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In Vivo Measurement of Calcium Ion with Solid-State Ion-Selective Electrode by Using Shelled Hollow Carbon Nanospheres as Transducing Layer

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ABSTRACT

*In vivo* monitoring of extracellular calcium ion (Ca$^{2+}$) is of great importance due to its significant contributions in different (patho)physiological processes. In this study, we develop a potentiometric method with solid-state ion-selective electrodes (ISEs) for *in vivo* monitoring of the dynamics of the extracellular Ca$^{2+}$ by using hollow carbon nanospheres (HCNs) as a transducing layer and solid contact to efficiently promote the ion-to-electron transduction between an ionophore-doped solvated polymeric membrane and a conducting substrate. We find that the use of HCNs essentially improves the stability of the signal response and minimize the potential drift of the as-prepared ISEs. With three-shelled HCNs (3s-HCNs) as the transducing layer, we fabricate a solid-state Ca$^{2+}$-selective microelectrode by forming Ca$^{2+}$-selective membrane with calcium ionophore II (ETH129) as the recognition unit, 2-nitrophenyl octyl ether (o-NPOE) as the plasticizer, sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate (NaTFPB) as the ion exchanger, and polyvinyl chloride polymeric (PVC) as the matrix onto the 3s-HCN modified carbon fiber electrodes (CFEs). The as-prepared electrode shows a high stability and a near Nernst response of 28 mV/decade toward Ca$^{2+}$ over a concentration range of $10^{-5}$ - 0.05 M as well as a good selectivity against species endogenously existing in the central nervous system. With these properties, the electrode is used for real-time recording of the dynamics of extracellular Ca$^{2+}$ during spreading depression induced by electrical stimulation, in which the extracellular Ca$^{2+}$ in rat cortex is found to decrease by $(50.0 \pm 7.5)\%$ ($n = 5$) during spreading depression. This study essentially offers a new platform to develop solid-state ISEs, which is particularly useful for *in vivo* measurements of metal ions and pH in live rat brain.
INTRODUCTION

In the central nervous system, Ca$^{2+}$ is widely involved in many physiological activities.$^{1-7}$ For instance, it acts as one of the signaling messengers and participates in a variety of physiological events such as regulation of neuronal survival, neurotransmitter release, gene expression, and cell communication.$^{1-4}$ In addition, Ca$^{2+}$ homeostasis is crucial to the survival of cells and thus it is essential to keep a tight control of Ca$^{2+}$ concentration.$^{5,6}$ Normally, extracellular Ca$^{2+}$ exists in a much higher level (ca. ~1 mM) compared to that of intracellular one (ca. ~100 nM), providing a sufficient source of Ca$^{2+}$ needed for brain functions.$^{7}$ In this context, monitoring the dynamics of extracellular Ca$^{2+}$ has a major impact on our understanding of the detailed molecular mechanisms of the Ca$^{2+}$ messenger system.$^{6,7}$

So far, analytical methods, including atomic absorption spectroscopy, nuclear magnetic resonance, and fluorescence imaging have been reported for the detection and imaging of Ca$^{2+}$; however, these methods are yet insufficient for in vivo monitoring of the extracellular Ca$^{2+}$ due to the lack of spatiotemporal resolution as well as the capability to perform in a living animal level.$^{12,13}$ As an alternative approach, the ion-selective electrode-based potentiometry, featuring great selectivity due to the use of ion-selective membranes (ISMs), and a high spatial resolution resulted from the use of micrometer sized-electrode can meet the requirements for in vivo analysis of Ca$^{2+}$. Moreover, the ISE-based potentiometric analysis could be performed with simple equipment with ease in operation and low cost and have thus been widely used in Ca$^{2+}$ studies.$^{16}$ Although sensitive in the detection of both intracellular and extracellular Ca$^{2+}$, their relatively large size and the involvement of preparing glass capillary with inner filling solutions necessitates the advancement of technology to be less invasive and easier handling.$^{17-26}$

To further explore the application of ISEs for in vivo monitoring of ions (metal ions and H$^+$) in the central nervous system, we recently have prepared solid-state ISEs where ion-selective membranes (ISMs) was formed on CFES.$^{12}$ The use of CFES as the substrate in these ISEs not only simplifies the electrode preparation and implantation into brain region but also accelerates the electrode response to ions. Moreover, as we recently demonstrated, the ISM is highly resistant to protein adsorption, which enables reliable measurement of the dynamics of pH in rat brain.$^{12}$ As reported previously, the ISM/substrate interface acts as a condenser when the ISM is directly deposited on the electronically conducting substrate, where the ion-to-electron transduction is mostly interrupted because one of phases is a pure ionic conductor and the other is electronic conductor.$^{27}$
Moreover, the formation of water layer between ISM and conducting substrate leads to potential drift.\textsuperscript{28,29} It is generally accepted that the measured electromotive force (EMF) is the sum of all the phase boundary potentials in a potentiometric cell, which consists of a solid-state ISE and a reference electrode.\textsuperscript{30} The boundary potential at the ISM/solution interface is established due to the electrochemical equilibrium involving ions of both contacting phases, and the electronically conducting substrate is an electronic conductor that is connected to a measuring instrument. Therefore, the potential should be constant at the interface of the ionically conducting membrane and electronically conducting substrate for stably and reproducibly outputting the measured EMF values.\textsuperscript{27,31} For this purpose, a transducing layer or solid contact remains essential to mediate the charge transfer of this interface and further stabilize the potential. An ideal transducing layer should meet the conditions including (a) a large redox capacity or double layer capacitance to facilitate the reversible ion-to-electron transfer; (b) relatively hydrophobic property to decrease an aqueous layer formation at the ISM/solid contact interface; (c) the absence of side reactions; and (d) ease in preparation.\textsuperscript{30} So far, hydrogels, redox-active self-assembled monolayer and polymers and carbon materials have been used as the transducing layers in ISEs, as reviewed systematically in several excellent reviews.\textsuperscript{16,30,32-36}

Herein, we demonstrate that HCNs can act as the transducing layer for the development of solid-state ISEs for \textit{in vivo} monitoring of Ca\textsuperscript{2+} (Scheme 1). With HCNs as the transducing layer (TEM images of HCNs are shown in Scheme 1, inset), the solid-state ISEs fabricated with CFEs as the substrate have a good stability, reliability, and capability for \textit{in vivo} monitoring of Ca\textsuperscript{2+}. Although the spherical and unique hollow structure as well as the chemical and mechanical stability of HCNs have enabled them to be useful in some fields such as energy storage, adsorbent and catalysis,\textsuperscript{37-41} we demonstrate here that HCNs can act as ion-to-electron transducing layer in solid-state ISEs that enable \textit{in vivo} monitoring of the dynamics of extracellular Ca\textsuperscript{2+} with a high reliability. This study offers a new platform to the development of micro-sized solid-state ISEs that are particularly useful for \textit{in vivo} monitoring of metal ions and pH in the central nervous system.
**Scheme 1.** Illustration of *in vivo* monitoring of Ca\(^{2+}\) in rat brain during spreading depression induced by electrical stimulation with micro-sized Ca\(^{2+}\)-ISE. Inset, TEM images of single-shelled (1s-HCNs), two-shelled (2s-HCNs), three-shelled (3s-HCNs), and four-shelled (4s-HCNs) hollow carbon nanospheres. Scale bar, 100 nm.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** Calcium ionophore II (ETH129), 2-nitrophenyl octyl ether (o-NPOE), sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate (NaTFPB), poly (vinyl chloride) (PVC, high molecular weight), ascorbic acid (AA), dopamine (DA), (±)-epinephrine (E), 5-hydroxytryptamine (5-HT), noradrenaline (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), and uric acid (UA) were all purchased from Sigma and used as supplied. Other reagents were of analytical grade and used without further purification. Artificial cerebral fluid (aCSF) was prepared by mixing KH\(_2\)PO\(_4\) (0.5 mM), NaCl (126 mM), KCl (2.4 mM), MgCl\(_2\) (0.85 mM), CaCl\(_2\) (1.1 mM) and Na\(_2\)SO\(_4\) (0.5 mM) and the solution pH was adjusted to 7.4. All aqueous solutions were prepared with Milli-Q water (18.2 MΩ·cm).

**Synthesis of Shelled Hollow Carbon Nanospheres.** Porous HCNs with different shells of 1, 2, 3 and 4 were synthesized according to previous report.\(^{37}\) Briefly, 3-aminophenol/formaldehyde (3-AF) resins were first synthesized using 3-aminophenol (3-AP, 0.1 g, 0.907 mmol), formaldehyde solution (HCHO (37 wt %), 0.1 mL, 1.331 mmol), and ammonia aqueous solution (NH\(_3\)OH, 25 wt %) as catalyst reacting 30 min at room temperature. After that, 3-AF particles were collected by centrifugation and purified with Milli-Q water. Resin
single-shelled hollow nanospheres (1s-HNs) were synthesized by dispersing 3-AF particles into 30 mL Milli-Q water, followed by adding 20 mL acetone to remove the interior part of particles. After centrifugation and purification with Milli-Q water, the obtained resin 1S-HNs were carbonized at 1200 °C for 6 h under an Ar atmosphere to yield single-shelled HCNs (1s-HNs). The synthesis of 3-AF resin multishelled hollow nanospheres (Ms-HNs) is similar to 1s-HNs that were obtained by repetitious processes of growth and removal and then carbonization to multishelled HCNs (Ms-HCNs). For example, the resin 2s-HNs were constructed as following: with resin 1s-HNs as growth seeds, 3-AP, HCHO and NH₄OH were mixed with Milli-Q water, reacted for 30 min at ambient temperature and removed the internal part using acetone. Following centrifugation and purification, resin 2s-HNs were proceeded for carbonization at 1200 °C for 6 h under Ar atmosphere to yield the 2s-HCNs.

Fabrication of Ca²⁺-ISEs. For in vitro and in vivo studies, Ca²⁺-ISEs were prepared with glassy carbon (GC) electrodes and CFEs as substrate, respectively. Glassy carbon (GC, 3 mm diameter, sealed in a solvent-resistant Teflon plastic body) electrodes were polished first with emery paper and then with aqueous slurries of fine alumina powder (0.3 and 0.05 μm) on a polishing cloth. The electrodes were finally rinsed with ethanol, acetone, and Milli-Q water under an ultrasonic bath for 5 min each. Prior to formation of calcium ion-selective membrane (Ca²⁺-ISM) onto substrate electrode, 1 mg/mL of HCN powder was dispersed into Milli-Q water, and 10 μL of the dispersion was coated onto GC electrodes. The electrodes (HCN-modified GC) were then air-dried to evaporate the solvent. For the formation of Ca²⁺-ISM onto the HCN-modified GC electrodes, a cocktail totally 200 mg containing ETH129 (1.0 wt. %), NaTFPB (0.6 wt. %), o-NPOE (65.6 wt. %) and PVC (32.8 wt. %) was dissolved into 1.5 mL tetrahydrofuran (THF), and 20 μL of the mixture was casted onto the HCN-modified GC electrodes. The Ca²⁺-ISEs were air-dried for 12 h and soaked in 0.1 M CaCl₂ solution for at least 24 h prior to use.

Micro-sized Ca²⁺-ISEs on CFE substrate with 3s-HCNs as the transducing layer were prepared with the procedure similar to that with GC electrode as the substrate. Briefly, 3s-HCNs was dispersed into Milli-Q water and the mixture was sonicated to form a homogeneous dispersion (2 mg/mL). CFEs were prepared and pretreated as demonstrated previously,¹² and photographic illustration of as-made electrodes are shown in Figure S1. Briefly, The glass capillaries (o.d. 1.5 mm, length 100 mm) were pulled into two capillaries (fine tip at 30-50 μm in diameter) with a microelectrode puller (WD-1, Chengdu Instrument Factory, Sichuan, China).
A single carbon fiber (CF, 7 μm diameter, Tokai Carbon Co., Tokai, Japan) was attached to a copper wire with silver conductive paste, and then carefully inserted into the capillary. Both open ends of the capillary were sealed with epoxy resin with 1:1 ethylenediamine and the electrodes were dried at 100 °C for 2 h. The exposed CF was cut to 200-500 μm in length with a surgery scalpel under a microscopy. After that, the CFEs were sequentially sonicated in acetone, 3 M HNO₃, 1.0 M KOH and Mili-Q water for 3 min. Prior to modification with 3s-HCNs, the electrodes were subjected to electrochemical activation in 0.5 M H₂SO₄ which is performed with potential-controlled amperometry at +2.0 V for 30 s, -1.0 V for 10 s, and cyclic voltammetry within a potential range from 0 to 1.0 V at a scan rate of 0.1 V/s sequentially until a stable cyclic voltammogram was obtained. Next, the CFEs were immersed and carefully rolled in 3s-HCN dispersion on a glass surface, and finally dried in ambient temperature. After that, the 3s-HCNs modified-electrodes were immersed and rolled in Ca²⁺-ISM droplet to form Ca²⁺-ISEs until the tip of CF was fully covered with Ca²⁺-ISM. The micro-sized electrodes were air-dried for 12 h and soaked in 0.1 M CaCl₂ solution for at least 24 h prior to use.

**Apparatus and Measurements.** Potentiometric measurements were performed by a computer-controlled electrochemical analyzer (CHI 832B, Shanghai, China) with a two-electrode system with the as-prepared Ca²⁺-ISEs as indicating electrode. For both *in vitro* and *in vivo* electrochemical measurements, a tissue-implantable Ag/AgCl microelectrode was prepared as described previously¹² and used as reference electrode. Electrochemical impedance spectra (EIS) were performed on an Autolab PGSTAT302 (Netherland) from 1 MHz to 0.1 Hz. The Ca²⁺-ISEs were used as working electrode, Pt wire as counter electrode and micro-sized Ag/AgCl electrode as reference electrode. Constant-current chronopotentiometry was performed by a computer-controlled electrochemical analyzer (CHI 660E, Shanghai, China) with the same electrode system as that employed for EIS. All experiments were performed at ambient temperature. Scanning electron microscopy (SEM) was performed on a Hitachi S8020 microscope (Japan). Transmission electron microscopy (TEM) images were recorded on a Hitachi JEOL-2100F microscope (Japan).

**In Vivo Experiments.** Adult male Sprague-Dawley rats (weighing 300-350 g) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. were housed on a 12:12 h light-dark schedule with food and water *ad libitum*. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee of National Center for Nanoscience and Technology of China. The animals were positioned onto a stereotaxic frame after 5 min of 4% halothane induced anesthesia and was maintained with 2% halothane during
*in vivo* experiments through gas pump (R520, RWD, Shenzhen, China). The micro-sized \( \text{Ca}^{2+} \)-ISE with 3s-HCNs as the transducing layer was implanted into the cerebral cortex (AP: -2.20 mm, L: 1.50 mm, DV: -1.50 mm from dura) using standard stereotaxic procedures. The micro-sized Ag/AgCl electrode was implanted into the dura of the brain. The stimulating electrode was implanted into the same depth as that of the \( \text{Ca}^{2+} \)-ISEs and within 1 mm away from each other and was connected to an ISO-flex stimulation box (AMPI, Jerusalem, Israel) to deliver electrical stimulation controlled by Master-9 (AMPI, Jerusalem, Israel). For each stimulation train, a continuous monophasic current pulse (600 \( \mu \text{A} \), 5 s) was applied and EMF response was recorded with the \( \text{Ca}^{2+} \)-ISEs and used for *in vivo* measurement of the dynamics of the extracellular \( \text{Ca}^{2+} \) (Scheme 1).

**RESULTS AND DISCUSSION**

**EMF Response of \( \text{Ca}^{2+} \)-ISEs with HCNs as Transducing Layer toward \( \text{Ca}^{2+} \).** As shown in Scheme 1 (inset), the HCNs with different shells well retain spherical shape and hollow structure. For the single-shelled HCNs (1s-HCNs), the thickness of the shell and the diameter of the cavity are 40 nm and 160 nm, respectively. The shell-shell interspaces size is *ca.* 5 nm in the multi-shelled HCNs. Moreover, during the synthesis of HCN, a carbonization procedure was introduced to generate the porous structure, which has been thoroughly characterized in previous reports.\(^{37}\) To study the capability of HCNs as the transducing layer for \( \text{Ca}^{2+} \)-ISEs, we investigated the response of the \( \text{Ca}^{2+} \)-ISEs prepared with HCNs as the transducing layers toward \( \text{Ca}^{2+} \). For comparison, the \( \text{Ca}^{2+} \)-ISEs were also prepared without an additional transducing layer (i.e., directly cast-coating ISM onto glassy carbon (GC) substrate) or with solid nanospheres as the transducing layer. As shown in Figure 1, all electrodes show a linear response toward the logarithm of the concentration of \( \text{Ca}^{2+} \) (insets in each figure) with the slopes close to the Nernstian slope. Notably, a close comparison of the electrode responses obtained at the HCNs-based electrodes (C-F) with those at the transducing layer-free (A) and carbon nanospheres-based (B) electrodes, especially after 400 s, have shown an obvious reduced potential drift with HCNs-ISE, suggesting the advantages of introducing HCNs as transducing layers.
Figure 1. EMF responses of the Ca$^{2+}$-ISEs without transducing layer (A) and with solid nanospheres (B), 1s-HCNs (C), 2s-HCNs (D), 3s-HCNs (E), or 4s-HCNs (F), as the transducing layer toward successive addition of Ca$^{2+}$ into the Ca$^{2+}$-free aCSF. Inset, the corresponding calibration equation for each electrode.

The stability enhancement with HCNs as the transducing layer was further verified by continuously running the measurements for more than 10 h, as depicted in Figure 2. For the Ca$^{2+}$-ISE without transducing layer, a large potential drift (2.2 mV/h) was observed. However, the potential drifts were greatly reduced when HCNs were used as transducing layers of the Ca$^{2+}$-ISEs. With solid nanospheres, 1s-HCNs, 2s-HCNs, 3s-HCNs, or 4s-HCNs as the transducing layer, the potential drifts were 0.30 mV/h, 0.25 mV/h, 0.14 mV/h, 0.020 mV/h and 0.13 mV/h, respectively. Even though we have observed differences in potential drift values with 1s, 2s, 3s, or 4s-HCNs, there was no obvious trend in the results. Taken together, these results highlight the key role of introducing HCNs as transducing layer in improving the stability of the solid-state ISEs.
Figure 2. The stability of the Ca$^{2+}$-ISEs without transducing layer (a) and with solid nanospheres (b), 1s-HCNs (c), 2s-HCNs (d), 3s-HCNs (e), or 4s-HCNs (f) as the transducing layer in 1 mM CaCl$_2$. Note that the curves represent the potential changes of electrodes, not the actual potential.

The stability enhancement was ascribed to the increase of the electrical capacity of the electrodes by introducing HCNs transducing layer, which was demonstrated with current reversal chronopotentiometry. Figure 3 shows typical chronopotentiograms ($E$ - $t$ curves) recorded at the Ca$^{2+}$-ISEs prepared with different procedures in 0.1 M CaCl$_2$ solution when a current of ±1 nA was applied, each for 60 s. At all Ca$^{2+}$-ISEs, the $E$ - $t$ curves show a potential jump when the current reversed, followed by a slow potential drift as a function of time, which was similar to the previous report. Without HCNs as the transducing layer, the as-prepared Ca$^{2+}$-ISE was easily polarized; when a constant current of ±1 nA was applied, a dramatic potential change of 40 mV was recorded at a short time of 60 s (black curve). The uses of HCNs as the transducing layer greatly reduce the potential drift of the as-prepared Ca$^{2+}$-ISEs under the same conditions (other color curves), indicating the improved stability of the HCNs-based Ca$^{2+}$-ISEs. Furthermore, the potential drift ($\Delta E/\Delta t$) was used to calculate capacitance ($C$) of the transducing layer with the equation of $\Delta E/\Delta t = I/C$, where $\Delta E$ and $I$ are the potential change at short time and the applied current, respectively. The results were summarized in Table 1.
Figure 3. Chronopotentiograms recorded in 0.1 M CaCl$_2$ solution with the Ca$^{2+}$-ISE prepared without (a, black) and with the transducing layer of solid nanospheres (b, red), 1s-HCNs (c, blue), 2s-HCNs (d, magenta), 3s-HCNs (e, olive) or 4s-HCNs (f, navy). Current applied, +1 nA for 60 s and -1 nA for 60 s.

Table 1. Capacitance and EMF drift of the Ca$^{2+}$-ISEs measured by chronopotentiometry at ±1nA.

<table>
<thead>
<tr>
<th>Transducer</th>
<th>ΔE (mV)</th>
<th>ΔE/Δt (μV/s)</th>
<th>C=I/(dE/dt) (μF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>40</td>
<td>660</td>
<td>1.5</td>
</tr>
<tr>
<td>Solid nanospheres</td>
<td>5.0</td>
<td>83</td>
<td>12</td>
</tr>
<tr>
<td>1s-HCNs</td>
<td>2.2</td>
<td>37</td>
<td>27</td>
</tr>
<tr>
<td>2s-HCNs</td>
<td>8.2</td>
<td>137</td>
<td>7.3</td>
</tr>
<tr>
<td>3s-HCNs</td>
<td>1.5</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>4s-HCNs</td>
<td>3.8</td>
<td>63</td>
<td>16</td>
</tr>
</tbody>
</table>

It could be seen in Table 1 that the high ΔE/Δt of the transducer-free Ca$^{2+}$-ISE (i.e., 660 μV/s) may be related to the low interface capacitance (1.5 μF); the use of HCNs as the transducing layer and the solid contact increases the capacitance of the Ca$^{2+}$-ISEs and thereby increases the EMF stability of the electrodes. Such property was considered to result from the high specific surface area, porous structure and the cavity of HCNs. In addition, it has been reported that the hollow structure favors metal ion transportation, which may be the other reason to mediate the charge transfer process.

It has been reported that a thin water layer (also called aqueous layer or water film) would unintentionally accumulate at the ISM/solid contact interface, which causes the potential drifts of ISEs. The water layer acts as an electrolyte reservoir that re-equilibrates on the change of ionic composition or some gases. The
thickness of aqueous layer was generally measured as 100 ± 10 Å at the interface of PVC sensing membrane and silicon wafer substrate in coated-wire ISEs. Although the volume of the water layer is often extremely small, even minute ion fluxes can cause large changes of ion concentration in water film. Therefore, the presence of such an aqueous layer was considered as disadvantages of the solid-state ISEs because the electrodes have to suffer from slow equilibration process, potential drift, sensitivity to osmolality changes, and ultimately mechanical failure because of membrane delamination.

The effective way to preventing the formation of aqueous layer is to decrease the water uptake of ISM or to increase the hydrophobicity of transducer layer. Here, water layer test was carried out to evaluate whether a water film exist between the ISM and HCNs layer. To do this, the EMF responses of the electrodes were continuously recorded when the electrodes were initially immersed into 1 mM CaCl$_2$ for 2 h, then 1 mM MgCl$_2$ for 2 h, and finally 1 mM CaCl$_2$ for 8 h. As shown in Figure 4, all the Ca$^{2+}$-ISEs show an obvious potential drop with a drift when the Ca$^{2+}$ solution was changed into MgCl$_2$ solution. While, the potential recovers to the initial value when the electrodes were placed back into the CaCl$_2$ solution, suggesting the formation of water film at the ISM/GC (without solid contact) interface. It is generally accepted that the transport of the relevant ions from the sample through the ISM into the water layer or the reverse process is the main reason for the slow drifts. When the test solution was replaced by MgCl$_2$, Ca$^{2+}$ in the water layer is diluted owing to the
diffusion of MgCl$_2$ from solution to water layer and the diffusion of CaCl$_2$ from layer to solution. Similarly, the drift was also observed after the electrode was immersed back to the Ca$^{2+}$ solution owing to the gradual diffusion of Ca$^{2+}$ to the layer. The striking difference in the potential drift recorded with the Ca$^{2+}$-ISEs lies in the quicker potential drops with a tiny drift when HCNs were used as the transducing layer and the solid contact compared with that without solid contact. This observation suggests that the introduction of HCNs into solid-state ISEs essentially allows the as-prepared electrodes to eliminate much formation of water layer at the interface of ISM and solid contact. This property was reasoned to result from the hydrophobicity and the hollow structure with cavities of HCNs.$^{37}$

By serial comparison, we found that the Ca$^{2+}$-ISE with 3s-HCNs as the transducing layer shows the best performance in the stability. The EMF drift of this electrode (25.0 $\mu$V/h) is lower than that obtained at the ISE with single-wall nanotubes as the transducing layer (220 $\mu$V/h)$^{89}$ and comparable to that of the potassium-selective electrode with porous carbon spheres-based as the transducing layer (15 $\mu$V/h)$^{90}$ even the amount of 3s-HCNs (10 $\mu$g) used here was much less than that of porous carbon spheres (160 $\mu$g) as summarized in Table S1. As mentioned above, the improved stability of the 3s-HCNs-based electrode was understood with the unique hollow structure of HCNs that is different from the tubular structure of SWNTs, in which the former structure can provide a large surface area to shorten the diffusion distance and thus facilitate ion transport.$^{37}$ Moreover, a proper proportion of cavities between carbon layers was reported to buffer the volume change that arises from the ion insertion/extraction and water layer accumulation.$^{51}$

**Application to In Vivo Monitoring the Dynamics of Extracellular Ca$^{2+}$.** Having demonstrated the good response and high stability of the 3s-HCNs-based Ca$^{2+}$-ISE, we next studied its application for in vivo analysis. To do this, we fabricated micro-sized solid-state Ca$^{2+}$-ISEs using 3s-HCNs as the transducing layer, ionophore-doped PVC membrane as the ISM, and CFEs as the conducting substrate. Figure 5 shows SEM images of the as-prepared electrodes (A, B and C). 3s-HCNs were successfully deposited onto the CFEs uniformly (B) by manually rolling CFEs in HCNs dispersion and the CFE modified with 3s-HCNs was totally covered with Ca$^{2+}$-ISM (C). In order to verify the 3s-HCNs works as the transducing layer of Ca$^{2+}$-ISEs on the carbon fiber substrate, the short-term potential stability of the electrode was studied with current reversal chronopotentiometry and the interfacial water film and potential stability were also assessed. As shown in Figure 5 (D, E and F), the micro-sized Ca$^{2+}$-ISE (red curves) exhibits better stability compared with the Ca$^{2+}$-
ISE without transducing layer (black curves). Moreover, the resistances of our micro-sized Ca\(^{2+}\)-ISE was measured to be 4.5 MΩ (Figure S2), which is significantly lower than that of conventional glass microelectrodes, holding a great promise for fast response in in vivo applications.

**Figure 5.** SEM images of (A) bare CFE, (B) 3s-HCN-modified CFE and (C) Ca\(^{2+}\)-ISE with 3s-HCNs as the transducing layer. (D) Chronopotentiograms obtained with the Ca\(^{2+}\)-ISEs using 3s-HCNs as the transducing layer (red curve) and without transducing layer (black curve) in 0.1 M CaCl\(_2\). Applied current +1 nA for 60 s, and -1 nA for 60 s. (E) Water layer test for the Ca\(^{2+}\)-ISE without transducing layer (black curve) and with 3s-HCNs as the transducing layer (red curve). The measurements were recorded in 1 mM CaCl\(_2\) for 1 h, 1 mM MgCl\(_2\) for 1 h, and back to 1 mM CaCl\(_2\) for 3 h. (F) The potential stability of the Ca\(^{2+}\)-ISEs with 3s-HCNs as the transducing layer (red curve) and without transducing layer (black curve) in aCSF.

Prior to _in vivo_ measurements, we studied the analytical properties of the electrodes in terms of response as well as the selectivity toward Ca\(^{2+}\). As showed in **Figure 6A**, the micro-sized Ca\(^{2+}\)-ISE shows a nearly Nernstian response of 28 mV/decade over the Ca\(^{2+}\) concentration range of 10\(^{-5}\) M - 0.05 M. As reported previously, O\(_2\) in the solution can diffuse through the ISM and reach the interface of solid contact, which may cause undesirable effects if the solid-state ISEs lack of appropriate solid contact.\(^{29}\) It is very likely that O\(_2\) would form an irreversible oxygen half-cell with these species, resulting in the change of the phase boundary potential.\(^{45}\) Therefore, the effect of O\(_2\) was investigated here by recording the EMF response of the micro-sized Ca\(^{2+}\)-ISE when the solution was first bubbled N\(_2\) to remove dissolved O\(_2\) and then purged O\(_2\) into in aCSF. As shown in **Figure 6B**, no significant potential drift was observed upon exposure to O\(_2\). Light interference has
been observed for several solid-state ISEs, especially with poly(3-octylthiophene) (POT) as solid contact.\textsuperscript{28,30} The effect of light was studied here by continuously recording the EMF response in a stirred aCSF when the ambient light was turned on or off. The EMF response (Figure 6C) was found to be almost constant during the measurement, suggesting that the 3s-HCNs-based Ca\textsuperscript{2+}-ISE has no light sensitivity.

![Image of Figure 6](image)

**Figure 6.** (A) EMF responses of the micro-sized Ca\textsuperscript{2+}-ISE with 3s-HCNs as the transducing layer and solid contact toward the successive addition of Ca\textsuperscript{2+} into the Ca\textsuperscript{2+}-free aCSF. Inset, calibration curve of the electrode. (B) Effect of O\textsubscript{2} on the potential stability of the micro-sized Ca\textsuperscript{2+}-ISE in aCSF. (C) Effect of light on the potential stability of the micro-sized Ca\textsuperscript{2+}-ISE in aCSF. (D) EMF responses at the micro-sized Ca\textsuperscript{2+}-ISE in aCSF (pH 7.4) toward the additions of AA (100 \(\mu\)M), DA (10 \(\mu\)M), DOPAC (20 \(\mu\)M), UA (50 \(\mu\)M), E (20 \(\mu\)M), NE (20 \(\mu\)M), 5-HT (10 \(\mu\)M), K\textsuperscript{+} (3 mM), Na\textsuperscript{+} (5 mM), Mg\textsuperscript{2+} (1 mM), or 10 mM CaCl\textsubscript{2}.

It is known that the change of Ca\textsuperscript{2+} in the brain usually accompanies with the change of inorganic ions like K\textsuperscript{+}, Na\textsuperscript{+} and H\textsuperscript{+}.\textsuperscript{52,53} The potentiometric method usually less interfered by the redox substances.\textsuperscript{12,54} However, for the ISEs we fabricated using polymeric membrane and carbon fiber as conductive substrate, many electroactive species, including AA and DA, can be easily oxidized or reduced at the substrate, producing potential interference toward Ca\textsuperscript{2+} measurement. To study the possible interference from these species, we recorded the EMF responses of the micro-sized Ca\textsuperscript{2+}-ISE toward these species (Figure 6D). The successive addition of electroactive redox species or metal ions (Mg\textsuperscript{2+}, K\textsuperscript{+} and Na\textsuperscript{+}) did not produce noticeable potential disturbance, suggesting these species did not interfere with the Ca\textsuperscript{2+} detection, further validating our method for
selectively monitoring Ca\(^{2+}\) in live brain of rats.

![Figure 7](image)

**Figure 7.** EMF responses of the micro-sized Ca\(^{2+}\)-ISE toward successive addition of Ca\(^{2+}\) before (black curve) and after (red curve) the electrode was implanted in the brain cortex for 2 h. Inset, pre- (black) and post- (red) calibration curves of the micro-sized Ca\(^{2+}\)-ISE.

Moreover, when the electrode is implanted into the brain of animals, it is inevitably subjected to the nonspecific adsorption of biological macromolecules, especially proteins onto its surface.\(^{12,55,56}\) This would deactivate the electrode and thus lead to a gradual loss of sensitivity and finally invalidate \textit{in vivo} measurements.\(^{55,57}\) To study the antifouling ability of the electrode in a real physiological environment, we implanted the micro-sized Ca\(^{2+}\)-ISE into the live brain of rats for 2 h and compared its response towards Ca\(^{2+}\) before its implantation into rat brain. As depicted in **Figure 7**, the EMF response toward Ca\(^{2+}\) was almost maintained after \textit{in vivo} implantation of the electrode, suggesting a high resistance of the electrode against the nonspecific adsorption of biomolecules in the cerebrospinal fluid.\(^{12}\) Taken together, the use of conduciveness and the hydrophobic nature of HCNs to stabilize the potential drift, and chemical stability of HCNs toward O\(_2\), CO\(_2\) and light, all synergistically contribute HCN-modified new sensor with better stability, holding great potential for practical in vivo applications.

With these properties, we finally used the micro-sized Ca\(^{2+}\)-ISE to monitor the Ca\(^{2+}\) dynamics in rat cortex during spreading depression (SD). As reported previously, SD is a transient propagating wave of neurons and glial depolarization in cerebral grey matter accompanied by massive changes of ionic concentrations, swelling of neurons and subsequent suppression of neuronal and glial activity.\(^{52,58,59}\) **Figure 8** shows the potential responses obtained with the electrodes in cerebral cortex following SD induced by local electrical stimulation.
(600 µA, 5s). In relative to the basal level of Ca\(^{2+}\) under normal conditions, electrical stimulation led to a decrease in the concentration of the extracellular Ca\(^{2+}\) in rat cortex by \((50 \pm 8\)% \((n = 5)\) during SD process, suggesting that a large amount of extracellular Ca\(^{2+}\) would influx into cells, which was consistent with the previous report.\(^{59}\) It is known that the activation of neurons could induce glutamate-evoked calcium influx in postsynaptic neurons, thus changes in extracellular ion concentration probably are natural consequences of the firing activity of neurons.\(^{59,60}\)

**Figure 8.** In vivo monitoring the dynamics of extracellular Ca\(^{2+}\) in cerebral cortex following SD induced by local electrical stimulation. Potential response was recorded with the micro-sized Ca\(^{2+}\)-ISE. A continuous monophasic current pulse (600 µA, 5 s) was applied for each stimulation train.

**CONCLUSIONS**

In summary, by using hollow carbon nanospheres as transducing layer and solid contact, we have successfully developed a potentiometric method for real-time monitoring of the extracellular Ca\(^{2+}\) in the living brain of rats. Electrochemical studies show that the HCNs can improve the signal stability of the solid-state ISEs resulted from the unique hollow structure and of their surface hydrophobicity. The ISEs exhibit a high stability and selectivity against the species endogenously existing in the brain as well as tolerance against O\(_2\) and light. These properties substantially enable the as-developed potentiometric method to real-time monitor the dynamics of the extracellular Ca\(^{2+}\) in the living brain of rats. This study offers a platform to development of micro-sized solid-state ISEs that are potentially used for in vivo monitoring of metal ions and pH in living brain of the animals during brain functions.
ASSOCIATED CONTENT

Supporting Information Available: Photographic illustration of microsized electrodes and additional experimental details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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