

Full Paper

Development of a Disposable Screen Printed Amperometric Biosensor Based on Glutamate Dehydrogenase, for the Determination of Glutamate in Clinical and Food Applications

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Abstract- A screen printed carbon electrode (SPCE) containing the electrocatalyst Meldola's Blue (MB) has been investigated as the base transducer for a glutamate biosensor. The sandwich biosensor was fabricated by firstly depositing a chitosan (CHIT) layer onto the surface of the transducer (MB-SPCE), followed by glutamate dehydrogenase (GLDH): this device is designated GLDH-CHIT-MB-SPCE. NAD⁺ was added to buffer solutions prior to the measurement of glutamate. This biosensor was used in conjunction with amperometry in stirred solution at an applied potential of +0.1 V (vs. Ag/AgCl). Optimum conditions for the analysis of glutamate were found to be as follows: temperature, 35 °C; buffer, pH 7; ionic strength, 75 mM; NAD⁺, 4 mM; CHIT 0.05% in 0.05 M HCl; GLDH, 30 U. The linear range of the biosensor was found to be 12.5 µM to 150 µM, the calculated limit of detection (based on three times signal to noise) was 1.5 µM and the sensitivity was 0.44 nA/µM. The proposed biosensor was used to measure glutamate in serum before and after fortification with glutamate. The endogenous concentration of glutamate was found to be 1.68 mM and the coefficient of variation (CV) was 4.1%. The serum was then fortified with 2 mM of glutamate, and the resulting mean recovery was 96% with a CV of 3.3% (n=6). An unfiltered beef OXO cube was analysed for monosodium glutamate (MSG) content. The endogenous

content of MSG was 125.43 mg/g with a CV of 8.98%. The OXO cube solution was fortified with 0.935 g (100 mM) of glutamate, the resulting mean recovery was 91% with a CV of 6.39%.

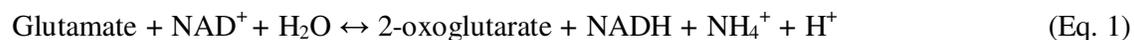
Keywords- Glutamate, Screen-printed biosensor, Amperometry, Clinical, Food

1. INTRODUCTION

Glutamate is one of the most abundant excitatory neurotransmitters and is involved in fundamental neurological processes such as the formation of memories and learning [1]. Deficiencies or abnormalities in the behaviour of neurological pathways that utilize glutamate and its receptors are associated with neurological disorders such as Alzheimers [2], schizophrenia [3] and depression [4].

Glutamate is also a key compound in nitrogen metabolism, protein synthesis and degradation, and is of great physiological importance. It is the most abundant intracellular amino acid with concentrations varying between 2 and 20 mM [5], whilst estimated extracellular levels of L-glutamate are around 150 μ M in plasma and 10 μ M in cerebrospinal fluid [6,7].

Glutamate metabolism is linked with the citric acid cycle [8], GABA synthesis [9] and urea cycle [10]. It is also linked to amino acid degradation as glutamate dehydrogenase (GLDH) catalyses the elimination of amino groups from amino acids. GLDH catalyses the oxidative deamination of glutamate to 2-oxoglutarate, using nicotinamide adenine dinucleotide (NAD^+) as a cofactor (Eq.1) [11]. It is a reversible reaction, which typically favours glutamate formation in mammals [12].



Genetically expressed defects in glutamate metabolism have been recently discovered and lead to hyperinsulinism/hyperammonemia syndrome [13]. L-glutamate, in the form of monosodium glutamate (MSG), also has widespread use as a flavour-enhancing food additive, commonly found in Chinese food and is linked to Chinese Restaurant Syndrome (CRS) [14]. Thus the analysis and determination of L-glutamate is important for both food and clinical applications.

The measurement of glutamate has been carried out using various analytical techniques such as HPLC [15,16], capillary electrophoresis [17] and enzyme recycling in conjunction with microfluidic [18] and spectrophotometric [19] techniques. In the present study we wished to explore the possibility of developing an amperometric biosensor for glutamate measurements. This device would offer reliability, convenience and low cost, particularly when fabrication was performed using screen-printing technology. We previously reported on the development of amperometric biosensors based on dehydrogenase enzymes, for lactate

[20,21], alcohol [22], glucose [23] and NH_4^+ [24,25]. These devices were based on a screen printed carbon electrode (SPCE) incorporating the electrocatalyst Meldola's Blue (MB-SPCE). MB reduces the overpotential for the detection of NADH, which is formed during the operation of the biosensor. Applied potentials close to 0 V vs. Ag/AgCl are possible. In a previous paper [25] we reported on a electrochemical NH_4^+ biosensor based on the reaction of this species with 2-oxoglutarate and NADH in the presence of the enzyme GLDH. Consequently, we considered that the development of a glutamate biosensor should be feasible by immobilizing GLDH onto the surface of a MB-SPCE and driving the overall reaction in the opposite direction by incorporating the oxidised cofactor (NAD^+) with the enzyme .

In this study we have investigated an immobilising procedure involving chitosan (CHIT). Chitosan is a linear hydrophilic polysaccharide composed of n-acetyl-D-glucosamine and D-glucosamine units which are linked with β -(1-4) glycosidic bonds. It is a biocompatible, inexpensive, non-toxic biopolymer with excellent film forming properties [26] and can therefore be employed for enzyme immobilisation. The application of chitosan as an enzyme immobilization matrix has been reported previously for many different enzymes [27].

In the present paper we describe the development and application of a GLDH-CHIT-MB-SPCE biosensor for the determination of glutamate in serum and glutamate as MSG in food (OXO cubes).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All chemicals were of analytical grade, purchased from Sigma Aldrich, UK, except glutamate dehydrogenase (CAT: 10197734001) which was purchased from Roche, UK. The 75 mM phosphate buffer (PB) was prepared by combining appropriate volumes of tri-sodium phosphate dodecahydrate, sodium dihydrogen orthophosphate dehydrate and disodium hydrogen orthophosphate dehydrate solutions to yield the desired pH. Glutamate and NADH/ NAD^+ solutions were prepared in 75 mM PB. 0.05% of chitosan was dissolved in 0.05 M HCl following up to 10 minutes sonication. Bovine serum albumin (BSA) (obtained from AbD Serotec) was used to create dummy electrodes by drop-coating the same equivalent protein mass as glutamate dehydrogenase. Fetal bovine serum (FBS) (South American Origin, CAT: S1810-500) obtained from Labtech Int. Ltd, was used for serum analysis. Food samples (Beef OXO cubes) were obtained from a local supermarket.

2.2. Apparatus

All electrochemical experiments were conducted with a three-electrode system consisting of a carbon working electrode containing MB-SPCE (Gwent Electronic Materials Ltd; Ink

Code: C2030519P5), Ag/AgCl reference electrode (GEM Product Code C61003P7): printed onto PVC, and a separate Pt counter electrode. The area of the working electrode was defined using insulating tape, into a 3×3 mm square area. The electrodes were then connected to the potentiostat using gold clips. Solutions, when required, were stirred using a circular magnetic stirring disk and stirrer (IKA® C-MAG HS IKAMAG, Germany) at a fixed speed. A μ Autolab II electrochemical analyser with general purpose electrochemical software GPES 4.9 was used to acquire data and experimentally control the voltage applied to the SPCE in the 10 ml electrochemical cell. An AMEL Model 466 polarographic analyser combined with a GOULD BS-271 chart recorder was used for some amperometric studies. Measurement and monitoring of the pH was conducted with a Fisherbrand Hydrus 400 pH meter (Orion Research Inc., USA). Sonications were performed with a Devon FS100 sonicator (Ultrasonics, Hove, Sussex, UK).

2.3. Procedures

2.3.1. Fabrication of glutamate biosensor

A 0.05% HCl solution containing CHIT was drop-coated onto the surface of the unmodified MB-SPCE 9 mm² working electrode and left to dry under vacuum. Once dried the enzyme GLDH with the appropriate quantity of units was drop-coated directly onto the CHIT layer. The same procedure was used to obtain dummy biosensors by using CHIT and the same mass of BSA as that of the enzyme. Fig. 1 illustrates the proposed GLDH-CHIT-MB-SPCE biosensor. The biosensors were stored in a desiccator, shielded from light at 4 °C overnight to allow the enzyme layer to dry.

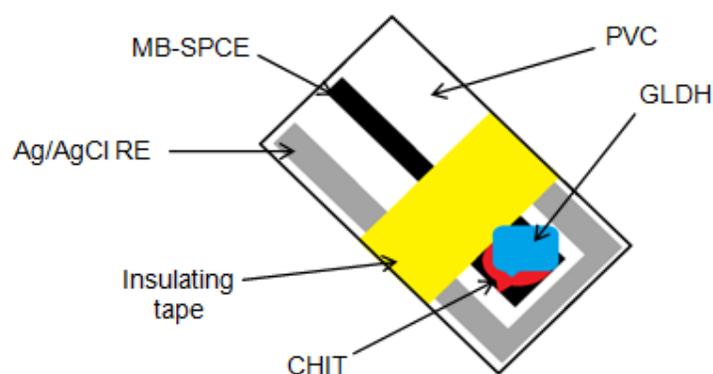


Fig. 1. Diagram of proposed biosensor strip; the insulating tape defines both the WE and RE. CHIT is then drop-coated and allowed to dry, followed by GLDH. All studies were conducted using a separate Pt wire counter electrode

Serum and food samples were analysed (n=3) for interferences using dummy biosensors. If interferences were detected, the average signal generated was deducted from the enzyme biosensor response.

2.3.2. Calibration studies of the biosensor using amperometry in stirred solution

All amperometric measurements were performed in stirred solutions using an applied potential of +0.1 V vs. Ag/AgCl. Measurements of glutamate were conducted in 75 mM PB (pH 7.0) containing 4 mM of NAD⁺ and 50 mM NaCl using the GLDH-CHIT-MB-SPCE. The biosensor was immersed into a stirred 10 mL buffer solution, the potential applied and sufficient time was allowed for a steady-state current to be obtained. The amperometric responses to additions of known concentrations of glutamate were then recorded. Amperometry was used to determine the effects of pH (5–9) and temperature (25–40 °C) by examining the performance characteristics over the concentration range of 25 µM to 300 µM glutamate.

2.3.3. Application of optimised amperometric biosensor (GLDH-CHIT-MB-SPCE) to the determination of glutamate in food

OXO cubes (5.9 g, average mass of three OXO cubes) were prepared by dissolving one cube in 50 mL of PB and sonicating for 15 minutes. The endogenous concentration of MSG was determined by using the method of standard addition. An initial 10 µL volume of the dissolved OXO cube was added to the stirred buffered solution (10 mL) in the voltammetric cell containing the biosensor, operated at +0.1 V with subsequent standard additions of 10 µL of 25 mM glutamate.

The reproducibility of the biosensor assay for MSG analysis in OXO cubes was determined by repeating the whole procedure six times with six individual biosensors. The effects of interferences from the OXO cube were established by using a dummy BSA biosensor and deducted from the enzyme biosensor signal.

The recovery of MSG added to OXO cubes was investigated by fortifying the solution containing one OXO cube with 100 mM of glutamate. The analysis of this solution was performed in a similar manner to that described for the unfortified solution. In this case, an aliquot of only 2 µL of the fortified OXO solution was added to 10 mL volumes of buffer solution. Standard additions of 2.5 µL of 100 mM glutamate were added to this mixture.

2.3.4. Application of optimised amperometric biosensor (GLDH-CHIT-MB-SPCE) to the determination of glutamate in serum

To determine the original glutamate concentration, an initial volume of 100 µL of serum was added to 9.9 mL of buffered solution. The serum solution was subjected to amperometry

in stirred solution using an applied potential of +0.1 V vs. Ag/AgCl. This was followed by additions of 10 μ L aliquots of 25 mM standard glutamate solution to the voltammetric cell. The currents resulting from the enzymatic generation of NADH were used to construct standard addition plots, from which the endogenous concentration of glutamate was determined. The reproducibility of the biosensor was deduced by repeating the studies six times with six individual biosensors.

The procedure was repeated using serum spiked with 2 mM glutamate ($n=6$) to determine the recovery of the assay. The effects of the interference from serum were established by using a dummy BSA biosensor. A dummy biosensor was constructed by drop coating the equivalent weight of the enzyme with BSA.

3. RESULTS AND DISCUSSION

3.1. Principle of Operation of the Biosensor

The overall principle of operation of the biosensor is shown in Fig. 2. Glutamate in solution is oxidised to form 2-oxoglutarate in the presence of the immobilized enzyme glutamate dehydrogenase (GLDH) and NAD^+ ; the product NADH and NH_4^+ are formed during this reaction (Eq. 1). The NADH diffuses through the CHIT layer to reach the electrode (Fig. 1), where it undergoes oxidation by the MB_{ox} which is reduced to MB_{red} . The electrochemical oxidation of MB_{red} to MB_{ox} occurs at an applied potential of +0.1 V vs. Ag/AgCl and produces the analytical response (Fig. 2). The value of +0.1 V was deduced by constructing a hydrodynamic voltammogram and selecting the potential from the position of the plateau (Fig. 3).

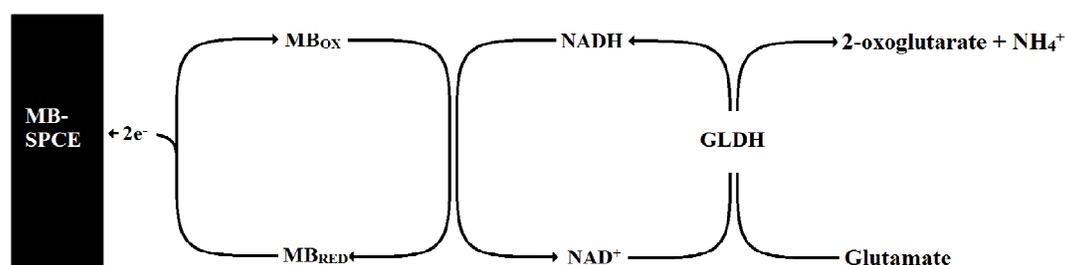


Fig. 2. Schematic displaying the interaction between the immobilized enzyme GLDH and glutamate at the surface of the electrode and the subsequent generation of the analytical response

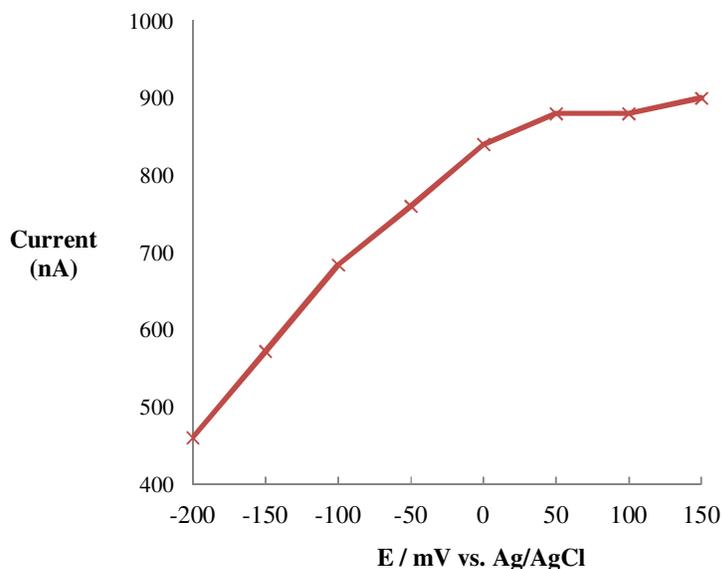


Fig. 3. Hydrodynamic voltammogram plot of GLDH-CHIT-MB-SPCE in the presence of 1 mM NADH, in 10 mL of supporting electrolyte; 75 mM, PB (pH 7.0) and 50 mM NaCl

3.2. Immobilisation of GLDH using chitosan

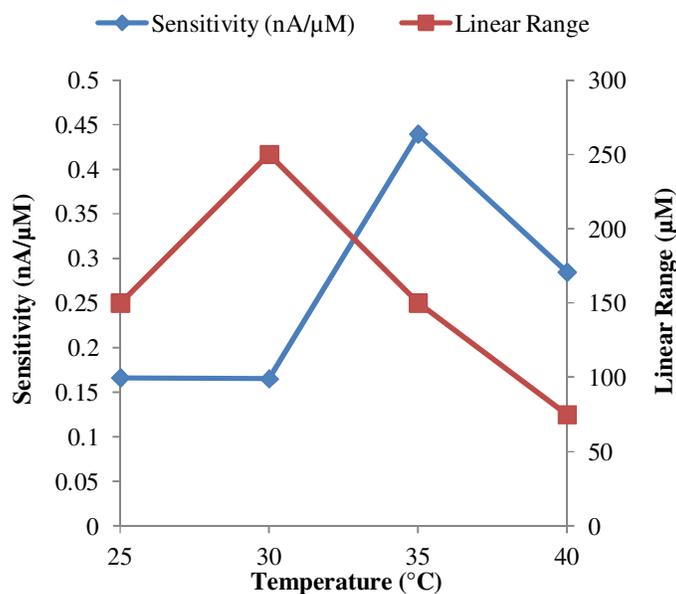
During initial studies when drop-coating the GLDH onto the surface of the MB-SPCE alone, steady-state currents were not produced. Additions of glutamate produced inconsistent currents, suggesting that the GLDH may have been dissipating into the free solution, producing sporadic current responses. Studies were conducted to investigate the use of glutaraldehyde and cellulose acetate; both commonly used enzyme binding agents, failed to generate steady-state currents or produced little to no signal response, indicating loss of enzyme into solution. CHIT was therefore applied to the surface of the biosensors to fabricate GLDH-CHIT-MB-SPCE devices; subsequent additions of glutamate produced consistent steady state responses with this approach.

3.3. Optimisation Studies

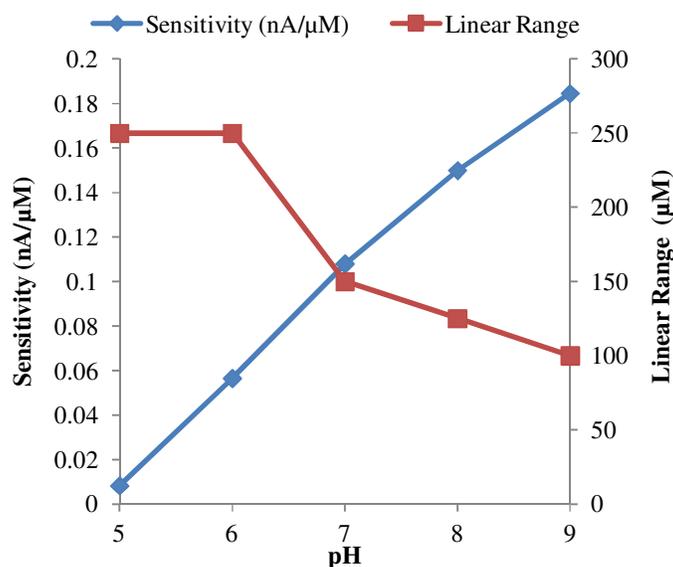
The response of the GLDH-CHIT-MB-SPCE biosensor to changes in temperature was investigated using concentrations of glutamate over the range 12.5 to 250 μ M prepared in 75 mM phosphate solution (pH 7.0). The effect of temperatures between 25 to 40 $^{\circ}$ C was studied. The maximum amperometric response was found to occur at 35 $^{\circ}$ C (Fig. 4A). The decrease occurring above 35 $^{\circ}$ C may be due to enzyme denaturing at higher temperatures. It may be noted that the linear range was highest using 30 $^{\circ}$ C which could be beneficial in some applications.

The effect of pH was investigated over the pH range 5–9 (Fig. 4B). The highest sensitivity (0.18 nA/ μ M) was obtained at pH 9, with a near-linear decrease down to a pH of

5. However, the linearity of the responses showed an inverse trend with the greatest linear range occurring at pH 5, decreasing down to a pH of 9.



(A)



(B)

Fig. 4. A. Illustrates the effects of temperature upon the current response of the biosensor to injections of glutamate up to 275 μM in 25 μM steps. **B.** Illustrates the effects of pH upon the current response of the biosensor to the addition of glutamate. An applied potential +0.1 V vs. Ag/AgCl in 10 ml of 75 mM phosphate buffer solution containing 50 mM NaCl was used.

3.4. Calibration Studies

Calibration studies with glutamate were conducted with the fully developed glutamate biosensor using the optimized experimental conditions over the concentration range 25 μ M–275 μ M. The biosensor exhibited a sensitivity of 0.44 nA/ μ M. The calculated limit of detection (based on three times signal-to-noise) was 1.5 μ M, thus the biosensor is clearly appropriate for analysing extracellular concentrations of glutamate in clinical applications.

3.4.1. Application of the optimum amperometric biosensor (GLDH-CHIT-MB-SPCE) to the determination of glutamate in unspiked and spiked food

Many food products are known to contain MSG as a flavour enhancer, therefore, we decided to explore the possibility of applying our new glutamate biosensor to a known brand of beef stock cube.

A standard addition study was conducted by dissolving one OXO cube (5.916 g mass) in 50 ml of PB using sonication for 15 minutes. Six replicate OXO cube samples were analysed using fresh biosensors for each measurement. The determination was performed by filling the cell with 9.99 mL of electrolyte, establishing a steady state current, and then injecting a 10 μ L volume of unfiltered OXO cube in PB solution with subsequent 10 μ L injections of 25 mM glutamate (Fig. 5A). The mean quantity of glutamate discovered in unspiked OXO cubes ($n=6$) was 125.43 mg/g with a coefficient of variation of 8.98%. Results are shown in Table 1. A standard addition calibration plot is shown in Fig 5B.

GLDH-CHIT-MB-SPCE biosensors were used to determine glutamate in spiked OXO cubes. The OXO cube was spiked with 100 mM of glutamate, doubling the endogenous level of glutamate found in OXO cube stock. The standard addition method employed for the unspiked OXO cubes was altered by changing the initial injection of spiked OXO cube solution to 2 μ L. The mean recovery ($n=6$) was a 91% with a CV of 6.39% (Table 1.) This result indicated high reproducibility for biosensors in what was a complex, unfiltered medium. Interestingly the endogenous content of MSG in OXO cubes measured by our biosensor, gave similar values for stock cubes containing MSG analysed by both HPTLC [28] and an optical biosensor [29]; values of 133.50 mg/g and 182.9 mg/g were detected by the named methods respectively.

3.4.2. Application of the optimum amperometric biosensor (GLDH-CHIT- MB-SPCE) to the determination of glutamate in both unspiked and spiked serum

Amperometry, in conjunction with the method of multiple standard additions was conducted to determine the endogenous levels of glutamate and the recovery for serum spiked with additional glutamate. The replicate serum samples were analysed using a fresh biosensor for each measurement.

The data obtained on serum samples using the glutamate biosensor are shown in Table 2. The endogenous levels of glutamate detected were 1.68 mM for the unspiked samples. The coefficient of variation was 4.09% for the six individual samples. This concentration is above that discovered by Ye and Sontheimer in 1998 [30] who discovered concentrations ranging from 808.2 μM to 1195.7 μM in fetal bovine (calf) sera (FBS) using a bioluminescence detection method. However, this study did not include the provider of serum nor the South American variety used in studies conducted using the GLDH-CHIT-MB-SPCE biosensor, thus glutamate concentrations may potentially be higher in different batches given the variation displayed. These results show promise due to the low coefficient of variation, small amount of serum required to carry out analysis and the lack of preparation steps.

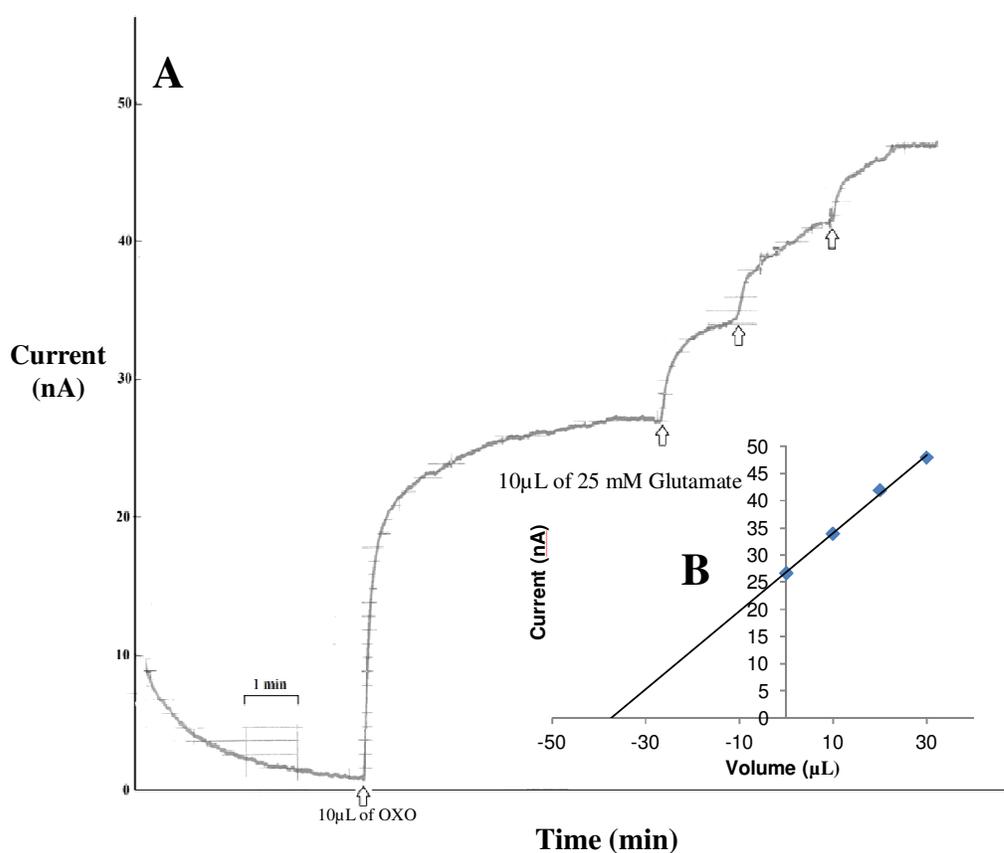


Fig. 5. A. Amperogram obtained using a GLDH-CHIT-MB-SPCE with a solution containing 10 μL of a dissolved OXO cube; arrows indicate additions of 10 μL of 25 mM glutamate into a 10 mL volume. Measurements taken 1.5 min after each addition. **B.** Typical standard addition calibration plot obtained with a solution containing a dissolved OXO cube using a GLDH-CHIT-MB-SPCE

Table 1. Data obtained from the unspiked and spiked OXO cube/food study. The mean endogenous concentration was 125.43 mg/g (n=6) in unspiked OXO cubes. The mean recovery was 91%, coefficient of variation of 6.39% indicating a good level of reproducibility in a complex unfiltered media (n=6)

Unspiked OXO Cube	
Sample	Quantity of Glutamate Detected (mg/g)
1	114.15
2	147.29
3	129.37
4	113.96
5	125.47
6	122.35
Mean (mg/g)	125.43
Std Dev	11.26
CV (%)	8.98

Spiked OXO Cube recovery				
Sample	Endogenous Concentration (mg/g)	Spike (mg/g)	Total Concentration Found (mg/g)	Recovery (%)
1	125.43	158.03	266.05	86
2	125.43	158.03	268.20	88
3	125.43	158.03	280.11	97
4	125.43	158.03	279.03	96
5	125.43	158.03	273.73	92
6	125.43	158.03	262.15	83
Mean (%)	91			
Std Dev	6.00			
CV (%)	6.39			

The GLDH-CHIT-MB-SPCE biosensors were then used to determine glutamate in spiked serum. The serum was spiked with 2.00 mM glutamate so as to roughly double the

endogenous levels of glutamate and allow a distinction between the spike and endogenous levels. The results are shown in Table 2. The mean recovery ($n=6$) was 96% with a CV of 3.29% indicating high reproducibility in serum. This method offers an economical and simple approach for the screening of glutamate levels in serum. It should be mentioned that the biosensors were stored in a desiccator in a refrigerator at 4 °C before use; under these conditions they were found to be stable for at least one week.

The GLDH-CHIT-MB-SPCE biosensor described in this paper possesses a superior limit of detection by comparison to other biosensors utilising glutamate dehydrogenase and can be fabricated simply in comparison to those reported previously (Table 3). Consequently, these devices hold promise for application quality control in clinical laboratories. It is interesting to note that a biosensor based on glutamate oxidase [31] has been reported for the determination of glutamate in soy sauce; a detection limit of 10 nM was achieved. However, it should be mentioned that the operating potential required to detect H_2O_2 was +950 mV, as a result the selectivity of the device may not be sufficient for the analysis of glutamate in serum.

Table 2. Data obtained from the unspiked and spiked serum study. In unspiked serum, 1.68 mM of glutamate was detected ($n=6$) with a coefficient of variation of 4.1%. In spiked serum, the mean recovery was 96%, coefficient of variation 3.29% ($n=6$).

Unspiked Serum	
Sample	Concentration of Glutamate detected (mM)
1	1.70
2	1.54
3	1.67
4	1.76
5	1.69
6	1.71
Mean (mM)	1.68
Std Dev	0.07
CV (%)	4.10

Spiked Serum				
Sample	Spike (mM)	Endogenous Concentration (mM)	Total Concentration Found (mM)	Recovery (%)
1	2.00	1.68	3.52	92
2	2.00	1.68	3.65	98
3	2.00	1.68	3.62	97
4	2.00	1.68	3.65	99
5	2.00	1.68	3.62	97
6	2.00	1.68	3.49	90
Mean (%)	96			
Std Dev	3.14			
CV (%)	3.29			

Table 3. A comparison of previously reported Glutamate biosensors including the one described in this paper

Immobilization Method	Ref.	LOD	Optimal pH	Applied Voltage	[NAD ⁺]	Sensitivity	Linear Range	Response Time
GDH attached to the inner surface of a 75 μ M i.d. capillary using biotin-avidin chemistry. NADH measured by fluorescence.	[32]	3 μ M	8.5, but saline 7.3 also used.	-	3 mM	-	-	450 ms
Glassy carbon covered in CNT-CHIT-MDB composite film. GDH in PBS was casted on the CNT composite electrode.	[33]	2 μ M	7.2	-100 mV	4 mM	0.71 \pm 0.08 nA/ μ M	N/A	~10 s
GDH and diaphorase immobilized on a nanocomposite electrode.	[34]	5.4 μ M	9.0	+300 mV	2 mM	28 nA μ M ⁻¹ cm ⁻²	10 - 3495 μ M	-
Polymer-modified electrode. MB entrapped into two polymers	[35]	2 μ M	7.4	0 mV	1 mM	0.70 nA μ M ⁻¹	0 - 100 μ M	
Nanomolar Detection of Glutamate at a biosensor based on Screen-printed Electrodes modified with Carbon Nanotubes	[31]	10 nM	7.4	+950 mV	N/A	0.72nA \pm 0.05 μ A μ M ⁻¹	0.01 - 10 μ M	<5 s
GLDH-CHIT-MB- SPCE	This study	1.5 μ M	7.0	+100 mV	4 mM	0.44 nA/ μ M	12.5 - 150 μ M	~2 s

4. CONCLUSION

This paper has demonstrated the effective use of CHIT to immobilise GLDH onto the surface of a MB-SPCE for the fabrication of a glutamate biosensor and the successful

application of the biosensor to the determination of glutamate, in both serum and food samples, without any sample pre-treatment, other than dilution.

This simple approach is attractive as it is based on an electrochemical biosensor fabricated by incorporating biological compounds with a chemically modified screen printed carbon electrode. SPCE's can be mass produced at low cost so biosensors based on this technology can be considered disposable. This latter feature may be of significance in biological fluid analysis where contamination may be an issue.

In summary our proposed biosensor approach offers the advantages of good selectivity owing to the combination of an enzyme integrated with a transducer operated at a low potential of only +100 mV, coupled with the simplicity of fabrication and analytical operation.

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