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Micropipet Tip-Based Miniaturized Electrochemical Device Combined with Ultramicroelectrode and Its Application in Immobilization-Free Enzyme Biosensor

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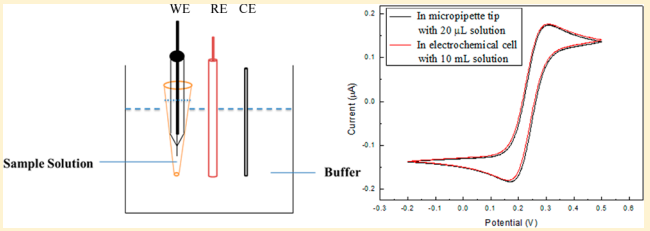
Supporting Information

ABSTRACT: In this study, a simple miniaturized microliter electrochemical device was constructed using a disposable micropipet tip and a reproducible carbon fiber ultramicroelectrode. The novel electrochemical device set the electrochemical reaction in a micropipet tip containing an ultramicroelectrode. We investigated the feasibility of the designed electrochemical device by cyclic voltammetric measurements of redox probe. Its application in an immobilization-free enzyme electrochemical biosensor was also evaluated. Horseradish peroxidase and glucose oxidase were selected to test sensor feasibility. Our results showed that the micropipet tip-based electrochemical device could detect low substrate or enzyme concentration or enzymatic reaction rate. The electrochemical device was applied to analyze the glucose content in human blood samples. With the advantages of low cost, easy operation, rapid detection and high reproducibility, this design provides a new approach in immobilization-free enzyme biosensor construction. Integrated with an ultramicroelectrode, our micropipet tip-based electrochemical device could replace most normal electrodes and electrochemical cells in common laboratories for electroanalysis.



Compared with normal electrodes, ultramicroelectrodes have numerous advantages, including enhanced mass transport, improved signal-to-noise ratio, rapid response time, steady or quasi-steady state current, and very small *iR* drop.[1,2] Theoretically, ultramicroelectrodes are more suitable for investigation of chemical thermodynamics and kinetics. Ultramicroelectrodes have been widely used in neuroscience,[3] single cell analysis,[4,5] and chemical kinetic parameter determination.[6,7] The effects of convection and fluid mechanics can almost be ignored for ultramicroelectrodes. The charge current decays very quickly due to the ultrasmall RC time constant, which brings more advantages in biosensing using ultramicroelectrodes.

The small size of the ultramicroelectrodes also brings difficulties in the fabrication of the matched miniaturized cell. Numerous studies focused on the miniaturization and integration of electrodes in small cells, including positioning needle-type microelectrodes in microvials[8] or microdroplets,[9] preparing two- or three-electrode integrated microcell tips,[10] and fabricating electrochemical microcells on chips based on nanogap electrodes.[11] These constructions of miniaturized electrochemical microsystems are always accompanied by sophisticated technologies such as high-resolution photolithography,[12,13] ion beam or plasma etching,[14,15] laser ablation,[16,17] and nanoimprint lithography.[18] These technologies increase the cost and operation of electrochemical devices. Thus, the complex electrochemical devices cannot be applied widely in common laboratories. This leads us to develop simple and economical miniaturized electrochemical devices coupled with ultramicroelectrodes that can be applied to small-volume (such as several microliters) electroanalysis and can easily be operated.

In this work, we designed a simple, novel miniaturized electrochemical device composed of a micropipet tip and a carbon fiber ultramicroelectrode (Figure 1a). The electrochemical reaction occurred inside the micropipet tip containing the ultramicroelectrode. The micropipet tip worked as a miniaturized electrochemical cell that could accurately aspirate the sample solution at the microliter level. The tip was also used to fix and protect the ultramicroelectrode inside. No special technique was required to fabricate the electrochemical device. The whole device is robust, easy to handle, and expected to detect different types of electrochemical systems in general laboratories. We also exploited solution-based enzymatic reactions such as horseradish peroxidase (HRP) and glucose oxidase (GOx) using this micropipet tip-based electrochemical device. The substrate (H_2O_2 or glucose) and enzyme concentrations and the enzymatic reaction rate were effectively detected with high sensitivity and reproducibility. Therefore, the device opens a new developmental direction for miniaturized electrochemical biosensors.

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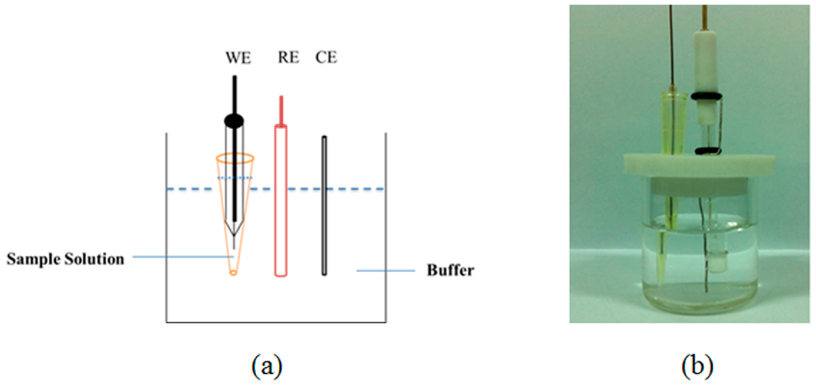

Figure 1. (a) Schematic and (b) photograph of micropipet tip-based miniaturized electrochemical device combined with a carbon fiber ultramicroelectrode.

EXPERIMENTAL SECTION

Chemicals. HRP (E.C.1.11.1.7, ≥ 250 units/mg, RZ 3.0), GOx (E.C.1.1.3.4, type X-S, lyophilized powder, 130 units/mg, from *Aspergillus niger*), D-(+)-glucose, $K_3Fe(CN)_6$, and $K_4Fe(CN)_6$ were obtained from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (H_2O_2) was purchased from Beijing Chemicals (Beijing, China). Hydroquinone (HQ) was purchased from Sinopharm Chemicals (Shanghai, China). Unless otherwise noted, all solutions were prepared using a freshly prepared phosphate buffer (PB, 0.1 M, pH 7.4). All samples and buffer solutions were prepared using ultrapure water from a Milli-Q water purification system (Millipore).

Fabrication of Carbon Fiber Ultramicroelectrode. A carbon fiber ultramicroelectrode was fabricated as previously described[2] with slight modifications. A carbon fiber (diameter: 7 μm) was connected to a copper wire with silver paint. This wire was inserted into a glass capillary with a tip (inner diameter: 20 μm), and the carbon fiber was exposed from the tip. The copper wire−glass capillary was sealed with epoxy. The tip was placed on the outer flame of the gas lamp. In a very short time, the capillary was fused. The protrudent carbon fiber was placed into the bottom of the inner flame. The carbon fiber was etched slowly until the desired length was obtained. The tip of the carbon fiber microelectrode could be controlled to submicrometer, as shown in Figure S1 of the Supporting Information.

Apparatus and Electrochemical Measurements. An AC-SHG1 inverted fluorescence microscope (Nikon, Japan) was used to photograph the carbon fiber ultramicroelectrode. A CHI 660C electrochemical workstation (Shanghai Chenhua Instruments Company, Shanghai, China) was employed to accomplish the electrochemical experiments. As shown in Figure 1, the electrochemical cell was a homemade glass tube combined with an inserted micropipet tip. The analyte solution with fixed volume was in the micropipet tip. The carbon fiber ultramicroelectrode as a working electrode (WE) was inserted into the micropipet tip and was held tightly inside the tip. Then, the micropipet tip was placed in a glass cell containing an electrolyte buffer. An Ag/AgCl reference electrode (RE) and a Pt counter electrode (CE) were also assembled in the cell to form a full three-electrode system with an ultramicroelectrode. The photograph of the device is shown in Figure 1b. All experiments were carried out in this designed electrochemical device.

RESULTS AND DISCUSSION

Fabrication of the Micropipet Tip-Based Electrochemical Device. We first investigated the feasibility of the designed micropipet tip-based electrochemical device by cyclic voltammetric measurements of the redox probe. As shown in Figure 1, a 20 μL buffer solution containing 5 mM $K_4Fe(CN)_6$/$K_3Fe(CN)_6$ was accurately aspirated into a micropipet tip of 10−100 μL. The ultramicroelectrode was inserted into the micropipet tip and positioned tightly. The solution in the tip did not leak out because of the effect of surface tension when the tip was placed in the glass cell. The results were compared with those acquired by dipping the ultramicroelectrode in the conventional electrochemical cell containing a 10 mL 5 mM $K_4Fe(CN)_6$/$K_3Fe(CN)_6$ solution. As shown in Figure 2, the

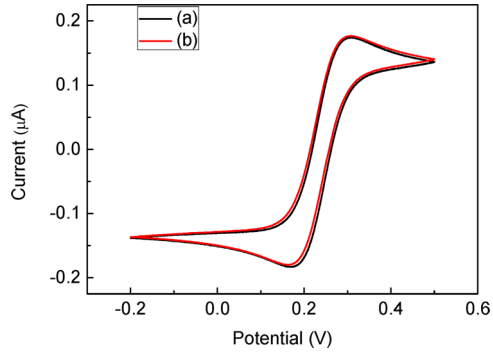

Figure 2. CVs of carbon fiber ultramicroelectrode (a) inserted in the micropipet tip with a 20 μL solution and (b) inserted directly in the traditional electrochemical cell with 10 mL solution containing 5 mM $K_4Fe(CN)_6$/$K_3Fe(CN)_6$; scan rate: 0.1 V/s.

cyclic voltammogram (CV) obtained at 0.1 V/s for 20 μL displayed the typical quasi-steady state current for multidimensional diffusion at a cylindrical microelectrode at slow scan rates, which was almost the same as that in the bulky solution. Given that the analyte only existed in the tip, the sample solution was almost reduced by a thousand times compared with that of a traditional electrochemical cell. If a micropipet tip of 0.5−10 μL was selected, the sample solution might be reduced to only several microliters.

The difference of analyte concentration between inside and outside the tip can cause the analyte to diffuse slowly into the cell, causing changes in the analyte concentration in the tip. The effect of analyte diffusion on the electrochemical signal was examined by a repetitive potential sweep at a scan rate of 0.1 V/

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s in the same micropipet tip containing a 5 mM $K_4Fe(CN)_6$/$K_3Fe(CN)_6$ solution. The result showed that the reduction and oxidation currents only decreased by 0.3% and 1.5%, respectively, after 20 min CV scans, indicating that the analyte leakage from the micropipet tip to the cell via diffusion was relatively slow. In consideration that electrochemical detection could be finished within 1 min, analyte diffusion was ignored. On the other hand, the stable current obtained indicated that the carbon fiber ultramicroelectrode was not contaminated during the CV scans and could be used repeatedly. The device can also be simplified to a two-electrode system by removing the homemade glass tube and RE. Both ultramicroelectrode and Pt wire were inserted into the same micropipet tip. But, it will increase the difficulty of the operation.

Investigation of the Solution-Based Enzymatic Reaction and Its Application in Biosensor. Enzyme-based electrochemical biosensors are usually fabricated through the immobilization of enzyme on the electrode surface. The kinetics, stability, and specificity of immobilized enzymes differ from those in the homogeneous solution because of the structural changes in immobilization. Retaining the specific biological functions of enzymes is highly desired in constructing the immobilized enzyme layer. Most published works focused on developing biocompatible materials such as conducting polymers,[19] sol–gel materials,[20,21] nanomaterials,[22] and nanocomposite materials[23] to immobilize recognition molecules. Our designed miniaturized device combined with an ultramicroelectrode provides a new developmental direction for the fabrication of electrochemical biosensors, which is constructing immobilization-free enzyme biosensors. The miniaturized device resulted in low enzyme consumption, and the ultramicroelectrode provided high mass transport, which made biomolecule immobilization unnecessary in biosensor construction.

We first exploited the solution-based reaction of HRP with H_2O_2 in the micropipet tip for immobilized-free enzymatic reaction sensing. HQ was used as an electron mediator to investigate HRP bioactivity because the direct electrochemistry behavior of HRP in the solution was not easily observed. In consideration that the operation procedures (solution mixing and aspiration) and electrochemical device setup could be finished within 1 min, measurements began at 1 min after mixing the solution. The CVs of the ultramicroelectrode in PB containing 1 mM HQ, 1 mM H_2O_2, and different HRP concentrations are shown in Figure S2 of the Supporting Information. When the HRP concentration was higher than 1 μg/mL, 1 mM H_2O_2 and 1 mM HQ could react completely in the first minute. The CV curves did not change with time. When the HRP concentration was reduced to 100 ng/mL, the reaction became incomplete, causing the signal change to decrease correspondingly. Obtaining a steady CV curve requires more time. Thus, the selected HRP concentration in solution was 1 μg/mL to sensitively and rapidly detect H_2O_2 in the presence of 1 mM HQ. HRP consumption was only 20 ng for one measurement in 20 μL solution. By contrast, the enzyme on the electrode was immobilized by depositing several microliters of milligrams per milliliter of enzyme solution, indicating that HRP consumption was in the grade of micrograms. Therefore, HRP consumption in our system was significantly less than that in HRP immobilization on the electrode. The CVs of ultramicroelectrode in PB containing 1 mM HQ, 1 μg/mL HRP, and different H_2O_2 concentrations are shown in Figure 3A. In the absence of H_2O_2, CV obtained at 50

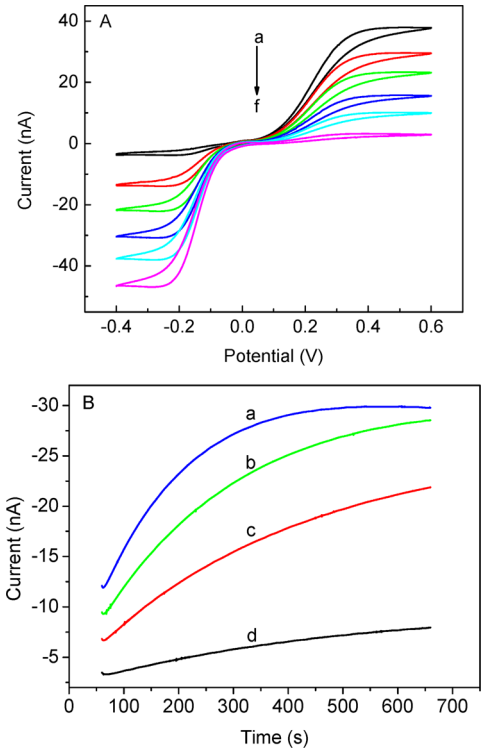

Figure 3. (A) CVs of 1 mM HQ and 1 μg/mL HRP in 0.1 M PB (pH 7.4) in the (a) absence and (b–f) presence of different H_2O_2 concentrations: 200, 400, 600, 800, and 1000 μM; scan rate: 50 mV/s. (B) Amperometric response curves of 1 mM HQ and 1 mM H_2O_2 in 0.1 M PB (pH 7.4) at an applied potential of −0.3 V in the presence of different HRP concentrations: (a) 75, (b) 50, (c) 30, and (d) 10 ng/mL.

mV/s displayed normal sigmoidal responses (Figure 3, panel A, curve a). After the addition of 0.2 mM H_2O_2, a significant increase in the reduction peak, accompanied by a decrease in the oxidation peak, was observed (Figure 3, panel A, curve b). Increased H_2O_2 in the buffer further increased the reduction peak intensity and decreased the oxidation peak intensity (Figure 3, panel A, curves c–f). The results showed that the reduction peak occupied the majority in the presence of 1 mM H_2O_2. The detection limit of H_2O_2 could be decreased by decreasing the HQ concentration. As shown in Figure S3A of the Supporting Information, 20 μM H_2O_2 could easily be detected in the presence of 100 μM HQ. However, the interference of dissolved O_2 appeared (Figure S3A–a of the Supporting Information). The lowest detectable concentration of H_2O_2 was 1 μM in the presence of 10 μM HQ (Figure S3B–b of the Supporting Information), which is comparable to many developed biosensors.[24−26] Our developed immobilization-free enzyme biosensor has many advantages. The avoidance of enzyme immobilization saves time and operation. The homogeneous reaction can maintain enzyme activity. The electrode is used only as the signal producer, which greatly increases stability, reliability, and reproducibility of the biosensor.

Both enzyme concentration and enzymatic reaction rate can be detected when fixing the concentrations of H_2O_2 and HQ. Amperometry at −0.3 V was carried out in PB containing 1 mM HQ, 1 mM H_2O_2, and different HRP concentrations (Figure 3B). As shown in Figure 3B, the slope of the curve in any time indicates the enzymatic reaction rate. The slope

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decreased gradually to zero when the reaction was complete. When the enzyme concentration was decreased (Figure 3, panel B, curves a–d), a longer time was required to obtain the saturated current. A calibration curve between the current and HRP concentration obtained at 100 s is shown in Figure S4 of the Supporting Information. HRP detection in microliter volumes is important because HRP is a common signal generator in many biosensors. The device can also be applied to detecting the HRP-mimicking DNAzyme, which can further be used as a signal generator in label-free aptamer-based biosensors.

Similarly, the detection of glucose by GOx using the same microdevice was conducted. GOx can catalyze glucose oxidation and produce gluconic acid and H_2O_2 in the presence of oxygen. The detected glucose signal is often derived from H_2O_2 or the decreased cathodic current during dissolved oxygen consumption. These two methods were investigated using the developed miniaturized device. Given that H_2O_2 was easily detected as described above, a combination of GOx and HRP was used to detect H_2O_2 produced by the catalyzed oxidation of glucose (Figure 4A). Our results showed that as

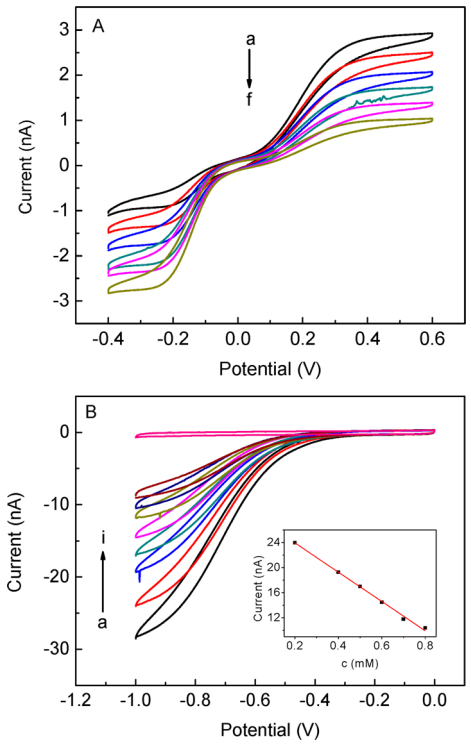

Figure 4. (A) CVs of 100 μM HQ, 10 μg/mL HRP, and 200 μg/mL GOx in 0.1 M PB (pH 7.4) in the (a) absence and (b–f) presence of different glucose concentrations: 20, 40, 60, 80, and 100 μM; scan rate: 50 mV/s. (B) CVs of 1 mg/mL GOx in 0.1 M PB (pH 7.4) in the (a) absence and (b–h) presence of different glucose concentrations: 200, 400, 500, 600, 700, 800, 1000, and 1500 μM; scan rate: 50 mV/s. Inset: linear range from 200 to 800 μM, R^2=0.995.

low as 20 μM glucose was detected in the presence of 100 μM HQ. The lower level of glucose could be detected in the presence of lower HQ concentration. Glucose detection by the cathodic current during dissolved oxygen reduction was also investigated (Figure 4B). As shown in the inset of Figure 4B, the calibration curve showed good linearity.

We used the device to detect glucose in human blood samples to demonstrate the practical application of the glucose enzyme sensor. The concentrations of two fresh human blood samples provided by a hospital attached to Peking University were 5.85 and 7.79 mM. The CVs of 1 mg/mL GOx in the presence of serum diluted 10 times are shown in Figure S5 of the Supporting Information. The results calculated from the calibration curve by this biosensor are 5.7 mM ± 0.5 mM and 7.6 mM ± 0.6 mM. This favorable agreement indicates that the electrochemical device can be applied to analyze biological samples.

The carbon fiber ultramicroelectrode can be used repeatedly as a detector. This ultramicroelectrode can easily be cleaned by ethanol immersion for 1 min and then water immersion for 1 min or by rinsing with water. If some organic chemicals are adsorbed on the surface, the ultramicroelectrode can be flamed rapidly by an alcohol lamp to be cleaned completely. Aside from the carbon fiber ultramicroelectrode, Pt or Au ultramicroelectrodes can also be used in this device.

Our device is a simple combination of an ultramicroelectrode and a micropipet. For comparison, most carbon fiber ultramicroelectrodes were used to detect brain-signaling molecules in vivo[27] or to monitor dopamine release from single living vesicles.[4] Several ultramicroelectrodes were used to study mercury-free measurements of metal speciation,[28] which can also be used by our device. Several decorated single carbon fiber microelectrodes can be used to detect H_2O_2.[29] Using our device for similar determination is more convenient and specific. As mentioned previously, developing small cells suitable for ultramicroelectrode is difficult. Most of the reported microsystems were costly and complex. The micropipet as a small-sized cell in this work was simple, cheap, and feasible. As an immobilization-free enzyme biosensor, the micropipet has several advantages such as rapidness and convenience compared with immobilized enzyme biosensors based on the microfluidic device.[30]

CONCLUSIONS

In summary, a simple and easily operated microliter electrochemical device combined with a carbon fiber ultramicroelectrode was developed for routine electrochemical detections. The homogeneous enzymatic reaction was investigated using the device, including HRP and GOx. Both H_2O_2 and glucose were rapidly and sensitively detected, indicating its potential application in an immobilized-free enzyme biosensor. The designed miniaturized electrochemical device presents numerous advantages. First, the micropipet tip as the detecting cell is economical, clean, disposable, and capable of accurately aspirating the fixed volume, promising good reproducibility to the system. Second, shortage of the vulnerable damage of the carbon fiber ultramicroelectrode was overcome by the protection of the micropipet tip. Third, the preparation and operation of our device are simple and rapid. The entire operation can be finished within 1 min. Therefore, the operation can easily be performed in general electrochemical laboratories. In addition, the device can replace conventional-sized electrodes and electrochemical cells for the detection of microliter volume samples. The designed miniaturized device also brings a new developmental direction for the construction of immobilization-free enzyme electrochemical biosensors.

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■ 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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Notes

The authors declare no competing financial interest.

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