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ENZYME-BASED AMPEROMETRIC BIOSENSORS FOR CONTINUOUS AND ON-LINE MONITORING OF CEREBRAL EXTRACELLULAR MICRODIALYSATE

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TABLE OF CONTENTS

1. ABSTRACT

Analytical systems integrating *in vivo* microdialysis sampling with enzyme-based electrochemical biosensor detection have been increasingly accepted to be a new technique for continuous and on-line monitoring of biologically important species. Extensive interests in such integrated on-line analytical systems have suggested that these systems are very useful for physiological and pathological investigations. This review mainly focuses on the principle, development and striking applications of the enzyme-based amperometric biosensors integrated with *in vivo* microdialysis for continuous and on-line monitoring of cerebral extracellular fluid in recent years.

2. INTRODUCTION

Understanding of chemical essence in physiological and pathological events has been a long-standing goal. Typical example can be given that understanding chemical process in brain functions has drawn extensive interests over last several decades (1). Attempt on brain chemistry has essentially revealed that chemical process is primarily involved and plays a great role in brain functions. It is known that the mammalian brain contains ~10^{11} nerve cells or neurons, which assimilate and process information. These neurons receive information from other cells through a treelike network called dendrites and transmit it in the form of electrical impulse or action potentials along a cable-like axon to other nerves. At the end of axon, the message is relayed to other cells at the end points of contact named synapses, where the electrical message eventually leads to the release of so-called chemical neurotransmitters that diffuse across a small gap to another neuron. The neurotransmitter binds to and activates a receptor on this second neuron and eventually results in another action potential. Repeating such cycles of chemical and electrical transformations substantially enables neurotransmission that plays a great role in brain function, such as learning and memory (1, 2).

It becomes widely accepted that knowledge of the change in the level of chemical species in cerebral extracellular fluid involved in the chemical process will largely facilitate understanding of brain function, e.g., the mechanism for the neurotransmitters to impact target neurons and ultimately to effect physiological events (2). To date, many methods including chemical and morphologic methods and molecular biology have been used for investigations on chemical essences involved in physiological process, of which the chemical strategies, such as fluorescence, chemiluminescence and mass spectrometry, are primarily based on the determination of biologically important species to probe the chemical process involved in physiological process (3-5). As one of most widely used chemical approaches, electrochemical methods principally based on redox process of analyte have been demonstrated to be very effective and facile route to brain chemistry primarily because of their high sensitivity, selectivity and method simplicity (6). Moreover, superior to other chemical methods used for probing brain chemistry, electrochemical methods are readily amenable for *in vivo* and real-time monitoring of chemical species in cerebral extracellular fluid.

In general, electrochemical methods employed for probing brain chemistry can be divided into two categories, *in vivo* monitoring and *in vitro* determinations. The former includes *in vivo* voltammetry that employs (ultra)microelectrodes directly implantable in brain region to real-time record the change in the level of chemical species, neurotransmitters in particular, in physiological process (7). *In vivo* voltammetry has been reviewed several times in literature (1-2, 7) and will not be reviewed here. Another technique that integrates *in vivo* microdialysis sampling with on-line detection could also be considered as another *in vivo* technique since the combination of *in vivo* sampling and on-line detection substantially provides nearly temporal and spatial information on the change in

the level of the chemical species (8). *In vitro* determination includes those for the samples extracted from cerebral systems, for example, the determinations performed with pre-separation, for instance, with high performance liquid chromatography (HPLC) or capillary electrophoresis, coupled with a subsequent electrochemical detection. We should note here that some neurotransmitters including dopamine, norepinephrine, 5-hydroxytryptamine, and their metabolites are electroactive, and consequently electrochemical methods are particularly useful for their determinations. So far electrochemical methods used for the determination of these neurotransmitters and their metabolites either by *in vivo* voltammetry or by integration of *in vivo* microdialysis sampling with pre-separation procedure have been widely used for physiological investigations (9-11). These determinations will not be reviewed here either. Other biologically important compounds, e.g., glucose, lactate, acetylcholine, glutamate, histamine and γ-aminobutyric acid (GABA), are, however, electroinactive and are not readily amenable for direct analysis. For these species, systems integrating *in vivo* microdialysis with enzyme-based amperometric biosensors would offer an effective and straightforward route to the measurements. This article will mainly focus on enzyme-based amperometric biosensors used in such integrated systems for continuous and on-line monitoring of the species in cerebral extracellular fluid.

3. INTEGRATION OF *IN VIVO* MICRODIALYSIS WITH ON-LINE AMPEROMETRIC BIOSENSOR DETECTION

Microdialysis, introduced in the early 1980s, is an *in vivo* sampling technique (12) and originally developed for *in vivo* sampling and detection of neurotransmitters combined with HPLC separation (8). The method, consisting of a tubular, semi-permeable membrane and a perfusion system, can sample the extracellular fluid and exclude large biological molecules, e.g., proteins and enzymes, by the dialysis membrane, whereas small molecules like neurotransmitters and energy metabolites can freely diffuse into the stream of perfusate (8). For the subsequent analysis of microdialysate, a variety of methods have been used, which can be primarily divided into two categories, on-line and off-line detection. The former directly couples the detecting system to the outlet of the microdialysis probe and can thus continuously and on-line record the level of analyte without need of sample pre-separation, while the latter requires a collection and pre-separation of the microdialysate before the microdialysate goes to the detector (13-16).

Among the methods used for on-line detection (3-5, 8), enzyme-based amperometric biosensors are most useful and widely used because of the high selectivity and sensitivity. So far the systems integrating *in vivo* microdialysis sampling with on-line amperometric detection have been proved to be relatively useful and readily amenable for physiological investigations, providing near real-time information of the change in biological species (8) although it is still only limited to some compounds, especially those with the oxidase or dehydrogenase as will be demonstrated below. Such integrated on-line analytical systems have several advantages over *in vivo* voltammetry and *in vivo* microdialysis sampling followed with off-line detection; for example, although *in vivo* voltammetry offers temporal and spatial information, especially on the change in the level of catecholamines and serotonin useful for understanding brain chemistry (17), it is limited for practical applications because of the unsatisfied stability and reproducibility of the electrode (generally carbon fiber microelectrodes) (18). It is known that the electrodes used for *in vivo* voltammetric measurements are easily fouled by the strong non-specific adsorption of biomolecules, e.g., collagen (19). Moreover, *in vivo* voltammetry requires a good technique for microelectrode fabrication and a basic knowledge on electrochemistry that may be far available for a physiologist. On the other hand, the integrated systems offer a higher time resolution in relative to off-line detection since the off-line detection requires additional procedures, e.g., sample collection and separation. These procedures also result in a remarkable fouling and dilution of the samples (8, 20-24).

Summarily, the integrated systems consisting of *in vivo* microdialysis sampling and on-line amperometric biosensing essentially provide advantages mainly in transient and near real-time nature, no sample collection and less sample dilution, simple operating procedure and are thus quite applicable for physiological investigations.

4. ENZYME-BASED AMPEROMETRIC BIOSENSORS FOR CONTINUOUS AND ON-LINE MEASUREMENTS

Taking account of the complexity of the microdialysate and the inherent features of the integrated on-line analytical systems without a pre-separation procedure, detectors used for continuous and on-line measurements of cerebral microdialysate should have: (I) high selectivity against other electroactive species coexisting in extracellular cerebral fluid, e.g., ascorbic acid (AA), uric acid (UA), catecholamine and their metabolites; (II) high sensitivity competent for the determination of the species at a low basal level, even after dilution during *in vivo* microdialysis sampling; (III) high stability capable for a long-time and continuous operating in a flowing system during animal experimentation; and (IV) high tolerance against cross-talk among each analyte while the system is used for multi-analyte analysis in some instances.

Enzyme-based amperometric biosensors that utilize bio-recognition of enzymes toward substrate to gain the specificity would mostly satisfy the above requirements. As a result, such kinds of amperometric biosensors have been demonstrated to be particularly useful for continuous and on-line monitoring of cerebral microdialysate (25-28). Moreover, fortunately, most of the electroinactive compounds mentioned above, e.g., glucose, lactate, acetylcholine, glutamate, histamine and γ-aminobutyric acid (GABA), have a counterpart enzyme, such as glucose oxidase, lactate oxidase, acetylcholine esterase and choline oxidase. These enzymes can be essentially used to convert these biologically important but

Table 1. Amperometric biosensors for on-line measurements of biological species

Species	Enzyme used	Linear range	ECF level	Reference
Glucose	Glucose oxidase	0.05 ~ 20 mM 0.01 ~ 10mM 1 ~ 400 μM 2.5 μM 0 ~ 30 mM 0 ~ 20 mM	~ 10 mM	25 38 39 41 43 44
Lactate	Lactate oxidase	0.05 ~ 20 mM 0 ~ 10 mM 0 ~ 30 mM	~ 1 mM	25 44 51
Acetylcholine	Acetylcholine-esterase and choline oxidase	2.5 nM ~ 2 μM	μM level	46
γ-Aminobutyric acid	Gabase and glutamate oxidase	0.1 ~ 10 μM	μM level	48
Glutamate	Glutamate oxidase	80 nM ~ 20 μM 50 nM ~ 10 μM 0.1 ~ 30 μM 10 nM ~ 1 μM	~ 10 μM	22 27 51 52
Histamine	Histamine oxidase	0.1 ~ 100 μM.	μM level	28
H_2O_2	Horseradish peroxidase	0.2 ~ 15 μM	~1 μM	65

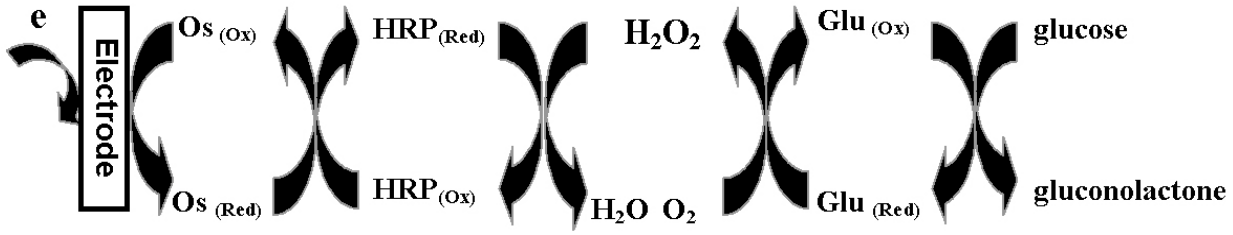
electroinactive species into electrochemically detectable signal and eventually enable them to be detectable with electrochemical approaches. So far the enzyme-based electrochemical biosensors have been increasingly used for continuous and on-line monitoring of the species in cerebral microdialysate as summarized in Table 1 and illustrated below.

4.1. Glucose and Lactate

Glucose not only represents the primary energy source for the brain (29), but also plays an important role in synaptic transmission (30). Earlier reports have suggested that either a large rise or fall in plasma glucose levels could cause the brain to activate the sympathetic nervous system (31). Moreover, recent evidence shows that systemic glucose treatment enhances memory (32). On the other hand, lactate is formed from glucose in an actively glycolyzing system under anaerobic and aerobic conditions. Besides, lactate is of interest in metabolic disorders, including cerebral ischemia and lactate acidosis in diabetic and it is also an energy source during neuronal activation (33). Therefore, continuous and on-line monitoring of glucose and lactate in the cerebral extracellular fluid would be of great importance in studies on energetics and metabolism of brain systems.

The basal concentration of glucose and lactate in cerebral systems is at mM level. Electrochemical biosensors for the determination of both species are generally based on the enzymes, i.e., glucose oxidase and lactate oxidase, which specifically catalyze the oxidation of glucose and lactate, respectively, in the presence of dissolved oxygen as electron donor. Since the O_2 donor is eventually converted into hydrogen peroxide (H_2O_2) and both O_2 and H_2O_2 are electrochemically detectable, the determination of O_2 or H_2O_2 would enable the electrochemical methods to be competent for the desired determinations. Such oxidase-based biosensors are termed electrochemically as first-generation biosensors of which the measurement is basically based on the determination of the consumption of O_2 (34) or the formation of H_2O_2 in the enzymetic reactions (35, 36). Similar to the limitations inherent in the first-generation amperometric biosensors, the determinations of glucose and lactate also suffer from a high overpotential for H_2O_2 oxidation, bearing remarkable interference from coexisting electroactive species, AA and UA in particular. Nevertheless, such kind of amperometric biosensors integrated with *in vivo* microdialysis are yet useful for the measurement of cerebral microdialysates on the condition that other coexisting compounds do not contribute amperometric interference. To gain a high specificity against AA, several strategies, e.g., coating the biosensor with a layer of an anionic polymer (35), an immobilized ascorbate oxidase layer (37) or electropolymerized membrane (38) have been reported. Moreover, the on-line analytical systems make it possible to readily introduce an electrochemical pre-oxidizing system either in the perfusing line or inside the electrochemical cell to consume AA in the sample and thus suppress its interference to the biosensors (39).

Another effective strategy to suppress the great interference in the first-generation amperometric biosensors is the utilization of horseradish peroxidase (HRP) to specifically catalyze H2O2 reduction with a low overpotential. In this case, the electron transfer between HRP and electrode can be sufficiently shuttled, e.g., with Ospoly(vinylpyridine) at 0.0 V (vs. Ag/AgCl) (40). HRP linked with Ospoly(vinylpyridine) gel, so-called Os-gel-HRP, has been currently commercially available and is very useful for the practical development of such bi-enzyme (oxidase and peroxidase)-based amperometric biosensors because of the ease in immobilization onto electrode surface, fast electron transfer rate of Ospoly(vinylpyridine) and high stability of the gel. In general, for practical development, Os-gel-HRP is first coated on electrode surface and a second cross-linking of oxidase enzyme on the electrode essentially produces the bi-enzyme-based amperometric biosensors that are competent for specific, stable and on-line measurements. The determination scheme for such bi-enzyme-based amperometric biosensors



Scheme 1 Schematic depiction of determination scheme of amperometric biosensors for glucose based on bi-enzyme of glucose oxidase and horseradish peroxidase.

can be illustrated in Scheme 1, with glucose oxidase (GOx) as an example. The demonstrated GOx/HRP-based glucose amperometric biosensors have been used for continuous and on-line measurements of glucose in cerebral microdialysate with excellent analytical properties, e.g., high specificity and stability and good linearity (41).

On the other hand, the above determination scheme (both through direct oxidation of H_2O_2 and based on HRP-catalyzed reduction of H_2O_2 shown in Scheme 1) is quite suitable for other oxidase-based amperometric biosensors provided GOx used in the glucose biosensor is simply replaced with other oxidases, such as lactate oxidase, glutamate oxidase or choline oxidase (39). Moreover, such a determination scheme can also be exploited for development of multi-channel amperometric biosensors for simultaneous measurements of multi-analyte in cerebral microdialysate on the condition that each biosensor is properly assembled so as to well avoid cross-talk between each other (42, 43). The cross-talk generally rises from the diffusion of electrochemically detectable enzymatic reaction products (H_2O_2 in this case) from one biosensor to another and eventually yields falsely elevated current response on each amperometric biosensor. Osborone *et al.* fabricated a split-disk plastic film carbon electrodes that allowed different oxidase enzymes, i.e., GOD and lactate oxidase, to be stably immobilized on each half of the split-disk, Os-gel-HRP-coated, plastic film carbon electrodes (39). Such split-disk electrode-based amperometric biosensors successfully enabled electrochemically independent, simultaneous and on-line determinations of glucose and lactate in cerebral microdialysate. Perdomo *et al.* developed microfabricated multi-enzyme-based first-generation amperometric biosensors on a silicon chip for simultaneous monitoring of lactate and glucose (44).

As another kind of electron transfer mediator used for HRP, instead of Ospoly(vinylpyridine) gel, Mao *et al.* employed electropolymerized film of Meldola's blue to shuttle the electronic communication between HRP and electrode (45). The as-prepared H_2O_2 biosensing system was further used to construct amperometric biosensors for glucose and choline at a more negative potential (-0.30 V *vs*. Ag/AgCl) than those based on Os-gel-HRP. The biosensors were therefore free from the interference from other electroactive species, such as AA and UA, and were demonstrated to be potential for on-line measurement of both species in cerebral microdialysate.

4.2. Acetylcholine

Acetylcholine (ACh) is widely distributed in the body, and in vertebrates only trace amount of ACh is needed to transfer important biological information through motor neurons in the spinal cord and at nerve skeletal junctions (46). Moreover, ACh has been considered as an important neurotransmitter involved in learning and memory in relation to the mammalian central nervous systems (47). ACh biosensor can be developed by a combination of acetylcholine-esterase (AChE) and choline oxidase (ChOx) that converts ACh to betaine aldehyde, producing electrochemically detectable H_2O_2, as shown below.

$$\mathrm{ACh + H_2O \xrightarrow{AChE} acetate + choline}$$

$$\mathrm{Choline + O_2 \xrightarrow{ChO_x} betaine\ aldehyde + 2H_2O_2}$$

Besides the interference inherent in other amperometric biosensors used for continuous and on-line measurements of cerebral microdialysate, ACh biosensor suffers from another serious interference from choline because choline is also detectable according to the determination scheme shown above. Moreover, the concentration of choline is around 1000 times higher than that of ACh in cerebral systems. Therefore, elimination the interference from choline becomes a key problem for the development of ACh on-line amperometric biosensors.

To avoid the interference from choline, Niwa *et al.* used a pre-reactor prepared with choline oxidase (ChOx) and catalase to remove choline from the sample and thereby eliminate its interference (46). The experimental protocol could be shown below.

$$\mathrm{Choline + O_2 \xrightarrow{ChO_x} betaine\ aldehyde + 2H_2O_2}$$

$$\mathrm{2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2}$$

The interference from AA at the ACh on-line biosensors could be suppressed by further coating the AChE-ChOx/ Os-gel-HRP biosensor with an additional film, e.g., Nafion.

4.3. γ-Aminobutyric acid.

γ-Aminobutyric acid (GABA) is well accepted as a neurotransmitter that regulates inhibitory neurotransmission in mammalian central nervous systems. Direct monitoring of GABA is very difficult because it is insensitive either to electrochemical or spectroscopic

methods. In addition, it is difficult to construct GABA biosensors in combination with an enzymatic reaction because neither oxidase nor dehydrogenase can be available for GABA.

Gabase, which mainly contains two enzymes, γ-aminobutylate ketoglutarate aminotransferase (GABA-T) and succinic semialdehyde dehydrogenase (SSDH), can convert GABA into glutamate. Glutamate can then be specifically oxidized to α-ketoglutarate and ammonia in the presence of glutamate oxidase (GluOx). The enzymatic reaction produces H_2O_2 that is electrochemically detectable either through direct oxidation or HRP-catalyzed reduction reactions. The Gabase, GluOx and HRP-based GABA determination scheme is shown below:

$$\text{GABA} + \alpha\text{-ketoglutarate} \xrightarrow[\text{SSDH}]{\text{GABA-T}} \text{succinicsemialdehyde(SSA)} + \text{glutamate}$$

$$\text{glutamate} + H_2O + O_2 \xrightarrow{\text{GluO}_x} \alpha\text{-ketoglutarate} + NH_3^+ + H_2O_2$$

$$H_2O_2 + \text{Ferric HRP} \rightarrow \text{HRP compound I} + H_2O$$

$$\text{HRP compound I} + \text{Os(II)} \rightarrow \text{HRP compound II} + \text{Os(III)}$$

$$\text{HRP compound II} + \text{Os(II)} \rightarrow \text{Ferric HRP} + \text{Os(III)}$$

$$\text{Os(III)} + e \rightarrow \text{Os(II)}$$

Niwa *et al.* first developed such a determination scheme and successfully constructed on-line amperometric biosensors for GABA (48). They first applied an inner layer film of Os-gel-HRP and then an outer layer film consisting of gabase and GluOx co-immobilized with bovine serum albumin on the electrode. The as-prepared tri-enzyme-based biosensor shows a low detection limit (0.1 μM) and good linearity (0.1~10 μM) toward GABA in a continuous-flow system, suggesting its potential use for on-line measurement of GABA in cerebral microdialysate. The main problem for such an amperometric biosensor for practical measurement of GABA in cerebral microdialysate is the overlapping of the response for GABA with that for glutamate because glutamate can also be specifically oxidized to produce H_2O_2 according to the same mechanism shown above. To overcome this limitation, Niwa *et al.* added a pre-reactor prepared with GluOx and catalase to totally consume glutamate molecules in the sample before the sample was perfused to the GABA biosensor. As a result, the system exhibits a sensitivity of 1.56 nA/μM for GABA, almost no response toward 10 μM glutamate under a continuous-flow condition. AA interference was also well suppressed either by further spin-coating of Nafion film on the surface of the biosensor or shifting the operating potential toward a negative direction, e.g., -0.10 V (*vs*. Ag/AgCl).

4.4. L-Glutamate.

L-Glutamate is one of the main neurotransmitters and plays an important role in synaptic plasticity. The long-term changes in synaptic efficacy, such as long-term potentiation (LTP) (49) and long-term depression (LTD) (50) of excitatory synaptic transmission, are considered to be the neuronal bases for learning and memory. The concentration of glutamate is at μM level in the extracellular fluid. Similarly to the on-line amperometric biosensors for glucose and lactate mentioned above, glutamate biosensors are generally based on the use of L-glutamate oxidase (GluOx) to catalyze the oxidation of glutamate into α-ketoglutarate, producing electrochemically detectable H_2O_2.

$$\text{glutamate} + H_2O + O_2 \xrightarrow{\text{GluO}_x} \alpha\text{-ketoglutarate} + NH_3^+ + H_2O_2$$

Zilkha (21) developed amperometric biosensor for on-line measurement of glutamate by co-immobilizing GluOx with electropolymerized film of 1, 2-diaminobenzene and detected glutamate through the direct oxidation of the produced H_2O_2. The interference from AA was eliminated with the polymerized film of 1, 2-diaminobenzene used. Similarly, Berners *et al.* developed amperometric biosensor for glutamate by co-immobilizing GluOx with electropolymerized film of o-phenylenediamine (51). To overcome the interference from AA, they added a pre-oxidation system to consume AA in the sample. With the experimental configuration demonstrated, they could sensitively and on-line measure glutamate virtually interference free from other species at their physiological levels. Niwa *et al.* developed a small-volume L-glutamate oxidase enzymatic reactor (0.75 mm i.d. and 2.5 cm long) that could be coupled with an electrochemical detector in a thin-layer radial flow cell with an active volume of 70~340 nL (52). They used glassy carbon bulk or carbon film ring-disk electrodes modified with Os-gel-HRP as the detector for the determination of H_2O_2 produced from the enzymatic reactor. The high conversion efficiency of glutamate in the enzymatic pre-reactor essentially yielded a high sensitivity for the determination. Moreover, the small volume of the thin-layer flow cell used largely improved the time resolution for continuous and on-line measurements.

As an alternative to the determination scheme demonstrated above, Yao *et al.* reported an amplified amperometric biosensor for glutamate with an enhanced sensitivity through substrate recycling (22). They co-immobilized L-glutamate oxidase and glutamate dehydrogenase (GIDH) in a PTFE reactor to recycle glutamate (shown below). The produced H_2O_2 was detected with a poly(1,2-diaminobenzene)-coated platinum electrode positioned in a downstream. With such a protocol, they achieved a 160-fold increase in sensitivity for glutamate compared with the unamplified system.

$$\text{glutamate} + H_2O + O_2 \xrightarrow{\text{GluO}_x} \text{2-oxoglutarate} + NH_3^+ + H_2O_2$$

$$\text{2-oxoglutarate} + NH_3^+ + \text{NADH} \xrightarrow{\text{GIDH}} \text{glutamate} + NAD^+$$

4.5. Hypoxanthine.

Hypoxanthine is one of the main metabolites of adenine nucleotide degradation that accumulates in biological tissues. Levels of hypoxanthine are used as an index of both the pathology of some processes in the human body and the freshness of meat in the food industry (53). Hypoxanthine biosensors can be constructed by using xanthine oxidase as a bio-recognition unit (54-55). Base on

a combination of Os-gel-HRP and xanthine oxidase (XOD), Mao *et al.* designed amperometric biosensor for on-line measurement of hypoxanthine (56). Hypoxanthine was specifically oxidized by XOD to produce H_2O_2 that was further selectively sensed with Os-gel-HRP. The operating potential was optimized at -0.20 V to well avoid the interference from AA and other species. In continuous-flow amperometric experiments, the biosensor was demonstrated to exhibit linear and sensitive response towards hypoxanthine.

4.6. Hydrogen peroxide.

H_2O_2, one of the main metabolites of reactive oxygen species, e.g., superoxide ($O_2^{\bullet-}$), in itself is a toxic compound. The direct determination of $O_2^{\bullet-}$ has been proved to be very difficult (57-59). Thus, a specific and technically undemanding method for reliable and durable measurement of H_2O_2 would be useful for investigations focusing on oxidative stress and lipid per-oxidation (60). Unlike other compounds mentioned above, H_2O_2 is electroactive and can be principally detected directly. However, the serious specificity problems associated with the direct electrochemical measurement eventually made it almost impossible for *in vivo* voltammetric or on-line amperometric measurement of H_2O_2 in the cerebral systems. Moreover, the changes in the endogenous levels of O_2 and pH that are essentially associated with the formation of H_2O_2 under patho-physiological conditions also render difficulty in the electrochemical determinations of H_2O_2.

Horseradish peroxidase (HRP), upon wired with electrode through electron transfer mediators or promoters, can be relatively useful for selective determination of H_2O_2 in most cases (61, 62), yet such HRP-based amperometric biosensors are not well competent for continuous and on-line measurement of cerebral H_2O_2 mainly because of the great interference from AA. AA interferes with the measurement of H_2O_2 through the reactions given below.

$$\text{Ascorbic acid} + 2e + 2H^+ \rightarrow \text{dehydroascrobic acid (DHA)}$$
$$H_2O_2 + AA \rightarrow 2H_2O + DHA$$
$$Os(III) + AA \rightarrow Os(II) + DHA$$

As shown, AA can be directly oxidized at the substrate electrode to produce dehydroascorbic acid (DAA). Besides, AA can chemically react with H_2O_2 under the catalysis of metal ions, e.g., Fe^{3+} or Cu^{2+} (63) or by HRP (64). Moreover, AA can possibly reacts with some mediators used for shuttle electron transfer of HRP, e.g., Ospoly(vinylpyridine) (64). All these by-reactions substantially make it difficult to simply use the HRP-based amperometric biosensor for continuous and on-line determination of cerebral H_2O_2.

To overcome the limitations inherent in the HRP-based amperometric H_2O_2 biosensors, Mao *et al.* (65) developed a continuous and on-line H_2O_2 biosensing system with ring-disk carbon film electrode positioned in a thin-layer radial flow cell. They coated the central disk electrode with ascorbate oxidase to pre-oxidize and thus consume most of AA molecules in the sample through an enzymatic mechanism. On the ring electrode, they co-immobilized HRP with electropolymerized film of pyrrole since the polypyrrole can be used to promote or mediate the electronic communication between HRP and electrode at a low potential (0.0 V). The HRP/polypyrrole-modified ring electrode was over-coated with a thin film of polyphenol to further improve the selectivity against AA. On the other hand, the homogeneous reaction between AA and H_2O_2 was suppressed by removing heavy metal ion catalysts with the chelating agent, EDTA, introduced in external phosphate buffer solution (PBS). The external buffer also delivered an excess of dissolved O_2 needed for the ascorbate oxidase-catalytic reaction and stabilized the pH level of the electrode environment. Further, unlike other mediates used for shuttle electron transfer between HRP and electrode, polypyrrole used did not react with AA chemically. This protocol successfully enabled trace levels of cerebral H_2O_2 to be readily monitored, virtually interference-free from physiological levels of AA, UA, electroactive neurotransmitters and their principle metabolites, in a continuous-flow system.

5. SUMMARY

In summary, enzyme-based amperometric biosensors integrated with *in vivo* microdialysis are relatively useful for continuous and on-line monitoring of biologically important species, providing reliable and near real-time information on the change in the level of extracellular species. Such a biosensor technology combined with *in vivo* microdialysis sampling technique is particularly useful for the continuous measurements of the species in the cerebral systems and could be very useful for the investigation of brain chemistry, especially upon further combination with other methods, e.g., electrophysiological methods, *in vivo* voltammetry and *in vivo* microdialysis with off-line detections.

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