Flow-Injection Biosensor System for In Vivo Analysis of Biomolecules in the Extracellular Space of Brain

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Abstract

The in vivo monitoring of the concentration changes of biomolecules at a variety of sites of the living systems including blood stream, subcutaneous tissue, muscle, internal organs, and brain, has been desired for the pharmacological, clinical, and biological studies. The most general approach to achieve such goals was the use of implanted biosensors, but it was limited because of the variations in sensitivity during the prolonged in vivo monitoring. Recently, microdialysis has been used as a sampling technique for in vivo analysis. This article reviews novel flow-injection biosensor systems which make possible on-line monitoring of glucose metabolism and pharmacological behavior of neurotransmitters in the extracellular space of brain. This approach was developed by coupling microdialysate sampling to the biosensor system using a flow-injection interface. The advances in these researches are reviewed with the results obtained in our recent works.

Keywords Amperometric flow injection analysis, in vivo monitoring, microdialysis sampling, biosensor, bioreactor, enzyme amplification, brain, serum, glucose, L-lactate, pyruvate, L-glutamate, acetylcholine.

1. Introduction

Microdialysis is a sampling technique for in vivo analysis [1] and has successfully been applied in pharmacological and biological studies of biomolecules in dialysate. In many cases, however, the dialysate was collected and usually assayed by liquid chromatography.

The use of miniaturized biosensors was the most simple approach for the in vivo monitoring, but it was limited because of the variations in sensitivity during the prolonged in vivo monitoring. In recent years, sensor systems combined microdialysis sampling have been developed for in vivo analysis and continuous monitoring of concentration changes of biomolecules in blood [2], subcutaneous tissue [3], and brain [4]. For such researches, some miniaturized enzyme-based amperometric biosensors have been proposed as specific detectors for the in vivo monitoring of glucose [3, 5-7], L-glutamate [8-10], acetylcholine[11], and L-lactate [3, 12]. A similar attempt was also achieved by using an enzyme reactor as a recognition element of a particular analyte in dialysate, e.g., glucose [13].

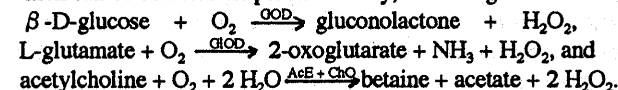
Glucose is a main nutrient in the brain, while L-lactate and pyruvate are formed from glucose in an actively glycolyzing system, and are of interest in metabolic disorders. Also, L-glutamate and acetylcholine are the most important excitatory neurotransmitters in the brain. Therefore, the in vivo monitoring of concentration changes in such analytes that take place rapidly in brain cells has been especially desired in the research field of brain chemistry.

This article reviews the in vivo flow-injection biosensor systems with an on-line microdialysis sampling which make possible the specific detection of a single analyte or the simultaneous monitoring of plural analytes in brain. This approach has been developed by coupling microdialysis sampling to the biosensor system using a flow-injection interface. The advances in these researches are reviewed with the results obtained in our recent works.

2. The use of the enzyme reactor as a recognition element

2.1. On-line amperometric assay of glucose, L-glutamate, and acetylcholine using immobilized enzyme reactors [14]

Here, we describe an on-line amperometric monitoring system of glucose, L-glutamate, and acetylcholine. These analytes are not detectable by direct electrochemical oxidation. Therefore, we chose to use the glucose oxidase (GOD), L-glutamate oxidase (GlOD), and acetylcholine esterase (AcE) + choline oxidase (ChO), to convert each of these analytes to hydrogen peroxide which can be detected amperometrically, according to



β-D-glucose + $O_2 \xrightarrow{GOD}$ gluconolactone + H_2O_2,
L-glutamate + $O_2 \xrightarrow{GlOD}$ 2-oxoglutarate + NH_3 + H_2O_2, and
acetylcholine + O_2 + 2 $H_2O \xrightarrow{AcE+ChO}$ betaine + acetate + 2 H_2O_2.

Each of the enzymes was loaded or coloaded onto reactor (PEFE coil; 20 mm×0.5 mm i.d.) packed with aminopropyl-CPG using glutaraldehyde as a cross-linking agent. The microdialysis system used in this work is outlined in Fig. 1.

The apparatus consisted of a two-channel microsyringe pump, potentiostat, and a strip chart recorder. Each of the immobilized enzyme reactors was positioned after a microdialysis probe shown schematically in Fig. 2 and in front of a poly(1,2-diaminobenzene) film-coated platinum electrode. A constant potential (+ 0.6 V vs. Ag/AgCl) was applied to the polymer film-coated electrode and the current was recorded. The Ringer's

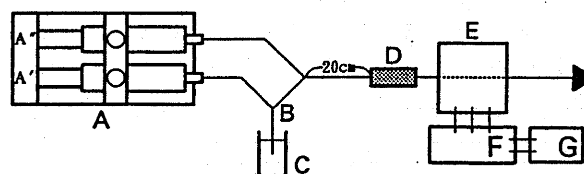


Fig. 1 Schematic diagram of microdialysis system with immobilized enzyme reactor. A: two-channel microsyringe pump, B: microdialysis probe, C: sample, D: immobilized enzyme reactor, E: poly(1,2-diaminobenzene) film-coated platinum electrode, F: potentiostat, G: recorder. All the parts were connected with PTFE coil (i.d. 0.1 mm).

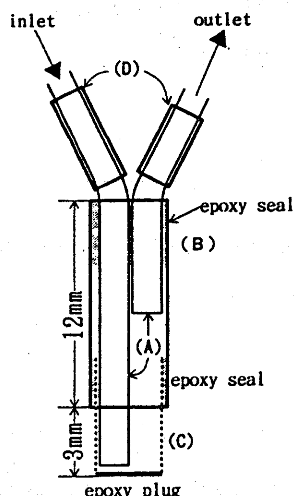


Fig. 2 Schematic diagram of a microdialysis probe. A: fused silica tube (o.d. 150 μm, i.d. 75 μm), B: fused silica tube (o.d. 470 μm, i.d. 350 μm), C: dialysis fiber (o.d. 220 μm, i.d. 200 μm), D: stainless steel tube (o.d. 440 μm, i.d. 270 μm).

solution was pumped at a constant flow (2.0 μl min^{-1}) with microsyringe pump.

For the glucose assay, the GOD immobilized reactor was used and the 0.1 M phosphate buffer at pH 7.0, containing 1 mM sodium azide was selected as a carrier buffer. Under the recommended flow conditions, a linear calibration plot for glucose assay was obtained over 2×10^{-6} – 1×10^{-2} M; the slope was 272 nA mM^{-1}.

For the L-glutamate assay, the GlOD immobilized reactor was used and 0.1 M phosphate buffer at pH 7.5 was selected as an optimum carrier buffer. Under the recommended flow conditions, the permeability of L-glutamate into the probe was 7.6%. However, the conversition efficiency of L-glutamate to hydrogen peroxide was ca. 100%. A linear calibration plot for L-glutamate assay was obtained over 2×10^{-6} – 5×10^{-3} M under the same flow conditions as the glucose assay; the slope was 181 nA mM^{-1}.

For the acetylcholine assay, the AcE+ChO coimmobilized reactor was used. However, choline produces hydrogen peroxide in this enzyme reactor and, therefore interferes with the measurement of acetylcholine. Because of this, the ChO-catalase (CT) coimmobilized reactor was inserted in series before the AcE+ChO coimmobilized reactor to remove choline in the dialysate according to choline $\xrightarrow{\text{ChO-CT}}$ betaine + H_2O. When the borate buffer (0.1 M) at pH 8.5 was selected as a carrier buffer, the ChO-CT coimmobilized reactor had an activity that was sufficient to decompose choline below 2 mM. Also, the permeability of acetylcholine into the probe was about 11%. A linear calibration plot for acetylcholine was obtained over 2×10^{-6} – 5×10^{-3} M; the slope was 82 nA mM^{-1}.

In this work, the poly(1,2-diaminobenzene) film-coated platinum electrode was used as an amperometric detector of hydrogen peroxide generated enzymatically in the enzyme reactors. This polymer-coated electrode can detect hydrogen peroxide selectively without any interference from electroactive interferents (e.g., ascorbate and urate) and proteins in serum. The usefulness of this polymer-coated electrode was evaluated in the proposed flow system with a microdialysis probe and the GOD immobilized reactor. The signal currents for glucose were monitored continuously for 3 h after a microdialysis probe was immersed in a control serum. The stability of the signal current for glucose in control serum was good; even after 2 h, the signal current retained 98% of its original current. Also, in the system without the GOD immobilized reactor, the base-line current did not vary after the probe was immersed in serum and even in 0.2 mM ascorbate solution. These results show that the poly(1,2-diaminobenzene)-coated platinum electrode prevents the electroactive interferents (ascorbate etc.) and proteins, commonly found in serum, from reaching the electrode surface and from interfering with the detection of the hydrogen peroxide generated in the enzyme reactor.

The in vivo monitoring of glucose in the rat brain was conducted by implanting a microdialysis probe into the frontal lobe. The rat was allowed to recover overnight in a large plastic box with free access to food and water. The probe was then connected to a microsyringe pump through a swivel to allow the rat free movement. After 12 min the signal current increased up to a steady value, corresponding to glucose content in rat brain. When a 1 ml aliquot of 20%(w/v) glucose was injected into abdomen of the rat, the signal current increased and then decreased to the value before glucose injection. It was found from these results that the present continuous flow method was useful for the in vivo monitoring of glucose. However, the sensitivity of the present method was not sufficient for the accurate in vivo monitoring of trace amounts of L-glutamate and acetylcholine in rat brain.

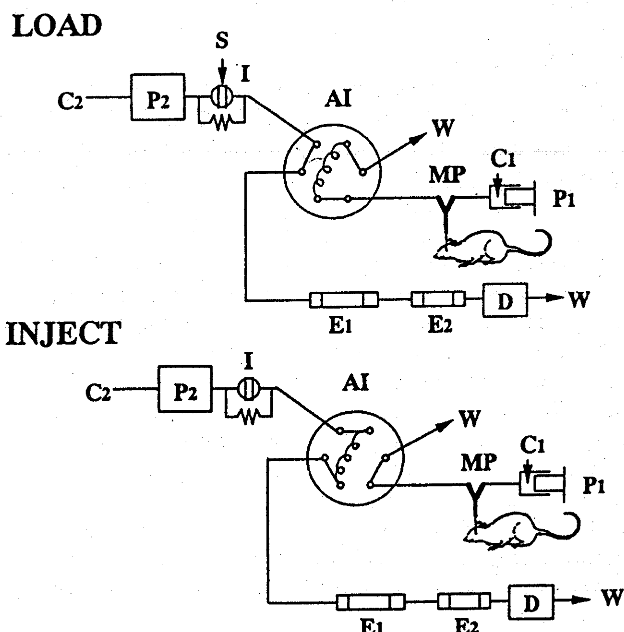


Fig. 3 Two stages (load and inject) of an FIA system used for in vivo experiments of L-glutamate. P_1: syringe pump, P_2: double plunger μl pump, MP: microdialysis probe, AI: six-way autoinjector with a sample loop of 2.5 μl, I: injector with a sample loop of 2.5 μl, D: electyrochemical detector with poly(1,2-diaminobenzene) film-coated platinum electrode, E_1: saccharopine dehydrogenase immobilized reactor, E_2: L-glutamate oxidase / glutamate dehydrogenase coimmobilized reactor, C_1: Ringer's solution, C_2: carrier buffer, S: standard L-glutamate solution (1 μM) to calibrate the detection system, W: waste.

2.2. An in vivo flow-injection system for highly selective and sensitive detection of L-glutamate using enzyme reactor involving amplification [15-17]

L-Glutamate is one of the most important excitatory neurotransmitters in brain. Some enzyme-based biosensors have

been proposed for the monitoring of the release of endogeneous glutamate into the extracellular space of the central nervous system. However, most of these methods were not sufficient in the sensitivity for the in vivo monitoring of L-glutamate or continuous calibrations of the sensor sensitivity during monitoring were not possible. In this work, a calibration method was proposed to improve these problems by coupling microdialysate sampling in short interval to the flow injection detection system with enzyme reactor involving amplification by substrate recycling, which allowed alternate detection of standard and dialysate sample and enhancement of the sensitivity for L-glutamate. The system includes a microdialysis probe, two immobilized enzyme reactors, a six-way autoinjector, and a poly(1,2-diaminobenzene) film-coated platinum electrode (see Fig. 3). In the first stage (load for 3 min, Fig. 3) of the operation, the dialysate from the probe was delivered to the sample loop of the six-way autoinjector by perfusing Ringer's solution at the flow rate of 2 μl min^{-1}. Simultaneously, a 1 μM L-glutamate standard solution (2.5 μl) for checking drifts in the sensitivity was injected in the FIA line. Optimized carrier buffer was 0.1 M, pH 6.7, ammonium phosphate buffer containing 0.5 mM NADH, 5 mM L-lysine, and 147 mM NaCl and pumped at 100 μl min^{-1}. In the second stage (inject for 30 s, Fig. 3), the dialysate collected in the sample loop was injected into the FIA flow line and assayed electrochemically. The enzyme column E_2 was employed to enhance the sensitivity of L-glutamate as an on-line amplifier based on the substrate recycling, as shown schematically in Fig.4.

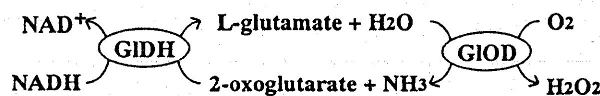


Fig. 4 Enzymatic substrate recycling of L-glutamate in the L-glutamate oxidase (GlOD)/glutamate dehydrogenase (GlDH) coimmobilized reactor, under the conditions in the presence of excess of NADH, oxygen, and ammonium.

To improve the substrate specificity of this reactor, acidic pretreatment and 6-diazo-5-oxo-L-norleucine treatment were done, because the trace amounts of NADH oxidase and glutaminase were contained as an impurity in the commercially available GlOD. The resulting enzyme reactor worked as a specific reactor of L-glutamate involving amplification (amplification factor was about 60). The cycle was also initiated with 2-oxoglutarate, and so enzyme column E_1 was positioned in series before the enzyme column E_2 to eliminate enzymatically 2-oxoglutarate in the presence of excess of NADH and L-lysine in the carrier buffer, corresponding to

2-oxoglutarate + L-lysine + NADH $\rightarrow$ saccharopine + NAD^+.

This column E_1 can decompose completely and selectively 2-oxoglutarate less than 0.3 mM added to the L-glutamate solutions when used as the eliminating column. By the present method, the variations of L-glutamate level released from rat brain cells were assayed in vivo. The average amounts of L-glutamate in the rat brain was 6.2 μM and the reproducibility of the measurement was 1.2% r.s.d (n=7) under the analytical speed of 20 dialysates h^{-1}. This method was also applied to monitor the change in the L-glutamate concentrations in the rat brain as a result of KCl stimulation. As a result, the increase of the L-glutamate concentration in the rat brain was caused by the physiological action of KCl permeated from the dialysis probe into the extracellular space.

3. The use of biosensors as molecular recognition sensor

3.1. Simultaneous in vivo monitoring of glucose, L-lactate, and pyruvate concentrations in rat brain [18, 19]

Most of FIA methods with microdialysis sampling have generally been used for the determination of a single analyte. In many cases, however, it is preferable to have the opportunity to measure plural analytes from the dialysate sample, e.g., in the study of metabolic disorders. Glucose is a main nutrient in the brain, while L-lactate and pyruvate are formed from glucose in an actively glycolyzing system under anaerobic and aerobic conditions, respectively, and are of interest in metabolic disorders. Therefore, the simultaneous on-line monitoring of glucose, L-lactate, and pyruvate in the brain would be of great benefit for studies on glucose metabolism in the brain.

The analytical system is similar to that shown in Fig. 3 and is based on the combination of a microdialysis sampling system and a flow-injection system with a triple enzyme electrode arranged in perpendicular for the flow direction. The triple enzyme electrode is constructed by hybridizing a poly(1,2-diaminobenzene) film to three sensing parts modified with the GOD, lactate oxidase (LOD), or pyruvate oxidase (POD) membrane cross-linked with gelatine using glutaraldehyde, which respond selectively to glucose, L-lactate, and pyruvate, respectively, without any cross-reactivity. As the optimum carrier buffer 0.1 M phosphate buffer at pH 6.5 was selected and pumped at a flow rate of 0.2 ml min^{-1}. Also, when the flow rate of the Ringer's solution was 2 μl min^{-1}, the permeability into the probe was 13.3% for glucose, 21.4% for L-lactate, and 22.1% for pyruvate, respectively. The differences of the permeability for three analytes are dependent on those in their molecular size, molecular structure, and hydrophilicity. Under these flow conditions, the optimum time intervals of the load and injection were 120 and 10 s, respectively, and up to 28 dialysates h^{-1} could be analyzed. The three sensing parts of the triple enzyme electrode responded linearly to the concentrations of glucose, L-lactate, and pyruvate between 0.1 and 10 mM, without any interference from oxidizable species (urate, ascorbate, and cysteine) present in serum and in rat brain.

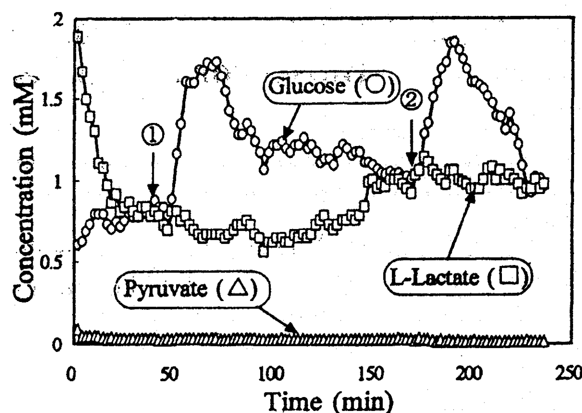


Fig. 5 Time-course of glucose, L-lactate, and pyruvate concentrations in rat brain. A 1.0 ml aliquot of 0.3 g ml^{-1} and 0.6 g ml^{-1} glucose solutions were injected into the abdomen of the same rat at arrow ① and arrow ②, respectively.

The proposed FIA method can be successfully applied to the simultaneous in vivo assay of three analytes in the rat brain, by implanting a microdialysis probe into the frontal lobe of the rat. The time courses of glucose, L-lactate, and pyruvate concentrations in the rat brain are shown in Fig. 5. The average amounts of glucose, L-lactate, and pyruvate in the extracellular

space of the rat brain were about 0.8 mM for each of glucose and L-lactate and less than 0.1 mM for pyruvate, respectively, although their concentrations varied much less with the elapse of time. When a 1 ml aliquot of 0.3 g ml^{-1} glucose was injected into the abdomen of the rat, the signal current of glucose increased up to a current corresponding to 1.7 mM, and then after 30 min it decreased as shown in Fig. 5. Furthermore, a 1 ml aliquot of 0.6 g ml^{-1} glucose was injected similarly into abdomen of the same rat, but the glucose concentration in the rat brain was not increased beyond 1.8 mM. This means the presence of a "blood brain barrier" system, which is one of the physiological protection systems of the brain. However, the L-lactate and pyruvate concentrations, although the L-lactate concentration increased much less when the rat was in motion, were not influenced by glucose injections, and kept almost constant during all periods of the measurement.

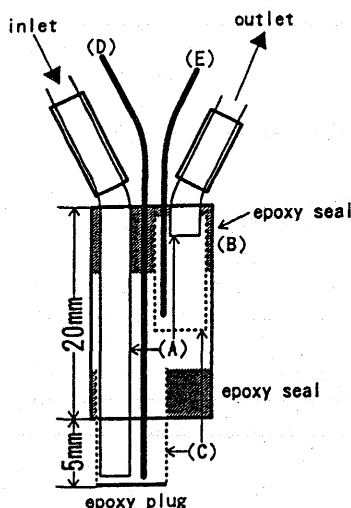


Fig. 6 Schematic diagram of microdialysis electrode. A: fused silica tube (o.d. 105 μm, i.d. 40 μm), B: fused silica tube (o.d. 440 μm, i.d. 350 μm), C: dialysis fiber (o.d. 220 μm, i.d. 200 μm), D: platinum fiber (50 μm), E: silver/silver chloride fiber (50 μm).

3.2. Microdialysis fiber enzyme electrode for in vivo monitoring [20, 21]

The flow-injection biosensor systems with a microdialysate sampling were well suited for the on-line assay of a particular analyte in the dialysate with complex matrices. However, such a flow-system is not strictly a real-time monitoring process, because of the delay time between sampling and detection. In this research, we prepared a microdialysis fiber enzyme electrode which makes the possible real-time and in vivo monitoring. The electrode consists of a platinum fiber as a working electrode and a silver / silver chloride fiber as a reference electrode inserted into the tip and in the upper compartment of the dialysis fiber, respectively, as shown in Fig. 6. The platinum fiber was coated electrochemically with a poly(1,2-diaminobenzene) film, which permitted the selective detection of low molecular weight hydrogen peroxide generated enzymatically. We selected glucose electrodes using GOD as a typical model of the sensor of this type. The measurement was first done by holding stationary a soluble enzyme solution in the probe cavity, and then by the electrode with the enzyme layer immobilized on the polymer-coated platinum fiber. For the fiber electrode with the soluble enzyme, the response current was proportional to the concentration of glucose over the range of 5×10^{-6} - 1×10^{-3} M. This type of sensor has the advantages that the lifetime of the sensor can be extended by infusing in fresh enzyme solution when required and that a variety of sensors can be easily constructed by infusing the solution of the suitable hydrogen peroxide-producing enzymes without tedious immobilization procedures. In contrast, the sensitivity of the fiber electrode with the immobilized enzyme to glucose was about 83% compared to that of glucose electrode with the soluble enzyme.

3. Conclusion

The present flow-injection biosensor systems with an on-line microdialysis sampling are very selective and useful for the simultaneous in vivo monitoring of biomolecules, without any interference from oxidizable species (such as ascorbate, urate, and cysteine) and proteins present in serum and in the extracellular space of the rat brain. Furthermore, these methods can be expected to be applied to the in vivo monitoring of a variety of biomolecules at other parts of the living cells such as blood stream, subcutaneous tissue, and muscle, and furthermore to the specific detection of a single analyte or the simultaneous detection of plural analytes in a variety of foods.

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