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Chapter 2

Enzyme-Based Electrochemical Glutamate Biosensors

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Additional information is available at the end of the chapter

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Abstract

Glutamate, a major excitatory neurotransmitter in the mammalian central nervous system, plays a vital role in many physiological processes and is one of the key neurotransmitters of interest in psychopharmacology. It is involved in many normal and abnormal behaviors related to neurological and psychiatric disorders. The glutamate system has been proposed to play a significant role in various neurological and psychiatric disorders such as Alzheimer's disease, autism, schizophrenia, depression, drug addiction, and more. The design, construction, and optimization of enzyme-based electrochemical biosensors for in vivo and in vitro detection of glutamate are active areas of interdisciplinary research. For example, various glutamate biosensors have been developed for monitoring dynamic levels of extracellular glutamate in the living brain tissue adding to the current medical knowledge of these complex neurotransmitter systems and ultimately impacting treatment plans. In addition to biological sciences and clinical medicine, glutamate biosensors have been used in environmental monitoring, in the fermentation industry, and in the food industry for determination of monosodium glutamate (MSG), a common flavor-enhancing food additive.

Keywords: biocatalytic sensors, L-glutamate, mediator, monosodium glutamate, carbon nanotubes, nanomaterials, neurological and psychiatric disorders, glutamate oxidase, glutamate dehydrogenase

1. Introduction

Glutamate is a nonessential amino acid, a precursor for gamma aminobutyric acid (GABA (the primary inhibitory neurotransmitter)), and an abundant excitatory neurotransmitter in the mammalian central nervous system that is produced by pyramidal cells located in the cerebral cortex and hippocampus. It has an important role in the formation and stabilization of synapses, long-term potentiation (i.e., the long-lasting enhancement in signal transmission

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between two neurons which results from synchronously stimulating them), neurodegenerative diseases, learning, and the formation of memories. In addition, maintaining a balance in glutamate levels in the central nervous system is important because increased levels lead to neurotoxicity and cell death, while decreased levels result in impaired long-term potentiation, impaired synaptic plasticity (i.e., the ability of synapses to change their structures in response to inputs and changes in their environment), and impaired cognitive performance [1]. Glutamate also plays a pivotal role in cellular metabolism as it is associated with transamination reaction, a key step in amino acid degradation, and is the product of deamination. Hence, quantifying glutamate levels in biological fluids and tissues reliably and reproducibly is of interest in many disciplines.

Glutamate dysregulation may induce excitotoxicity, which is closely associated with multiple psychiatric and cognitive disorders. Glutamate is hypothesized to have a key role in the pathophysiology of psychiatric disorders such as schizophrenia [2] and depression [3]. It is also currently a target of novel drugs for potential treatments of schizophrenia and depression. Briefly, the glutamate hypothesis of schizophrenia suggests that there is a hypofunction in *N*-methyl-D-Aspartate (NMDA)-type glutamate receptors within the glutamate system wherein the normal balance between excitatory glutamate and inhibitory GABA is destabilized resulting in a combination of excitotoxicity and impaired neuroplasticity, which results in the psychotic symptoms of schizophrenia. As stated earlier, glutamate or glutamic acid is an excitatory amino acid neurotransmitter that is synthesized from the precursor amino acid glutamine, in cells called glia which protect and support neurons in the nervous system. In addition, glia recycle and regenerate glutamate after its release during neurotransmission. After glutamate is released from glutamate neurons, it binds to synaptic receptors and is then pumped into glia by an excitatory amino acid transporter (EAAT). Once in the glia, glutamate is converted into glutamine by the enzyme glutamate synthetase. Glutamine is released from glia via reverse transport either by the specific neutral amino acid transporter (SNAT) or by the alanine-serine-cysteine transporter (ASC-T) and transported into the neuron. Inside the neuron, glutamine is converted to glutamate by a mitochondrial enzyme called glutaminase for use as a neurotransmitter. After use, glutamate is stored in synaptic vesicles for subsequent release during neurotransmission, and its actions are terminated via removal by EAATs [4]. The next section will outline the proposed role of glutamate in schizophrenia.

2. Glutamate and schizophrenia

In the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), schizophrenia is defined by a group of characteristic symptoms which consist of (1) psychotic or positive symptoms (i.e., symptoms characterized by the presence of something that should be absent, such as hallucinations or delusions), (2) disorganized symptoms (such as disorganized speech–e.g., incoherence or frequent derailment, disorganized or catatonic behavior, and inappropriate affect), (3) negative symptoms (i.e., symptoms characterized by the absence of something that should be present, such as avolition [lack of motivation], affective flattening [diminished emotional expression], or alogia [poverty of speech]), (4) deterioration

in social, occupational, or interpersonal relationships, and (5) continuous signs of the disturbance for at least 6 months [1].

Glutamate-based theories of schizophrenia resulted from the observation that two related compounds—phencyclidine (PCP) and ketamine—produced symptoms and cognitive deficits in healthy subjects which resembled those found in schizophrenia. These symptoms, including negative as well as positive symptoms, resolved following elimination of the compounds. Years later, further research revealed that these compounds produced their effects by blocking neurotransmission at NMDA-type glutamate receptors which in turn gave rise to current glutamate-based models of schizophrenia. In addition, it has been found that many suspected genes for schizophrenia affect glutamate neurotransmission. Also, NMDA receptors are regulated by brain levels of glycine, D-serine, and glutathione such that disturbances in concentrations of these compounds and of the genes related to their synthesis may all contribute to schizophrenia. Since available data to date suggests that there is more than one single cause of NMDA receptor dysfunction among different individuals, perhaps threshold models are more useful in that for each individual, the sum of genetic and environmental factors determines whether NMDA receptor functions will fall below a critical level. In schizophrenia, it is postulated that psychosis may emerge once the level of NMDA receptor function decreases by approximately 20%, with worsening symptom severity thereafter. Furthermore, since glutamate and NMDA receptors are widely distributed throughout the brain, this has led to a concept of "whole brain" dysfunction, which involves the prefrontal, limbic, auditory, and visual cortices among others. Some specific examples of the consequences of glutamate deficits in certain brain regions include (1) working memory impairments and inability to unlearn dysfunctional behavior patterns due to glutamate deficits in the dorsolateral prefrontal cortex, (2) impaired response inhibition and hence an increased tendency for impulsivity and impulsive aggression due to glutamate deficits in the inferior prefrontal cortex, (3) impairments in learning and memory formation which contribute to psychosis and delusion formation due to glutamate deficits in the medial temporal cortex, (4) perceptual changes including impaired reading ability due to glutamate deficits in the visual cortex, and (5) impaired ability to detect vocal intonation which leads to impairments in emotional recognition and social cognition due to glutamate deficits in the primary auditory cortex. Thus, widespread cognitive dysfunction in these brain regions due to dysfunctional glutamate and NMDA-type glutamate receptors represents a formidable obstacle that prevents individuals with schizophrenia from returning to premorbid levels of functioning [5].

3. Other applications of glutamate biosensors

In addition to its key roles in fundamental neurological processes and neurological disorders, glutamate is important in protein synthesis, protein degradation, and nitrogen metabolism. The concentrations of glutamate in intracellular environment vary from 2 to 20 mM [6]. Usual glutamate concentrations in plasma are approximately 150 and 10 μM in cerebrospinal fluid [7, 8]. Normal glutamate concentration in the extracellular space ranges between 1 and 80 μM [9]. The quantification of L-glutamate is also important in food analysis due to

questions about its safety as a food additive. Monosodium glutamate (MSG, $C_5H_8NO_4Na$), invented by Dr. Kikunae Ikeda in 1908, is a commonly used flavor-enhancing additive found in Chinese restaurant food [10, 11], canned soups, canned vegetables, and processed meats. Interestingly, there has been controversy surrounding the use and safety of MSG as a food additive. According to some studies, excessive intake of MSG may cause headaches and stomach pain in certain individuals as well as neuronal excitotoxicity. However, use of MSG as a food additive is generally regarded as harmless. Still, many food manufacturers choose to advertise their products as being MSG-free. Development of MSG biosensors for food applications is also an active area of research. For example, Monošík et al. recently prepared and characterized a bienzymatic nanocomposite electrode for quantification of MSG in food samples, utilizing L-glutamate dehydrogenase and diaphorase enzymes immobilized between chitosan layers on nanocomposite electrodes consisting of multiwalled carbon nanotubes (MWCNTs) [12]. The structure of monosodium glutamate is shown in **Figure 1**.

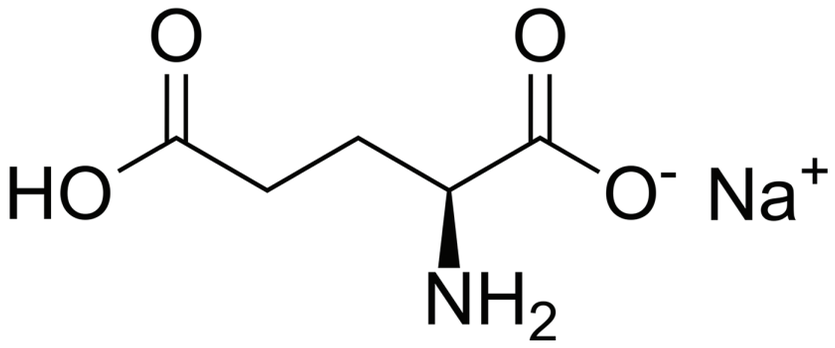

Figure 1. The chemical structure of monosodium glutamate.

4. Glutamate biosensors

This chapter will primarily focus on providing an overview about various enzyme-based glutamate biosensors that utilize sensitive electrochemical detection methods. In addition, the characterization and optimization of newly developed enzyme-based electrochemical biosensors will be discussed briefly. A brief overview of the most common electrochemical detection methods utilized in biocatalytic glutamate sensor characterization, testing, and in quantitative analysis will also be provided. We will begin with a brief introduction to electrochemical biocatalytic sensors.

In general terms, biosensors are devices that register a biochemical reaction which is then converted into a signal that can be detected and quantified [13]. A typical biosensor contains biological recognition molecules, such as enzymes, that are highly selective and specific for a given analyte. In electrochemical biocatalytic sensors, the biological recognition molecules bind reversibly to a particular analyte on or near an electrochemically active interface which may incorporate nanomaterials, giving rise to a measurable signal [13]. An electric transducer, usually a modified electrode, which is in contact with the electrochemically active

interface, converts the biochemical reaction into an electrical signal that is further amplified by a signal processor into a useful form. The development of electrochemical biosensors for determination of glutamate is an active area of research as these sensors are typically easy to use, fast, reliable, convenient, portable, and affordable. Various instrumental analysis techniques such as spectrophotometry [14, 15], chemiluminescence [16], capillary electrophoresis [17–19], gas chromatography [20], and high-performance liquid chromatography [21–23] have also been utilized in detecting glutamate. However, these methods can be expensive, time-consuming, labor intensive, often require sample preparation, and utilize sophisticated instruments which require trained personnel to operate. Specifically, chromatography methods often require analyte derivatization, while spectrophotometry requires tedious sample pretreatment procedures.

Glutamate biosensors have been developed for both in vitro and in vivo applications. One of the challenges surrounding the utilization of sensitive and selective in vivo monitoring of dynamic levels of extracellular glutamate in living tissues using biosensors is that unlike an electroactive analyte such as dopamine, glutamate is nonelectroactive. Therefore, the direct measurement of glutamate using voltammetric electroanalytical techniques is not possible. However, if a biological recognition component such as an enzyme is added onto a physical transducer, in this case the electrode, the glutamate levels may be measured indirectly by quantifying one of the enzymatically generated products at the biocatalytic sensor [13]. A few commercial biosensors for routine glutamate measurements in the food industry are available from companies such as Yellow Springs Instruments (Yellow Springs, OH, USA).

Microdialysis methods have been widely used in monitoring extracellular glutamate levels in the brains of conscious animals. Yao and Okano described an in vivo flow-injection biosensor system with online microdialysis samples attempting to simultaneously determine the concentrations of L-glutamate, dopamine, and acetylcholine [24]. The triple electrode measured an average of 6 μM of L-glutamate in rat brain, whereas dopamine and acetylcholine levels were below the detection limit of the biosensor.

Furthermore, studies have shown that the glutamate detected using microdialysis is not or is only partly derived from synaptic transmission. As a result, development of appropriate biosensors for the accurate detection and monitoring of glutamate in vivo remains an ongoing endeavor. The ideal in vivo biosensors would be enzyme-based electrochemical biosensors in which a highly specific enzyme is immobilized on the surface of a sensitive electrochemical transducer. Indeed, certain authors have reported the fabrication and utilization of glutamate biosensors capable of second-by-second in vivo monitoring of glutamate levels in freely moving rats and mice [25, 26].

Nanotechnology and advances in microfabrication technology have played a crucial role in the fabrication of many biosensors. Nanoscale materials, with at least one dimension ranging in size from 10^{-7} to 10^{-9} m, have been incorporated into various enzymatic biosensors including many of the more recent L-glutamate sensors. The trend to manufacture smaller and more portable biosensor devices with improved performance has in part led to the incorporation of nanomaterials into biosensors. The unique chemical and physical properties of the nanomaterials

also enhance the analytical performance of these biosensors. The nanomaterials utilized include carbon nanotubes (CNTs), graphene, nanowires, carbon fibers, and metal nanoparticles (NPs). Electrochemical biosensors that incorporate nanomaterials such as CNTs on the transducer surface generally have very good conductivities and much greater surface areas onto which enzymes and other molecules may be immobilized.

Recently, combining the high bioselectivity and specificity of oxidoreductase enzymes with the numerous and advantageous chemical and physical properties of organic and inorganic nanomaterials such as graphene, carbon nanotubes, carbon fibers, and gold has resulted in the development of highly sensitive and stable electrochemical biosensors with significantly improved performance for many analytes of medical interest, including glutamate.

5. Glutamate oxidase–based biosensors

Many glutamate biosensors are based on quantifying the oxidation of hydrogen peroxide (H_2O_2) liberated in a chemical reaction between glutamate oxidase (GmOx, an oxidoreductase enzyme, EC 1.4.3.11) and L-glutamate in the presence of oxygen (O_2), water (H_2O), and a flavin adenine dinucleotide (FAD) cofactor [27–45]. GmOx enzyme catalyzes the oxidative deamination of glutamate resulting in the formation of 2-oxoglutarate (i.e., α-ketoglutarate, α-KG), ammonia (NH_3), and H_2O_2 [46].

$$\text{L-glutamate} + O_2 + H_2O \rightarrow \text{2-oxoglutarate} + NH_3 + H_2O_2 \quad (1)$$

The oxidation of hydrogen peroxide occurs at the electrode surface or at very short distances away within the sample solution. The resulting current (i.e., detectable signal) which is proportional to the concentration of redox active species is then quantified by the transducer.

GmOx is a highly selective enzyme [47] and therefore unlike for many catalytic enzyme-based biosensors, interference from unwanted enzymatic reactions with similar molecular species is not a major concern for these glutamate biosensors. However, there is one study which mentions that a GmOx enzyme had a slight sensitivity (0.6 %) for L-aspartate, an amino acid with a similar side-chain group to L-glutamate [46].

In addition, electrooxidation of the GmOx-generated hydrogen peroxide requires relatively high positive potential at which common electroactive interferents, such as ascorbic acid and dopamine, also undergo oxidation thereby adding to the current (i.e., the signal) [48]. Thus, the elimination of interference in glutamate biosensors that utilize GmOx as the biorecognition molecule is critical. Strategies for the elimination of this interference by other electroactive species (which are commonly found in the sample matrix) include coating the biosensor with permselective nonconductive or conductive polymers such as Nafion, cellulose acetate, o-polyphenylenediamine (PPD), polypolyaniline, polythiophene, or polypyrrole [9, 48, 49]. The idea behind the addition of a permselective membrane is that the small pores within the membrane or film will only allow certain small molecules to pass through therefore minimizing interference by other larger electroactive species (see **Figure 2**). Also, positively

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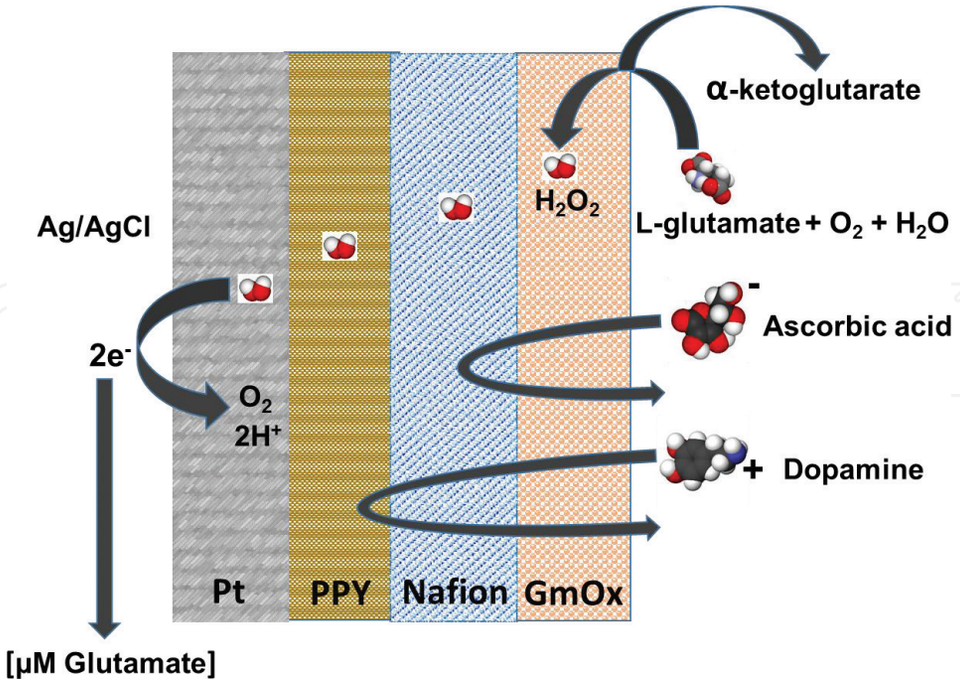

Figure 2. Elimination of interference in glutamate biosensors based on GmOx enzyme.

charged groups such as those found in sulfonated tetrafluoroethylene copolymer (Nafion) will prevent or minimize the diffusion of anionic sample components, such as ascorbic acid, across the membrane and onto the electrode surface where the redox reactions are detected. Furthermore, though membranes and composites incorporating permselective conducting polymers such as polypyrrole (PPY) have other desirable properties (such as high conductivity and being redox active), it should be noted that coating the biosensor transducer with these polymeric films can lead to longer response times and lower signals due to the added diffusion barrier for both the substrate for the enzyme and the redox active species produced in the enzyme-catalyzed reaction. Co-immobilization of peroxidase with a redox polymer [50], immobilization of ascorbate oxidase [9], and self-referencing [51] have also been utilized as strategies to minimize interference from other electroactive species. On the other hand, performing electrochemical peroxidation, a process that utilizes sacrificial electrodes and stoichiometrically balanced applications of hydrogen peroxide to efficiently destroy interfering species in the aqueous phase, poses a risk of also oxidizing the analyte of interest.

Many glutamate biosensors based on the GmOx-catalyzed reaction also include redox mediators or a second enzyme that reacts with H_2O_2 and are often referred to as second-generation biosensors. By incorporating a redox mediator, it is possible to lower the potential required for the H_2O_2 oxidation, thereby further limiting interference in complex biological samples by other species present, such as ascorbic acid or uric acid, which may also be redox active at the higher detection potential. Commonly used redox mediators in enzymatic biosensors include ferrocyanide [12], ferrocene and ferrocene derivatives [52–54], osmium complexes [55], quinine derivatives, and hexacyanoferrates, such as Prussian blue [45, 56, 57] and Ruthenium purple [58].

Prussian blue is the oldest coordination compound and was serendipitously discovered by Diesbach, an artist in 1704. It possesses excellent electrochemical characteristics and exceptional catalytic properties, making it a popular redox mediator. It is often referred to as the "artificial peroxidase" and has been well characterized and incorporated into various high-performing enzyme-based biosensors due to its excellent electrocatalysis toward the reduction of enzyme-generated H_2O_2 [45, 57, 59, 60]. Prussian blue is electrochemically reduced to form Prussian white (PW) which can then catalyze the reduction of H_2O_2 at low potentials of 0 V versus Ag/AgCl reference electrode [59].

6. Glutamate dehydrogenase–based biosensors

Another commonly used enzyme utilized in glutamate biosensors is L-glutamate dehydrogenase (GLDH) [49, 61–67]. GLDH (EC 1.4.1.2) catalyzes the deamination of amino acids, specifically the oxidative deamination of L-glutamate to 2-oxoglutarate (i.e., α-ketoglutarate) in the presence of nicotinamide adenine dinucleotide (NAD^+) which serves as an oxidized cofactor for the enzyme. In mammals, this reversible enzyme-catalyzed reaction strongly favors the formation of ammonium (NH_4^+) and 2-oxoglutarate.

$$\text{L-glutamate} + NAD^+ + H_2O \leftrightarrow \text{2-oxoglutarate} + NADH + NH_4^+ + H^+ \quad (2)$$

Glutamate biosensors have also been fabricated wherein both GLDH and GmOx are co-immobilized on the transducer surface [68]. Basu et al. co-immobilized both enzymes on a polycarbonate membrane by cross-linking procedures, involving glutaraldehyde, in the presence of a bovine serum albumin (BSA) spacer molecule in order to develop a biosensor for quantification of MSG in food. The MSG biosensor utilized substrate recycling which resulted in the amplification of the transducer response, thereby increasing the sensitivity [68].

On the other hand, rather than directly detecting the glutamate levels, Meng et al. quantified glutamate by the detection of the anodic current of enzymatically generated NADH [67]. The electron transfer kinetics of the oxidation of NADH is sluggish and the direct oxidation of NADH at bare electrodes requires a high overpotential (0.7–1.0 V), where many interferences can occur. Also, bare electrodes are more likely to be affected by fouling which is caused by the adsorption of oxidation products onto their surfaces. The authors overcame these challenges by preparing biocompatible biosensors utilizing thionine (Th) and single-walled carbon nanotubes (SWCNT) nanocomposite to catalyze the electrochemical oxidation of NADH at an anodic potential of less than 0.19 V versus a standard hydrogen electrode (SHE) [67].

Azmi et al. also developed a spectrophotometric biosensor based on GLDH that was immobilized in chitosan for the determination of ammonium in water samples [69]. Ammonium, which is known to be toxic even at low concentrations to various organisms, is widely used

in the farming, chemical, and automotive industries. In addition, ammonium is used as a parameter in the assessment of drinking and industrial water quality. The authors immobilized GLDH in a chitosan film (a natural biopolymer which can found in the exoskeleton of crustaceans) and measured ammonium in water based on NADH oxidation in the presence of α-ketoglutaric acid. The biosensor had a detection limit of 0.005 mM and a linear range of 0.005–0.5 mM NH_4^+ [69].

Chitosan GLDH film is a popular enzyme immobilization matrix for biosensors due to certain advantageous properties of chitosan such as being nontoxic, biocompatible, biodegradable, an effective antibacterial, having high mechanical strength, good adhesion, and containing numerous amino and hydroxyl groups. Other glutamate sensing materials include polymer/enzyme composites [70, 71], nanoparticle iridium/carbon film [72], DNA-Cu(II)/polyamine membrane [73], nanoneedles [74], ferrocene functionalized SWCNT interdigitated construction film [52], Prussian blue film [45], and others.

7. Optimization of experimental conditions and measurement platforms

The topography of the prepared electrodes is usually studied using scanning electron microscope (SEM). SEM imaging allows for evaluation of uniformity and dispersity of the materials within the hybrid films. For example, it is possible to see if the composite materials are distributed uniformly over the entire surface of the electrode transducer. Agglomeration (i.e., sticking of particles to one another forming large groups) is sometimes observed in SEM images when the composites do not cover the surface uniformly. Porosity of the composite materials may also be assessed based on SEM images. The three-dimensional nanostructure morphology gives an indication whether the enzymes in the hybrid film will be accessible to the substrates. Modified electrodes are also much less likely to suffer from surface fouling, which results from adsorption of oxidation products to electrode surfaces when compared to bare electrodes.

As stated earlier, the incorporation of conductive nanomaterials, such as CNTs, into the hybrid film on a modified electrode surface is gaining popularity. CNTs often significantly improve electron transfer and kinetics [75]. This is due to the electrical properties of CNTs which vary significantly and depend on the structural differences between CNTs, resulting in some CNTs being highly conductive like metals, while others act more like semiconductors [76]. The current carrying capacity of certain CNTs can be up to 1000 times greater than that of a copper wire [77]. Furthermore, CNTs provide a tremendous increase in the surface area onto which enzymes or other biomolecules may be immobilized, which ultimately improves quantification of the chemical reaction that is being analyzed. CNTs are also ideal for use in biosensors as they are nontoxic, nanometer sized, strong, and chemically stable [76].

The electrochemical performance of the modified electrodes is often evaluated using redox reactions of benchmark species such as $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox pair in cyclic voltammetry

(CV) studies. CV of this redox pair can be obtained using –300 mV initial potential, +800 mV switching potential, and –300 mV final potential with a carbon-based electrode against an Ag/AgCl reference electrode. The effect of changing scan rates (e.g., ranging from 30 to 120 mV/s) on electrochemical behavior is often also studied. Moreover, other neurotransmitters such as dopamine, which can directly undergo oxidation to dopamine-o-quinone at the electrode surface, may be detected in the living brain using voltammetry.

Characterization of modified electrode nanomaterials such as CNTs may be carried out by UV/Vis spectroscopy or Fourier transform infra-red (FTIR) spectroscopy. For example, Meng et al. utilized UV/Vis spectroscopy from 200 –800 nm [67] in their investigations. To confirm that thionine (Th) had adsorbed onto the SWCNT surface, the authors observed the occurrence of a notable absorption peak at about 600 nm as well as a small shoulder peak at circa 560 nm. To determine whether GLDH, the biocatalytic molecule, had been successfully immobilized on the Th/SWCNT nanocomposite, a peak at about 275 nm, which is an absorption peak characteristic for proteins, was monitored using UV spectroscopy.

When preparing a new biocatalytic sensor, parameters such as origin and availability of the enzyme, its operational and storage stability as well as immobilization procedure should also be carefully considered [78]. In addition, the influence of experiment conditions such as pH, temperature, ionic strength, and stirring on the enzyme-catalyzed reaction can be minimized by optimizing and keeping these conditions constant throughout the biosensor use. For example, GLDH-based biocatalytic sensors have utilized pHs ranging from 7.0 to 9.0 during the detection step [12, 67, 79]. Also, GmOx-based biosensors appear to utilize pHs from 7.0 to 7.4 [40, 68]. Of note, the optimal pH of the immobilized enzyme may be slightly different from the free enzyme in buffered aqueous solution. Furthermore, the detection temperatures which the enzymes are exposed to may vary depending on the specific application of the L-glutamate biosensor. The concentration of coenzyme NAD^+ will also need to be optimized for GLDH-based biosensors. Furthermore, the activity of enzymes such as GLDH (a hexameric enzyme from bovine liver) is affected by various cations and anions. Specifically, lanthanide (La^{3+}) and europium (Eu^{3+}) ions can enhance the activity of bovine GLDH, at least in solution [80]. Also, Meng et al. observed a decrease in catalytic currents generated by GLDH when Zn^{2+} was added to the solution with the inhibitory effect increasing with increasing concentrations of the zinc ions [67].

Amperometry is perhaps the most common electrochemical detection method used in quantitative analysis of an analyte once the biosensor has been characterized and optimized. It is a very popular electroanalytical detection method for quantitative analysis due to its simplicity and the low detection limits that can be achieved. In amperometry, the analyte concentration is determined by measurement of the signal—the current produced in a redox reaction as a function of time when a constant potential is applied to the electrodes. Amperometry results in current versus time plots where the current increases stepwise with each successive addition or formation of the redox active species. Amperometric signal response consisting of back-to-back steps in a "staircase" makes it relatively easy to

identify the starting and final current for each analyte addition or the formation of redox active species. The response in amperometry is usually rapid and reaches a dynamic equilibrium which results in a steady-state current signal within seconds. The electron transfer of reactants during electrochemical oxidation is mainly determined by the conductivity of the working electrode material and the active functional groups on its surface. Three electrode systems with working, reference, and auxiliary electrodes are typically used in amperometry. In amperometry, the oxidation or reduction potential used for the detection step is characteristic of the analyte species, thus adding to selectivity of the method by eliminating interferences from other redox active species that may also be present in the sample. Also, the detection potential is stepped directly to the desired, optimum value, and current resulting from the redox reaction is detected by the transducer (the working electrode in the biosensor). Current generated by the reaction (i.e., the current passing through the electrochemical cell over time) is proportional to the concentration of the electroactive species in the sample. Sometimes, the charging current or background current (i.e., the current needed to apply the potential to the system) present at the beginning of each measurement requires some time in order to stabilize before quantitative measurements can be made using amperometry.

In order to be considered as an alternative to any existing and well-established instrumental analysis methods, such as high-performance liquid chromatography (HPLC) or spectrophotometry for quantification of L-glutamate in clinical or food applications, the performance of new biosensor devices has to be tested in serum or food samples, respectively. Ideally, no sample pretreatment other than dilution should be required. Also, the use of low-cost and disposable devices such as screen printed carbon electrodes (SPCE) is advantageous in the analysis of biological fluids where contamination may be a problem. Hughes et al. described the development and optimization of a disposable screen-printed amperometric biosensor for glutamate based on GLDH [79]. The authors also developed a stable, reagentless amperometric glutamate biosensor by incorporating the GLDH biorecognition components using a layer-by-layer deposition involving chitosan and MWCNTs on SPCE [81]. The new reagentless biosensor was applied to the measurement of glutamate in beef stock cubes and serum samples.

8. Figures of merit and the performance of glutamate biosensors

When new biosensors are being developed and characterized, it is common to report their detection limit, sensitivity, specificity, accuracy, reproducibility (i.e., precision), response times, reusability including recovery times, long-term stability (i.e., shelf-life), lifetime, and performance in real samples such as serum or soup [13, 82]. For electrochemical biosensors, reporting the electrode material and type as well as the transducer's surface area and detection potential versus a reference electrode are also important experimental details. **Table 1** summarizes many of these analytical figures of merit for selected glutamate biosensors allowing comparisons to be made between various enzyme-based biosensors.

Electrode	Configuration	Application	Enzyme	Optimal pH	Applied potential	Response time	LOD	Linear range	Ref.
Screen-printed graphite	CHIT/MB/SPCE	In vitro	GLDH	7	0.1 V vs. Ag/AgCl	2 s	1.5 μM	12.5–150 μM	Hughes [79]
Platinum microelectrode array	Silicon wafer/ polypyrrole/ Nafion	In vivo	GmOx	7.4	0.7 V vs. Ag/AgCl	<1 s	<1 μM	10–100 μM	Wassum [40]
Oxygen electrode	Glutaraldehyde/ bovine serum albumin	In vitro (food)	GmOx and GLDH	7		120 d	0.02 mg/L	0.02–1.2 mg/L	Basu [68]
Glassy carbon electrode	SWCNT/thionine	NADH	GLDH	8.3	0.19 V vs. Ag/ AgCl	5 s	0.1 μM	0.5–400 μM	Meng [67]
Au planar nanocomposite	CHIT/MWCNT/ ferricyanide	In vitro (food)	GLDH and diaphorase	9		<60 s	5.4 μM	10–3495 μM	Monošík [12]
SWCNT bundles	Ferrocene/SWCNT	In vitro			0.2 V vs. Ag/AgCl	<300 s	1 μM	1–7 μM	Huang [52]
Pt. microelectrode	CHIT/ceria & titania NPs	In vitro (hypoxic brain tissue)	GmOx		0.6 V vs. Ag/AgCl	2–5 s	0.594/0.493 μM		Özel [44]
Graphite	PB/graphite	Proof of concept	GmOx	6.5	−0.05 V vs. Ag/ AgCl	3 s	0.01 μM	0.01–0.1 mM	Liu [45]
Au electrode	cMWCNT/AuNP/ chitosan	In vitro (sera)	GmOx	7.5	0.135 V vs. Ag/ AgCl	2 s	1.6 μM	5–500 μM	Batra [83]
Vertically aligned CNT nanoelectrode array	VACNT-NEA	Proof of concept	GLDH		0 V vs. Ag/AgCl		57 nM	0.1–300 μM	Gholizadeh [75]
Pt electrode	Chitosan-glutaric dialdehyde gels	Proof of concept	GmOx		0.6 V vs. Ag/AgCl	2 s	0.10 μM	0.10–500 μM	Zhang [37]
Pt electrode	Chitosan	In vitro (food)	GmOx		0.4 V vs. Ag/AgCl	2 s	0.10 μM	1–10 μM	Zhang [38]
Patterned Pt thin film electrodes	Glutaraldehyde/ SiO_2/PCB	Cell culture fermentation	GmOx		0.6 V vs. Ag/AgCl		0.0002 μM	0.00022500 μM	Bäcker [41]

Electrode	Configuration	Application	Enzyme	Optimal pH	Applied potential	Response time	LOD	Linear range	Ref.
SAM on smart biodevice	ECD/thioglycolic acid	Proof of concept	GmOx		NA		0.089 μM	0.1–10,000 μM	Rahman [42]
Pt electrode	Glutaraldehyde	In vitro (brain tissue) uptake	GmOx	7.4	0.6 V vs. Ag/AgCl	15–20 s	0.5 μM	2–800 μM	Soldatkin [84]
Screen-printed graphite	MWCNT-CHIT-MB/ CHIT-NAD^+-MB/ MWCNT-CHIT-MB/ MB-SPCE	In vitro proof of concept	GLDH	7	0.1 V vs. Ag/AgCl	<60 s	3 μM	7.5–105 μM	Hughes [81]

Table 1. A summary of previously published electrochemical glutamate biosensors, their electrode material, surface modification configuration, analytical figures of merit, and authors.

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9. Conclusion

Enzyme-based electrochemical glutamate biosensors have tremendous potential for manufacturing of cost-efficient, easy-to-use, fast, and portable alternatives for a wide range of applications from medical/clinical testing or neurological studies for diagnostics involving this important neurotransmitter in vivo or in vitro to environmental monitoring, process monitoring, and food-sensing applications [13, 82]. Electrochemical detection schemes are also typically very simple, sensitive, independent of sample volume, and well suited for monitoring glutamate from nM to μM in real samples such as biological fluids or processed foods. The incorporation of the oxidase or dehydrogenase enzyme as the biorecognition component on the electrochemical transducer provides additional selectivity and in some detection schemes even significant signal enhancement. Many glutamate biosensors are label free while others incorporate various redox active mediator molecules into a composite material on the biosensor surface. An increasing number of enzyme-based glutamate biosensors also utilize the advantageous properties of nanomaterials such as biocompatible CNTs or metal nanoparticles. It is likely to take years or a decade before many of these biosensors described in this chapter go from proof-of-concept stage to mass production of inexpensive, small, and reliable devices capable of competing with existing instrument-intensive laboratory methods for glutamate quantification such as spectroscopy or chromatography. However, with continuous developments in molecular biology, nanofabrication methods, immobilization methods of biomolecules, and multiplexing capabilities, the production of sensitive, selective, fast, and easy-to-use biosensors for quantification of glutamate and other neurotransmitters will be feasible in the not too distant future.

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