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In vivo Electrochemical Biosensor for Brain Glutamate Detection: A Mini Review

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Abstract

Glutamate is one of the most prominent neurotransmitters in mammalian brains, which plays an important role in neuronal excitation. High levels of neurotransmitter cause numerous alterations, such as calcium overload and the dysfunction of mitochondrial and oxidative stress. These alterations may lead to excitotoxicity and may trigger multiple neuronal diseases, such as Alzheimer's disease, stroke, and epilepsy. Excitotoxicity is a pathological process that damages nerve cells and kills cells via excessive stimulation by neurotransmitters. Monitoring the concentration level of brain glutamate via an implantable microbiosensor is a promising alternative approach to closely investigate in the function of glutamate as a neurotransmitter. This review outlines glutamate microbiosensor designs to enhance the sensitivity of glutamate detection with less biofouling occurrence and minimal detection of interference species. There are many challenges in the development of a reproducible and stable implantable microbiosensor because many factors and limitations may affect the detection performance. However, the incorporation of multiple scales is needed to address the basic issues and combinations across the various disciplines needed to achieve the success of the system to overcome the challenges in the development of an implantable glutamate biosensor.

Keywords: *brain glutamate, biosensing techniques, microelectrode*

Introduction

Glutamate

Glutamate (Glu) is an important neurotransmitter in the mammalian brain. Glutamate plays a main role in brain development, neurotransmission, synaptic plasticity and neurotoxicity; glutamate is also involved in neurological disorders, such as ischemia (1,2), schizophrenia (3,4), epilepsy (5), Alzheimer's disease (AD) (6–8), and Parkinson's disease (PD) (9,10). Moreover, brain injury (11,12) can also increase glutamate levels. Thus, it is very important to identify the levels of extracellular glutamate concentration in the affected brain because of their importance in neurotransmission. Recent discoveries have demonstrated that glutamatergic neurotransmission in the central nervous system (CNS) is mediated by a dynamic interaction between neurons and astrocytes. Thus, to control the concentration of glutamate that triggers excitotoxicity, a complex system is required to regulate glutamate metabolism (13). Lee et al. 2007, (14) located potential sources of glutamate using high frequency stimulation (HFS) with deep brain stimulation (DBS), which affected the neurotransmitter levels in ferret thalamic slices, to determine a pool of glutamate in neurons and astrocytes for PD. As a result of many experiments, they concluded that glutamate was released from astrocytes, where calcium-dependent and some independent glutamate was released from axonally driven synapses (neurons). Another DBS study using HFS that focused on glutamate release in the subthalamic nucleus (STN) (15) proposed that DBS increases neurotransmitter levels within the basal ganglia, which is used for the feedback signal that produces the closed group for the DBS system and the selection for the site of stimulation to be optimised. Although this method is difficult to handle because of troubleshooting the glutamate level, they have identified a constant relationship between HFS and extracellular glutamate release using a duty cycle technique to overcome the overshoot in the desired glutamate concentration during stimulation.

The main motivation for the ongoing worldwide research on glutamate is a result of glutamate's role in the signal transduction of the nervous systems of apparently all complex living organisms, including humans. Glutamate is considered the major mediator of excitatory signals in the mammalian central nervous

system and is involved in most aspects of normal brain function, including cognition, memory and learning. Glutamate is one of the principal neurotransmitters in the mammalian brain; thus, it has been the subject of considerable medical interest because of the elevated concentration of extracellular glutamate release as a result of neural injury, which is toxic to neurons (16). It is implicated in a variety of disorders in humans, particularly selective neuronal injury and epilepsy. As research regarding new drug models for the modulation of glutamate release continues, there is a perceived need to be able to measure this neurotransmitter during surgery. To date, under these circumstances, glutamate is measured by high performance liquid chromatography (HPLC) (17) however, this techniques is time consuming and results in a restriction of real time information that can be obtained on the dynamics of drug response.

Glutamate is a toxic, but it is important because it not only mediates substantial information but also regulates brain development, which determines cell survival and differentiation and the formation and elimination of nerve contacts (synapses). Thus, glutamate must be present in the right concentration in the right place at the right time. Both too much and too little glutamate is harmful, which implies glutamate is simultaneously essential and highly toxic.

Regulation of brain glutamate

Similar to other signalling substances (neurotransmitters and hormones), the signalling effect of glutamate is not dependent on the chemical nature of glutamate but on how cells are programmed to respond when exposed to glutamate. Only cells with glutamate receptor proteins (glutamate receivers) on their surfaces are sensitive to glutamate. Glutamate exerts its signalling function by binding to and thereby activating these receptor proteins. Several subtypes of glutamate receptors have been identified: NMDA, AMPA/kainate and metabotropic receptors (mGluR). Although the individual receptor subtypes exhibit specific (restricted) localisations, glutamate receptors of one type or another are found virtually everywhere. Most nerve cells, and even glial cells, have glutamate receptors.

Glutamate must be maintained inside the cells (intracellular). At first glance, this appears to be an impossible system. A closer look, however, indicates that glutamate is not present everywhere. It is almost exclusively located inside cells. The intracellular location of approximately 99.99% of brain glutamate is the reason why this system can work. This is essential because glutamate receptors can only be activated by glutamate that binds to them from the outside. Thus, glutamate is relatively inactive as long as it is intracellular. The volume of brain cells and meshwork formed by their intermingled extensions constitutes approximately 80% of brain tissue volume. This network is submerged in extracellular fluid (ECF), which represents the remaining 20% of brain tissue volume. The normal (resting) concentration of glutamate in ECF is low on the order of a few micromolar (μM). In contrast, the glutamate concentration inside the cells is several thousand times higher at approximately 1 - 10 mM. The highest glutamate concentrations are located in nerve terminals, and the concentration inside synaptic vesicles may be as high as 100 mM (16).

Glutamate Excitotoxicity

Excitotoxicity is the pathological process by which neurons are damaged and killed by excessive activations of excitatory neurotransmitter glutamate receptors, such as the NMDA and AMPA receptors. For a normal glutamate concentration released into a synaptic cleft, it increases to a high level of approximately 1 mM; however, it only remains momentarily (a few milliseconds) at this concentration. At an abnormal glutamate concentration, the accumulation of neurotransmitter is high in the synaptic cleft and causes neuronal death.

There are several methods used for glutamate detection, such as magnetic resonance, capillary electrophoresis (18), high performance liquid chromatography (17) liquid chromatography coupled electrochemistry (19) and microdialysis (20). To date, many studies have focused on the use of microelectrodes, especially a microbiosensor, because electrochemical methods have advantages, including high sensitivity and selectivity, reproducibility, inexpensive, fast and accurate results (20–21). Research in the field of biosensors is particularly helpful in understanding the physiology of neurotransmitters in the brain when applied using an in vivo method (23–25).

Biosensor

A biosensor is defined as a sensor that transforms chemical information from a biochemical reaction concentration of a sample component to an analysis composition into an analytically helpful signal (26). The term biosensor has been widely applied to a number of devices

used to monitor living systems or incorporate biotic elements. A biosensor is composed of two elements: a biological recognition unit that specifically interact with a target and a transducer that is able to convert a change in the property of the solution or electrode surface, as a result of complex formation, into a recordable signal.

According to Altschuh (27), a biosensor is a device for the detection of an analyte that combines a biological component with a physicochemical detector component. According to the IUPAC definition, an electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) that is retained in direct spatial contact with an electrochemical transduction element (26).

The detection of a biological system in the form of a biochemical as an example analyte concentration will translate the information into a chemical or physical output, which is referred to as the sensitivity. The key idea of this identification system is to provide a high degree of selectivity for the analyte to be measured. Whereas all biosensors are more or less sensitive for a particular analyte, some biosensors are affected by the architecture and construction of the electrode categorised as a specific detection because they use a specific enzyme to detect a specific analyte.

A transducer is part of a sensor, also referred to as a detector or electrode, which transfers the signal from the analyte or the output of the reaction product into an electrical signal. Thus, a transducer provides a bidirectional signal transfer (non-electrical to electrical and vice versa). It consists of 3 parts: a "sensitive biological element" (a biologically derived material or biomimic) in which the sensitive elements can be created by biological engineering. Next, the "transducer" or the "detector element" (works in a physicochemical way; e.g., optical, piezoelectric, or electrochemical) that transforms the signal that results from the interaction of the analyte with the biological element into another signal (i.e., transducers) that can be more easily measured and quantified. An associated electronic or signal processor displays the results in a user-friendly way. A common example of a commercial biosensor is the blood glucose biosensor, which uses the enzyme glucose oxidase to break down blood glucose.

Amperometry is based on the current measurement that results from electrochemical oxidation or reduction of an electroactive species; for a non-electroactive species, it will require an enzyme present to produce a signal. It is typically performed by maintaining a constant potential at Pt, Au or C based working or array electrode with respect to a reference electrode, which serves as an auxiliary electrode if the currents are low, i.e., in the range of 10^{-9}–10^{-6} A. The resulting current is directly correlated with the bulk concentration of the electroactive compound species or the production or consumption rate within the adjacent biocatalytic layer. The biocatalytic reaction rate is often first order dependent on the bulk analyte concentration, similar to steady-state currents, and are typically proportional to the bulk analyte concentration.

Potentiometric measurements are involved in the determination of potential differences between an indicator and reference electrode or two reference electrodes separated by a permselective membrane when there is no significant current flowing between them. The transducer could be an ion selective electrode (ISE), which is an electrochemical sensor based on a thin film or selective membrane coating. The potential differences between indicators and the reference electrode is proportional to the logarithm of the ion activity or concentration, which has been described by the Nerst-Donnan equation. This case only occurs as follows:

(i) The membrane or layer selectivity is infinite or if there is a constant or sufficiently low enough concentration of interfering ion

(ii) The potential differences at various phase boundaries are either negligible or constant, with the exception at the membrane sample solution boundary

When the permselective membrane layer is placed adjacent to the potentiometric detector, several points must be considered:

(i) Transportation of the substrate or analyte to be analysed to the electrode or biosensor surface

(ii) Diffusion of the analyte to the reacting membrane layer

(iii) Reaction of the analyte in the presence of enzyme

(iv) Diffusion of the product reaction towards the detector and solution

Monitoring of Analyte

Hydrogen peroxide

A recent study involved substantial effort regarding the development of a biosensor that is able to detect neurotransmitters directly in brain tissue with less damage (28). Highly sensitive H_2O_2 electrodes have been combined with H_2O_2 producing oxidisation to construct useful biosensors for various analytes. This approach is typically conducted at a relatively high applied potential (+700 mV vs Ag/AgCl) for direct oxidation on the electrode surface for this study. The H_2O_2 generated as a result of the enzyme-catalysed oxidation of substrate (i.e., analyte) and the transfer of electrons to oxygen is subsequently oxidised or reduced at the electrode surface to provide a measurable current that can be correlated with the analyte concentration. These biosensor constructs based on glutamate oxidase, for example, have been increasingly applied for measurements of the neurotransmitter L-glutamate in living brain tissue (29).

$$H_2O_2 \rightarrow O_2 + 2\,H^+ + 2e \quad (1)$$

The focus of this study is the platinum electrode, which is involved in the oxidation H2O2 with the interaction of platinum oxides, such as $Pt(OH)_2$.

$$H_2O_2 + Pt(OH)_2 \rightarrow Pt(OH)_2.H_2O_2 \quad (2)$$

$$Pt(OH)_2.H_2O_2 \rightarrow Pt + 2\,H_2O + O_2 \quad (3)$$

$$Pt + 2\,H_2O \rightarrow Pt(OH)_2 + 2\,H^+ + 2e \quad (4)$$

Furthermore, it undergoes a non-redox platinum catalysed disproportionate reaction at the electrode surface. The extent of this reaction varies with the electrode material and its pre-treatment (30).

Ascorbic Acid

L-ascorbic acid is a common biosensor interference species that is oxidised on platinum electrode surfaces to form dehydroascorbic acid with the loss of two electrons. L-dehydroascorbic acid is unstable and undergoes rapid hydrolysis through an open chain electro-inactive product, L-2, 3-diketogulonic acid. Several electrode modifications have been performed to reject the detection of interference regardless of the increase in the sensitivity of the target analyte.

Type of Electrode

Many types of electrodes have been designed to develop an increased sensitivity towards a target analyte. Furthermore, the stability, persistency, flexibility, simple and low cost architecture of the electrode are key factors for the development of a biosensor. The first-generation biosensor methods of H_2O_2 detection that utilise potentials of +700 mV vs an Ag/AgCl reference electrode are more prone to interferences as a result of higher recording potentials. To improve the selectivity of the first-generation glutamate biosensors, several techniques have been used, such as polymer coatings on platinum, gold (31,32), palladium (33), or glassified carbon electrodes(34,35), and include, for example, overoxidised polypyrrole (25,28,36,37), polyphenylenediamines (38,39), polyurethanes (40,41), polyaniline (42,43) and Nafion (44–46) .

Glassy carbon electrode (GC)

Most electrode studies of neurotransmitters have used a GC electrode because it is cheaper compared with other types of electrodes, such as palladium, aurum and platinum. A modification of the electrode is required to improve its sensitivity to target species and selectivity towards interference species. A study of glutamate detection using glassy carbon requires a further deposition of a multi-wall carbon nanotube (MWNT) and long-time consumption to produce a modified electrode (47). Carbon electrodes are able to produce a stable and reproducible biosensor, which is an improvement regarding electrode sensitivity by electropolymerisation; however, too many procedures for modification result in a time consuming electrode preparation.

Nanomaterials for biosensing applications have recently received more attention in this field. The most popular electrode modification has used carbon nanotubes because of their fascinating properties (48–50). The present study demonstrated that a new modification using nanocomposites can be incorporated with a suitable membrane layer or film using the electropolymerisation method, which thus increases the performance of the biosensor, including the selectivity and sensitivity with a low limit of detection and fast response (48).

Platinum (Pt) electrode

Platinum is a very useful electrode for the detection of glucose and other neurotransmitters, and some modifications have been performed for

the Pt electrode with carbon for improvement (51). The advantage of using a Pt electrode is that it permits a fast measurement and very fast preparation, which uses less carbon powder (40) or carbon paste (52) to enhance the sensitivity of the electrode (25).

Recent technology adopted biocompatible nanoparticles such as chitosan to sustain the enzymatic reaction in conditions of oxygen depletion. The method utilises oxygen storage and release capacity of nanoparticles, which enhances the performance of the biosensor. Other researcher used ceria and titania nanoparticles (53) in detecting glutamate at low oxygen condition. A Pt electrode was used in this research, which provides a simple preparation of the nanocomposite that resulted in increased selectivity towards interference species after Pt electrode modification.

Gold

Many researchers have used gold as a working electrode for a specific target species. In this work, using a gold electrode for glutamate detection is not advisable in terms of the sensitivity to H2O2, which is lower compared with a platinum electrode (23); however, gold could represent a better electrode if the target species are AA or DA. Some studies have used a gold electrode modified with MWNT for the detection of dopamine to enhance the sensitivity of the neurotransmitter (32,54). In this case, glucose and AA act as interference species in the detection of dopamine. However, a gold electrode is also an important tool regarding biosensors; many steps of electrode fabrication might produce a different thickness as compared to carbon powder and electropolymerisation of PPD or PANi, which could affect the in vitro and in vivo current signals of the neurotransmitter.

There is an additional new electrochemical method using a glutamate biosensor, which is more simple and rapid and has high sensitivity and selectivity. Friendly nanomaterials, referred to as carbon nanotubes (CNTs), have widened this field towards the development of a third generation biosensor based on electron transfer between an enzyme and the electrode surface. CNTs exhibit a better potential for sensing electrode conductivity and assist the electron transfer that could improve analytical sensitivity and selectivity. Chitosan (CHIT) has been used in electrode modification because of its attractive properties, which include improved film forming, high permeability, non-toxic, and vulnerable to chemical modification. This design has improved glutamate detection via the immobilisation of glutamate oxidase (GluOx) on carboxylated multiwalled carbon nanotubes (cMWCNT), as well as the electrodeposition of CHIT and gold nanoparticles on an Au electrode (50).

Type of Membrane Layer

Recent biosensor research has designed an immobilisation and modification of electrodes. It occurs where the entrapment of enzymes within non-conducting polymer membranes has been shown to present a degree of permselectivity at the sensor surface (55). A suitable polymer must be selected as the immobiliser that may eliminate a variety of electroactive species, such as ascorbic acid and uric acid, which have the potential to act as interferents. The feature of electropolymerisation as a method of enzyme immobilisation is of importance if the sensor is used in vivo, and it provides a high sensitivity to the signal response.

There are several types of membrane layers or polymers that act as a barrier on an electrode surface to reject interference species, such as ascorbic acid (AA), dopamine (DA), serotonin (HT-5), acetylcholine (Ach), epinephrine and norepinephrine (NE), which are present in the extracellular fluid (ECF). Thus, a low signal response is produced for interference species.

Biosensor Architecture

For the development of a glutamate sensor based on enzymes, glutamate oxidase was immobilised on a thin layer at the transducer or electrode surface using various procedures. The following procedures are most generally employed.

(a) Entrapment behind a membrane: a solution of enzyme, a suspension of cells or a slice of tissue is confined by an analyte permeable membrane as a thin film that covers the electrochemical detector

(b) Entrapment of biological receptors within a polymeric matrix, such as polyacrylonitrile, agar gel, polyurethane (PU) or poly (vinyl) alcohol (PVAL) membranes, sol gels or redox hydrogels with redox centres

(c) Entrapment of biological receptors within self-assembled monolayers (SAMs) or bilayer lipid membranes (BLMs)

(d) Covalent bonding of receptors on membranes or surfaces activated by bifunctional groups or spacers, such as glutaraldehyde

(e) Bulk modification of the entire electrode material, e.g., enzyme-modified carbon paste or graphite epoxy resin

Receptors are immobilised alone or mixed with other proteins, such as bovine serum albumin (BSA), either directly on the transducer surface or on a polymer membrane that covers it. In the latter case, preactivated membranes can be directly used for the enzyme or antibody immobilisation without further chemical modification of the membrane or macromolecule.

Apart from the previous example, reticulation and covalent attachment procedures are more complicated than entrapment; however, they are especially useful in cases where the sensor is so small that the appropriate membrane must be directly fabricated on the transducer. In these conditions, more stable and reproducible activities can be obtained with covalent attachment.

Inner and outer membranes

In addition to the reacting layer or membrane, many biosensors, especially biosensors designed for biological or clinical applications, incorporate one or several inner or outer layers. These membranes serve three important functions.

(a) Protective barrier. The outer membrane prevents large molecules, such as proteins or cells of biological samples, from entering and interfering with the reaction layer. It also reduces the leakage of the reacting layer components into the sample solution. This outer membrane function is important. Furthermore, a properly chosen membrane exhibits permselective properties, which may also be beneficial to the biosensor function. It may decrease the influence of potential interfering species detected by the transducer

(b) Diffusional outer barrier for the substrate. Because most enzymes follow some form of Michaelis-Menten kinetics, enzymatic reaction rates are largely non-linear with concentration. Nevertheless, linear dynamic ranges may be large if the sensor response is controlled by the substrate diffusion through the membrane and not by the enzyme kinetics. This control is achieved by placing a thin outer membrane over a highly active enzyme layer; the thinner the membrane, the shorter the biosensor response time. Furthermore, this diffusion barrier also makes the sensor response independent of the amount of active enzyme present and improves the sensor response stability

(c) Biocompatible and biostable surfaces. Biosensors are subject to two sets of modifications when they are in direct contact with biological tissues or liquids, i.e., implanted in vivo or, more generally, in biologically active matrices, such as cell cultures:

- Modification of the host biological sample by various reactions caused by biosensor introduction and toxicity
- Modification of the biosensor operating properties by sample components or structure, which includes external layer or inner detector fouling, inhibition of the biorecognition reaction, and substrate and/or co-substrate transport rate towards the biorecognition area

Apart from molecular recognition systems or transducers, which require direct contact between a sample and biological receptor, the choice of an outer layer is generally essential for the stability of the response after implantation. Depending on the sensor diameter, i.e., centimetre or sub-millimetre range, pre-cast membranes, such as those made of collagen, polycarbonate or cellulose acetate, or, alternatively, polymeric materials deposited by dip- or spin-coating (cellulose acetate, Nafion or polyurethane) may be used. Micro size biosensors are often prepared by entrapping the enzyme via an electropolymerisation step.

The electropolymerisation can be performed in amperometry or cyclic voltammetry. This technique involves the anodic oxidation of a suitable monomer from a buffered solution that contains enzyme to form a polymer on the electrode surface. The changes in the applied potential and size of the electrode during electropolymerisation could affect the rate of polymer deposition by the thickness of the film. The polymer will act as a polymer membrane that covers the electrode. A membrane that protects the film, which is an electropolymerised electrode, contains charges that will reject species that contain the same charge on the membranes

deposited on the electrode. Thus, the species molecule or electroactive species cannot penetrate the layer, and no response arises if the compound does not diffuse onto the electrode.

Poly-o-phenylenediamine (PPD)

Electropolymerisation of o-phenylenediamine (o-PD) at platinum electrodes has been shown to provide a high level of selectivity at platinum cylinders while maintaining a high sensitivity to the target analyte (39,56). Poly-o-phenylenediamine (PPD) is a useful polymer for fabrication electrodes and also the most popular permselectivity membrane that has a property of blocking interference species in vivo. There are several methods to produce a monomer *o*-PD to form PPD using cyclic voltammetry (CV) and constant potential amperometry (CPA). CV is the most common method for analytical study because voltammetry involves applying potential on working electrode to evoke redox reaction of species in electrolytes. CPA involves holding the working electrode at a fixed potential while monitoring the current flow over time. Following electropolymerisation, this will produce an electrode design of Pt/PPD.

There are advantages of using PPD as a polymer in this technique because it has a high permeability to the oxidase transduction molecule hydrogen peroxide (H_2O_2). It is also formed in an ultra-thin layer (57) in neutral media deposited electrochemically from *o*-PD (58), which allows the immobilisation of enzyme to work efficiently (59). On top of that, PPD not only provides high H_2O_2 permeability but also low AA permeability following PPD electropolymerisation by CV (39). Hence, it is an ideal for in vivo biosensor applications which was reported to be stable over a week without any change in sensitivity (60).

Polyethylenimine (PEI)

The polycation PEI has been used in many studies; it has typically been used on carbon electrodes to neutralise negative charges. PEI is a positive charge film; therefore, it is an ideal candidate for the detection of glutamate because the attraction of different charges is stronger. Thus, it helps to enhance the sensitivity of the electrode.

Nafion

An author from Watanabe et al., (61) performed an experiment that detected H2 oxidation and O2 reduction using a platinum electrode coated with a nafion layer. The findings indicate that the membrane thickness, which is less than 0.2 μm, does not affect the sensitivity. This could be an initial step for the introduction of using nafion. One strategy to reduce the signal of interference species is to use nafion. Nafion consists of negative charges and rejects AA and uric acid (UA), which will be oxidised and form a negative charge. Thus, nafion is also a good candidate; however, there are some studies that demonstrated a decreased sensitivity when coated with nafion after electropolymerisation of PPD (28). However, nafion film is unable to reject DA (25) that consists of a positive charge and produces a high signal compared with AA and UA. Nevertheless, nafion is a good permselectivity membrane, but requires improvement for electrode fabrication because of the ability of nafion to reject many interferent species, with the exception of DA.

Overoxidised polypyrrole (OPPy)

The use of electrochemically deposited polymers, such as polypyrrole (PP) and overoxidised polypyrrole (OPPy), as permselective layers in biosensor construction has been discussed in several studies. Many studies have frequently used PPy and its derivatives to conduct polymers because of their versatility and ability to covalently bind a variety of redox species. OPPy is produced from the polymerisation of PPy, which applies more potential with a longer duration (25) and has been identified as the best permselectivity membrane (28) in which it was able to reject interferent species as a good conductive.

Chitosan (CHIT)

Chitosan, which is also known as poly-[1-4]-β-D-glucosamine, is a derivative of chitin and a naturally occurring polysaccharide found in insects, arthropods and crustaceans. A quantitative amount of chitosan is obtained by deacetylation of chitin's acetamide groups in a strong alkaline solution. It is also well known regarding complex transition metal ions through chelating at its amino group (62). One technique of a permselective membrane to reject the most abundance interference species, such as AA, is to use chitosan. Chitosan is a chain polycation on the Pt surface electrode (63), which exerted a strong modification of the electrode in the detection of glutamate. The advantage of using chitosan in biosensor techniques is because of its non-toxic component, which has been used for healing medications in living tissues (64). This might be a good property for use in in vivo techniques. However, a report using chitosan by Liu et al. (54) reported an increased detection of DA and UA and

a decreased AA signal using AA as a coexistence for the detection of DA and UA. Therefore, the enhancement of DA and AA increases, and the value of the limit of detection (LOD) is very low for 0.03 μM DA and 0.07 μM UA.

Enzyme immobilisation

Glutamate is a key target analyte in this work. In the presence of glutamate oxidase (GluOx), glutamate reacts to form α-ketoglutarate. This oxidative deamination results in the removal of the amino group from the alpha carbon and the production of ammonia. There are many different mechanisms involved in the immobilisation of an enzyme; however, the most effective way is via cross-linkage. The enzyme is covalently bonded to a matrix through a chemical reaction, and it is the most popular method because the binding site does not cover the enzyme's active site. It also allows enzymes to be maintained in place throughout the reaction.

The sensitivity of an electrode is different when the arrangement of PPD and glucose oxidase (GO), as example of enzyme, are in different positions. When the design of the electrode Pt/PPD/GO is formed after a Pt bare electrode has been through electropolymerisation and subsequent enzyme immobilisation, the result exhibits more sensitivity compared with the design Pt/GO/PPD. This Pt/GO/PPD is designed because of the inactivation of the enzyme, which thus produces a low signal response when the polymer is formed after enzyme immobilisation (65).

Glutaraldehyde Cross-linking

Cross linking is a widely used method of enzyme immobilisation. It is used to both stabilise the enzyme and provide a higher loading of active enzyme (66). At a neutral or basic pH, the dialdehyde undergoes an aldol condensation with itself, followed by dehydration to generate α, β unsaturated polymers.

Effect of pH

pH is important for the electropolymerisation and activation of the enzyme. Most of the enzyme is active at a neutral medium during experiments using a phosphate buffer as a medium; the pH of the ECF was also obtained for the in vivo determination of the glutamate level in the hippocampus in the brain. A study conducted by Hall et al. (67) demonstrated the importance of pH for the reaction of phosphate buffer in terms of the development of a surface binding site for H_2O_2 from a precursor site through an interaction with $H_2PO_4^-$ from the electrolyte. A steady state response of the Pt electrode for H_2O_2 detection at a fixed potential (E = + 584 mV vs Ag/AgCl) with the concentration of 14 mM, pH range from 6 to 8, has the optimised current densities.

Effect of Current Potential in Electropolymerisation

Many researchers have studied the optimum current potential for electropolymerisation. A constant 300 mM concentration of PPD in PBS and Pt electrode serve as a working electrode for biosensor development, which suggests the best potential current for CPA is + 700 mV vs. SCE (39) for H_2O_2 sensitivity. This causes the selectivity against AA to decrease as the current potential increases up to +700 mV. According Govindarajan et al. (68), decreasing the potential current improves the selectivity of Pt/PPD against AA (interference species) improves because a slow diffusion of monomer to the electrode surface has formed a compact PPD layer. Although each study proposed a different optimum current potential, an investigation has determined the best current density range is from + 650 mV to +800 mV for H_2O_2 to oxidise.

Effects of Geometry and Size of Electrode

Geometry

Diffusion can be defined as the movement of a species via a different gradient concentration in a solution as a result of a chemical potential gradient. If a linear diffusion at a planar electrode over a concentration gradient is assumed, diffusion can be described by Fick's first law, which relates the diffusive flux to the concentration under the assumption of a steady state. It postulates that the flux goes from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient.

$$J_i = -D\frac{\delta C_i}{\delta x} \qquad (5)$$

Where J represents the flux of the species that measures the amount of substance that will flow through a small area during a small time interval. The constant D is referred to as the diffusion coefficient; n is the concentration in dimensions,

and χi is the direction. Unfortunately, Fick's First Law (Eqn. 5) is only applied for one dimension and is not suitable for a complicated system, such as two or three-dimensional systems. For example, diffusion to a disk electrode is different than that involved to a cylindrical electrode. The Fick's Law equation has been reformulated for multidimensional diffusion. The corresponding diffusion (Eqn 6) predicts how diffusion causes the concentration to change with time for any geometry where ∇^2 is the Laplace operator.

$$\frac{\delta C_i}{\delta x} = D_i \overline{V}_i^2 = D\left(\frac{\delta C}{\delta x}\right)^2 \qquad (6)$$

Diffusion to a disc surface occurs in three dimensions and is referred to as a hemispherical diffusion. Diffusion to a cylindrical surface is less efficient and occurs in only two dimensions. As a molecule diffuses towards a micro-disk, it can arrive at the disk surface from three dimensions. Depending on the proximity of the molecule's starting point from the electrode, the cylinder can appear as a planar electrode. This difference in diffusion accounts for some differences in the characteristics of the cylinder and disk biosensors. McMahon and co-workers demonstrated that platinum micro-discs had more oxygen tolerance than platinum cylinders (65). They have determined that the sequential stability of the glutamate response was higher for micro-discs than cylinder electrodes. This study demonstrated that the selectivity of poly (*o*-phenylenediamine) (PPD) covered micro-discs is quite low compared with 1 mm cylinders and the efficiency of the polymer formation decreases with a decrease in the surface area of the electrode (69).

Size

Their small size allows an unhampered approach to the analyte and provides lateral resolution. Theoretically, the smaller the diameter of the electrode size, the higher the sensitivity level of the response. This relationship occurs because a smaller electrode is required for small regions and to study layers of cells within regions, such as the cerebral cortex. Thus, some research has been conducted using various electrode sizes. Table 1 shows the highest sensitivity of different electrode sizes with a modification of the electrode to enhance the sensitivity in the detection of glutamate. Overall, in terms of the size of the electrode, the smaller the size, the higher the level of sensitivity; however, it also depends on the modification of electrodes that affect the response signal. A good modification of an electrode also enables each electrode to detect glutamate and simultaneously reduce the selection of interference. Therefore, determining the modified electrode selections are important and not necessarily based on the theory if several modifications can be changed and improve the sensitivity of the electrode.

Biosensor Application

Detection of in vivo glutamate

Many studies have been conducted to detect the level of glutamate in some parts of the brain that are only focused on the forebrain, which functions in memory. Many other studies have focused on detecting the level of glutamate in some parts of the brain that are only focused on the function of the frontal and temporal lobes for cognitive and memory processes, respectively. To perform reliable measurements with an in vivo microbiosensor, a number of steps are necessary. Great care should be placed in the selection of a microbiosensor and a suitable modification of the electrode that can not only perform on the desired timescale but also has sufficient selectivity for the analyte of interest for the species' experimental conditions.

This selectivity should be confirmed by the in vitro characterisation of the microbiosensor with the analyte of interest and other species that could interfere in vivo. When a microbiosensor is used experimentally, it should be used in a region (Table 2) where there is conditional evidence for the presence of the analyte of interest, and the electrochemical signal should be consistent with the species. There should also be independent corroborative evidence for the signals identified, which is determined using alternative technology.

Finally, the signal should be characterised by appropriate pharmacological intervention. Once all steps are taken to avoid the mistaken identification of analytes, the benefits of an in vivo chemical microbiosensor can be interesting.

Calibration Characteristics: Sensitivity, Working and Linear Concentration Ranges, Detection and Quantitative Determination Limits

In general, sensor calibration is performed by adding standard solutions of the analyte and plotting the steady-state responses, R_{ss}, possibly corrected for a blank (often referred to as the background) signal R_{bl} versus the analyte concentration, c, or its logarithm, log c/c^o; c^o

refers to a reference concentration, typically 1 mol/l, although such a high concentration value is never used, and the highest value typically reaches 1-10 mmol/l. Transient responses are important for sequential samples, but are less significant for continuous monitoring: within several possibilities, they are generally defined as the maximum rates of variation of the sensor response (dR/dt)max after the addition of the analyte into the measurement cell.

The sensitivity and linear concentration range of the steady-state calibration curves are determined by plotting the ratio $(R_{ss}-R_{bl})/c$ or $(R_{ss}-R_{bl})/\log c/c^o$ versus $\log c/c^o$. This method is substantially more concise than plotting the typical calibration curves $(R_{ss}-R_{bl})$ versus c or $\log c/c^o$ because it assigns the same weight to low and high analyte concentration results. Similarly, the sensitivity and linear range of the transient calibration curves are determined by plotting the ratio (dR/dt)max/c or (dR/dt)max/$\log c/c^o$ versus $\log c/c^o$. In both cases, the sensitivity is determined within the linear concentration range of the biosensor calibration curve.

Table 1: Detection of glutamate of different electrode sizes

Type of electrode	Size of electrode (diameter)	Sensitivity	Reference
Pt_D/PPD-BSA/PEA/PEI/GluOx/ PPD-BSA (Platinum disk)	125 µm	71 ± 1 mA $M^{-1}cm^{-2}$	Govindarajan et al., 2013
Pt Blk/OPPy (Platinum disk)	125 µm	80 ± 10 nA $\mu M^{-1}cm^{-2}$	Hamdi et al., 2006
Pt/PPD/GluOx (Platinum disk)	125 µm	100 ± 13 nA $\mu M^{-1}cm^{-2}$	McMahon et al., 2006b
Pt/CHIT/GluOx (Platinum disk)	1.6 mm	85 mA $M^{-1}cm^{-2}$	Zhang et al., 2006
Pt electrode with bienzyme salicylate hydroxylase/l-glutamate dehydrogenase	0.50 mm	86.6 nA mM^{-1}	Cui et al., 2007
CNT composite electrode	0.07 cm^2	0.71 ± 0.08 nA μM^{-1}	Chakraborty & Raj, 2007

Table 2: Detection of brain glutamate in-vivo. Note; anterior–posterior (AP), medial–lateral (ML) and dorso-ventral (V)

Type of electrode	Site	Coordinate	Reference
MWCNTs/PAMAM/Pt/ Nafion	Striatum	2.5 mm anterior to bregma 2.5 mm lateral from midline 7.0 mm below dura	Yu et al., 2011b
$[C_3(OH)_2mim][BF_4]$-Au/ Pt-Nafion	Subthalamic nucleus area (STN)	3.5 mm posterior to bregma 2.4 mm lateral from midline 7.0 mm below dura	Yu et al., 2011a
Pt/PPD/GluOx-silica gel layer Sol-gel film	Nucleus tractus solitarus (NTS) regions	0.0 mm rostral 0.5–0.7 mm lateral 0.6–1.0 mm ventral of obex	Tian et al., 2009
Pt Blk/OPPy/GluOx/GA	Striatum	AP +0.8 mm ML +2.0 mm VD 4.5 mm	Hamdi et al., 2006
Silica-based platinum	Prefrontal cortex (PFC)	AP +3.4 mm ML +0.8 mm VD –5.0 mm relative to bregma	Wahono et al., 2012
Pt/IrOx	Striatum	anterior–posterior +0.7 mm medial–lateral +2.4 mm dorsal–ventral 7.0 mm	Tolosa et al., 2013

Electrochemical biosensors always have an upper limit of the linear concentration range. This limit is directly related to the biocatalytic or biocomplexing properties of the biochemical or biological receptor; however, in the case of enzyme-based biosensors, it may be significantly extended via an outer layer diffusion barrier to substrate S. The compromise for this extension in the linear concentration range is a decrease in sensor sensitivity. The local substrate concentration, within the reaction layer, can be at least two orders of magnitude lower than the bulk solution. In relation to the typical parameters for Michaelis-Menten kinetics, i.e., K_M and V_{max}, enzyme based biosensors are often characterised by their apparent K_M and $(R_{ss}-R_{bl})_{max}$: the first parameter represents the analyte concentration, which yields a response equal to half of its maximum value, $(R_{ss}-R_{bl})_{max}$, for an infinite analyte concentration. When the apparent KM is substantially larger than its value for soluble enzyme, it indicates that a significant substrate diffusion barrier is present between the sample and the reaction layer or the rate of the reaction to the co-substrate S' with the enzyme is increased. As for the enzyme solution kinetics, the apparent K_M is typically determined by Lineweaver-Burk reciprocal plots, i.e., $1/(R_{ss}-R_{bl})$ versus 1/c. As for any electrochemical sensor, one should state the composition and the number of standards used and how the sample matrix is simulated or duplicated. It may be necessary to specify procedures for each biosensor type and application. The sensitivity is the slope of the calibration curve, i.e., $(R_{ss}-R_{bl})$ versus c or log c/co. One should always avoid confusion between sensitivity and detection limits. The limits of detection (LOD) and quantification (LOQ) take into account the blank and signal fluctuation (noise). Their definition is not specific to biosensors, and the IUPAC recommendations should be used. The working concentration range, which may considerably extend the linear concentration range, is determined by the lower and upper limits of quantification.

Selectivity and Reliability

Biosensor selectivity is determined and expressed as for other amperometric or potentiometric sensors. It depends on the choice of the biological receptor and transducer. Many enzymes are specific.

Nevertheless, class (non-selective) enzymes, such as alcohol, group sugar or amino-acid oxidases, peroxidases, laccase, tyrosinase, ceruloplasmin, alcohol or glucose NAD-dehydrogenase, have been used for the development of class biosensors, such as those for the determination of phenols, which are used in environmental monitoring or food analysis. Bacteria, yeast or tissue cultures are naturally non-specific.

When transducer interfering substances are well identified, their influence may be restricted by the application of appropriate inner or outer membranes. Alternatively, a compensating sensor may be introduced in the set-up without a biological receptor on its surface. Of the various methods for biosensor selectivity determination, two methods are recommended depending on the aim of the measurement. The first method consists of measuring the biosensor response to interfering substance addition: a calibration curve for each interfering substance is plotted and compared with the analyte calibration curve under identical operating conditions. The selectivity is expressed as the ratio of the signal output with the analyte alone to that with the interfering substance alone at the same concentration as the analyte. In the second procedure, interfering substances are added, at their expected concentration, into the measuring cell, which contains the typical analyte concentration at the mid-range of its expected value. The selectivity is subsequently expressed as the percentage of variation of the biosensor response.

Although more easily quantified than the calibration curve comparison performed in the first procedure, the second method is characteristic of each application and presents a more restricted significance. This selectivity may depend on the analyte concentration range, which is determined. The reliability of biosensors for given samples depends on both their selectivity and reproducibility. It must be determined under actual operating conditions, i.e., in the presence of potential interfering substances. A reliable biosensor response means that analyte concentration should not fluctuates with any interfering species within the sample matrix. Thus, for each type of biosensor and sample matrix, one should clearly quantify specific interference species that should be eliminated. This reliability determination is necessary for the accuracy assessment of biosensor application.

Reproducibility, Stability, and Lifetime

The definition of reproducibility is the same for electrochemical biosensors as any other analytical device: reproducibility is a measure of the scatter or drift in a series of observations or

results performed over a period of time. In general, it is determined for the analyte concentrations within the usable range.

The operational stability of a biosensor response may vary considerably depending on the sensor geometry and method of preparation, as well as the applied receptor and transducer. Furthermore, it is strongly dependent on the response rate-limiting factor, i.e., a substrate external or inner diffusion or biological recognition reaction. Finally, it may vary considerably depending on the operational conditions. For operational stability determination, we recommend consideration of the analyte concentration, the continuous or sequential contact of the biosensor with the analyte solution, temperature, pH, buffer composition, presence of organic solvents, and sample matrix composition.

Although some biosensors have been reported to be usable under laboratory conditions for more than one year, their practical lifetime is either unknown or limited to days or weeks when they are incorporated in industrial processes or biological tissue, such as glucose biosensors implanted in vivo. For storage stability assessment, the significant parameters include the state of storage, i.e., dry or wet, atmosphere composition, i.e., air or nitrogen, pH, buffer composition and presence of additives. Knowledge of the biosensor rate-limiting step or factor is especially important for understanding the stability properties.

Finally, the mode of assessment of the lifetime should be specified, i.e., by reference to the initial sensitivity, upper limit of the linear concentration range for the calibration curve, accuracy or reproducibility. It is recommended that the definition of the lifetime, denoted as t_L, represents the storage or operational time necessary for the sensitivity, within the linear concentration range, to decrease by a factor of 10% (t_L10) or 50% (t_L50). For the determination of the storage lifetime, a comparison of the sensitivities of different biosensors, derived from the same production batch, after different storage times under identical conditions is suggested. Biosensor stability may also be quantified as the flow, when the sensitivity evolution is monitored during either storage or operational conditions. It is useful for biosensors for which evolution is either very slow or studied during a rather short period of time.

Conclusion

The technical fundamentals of electrode design, properties and performance must be understood. It will be a challenge to extend the stability of the electrode for more than a week following brain implantation. Some characteristics of biosensors are common to different types of electrochemical sensors. Many modern nano-designs have been used to enhance the stability of the electrode. However, inherent factors that limit the biosensor performance must be addressed. Others factors are more specific to biosensor principles, but may be common to different types of transducers. Biosensor responses will be controlled by the kinetics of recognition and transduction reactions or by mass transfer rates. Determination of the rate-limiting step is clearly essential for the understanding, optimisation and control of the biosensor performance criteria.

Nevertheless, this is a living document and, as such, will be revised periodically as needed to address ambiguities and new technological developments as they arise in the evolution of electrochemical biosensors. Comments on this document are actively solicited from scientists working in this, and related, fields of research.

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Conflict of Interest

None.

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