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Ratiometric Fluorescent Probe Based on Gold Nanoclusters and Alizarin Red-Boronic Acid for Monitoring Glucose in Brain Microdialysate

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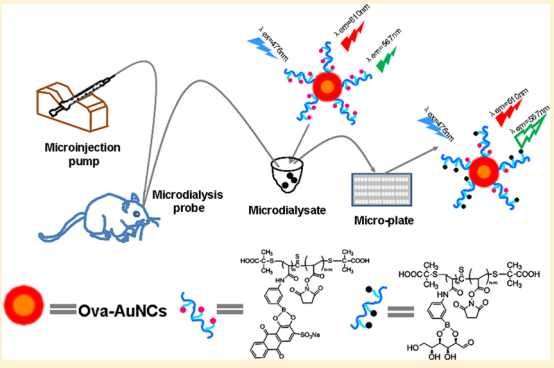
Supporting Information

ABSTRACT: Glucose monitoring with high sensitivity and accuracy in the cerebrospinal fluid is a challenge for evaluating the role of glucose in the physiological and pathological processes. In this work, a ratiometric fluorescent probe for sensing glucose was developed. In the probe, the gold nanoclusters protected by ovalbumin played the role as the reference of fluorophore and the Alizarin Red S-3-aminophenyl boronic acid immobilized on the poly(*N*-acryloxysuccinimide) acted as both the response signal and specific recognition unit for sensing glucose. Once the ratiometric fluorescent probe reacted with glucose in the biological system, its fluorescence intensity at 567 nm was quenched, while the fluorescence intensity at 610 nm was essentially unchanged. In addition, the prepared ratiometric fluorescent probe showed higher stability against environmental effects. As a result, the present ratiometric fluorescent probe was successfully used for monitoring of glucose in the rat brain following the cerebral calm/ischemia.



Glucose is one of the most significant brain components in the biological systems.[1] It does not only provide the brain cells with energy but also joining in synthesizing neurotransmitter as the metabolic intermediate and synaptic neurotransmission. The level of brain glucose in vivo can provide useful information on brain activity related to brain energy metabolism and be an indicator of acute human brain injuries, such as stoke.[2]

Nowadays, various methods for determination of glucose in the brain of rats have been reported, such as colorimetry[3] and electrochemistry.[4] Among them, there are two monitoring systems to detect glucose. One is a biological system related to the enzyme and enzymatic hydrolysate,[5] such as applying glucose oxidase to monitor glucose. The other is a biological system without the enzyme, such as designing and synthesizing probe for glucose.[6] Compared with the enzymatic system, the system without enzyme has many advantages, such as higher stability, lower cost, and easier manufacturing. Notably, nonenzymatic glucose sensors can carry out to be continuous glucose monitoring.[7]

Among the nonenzymatic glucose systems, the chemistry of boronic acid and diol compounds has been reported and successfully used as a molecular recognition tool in chemical sensors and biosensors.[8−14] Boronic acid can react with compounds containing 1,2-diols or 1,3-diols through a reversible ester formation with high affinity. It is worth noting that boronic acid derivatives and diols containing fluorescent dyes, such as alizarin or Alizarin Red S (ARS) can make up the fluorescent probe for sensing saccharides.[15,16]

To improve the detection sensitivity and selectivity of the assay, lots of effort has been made. For example, it has been reported that adding surfactants to the alizarin−boronic acid solution can improve the detection sensitivity and selectivity of the method.[17] However, most of the previous strategies usually need either complicated modification or a harsh detection environment. In addition, the cytotoxic effect of some reported probes for detecting glucose should not be neglected.

Recently, the ratiometric fluorescent probe has widely attracted attention due to its sensitivity and the built-in correction for avoiding environmental effects. To the best of our knowledge, the ratiometric fluorescent probe for sensing glucose is rare. It is well-known that an attractive ratiometric fluorescent probe should consist of three parts: a signal response or signal output section, a proportional part, and a linker. In this work: (1) We considered it is extremely simple that 3-aminophenyl boronic acid (APBA) could be modified by

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ARS via an amide reaction. Then APBA-ARS could be taken as the signal response section for monitoring glucose easily and continuously. (2) Because of the multifunctional groups, the homopolymer of *N*-acryloxysuccinimide (PNAS) could be selected and served as the linker of the probe, which could be used easily for combining the boronic acid−alizarin and gold nanoclusters (AuNCs). Moreover, the PNAS has been successfully used for combining the targeting imaging and therapeutic moieties to cancer imaging in our previous work. (3) The biomolecule-stabilized AuNCs were regarded as the proportional part due to their strong fluorescent emission, high brightness, and excellent biocompatibility.[18] Notably, the AuNCs protected by bovine serum albumin was employed as a reference fluorophore.

Herein, on the basis of our previous work,[19] ovalbumin protected AuNCs (Ova-AuNCs) were synthesized, which have strong emission and excellent biocompatibility. Then APBA was linked to PNAS, the PNAS-APBA with ARS constituted the fluorescent probe for monitoring glucose. Ultimately, the Ova-AuNCs emitting at 610 nm served as the reference signal for providing built-in correction to avoid environmental effects because of its good stability, while PNAS-APBA-ARS was employed as both the specific recognition element and the response signal for determination of glucose due to the weakening fluorescence of the APBA-ARS at 567 nm after specifically being reacted with glucose. Further, the proposed ratiometric fluorescent probe was applied for quantitative determination of glucose in the rat brain following the cerebral calm/ischemia to prove the role of glucose in the physiological and pathological process.

EXPERIMENTAL SECTION

Chemicals and Materials. APBA, ARS, and peroxidase, horseradish (HRP) were purchased from Aladdin Chemistry Company (Shanghai, China). D-Glucose, ovalbumin (Ova), and transferrin (TF) were provided by Beijing Xinjingke Biotech. Ltd. (Beijing, China). Dopamine (DA), ascorbate acid (AA), uric acid (UA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxytryptamine (5-HT) were bought from Sigma-Aldrich (St. Louis, MO). Chloroauric acid ($HAuCl_4{\cdot}3H_2O$) was obtained from Shenyang Jinke Reagent Factory (Shenyang, China). *S*,*S*′-Bis(α,α′-dimethylacetic acid) trithiocarbonate (DATB) were synthesized according to the reference.[20] *N*-Acryloxysuccinimide (NAS) was purchased from TCI Development Co., Ltd. (Shanghai, China). Azo-bis-isobutryonitrile (AIBN) and other chemicals were obtained from Beijing Chemical Corporation (Beijing, China). Glucose oxidase and peroxidase (GOD-POD) method (GOD-POD kit, E1010) were bought from Beijing Applygen Technologies Inc. (Beijing, China). All chemicals used in this work were of analytical grade and used as received. Water was purified using a Milli-Q-system (Millipore, Bedford, MA). Artificial cerebrospinal fluid (aCSF, pH = 7.3) was prepared by mixing sodium chloride (126.0 mM), potassium chloride (2.4 mM), monopotassium phosphate (0.5 mM), magnesium chloride (0.85 mM), sodium bicarbonate (27.5 mM), sodium sulfate (0.5 mM), and calcium chloride (1.1 mM) into ultrapure water. A stock solution of PNAS-APBA-ARS was prepared by dissolving PNAS-APBA-ARS in aCSF solution. The solutions of DA, AA, UA, DOPAC, 5-HT, D-glucose were freshly prepared with aCSF solution prior to use.

Apparatus. Fluorescence measurements were performed using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). The fluorescence measurements of striatum glucose were carried out on a microplate reader (model SpectraMax M5). The size and zeta potentials of the AuNCs were measured by using a Zetasizer Nano ZS (Malvern Instruments, Southborough, U.K.). Fourier transform-infrared (FT-IR) spectra were recorded on a TENSOR-27 spectrometer (Bruker, Germany) in the range of 4000−400 cm^{-1}. Transmission electron microscopy (TEM) images were taken on a JEM-2010 (Jeol Ltd., Japan) at an accelerating voltage of 200 kV. The TEM specimens were prepared by dropping the sample solutions onto 50 Å carbon coated copper grids with the excess solution being immediately wicked away.

Synthesis of Ova-AuNCs. All glassware was washed with freshly aqua regia (hydrochloric acid/nitric acid = 3:1, v/v), and rinsed with ethanol and ultrapure water prior to use. AuNCs protected by the Ova were synthesized following previous strategy.[19] In a typical synthesis, Ova solution (5.0 mL, 50.0 mg/mL) was added to aqueous chloroauric acid solution (5.0 mL, 10 mM) under vigorous stirring. At a time 2 min later, sodium hydroxide solution (0.5 mL, 1.0 M) was introduced. The mixture was incubated at 80 °C. The color of the solution changed from light yellow to light brown and then to deep brown. The reaction was completed in 5 min.

Synthesis of PNAS, PNAS-APBA, PNAS-APBA-ARS. Following previous strategy,[19] the polymer of PNAS was synthesized by the radical reversible addition−fragmentation chain transfer (RAFT) method. NAS (10.0 mmol, 1.7 g), DATB (100.0 μmol, 28.0 mg), and AIBN (50.0 μmol, 8.0 mg) were added into 16.0 mL of dioxane. Then the flask was sealed under nitrogen after three freeze−evacuate−thaw cycles and then placed in an oil bath thermostated for RAFT polymerization at 60 °C for different times. The final polymer PNAS was obtained by pouring the reaction mixture into excess diethyl ether while stirring, recovered by filtration, washed several times with the diethyl ether, and finally dried under vacuum at room temperature overnight.

PNAS (100.0 mg) synthesized in different times and APBA (50.0 mg) were mixed into DMSO (8.0 mL) solution, respectively. Then the flask was sealed under nitrogen and reacted for 24 h at room temperature. The final PNAS-APBA was obtained by pouring the reaction mixture into excess diethyl ether while stirring, recovered by filtration, washed several times with the same solvent, and finally dried under vacuum at 50 °C overnight.

According to the previous report,[17] the molar ratio of boronic acid and ARS was 1:2 and the alizarin−boronic acid adducts can be used as the saccharides probe. So we added ARS (69.0 mg) and the previous synthesized PNAS-APBA into DMSO (8.0 mL) solution. Then the flask was sealed under nitrogen and reacted for 6 h at room temperature. The final PNAS-APBA-ARS was separated from the unreacted ARS by dialysis in ultrapure water for 48 h using dialysis tube (molecular weight cutoff (MWCO) 3500) with stirring. Then the product was recovered by filtration, washed several times with the same solvent, and finally dried under vacuum at 50 °C overnight. The schematic diagram of synthetic route for PNAS-APBA-ARS was shown in Scheme 1.

Preparation of Ova-AuNCs@PNAS-APBA-ARS. PNAS-APBA-ARS (33.9 mg) was dissolved into 5 mL aCSF (pH = 7.3) solution. Then 100 μL of PNAS-APBA-ARS solution and different volumes of Ova-AuNCs (0−500 μL) were added into the round-bottom flask with stirring for 3 h.

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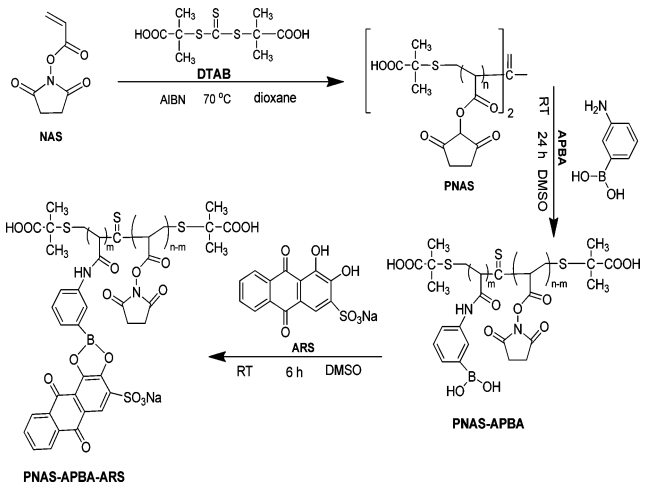
Scheme 1. Synthetic Route of the Fluorescent Probe (PNAS-APBA-ARS)



Fluorescent Detection for Glucose. In the fluorescent experiment, a 1.0 cm quartz cell was used. The sample was excited at 476 nm, and the emission was collected from 500 to 750 nm. Volumes of 100 μL of PNAS-APBA-ARS solution and 150 μL of Ova-AuNCs were added into a 10.0 mL volumetric flask. Then different glucose solutions were added with the aCSF solution (pH = 7.3) to the volume. Fluorescent spectrum was performed using an F-4500 fluorescence spectrophotometer.

Animals and in Vivo Microdialysis. Adult male Sprague–Dawley rats (300–350 g) provided from Health Science Center of Peking University were maintained on a 12/12 h light-dark schedule with water and food ad libitum. The procedures of animal surgery for in vivo microdialysis were performed with the methods described previously.[21] To carry out an in vivo microdialysis experiment, the animal was first anaesthetized with chloral hydrate (350 mg/kg IP) and then positioned onto a stereotaxic frame. A microdialysis guide cannula (BAS) was implanted in the striatum (AP = 0 mm, V = 4.0 mm from dura, L = 3.0 mm from bregma) using standard stereotaxic procedures. The BAS was fastened with three skull screws and dental cement. The stainless steel dummy blocker was inserted into the guide cannula and maintained fixed until the microdialysis probe (BAS, dialysis diameter, 0.24 mm; length, 4 mm) was inserted. In the whole experiment procedure the body temperature of the rats was kept at 37 °C with a heating pad. Immediately after the experiment, the rats were placed into a warm incubator individually to recover from the anesthesia. The rats recovered for at least 24 h before the in vivo microdialysis sampling.

Then the microdialysis probe, BAS, was implanted in the rat brain and was equilibrated through perfusing the aCSF solution (pH = 7.3) for at least 90 min at 1.0 μL/min. Also after sampling the microdialysates for 60 min, the cerebral ischemia surgery was performed following the simple procedures with minor modification in the previous reports.[21,22] In brief, the right common carotid artery, internal carotid artery, and external carotid artery were exposed surgically. A 4-0 monofilament nylon suture with a rounded tip was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the middle cerebral artery. Then the middle cerebral artery occlusion (MCAO) was completed. It was noteworthy that all microdialysates were gathered every 20 min and carried out to fluorescent analysis directly.

Fluorescence Detection for Striatum Glucose. For quantitative sensing of glucose in the microdialysate sampled from the striatum of the rat brain, 15.0 μL of the brain microdialysate was added into 200 μL of the mixing solution (PNAS-APBA-ARS and Ova-AuNCs). Then the fluorescence spectra were measured by a microplate reader (model SpectraMax M5).

RESULTS AND DISCUSSION

Characterization of Ova-AuNCs. AuNCs protected by Ova were synthesized according to our previous work.[19] Ova solution (5.0 mL, 50.0 mg/mL) was added to aqueous chloroauric acid solution (5.0 mL, 10.0 mM) under vigorous stirring for 2.0 min. Then NaOH solution (0.5 mL, 1.0 M) was introduced into the mixture solution. Figure 1 shows the typical

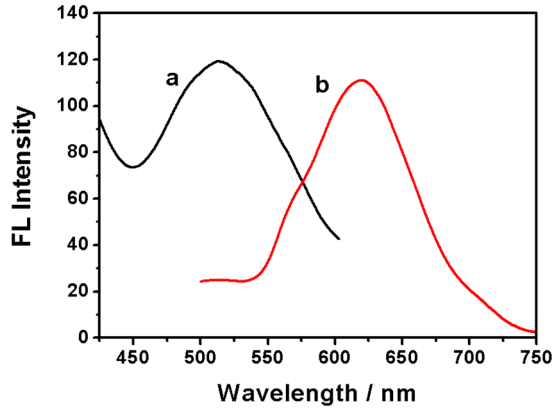


Figure 1. Excitation (a) and emission (b) spectra of Ova-AuNCs in the aCSF solution, the excitation wavelength is at 513 nm, and the emission wavelength is at 625 nm.

excitation (curve a) and emission (curve b) spectra of Ova-Au NCs in aCSF solution. The emission spectrum of AuNCs displayed an approaching near-IR emission around 625 nm upon excitation at 513 nm. The spectra and average zeta potential (−24.0 mV) were consistent with the literature,[19] which suggested the successful preparation of Ova-stabilized fluorescent AuNCs. Then the size and size distribution of Ova-AuNCs were measured by Zetasizer Nano ZS and transmission electron microscopy (TEM), as exhibited in Figures S1 and S2 in the Supporting Information, it was almost 2.5 nm.

A rational selection of time and temperature was vital to synthesize Ova-AuNCs. Thus, the influence of the incubation time and temperature on the fluorescence intensity was investigated. The fluorescence intensity of the Ova-AuNCs solution gradually enhanced from 25 to 80 °C. However, as shown in Figure S3 in the Supporting Information, from 80 to 100 °C, the fluorescence intensity of the Ova-AuNCs decreased. It indicated the structure of Ova was destroyed when the temperature was higher than 80 °C. Finally, the Ova-AuNCs was synthesized at 80 °C in 5 min. We further examined the response of AuNCs at different pH. Figure S4 in the Supporting Information showed the emission intensity of the AuNCs could obtain the highest value at pH 2.0. Moreover, it indicated the Ova-AuNCs have good stability.

Characterization of PNAS, PNAS-APBA and PNAS-APBA-ARS. According to our previous work, the polymer,

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PNAS, was synthesized by the RAFT method at the polymerization time of 10 h for the subsequent experiments.[19] As displayed in Figure 2, the successful binding of APBA with

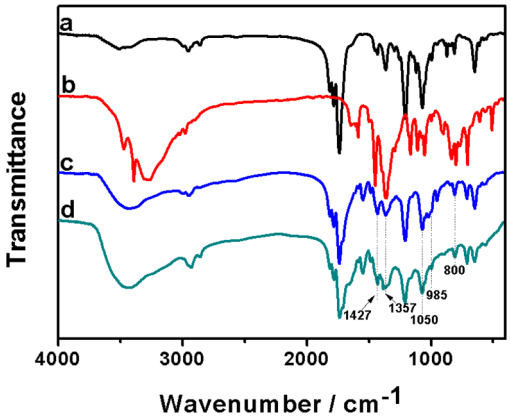

Figure 2. FT-IR spectra of the (a) PNAS, (b) APBA, (c) PNAS-APBA, and (d) PNAS-APBA-ARS.

PNAS was supported by FT-IR spectra. The vibrations of aromatic boronic acids lied at 985 and 1050 cm^{-1}, which are assigned to B−OH bending modes, while the vibrations correspond to the asymmetric B−O stretching mode were observed at 1357 and 1427 cm^{-1}. The peak of 800 cm^{-1} was attributed to benzene ring vibration. In addition, the binding of PNAS-APBA with ARS was supported by the enhancement of the B−O vibration (1357 and 1427 cm^{-1}) and it was also clearly evidenced by the fluorescent spectra. As shown in Figure 3, at the excitation wavelength of 567 nm, PNAS-APBA had no

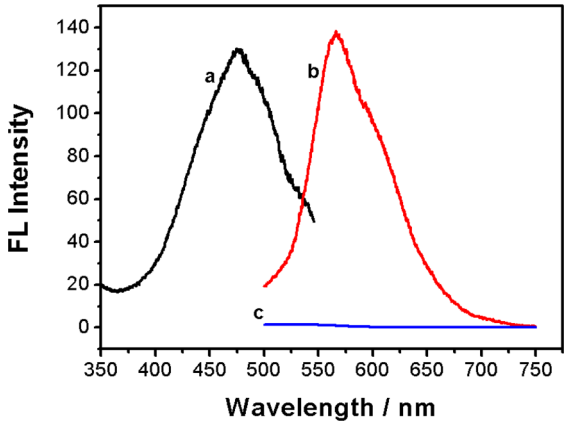

Figure 3. Excitation (a) and emission (b) spectra of PNAS-APBA-ARS in the aCSF solution, the excitation wavelength is at 476 nm, and the emission wavelength is at 567 nm. (c) The fluorescence spectra of PNAS-APBA at the excitation wavelength of 476 nm.

fluorescent emission. However, PNAS-APBA-ARS had a strong fluorescent emission. Therefore, the glucose probe, PNAS-APBA-ARS, was successfully synthesized. Then, different PNAS-APBA-ARS were synthesized and their fluorescent intensities were measured by the F-4500 fluorescence spectrophotometer. The results were shown in Figure S5 in the Supporting Information, and it was found that different chain lengths of PNAS had no influence to the intensity of the APBA-ARS fluorescence (λ_{em} = 567 nm) because the linker PNAS had enough multifunctional groups.

The stability of PNAS-APBA-ARS in the aCSF solution (pH = 7.3) was investigated as shown in Figure S6 in the Supporting Information. The results demonstrated that PNAS-APBA-ARS could be stable for at least 1 week in aqueous media. The fluorescence spectra decay of PNAS-APBA-ARS in aqueous solutions with and without glucose was also studied. The results (Figure S7 in the Supporting Information) exhibited that the reaction between the probe and glucose could be completed within 10 min. Meanwhile, the influence of pH on the fluorescence intensity of the ratiometric fluorescent probe with and without glucose were also studied (Figure S8 in the Supporting Information). The data demonstrated that although the largest decline in fluorescent intensity could be obtained when glucose was added at pH 7.0 or 6.0, considering the pH value of the aCSF solution, thus pH at 7.3 was finally chosen in the further experiment for glucose sensing with the ratiometric fluorescent probe in the cerebral system.

Characterization of Ova-AuNCs@PNAS-APBA-ARS. Because the fluorescence intensity PNAS-APBA-ARS (λ_{em} = 567 nm) was almost equal with the fluorescence intensity of Ova-AuNCs (λ_{em} = 610 nm) at the excitation wavelength of 476 nm (Figure 4), 150 μL of Ova-AuNCs was used for further

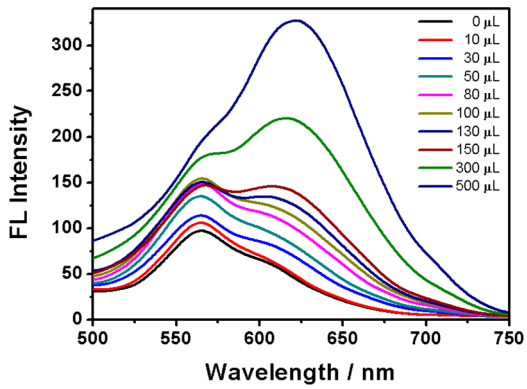

Figure 4. Emission spectra of 100 μL of PNAS-APBA-ARS with different Ova-AuNCs from 0 to 500 μL at the excitation wavelength 476 nm in the aCSF solution.

study. Finally, 100 μL of PNAS-APBA-ARS and 150 μL of Ova-AuNCs constituted in the ratiometric fluorescent probe for sensing glucose, as shown in Figure S9 in the Supporting Information.

After adding glucose, the fluorescent probe PNAS-APBA-ARS reacted with glucose by displacing ARS, which made the fluorescence intensity of PNAS-APBA-ARS weakened, while the fluorescence intensity of Ova-AuNCs emission peak at 610 nm stayed constant (Figure 5). The dual-emission fluorescence intensity ratio (I_{610}/I_{567}) increased linearly $Y = 1.56 \times 10^{-2}X + 1.01$ with the concentration of glucose in the range of 0.50−10.0 mM (R^2 = 0.998) with a detection limit of 0.10 mM, as displayed in Figure 6. The fluorescent response of the ratiometric probe toward glucose was carried out to prove the assay principle, as shown in Scheme 2. The glucose can displace the ARS in the fluorescent probe PNAS-APBA-ARS. In this ratiometric fluorescent probe, the Ova-AuNCs emitting at 610 nm served as the reference signal for providing built-in correction to avoid environmental effects, while PNAS-APBA-ARS was employed as both the specific recognition element and response signal for determination of glucose due to the weakening fluorescence of the APBA-ARS at 567 nm after

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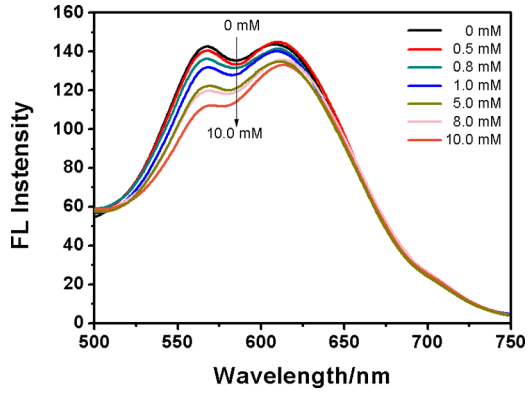


Figure 5. Fluorescence spectra of the ratiometric fluorescent probe upon the exposure to different concentrations of glucose (0, 0.5, 0.8, 1.0, 5.0, 8.0, 5.0, 10.0 mM) at excitation of 476 nm.

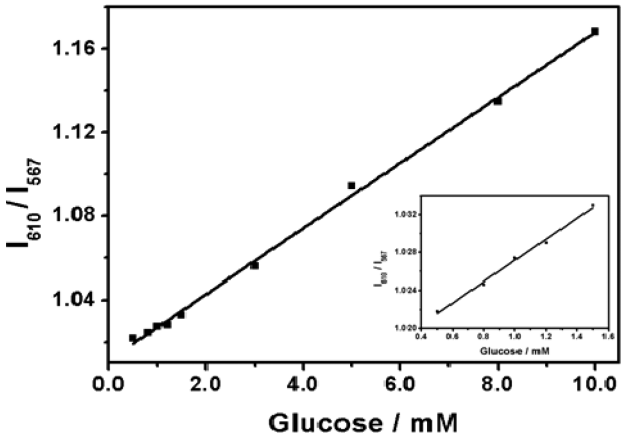
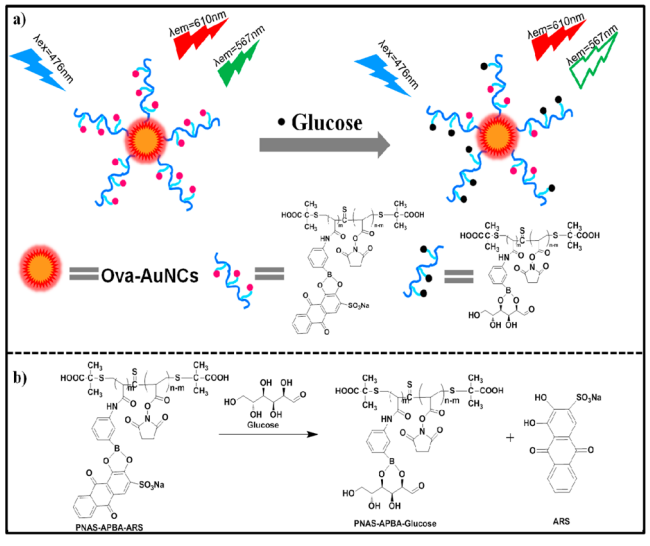


Figure 6. Relationship between I_{610}/I_{567} and the concentration of glucose from 0.5 mM to 10.0 mM. The inset is the enlargement of relationship between I_{610}/I_{567} and the concentration of glucose from 0.5 mM to 1.5 mM.

Scheme 2. (a) Principle of the Developed PNAS-APBA-ARS@AuNCs Fluorescent Probe for Monitoring Glucose and (b) Displacement Reaction Mechanism of PNAS-APBA-ARS with Glucose



specifically being reacted with glucose. Comparing with the reported boronic acid based probes for sensing glucose, the developed ratiometric fluorescent probe can provide a built-in correction for avoiding the environmental effects and show a higher sensitivity.

Interference. The selectivity of the ratiometric fluorescent probe was evaluated by testing the response of the probe toward other species that coexist in the cerebral systems, including AA, LA, DOPAC, DA, UA, and 5-HT.[3,23−26] Among them, AA, one of the most physiologically important small molecules, is around the millimolar level and has been found to be related in many physiological and pathological processes. LA is the main decomposition product in the process of global cerebral calm/ischemia. Thus, the effect of the AA, LA, DOPAC, UA, and 5-HT on the selectivity of the ratiometric fluorescent probe was investigated (Figure 7). Meanwhile, we

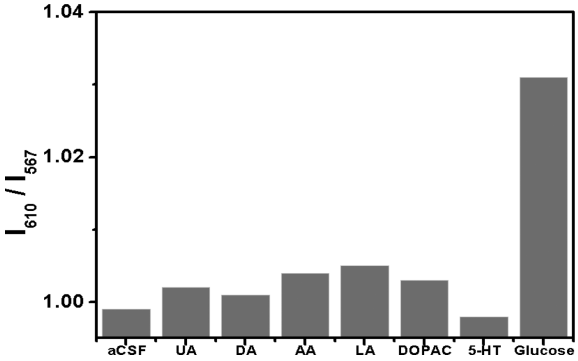


Figure 7. Selectivity tests of the glucose detection (20 μM UA, 20 μM DA, 50 μM AA, 5.0 mM LA, 50 μM DOPAC, 20 μM 5-HT, and 1.0 mM glucose).

also evaluated different concentration of LA in the presence of 0.5 mM glucose response toward the ratiometric fluorescent probe (Figure S10 in the Supporting Information). Moreover, the interference of two kinds of glycoprotein has been studied (Figure S11 in the Supporting Information). The results displayed that the ratiometric fluorescent probe could react with the glycoproteins; however, the in vivo microdialysis method could avoid the glycoproteins appear in the cerebrospinal fluid samples. Thus, the glycoproteins would not influence the glucose detection. Above all, it exhibited that the ratiometric fluorescent assay can be free from the interference of LA, AA, DOPAC, UA, and 5-HT. Then the proposed assay technique was further validated for the detection of cerebral glucose.

Monitoring of Striatum Glucose in the Calm/Ischemia Process. The development of valid methods for glucose sensing in the neurochemical processes is of great importance in neuroprotective therapeutics for the ischemic injury. The ratiometric fluorescent probe (PNAS-APBA-ARS@Ova-AuNCs) has showed a good sensitivity, selectivity, and stability for sensing glucose. Together with the striking properties of the Ova-AuNCs, the ratiometric fluorescent probe can avoid the environmental effects to provide a new approach to assay the stratum glucose.

In this application, the brain microdialysate was collected every 20 min in the surgeries of global cerebral calm/ischemia. The analytical process was demonstrated in Scheme 3. The fluorescence measurements were carried out on a microplate reader (model SpectraMax M5) and the results are shown in

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Scheme 3. Schematic Diagram of the Ratiometric Fluorescent Assay for Continuous Monitoring of Glucose in the Rat Brain

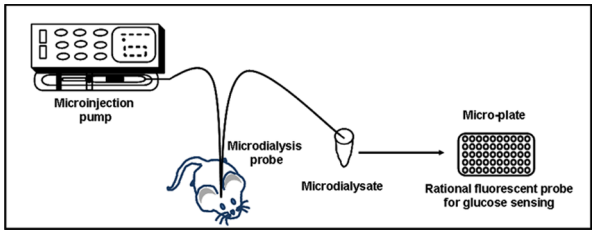

Figure 8. It has been observed that in the calm period, the basal level of glucose was 1.05 ± 0.05 mM, which was approximately

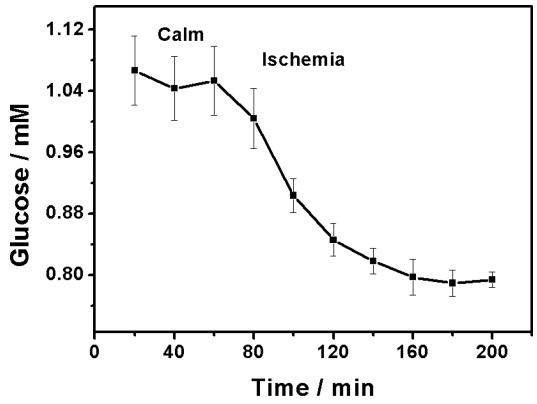

Figure 8. Time-dependent changes of the concentration of glucose in the microdialysates of a rat brain under different physiological conditions: calm, ischemia. Error bars represent the standard deviations of replicate measurements with n = 5.

consistent with the previous reports.[3,23] However, the level of glucose then decreased about 30.1% ± 3.0% after the surgeries of global cerebral calm/ischemia, which was in accordance with the published studies.[2,27] Moreover, the time-dependent changes of the concentration of glucose in the calm/ischemia process is in good agreement with the data (Figure S12 in the Supporting Information) measured with the commercial glucose kit (GOD-POD method). The results indicated the practical application of sensing striatum glucose, demonstrating its great potential for physiological and pathological study.

CONCLUSIONS

By making sufficient use of the unique fluorescence properties of Ova-AuNCs and the displacement reactions between glucose with the ARS on the fluorescent probe PNAS-APBA-ARS, the ratiometric fluorescent probe was proved to be reliable and effective for glucose determination in rat brain microdialysate, avoiding the environmental effects. Moreover, it has displayed its high sensitivity. The continuously quantitative determination of glucose in the global cerebral calm/ischemia surgeries of the rat brain indicated the practical application of sensing striatum glucose. This work not only exhibited that the ratiometric fluorescent probe could be potentially applied in the physiological and pathological analysis but also provided a methodology for designing new ratiometric probes for sensing glucose involving biological processes in the future.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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