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ARTICLE TYPE

In-vivo and continuous measurement of bisulfide in the hippocampus of rat's brain by on-line integrated microdialysis/droplet-based microfluidic system

Feidan Gu[a], Xiaoyu Zhou[a], Xiaocui Zhu[a], Meiping Zhao*[a], Jie Hao[b], Ping Yu[b], and Lanqun Mao*[b]

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An on-line and continuous approach was demonstrated for in vivo measurement of bisulfide in rat's brain. A modified droplet-based microfluidic system was constructed which allowed on-line qualification of the fluorescence responses of the gold nanoparticle-glutathione-fluorescein isothiocyanate probe to the variation of bisulfide in the presence of the cerebral microdialysate background. The on-line method achieved a dynamic working range from 5.0 μM to 40 μM and a detection limit of 2.5 μM. The in-vivo bisulfide concentration in the hippocampus of rat's brain was measured under different physiological conditions. The on-line method may facilitate the study of H_2S biology by providing a previously unattainable continuous record of H_2S variation in living animals. It also provides a practical platform for in-vivo and continuous monitoring of other important species in cerebral systems.

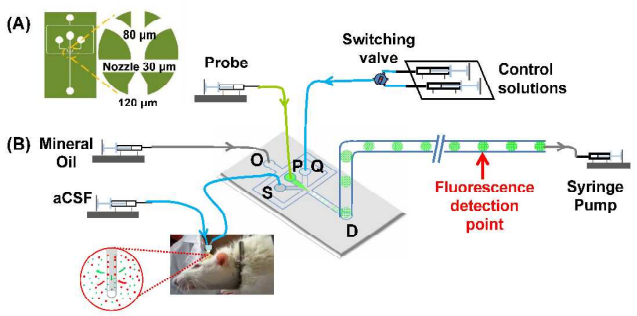
Introduction

Hydrogen sulfide (H_2S), known as a toxic gas and an environmental hazard, has been recently identified as the third endogenously produced gaseous transmitter, along with nitric oxide (NO) and carbon monoxide (CO).[1, 2] It functions both in the cardiovascular system and in the central nervous system (CNS).[3-5] In the CNS, H_2S is mainly produced from L-cysteine (Cys) and homocysteine (Hcy) by the actions of cystathionine b-synthase. In addition to biosynthesis, there are two forms of sulfur stores in mammals, acid-labile sulfur and bound sulfane sulfur, which release H_2S under acidic conditions and reducing conditions, respectively. Bound sulfur may be a source of H_2S in brain and it can immediately release H_2S in response to physiologic stimulation.[6] In the CNS, H_2S facilitates long-term potentiation and regulates intracellular calcium concentration and pH level in brain cells. Abnormal generation and metabolism of H_2S have been reported in the pathogenesis of ischemic stroke, Alzheimer's disease, Parkinson's disease, and recurrent febrile seizure.[7] On the other hand, exogenously applied H_2S is demonstrated to have value for the treatment of febrile seizure and Parkinson's disease. Thus, it is assumed that H_2S may play a neuroprotective role at physiological concentrations but may exhibit neurotoxic effects at significantly higher concentrations.

Under physiological conditions, >80% of hydrogen sulfide exists as bisulfide (HS^-). Free H_2S usually refers to the sum of dissociated and associated forms of H_2S. Polarographic sensor has been developed for real-time measurement of H_2S production in biological samples, including cells, organ homogenates, and intact tissues.[8] The gas/ion chromatography combined with electrochemical detection has also been applied for measurement of H_2S levels in brain.[9] However, no approaches have been reported for on-line and continuous monitoring of the in-vivo concentrations of H_2S in the brain tissues of living animals. A number of factors such as the instability and high volatility of bisulfide make it difficult to obtain the actual value of H_2S level in the brain tissues. To explore the functions of H_2S in nervous systems, it is of great importance to develop more r eliable and validated methods for in-vivo H_2S detection.

In-vivo microdialysis is a minimally-invasive technique for continuously sampling small molecules or ions from extracellular fluids of various tissues.[10] Droplet-based microfluidic systems are useful tools for measurement of the small-volume samples with high temporal resolution.[11-16] In our previous work, we have synthesized a gold nanoparticle-glutathione-fluorescein isothiocyanate (AuNP-GSH-FITC) probe for rapid and sensitive detection of bisulfide through a specific anion-for-molecule ligand exchange reaction.[17] By co-immobilization of thiol-polyethylene glycol (HS-PEG-COOH) onto the surface of the AuNPs, the probe showed high stability in high salty solution such as artificial cerebrospinal fluids (aCSF). Using the AuNP-GSH-FITC probe, a preliminary droplet-based microfluidic fluorescent sensor was further constructed for online monitoring of bisulfide variation in the microdialysate in aCSF.

In this work, we attempt to further improve the integrated microdialysis/droplet-based microfluidic chip system for in-vivo measurement of the HS^- concentration in rat's brain. As displayed in Scheme 1, a new droplet-generation design was employed. In addition to the inlets of the sample solution and the probe solution, a third inlet was added for on-line measurement quality control. The flow rates and other experimental conditions were optimized and the integrated system was successfully applied to track the variation of in vivo bisulfide concentration in the rat's brain under different physiological conditions. To the best of our knowledge, this is the first report on on-line and continuous measurement of in-vivo concentration of bisulfide in the brain of living animals.



Scheme 1. (A) CAD design of the droplet-formation microfluidic chip; (B) Illustration of the integrated microdialysis/droplet-based microfluidic chip system for in-vivo measurement of the HS^- concentration in rat's brain.

Experimental

Reagents and Instrumentation

Sodium sulfide nonahydrate ($Na_2S{\cdot}9H_2O$), hydrogen tetrachloroaurate trihydrate ($HAuCl_4$), sodium citrate, and other inorganic chemicals were purchased from Beijing Chemical Reagent Corporation (Beijing, China). L-glutathione, reduced (purity 499.5%) (GSH) and fluorescein isothiocyanate (FITC Isomer I (TLC 495%)) were purchased from Merck (Frankfurter, Germany). Mineral oil (light oil, neat) was purchased from Sigma (St. Louis, USA). Thiol-polyethylene glycol (HS-PEG-COOH) was purchased from Taiyuan Pegchem. Co., Ltd. (Taiyuan, China). All chemicals were of analytical reagent grade and used as received without any further purification.

Sylgard 184 prepolymer and its curing agent were purchased from Dow Corning (Midland, USA). A film mask from Shenzhen Microcad Photo-mask Ltd. was used as the photomask. The 40-μm negative dry film photoresist (FX540) was obtained from DuPont Comp (Wilmington, USA). The artificial cerebrospinal fluid (aCSF) (126 mM NaCl, 2.4 mM KCl, 1.1 mM $CaCl_2$, 0.85 mM $MgCl_2$, 27.5 mM $NaHCO_3$, 0.5 mM Na_2SO_4, 0.5 mM KH_2PO_4, pH 7.4) was prepared with pure water, filtered by a 0.45 μm millipore filter, and used as a perfusion solution for on-line measurements.

Microdialysis probes CMA 12 (20 kD, 4 mm) and MAB6 (15 kD, 2mm) were purchased from CMA Microdialysis AB (Stockholm, Sweden). The droplet fluorescence intensity was observed and recorded by an Inverted Fluorescence Microscopy (IX71, Olympus, USA) with U-MWIBA module (excitation filter BP460-495, emission filter BA510-550, and dichromatic filter DM505) equipped with an EMCCD (Evolve512, Photometric, USA). The droplet images were analyzed by ImageJ software.

Modification of the droplet-based microfluidic chip

As shown in Scheme 1, the droplet-based microfluidic chip was fabricated by modification of a previous design.[17] Liquids were injected into the device via four inlets: P, Q and S for the probe solution, the control solution and the sample solution (i.e. the effluent from microdialysis), respectively, and O for the oil phase. Outlet D was connected to a 10-cm long poly(tetrafluoroethylene) (PTFE) tube (0.6 mm i.d./1.0 mm o.d.). The microfluidic channels were created by using a dry film photolithography fabrication technique as previously described.[17] The channels for oil phase and aqueous solution were both 80 μm wide, and the nozzle where the droplets were formed was 30 μm wide (Scheme 1A). The main channel where the droplets flew through was 120 μm wide. The depth was 40 μm for all the channels. Standard solutions of Na_2S in aCSF at different concentrations were directly injected into the microchip via inlet S to calibrate the droplet fluorescence and the bisulfide concentration.

Integration of the droplet-based microfluidic chip with microdialysis sampling system for continuous on-line measurement

For the on-line monitoring of bisulfide in the effluent of microdialysis, the microdialysis probe was inserted into the standard solution of Na_2S in aCSF or the rat brain (Scheme 1B). Then the microdialysis probe was perfused with aCSF driven by a microinjection pump through fluorinated ethylene propylene (FEP) tube. The flow rates of microdialysis probe injection and oil phase delivering were set at 1.0 μL/min and 9.0 μL/min, respectively. A pump was attached to the outlets of the PTFE tube to reduce the pressure and the flow rate was set at 19 μL/min. The microdialysis recovery was determined by comparing the droplet fluorescent intensity of the same concentration of bisulfide in aCSF solution with and without microdialysis. For the in vivo measurements, brain microdialysates were sampled from the rat brain with MAB6 (15 kD, 2 mm) because of the size of hippocampus.

Animal surgery

Animal surgery was carried out following the procedures as described previously.[18] Briefly, adult male Sprague-Dawley rats (3 months of age, weighing 300±50 g at the time of surgery) were obtained from Experimental Animal Center of Peking University. The rats were housed individually in cages with free access to food and water in a light cycle of 12/12 hours light/dark. The animals were anesthetized with chloral hydrate (350 mg/kg i.p.) and were fixed in a stereotaxic frame with the incisor-bar set at 3.3 mm below the interaural line for the flat skull position.

A microdialysis guide cannula (BAS/MD-2250, BAS) was implanted into the hippocampus (4.0 mm caudal to bregma, 2.0 mm lateral to the midline, and 2.0 mm below the skull) in the rat brain using standard stereotaxic procedures. The guide cannula was kept in place with three skull screws and dental cement. Stainless steel dummy blockers were inserted into the guide cannula and fixed until the insertion of the microdialysis probe. Throughout the surgery, the body temperature of the rats was maintained at 37°C with a heating pad. After the surgery, the rats were placed into a warm incubator individually until they recovered from anesthesia. The rats were allowed to recover for 24 h before microdialysis sampling and on-line detection.

The surgical procedures for global two-vessel occlusion (2-VO) cerebral ischemia/reperfusion were undertaken during the period of in vivo microdialysis and on-line measurements. The 2-VO ischemia model was constructed with ligation of the bilateral common carotids arteries (CCAs) with a 3-0 suture to induce

permanent forebrain ischemia, with the methods reported previously.[19, 20] Briefly, through a midline cervical incision, both common carotid arteries (CCA) were exposed and isolated from surrounding connective tissue. Global ischemia and reperfusion were induced by first occluding both carotid arteries with nontraumatic arterial clips for ischemia and then removing the clips for reperfusion. Throughout the operation, supplements of chloral hydrate (100 mg/kg) were given as required, and the body temperature of the animals was maintained at 37 °C using a heating pad. The wound was sutured immediately after the ischemia procedure was completed.

All experimental procedures were performed in compliance with the relevant laws and institutional guidelines for the care and use of laboratory animals in Peking University, Beijing, China, and the experiments were also approved by the animal ethics committee of Peking University.

Results and Discussions

Modification of the droplet-based microfluidic chip for on-line measurement of bisulfide in the microdialysate

In our previous work, we have developed an AuNP-GSH-FITC probe which can rapidly response to the variation of bisulfide concentration in the droplets of microdialysate.[17] The probe showed high selectivity for bisulfide over other possibly coexisting anions, including sulfate, sulfite, bisulfite, thiosulfate, persulfate, citrate, thiocyanate, pyrophosphate, nitrite, oxalate, bicarbonate, nitrate and bromide. Because of the steric hindrance of the HS-PEG-COOH on the surface of AuNPs, the probe also successfully resists the interferences from relatively large co-existing thiol-containing biological molecules, such as GSH and bovine serum albumin. With the addition of proper amount of glyoxal, the interferences from small thiol-containing biological molecules such as Cys and Hcy could be also be effectively prohibited. These off-line experimental results suggested that under normal biological concentration conditions of these possible interfering species, their influences on bisulfide detection are negligible.

However, unlike ex-vivo measurements, the compositions of in-vivo biofluidic samples are variable, depending on the tested animal and specific experimental conditions. To avoid unexpected elevation of the level of interferences under certain circumstances, it is desirable to be able to perform on-line spiking or examination of the reaction solutions in the droplets. To this end, we modified the pattern of the channels for the droplets formation. As illustrated in Scheme 1, in addition to the inlets of the sample solution (S) and the probe solution (P), a third inlet (Q) was added to allow injection of the quality control solution to the droplets. A dual-channel switching valve were employed to smoothly change the composition of the control solutions without affecting the flow rate of the stream.

The microdialysis rate is usually between 1.0 and 5.0 μL/min. To ensure relatively high microdialysis efficiency and stable mixing ratio, we chose to fix the flow rates of the sample solution (i.e. the perfusion rate of the microdialysis probe), the probe solution and the control solution at 1.0 μL/min. Then we tested different flow rates of the oil phase in order to obtain stable and continuous spherical droplets in the PTFE tube. The optimum flow rate of the oil phase was observed to be 9.0 μL/min, at which the generated droplets were uniform and reproducible. The volume of the droplets are estimated to be 0.23±0.02 μL and the time intervals between the two neighboring droplets are 4.6±0.3 s. The flow rate of the droplets is estimated to be 7.5×10^{-4} m/s. The bisulfide concentration was quantitated based on the fluorescence intensity of the droplets recorded at the detection point with CCD camera, as described previously. The physical lag time between the microdialysis probe and the fluorescence detect point was approximately 10 min.

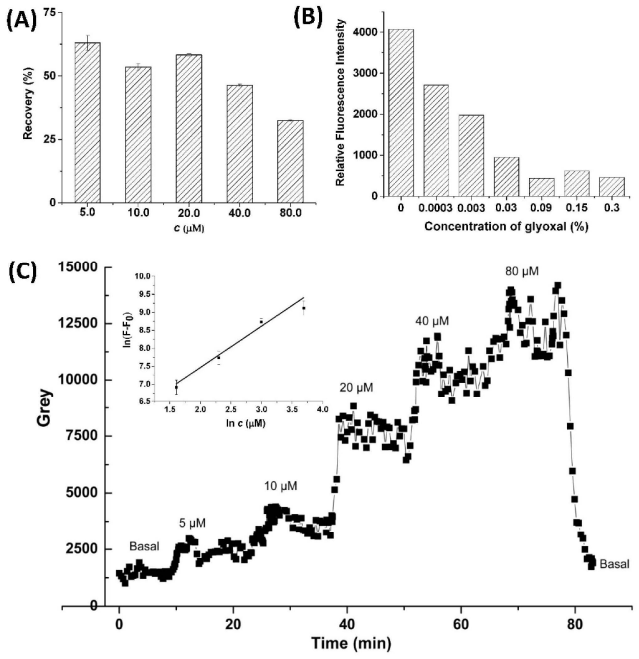


Fig 1. (A) The relative recoveries of microdialysis probe CMA 12 (20 kD, 4 mm) at different concentrations of bisulfide in aCSF perfusion solution. (B) Optimization of the glyoxal concentration for elimination of the influences from Cys and other biothiol analogues. (C) On-line, continuous monitoring of the variation of bisulfide in aCSF microdialysate by using the modified droplet-based microfluidic system. The inset shows the calibration curve for quantification of the bisulfide concentration. The flow rates of the he sample solution, probe solution and control solution are 1.0 μL/min. The flow rate of the oil phase is 9.0 μL/min.

Under above flow rate conditions, we determined the relative recovery of the microdialysis probe integrated with the droplet-based microfluidic system (defined as the percentage of the bisulfide concentration in the droplets containing the dialysate account for the original concentration of bisulfide in the perfusion solution). Fig. 1A showed the relative recoveries of microdialysis for bisulfide when using the microdialysis probe CMA 12 (20 kD, 4 mm). It can be seen that the recoveries were all above 50% at bisulfide concentrations varying from 5.0 μM to 40 μM in the perfusion, indicating that CMA 12 microdialysis probe was well suitable for measurement of bisulfide in brain regions with depth larger than 4 mm. For bisulfide concentrations higher than 80 μM, the recovery may be improved by applying slower flow

rates. For other brain regions with smaller size, microdialysis probe with shorter membrane length should be used. Therefore, we further tested another microdialysis probe MAB6 (15 kD, 2 mm) with a smaller cut-off and shorter membrane length, which showed an average recovery of 25±3%. For comparison, we also measured the recoveries of Cys in the concentration range from 50 μM to 200 μM under the same experimental conditions, which were found to be lower than 10%. These results suggested that the microdialysis probe could effectively exclude Cys and other thiol-containing analogues and reduce their influences on the bisulfide detection.

Previous off-line experimental results showed that addition of 0.1% glyoxal to the reaction solution can effectively eliminate the influence of Cys at concentration as high as 20 μM.[17] To further confirm that Cys and Hcy won't influence the bisulfide detection even at higher concentrations, we tested to add glyoxal to the reaction solution in the droplets to eliminate their interferences. Since long-time co-incubation of the AuNP–GSH-FITC probe with glyoxal may cause increase of the fluorescence background, we added glyoxal to the control solution which was included in the reaction solution as soon as the sample and the probe were mixed together. By fixing the concentration of Cys at 50 μM, we optimized the amount of glyoxal in the control solution. As shown in Fig. 1B, addition of 0.03% glyoxal in aCSF to the droplets could reduce the signal of 50 μM Cys to less than 15% of the original value. As the recovery of Cys was less than 10%, its concentration in the microdialysate would be far less than 50 μM. Hence, addition of 0.03% glyoxal to the droplets was sufficient to prohibit the interferences from Cys and other biothiol analogues. Fig. 1C displays the on-line fluorescent responses to the variation of concentrations of bisulfide in the perfusion solution in the presence of 0.03% glyoxal. The inset of Fig. 1C shows the calibration curve on a log–log scale for quantification of bisulfide based on the increase of fluorescence intensity. The linear working range was from 5.0 μM to 40 μM and the limit of detection (based on a signal-to-noise ratio of 3) was calculated to be 2.5 μM.

On-line monitoring of the variation of bisulfide in the brain of awake rats by using the integrated microdialysis/microfluidic chip system

For in-vivo measurement, a syringe pump in withdraw mode was attached to the outlet of the PTFE tube to help reduce the pressure of the liquid streams in the channels of the microfluidic chip caused by insertion of the microdialysis probe into the tissue. The withdraw flow rate was set at 19.0 μL/min. To measure the basal level of H_2S in awake rats, we anesthetized the rat with ether and fixed it on a home-made rat holder before it woke up. Then we inserted the microdialysis probe into the guide cannula and the bisulfide concentration in the cerebral microdialysate was continuously monitored by the microfluidic droplet system. Fig. 2A compares the on-line fluorescence signals of the bisulfide in the microdialysate of three different rats. As can be seen, the fluorescence signal showed a significant increase when the probe was inserted into the brain tissue through the cannula, indicating a notable elevation of bisulfide concentration in response to the slight injury of the brain tissue. Then the fluorescent signals gradually declined to a stable level. Interestingly, the smallest one among the three tested rats exhibited relatively higher responses to the insertion of the microdialysis probe. Whether or not these signal variations are associated with the age or weight or other conditions of the test animals merits further detailed investigations.

To verify that the detection system works well during the microdialysis processes, we spiked the droplets with known amount of Na_2S standard solutions (10 μM). As expected, the on-line system showed a large fluorescence response to the addition of bisulfide (Fig. 2B), indicating that the reaction between the AuNP–GSH-FITC probe and bisulfide were not affected by the matrices present in the cerebral microdialyate solution. We also compared the detection results of the same cerebral microdialyate solution with or without the addition of 0.03% glyoxal to the control solution. No significant differences were observed between the fluorescence signals of bisulfide in the cerebral microdialyate solution, indicating that the influences of Cys and other thiol-containing molecules on the on-line detection were negligible. To further rule out the false positive signals possibly induced by other ions, more negative control experiments can be performed by selectively masking bisulfide in the sample solution and monitoring whether the signals are inhibited.

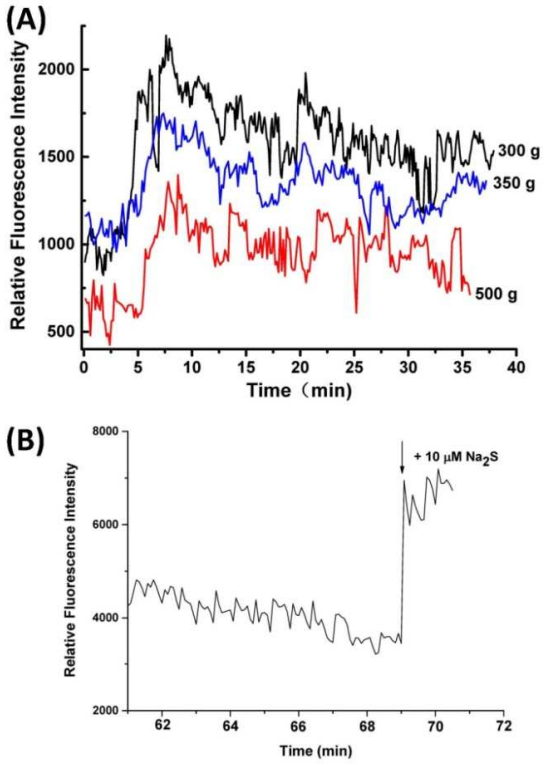


Fig. 2. (A) On-line fluorescence signals of the bisulfide in the hippocampus of rat's brain under awakened state. (B) Fluorescent responses to the on-line spiking of the microdialysate with Na_2S standard solutions. Microdialysis probe MAB6 (15 kD, 2 mm) was used for the in vivo microdialysis. The flow rates of the sample solution, probe solution and control solution are all 1.0 μL/min. The flow rates of oil phase and the outflow solution are 9.0 μL/min and 19.0 μL/min, respectively.

Quantitative measurement of the bisulfide concentration in rat's brain under different physiological conditions

To measure the bisulfide level in the rat's brain under different physiological conditions, we used chloral hydrate to anesthetize the rats. First, we compared the differences between the bisulfide concentrations in the hippocampus of the same rat under anesthetized state and awakened state. Fig. 3A shows the on-line fluorescence signals of the bisulfide in the cerebral microdialysate. As can be seen, under anesthetized state, the concentration of bisulfide didn't show similar dramatic increase as that observed in the awakened rats (see Fig. 2A). By contrast, the fluorescence signals only showed a moderate increase from the baseline level, indicating weak responses of the rat under anesthetized state to the slight brain tissue injury caused by insertion of the probe. After about 30 min, the rat woke up, and the fluorescence signals gradually increased to a relatively high concentration level, indicating production of larger amount of H_2S by the rat under awakened state. Based on the calibration curve and the microdialysis recovery measured in the same experiment, the in-vivo concentration levels of bisulfide in the hippocampus of the tested rat under anesthetized state and awakened state were observed to be 7.8±4.0 μM and 12.2±3.4 μM, respectively.

Ischemic stroke is caused by an interruption of blood supply to the brain (global) or part of the brain (focal) either by thrombosis or embolism. Previous studies suggested that H_2S was involved in the pathogenesis of ischemic stroke.[21] We investigated the dynamic changes of bisulfide before and after global cerebral ischemia by using the established on-line detection system. The rat was kept anesthetized with chloral hydrate throughout the whole experiments. We started to monitor the fluorescence signals of bisulfide in the hippocampus right after the two common carotid arteries of the tested rat were exposed and isolated from surrounding connective tissues through a midline cervical incision. After 110 min, a 10 min 2-VO ischemia was induced, followed by reperfusion. The results were shown in Fig. 3B. The average concentrations of bisulfide in the hippocampus of the tested rat during the first 110 min were found to be 22.1±3.5 μM. These values were much higher than those shown in Fig. 3A which were observed under the same anesthetized conditions, indicating that large amount of H_2S had been produced by the tested rat after it undertook the incision surgery. Then the bisulfide concentration was observed to decline to 13.9±3.7 μM during the 10-min 2-VO ischemia and subsequent reperfusion. The reason for these variations of the H_2S level in hippocampus remain to be further investigated.

Previous studies reported that the concentration of H_2S in brain was in the range from 50 to 160 μM.[9, 22] More recently, some other studies revealed that the concentration of H_2S in brain was much lower, which was in the nanomolar range.[6, 23] Our study showed that the bisulfide concentrations in the brain were less than 30 μM under all the tested conditions, suggesting that the H_2S in rat's brain are in the range below 40 μM. This agrees with the general consensus that the earlier measurements of 50-160 μM were overestimated, most likely due to unintended conversions during tissue process.

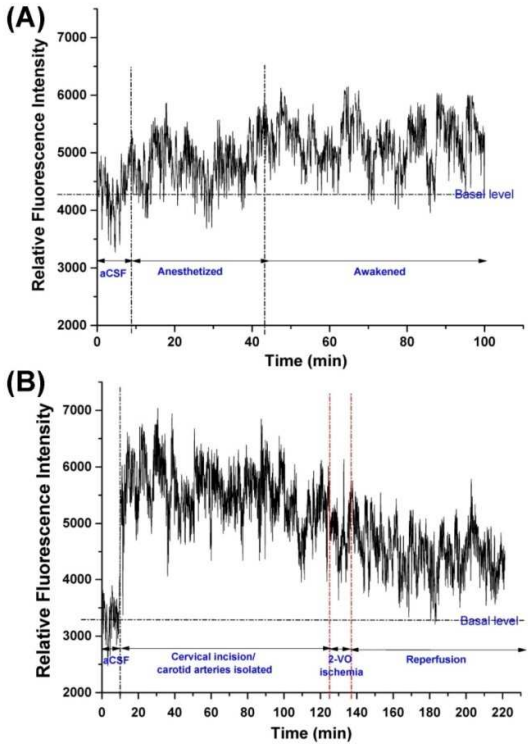


Fig. 3. Fluorescence-time responses recorded for the bisulfide in the microdialysates continuously sampled from the hippocampus of the same rat under different physiological conditions. (A) The basal level of bisulfide in the hippocampus under anesthetized and awakened state. (B) Dynamic variation of the bisulfide concentration in the hippocampus before and during 2-VO global cerebral ischemia/reperfusion. Microdialysis probe MAB6 (15 kD, 2 mm) was used for the in vivo microdialysis. The flow rates of the sample solution, probe solution and control solution are all 1.0 μL/min. The flow rates of oil phase and the outflow solution are 9.0 μL/min and 19.0 μL/min, respectively.

In comparison with the existing approaches for H_2S detection, the established on-line, in-vivo approach may prevent unwanted conversions during sample pretreatment and undesirable volatilization of the target during detection process.[24, 25] Continuous monitoring may provide much better insight into the changes of H_2S concentration under different physiological conditions. The method will greatly facilitate the investigations of the broad brain functions of H_2S

Conclusions

In this work, we have demonstrated an effective online approach for in-vivo, continuous measurements of cerebral bisulfide in rat's brain. The method was based on an integrated system of in-vivo microdialysis and a modified droplet-based microfluidic chip. The system was selective, reliable and reproducible, which enabled continuous measurement of cerebral bisulfide under various physiological conditions. The endogenous levels of H_2S were for the first time measured in the brain of living animals.

The results of this study not only offers a useful approach for physiological and pathological investigations associated with H_2S, but also provides a practical platform for on-line, in-vivo and continuous monitoring of other important species in cerebral systems.

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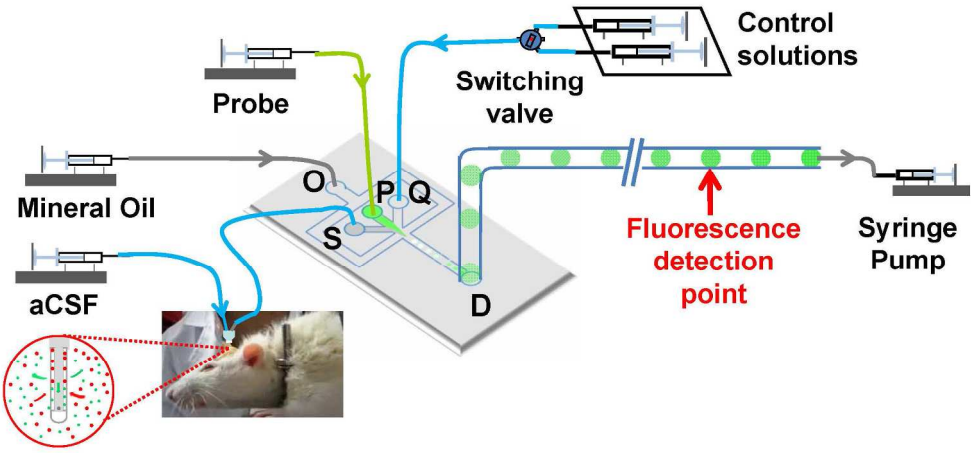
Notes and references

[a] *Beijing National Laboratory for Molecular Sciences, MOE Key Laboratory of Bioorganic Chemistry and Molecular Engineering, College of Chemistry and Molecular Engineering, Peking University, Beijing, 100871, China. E-mail: mpzhao@pku.edu.cn*

[b] *Beijing National Laboratory for Molecular Sciences, Key Laboratory of Analytical Chemistry for Living Biosystems, Institute of Chemistry, the Chinese Academy of Sciences (CAS), Beijing 100190, China. E-mail: lqmao@iccas.ac.cn*

1. K. Abe and H. Kimura, *J Neurosci*, 1996, **16**, 1066-1071.
2. R. Wang, *Physiol Rev*, 2012, **92**, 791-896.
3. J. Z. Weimin Zhao, Yanjie Lu and and R. Wang, *EMBO J.*, 2001, **20**, 6008-6016.
4. J. W. Elrod, J. W. Calvert, J. Morrison, J. E. Doeller, D. W. Kraus, L. Tao, X. Jiao, R. Scalia, L. Kiss, C. Szabo, H. Kimura, C. W. Chow and D. J. Lefer, *Proc Natl Acad Sci U S A*, 2007, **104**, 15560-15565.
5. B. H. Tan, P. T. H. Wong and J.-S. Bian, *Neurochem Int*, 2010, **56**, 3-10.
6. M. Ishigami, K. Hiraki, K. Umemura, Y. Ogasawara, K. Ishii and H. Kimura, *Antioxid Redox Signal*, 2009, **11**, 205-214.
7. L. F. Hu, M. Lu, P. T. Hon Wong and J. S. Bian, *Antioxid Redox Signal*, 2011, **15**, 405-419.
8. J. E. Doeller, T. S. Isbell, G. Benavides, J. Koenitzer, H. Patel, R. P. Patel, J. R. Lancaster, Jr., V. M. Darley-Usmar and D. W. Kraus, *Anal Biochem*, 2005, **341**, 40-51.
9. L. R. Goodwin, D. Francom, F. P. Dieken, J. D. Taylor, M. W. Warenycia, R. J. Reiffenstein and G. Dowling, *J Anal Toxicol*, 1989, **13**, 105-109.
10. J. J. E.-G. J. Revuelto-Rey, M.A. Munoz-Sánchez, F. Murillo-Cabezas, *Medicina intensiva*, 2012, **36**, 213-219.
11. A. Gunther, S. A. Khan, M. Thalmann, F. Trachsel and K. F. Jensen, *Lab on a Chip*, 2004, **4**, 278-286.
12. A. Gunther, M. Jhunjhunwala, M. Thalmann, M. A. Schmidt and K. F. Jensen, *Langmuir*, 2005, **21**, 1547-1555.
13. A. Gunther and K. F. Jensen, *Lab on a Chip*, 2006, **6**, 1487-1503.
14. J. G. Kralj, H. R. Sahoo and K. F. Jensen, *Lab on a Chip*, 2007, **7**, 256-263.
15. L. W. Norton, F. Yuan and W. M. Reichert, *Anal Chem*, 2007, **79**, 445-452.
16. G. T. R. Meng Wang, Kristin Schultz, Colin Jennings, Robert T. Kennedy, *Anal Chem*, 2008, **80**, 5607–5615.
17. X. Zhu, L. Xu, T. Wu, A. Xu, M. Zhao and S. Liu, *Biosens Bioelectron*, 2014, **55**, 438-445.
18. K. Liu, Y. Lin, P. Yu and L. Mao, *Brain Res*, 2009, **1253**, 161-168.
19. M. De Butte, T. Fortin and B. A. Pappas, *Neurobiol Aging*, 2002, **23**, 309-317.
20. I. Yonekura, N. Kawahara, H. Nakatomi, K. Furuya and T. Kirino, *J Cereb Blood Flow Metab*, 2004, **24**, 151-158.
21. A. S. Tay, L. F. Hu, M. Lu, P. T. Wong and J. S. Bian, *Neuroscience*, 2010, **167**, 277-286.
22. J. C. Savage and D. H. Gould, *J Chromatogr*, 1990, **526**, 540-545.
23. J. Furne, A. Saeed and M. D. Levitt, *Am J Physiol Regul Integr Comp Physiol*, 2008, **295**, R1479-1485.
24. S. K. Pandey, K. H. Kim and K. T. Tang, *Trac-Trends Anal Chem*, 2012, **32**, 87-99.
25. T. S. Bailey and M. D. Pluth, *J. Am. Chem. Soc.*, 2013, **135**, 16697-16704.



Graphical Abstract
208x98mm (300 x 300 DPI)