The design, development and application of electrochemical glutamate biosensors

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Abstract

The development of biosensors for the determination of glutamate has been of great scientific interest over the past twenty five years owing to its importance in biomedical and food studies. This review will focus on the various strategies employed in the fabrication of glutamate biosensors together with their performance characteristics. A brief comparison of the enzyme immobilisation method employed, as well as the performance characteristics of a range of glutamate biosensors are described in tabular form and then described in greater detail throughout the review: some selected examples have been included to demonstrate the ways in which these biosensors may be applied to real samples.

1. Introduction

Glutamate is considered to be the primary neurotransmitter in the mammalian brain and facilitates normal brain function [1]. Neurotoxicity, which causes damage to brain tissue, can be induced by glutamate at high concentrations, which may link it to a number of neurodegenerative disorders such as Parkinson's disease, multiple sclerosis [2] and Alzheimer's disease [3]. 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^+ [4]. Intracellular glutamate levels outside of the brain are typically 2–5 mM/L, whilst extracellular concentrations are ~0.05 mM/L [5]. It is also present in high concentrations throughout the liver, kidney and skeletal muscle [6].

Glutamate is also found in many foods in the form of monosodium glutamate (MSG) as a means to reduce salt intake and enhance flavour [7]. MSG is the source of some controversy, despite the fact that the EU limit of MSG in foods is 10g/kg of product, it is typically found in high concentrations in food claiming to contain no added MSG [8]. It can also be used to mask ingredients of poor freshness. The concentration of MSG in foods can vary significantly. The presence of monosodium glutamate in wastewater is also a concern due to its inhibitory effects on wheat seed germination and root elongation [9].

Electrochemical biosensors for the detection of glutamate offer a faster, more user-friendly and cheaper method of analysis in comparison to classical techniques such as high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). This review discusses electrochemical biosensors fabricated based on glutamate oxidase or glutamate dehydrogenase. The method of enzyme immobilization and, where applicable, the application of glutamate biosensors to biological and food samples.

2. Biosensors based on glutamate oxidase

In this section, the fabrication methods are sub-divided according to the technique of enzyme immobilization. The electrochemical response can generally be described by the following equations:

$$\text{Glutamate} + O_2 \xrightarrow{\text{GluOx}} H_2O_2 + \alpha\text{-ketoglutarate} \qquad (1)$$

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^- \qquad (2)$$
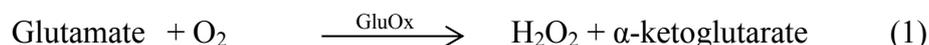
Equation 1 represents the enzymatic oxidation of the glutamate to form α-ketoglutarate and H_2O_2. Equation 2 describes the electrochemical detection of hydrogen peroxide at the base transducer which generates the analytical response.

2.1. Entrapment

The entrapment of enzymes is defined as the integration of an enzyme within the lattice of a polymer matrix or a membrane, whilst retaining the protein structure of the enzyme [10]. In addition to the immobilization of enzymes, membranes can also eliminate potential interfering species that may be present in complex media such as serum and food.

A selective biosensor for the determination of glutamate in food seasoning was developed by incorporating glutamate oxidase into a poly(carbamoyl) sulfonate (PCS) hydrogel. The GluOx-PCS mixture was then drop-coated onto the surface of a thick-film platinum electrode [11]. Liquid samples (1, 10 and 100μL) were diluted to 10mL with phosphate buffer. The biosensor was then utilised to determine the glutamate recovery from different concentrations of the sample. The results generated correlated favourably with a L-glutamate colorimetric test kit.

A recent application of a micro glutamate biosensor for investigating artificial cerebrospinal fluid (CSF) under hypoxic conditions was described [12]. The fabrication method is a complex, multi-step process whereby glutamate oxidase is incorporated with chitosan and ceria-titania nanoparticles (Figure 1).

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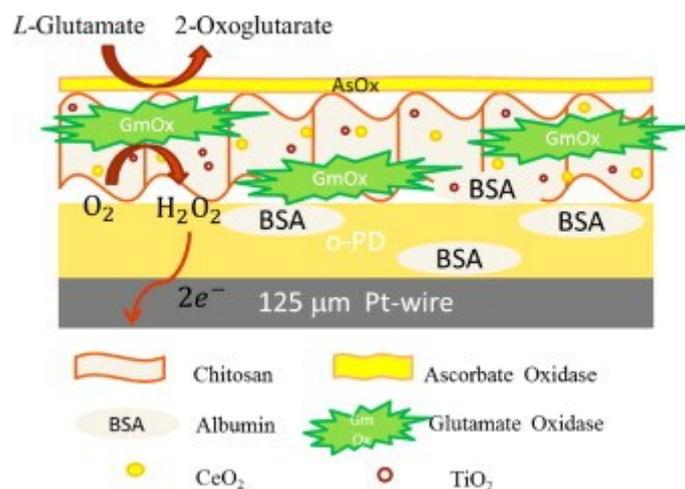
Figure 1: Schematic illustration of the biosensor design and the GluA detection principle. (Reprinted with permission from [12], Elsevier)

The nanoparticles are able to store and release oxygen in its crystalline structure; it can supply O_2 to GluOx to generate H_2O_2 in the absence of environmental oxygen. The biosensor was evaluated with artificial CSF which had been fortified with glutamate over the physiological range; the device was found to operate over the concentration of interest under anaerobic conditions.

A device for the measurement of glutamate in brain extracellular fluid utilising a relatively simple fabrication procedure has been reported [13]. The procedure involved dipping a 60-µm radius Teflon coated platinum wire into a buffered solution containing glutamate oxidase and *o*-phenylenediamine (PPD), followed by a solution containing phosphatidylethanolamine (PEA) and bovine serum albumin (BSA). The glutamate oxidase was entrapped by the electropolymerization of PPD on the surface of the electrode. The PPD and PEA was used to block out interferences.

An interesting entrapment approach employing polymers to encapsulate GluOx onto a gold electrode has been reported [14]. The first step involved the immersion of a gold disc electrode in 3-mercaptopropionic acid (MPA) solution, followed by drop-coating layers of poly-L-lysine and poly(4-styrenesulfonate). Once dry, a mixture of GluOx and glutaraldehyde was drop-coated on to the surface to form a bilayer. The authors suggested that MPA increases the adhesion of the polyion complex to the gold surface by the electrostatic interaction between the carboxyl groups

present on the MPA and the amino groups present on the poly-L-lysine. A response time of only 3 seconds was achieved after an addition of 20nM glutamic acid, which gave a current of 0.037 nA (1.85 nA/µM). A linear response was observed between 20µM and 200µM. Both the response time and limit of detection are superior to previously discussed biosensors. It was suggested that the rapid response was due to the close proximity of the enzymatic reaction to the surface of the electrode. For this method of fabrication of glutamate oxidase based biosensors, the latter approach leads to the lowest limit of detection.

The increased interest in glutamate measurement has led to the commercial development of an *in vivo* glutamate biosensor by Pinnacle Technology Inc. [15]; this has been successfully used for monitoring of real-time changes of glutamate concentrations in rodent brain. The biosensor employs an enzyme layer composed of GluOx and an "inner-selective" membrane, composed of an undisclosed material that eliminates interferences. The enzymatically generated hydrogen peroxide is monitored using a platinum-iridium electrode. The biosensor possesses a linear-range up to 50µM. The manufacturers indicate that the miniaturised biosensor requires calibration upon completion of an experiment in order to ensure the selectivity and integrity of the sensors.

2.2.   Covalent-bonding

The application of a glutamate oxidase based biosensor for the measurement of glutamate in the serum of healthy and epileptic patients has been described [16]. The fabrication method consists of electrodepositing chitosan (CHIT), gold nanoparticles (AuNP) and multiwalled carbon nanotubes (MWCNTs) on the surface of a gold electrode.

The serum sample was diluted with phosphate buffer solution (PBS) before analysis. The concentration of the glutamate in the sample was determined using a standard calibration curve constructed from the amperometric responses obtained with glutamate in PBS. The results compared favourably with a colorimetric test kit. A low operating potential of +0.135 V vs. Ag/AgCl for measuring the enzymatically generated H_2O_2 was significantly lower than other

biosensors based on GluOx [13,17], The time taken to reach 95% of the maximum steady state response was 2 seconds after the initial injection. <mark>This method of fabrication whilst complex, possesses a significantly lower operating potential when compared to other biosensors fabricated using entrapment techniques.</mark>

2.3. Cross-linking

Enzyme immobilization can be achieved by intermolecular cross-linking of the protein structure of the enzyme to other protein molecules or within an insoluble support matrix. Jamal et. al. [17] have described a complex entrapment method which consisted of drop-coating a 10µL mixture of GluOx (25U in 205µL), 2mg of BSA, 20µL of glutaraldehyde (2.5% w/v) and 10µL of Nafion (0.5%) onto a platinum nanoparticle modified gold nanowire array (PtNP-NAE) and allowing it to dry overnight under ambient conditions. The fabrication technique is illustrated in Figure 2. The analytical response results from the oxidation of H_2O_2 at the gold nanowire electrode as illustrated in Equation 2.
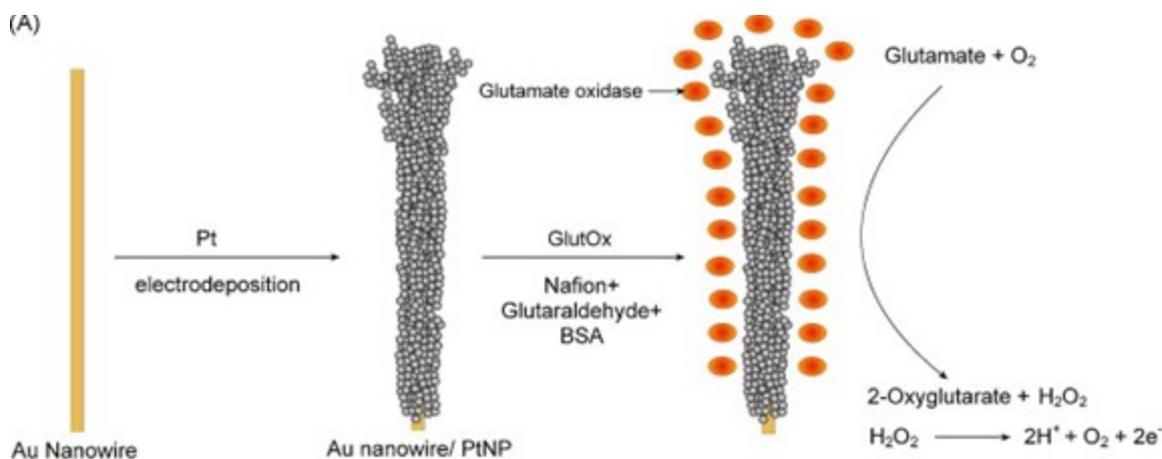
Figure 2: Schematic illustration of stepwise fabrication of the GlutOx/PtNP/NAEs electrodes. Reprinted with permission from [18], Elsevier.

The high sensitivity obtained appears to be related to the presence of the nanoparticles at the gold electrode surface. The nanoparticles act as conduction centres and facilitate the transfer of electrons towards the gold electrode. Additionally, a high enzyme loading was utilised, which in

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combination with the nanoparticles, resulted in increased enzyme immobilisation to the surface. However, given the high enzyme loading and use of both a gold nanowire electrode and platinum nanoparticles, the biosensor is unlikely to be commercially viable due to its high cost.

GluOx was immobilized to the surface of a palladium-electrodeposited screen printed carbon strip by a simple crosslinking immobilisation technique using a photo-crosslinkable polymer (PVA-SbQ) [18]. The biosensor exhibited a stable steady state response for six hours in a stirred solution indicating that the enzyme was fully retained within the polymer membrane.

A glutamate biosensor [19] was successfully applied to the determination of MSG in soy sauce, tomato sauce, chicken thai soup and chilli chicken. MSG levels compared very favourably with a spectrophotometric method (2 - 5% CoV based on n = 5). The biosensor was fabricated by mixing glutamate oxidase, BSA and glutaraldehyde, then spreading the mixture onto the surface of an O_2 permeable poly-carbonate membrane. The membrane was then attached to an oxygen probe using a push cap system and oxygen consumption was measured at an applied potential of -0.7 V; this is a considerably more negative operating potential compared to previously discussed biosensors. In this case, the response is a result of the reduction reaction shown in Equation 3.

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2 \qquad (3)$$
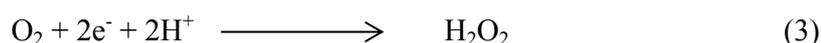
The lowest limit of detection achieved with a glutamate biosensor was fabricated by covalently immobilizing glutamate oxidase onto polypyrrole nanoparticles and polyaniline composite film (PPyNPs/PANI) [20]. Cyclic voltammetry was used to co-electropolymerize the compounds onto the surface. The PPyNPs act as an electron transfer mediator which allows a low operating potential to be employed (+85 mVs), that reduces the likelihood of oxidising interferences. The authors also claim that this leads to an increase in the sensitivity of the biosensor. The biosensor was successfully applied to the determination of glutamate in food samples including tomato soup and noodles. High recoveries of 95% - 97% were achieved which compares favourably with values attained by previously discussed biosensors [19].

3. Biosensors based on glutamate dehydrogenase

This section is subdivided in a similar way to section 2, ie: according to the method of immobilization. The electrochemical response can generally be described by the following equations:

$$\text{Glutamate} + \text{NAD}^+ \xrightarrow{\text{GLDH}} \text{NADH} + \alpha\text{-ketoglutarate} \quad (4)$$

$$\text{NADH} + \text{Mediator}_{ox} \longrightarrow \text{NAD}^+ + \text{Mediator}_{red} \quad (5)$$

$$\text{Mediator}_{red} \longrightarrow n e^- + m H^+ + \text{Mediator}_{ox} \quad (6)$$
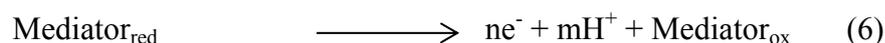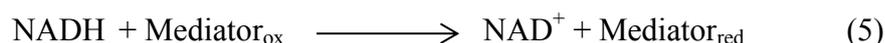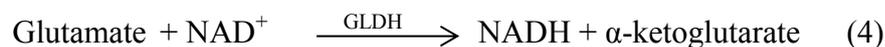
Equation 4 represents the enzymatic reduction of the cofactor NAD^+ to NADH and the oxidation of glutamate to α-ketoglutarate. Equation 5 represents the electrochemical reduction of the oxidised mediator to the reduced form (Mediator_{red}). Equation 6 describes the electrochemical oxidation of Mediator_{red} at the base transducer which generates the analytical response; the regenerated mediator $_{ox}$, can then undergo further reactions with NADH. Equations 4 and 5 represent the electrocatalytic oxidation of NADH, which occurs at lower applied potentials than obtained by the direct electrochemical oxidation of NADH at an unmodified electrode.

3.1. Entrapment

A simple fabrication method based on the integration of a liver mitochondrial fraction containing glutamate dehydrogenase was employed for the fabrication of a novel glutamate biosensor. The liver mitochondrial fraction was utilised in an attempt to reduce the cost of the biosensor, however, the activity of the biosensor appeared to be compromised in comparison to the biosensor incorporating purified glutamate dehydrogenase. The biological recognition element was mixed with a carbon paste, packed into an tube, and used in the determination of MSG in chicken bouillon cubes [21]. The enzymatically generated NADH was oxidised using ferricyanide as a electrochemical mediator. High recoveries of MSG were achieved. Several amino acids, commonly found in food products, did not interfere with the determination. Extensive pre-treatment of the food sample, consisting of dissolving, vacuum-filtering, washing

and then further diluting the sample in buffer, was required before analysis, in contrast to simpler food preparation methods previously discussed [19].

A novel biosensor fabrication technique was developed by Tang et. al. [22] which consisted of entrapping GLDH between layers of alternating poly(amidoamine) dendrimer-encapsulated platinum nanoparticles (Pt-PAMAM) with multi-walled carbon nanotubes. PAMAM's were used to modify the surface of the glassy carbon electrode due to their excellent biocompatibility and chemical fixation properties. The procedure was repeated using positively charged Pt-PAMAM and negatively charged GLDH which were alternatively adsorbed onto the CNTs in a layer-by-layer process. The assembly process and enzyme immobilisation process is illustrated in Figure 3.
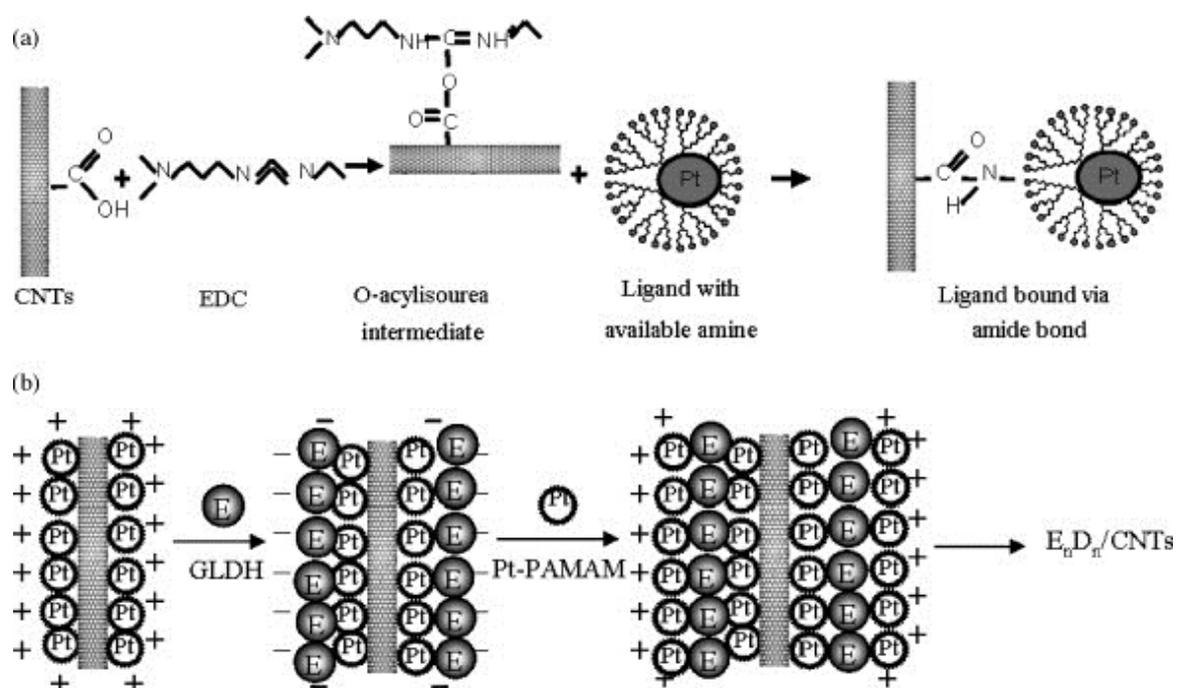
Figure 3: Schematic showing the procedure of immobilizing Pt-PAMAM onto CNTs (a) and the layer-by-layer self-assembly of GLDH and Pt-PAMAM onto CNTs (b). Pt-PAMAM/CNTs heterostructures were attached covalently via EDC, Pt-PAMAM and GLDH were alternately deposited. Reprinted with permission from [24], Elsevier.

Whilst the fabrication procedure is complex, the biosensor possesses superior sensitivity to previously discussed biosensors.

In contrast to the above, a simpler and less time consuming method of fabricating a glutamate biosensor has been described [23] which involved incorporating GLDH and NAD^+ into carbon paste. The mixture was inserted in a holder, placed in to a solution containing O-phenylenediamine and subsequently electropolymerized. The o-phenylenediamine film is simultaneously able to prevent interferences and facilitate the amperometric detection of NADH at low applied potentials by acting as an electron mediator. The biosensor was used to determine glutamate in chicken bouillon cubes; the results compared favourably to those obtained using a spectrophotometric method ($12.6 \pm 0.3\%$ (n = 5) and 12.3% respectively). Despite the simpler fabrication method, the linear range and sensitivity of the biosensor was lower than the sensor described by Tang et. al. [22].

In recent years, the biopolymer known as chitosan has been investigated in a variety of studies as a method of entrapping enzymes for biosensor construction [24]. CHIT appears to offer improved enzyme stability and is easy to immobilize onto a variety of materials. A biosensor for glutamate [25] has been constructed by depositing a mixture of purified CNTs, CHIT and MB onto a glassy carbon electrode and dried. The surface was then treated with an aliquot of GLDH in PBS, and dried at 4ºC. The cofactor was present in free solution at a concentration of 4mM. The selectivity of the biosensor was determined by the addition of interferences (AA, UA), with no apparent amperometric responses being generated. However, the application of the biosensor to clinical and food samples was not described in this paper.

Screen-printing technology has offered the possibility of the mass production at low cost of glutamate biosensors; these have been successfully applied to the measurement of glutamate in serum and food samples [26]. As screen-printed devices are inexpensive to manufacture they can be considered disposable, in comparison to glassy carbon electrodes, which are expensive and are not considered disposable devices. Consequently, the former are much convenient devices for fabricating biosensors and have become a popular route to commercialisation. The fabrication method involved drop-coating CHIT (0.05%) onto the surface of a Meldola's Blue (MB) SPCE

(MB-SPCE), followed by an aliquot of glutamate dehydrogenase (3U/µL). This device is designated GLDH-CHIT-MB-SPCE. The biosensor was then left to dry under vacuum at 4ºC overnight. NAD$^+$ was present in free solution at a concentration of 4mM.

The electro-catalyst Meldola's Blue allowed an operating potential of only +100mV to be employed; the response occurred as a result of the electrocatalytic oxidation of enzymatically generated NADH. The biosensor was successfully applied to the determination of MSG in Beef OXO cubes and endogenous glutamate in serum. The beef OXO cube was dissolved by sonication in PBS and the endogenous content of both the OXO cube and serum were determined. Recoveries of 91% were attained for the spiked OXO cube (n = 6) and 96% for the spiked serum test (n = 6). These results compare favourably to those reported by Alvarez-Crespo et. al. [23] and Basu et.al [19]. This device improves upon the linear range of previously discussed biosensors, [23,25].

In order to further develop this biosensor for potential commercial development, all of the components needed to be immobilized onto the surface of the transducer. The layer-by-layer fabrication process is described in the paper [27]. The schematic shown in Figure 4 summarises the important steps involved in this process.
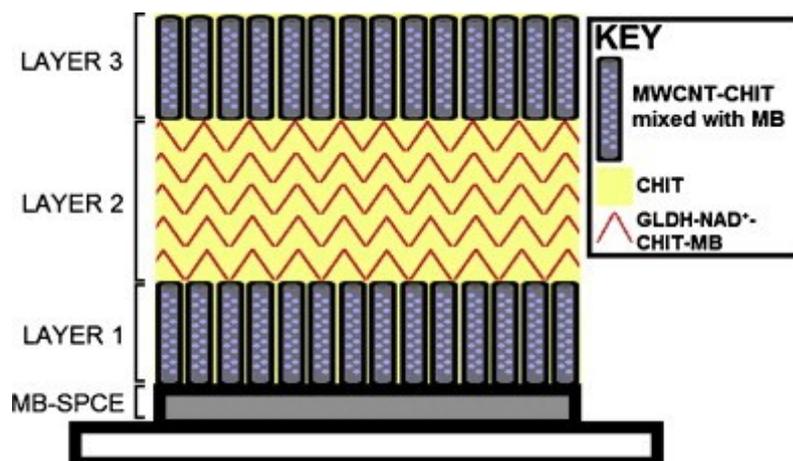
Figure 4: A schematic diagram displaying the layer-by-layer drop coating fabrication procedure used to construct the reagentless glutamate biosensor, based on a MB-SPCE electrode. Reprinted with permission from [27], Elsevier.

An amperometric plot using the reagentless biosensor are shown in Figure 5. The biosensor was successfully applied to the determination of glutamate in spiked serum. A recovery of 104% (n = 5, CoV: 2.91%) was determined, which compares favourably to previously discussed biosensors [19,23,26]. An interference study was conducted in both serum and food samples (stock cubes) and no interfering signals were generated. Such reagentless biosensors offer the advantage of being low cost, simple to use and requires no additional cofactor to be added to the sample solution. Clearly, this is a prerequisite for commercial devices.
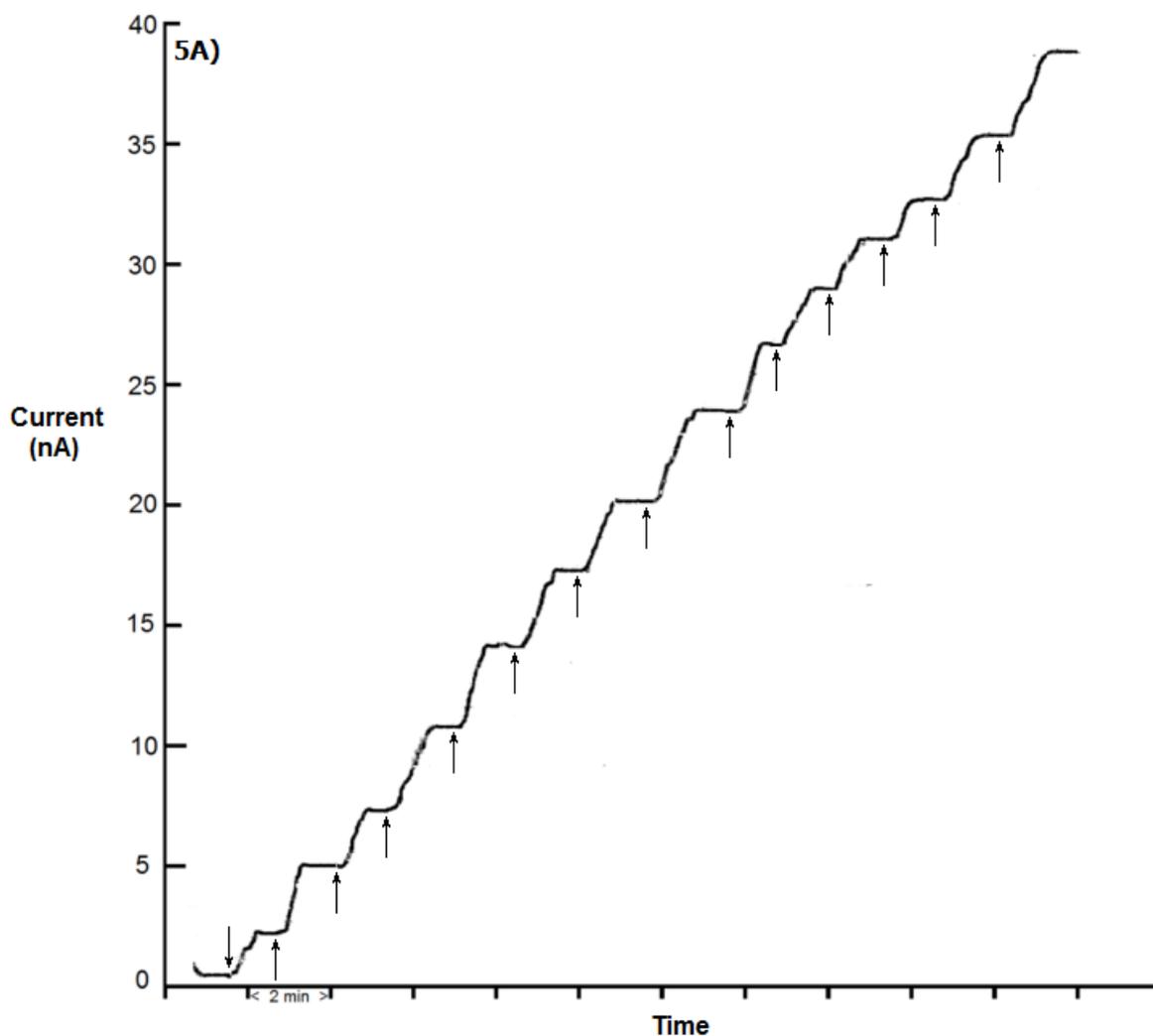
Figure 5: Amperogram conducted with the reagentless glutamate biosensor. Each arrow represents an injection of 3 μL of 25 mM glutamate in a 10 mL stirred solution containing supporting electrolyte; 75 mM, PB (pH 7.0), with 50 mM NaCl at an applied potential of +0.1 V vs. Ag/AgCl. Adapted with permission from [27], Elsevier.

3.2. Covalent Bonding

The electrochemical technique known as different pulse voltammetry was used in conjunction with a glutamate biosensor to develop a novel assay for measuring glutamate in a mixture of naturally occurring biomolecules commonly found in biological samples [28]. The biosensor fabrication procedure involves vertically aligned carbon nanotubes (VACNTs) which are treated in order to convert the tips of the CNTs into carboxylic acid groups, in order to covalently bind the enzyme. GLDH was bound to the CNTs using 1-(3-dimethylamino propyl)-3-

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ethylcarbondiimide hydrochloride (EDC) and hydroxyl-sulfosuccinimide sodium salt (sulfo-NHS) to promote amide linkages between the carboxylic tips of the CNTs and the lysine residue present on the enzyme. This was achieved by immersing the electrode in a solution containing EDC/sulfo-NHS and mixed. Once dried, the enzyme was drop-coated onto the electrode, dried for 2 hours and washed with PBS/BSA mixture. The DPV obtained with the synthetic samples indicated that over the concentration range studied, no significant interferences should be expected. However, the biosensor was not applied to a real sample.

3.3. Crosslinking

Carbon nanotubes have been successfully employed to produce cross linking matrixes. Single wall carbon nanotubes have been treated with thionine (Th) to produce a Th-SWCNT nanocomposite on the surface of a glassy carbon electrode [29]. The nanocomposite acts as both an electron mediator and enzyme immobilization matrix. The GLDH was mixed with BSA and crossed linked with glutaraldehyde and coated onto the Th-SWCNT layer. An applied potential of +190mV was utilised for amperometric measurements. The linear range was found to superior to that which was achieved by Gholiazadeh et. al. [28] and Tang et. al. [22]. Ascorbic acid, uric acid and 4-acetamidophenol were examined as possible interferents; no discernible current responses were seen.

4. Conclusions

The review has highlighted some novel approaches to the fabrication of electrochemical glutamate biosensors. The performance characteristics of the glutamate biosensors discussed are summarised within Table 1. The most commonly reported method for the immobilization of both glutamate oxidase and dehydrogenase is entrapment.

The utilisation of glutamate oxidase offers an advantage over glutamate dehydrogenase as the latter requires the cofactor NAD^+ to be co-immobilised onto the appropriate transducer. In addition the response times are also generally shorter for glutamate oxidase based biosensors. However, there are several drawbacks with the use of these biosensors. Significantly higher applied potentials must be utilised in order to generate an amperometric response from the enzymatic generation of H_2O_2. In contrast low operational potentials of around 0 V can be applied with dehydrogenase based biosensors; which is advantageous when measuring glutamate in complex media.

Expensive electrode materials such as gold and platinum are often used in the development of glutamate biosensors utilising glutamate oxidase. In contrast, three glutamate biosensors employing GLDH immobilized onto SPCE's as the electrode material have been described. SPCE's offer an inexpensive approach to fabricating glutamate biosensors which is clearly an important consideration in the commercialisation of such devices.

Finally, the cost of glutamate dehydrogenase is far lower than that of glutamate oxidase (as of August 2015). 100mg (20U per mg) of glutamate dehydrogenase costs £175. By contrast, glutamate oxidase is sold by Sigma for £88.20 per 1U. This is clearly an important consideration for commercialisation.

Table 1: Details of the enzyme immobilisation technique and performance characteristics of amperometric glutamate biosensors based on glutamate oxidase and glutamate dehydrogenase

Legend: NS – Not specified, N/A – Not applicable * - Voltametric Measurement, ~ - Polarographic Measurement

Glutamate Oxidase based single enzyme systems									
Immobilisation Technique	Type	Ref	LOD	Optimum pH	V (vs. Ag/AgCl)	Sensitivity	Linear Range	Stability	Response Time
Poly(carbamoylsulphonate) (PCS) hydrogel mixed with GluOx	Entrapment	[11]	1.01µM	6.86	+400mV	1.94 nA/µM	100 - 5000µM	90% of activity retained after 2 weeks.	NS
Mixed ceria and titania nanoparticles for the detection of glutamate in hypoxic environments	Entrapment	[12]	0.594µM	7.4	+600mV	0.7937 nA/µM	5 – 50µM	75% of signal after 70 assays in aerobic environment. Anaerobic enviroment, stable for 8 assays.	~5
Biosensor for Neurotransmitter L-Glutamic Acid Designed for Efficient Use of L-Glutamate Oxidase and Effective Rejection of Interferences	Entrapment	[13]	0.27 µM	7.4	+700 mV	3.8 ± 1.3 nA cm^{-2} mmol^{-1} L	0 - 100µM	NS	<10s

Polyion complex-bilayer membrane	Entrapment	[14]	0.2 nM	7.0	+800 mV	1.85 nA/μM	3 – 500 μM	Stable over 4 weeks with daily usage.	NS
Carboxylated multiwalled carbon nanotubes/gold nanoparticles/chitosan composite film modified Au electrode	Covalent-bonding	[16]	1.6μM	7.4	+135mV	155 nA/μM/cm^2	5 – 500 μM	4 months, no data shown.	2s
Pt nanoparticles modified Au nanowire array electrode	Entrapment	[17]	14μM	7.4	+650mV	194.6 μA mM^{-1} cm^{-2}	200 - 800 μM	98% of its initial response retained after 2 weeks.	4.8s
Photo-crosslinkable polymer membrane on a palladium-deposited screen-printed carbon electrode	Cross linking	[18]	0.05μM	7.0	+400mV	12.8 nA/μM	0.05μM - 100μM	Stored dry – 5 months, no change in response.	30 – 50s Not stated.
Cross linking with Glutaraldehyde and BSA as a spacing agent. ~	Cross linking	[19]	N/A	7.0	-700mV	NS	NS	84% of initial signal after 48 days of use every 3 days.	120s
Polypyrrole nanoparticles/polyaniline modified gold electrode.	Cross linking	[20]	0.1nM	7.5	-130mV	533 nA/ μM/cm^2	0.02 - 400μm	60 days at 4ºC	3s

Glutamate Dehydrogenase based single enzyme systems										
Immobilisation Technique	Type	Ref	LOD	pH	V (vs. Ag/AgCl)	[NAD+/NAD(P)+]	Sensitivity	Linear Range	Stability	Response Time
A mixture of carbon paste, octadecylamine and mitochondria fraction, packed into the working electrode.	Entrapment	[21]	100μM	7.5	+350mV	1mM	0.189μA/mM	0.4 – 10mM	60% after 10 days of use.	N/A
Alternatively assembling layers of glutamate dehydrogenase and Pt-PAMAM.	Entrapment	[22]	100 μM	7.4	+200mV	0.1mM	433 μA/mM^{-1} cm^2	0.2 - 250 μM	85% after four weeks	3s
Unmodified carbon paste mixed with lyophilized enzyme and coenzyme, packed into the well of a working electrode.	Entrapment	[23]	3.8μM	8.0	+150 mV	N/A	$4·6×10^5$ nA l mol^{-1}	5 - 78 μM	Signal decrease by 50% after 3 days	120s
GLDH dropcoated onto the surface of a CNT-CHIT-MDB modified GC electrode.	Entrapment	[25]	2 μM	7.0	-140mV	4mM	0.71 ± 0.08 nA/μM	25 – 100 μM	NS	NS
Immobilisation of GLDH with CHIT, drop coated onto the surface of a SPCE.	Entrapment	[26]	1.5 μM	7.0	+100mV	4mM	0.44nA/μM	12.5 - 150μM	NS	2s

Description	Immobilisation method	Ref	LOD	pH	Potential		Sensitivity	Linear range	Stability	Response time
Reagentless biosensor. Immobilisation of both NAD^+ and GLDH by utilising a mixture CHIT/MWCNT/MB dropcoated onto the surface of a SPCE in a layer by layer fashion.	Entrapment	[27]	3µM	7.0	+100mV	N/A	0.39 nA/µM	7.5 - 105µM	100% of original response retained after 2 weeks	20 – 30s
GLDH attached to CNTs utilising EDC and sulfo-NHS, by immersing electrode in PBS containing both compounds then dropcoating GLDH. *	Covalent bonding	[28]	0.57 µM	7.4	NS	2mM	With mediator: 1.17 mA mM^{-1} cm^{-2} / 0.153 mA mM^{-1} cm^{-2} Without mediator: 0.976 mA mM^{-1} cm^{-2} / 0.182 mA mM^{-1} cm^{-2}	With mediator: 0.1 - 20µM / 20 - 500µM Without mediator 0.1 - 20µM / 20 - 300µM	80.5% of original response after two weeks of use.	NS
GLDH is mixed with BSA then coated onto the surface of a Th-SWNTs/GC electrode, then followed by glutaraldehyde.	Crosslinking	[29]	0.1 µM	8.3	+190mV vs NHE	0.1µM	137.3 µA mM^{-1}	0.5 – 400µM	93% after 2 weeks	5s

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