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Online electrochemical systems for continuous neurochemical measurements with low-potential mediator-based electrochemical biosensors as selective detectors

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Abstract

This study demonstrates a new strategy to develop online electrochemical systems (OECSs) for continuously monitoring neurochemicals by efficiently integrating in vivo microdialysis with an oxidase-based electrochemical biosensor with low-potential electron mediators to shuttle the electron transfer of the oxidases. By using thionine and xanthine oxidase (XOD) as the examples for low-potential mediators and oxidases, respectively, we demonstrate that the use of low-potential mediators to shuttle the electron transfer of oxidases would offer a new approach to the development of oxidase-based biosensors with theoretical and technical simplicity. To construct the XOD-based biosensor, thionine was adsorbed at carbon nanotubes and used to shuttle the electron transfer of XOD. The XOD-based biosensor was positioned into an electrochemical cell that was directly coupled with in vivo microdialysis to form an online electrochemical system (OECS) for continuous and selective measurements of the substrate of XOD (with hypoxanthine as an example). The OECS based on the low-potential mediators is highly selective against the species endogenously existing in the brain system, which is attributed to the low operation potential benefited from the low redox potentials of the mediators. Moreover, the OECS demonstrated here is stable and reproducible and could thus be envisaged to find some interesting applications in physiological and pathological investigations. This study essentially offers a new strategy to develop online electrochemical systems, which is of great importance in understanding the molecular basis of physiological and pathological events.

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Introduction

In vivo quantitative monitoring of neurochemicals involved in physiological and pathological events in the cerebral systems is of great importance in understanding of the molecular basis of brain functions.[1-7] In general, two categories of analytical methods have been employed to pursuit such a purpose. One is in vivo (bio)sensing that employs electrochemical or optical probes with a small size to directly real-time monitor neurochemical change in the brain.[6, 8-11] The other is in vivo sampling-based neurochemical analysis, which is carried out through the combination of in vivo sampling either with sample separation and subsequent detection or directly with selective online detection.[7, 12, 13] Motivated by the long-term interests in understanding of the chemical essence underlying the physiological and pathological processes, we have been interested in the development online electrochemical systems (OECSs) for continuous measurements of physiologically important species in the brain of living animals by integrating in vivo microdialysis with direct electrochemical detection (i.e., no sample collection or separation).[14-18] Compared with the separation-based in vivo methods, OECSs have been demonstrated to be particularly attractive because OECSs possess near real-time nature, short analysis time but with less technique demanding owing to the avoidance of sample collection and separation. Compared with in vivo (bio)sensing for the neurochemical measurements, OECSs could be developed for simultaneous monitoring of multiple neurochemicals with easy physiologist-adaptability and compatibility with other neurotechnologies, such as electrophysiology and optical genetics.

Although the striking properties of OECSs enable them physiologically and pathologically useful, the avoidance of sample separation procedures essentially makes the selectivity a challenge in the development of such methodology. Enzyme-based electrochemical biosensors

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could provide a high selectivity for the electrochemical detection in the OECSs because this kind of biosensors combines the advantages benefited from the high substrate specificity of enzymes and from the electrochemical signal readout systems. For example, by taking advantages of high selectivity of enzyme-based biosensors, we and others have successfully developed OECSs for continuous monitoring of physiologically important species such as glucose/lactate,[14-16] acetylcholine,[19] and so forth in the cerebral systems. Among all kinds of enzymes, flavin adenine dinucleotide (FAD)-containing oxidases are most commonly used for the construction of the electrochemical biosensors with different electron transducing pathways, of which the effective biosensing of substrate necessitates an efficient electronic communication/connection between oxidases and electrode.[20-24] Due to the difficulties in conducting the direct electron transfer of the oxidases since the redox center is deeply embedded in the oxidases, the oxidase-based electrochemical biosensors have been mainly developed by using O_2 as the co-substrate (i.e. so-called first-generation biosensors) or artificial mediators as electron acceptors for the oxidases (i.e. so-called second-generation biosensors). The first-generation biosensors have been developed based on detection of consumed oxygen or enzymatic reaction products, i.e., hydrogen peroxide.[14, 25] Compared with the first-generation biosensors, the mediated biosensors operate at low potentials through smartly designing electrode surface or choosing the appropriate artificial mediators[20, 23, 26] and, as a consequence, are more useful for in vivo selective neurochemical measurements.

In terms of the formal potentials of FAD/$FADH_2$ in most oxidases such as glucose oxidase and xanthine oxidase (less than -0.30 V *vs*. Ag/AgCl),[27-31] ferricyanide, ferrocence and its derivatives, and so forth are capable of mediating FAD/$FADH_2$ oxidation and these molecules have been used to shuttle the electron transfer of FAD-containing oxidases to construct mediated

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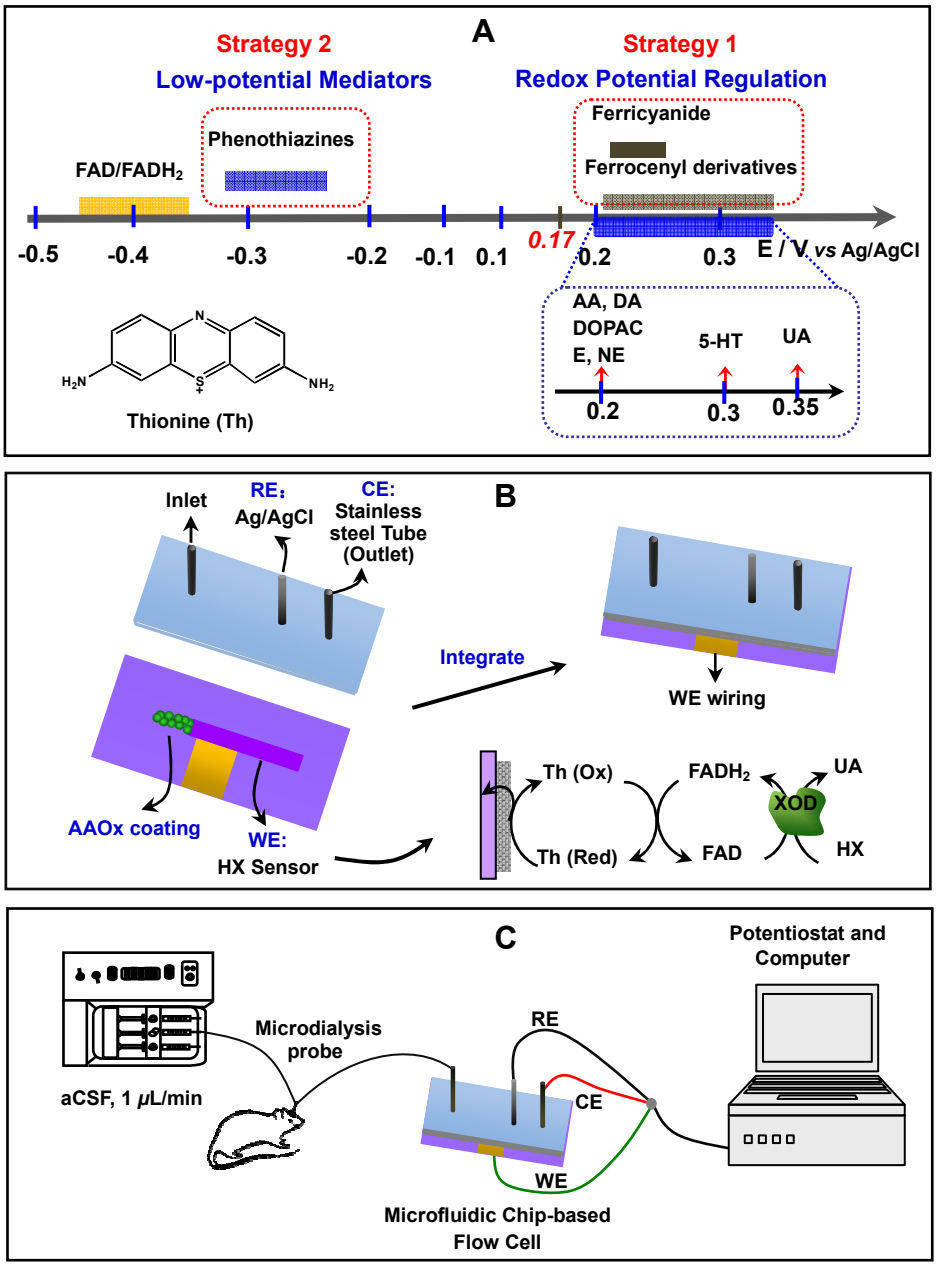

Scheme 1. Schematic illustration of A) strategies for construction of oxidase-based electrochemical biosensors in online electrochemical systems (OECSs) for continuous neurochemical monitoring. B) Figuration of microfluidic chip-based electrochemical flow cell and electrochemical and chemical reactions involved in the thionine-based biosensing of hypoxanthine. C) Continuous measurement of neurochemicals in rat brain with oxidase-based OECS by integrating the microfluidic chip-based flow cell with in vivo microdialysis.

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electrochemical biosensors. Nevertheless, the simple uses of these artificial mediators could not well meet the requirements of OECSs for continuous monitoring of neurochemicals in the brain since the formal potentials of these mediators generally overlap with, or even more positive than, those of the electroactive species commonly existing in the cerebral systems,[5] such as ascorbic acid (AA), uric acid (UA), catecholamine and their metabolites, as shown in Scheme 1. In such a case, strategies to negatively shift the redox potentials of the mediators remain very essential for developing OECSs. In this context, two strategies could be considered to lower the potentials of the artificial mediators; one is to regulate the potentials of the commonly used mediators. In such a way that the potentials could be negatively shifted, but still more positive than that of $FAD/FADH_2$ (Strategy **1**, Scheme 1). The other is to screen and thus use low-potential mediators (Strategy **2**, Scheme 1). For the Strategy **1**, we have demonstrated an approach to validate ferricyanide-based biosensors for selective online measurement of striatum glucose of guinea pigs based on regulation of the redox potential of ferricyanide through carefully controlling the different adsorption ability of ferricyanide and ferrocyanide on electrode surface with synthetic imidazolium-based polymer.[26] The good separation of the operation potential of the as-prepared biosensor from those for the oxidation of electroactive neurochemicals successfully enabled the ferricyanide-based biosensor as a selective detector for the OECSs.

To validate the Strategy **2** for the development of OECSs, we demonstrate in this study that the uses of low-potential mediators such as phenothiazines to enable the as-prepared biosensors to well serve as the selective detectors for the OECSs. As shown in Scheme 1 (Strategy **2**), some redox species, such as phenothiazine compounds have more positive potentials than that of $FAD/FADH_2$ and more negative potentials than the electroactive species in the cerebral system. The former property essentially enables these compounds as mediators to shuttle the electron

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transfer of oxidases at a low potential. The latter one leads to the online detection free from the interference from the electroactive species coexisting in the cerebral system. After integrating the oxidase-based biosensor into a microfluidic chip to form a microchip detector and directly coupling the as-formed microchip detector with in vivo microdialysis to form a new OECS, we find that the OECS demonstrated here with low-potential mediators for oxidases is selective, stable, and reproducible, and could thus be envisaged to find interesting applications in some physiological and pathological investigations. Moreover, the strategy demonstrated here may be developed to be versatile for the construction of new OECSs for continuous monitoring of neurochemicals in the cerebral systems, which is of great importance in understanding the molecular basis involved in brain functions.

Experimental

Chemicals and solutions

Hypoxanthine (HX), uric acid (UA), xanthine oxidase (XOD, EC 1. 1. 3. 22, from butter milk), ascorbate oxidase (AA_{OX}, EC 1. 10. 3. 3 from cucurbita species), and bovine serum albumin (BSA) were all purchased from Sigma and used as supplied. Thionine acetate was purchased from Alfa Aesar. Single-walled carbon nanotubes (SWNTs) were purchased from Shenzhen Nanoport Co. Ltd. (Shenzhen, China). Prior to use, the nanotubes were purified by refluxing the as-received samples in 2.6 M HNO_3 for 10 h, followed by centrifugation, re-suspension, and filtration and air-dry to evaporate the solvent. The purified SWNTs were further heated under Ar at 600 °C for 2 h. Artificial cerebrospinal fluid (aCSF) 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 and adjusting pH of the

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resulting solution to 7.4. Other chemicals were of at least analytical reagent and were used as received. All aqueous solutions were prepared with doubly distilled water.

Fabrication of microfluidic chip and preparation of XOD-based biosensor

Procedures for the microfluidic chip fabrication were described in Scheme 1 B. Pattern used in these experiments was designed by L-edit software, and a poly(methyl methacrylate) (PMMA) glass (60 mm × 60 mm) was used as the master of chip with desired pattern fabricated by mechanical microfabrication in Beijing Tonggongruidao Co. Ltd. A polydimethylsiloxane (PDMS) (Dow Corning, USA) master with positive surface patterns was produced by molding against the as-prepared PMMA glass master, as described previously.[32-34] The microfluidic channels were fabricated by replica molding from the master. The microchannels used in the experiments were of the following dimensions: width, 1 mm; depth, 0.5 mm; length, 30 mm. Inlets, outlets, and holes for placing reference electrode in the microfluidic channels were received by a sharpened needle tip.

An indium tin oxide (ITO)-coated glass plate was used as the substrate to prepare the XOD-based biosensor. Prior to the preparation, the ITO glass plates were first rinsed with ethanol, acetone, and doubly distilled water in an ultrasonic bath, each for 2 min, to provide a clean surface. After that, the parts for the biosensing and for wiring on the ITO glass were first protected by scotch tape, and then uniformly sprinkled with dry zinc powder. These parts were then subjected to an etching process by horizontally immersing the ITO glass into 4 mM hydrochloride acid at room temperature for about 20 s until the zinc layer was observed to lift off from the ITO glass. After that, the scotch tape was removed from ITO surface and the resulting ITO plate was sequentially rinsed in acetone and distilled water. A 2 mg/mL of the heat-treated SWNTs was dispersed into *N*, *N*-dimethylformmide, and 2 μL of the resulting

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dispersion was drop-coated onto ITO electrode. The SWNT-modified ITO was dried at ambient temperature and subsequently immersed in the aqueous solution of 200 μM thionine for about 2 h for saturated adsorption. The thionine/SWNT-modified ITO electrode was then rinsed with doubly distilled water and dried at ambient temperature. To prepare the XOD-based biosensor, 4 μL of aqueous solution of XOD (10 U/ mL) in 0.1 M phosphate buffer (pH 7.0) was mixed with 2 μL of aqueous solution of BSA (1%), and 2 μL of aqueous solution of glutaraldehyde (1%). The resulting mixture was totally coated onto the thionine/SWNT-modified ITO electrode. The electrode was then dried in air to obtain the XOD-based biosensor. To eliminate the interference from AA in the online measurements of cerebral hypoxanthine, on the upstream of the XOD-based biosensor, a mixture containing 4 μL AA_{OX} (1000 U/mL) dissolved in 0.1 M phosphate buffer (pH 7.0), 2 μL BSA dissolved in water (1%), and 2 μL glutaraldehyde dissolved in water (1%) was immobilized onto the blank ITO. After being air-dried, the PDMS replica with I shape microchannel was combined with the XOD/thionine/SWNT-modified ITO plate using extra pressing force to form a microfluidic chip-based electrochemical flow cell (Scheme 1B). The microfluidic electrochemical cell was used as the detector for the online electrochemical system for continuous measurements coupled with in vivo microdialysis (Scheme 1C). The microfluidic electrochemical flow cell was stored at +4 °C while not in use.

Apparatus and online experimental setup

Electrochemical experiments were carried out with a computer-controlled electrochemical analyzer (CHI 832B, Chenhua, Shanghai, China). For offline voltammetry experiments, the thionine/SWNT-modified and XOD/thionine/SWNT-modified glassy carbon (GC) or ITO electrodes were used as working electrode, a platinum spiral wire as auxiliary electrode, and an Ag/AgCl electrode (KCl-saturated) as reference electrode. The microfluidic chip

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electrochemical flow cell was integrated with the XOD-based biosensor as working electrode, micro-sized Ag/AgCl electrode prepared by polarizing Ag wire (diameter, 0.2 mm) at +0.60 V in 0.10 M hydrochloride acid for 30 min as reference electrode, stainless tubing as auxiliary electrode and outlet of the flow cell. The microfluidic chip was directly coupled to in vivo microdialysis to form an OECS for the measurement of cerebral HX continuously sampled from rat brain after local microinjection of hypoxanthine, as shown in Scheme 1C. Standard solutions or brain microdialysates were delivered from a syringe (BAS) and pumped through FEP tubing by a microinjection pump (CMA 100, CMA Microdialysis AB, Stockholm, Sweden) to the microfluidic chip-based flow cell, in which the XOD-biosensor was polarized at 0.0 V (*vs*. Ag/AgCl) for continuously monitoring hypoxanthine.

Online measurements of cerebral hypoxanthine spiked by local microinjection of hypoxanthine

The procedures for animal surgery and for in vivo microdialysis as well as for online measurements of brain microdialysates were similar to those described in our earlier studies.[35, 36] Briefly, adult male Sprague-Dawley rats (250-300 g) purchased from Health Science Center, Peking University were housed on a 12:12 h light-dark schedule with food and water *ad libitum*. 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.) and put onto a stereotaxic frame. The microdialysis guide cannula (BAS/MD-2250, BAS) was implanted into the striatum (0 mm caudal to bregma, 3.0 mm medial-lateral, and 4 mm ventral from the surface of the skull) using standard stereotaxic procedures. The guide cannula was kept in place with three skull screws and dental cement.

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Stainless steel dummy blockers were inserted into the guide cannula and fixed until the insertion of the microdialysis probe. For local microinjection experiments, lateral ventricle was used as a microinjection site. A single home-made cannula (diameter 0.3 mm) was implanted in this structure (0 mm caudal to bregma, 1.8 mm medial-lateral, and 4 mm ventral from the surface of the skull), and a dummy stylet was inserted into the cannula and fixed before the microinjection experiment. Throughout the surgery, the body temperature of the animals was maintained at 37 °C with a heating pad. Immediately after surgery, the rats were placed into a warm incubator individually until they recovered from the anesthesia. The rats were allowed to recover for at least 24 h before in vivo microdialysis sampling.

Before the online measurements, the microdialysis probes (CMA; dialysis length, 4 mm; diameter, 0.24 mm) were implanted into rat striatum and were perfused with aCSF solution at 1 μL/min for at least 90 min for equilibration. The outlet of the probe was directly connected to the microfluidic chip-based electrochemical flow cell, in which the XOD-based electrochemical biosensor was poised at 0.0 V for the online selective continuous measurements. The local microinjection experiments were performed when the electrochemical responses obtained for the brain microdialysate were stable. Dummy stylets were removed and 100 μM hypoxanthine was infused at a rate of 2 μL /min over a period of 5 min into the lateral ventricle. After the experiments, the animals were sacrificed with an overdose of chloral hydrate.

Results and discussion

Thionine-mediated electron transfer of XOD

To investigate if thionine could sever as a low-potential mediator to shuttle the electron transfer of XOD, we immobilized XOD onto the thionine/SWNT-modified ITO electrode and

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then compared the cyclic voltammograms of the resulting electrodes before and after the addition of substrate of XOD (typically hypoxanthine) into the aCSF. As depicted in Fig. 1, a pair of well-defined redox wave were recorded at the XOD/thionine/SWNT-modified electrode (black curve) with a redox potential of -0.23 V (*vs*. Ag/AgCl), which could be ascribed to the redox process of thionine adsorbed onto SWNTs. Upon addition of 2 mM hypoxanthine in solution, the oxidation peak current of thionine increases, while the reversed reduction peak current decreases (blue curve), suggesting that thionine shuttles electron transfer between FAD centers in XOD and the electrode and thus facilitate the oxidation of hypoxanthine at the electrode through the XOD-based catalytic cycle (Scheme 1B, inset). Note that, the oxidation observed here could neither be attributed to the direct electrochemistry of XOD nor the oxidation of hypoxanthine at the SWNT-modified electrode because that the direct electron transfer of FAD/$FADH_2$ of XOD and the direct oxidation of hypoxanthine at carbon nanotube-modified electrodes or other kinds of electrodes occurred at more negative potentials (approximately -0.40 V *vs*. Ag/AgCl) [37-40] and more positive potentials (usually higher than 0.80 V *vs*. Ag/AgCl),[41-43] respectively.

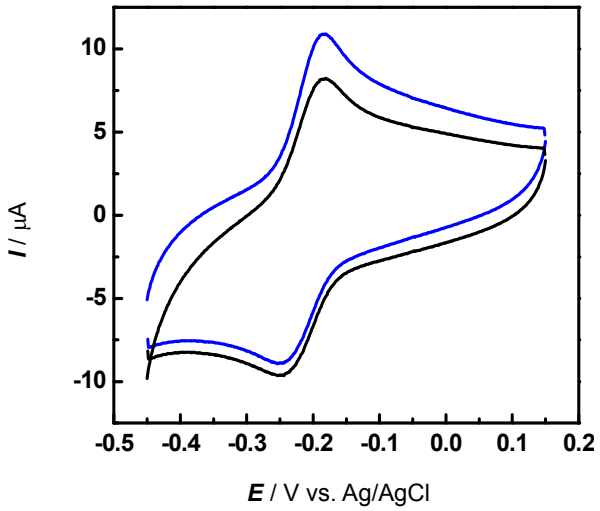

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Fig. 1 Typical CVs obtained at the XOD/thionine/SWNT-modified ITO electrode in aCSF in the absence (black curve) and presence (blue curve) of 2 mM hypoxanthine. Scan rate, 10 mV s^{-1}.

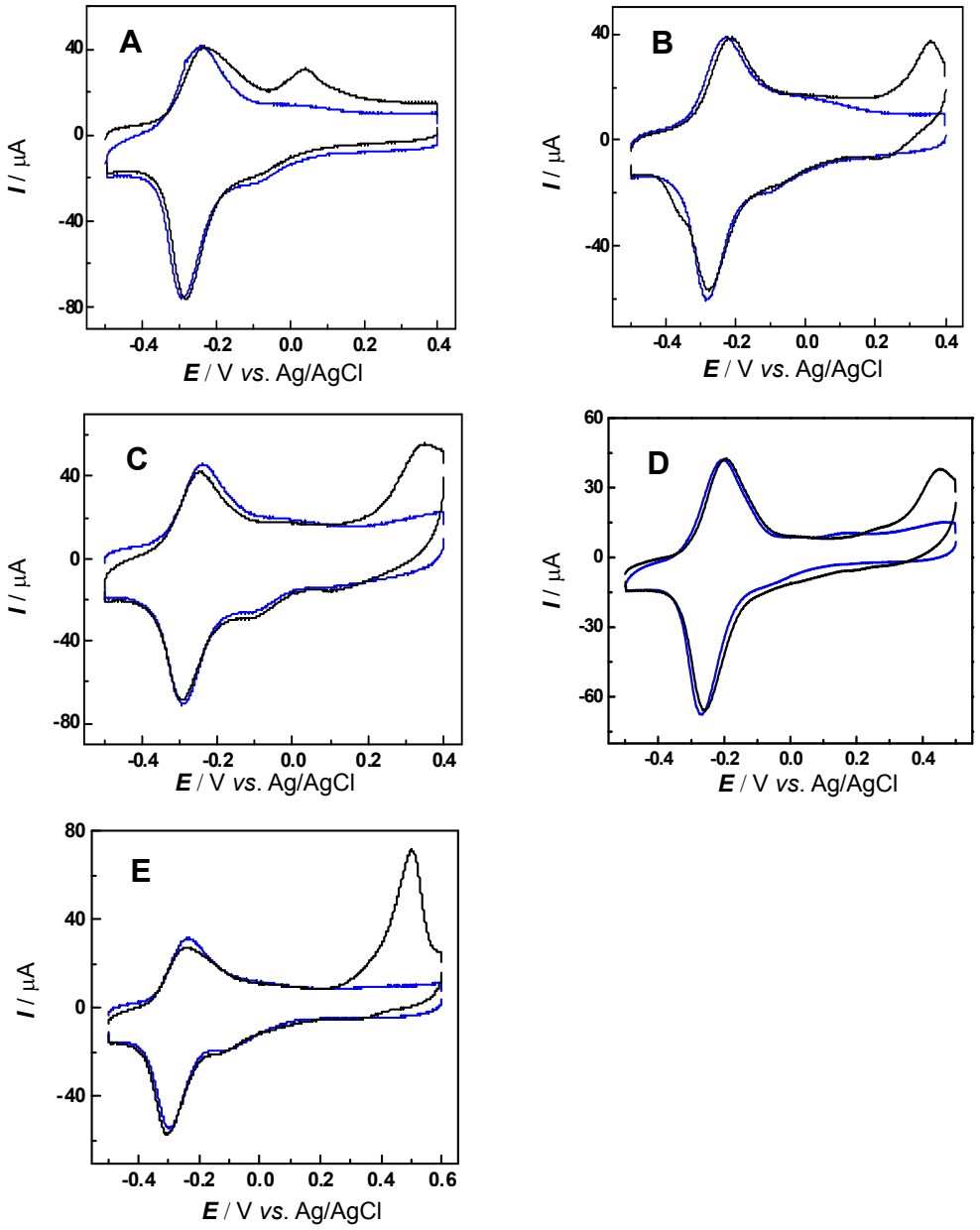

Fig. 2 CVs at the thionine/SWNT-modified GC electrode in aCSF in the absence (blue curves) and presence (black curves) of AA (A), DA (B), DOPAC (C), 5-HT (D), and UA (E). The concentration of each species was 1 mM. Scan rate, 20 mV s^{-1}.

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Compared with other kinds of artificial electron transfer mediators such as ferricyanide, ferrocenyl derivatives and hexacyanoferrates, the mediator employed here possesses a lower redox potential, which is more negative than those for the oxidation of electroactive species in the cerebral systems such as AA, UA, 3,4-dihydroxyphenylacetic acid (DOPAC) as mentioned above. Such a property substantially enables the oxidase-based biosensors based on low-potential mediators to be particularly attractive for effective biosensing of the neurochemicals free from the interference from the electroactive species coexisting in the brain, as described below.

Selectivity

To demonstrate the feature of being interference-free from the endogenous electroactive species benefited from the low redox potential of thionine, we performed cyclic voltammetry with the thionine/SWNT-modified glassy carbon (GC) electrodes (denoted as thionine/SWNT/GC electrodes) in aCSF containing the species commonly coexisting in the cerebral systems. As shown in Fig. 2A, the thionine/SWNT-modified GC electrode exhibits an oxidation peak for AA at a peak potential of 0.0 V, which was explained by the oxidation of AA at SWNTs, as reported previously.[17] The CVs display that the oxidation of DA (B), DOPAC (C), 5-HT (D), and UA (E) at thionine/SWNT-modified GC electrode started from 0.20 V, 0.20 V, 0.25 V, and 0.30 V, respectively, of which all these potentials were well-separated from that for the oxidation of thionine, suggesting the oxidase-based electrochemical biosensors with low-potential electron transfer mediators could be free from the interference- from these species.

These results described above demonstrate that the use of low-potential mediators (typically as thionine) could efficiently shuttle the electron transfer of oxidases and such a property essentially constitutes a consequence for the high selectivity of the as-prepared

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oxidase-based biosensors, forming a basis for the development of OECSs based on oxidase-based electrochemical biosensors by using low-potential electron mediators for online continuous neurochemical measurements.

To further study the selectivity, we in vitro evaluated the selectivity in an online continuous-flow system by assembling XOD-based electrochemical biosensor into the microfluidic chip-based electrochemical flow cell (Scheme 1 C). The detection potential for the online measurements was optimized to be 0.0 V, which makes the measurements be interference-free from O_2 and H_2O_2 since the reduction of O_2 and oxidation of H_2O_2, which could probably be involved in the enzymatic reaction, commence from -0.05 V and 0.20 V in aCSF at the thionine/SWNT-modified electrode, respectively (data not shown). As described above, although the utilization of the thionine/SWNT as the electrochemical transducer could well avoid the interferences from most kinds of electroactive species, AA could be oxidized at the thionine/SWNT-modified electrode at a low potential, as shown in Fig. 2A. In the online continuous-flow system, perfusion of 20 μM AA produced about 2 times larger current response than that of 10 μM hypoxanthine, as described in Fig. 3A (black line). In this case, by taking advantage of the flexible structure design ability of microfluidic chips, we immobilized ascorbate oxidase (AAox) in the upstream channel of the XOD-based biosensor in the microfluidic chip-based flow electrochemical cell for the purpose of elimination of the interference from AA. As depicted with red line in Fig. 3B (red line), after immobilization of AAox in the upstream channel of hypoxanthine biosensor, the current signal of 50 μM AA was fully suppressed by the enzymatic oxidation reaction of AAox, suggesting the introduction of AAox in the upstream of the biosensor could effectively remove AA before the biosensing process. Fig. 3C displays online current responses obtained for the electroactive species

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coexisting in the cerebral systems at the concentrations beyond the physiological level in brain microdialysate in the OECS at 0.0 V. The perfusion of AA (20 μM), DA (1 μM), DOPAC (2 μM), UA (20 μM), or 5-HT (2 μM) did not produce an observable current response (Fig. 3C), demonstrating that, with the immobilization of the AA_{OX} in the upstream of the biosensor to remove AA, the electrochemical biosensor with thionine as the low-potential electron transfer

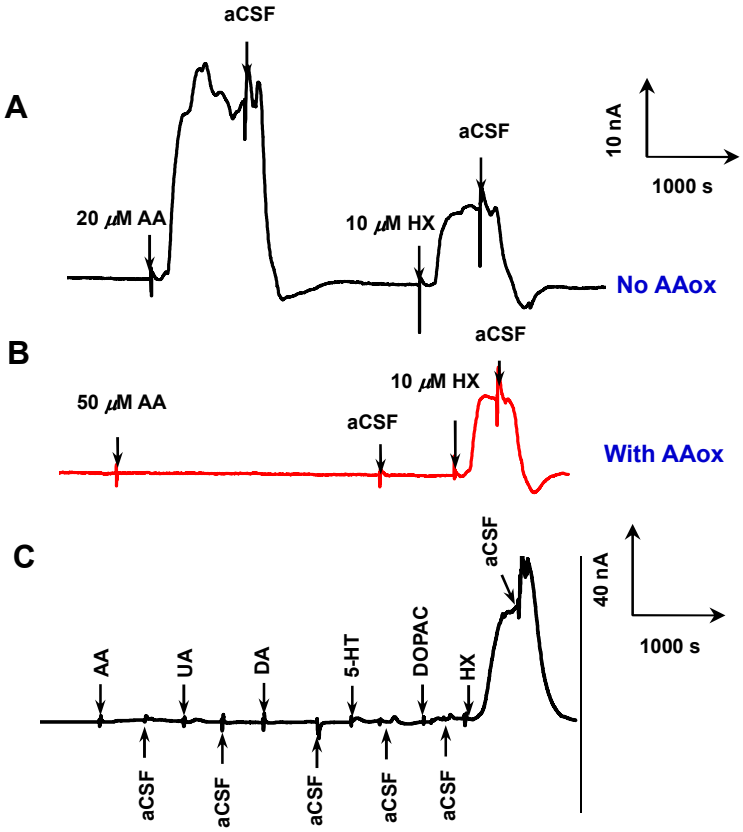

Fig. 3 (A, B) Online current-time responses towards AA and hypoxanthine recorded in the microfluidic chip-based flow cell without (A, black line) and with (B, red line) immobilization of AAox in the upstream of with the XOD-based biosensor. (C) Typical online current response for AA (20 μM), UA (10 μM), DA (2 μM), 5-HT (2 μM), DOPAC (2 μM) and hypoxanthine (50 μM) by the microfluidic chip-based flow cell with the XOD-based biosensor as the detector. aCSF was used as the perfusion solution. Flow rate, 1 μL /min. Potential applied, 0.0 V.

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mediator bears a high selectivity against the endogenous electroactive species, and such a property eventually enables the online OECS virtually interference-free.

Stability, reproducibility, and linearity

The stability of the OECS with the XOD-based biosensor as the detector was evaluated by continuously sensing hypoxanthine (50 μM) in the microfluidic chip-based continuous-flow system, as depicted in Fig. 4A. The current response obtained for hypoxanthine almost remained unchanged after continuously running the measurements for at least 2 h. For the discontinuous measurements, the OECS was stable without observable day-to-day variations for at least one week, with the continuous measurements for 2-3 h each day. The reproducibility of the OECS was investigated by repeatedly sensing hypoxanthine standards (50 μM) for 7 times (Fig. 4B) and the relative standard deviation was calculated to be 2.1%.

Fig. 4C shows a typical trace of online current response of hypoxanthine standards in the OECS. The current response was linear with the concentration of hypoxanthine within a dynamic concentration range from 1 to 100 μM (I (nA) = 0.81C_{HX} (μM) + 0.41, γ =0.999). The detection limit, based on a signal-to-noise ratio of 3, was calculated to be 0.40 μM. These results strongly demonstrate that the microfluidic chip-based OECS possesses a high selectivity, good linearity, high stability and reproducibility and could thus be used for continuous monitoring of hypoxanthine in the cerebral system.

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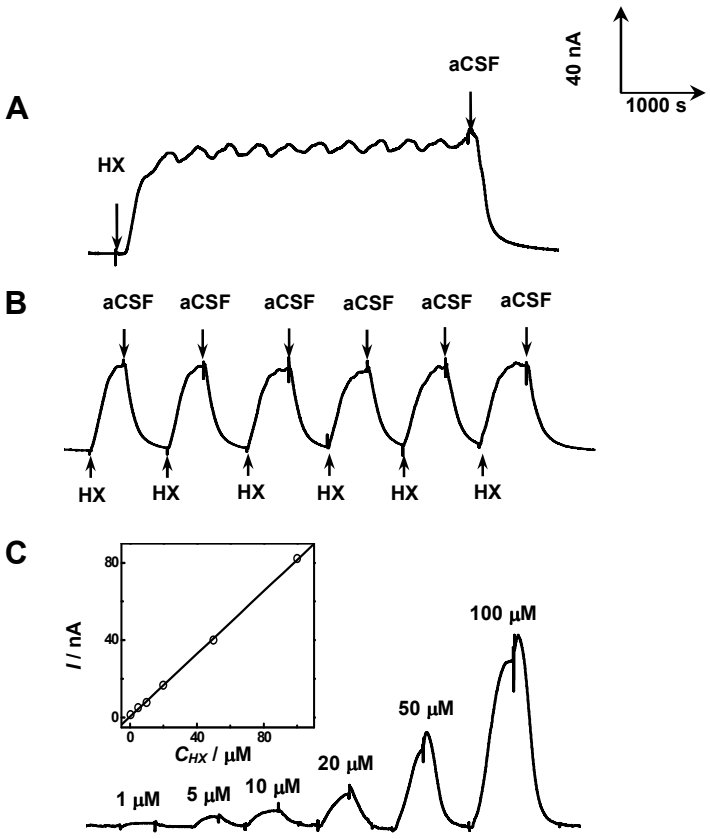

Fig. 4 Typical current-time responses continuously (A) and repeatedly (B) recorded for 50 μM standard solution of hypoxanthine, and for (C) standard solutions with different concentrations of hypoxanthine (concentrations were indicated in the figure) by the microfluidic chip-based flow electrochemical cell with the XOD-based biosensor as the detector. Other conditions were the same as those in Fig. 3.

Online monitoring of striatum hypoxanthine spiked by local microinjection of hypoxanthine

Considering the relatively complicated chemical environments involved in the cerebral systems, it remains very essential to investigating the selectivity of the method with the microdialysates in vivo continuously sampled from rat brain. To accomplish such a purpose, the ITO plate was modified only with SWNTs and thionine (i.e., without XOD). As could be seen

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from Fig. 5A, with the microfluidic chip-based flow cell with the thionine/SWNT-modified electrode as working electrode, the perfusion of brain microdialysate sampled form the striatum of rats did not yield an observable current response, suggesting that no species in the microdialysate could be electrochemically oxidized or reduced at the thionine/SWNT-modified electrode under the conditions employed here. Based on this, online continuous measurements of hypoxanthine in the microdialysate continuously sampled from the rats were conducted with the system shown in Scheme 1C. As reported previously, the physiological concentration of hypoxanthine in rat brain was about 3-5 μM.[44] After being dialyzed into the microdialysis probe, the physiological level of hypoxanthine in the brain microdialysate became quite low and was thus not detectable with the OECS demonstrated here, as displayed in Fig. 5B. However, the extracellular concentration of hypoxanthine was reported to increase significantly in some pathological conditions such as brain ischemia and hypoxia.[45-47] To further validate the OECS developed here for the continuous measurements of cerebral hypoxanthine, the measurements were performed with continuous measuring the brain microdialysate following a local microinjection of hypoxanthine into the ventricle. As shown in Fig. 5C, the current responses recorded for the brain microdialysate was observed after the local microinjection of hypoxanthine, demonstrating that the OECS developed with the strategy demonstrated in this study (i.e., Strategy **2**, Scheme 1A) could be potentially used for continuously and selectively monitoring cerebral hypoxanthine increase in some physiological and pathological processes.

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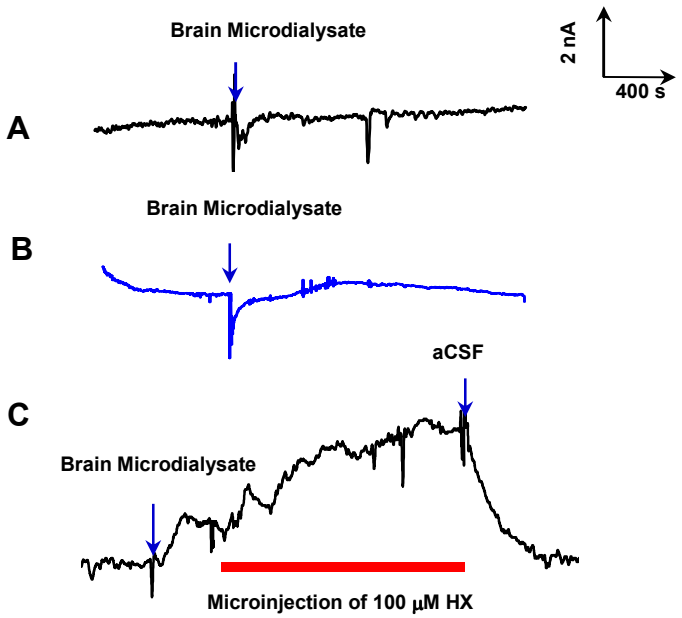

Fig. 5 Online current-time response obtained in the microfluid chip-based flow cell for the microdialysate in vivo sampled from rat brain with the thionine/SWNT-modified (A) and XOD/thionine/SWNT-modified ITO electrodes (B, C) as the detectors, and under the conditions of normal (A, B) and microinjection of hypoxanthine in lateral ventricle (C). The aCSF was used as the perfusion solution. Flow rate, 1 μL/min. The flow rate for microinjection of hypoxanthine was 2 μL/min. Other conditions were the same as those in Fig. 3.

Conclusion

By using thionine and xanthine oxidase (XOD) as typical examples for low-potential mediators and oxidases, respectively, we have successfully demonstrated a new strategy to develop oxidase-based OECSs for continuous and selective online measurements of neurochemicals. The OECS demonstrated here is selective, stable and reproducible and could thus be envisaged to find some interesting applications in physiological and pathological investigations. This study essentially offers a new strategy to develop online electrochemical

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systems, which is of great importance in understanding the molecular basis of physiological and pathological events.

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