4.

3.2 Scanning Electron Microscopy

Figure 5 displays SEM images of the different layers deposited on top of the original Meldola's Blue SPCE (MB-SPCE). The only treatment of the biosensor specimens is a drying procedure.

3.3 Hydrodynamic Voltammetry

1. Hydrodynamic voltammetry is performed using the complete biosensor with 400 μM of glutamate, in 0.75 mM phosphate buffer (pH 7.0) containing 50 mM NaCl.
2. An initial potential of −120 mV is applied to the biosensor and the resulting steady state current is measured.

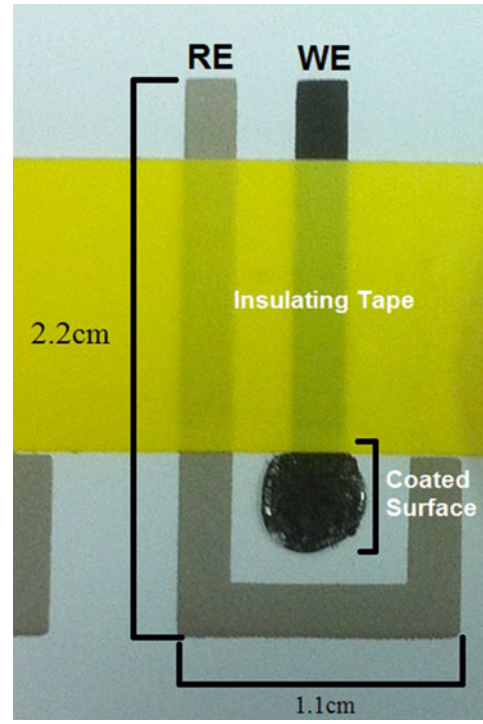

Fig. 4 Picture of the final biosensor

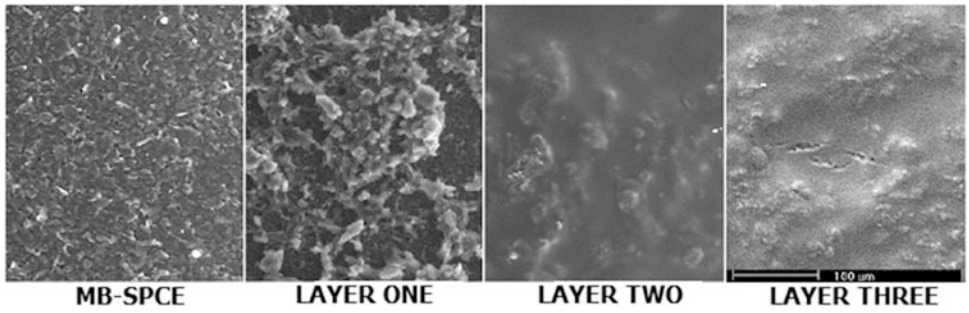

Fig. 5 SEM imaging of each individual layer of the reagentless biosensor. The scale is the same for all SEM images

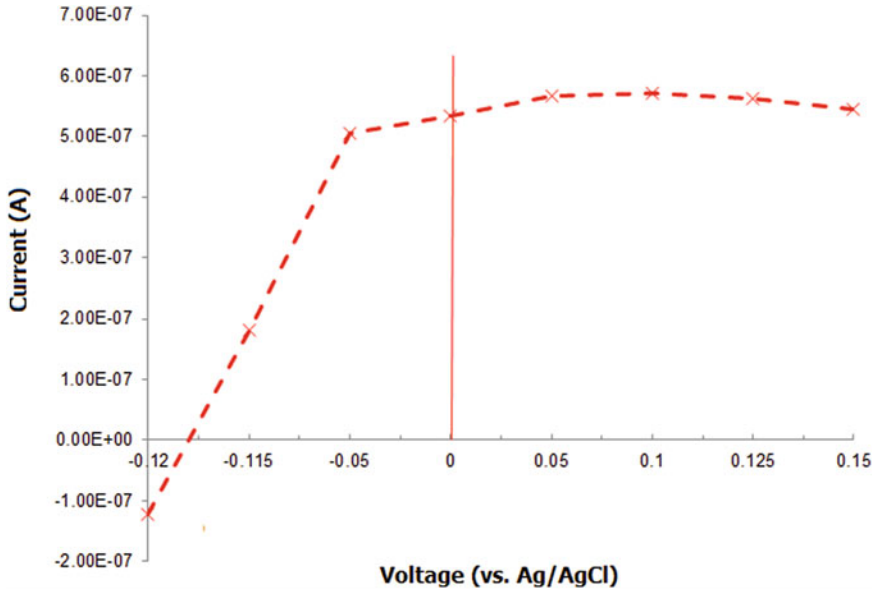

Fig. 6 Hydrodynamic voltammogram obtained using MB-SPCE/MWCNT-CHIT-MB/GLDH-NAD+−CHIT-MB/MWCNT-CHIT-MB biosensor in the presence of 400 µM glutamate in 75 mM phosphate buffer (pH 7.0) containing 50 mM NaCl

3. The potential is then changed to −115 mV and again a steady state current is measured.
4. The procedure is continued by changing the potential by 50 mV steps to a potential of +100 mV.
5. Subsequent steps are carried out by increasing by 25 mV up to a final potential of +150 mV.
6. The steady state currents are measured at each potential.
7. A hydrodynamic voltammogram is constructed by plotting the steady state currents against the corresponding potentials.
8. The resulting HDV plot is illustrated in Fig. 6.

3.4 Amperometry in Stirred Solution

1. All amperometric studies are performed in a fresh 10 mL solution containing of 75 mM PB pH 7.0 with 50 mM NaCl (PBS). Stir the solution with a magnetic stirrer.
2. A potential of +0.1 V vs. Ag/AgCl is applied. The charging current to is allowed to dissipate and for a steady state current to be attained.
3. An example amperogram for the calibration of the glutamate biosensor is shown in Fig. 7a. Subsequent calibration plots for five different sensors are shown in Fig. 7b.

3.5 Application of Optimized Amperometric Biosensor to the Determination of Glutamate in Food

1. An OXO cube is weighed before dissolving (approximately weight: 5.9 g) in 50 mL of phosphate buffer using sonication for 15 min.
2. The endogenous concentration of MSG is determined by using the method of standard addition.
3. The 5 μL volume of the dissolved OXO cube is inserted into the stirred buffered solution (10 mL) in the voltammetric cell containing the biosensor. A applied potential of +0.1 V (vs. Ag/AgCl) is used. Subsequent standard additions of 3 μL of 25 mM glutamate (final concentration: 7.5 μM) are injected into the solution. An amperogram of the standard addition procedure is shown in Fig. 8.
4. The reproducibility of the biosensor assay for MSG analysis in OXO cubes is determined by repeating the whole procedure five times with five individual biosensors.

3.6 Application of Optimized Amperometric Biosensor to the Determination of Glutamate in Serum

1. An initial volume of 150 μL of serum is injected into 9.85 mL buffer solution.
2. Once the current generated as a result of the serum had reached steady state, additions of 3 μL aliquots of 25 mM standard glutamate solution are added to the voltammetric cell.
3. The resulting currents are plotted in order to determine the endogenous concentration of glutamate. An amperogram of the standard addition procedure is shown in Fig. 8.
4. The reproducibility of the biosensor measurement is deduced by repeating the studies five times on a freshly diluted solution of the same serum with a fresh biosensor for each measurement.
5. The procedure is repeated using 50 μL of serum spiked with 1.5 mM glutamate ($n = 5$) to determine to the recovery of the assay.
6. A typical amperogram is shown in Fig. 9.

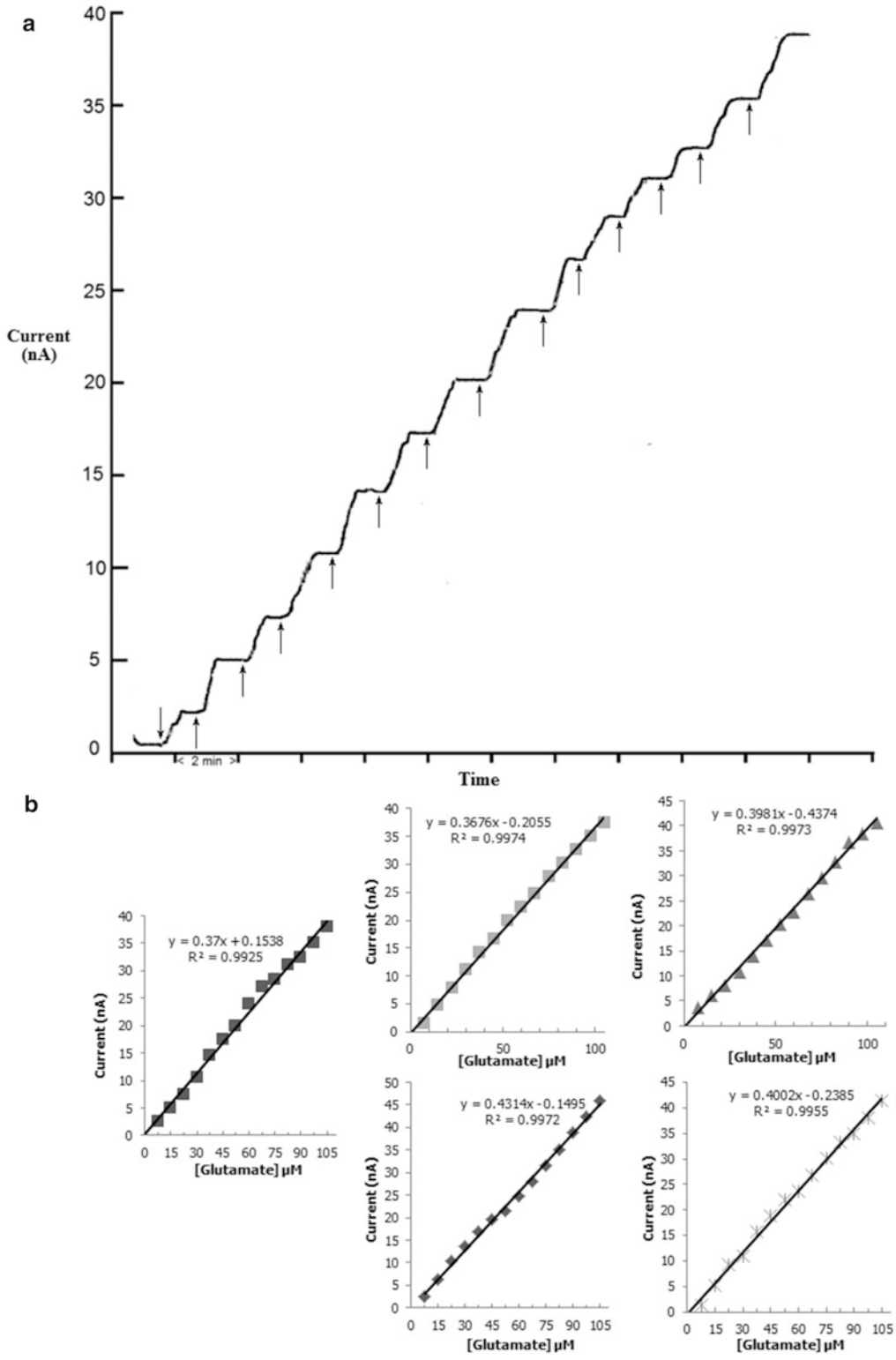

Fig. 7 (**a**) Amperogram conducted with the proposed final biosensor. Each arrow represents an injection of 3 µL of 25 mM glutamate in a 10 mL stirred solution containing supporting electrolyte; 75 mM, PB (pH 7.0), with 50 mM NaCl at an applied potential of +0.1 V vs. Ag/AgCl. (**b**) Calibration plots of five individually tested biosensors. The amperogram is depicted in the first calibration plot

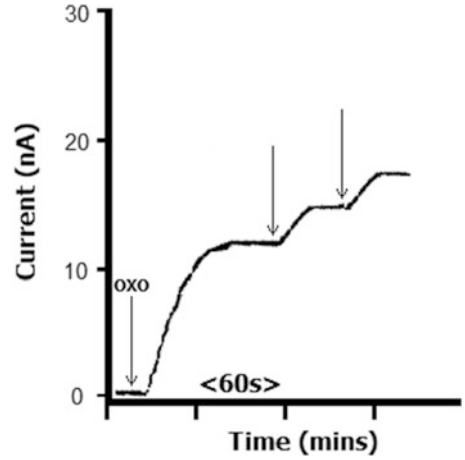

Fig. 8 A typical amperogram for the determination of the glutamate content of an OXO cube utilizing standard addition and subsequent injections of 3 μL glutamate (25 mM). The *first arrow* represents the injection of the OXO cube solution, with *subsequent arrows* denoting injections of glutamate

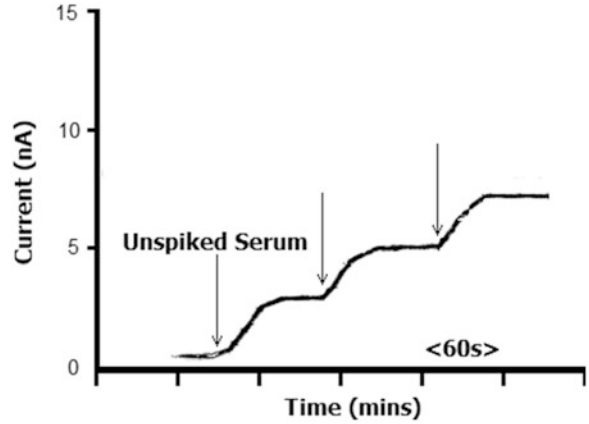

Fig. 9 A typical amperogram for the determination of the glutamate content of unspiked serum utilizing standard addition and subsequent injections of 3 μL glutamate (25 mM). The *first arrow* represents the injection of the serum solution, with *subsequent arrows* denoting injections of glutamate

4 Notes

1. During the drop coating procedure described in Subheading 3, difficulties may arise if the biosensors are allowed to dry completely after depositing the first layer of the biosensor. To ensure that this does not happen, the second layer of the biosensor must be drop-coated within 10 min of the drop-coating of

the first layer to ensure the layer has not dried completely. If layer one completely dries, it forms a layer that is typically indistinguishable from the original electrode ink. If this occurs, the procedure must be restarted using a fresh electrode, as subsequent layers deposited on the surface will not bind to the electrode.

2. During the operation of the biosensor it is found that modest stirring rates resulted in the most reliable analytical responses.
3. It should be noted that an initial charging current occurs when switching from open circuit to the operating potential (+100 mV). This charging current decreases quickly with time and a steady state response is obtained after 20 min
4. When injecting the analyte of interest into the solution, injecting behind the biosensor reduces the likelihood of disrupting the diffusion layer at the surface of the working electrode.

References

1. Pemberton RM, Pittson R, Biddle N, Hart JP (2009) Fabrication of microband glucose biosensors using a screen-printing water-based carbon ink and their application in serum analysis. Biosens Bioelectron 24:1246–1252
2. Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia A-S, McNamara JO, Williams SM (2001) Glutamate. Sinauer Associates, Sunderland, MA
3. Vornov JJ, Tasker RC, Park J (1995) Neurotoxicity of acute glutamate transport blockade depends on coactivation of both NMDA and AMPA/Kainate receptors in organotypic hippocampal cultures. Exp Neurol 133:7–17
4. Lau A, Tymianski M (2010) Glutamate receptors, neurotoxicity and neurodegeneration. Pflugers Arch 460:525–542
5. Butterfield DA, Pocernich CB (2003) The glutamatergic system and Alzheimer's disease: therapeutic implications. CNS Drugs 17:641–652
6. Berg JM, Tymoczko JL, Stryer L (2002) The first step in amino acid degradation is the removal of nitrogen. W H Freeman, New York
7. Brosnan JT (2000) Glutamate, at the interface between amino acid and carbohydrate metabolism. J Nutr 130:988S–990S
8. Brosnan JT, Man KC, Hall DE, Colbourne SA, Brosnan ME (1983) Interorgan metabolism of amino acids in streptozotocin-diabetic ketoacidotic rat. Am J Physiol 244:E151–E158
9. Schaumburg HH, Byck R, Gerstl R, Mashman JH (1969) Monosodium L-glutamate: its pharmacology and role in the Chinese restaurant syndrome. Science 163:826–828
10. Hughes G, Pemberton RM, Fielden PR, Hart JP (2016) The design, development and application of electrochemical glutamate biosensors. Trends Anal Chem 79:106–113
11. Alkire RC, Kolb DM, Lipkowski J (eds) (2011) Advances in electrochemical science and engineering. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim
12. Cho EJ, Lee J-W, Rajdendran M, Ellington AD (2011) Optical Biosensors: Today and Tomorrow. Elsevier, Amsterdam
13. Hughes G, Pemberton RM, Fielden PR, Hart JP (2015) Development of a novel reagentless, screen–printed amperometric biosensor based on glutamate dehydrogenase and NAD+, integrated with multi-walled carbon nanotubes for the determination of glutamate in food and clinical applications. Sens Actuators B 216:614–621