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Evaluation of an amperometric glucose biosensor based on a ruthenium complex mediator of low redox potential

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Abstract

An amperometric glucose ring-disk biosensor based on a ruthenium complex mediator of low redox potential was fabricated and evaluated. This thin-layer radial flow microsensor (10 μl) with ring-disk working electrode displayed remarkable amperometric sensitivity. For $Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$ (Ru-Py), a trinuclear oxo-acetate bridged cluster, a reversible redox curve of low redox potential and narrow potential window (redox potentials were −0.190 and −0.106 V versus Ag/AgCl wire, respectively) was observed, which is comparable to many reported mediators such as ferrocene derivatives and other ruthenium complexes. The glucose and hydrogen peroxide assays were carried out with this complex-modified electrode Ru-Py-HRP-GOx/Nafion. The sensitivity was obtained 24 nA (15.4 mA M^{-1} cm^{-2}) for 10 μM glucose and 126 nA (160 mA M^{-1} cm^{-2}) for 5 μM H_2O_2, respectively with a working potential at 0 V versus Ag/AgCl. Ascorbic acid was studied as interference to the glucose assay. The application of 0 V potential versus Ag/AgCl did not avoid the occurrence of the oxidation of ascorbic acid, however, the pre-coating of ascorbate oxidase on the disk part of the ring-disk working electrode efficiently pre-oxidized the ascorbic acid and hence eliminated its interference on the glucose response. The practical reliability was also evaluated by assaying the dialysate from the prefrontal cortex of Wistar rats.

Keywords: Micro-biosensor; Glucose; Ruthenium complex; Mediator

1. Introduction

Enzyme-based amperometric biosensors have been widely used for industrial, environmental and clinical applications, as they allow transferring the chemical reaction rate to the analyzable current signal [1,2]. They offer the advantages of low cost, simple handling, real-time analysis, fast response, high sensitivity and selectivity, insensitivity toward turbidity, comparable instrumental sensitivity, miniaturization, and so on when compared to the optical, calorimetric, piezoelectric and other related sensors [1].

According to the working mechanism (Fig. 1), searching for an ideal mediator is always a key point in fabricating a biosensor. The use of artificial electron mediators will allow the shuttle of electron from the redox center of enzyme to the surface of working electrode, reducing the operating potential and hopefully avoiding the interference from some electrooxidizable species in blood such as ascorbate, hence enhance the selectivity and sensitivity [1–3]. It is usually a low molecular weight redox couple that can access the active center of the enzyme. It should be chosen in such a way that it has a lower redox potential than the other electrochemically active interfering compounds in the sample. In addition, a high electrochemical rate constant is much desirable, which is important to ensure the response of the biosensor not limited by electrode kinetics and oxygen interference [1,2]. An ideal mediator is also characterized by a reversible heterogeneous kinetics, a stable oxidized and reduced forms, the inactivity towards oxygen [1,2]. So far, various metal complexes

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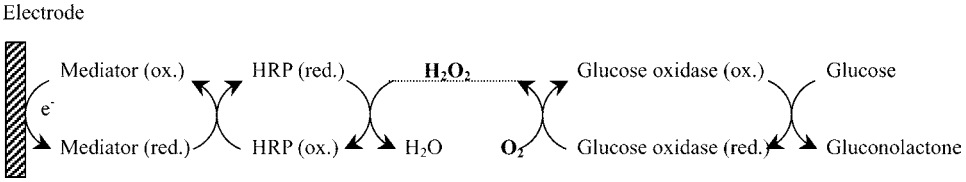

Fig. 1. Electron transfer pathway in a mediated bi-enzyme biosensor. HRP: horseradish peroxidase; ox.: oxidized; red.: reduced.

such as ferrocene and its derivatives [1], conducting polymers such as polyaniline and polypyrrole [1,4,5], conducting salts such as tetrathiafulvalene- or *N*-methylphenazinium-tetracyanoquinodimethane etc. [1,6] have been investigated as mediator or bi-functional cross-linker at the same time. In recent years, intensive research has been devoted to the complexes of metal group VIII (iron, ruthenium and osmium) [1,2,7–10]. According to the above principles, we explored a trinuclear oxo-acetate bridged ruthenium cluster $Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$, of interesting electronic structure and properties, as mediator for glucose biosensor in this work.

Immobilizing and keeping the glucose oxidase on the electrode surface is always another key point in fabricating a biosensor (Fig. 1) [4,11]. A variety of immobilization techniques have been explored, such as adsorption, covalent binding, cross-linking, entrapment in a porous matrix, coating behind a dialysis polymer membrane etc [1,10,12]. Heller and co-workers. contributed to this by wiring the enzyme in the hydrogel of an osmium redox mediator bound to a polyvinylpyridine and polyvinylimidazole backbone [13,14]. In general, immobilizing the enzyme and the mediator by applying a polymer film with an organic reagent as solvent, on one hand, could reject the interference and decrease the noise, but on the other hand, may lead to changes in the enzyme active structure and hence cause the kinetics, stability, and specificity of the enzyme to differ from what is observed in solution [4,11].

Among these immobilization methods and materials, Nafion was widely used and was suggested to be a biocompatible, highly stable, negatively charged matrix [9,15–17]. It could exclude negatively charged interfering substances from the biosensor response. According to the non-aqueous enzymological approach [9,16], immobilization of an enzyme in Nafion membranes was carried out in water-organic mixture with a high content of organic solvent. The enzyme with the mediator together cross-linked onto the surface of Nafion polymer, was syringed as a mixture onto the electrode. After solvent evaporation, the enzyme-mediator-Nafion membrane was formed and a reagentless mediator-based biosensor could be developed. The resulting membrane possessed both high adhesion to the surface and low swelling in aqueous media. The biological activity of the enzyme was high because no harsh conditions were adopted. Besides the advantage of simple handling, the Nafion polymer is commercially available. Thus, in this work, a similar enzyme immobilization method was applied.

In this study, besides applying the above-mentioned sensitive mediator of low redox potential and the selectively permeable Nafion membrane on the electrode surface, a low working potential (0 V versus Ag/AgCl) was applied as working potential to avoid the ascorbate interference [18]. At the same time, a ring-disk working electrode was designed to pre-oxidize the ascorbic acid by pre-coating the ascorbate oxidase on the disk part. In order to decrease the volume of sample, the amount of expensive enzymes and the response time for any possible in vivo monitoring [8,18], a thin-layer radial flow micro sampling system was fabricated and evaluated.

2. Experimental

2.1. Reagents

Glucose oxidase (GOx, from Aspergillus niger, 166.1 units mg^{-1}, Sigma), β-D(+)-glucose (corn sugar, Sigma), hydrogen-peroxide oxidoreductase (HRP, from Horseradish, 250 units mg^{-1} solid, Sigma), hydrogen peroxide (35% aqueous solution, Katayama Chemicals), ascorbate oxidase (AOx, from cucurbita sp., 102.3 units mg^{-1} solid, Sigma), ascorbic acid (AA, Katayama Chemicals), Nafion (5 wt.% solution in mixture of lower aliphatic alcohols and 10% water, Aldrich), Dulbecco's phosphate buffered saline (PBS, KCl 200 mg L^{-1}, KH_2PO_4 200 mg L^{-1}, NaCl 800 mg L^{-1}, Na_2HPO_4 1150 mg L^{-1}, Sigma) were used as received. PBS was used both as solvent for preparing glucose and H_2O_2 stock solutions and as background solution for electrochemical analytical measurement. The aqueous solutions of GOx, HRP and AOx in 2 units μl^{-1} were prepared using doubly distilled water. The aqueous solution of 2 mM $K_3Fe(CN)_6$ and 0.1 M KNO_3 served for cyclic voltammetric investigation. Ruthenium complex $Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$ (Ru-Py) was used as received from other research group.

2.2. Fabrication of microsensor

A thin-layer radial flow ring-disk microsensor (Fig. 2), consisting of the glass base and the plastic cover, was designed for the conventional three-electrode electrochemical analysis. A screen-printed Au ring-disk electrode (disk $\phi = 3$ mm, $S = 7.06\ mm^2$; ring inner $\phi = 4$ mm, ring width=1 mm, $S = 15.7\ mm^2$) was applied as base. The disk

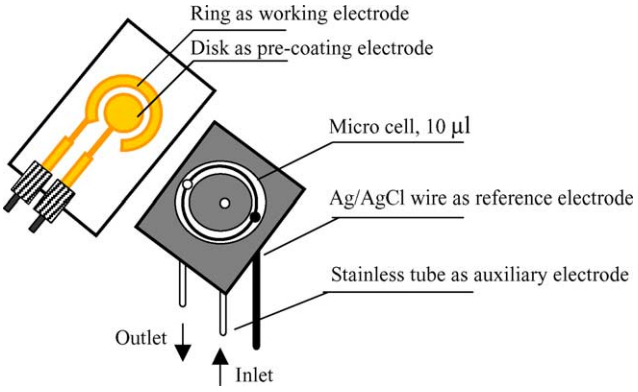

Fig. 2. Schematic representation of the thin-layer radial flow microsensor: glass-based screen-sputtered Au ring-disk electrode (left), plastic cover with micro-flow injection system and conventional electrodes (right).

part was coated with ascorbate oxidase in order to pre-oxidize the ascorbic acid. The ring part was modified by GOx and the mediator as the working electrode. On the cover, there were the micro cell (about 10 μl with the suitable diameter to the ring-disk electrode), a Ag/AgCl wire (0.1 mm diameter, prepared by plating a Ag wire in 3 M NaCl + AgCl following standard procedure) as ring reference electrode, a stainless tube ($\phi = 0.55$ mm) as both the auxiliary electrode and the liquid inlet, and the liquid outlet tube ($\phi = 0.25$ mm, resin). The stock solution was fed into the microsensor by a microinjection pump (CMA/100, Stockholm, Sweden).

2.3. Preparation of the bi-enzyme based electrode

The ring-disk electrode was first treated by UVO-Cleaner (Model 144A-100, Jelight Company, Inc., USA) at 120 °C for 20 min in order to remove the impurities adsorbed on the gold surface. For the Ru-Py-HRP-GOx/Nafion microsensor, it was prepared by a one-step procedure according to the non-aqueous enzymology approach [9,16] as follows. First, 0.1 mg Ru-Py was mixed with 5 μl HRP (2 units μl^{-1}) and 5 μl GOx (2 units μl^{-1}), then 4 μl of this mixture was cast on the electrode surface. After drying in air, 2 μl of 0.5 wt.% Nafion was further coated on the layer. In order to investigate the interference effect of ascorbic acid, 2 μl AOx was alternatively dropped on the disk part. This prepared electrode, coupled with the cover part, is indicated as Ru-Py-HRP-GOx/Nafion microsensor. Finally it was dried at room temperature for several hours and stored at 4 °C in a refrigerator before use.

2.4. Flow injection analysis

Cyclic voltammetry and amperometry were performed using ALS/CH Instruments (Electrochemical Analyzer Model 1232) with the data acquisition software package. The common three-electrode system was applied and the cathodic current was set to be positive. PBS was first passed to obtain a stable base line. The analyte stock solution in the syringe was fed into the microsensor by the microinjection pump with a feeding speed of 3 μl min^{-1}. For amperometric measurement, all the potentials were poised at 0 V versus Ag/AgCl. If not otherwise indicated, all the potentials hereafter refer to Ag/AgCl. Dialysate samples from the frontal cortex of five Wistar rats (CMA12 probe, CMA midrodialysis AB) were also assayed on the as-prepared glucose biosensor without further pretreatment. For confirmation, the glucose concentrations of these biosamples were also measured on CMA 600 Microdialysis Analyzer (CMA microdialysis, Sweden).

3. Results and discussion

3.1. Evaluation of the ruthenium complex

Ru-Py was reported to be a trinuclear oxo-acetate bridged cluster (Fig. 3), its electronic structure, redox property, including rate of electron self-exchange had been well investigated [19–23]. It was reported to be useful for the catalytic oxidation of organic compounds [24,25]. Due to its interesting electronic structure and properties, we applied it as the mediator for the glucose sensor in this work.

Fig. 4 shows that the cyclic voltammogram of the background solution was featureless and that the Ru-Py complex gave an ideal redox curve. Its reduction and oxidation potentials were −0.190 V and −0.106 V, respectively. The redox window ($\Delta E = E_{ap} - E_{cp} = 0.084$ V) was narrow. It is said that an ideal mediator should have a low redox potential [1]. The redox potential of Ru-Py complex seemed to be perfect (recalibrated to be −0.067 V and 0.017 V versus SCE, respectively) and comparable to that of many mediators such as, ferrocene and ferrocene derivatives [1,9]: 1,1-dimethyl ferrocene (0.100 V), ferrocene (0.165 V), vinyl ferrocene (0.250 V), ferrocene carboxylic acid (0.275 V), hydroxy methyl ferrocene (0.185 V); ruthenium complex [7,8,26]: $[Ru(CN)_6]^{4-}$ (0.685 V), Ru-bpy (1.085 V); Os(III/II) complex [2,7]. This comparison reveals that Ru-Py is a good mediator for glucose biosensor, as also verified by the results below.

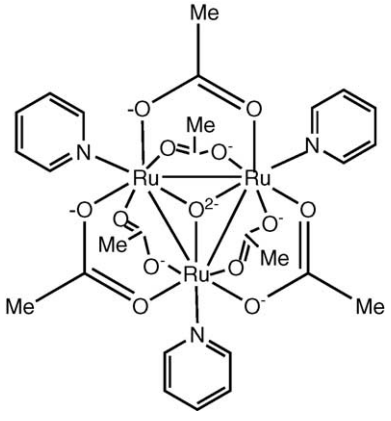

Fig. 3. Chemical structure of $Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$ complex.

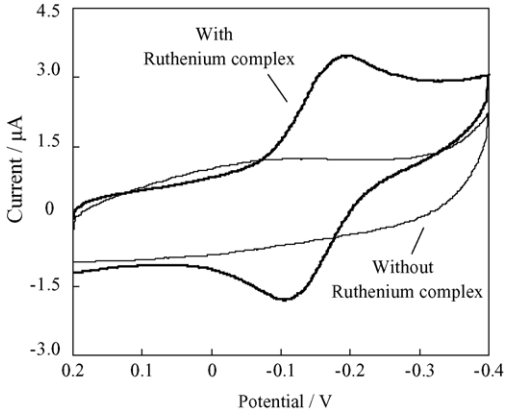

Fig. 4. Cyclic voltammograms for mixture solution (a) with and (b) without $Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$ complex, respectively. 0.1 M tetrabutylammonium perchlorate as support electrolyte in the ethanol+PBS solution, scan rate of 0.1 V s^{-1}, Ag/AgCl wire as reference electrode.

3.2. Evaluation of the Ru-Py-HRP-GOx/Nafion microsensor

Fig. 5a shows the glucose response current on Ru-Py-HRP-GOx/Nafion microsensor. For 10 μM glucose, the response current was high to 24.2 nA (corresponding to 15.4 mA M^{-1} cm^{-2}), and it greatly increased and reached 137 nA (0.873 mA M^{-1} cm^{-2}) for 1 mM glucose. For higher concentration, the current response did not further increase following a typical Michaelis-Menten curve. The linear range was relatively narrow (10–500 μM) according to the linear equation of (Fig. 5a)

$$I\,(\text{nA}) = 0.1597C\,(\mu\text{M}) + 20.975\,(R^2 = 0.991)$$

which may be owing to the low applied potential. The low applied potential, on one hand, is capable of avoiding the noise or bias signals from the electrooxidizable interfering substances, but on the other hand, also leads to a decreased sensitivity [18]. Although in this work the Ru-Py-HRP-GOx/Nafion microsensor shown a low current density when comparing to the one modified by the osmium-poly(4-vinylpyridine)gel-HRP [13,14], since the glucose concentration in human blood lies within the limits of 3.5–5 mM [8,27,28], this Ru-Py-HRP-GOx/Nafion microsensor can meet the demands and no further improvement of the preparation of this film electrode is needed. The cathodic current plateau was stable. For 1 mM glucose, the amperometric trace shows that the response time, defined as the time of 90% of the full signal, was about 240 s. The performance of Ru-Py-HRP-GOx/Nafion microsensor in this work was comparable to some similar studies [17,29,30], indicating that the Ru-Py complex is a satisfying mediator for glucose biosensor. Although its life was limited to few days and it needs to further improve the coating in the future work, the obtained good sensitivity of the biosensor towards glucose indicates that, the design and fabrication of the thin-layer radial flow microsensor was successful and could be practically applied.

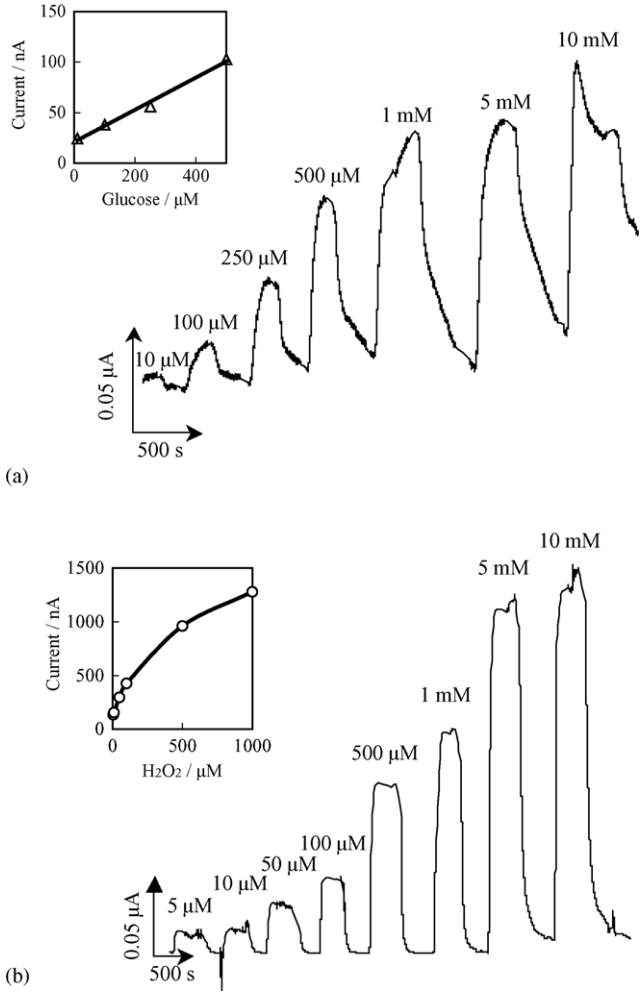

Fig. 5. Amperometric response of (a) glucose solution and (b) H_2O_2 solution on Ru-Py-HRP-GOx/Nafion microsensor polarized at 0 V vs. Ag/AgCl wire, with their linear plot insets, respectively.

Actually this Ru-Py-HRP-GOx/Nafion microsensor can also act as the H_2O_2 sensor (Fig. 1). In order to understand the Nafion membrane behavior and improve the immobilization technique as well, the amperometric response of H_2O_2 on Ru-Py-HRP-GOx/Nafion microsensor was also carried out in the concentration range from 5 to 10 mM (Fig. 5b), with the corresponding response current from 126 nA (160 mA M^{-1} cm^{-2}) to 2066 nA (1.32 mA M^{-1} cm^{-2}) obtained, respectively. The calibration range was considered within 5–100 μM according to the linear equation of (Fig. 5b)

$$I\,(\text{nA}) = 3.0713C\,(\mu\text{M}) + 126.06\,(R^2 = 0.994)$$

Actually the detection limit of H_2O_2 could be extended to be much lower limit from the theoretic point of view. This performance further confirms that the Ru-Py-HRP-GOx/Nafion microsensor was comparable to other modified electrode. The good performance may be related to two factors. One is the sensitive Ru-Py mediator at this low applied redox potential. Another is the thin-layer radial flow design. The thin-layer

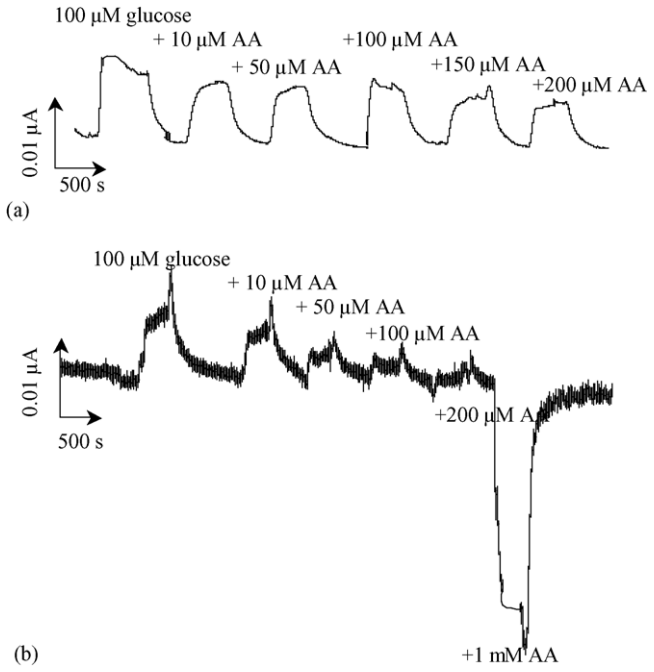

Fig. 6. Effect of ascorbic acid interference on the glucose response on Ru-Py-HRP-GOx/Nafion microsensor (a) with and (b) without AOx pre-coating, polarized at 0 V vs. Ag/AgCl wire, respectively. Symbols "+" mean the addition of the ascorbic acid into 100 μM glucose.

radial flow is thought to offer similar characteristic as rotating disk electrode. Although the flow rate was only 3 μl min^{-1}, it was enough to decrease the diffusion layer and hence a larger response current was obtained. If comparing the response current between glucose (Fig. 5a) and H_2O_2 (Fig. 5b) on this same microsensor, it can be reasonably seen that the modified electrode has much better sensitivity toward H_2O_2 than toward glucose. The relatively low amperometric response of glucose might be caused by the diffusion limitation in the Nafion film or the part loss of GOx enzyme function during the film modification.

3.3. Effect of ascorbic acid interference

As above-mentioned, the electrooxidizable components of blood such as ascorbate, urate, and cysteine often interfere with glucose and lactate assays [3]. The physiological concentration of ascorbic acid (AA) varies significantly from 36 to 85 μM [28]. In this study, therefore, AA in a similar concentration range was investigated as the interfering substance.

Fig. 6a shows the effect of AA interference on the response current of glucose. It could be obviously observed that the addition of 10, 50 and 100 μM of AA into the 100 μM glucose almost did not result in a significant interference. When the molar ratio of AA to glucose was further increased to 1.5 and 2.0, respectively, the cathodic current plateaus gradually decreased to 70% of its value. Namely, for the microsensor with AOx pre-coating, the AA almost did not interfere with

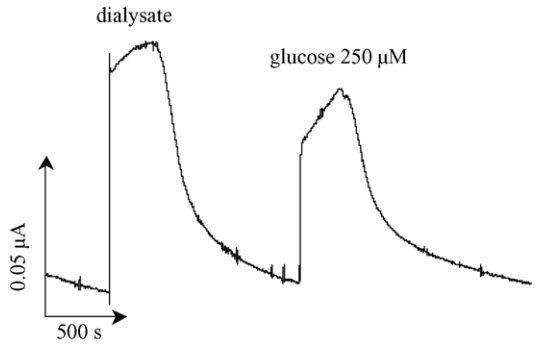

Fig. 7. Amperometric responses of the dialysate sample and glucose standard (250 μM) on the Ru-Py-HRP-GOx/Nafion microsensor.

the glucose current response. It may be due to few factors: one is due to the fact that the AOx pre-coating in the disk part pre-oxidized the AA. Another is the 0 V potential applied. However, when the concentration of AA was high, it seemed that this low potential was not safe enough to keep from all the interference of AA, as it was further verified by the result in Fig. 6b.

Fig. 6b shows the case of Ru-Py-HRP-GOx/Nafion microsensor without AOx pre-coating on the disk. For the 100 μM glucose, the corresponding current response was about 13.7 nA. When 10, 50, 100 and 200 μM of AA was further added into the 100 μM glucose, the current was changed to 11.5, 9.0, 6.3 and 4.8 nA that close to the base line, respectively. The decrease of cathodic current plateaus was clearly caused by the oxidation of AA on the electrode surface, which was proved by the phenomenon that, after the addition of 1 mM AA to the 100 μM glucose, instead of the observation of cathodic current, an anodic current peak of −26.6 nA appeared. In this case, the signal of H_2O_2 reduction was completed vanished by the oxidation current resulting from the high amount of AA on the electrode surface.

According to the above results, it can be seen that even if the potential was positioned to 0 V, it was not safe enough to eliminate the interference from AA. The pre-coating of AOx in the disk part would efficiently keep from the interference from the AA, confirming the interest in design the ring-disk working electrode. The latter offers advantages over some similar models, for example, by employing lead oxide as an interference-removing agent [31], or eliminating ascorbate interference by constructing the ascorbate oxidase layer on the glucose enzyme layer [32].

3.4. Assay of dialysate sample

Finally, in order to evaluate the practical reliability of the as-prepared Ru-Py-HRP-GOx/Nafion microsensor, five dialysate samples from the frontal cortex of five Wistar rats were also assayed without further pretreatment (Fig. 7). It can be seen that, the dialysate sample from rat brain successfully showed the strong amperometric responses on the

as-prepared Ru-Py-HRP-GOx/Nafion microsensor. Taken glucose standards as reference, the glucose concentration is calibrated to be $345 \pm 64\,\mu M$ (mean ± S.D., $n = 5$), which were close to the concentration $384 \pm 62\,\mu M$ ($n = 5$) that was obtained on the CMA 600 Microdialysis Analyzer for confirmation. These results suggest that the prepared novel glucose sensor by Ru-Py-HRP-GOx/Nafion modification can be used as biosensors for continuous and reliable measurement of glucose in biological samples. Further efforts for durable measurements using this novel sensor are in progress.

4. Conclusions

The thin-layer radial flow glucose micro biosensor with the trinuclear oxo-acetate bridged cluster $Ru_3(\mu_3\text{-}O)(AcO)_6(Py)_3(ClO_4)$ as mediator was designed and evaluated. This ruthenium complex has a low redox potential and narrow potential window, and proved to have comparable performances to some reported mediators. The fabricated microsensor with the ring-disk working electrode displayed remarkable amperometric sensitivity towards glucose and H_2O_2, as well as the anti-interference function against ascorbic acid. It has advantages over some microsensors designed for the similar purpose, showing a possible use in the in vivo microdialysis.

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