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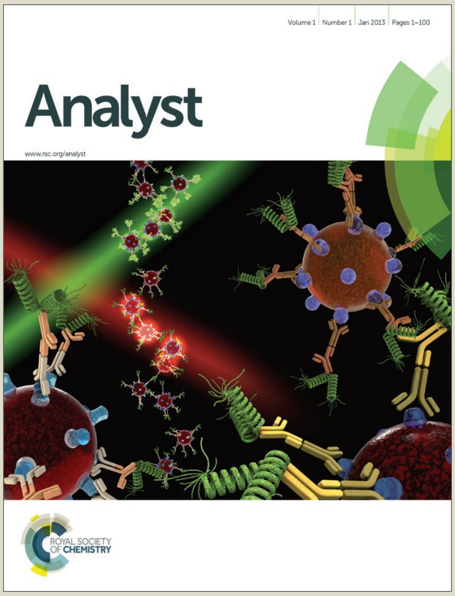

ROYAL SOCIETY OF CHEMISTRY

Ferricyanide-backfilled cylindrical carbon fiber microelectrodes for in vivo analysis with high stability and low polarized potential

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Abstract. Development of stable and reproducible method for in vivo electrochemical monitoring of neurochemicals is of great physiological importance. In this study, we demonstrate ferricyanide-filled cylindrical carbon fiber microelectrodes (CFEs) for in vivo electrochemical analysis with a high stability and a low polarized potential. We first studied the voltammetric behavior of a cylindrical CFEs by using a model system consisting of two separated cells each containing potassium ferricyanide ($K_3Fe(CN)_6$) or potassium ferrocyanide ($K_4Fe(CN)_6$). We observed that $E_{1/2}$ values of the system were dependent on the ratio of the lengths of the cylindrical CFEs and of the concentrations of the redox species on both poles. Based on this property, we prepared the ferricyanide-backfilled cylindrical CFEs, and found that this kind of electrodes exhibit more stable current response and lower polarized potential than the CFEs backfilled with KCl or with $Ru(NH_3)_6Cl_3$. Animal experiments with the ferricyanide-backfilled cylindrical CFEs demonstrate that this kind of electrodes could be used for in vivo monitoring of neurochemical release with a high stability under some physiological conditions.

Keywords: In vivo amperometry, Ferricyanide-backfilled cylindrical carbon fiber electrodes, Electrode kinetics.

Introduction

Many biological molecules (e.g., catecholamines, ascorbate, histamine and 5-hydroxytryptamine) related to signal transmission bears good electrochemical properties, which enables the electrochemical microsensors designed from carbon fibers quite useful to probe the change of these molecules.[1-4] On one hand, the uses of CFEs as the microsensors actually endow the as-formed in vivo electrochemical methods with high spatial/temporal resolution owing to their small size and mechanical rigidity.[1,5] On the other hand, the rich surface chemistry of carbon materials makes it possible to easily functionalize CFEs into in vivo electrochemical sensors and biosensors with various methods, such as covalent and noncovalent surface modification.[2,6] So far, these CFE-based microsensors have been widely used for studying single-cell exocytosis and brain functions such as intact neurons, vesicles, brain slices and in vivo analysis.[5,7-12] More remarkably, the CFE microsensors with a high selectivity and sensitivity have been developed over the past decade and demonstrated to be potentially useful to study the neurochemical processes involved in physiological and pathological events.[9-15]

Electrolyte-backfilled CFEs were firstly developed by Chow et al. by pulling a glass capillary to encase a small piece of carbon fiber and establishing an electrical contact by backfilling the inner electrode with electrolyte (i.e., 3 M KCl).[16] This method was later developed by backfilling the inner electrode with other kinds of electrolyte solution (e.g., potassium acetate).[17] Recently, Zhang et al. systematically studied the electrochemical behavior of this kind of system with disk microelectrodes, and concluded that the electrolyte-backfilled CFEs behavior like closed bipolar electrodes by coupling electrochemical reactions on the two sides of the disk microelectrodes. [5,18,19] They further proposed that the electrolyte-backfilled carbon fiber disk microelectrode was electrically coupled to the reduction of soluble oxygen at the interior fiber and the oxidation of neurotransmitter (e.g., dopamine) at the exterior fiber. These studies are not only useful for fundamental understanding of the electrochemical processes in closed bipolar cell but also offer a new approach to biological sensing.[20-22]

We are interested in the electrolyte-backfilled cylindrical CFEs because this kind of electrodes are more suitable for in vivo analysis than the disk CFEs due to the robust current output originated from the larger surface area of the cylindrical CFEs.[23,24] Traditional electrolyte-backfilled cylindrical CFEs have been prepared by filling the inner CFEs with 3 M KCl solution.[16,25-27] In this case, reduction of the dissolved oxygen is used for electric conduction. However, the fluctuation of the dissolved oxygen eventually renders poor stability and reproducibility of the as-formed electrochemical methods for in vivo analysis. In this study, we demonstrate the cylindrical CFEs backfilled with electrolyte containing ferricyanide. We observed that $E_{1/2}$ values of the system were dependent on the ratio of the lengths of the cylindrical CFEs and of the concentrations of the redox species on both poles. We further observed that the presence of ferricyanide in the electrolyte to backfill the inner electrode results in the improvement of stability and reproducibility of the as-formed cylindrical CFEs as compared with those of the electrodes backfilled with 3 M KCl or $Ru(NH_3)_6Cl_3$, which enabled in vivo electrochemical monitoring of neurochemical release at a relatively low potential with a high stability.

Experimental

Chemicals and reagents

Dopamine (DA), hexaammineruthenium (III) chloride ($Ru(NH_3)_6Cl_3$) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma and used as supplied, and the solutions were prepared before use. Potassium ferrocyanide ($K_4Fe(CN)_6$) was purchased from Kanto Chem. Co. Inc. (Japan). Potassium ferricyanide

($K_3Fe(CN)_6$) was obtained from Beijing Chemical Co. (Beijing, China). 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 Milli-Q water, and the solution pH was adjusted to pH 7.4. The high-K^+ solution used to stimulate neurochemical release was prepared by mixing NaCl (78 mM), KCl (70 mM), $MgCl_2$ (1 mM), and $CaCl_2$ (2 mM) and HEPES (10 mM) into Milli-Q water, and the solution pH was adjusted to pH 7.4. Other chemicals were of at least analytical reagent grade and were used without further purification.

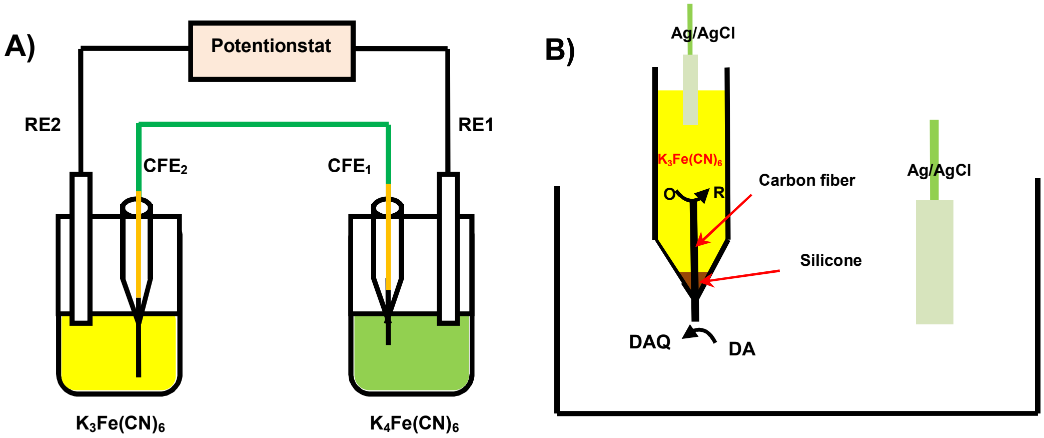
Fabrication of cylindrical CFEs

Two kinds of CFEs were used in this study, as schematically shown in Scheme 1. The CFEs used in Scheme 1 A were fabricated as reported previously.[28] Briefly, a glass capillary (o.d. 1.5 mm, length 10 cm) was pulled on a microelectrode puller (WD-1, Chengdu Instrument Factory, Sichuan, China) into two fine capillaries. The pulled capillary was used as the sheath of CFEs. A single carbon fiber (7 μm in diameter, Tokai Carbon Co., Tokai, Japan) was attached to a copper wire with silver conducting paste. Then, the carbon fiber-attached copper wire was carefully inserted into the capillary. Both open ends of the capillary were sealed with epoxy resin with 1:1 ethylenediamine as the harder and the excess epoxy on the fiber was carefully removed with acetone.

The CFEs used in Scheme 1B was fabricated according to the previous report with a minor modification.[16] Briefly, a single carbon fiber was inserted into a glass capillary and then the capillary was pulled on a laser puller (Model P-2000, Sutter Instrument Co). The CFEs fabricated with both methods were first sequentially sonicated in acetone, 3 M HNO_3, 1.0 M KOH, and distilled water each for 3 min, and then electrochemically pretreated in 1.0 M NaOH at +1.5 V for 80 s.[28]

Electrochemical experiments

Cyclic voltammetry (CV) was performed on a computer-controlled electrochemical analyzer (CHI 660E, Shanghai, China). For the electrochemical system shown in Scheme 1A, a four-electrode, two-cell setup equipped with two cylindrical CFEs and two Ag/AgCl electrodes was used, and the two cylindrical CFEs were directly connected by conducting wire. Each of two separated cells was filled with $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$. Ag/AgCl electrodes were used as reference electrode 1 (RE1) and reference electrode 2 (RE2), as shown in Scheme 1A. For the electrochemical system shown in Scheme 1B, the inner solution was filled with electrolyte solution to construct the conductivity, and the outer solution was aCSF containing DA. It should be noted that the inner length of the CFE (ca. 7.



Scheme 1. A) Schematic diagram of electrochemical cells with two cylindrical CFEs in 3 M KCl solution containing $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$ as a model for studying cyclic voltammetric response of the electrolyte-backfilled cylindrical CFEs. B) Schematic diagram of an electrochemical cell with an electrolyte-backfilled cylindrical CFE. Here, $K_3Fe(CN)_6$ dissolved in 3 M KCl solution was backfilled to the inner CFE to establish the conductivity in the capillary.

mm) were much longer than that of outer part (ca. 500 μm). In this case, the longer one could be considered as the cathode and the other one as the anode. By doing so, the anodic pole could be regarded as the dominating pole and thus the current flow through the whole system depended on the anodic pole. A 3 M KCl solution with or without 5 mM $K_3Fe(CN)_6$ or 5 mM $Ru(NH_3)_6Cl_3$ was used as supporting electrolyte as indicated. A scan rate of 10 mV/s was used for all CV experiments unless noted otherwise

In vivo experiment

Adult male Sprague-Dawley rats (300-350 g) were purchased from Center for Health Science, Peking University. The animals were housed on a 12/12 h light-dark schedule with food and water *ad libitum*. Animal experiments were performed as reported previously.[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 anesthetized with chloral hydrate (345 mg/kg, ip) and positioned onto a stereotaxic frame. The CFE backfilled with $K_3Fe(CN)_6$ solution was implanted into striatum (AP = 0 mm, L = 3.0 mm from bregma, V = 4.0 mm from dura) by standard stereotaxic procedures.[30] The reference electrode of the Ag/AgCl was positioned into the dura of brain and fabricated as reported previously.[2] Briefly, the Ag/AgCl reference electrode was prepared by first polarizing Ag wire (diameter, 1 mm) at 0.6 V in 0.1 M hydrochloride acid for 30 min to produce an Ag/AgCl wire and then inserting the as-prepared Ag/AgCl wire into a pulled glass capillary, in which aCSF was aspirated from the fine end of the capillary and used as the inner solution for the reference electrode. The other end of the capillary with Ag wire exposed was sealed with epoxy. The high-K^+ solution was injected into the same deep with the CFE by micro-injection pump (RS-232, Harvard) as demonstrated previously,[10,31,32] and the distance between CFE and syringe tip was 0.98 mm. The volume of KCl solution injected was 5 μL.

Results and Discussion

Cyclic voltammetric response of the coupled cylindrical CFEs

To investigate the cyclic voltammetric response of the cylindrical CFEs, a simplified experimental setup was chosen, which connects two separate two-electrode cells in series as shown in Scheme 1A. The two cylindrical CFEs denoted as CFE1 and CFE2 were used as the two ends of the coupled electrochemical reaction. With this setup, the solution composition and the relative size of the CFEs could be easily changed. Herein, the $K_3Fe(CN)_6$ was reduced into $K_4Fe(CN)_6$ at the cathodic pole, and the $K_4Fe(CN)_6$ was oxidized into $K_3Fe(CN)_6$ at the anodic pole. In the present system, the $E_{1/2}$ value was observed to be dependent on the ratio of the length of the cylindrical CFEs used as the cathode and anode (L_c/L_a) in two poles. As depicted in Fig. 1A, with the shortening of the length of anodic cylindrical CFEs and keeping the length of cathodic cylindrical CFEs as the constant, the $E_{1/2}$ value of the steady-state voltammogram was negatively shifted. The plot of $E_{1/2}$ versus $\ln(L_c/L_a)$ gave a good linearity under the conditions employed here (inset, Fig. 1A). We also observed the $E_{1/2}$ value of the steady-state voltammogram depended on the ratio of the concentrations of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ used in the cathodic and anodic poles (C_c/C_a), respectively. As typically shown in Fig. 1B, with decreasing the concentration of $K_4Fe(CN)_6$ (C_a) and keeping the concentration of $K_3Fe(CN)_6$ (C_c) as constant, the $E_{1/2}$ value of the steady-state voltammograms was negatively shifted. The $E_{1/2}$ value was also in a good linearity with $\ln(C_c/C_a)$ (inset, Fig. 1B).

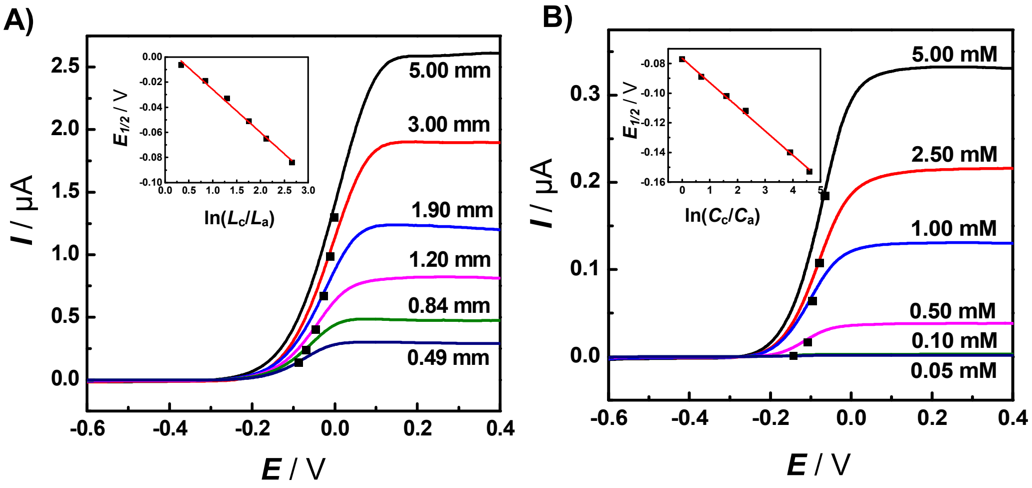


Fig. 1 Voltammograms obtained with the electrochemical cell with two cylindrical CFEs in the anodic and cathodic poles shown in Scheme 1A. (A) The length of the cylindrical CFE used in the cathodic pole (L_c) was kept as 7 mm, while the length of the cylindrical CFE used in the anodic pole (L_a) was changed from 5.00 mm to 0.49 mm as indicated in the figure. A 3 M KCl solution containing 5 mM $K_3Fe(CN)_6$ or 5 mM $K_4Fe(CN)_6$ was used as the electrolyte in the cathodic and anodic poles, respectively. Inset, plot of $E_{1/2}$ versus $\ln(L_c/L_a)$. (B) The concentration of the cathodic pole (C_c) was kept as 5 mM $K_3Fe(CN)_6$ in 3 M KCl and the concentration of $K_4Fe(CN)_6$ in the anodic pole (C_a) was changed from 5.00 mM to 0.05 mM in 3 M KCl as indicated in the figure. L_a and L_c were 0.49 mm and 7.00 mm, respectively. Inset, plot of $E_{1/2}$ versus $\ln(C_c/C_a)$.

The dependences observed above could be understood by Equation 1 reported previously by Zhang *et al.*[19]

$$E_{1/2} = \left(E_a^{0'} - E_c^{0'}\right) - \frac{RT}{nF}\ln\left[-2\left(\frac{i_d^c}{i_d^a}\right) - 1\right] \qquad (1)$$

Where, $E_a^{0'}$ and $E_c^{0'}$ are the formal potentials for the anodic and cathodic poles, respectively. i_d^a and i_d^c are the limiting currents on the anode and cathode, respectively. R is the gas constant. T is the absolute temperature. n is the number of electrons transferred per redox species, F is the faraday constant.

In this study with cylindrical CFEs as working electrodes, the quasi-steady-state limiting current of the cylindrical CFE could be evaluated by Equation 2.[33]

$$i_{\mathrm{qss}} = \frac{2\mathrm{nFADC}^*}{\mathrm{r_0 ln\tau}} \qquad (2)$$

Where, r_0 is the radius of the electrode, C^* is the bulk concentration of the redox species. A is the surface area of the electrode. τ is the expression of the time t: $\tau = 4\mathrm{Dt}/\mathrm{r}_0^2$.[33] If we consider 1) two poles bear almost the same mass transfer coefficient ($\mathrm{m} = 2\mathrm{D}/\mathrm{r_0 ln\tau}$),[33] and 2) the length of cylindrical CFE is much larger than the radius, then, the $E_{1/2}$ values for cylindrical CFEs could be derived from Equations 1 and 2:

$$E_{1/2} = \left(E_a^{0'} - E_c^{0'}\right) - \frac{RT}{nF}\ln\left[2\left(\frac{l_\mathrm{c}C_\mathrm{c}}{l_\mathrm{a}C_\mathrm{a}}\right) - 1\right] \qquad (3)$$

Where, L_a and L_c are the length of anodic and cathodic electrodes, respectively. In our experiments, $2L_\mathrm{c}C_\mathrm{c}/L_\mathrm{a}C_\mathrm{a}$ was controlled to be much larger than 1. From Equation 3, we could easily understand the relationship between $E_{1/2}$ values and the ratios of the lengths of the cylindrical CFEs and of the concentrations of the redox species in the cathodic and anodic poles.

From Equation 3, one may understand that the $E_{1/2}$ is changeable by changing the concentration of the different species in the coupled electrochemical reaction. It occurs to us that, to well enable the electrolyte-backfilled cylindrical CFEs (Scheme 1B) for practical analysis, the redox species used in the cathodic pole should have fast electron transfer kinetics and high concentration as well as the good tolerance against the fluctuation of temperature and pressure. In this sense, oxygen dissolved in the 3 M KCl used in conventional CFEs is not likely the best choice as the redox species in the cathodic pole because it bears sluggish kinetic process on carbon electrode and the relatively low concentration. Therefore, the uses of additionally added redox species to replace oxygen as the redox species in the cathodic pole could improve the analytical properties of the electrolyte-backfilled cylindrical CFEs, as described below.

Electrochemical properties of the ferricyanide-backfilled cylindrical CFEs

To investigate the properties of the cylindrical CFEs backfilled with additionally added redox species, two redox species, i.e., $K_3Fe(CN)_6$ and $Ru(NH_3)_6Cl_3$ with fast kinetic process were separately backfilled into the cylindrical CFEs (Scheme 1B) and the electrochemical properties of the as-formed electrodes were studied along with the CFE backfilled with pure 3 M KCl solution (i.e., with dissolved oxygen as the redox species). Fig. 2 compares CVs of the oxidation of 10 μM DA at different electrodes. All electrodes show similar sigmoidal voltammetric responses, which were ascribed for the oxidation of DA. However, $E_{1/2}$ values for the oxidation of DA largely vary with the electrodes, in which the cylindrical CFE

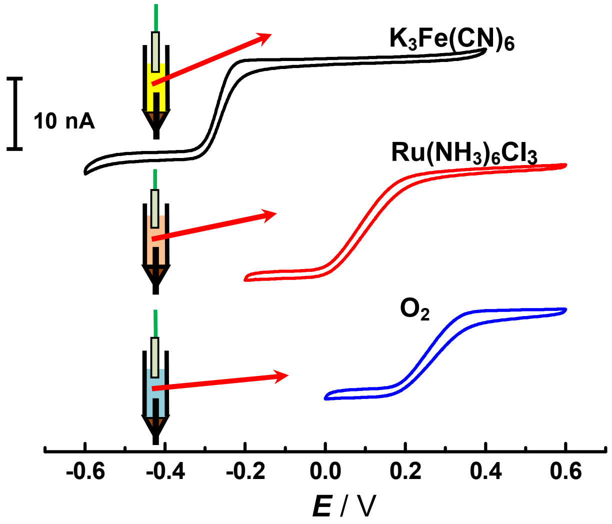


Fig. 2 CVs obtained with the cylindrical CFEs backfilled with 3.0 M KCl solution in the absence (blue curve) and presence of 5 mM $K_3Fe(CN)_6$ (black curve) and 5 mM $Ru(NH_3)_6Cl_3$ (red curve) in aCSF containing 10 μM DA. Scan rate, 10 mV/s. The lengths of cylindrical CFEs in the cathodic and anodic poles were 7 mm and 500 μm, respectively.

backfilled with $K_3Fe(CN)_6$ exhibit the most negative $E_{1/2}$ value among all the electrodes investigated here. This variation was elucidated in terms of the dependence of the $E_{1/2}$ values on the formal potentials of redox species presented in the cathodic poles when the redox reaction in the anodic pole was kept the same, i.e., DA oxidation.[5,18,19] On the other hand, we observed the kinetics for DA oxidation, which could be simply judged by $|E_{3/4}-E_{1/4}|$ values, also vary with the electrodes; the cylindrical CFE backfilled with $K_3Fe(CN)_6$ exhibits the smallest $|E_{3/4}-E_{1/4}|$ value (ca. 69 mV) as compared with those of the electrodes backfilled with $Ru(NH_3)_6Cl_3$ (ca. 90 mV) and 3 M KCl (ca. 95 mV), essentially demonstrating that the $K_3Fe(CN)_6$-backfilled CFE possesses a fast kinetic process for DA oxidation. The variation could be understood by the difference in the cathodic limiting current[19] and the electron transfer kinetics of the redox species in the cathodic poles. Interestingly, we found that the use of $Ru(NH_3)_6Cl_3$ in this case did not provide a fast kinetics as expected under the conditions employed here. The reason for this remains unclear at the current stage.

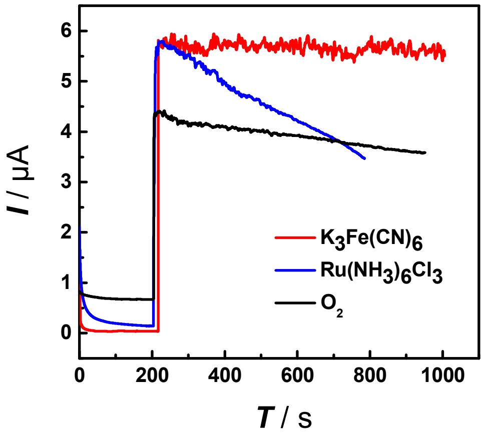


Fig. 3 Amperometric responses obtained with the cylindrical CFEs backfilled with 3.0 M KCl solution in the absence (black curve) and presence of 5 mM $K_3Fe(CN)_6$ (red curve) and 5 mM $Ru(NH_3)_6Cl_3$ (blue curve) in aCSF containing 10 μM DA. The potentials for the cylindrical CFEs backfilled with 3 M KCl solution in the absence (black curve) and presence of 5 mM $K_3Fe(CN)_6$ (red curve) and 5 mM $Ru(NH_3)_6Cl_3$ (blue curve) were 0.78, 0.10 and 0.40 V, respectively.

We have also studied the stability for DA oxidation at different electrodes. Fig. 3 compares the amperometric responses obtained at the cylindrical CFEs backfilled with different solutions. Compared with the CFEs backfilled with pure KCl solution (black curve) and KCl solution containing $Ru(NH_3)_6Cl_3$ (blue curve), the CFE backfilled with KCl solution containing $K_3Fe(CN)_6$ exhibited highest stability for DA oxidation. The current responses for DA oxidation at the $K_3Fe(CN)_6$-backfilled electrodes were even well maintained at the time points of 4 and 8 h after the electrodes were backfilled with freshly prepared 3 M KCl solution containing $K_3Fe(CN)_6$ (data not shown). Although the CFEs backfilled with pure KCl solution also exhibits a relatively stable response toward DA oxidation, such responses differ largely with the time after the electrodes were freshly backfilled and with repeat backfilling (data not shown). These variations were considered to presumably result from the fluctuation of dissolved oxygen concentration with temperature and pressure. The $Ru(NH_3)_6Cl_3$-backfilled CFEs also unexpectedly show a poor stability and reproducibility for DA oxidation (blue curve). The reason for this phenomenon remains unclear in the present stage. Moreover, the $K_3Fe(CN)_6$-backfilled CFEs show stable current response toward DA oxidation at a potential of *ca.* 0.1 V, which was much lower than those of KCl-backfilled (*ca.* 0.78 V) and $Ru(NH_3)_6Cl_3$-backfilled (ca. 0.40 V) CFEs. This low operation potential is believed to minimize electric distortion to neurons[5] and could thus well validate the as-prepared electrochemical methods for in vivo analysis. At such a low operation potential, the $K_3Fe(CN)_6$-backfilled CFEs were well responsive to DA; the current (I) increases with increasing the concentration of DA (C_{DA}) in the solution (Fig. 4) and was linear with C_{DA} within the concentration range from 1 to 45 μM (I/nA = 0.561C_{DA}/μM + 0.0522, $R^2 = 0.999$) (inset, Fig. 4). Such a linearity, along with the low operation potential, high stability and reproducibility of the $K_3Fe(CN)_6$-backfilled cylindrical CFEs essentially enables their application for in vivo monitoring of neurochemical release.

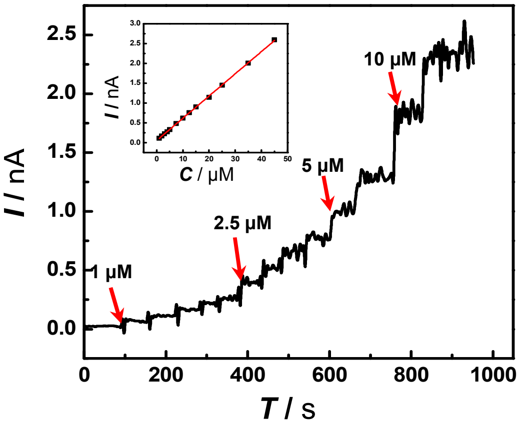


Fig. 4 Current response obtained with the concentration of DA at $K_3Fe(CN)_6$-backfilled CFEs with the adding of different concentrations of DA indicated in the figure. Insert, the plot of the current response versus the concentration of DA.

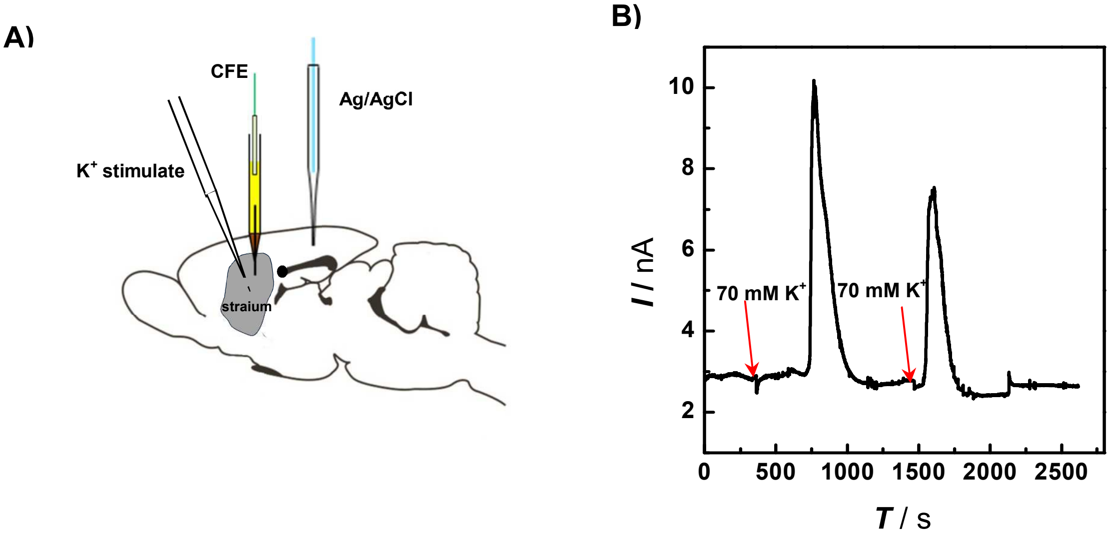


Fig. 5 (A) A diagram of the animal experiment with the $K_3Fe(CN)_6$-backfilled CFE. (B) The current-time curve obtained at the ferricyanide-backfilled CFEs in vivo. The stimulation was implemented at 400 s and 1500 s, respectively. The polarized potential was set at 0.1 V.

To demonstrate the application of the $K_3Fe(CN)_6$-backfilled CFEs for in vivo monitoring of neurochemical release, we implanted the electrodes into the striatum, as schematically shown in Fig. 5A. Fig. 5B shows the typical result for real time recording of neurochemical release stimulated by local injection of 70 mM KCl with the $K_3Fe(CN)_6$-backfilled CFE. Consistent with the previous reports,[10,31,32] KCl stimulation clearly results in the release of catecholamines in striatum, demonstrating that the strategy described here for the preparation of cylindrical CFEs could be useful for the development of effective electrochemical methods for reliable real-time monitoring of neurochemicals in vivo.

Conclusion

In summary, we have demonstrated an electrochemical method for in vivo monitoring neurochemical release with ferricyanide-backfilled cylindrical carbon fiber electrodes with high stability and low polarized potential. We observed the $E_{1/2}$ values of the cylindrical CFEs strongly depend on the ratios of the lengths of the cylindrical CFEs and of the concentrations of the redox species on both poles. More remarkably, we found that the cylindrical CFEs backfilled with ferricyanide exhibited more stable current response and lower polarized potential as compared with those backfilled with pure KCl solution and with $Ru(NH_3)_6Cl_3$. These properties essentially enable the electrochemical method with the $K_3Fe(CN)_6$-backfilled CFEs to be useful for in vivo analysis. This study provides fundamental and experimental evidences to improve the performance of the electrolyte-backfilled CFEs for in vivo analysis.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant No. 21321003, 21127901, 21435007, 91213305 for L. Mao, 21475138 and 21322503 for P. Yu, 21475114 and 21275123 for J. Fei), the National Basic Research Program of China (973 programs, 2013 CB933704), and the Chinese Academy of Sciences.

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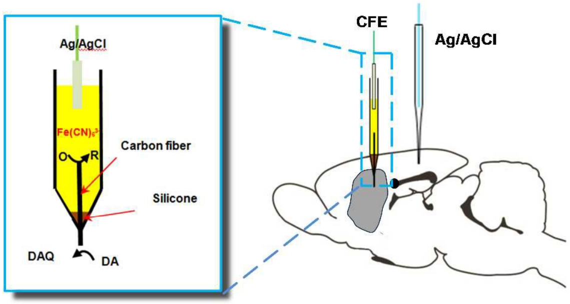

A ferricyanide-backfilled cylindrical carbon fiber microelectrodes was fabricated and studied for in vivo analysis with a high stability and a low polarized potential.