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Multi-enzyme microreactor-based online electrochemical system for selective and continuous monitoring of acetylcholine

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Abstract. This study demonstrates an online electrochemical system (OECS) for selective and continuous measurement of acetylcholine (ACh) through efficiently integrating in vivo microdialysis, multi-enzyme microreactor and electrochemical detector. The multi-enzyme microreactor was prepared by first co-immobilizing two kinds of enzymes, i.e., choline oxidase (ChOx) and catalase (Cat) onto magnetite nanoparticles and then confining the as-formed nanoparticles into a fused-silica capillary with the assistance of external magnet. The multi-enzyme microreactor was settled between in vivo microdialysis sampling system and electrochemical detector to suppress the interference from choline toward ACh detection. Selective detection of ACh was accomplished in the electrochemical detector with ACh esterase (AChE) and ChOx as the recognition units for ACh and Prussian blue (PB) as the electrocatalyst for the reduction of hydrogen peroxide (H_2O_2). The current recorded with the OECS was linear with the concentration of ACh (I / nA = -3.90C_{ACh}/μM + 1.21, γ = 0.998) within a concentration range from 5 μM to 100 μM. The detection limit, based on a signal-to-noise ratio of 3, was calculated to be 1 μM. Interference investigation demonstrates that the OECS did not produce an observable current response toward physiological levels of common electroactive species, such as ascorbic acid (AA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and uric acid (UA). The high selectivity and the good linearity in combination with the high stability may enable the OECS developed here as a potential protocol for continuous monitoring of cerebral ACh release in some physiological and pathological processes.

Introduction

Acetylcholine (ACh) has been known as one kind of the most important neurotransmitters, which is involved in neurotransmission processes in both the peripheral and central nervous systems.[1-3] Besides, it plays an important role in neurological diseases and cognitive functions. For instance, researches have demonstrated that the decrease of ACh in the cerebral system made individuals prone to various nerve disorders including Alzheimer's disease and multiple sclerosis.[4-6] To date, liquid chromatography and capillary electrophoresis combined with different detectors (mainly electrochemical detector) have been employed for the measurements of ACh in the cerebral systems with the coexistence of physiological levels of Ch and other kinds of physiologically import species.[7-10] In addition to the involvement of separation procedure for differentiating ACh in the extracellular fluid, the poor electrochemical property of ACh essentially necessitated an online enzyme column to convert ACh into electrochemically detectable species.[11-13] These procedures, on one hand, make the analytical systems complicated and, on the other hand, lower the time resolution of the analysis.[14-18] In this context, a non-separation method for continuous measurement of ACh in the cerebral system is highly demanded.

In order to continuously monitor physiologically important species in rat brain such as glucose/lactate,[17] and Ca^{2+}/Mg^{2+},[18] in the cerebral systems, we have recently developed online electrochemical systems (OECSs) by efficiently combining in vivo microdialysis sampling with selective electrochemical detection. Compared with separation-based methods used for the neurochemical measurements, OECSs possess near real-time nature and short analysis time.[14] Although the striking properties of OECSs enable them to be physiologically and pathologically useful, the avoidance of sample separation procedures makes the selectivity for this analytical protocol a great challenge. This is the case particularly for the ACh measurement since the concentration of Ch is much higher than that of ACh, and both

ACh and Ch react enzymatically at an AChE and ChOx-modified electrode or an immobilized reactor. Generally, Ch and ACh could be removed and monitored, respectively, through following serial reactions under the catalysis of enzymes.[19]

$$ACh + H_2O \xrightarrow{AChE} Ch + CH_3COOH \quad (1)$$

$$Ch + O_2 + 2H_2O \xrightarrow{ChOx} Betaine + 2H_2O_2 \quad (2)$$

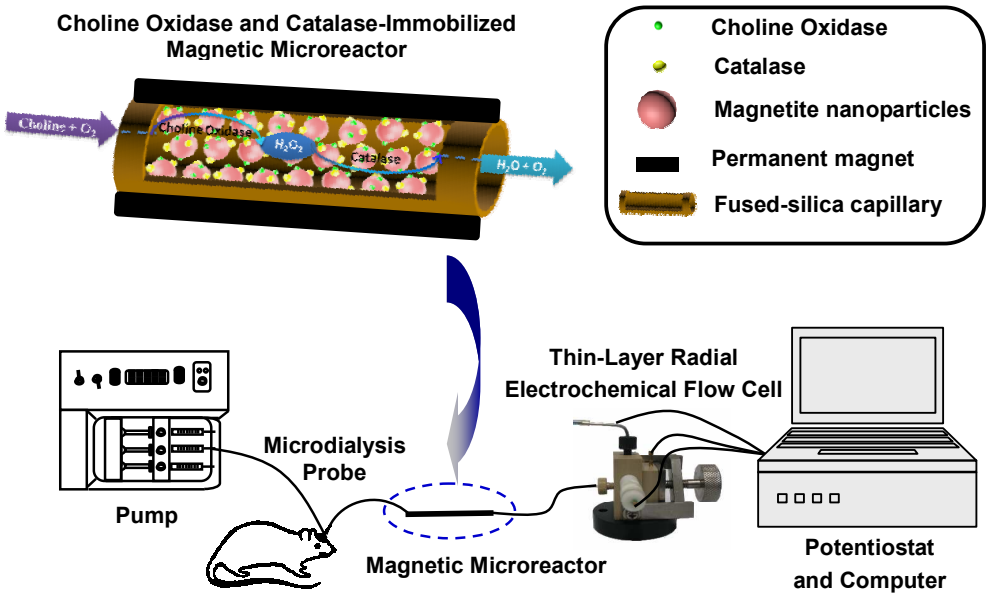
$$2H_2O_2 \xrightarrow{Cat} 2H_2O + O_2 \quad (3)$$

Based on reactions 2 and 3, Niwa, *et al*. have successfully developed an online analytical system for selective continuous measurement of ACh in the cultured brain slice, in which a small-volume enzymatic pre-reactor in a 5-cm-long Teflon tube packed with ChOx and Cat separately immobilized on beads and serially packed in the tube to remove Ch.[20] Although such a pre-reactor enabled ACh detection was advantageous over the conventional ACh measurement with LC, which normally required half an hour and was not a continuous measurement, it still suffered from relatively long, i.e., about 6 min time delay caused by the dead volume between a sampling capillary and the electrode in the flow cell, which mainly came from the dead volume of the prereactor (22 μL). Such a dead volume might made this enzymatic pre-reactor not fit well with microdialysis sampling technique to form an OECS for sensitivity measurements of neurochemical in a near real time nature.

In our early study, we have described an effective method to fabricate an enzyme microreactor to remove ascorbic acid and 3,4-dihydroxyphenylacetic acid by immobilizing laccase onto magnetic Fe_3O_4 nanoparticles and confining the laccase-immobilized nanoparticles into a fused-silica capillary with an external magnet.[21, 22] The laccase-based microreactor was demonstrated to show a high efficiency to remove the substrate from the systems with a readily renewable feature. Inspired by this, this study demonstrates a

multi-enzyme microreactor by immobilizing two kinds of enzymes, i.e., ChOx and Cat onto Fe_3O_4 nanoparticles and confining the magnetic nanoparticles into a fused-silica capillary for removing Ch and thus eliminating its interference toward ACh measurement. In addition, unlike the methods described previously for the electrochemical reduction and sensing of H_2O_2 for ACh measurements with the use of horseradish peroxidase (HRP) that can catalyze the reaction between ascorbic acid (AA) and H_2O_2 and thus constitute interference towards the determination of ACh,[20, 23, 24] this study utilizes Prussian blue (PB) as the electrocatalyst for the detection of H_2O_2 produced from two enzymatic reactions. As the artificial peroxidase, PB efficiently catalyzes the reduction of H_2O_2, allowing the detection of H_2O_2 occur at a potential far from the redox potentials of electroactive species commonly existing in the cerebral systems, such as AA, uric acid, and 3,4-dihydroxyphenylacetic acid, as reported previously.[25-28]

By using the multi-enzyme prereactor and the PB-based biosensor, we demonstrate here an OECS for continuous and selective monitoring ACh by efficiently converting Ch into electrochemically inert species into a multi-enzyme microreactor and using an enzyme-based biosensor as a selective detector for ACh. The novelty of this study lies in the fabrication of a highly efficient and readily renewable multi-enzyme microreactor containing the immobilized choline oxidase and catalase to remove Ch and in the use of PB as the electrocatalyst to replace horseradish peroxidase (HRP) for the reduction of H_2O_2 for ACh measurement, as shown in Scheme 1. The OECS demonstrated here is selective, reproducible and stable and could find some interesting physiological and pathological application associated with ACh release.



Scheme 1. Schematic illustration of conversion of Ch in the multi-enzyme microreactor containing choline oxidase and catalase and of an OECS for continuous ACh measurement consisting of in vivo microdialysis, multi-enzyme microreactor, and electrochemical biosensor detector.

Experimental

Chemicals and reagents

Acetylcholine (ACh), choline (Ch), dopamine (DA), sodium ascorbate (AA), uric acid (UA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-aminopropyl triethoxysilane, bovine serum albumin (BSA), choline oxidase (ChOx, from Alcaligenes species, EC 1.1.3.17), acetylcholine esterase (AChE, from bovine erythrocytes, EC 3.1.1.7) and catalase (Cat, from bovine liver, EC 1.11.1.6) were all purchased from Sigma. H_2O_2 was purchased from Beijing Chemical Company (Beijing, China). Other chemicals were of at least analytical reagent and were used as received. Aqueous solutions were prepared with doubly distilled water. Artificial cerebrospinal fluid (aCSF) used as the perfusion solution for in vivo microdialysis

was prepared by mixing NaCl (126 mM), KCl (2.4 mM), KH_2PO_4 (0.5 mM), $MgCl_2$ (0.85 mM), $NaHCO_3$ (27.5 mM), Na_2SO_4 (0.5 mM), and $CaCl_2$ (1.1 mM) into doubly distilled water.

Preparation of ChOx and Cat-immobilized magnetic microreactor

Magnetite nanoparticles used for co-immobilization of ChOx and Cat were prepared with a method reported in our study previously.[21] Briefly, under magnetic stirring, 50 mL of aqueous suspension of $Fe(OH)_2$ was prepared by adjusting the pH value of aqueous solution of 0.10 M $FeCl_2$ to 7.8 with 1.0 M KOH solution. Then, 500 μL of the aqueous solution of H_2O_2 (3 wt%) was added into the $Fe(OH)_2$ suspension, producing black and magnetic precipitate. With the assistance of magnetic separation, the magnetic particles were washed with 3 × 50 mL distilled water and 3 × 50 mL acetone, and finally dried at room temperature. The synthesized iron oxide nanoparticles were functionalized with amino groups by mixing 200 μL of 3-aminopropyl triethoxysilane into 200 mL of aqueous dispersion of iron oxide nanoparticles (0.5 mg/mL), and the resulting mixture was continuously stirred for 8 h at room temperature. The nanoparticles were separated by magnetic decantation, rinsed with 3 × 70 mL ethanol, and dried in air to form amino-functionalized iron oxide nanoparticles. The amino-functionalized magnetic nanoparticles (10 mg) were dispersed into 2 mL of 10% glutaraldehyde solution in phosphate buffer (0.10 M, pH 7.4) and the mixture was continuously sonicated for 4 h. After that, the nanoparticles were separated by magnetic decantation, washed with distilled water and dispersed into 2 mL phosphate buffer (0.10 M, pH 7.0). To immobilize ChOx and Cat onto the magnetic nanoparticles, 1 mg ChOx and 5 mg Cat were mixed into the suspension of the as-prepared nanoparticles and the resulting mixture was gently stirred overnight. The nanoparticles were separated by magnetic decantation and washed with phosphate buffer (4 × 2 mL) to give ChOx and Cat-immobilized magnetite nanoparticles. Finally, the ChOx and Cat-immobilized

nanoparticles were dispersed into 1 mL phosphate buffer (0.10 M, pH 7.0) and the suspension was directly perfused into a capillary to prepare the magnetic microreactor.

A fused-silica capillary (75-μm i.d., 150-μm o.d., 2.5-cm long) was used to accommodate the ChOx and Cat-immobilized magnetite nanoparticles to form a magnetic microreactor. One pair of Nd-Fe-B permanent magnet (3 mm × 3 mm × 30 mm) was assembled on both side of the fused-silica capillary to firmly attract and fix ChOx and Cat-immobilized magnetite nanoparticles onto the inside of the capillary (Scheme 1). A 1 mL of the prepared ChOx and Cat-immobilized magnetite nanoparticles was pumped into the capillary and the nanoparticles were stably packed onto the inner wall of the capillary.

Electrodes and thin-layer flow cells

The electrode polishing and pretreatment, PB modification on glassy carbon (GC, 6-mm diameter) electrode, and thin-layer radial electrochemical flow cell equipment processes were performed as reported in our previous studies.[21,25] Briefly, GC electrodes were polished with aqueous slurries of fine alumina powder (0.3 and 0.05 μm) on a polishing cloth and rinsed with acetone and doubly distilled water under an ultrasonic bath, each for 5 min. Before PB modification, GC electrodes were electrochemically activated at +1.7 V for 180 s in 0.10 M KCl solution buffered with 0.05 M phosphate buffer (pH 7.4). The PB-modified GC electrodes were prepared by drop-coating 5 μL aliquot of 10 mM HCl solution containing 50 mM potassium ferricyanide and 50 mM ferric chloride onto the electrodes. After drying under ambient temperature, the electrodes were sequentially rinsed with 10 mM HCl and doubly distilled water, and then heated at 100 ºC for 1 h. After that, the electrodes (PB-modified electrodes) were performed with cyclic voltammetry in 0.10 M KCl solution buffered with 0.05 M phosphate buffer (pH 7.4) until a stable voltammetric response was obtained.[25] For co-immobilization of AChE and ChOx onto the PB-modified GC electrode, 10 µL of AChE (10 mg/mL) in 0.10 M phosphate buffer (pH 7.0), 10 µL of

ChOx (10 mg/mL) solution in 0.10 M phosphate buffer (pH 7.0), 5 μL of 1% BSA, and 7 μL of 1% gluteraldehyde aqueous solution were gently mixed and the resulting mixture was totally coated onto the PB-modified GC electrodes. After being air-dried and rinsed with distilled water, the electrodes (denoted as AChE/ChOx/PB/GC electrodes) were fixed into the thin-layer radial electrochemical flow cell for online electrochemical measurements. The thin-layer radial electrochemical flow cell consists of a thin-layer radial flow block with a 50-μm gasket, the AChE/ChOx/PB/GC electrode as working electrode, stainless steel as auxiliary electrode, and Ag/AgCl electrode (3 M NaCl) as reference electrode. The thin-layer radial electrochemical flow cell was stored at 4 °C while not in use.

Apparatus and OECS setup

The integration of magnetic microreactor, electrochemical detection, and in vivo microdialysis to form an OECS for continuous measurements of ACh in the brain of freely moving rats was schematically shown in Scheme 1. Standard solutions or brain microdialysates were delivered from gas-impermeable syringe (BAS) and pumped through tetrafluoroethylene hexafluoropropene (FEP) tubing by a microinjection pump (CMA 100, CMA Microdialysis AB, Stockholm, Sweden) to the radial flow cell. Online electrochemical measurements of ACh was performed with a computer-controlled electrochemical analyzer (CHI 1030, Shanghai, China) in a thin-layer radial flow cell with AChE/ChOx/PB/GC electrode as working electrode at -0.05 V *vs*. Ag/AgCl.

Online measurements of ACh in brain microdialysate

The procedures for animal surgery and in vivo microdialysis as well as online measurements of brain microdialysate were similar to those reported in our early study.[29] Briefly, adult male Sprague-Dawley (250-300 g) rats were obtained from Center for Health Science, Peking University. Animal experiments were performed under the collaboration with our collaborators from Peking University Third Hospital and were conformed to the

"Guide for Care and Use of Laboratory Animals" of Peking University biomedical ethics committee. The animals were anaesthetized with chloral hydrate (345 mg/ kg, i.p.). The microdialysis guide cannulas (BAS/MD-2250, BAS) was implanted into the striatum (AP = 0 mm, L = 3.0 mm from bregma, V = 4.5 mm from the surface of the skull) using standard stereotaxic procedures.[30] The guide cannula was fixed with three skull screws and dental cement. During the whole surgery, body temperature of the animals was maintained at 37 °C with a heating pad and, after surgery, the rats were placed into a warm incubator until they recovered from the anesthesia. After at least 24 h recovery of rats, the microdialysis probes (BAS; dialysis length, 4 mm; diameter, 0.24 mm) were implanted into rat striatum and were perfused with aCSF at 1 μL/min for at least 90 min for equilibration and then online measurements were performed.

Results and discussion

The surface morphologies of the synthesized magnetic nanoparticles were characterized by scanning electron microscope imaging (SEM), which shows that the synthesized Fe_3O_4 particles have a size of 20-50 nm (data not shown). The large surface area of the magnetic nanoparticles facilitates a large amount of ChOx and Cat enzyme loading onto the surface of the magnetic nanoparticles, resulting in a high Ch converting efficiency. In addition, the three-dimensional structure of the synthesized Fe_3O_4 particles enables a large pore and channel structures among the magnetic nanoparticles, greatly enhancing the permeability and reducing the flow resistance when Ch solution flowing through the magnetic microreactor with the ChOx and Cat-modified magnetic nanoparticles packed inside. The enzyme-modified magnetic particles could be easily removed from the microreactor by removing the external magnet and pumping pure water through the microreactor at a high flow rate, for example, 20 μL/min.

In mammalian central nervous system (CNS), Ch is considered as one of the main products of ACh degradation by the AChE and ChOx, and exists at a much higher level (micromolar range) than ACh (nanomolar range) in the CNS.[19, 31, 32] As a result, the selective online electrochemical determination of ACh with the presence of Ch remain a great challenge since, at an AChE and ChOx-modified electrode, Ch can also be oxidized to produce H_2O_2 and thus interfere with ACh detection, according to the reactions in equation 2. Thus, the removal of Ch in the brain microdialysates remains very essential for ACh measurement. In this study, the efficiency of the enzyme microreactor for Ch removal was investigated. As displayed in Fig. 1, the perfusion of 10 μM Ch into the OECS without the ChOx and Cat-immobilized magnetic microreactor obviously results in a large current response, demonstrating Ch is enzymatically oxidized by ChOx and the produced H_2O_2 is electrochemically reduced at the AChE/ChOx/PB/GC electrode. On the contrary, the introduction of the ChOx and Cat-immobilized magnetic microreactor into the OECS results in almost no obvious current response for 10 μM Ch, demonstrating an efficient consumption and thereby removal of Ch by the microreactor, in which Ch is converted into H_2O_2 by ChOx and H_2O_2 is subsequently converted into H_2O by Cat. Such a property of the microreactor demonstrates that ChOx and Cat were successfully immobilized on the surface of magnetic particles for complete removal of high concentration of Ch before the online measurement of ACh. In addition, no current response was recorded for Ch (10 μM) after continuously running the experiments with the microreactor for at least 1 h (data not shown). Moreover, the microreactor could be used discontinuously for the removal of Ch at least 10 days (3-6 hours each day) or after its storage at 4 °C for two weeks (data not shown). These results demonstrate that the microreactor was quite stable for Ch removal, which eventually validates the selective ACh measurement.

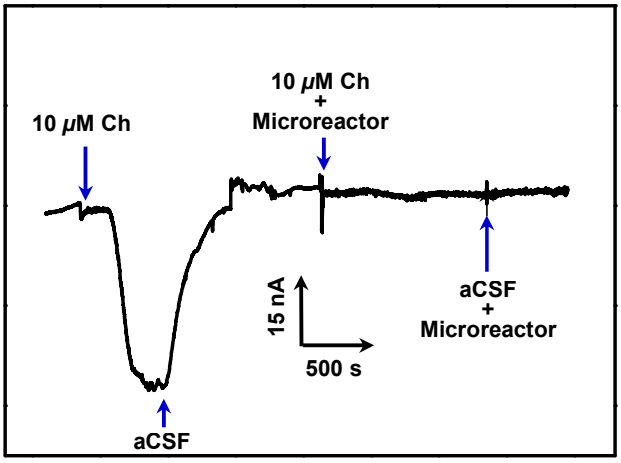


Fig. 1 Typical current-time response recorded for Ch (10 μM) at the AChE/ChOx/PB/GC electrode fixed in the thin-layer radial flow cell with ChOx and Cat-immobilized magnetic microreactor in the upstream. The electrode was polarized at -0.05 V *vs*. Ag/AgCl electrode. The aCSF was used as the perfusion solution. Flow rate, 1 μL/min.

In addition to Ch, other kinds of neurochemicals coexisting in the cerebral systems including AA, DA, DOPAC, and UA may also interfere with the ACh measurements in the OECS.[33-35] Thus, the selectivity of multi-enzyme microreactor-based OECS against these species was investigated. As displayed in Fig. 2, when the AChE/ChOx/PB/GC electrode was polarized at -0.05 V, the OECS did not produce an obvious current response toward 20 μM AA, 5 μM DA, 10 μM DOPAC, or 20 μM UA, but a relatively large reduction current response toward 10 μM ACh, indicating that the OECS developed here for ACh measurements is essentially interference-free.

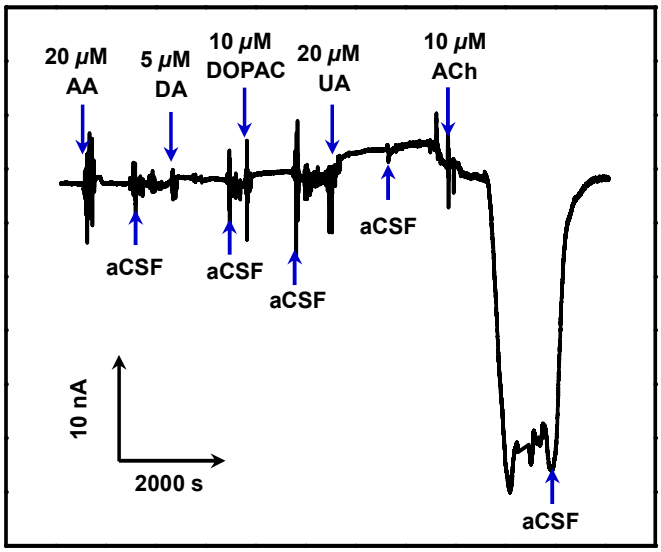


Fig. 2 Typical amperometric response recorded for AA (20 μM), DA (5 μM), DOPAC (20 μM), UA (20 μM) and acetylcholine (20 μM) at the AChE/ChOx/PB/GC electrode fixed in the thin-layer radial flow cell with ChOx and Cat-immobilized magnetic microreactor in the upstream. Other conditions were the same as those in Fig. 1.

Fig. 3 displays typical current-time responses for ACh produced by the OECS with ChOx and Cat-immobilized magnetic microreactor in the upstream of the thin-layer flow cell detector. Well-defined current responses were recorded for ACh, suggesting the good conversion efficiency of AChE and ChOx toward ACh and high electrocatalytic activity of Prussian blue (PB) for the reduction of H_2O_2 produced from the enzymatic reactions. The current was linear with the concentration of ACh (I / nA = -3.90C_{ACh}/μM + 1.21, γ = 0.998) within a concentration range from 5 μM to 100 μM. The limit of detection was calculated to be 1 μM, based on a signal-to-noise ratio of 3.

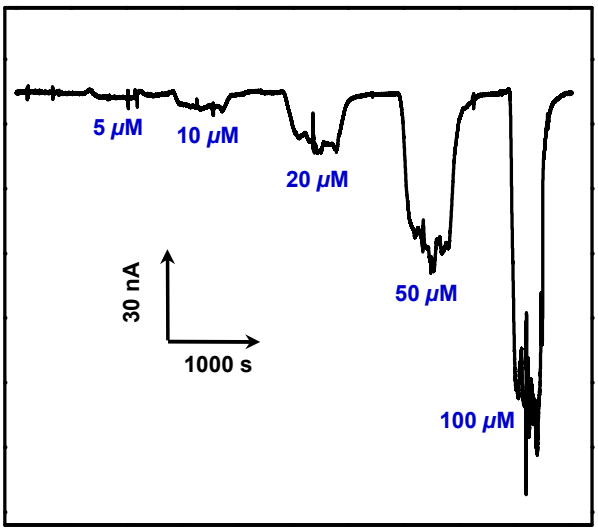


Fig. 3 Typical current-time response for ACh standards (concentrations indicated in the figure) produced by the OECS with the AChE/ChOx/PB/GC electrode fixed in the thin-layer radial flow cell as the detector and with a ChOx and Cat-immobilized magnetic microreactor in the upstream. Other conditions were the same as those in Fig. 1.

In addition to the good linearity, the OECS also shows a good reproducibility, which was estimated through consecutively measuring ACh (50 μM) in the continuous-flow cell with the ChOx and Cat-immobilized magnetic microreactor in the upstream for 7 times, as displayed in Fig. 4 A. The relative standard deviation was calculated to be 0.8%. Stability of the OECS was investigated by continuously sensing ACh in the continuous-flow electrochemical cell with the ChOx and Cat-immobilized magnetic microreactor, as depicted in Fig. 4 B. The current responses recorded for ACh (50 μM) remain unchanged after continuously running the measurements for more than 1 h, indicating that the high reliability of the AChE/ChOx/PB/GC electrode as the detector in the continuous-flow system. These properties substantially enable the OECS developed here useful for selective and reliable monitoring of ACh release in the rat brain, as demonstrated below.

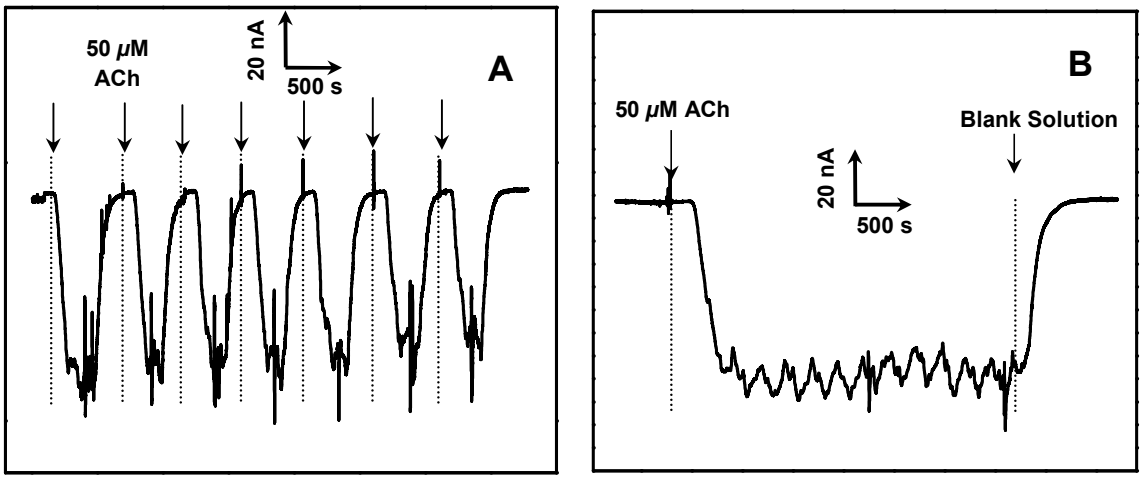


Fig. 4 Typical amperometric responses repeatedly (A) and continuously (B) recorded for 50 μM ACh standards with the OECS with the AChE/ChOx/PB/GC electrode equipped in the thin-layer radial flow cell and with a ChOx and Cat-immobilized magnetic microreactor in the upstream. Other conditions were the same as those in Fig. 1.

Fig. 5 shows a typical recording for the microdialysate continuously sampled from the striatum of the rat brain with the OECS. Only a small current response was obtained for the microdialysate, implying the basal level of ACh in the striatum microdialysate was not detectable with our OECS mainly because of the low basal level of ACh in the cerebral systems.[36,37] To demonstrate the potential application of the OECS for the measurements of ACh release, the microdialysates were in vitro spiked with ACh standards. As shown in Fig. 5, the current responses recorded for the in vitro spiked samples were almost the same as those for ACh standards, suggesting that the OECS demonstrated here was selective for the measurements of microdialysate ACh. These demonstrations further suggest the OECS developed in this study could be potentially used for continuously monitoring cerebral ACh release in some physiological and pathological processes, such as the acquisition of important neurosteroids, cocaine and amphetamine, in which the concentration of ACh was largely increased, in relative to the basal level.[36,37]

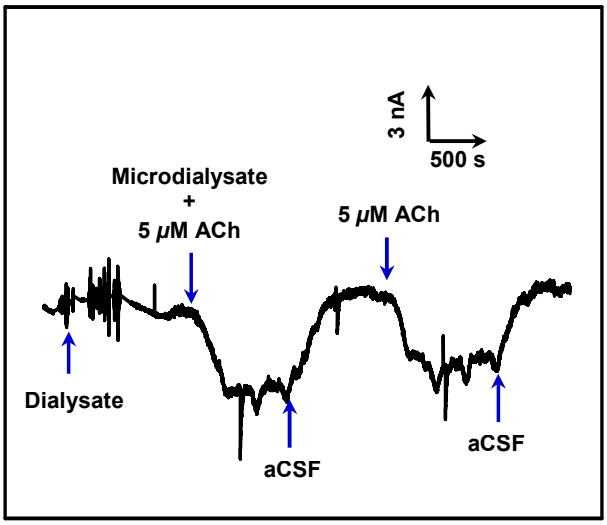


Fig. 5. Typical amperometric responses obtained with the OECS for pure brain microdialysate continuously sampled from the striatum of rats, microdialysate spiked with ACh, and ACh standard. Other conditions were the same as those in Fig. 1.

The use of ChOx and Cat-immobilized magnetic microreactor to eliminate Ch employed for the OECS of ACh in this work is remarkable since the use of magnetic nanoparticles enables the easy and stable immobilization of enzymes on the inner walls of the microreactor, and thus allows an easy renewal of enzyme-modified magnetic nanoparticles. Most importantly, the dead volume of the ChOx and Cat-immobilized magnetic microreactor remains very small, which greatly shortens the time delay to less than 1 min between a microdialysis probe and the electrode in the flow cell. Though biomolecules such as enzyme, oligonucleotides and antibody can be connected to the magnetic nanoparticles and enzyme-based microreactors offer several useful properties in terms of high conversion efficiency and biocomponent stability for numerous analytical applications, few research using enzyme-immobilized magnetic microparticles for OECS have been explored for in vivo analysis.[38,39] Accordingly, the strategy demonstrated here could be used as a new and

effective platform for development of new OECSs with various enzyme-modified magnetic microreactors for continuous measurements of physiologically important species.

Conclusion

By efficiently integrating the ChOx and Cat-immobilized magnetic microreactor, AChE and ChOx/PB-modifed GC electrode, and in vivo microdialysis sampling technique, we have demonstrated a new OECS for selective measurements of ACh in the presence of Ch and other kinds of neurochemicals. The introduction of the ChOx and Cat-immobilized magnetic microreactor into the OECS system successfully eliminates the interference of Ch to ACh by sequential enzymatic reactions. The OECS demonstrated in this study is selective, stable and reliable and could find interesting applications in some physiological and pathological investigations.

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