



Short communication

Development of amperometric α -ketoglutarate biosensor based on ruthenium–rhodium modified carbon fiber enzyme microelectrodeSujittra Poorahong^{a,b}, Padmanabhan Santhosh^a, Gabriela Valdés Ramírez^a, Ta-Feng Tseng^{a,c}, Joseph Isaac Wong^a, Proespichaya Kanatharana^b, Panote Thavarungkul^b, Joseph Wang^{a,*}^a Department of NanoEngineering, University of California San Diego, La Jolla, CA 92093, USA^b Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand^c Department of Biomedical Engineering, Chung-Yuan Christian University, Chung Li 32023, Taiwan, ROC

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ABSTRACT

A rapid and highly sensitive miniaturized amperometric biosensor for the detection of α -ketoglutarate (α -KG) based on a carbon fiber electrode (CFE) is presented. The biosensor is constructed by immobilizing the enzyme, glutamate dehydrogenase (GLUD) on the surface of single carbon fiber modified by co-deposition of ruthenium (Ru) and rhodium (Rh) nanoparticles. SEM and EDX shed useful insights into the morphology and composition of the modified microelectrode. The mixed Ru/Rh coating offers a greatly enhanced electrocatalytic activity towards the detection of β -nicotinamide adenine dinucleotide (NADH), with a substantial decrease in overpotential of ~ 400 mV compared to the unmodified CFE. It also imparts higher stability with minimal surface fouling, common to NADH oxidation. Further modification with the enzyme, GLUD leads to effective amperometric biosensing of α -KG through monitoring of the NADH consumption. A very rapid response to dynamic changes in the α -KG concentrations is observed with a response time of 6 s. The current response is linear between 100 and 600 μ M with a sensitivity of 42 μ A M⁻¹ and a detection limit of 20 μ M. This proof of concept study indicates that the GLUD-Ru/Rh-CFE biosensor holds great promise for real-time electrochemical measurements of α -KG.

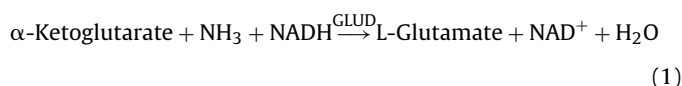
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1. Introduction

α -Ketoglutarate (α -KG) is a key intermediate in Krebs cycle, and is one of the major precursors for the synthesis of most biochemical substances. It is produced from the oxidative decarboxylation of isocitrate, catalyzed by the enzyme, isocitrate dehydrogenase 1 (IDH1). It has been recently demonstrated that IDH1 is frequently mutated in low grade gliomas, a common form of brain tumor in humans to form single amino acid substitution at Arg¹³² (R132) (Ichimura et al., 2009). These monoallelic mutations (IDH1-R132) inactivate the enzyme and inhibit the production of α -KG. This in result increases the Hypoxia Inducible Factor 1, an angiogenic factor which is responsible for the growth of tumor cells (Zhao et al., 2009). α -KG has been emphasized to possess exciting angiogenesis suppressor activity (Schaub et al., 1979). Moreover, micronutrient application of α -KG has been shown to have beneficial effects on several malignant tumors (Wagner et al., 2010). Hence, the ability to follow the kinetics of α -KG formation in such gliomas would

be useful in analyzing cellular activities. To our knowledge there are no reports on electrochemical biosensors for the measurement of α -KG. A flow injection system with a bi-enzymes (glutamate dehydrogenase and glutamate oxidase) reactor and a downstream electrochemical detector was developed for fermentation monitoring of α -KG (Collins et al., 2001).

This proof of concept study describes the development, optimization and characterization of an amperometric microsensor for α -KG based on a GLUD-Ru/Rh modified single carbon fiber electrode. The biocatalytic detection of α -KG at the modified CFE transducer involves the following reaction:



where the enzyme, GLUD catalyzes the conversion of α -KG into L-glutamate in the presence of ammonia and NADH. The quantitation of α -KG is achieved through a low-potential anodic detection of the depleted NADH at the GLUD-Ru/Rh-CFE. CFE transducers have been widely used for exploring microscopic domains and measurements of local concentration profiles in living cells (Huffman and Venton, 2009). Moreover, these microelectrodes are widely used in biomedical sciences owing to their well-resolved spatially and temporally response in biological media for *in vivo* applications.

* Corresponding author at: Department of NanoEngineering, University of California, San Diego, Mail Box 0448, La Jolla, CA 92093, USA. Tel.: +1 858 246 0128; fax: +1 858 534 9553.

E-mail address: josephwang@ucsd.edu (J. Wang).

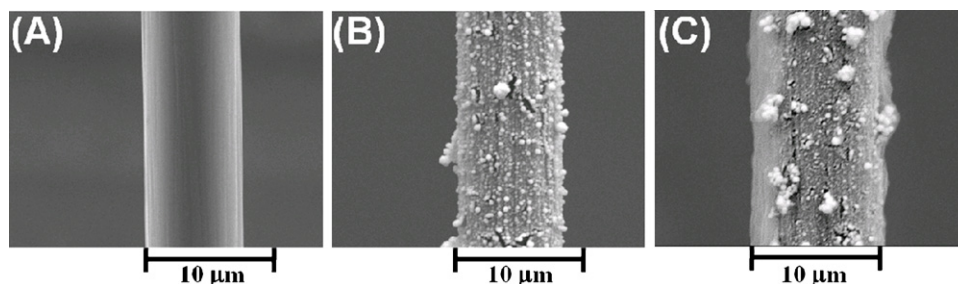


Fig. 1. SEM images of bare (A), Ru/Rh (B), and GLUD-Ru/Rh coated with glutaraldehyde (C) CFEs; accelerating voltage: 20 kV.

However, like most carbonaceous electrodes, CFE requires high overvoltage for the oxidation of NADH. In addition, a serious problem associated with amperometric detection of NADH at carbon electrodes is the fouling of the electrode surface by the adsorption of reaction intermediates. Significant efforts have been directed towards developing new or modified electrode materials that lower the overpotential for NADH oxidation and minimize surface passivation effects. Mediator compounds that include polyazine dyes (Karyakin et al., 1999), catechol moieties (Jaegfeldt et al., 1981), metal complexes (Wu et al., 1996), or conducting polymer (Bartlett et al., 1997), have been used to modify the electrode surface to address these overpotential and passivation effects. One such effort is the modification of electrode by metal particles owing to their remarkable electrocatalytic activity (Wang, 2005). In the following sections, we demonstrate that the co-deposition of Ru and Rh metal nanoparticles onto the single CFE offers an effective electrocatalytic detection of NADH (compared to the individual metals) along with a highly stable response. This was followed by the immobilization of the GLUD enzyme and a low-potential anodic detection of α -KG via the NADH consumption (Eq. (1)).

2. Experimental

2.1. Materials and chemicals

L-Glutamic dehydrogenase from bovine liver-Type III (GLUD; EC 1.4.1.3), α -ketoglutaric acid sodium salt (α -KG), β -nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), ruthenium (Ru; 990 ppm), rhodium (Rh; 996 ppm), and ammonium chloride were obtained from Sigma–Aldrich and used as received. Carbon fibers (diameter: 8 μ m and length: 25 mm) were procured from Alfa Aesar. All other reagents were analytical grade from Sigma–Aldrich and used without further purification. Ultra pure deionized water (18.2 M Ω cm) from a NANOpure Diamond (Barnstead) source was used in all experiments. All measurements were performed at 25 ± 1 °C in 0.1 M phosphate buffer (pH 8.0).

2.2. Instrumentation

All the electrochemical measurements were performed with a μ -Autolab type II (Metrohm, Netherlands) interfaced with computer using GPES 4.9 software (Eco Chemie, Switzerland). The typical three-electrode cell assembly was used with Ag/AgCl and platinum wire as reference and counter electrodes, respectively. Scanning electron microscope (SEM) image was obtained with a Phillips XL30 ESEM, coupled with Energy-dispersive X-ray spectroscopy (EDX).

2.3. Fabrication of α -KG microsensor

A single carbon fiber was inserted to 0.60 mm outer diameter tip of a polypropylene tube. Electrical contact was provided with

a copper wire and silver conductive epoxy, the open end of the polypropylene tube was sealed with epoxy resin. Finally, the carbon fiber was cut down at 1000 μ m. The CFE was modified by electrochemical co-deposition of the GLUD enzyme and Ru and Rh particles in phosphate buffer. Prior to deposition, the electrodes were electrochemically cleaned in phosphate buffer by cycling the potential between -1.4 and $+1.4$ V for 10 cycles at a scan rate of 50 mV s^{-1} . The enzyme and metals deposition (Ru and Rh) was accomplished by cycling the potential between 0 and -1.5 V at scan rate 50 mV s^{-1} for 30 cycles in a 200 μ L beaker containing Ru and Rh (100 ppm each) and 3 kU mL^{-1} of GLUD. The pH of the solution was adjusted to 5.0 using 1.5 M KOH before adding the enzyme. After deposition, the electrodes were cleaned electrochemically by cycling the potential in the range of -0.5 to 0.5 V at a scan rate 50 mV s^{-1} in phosphate buffer until constant current on the CV was obtained (~ 10 cycles). Further, the enzyme-modified electrode was dipped in 2% glutaraldehyde for 3 s to form a protective coating. The electrode was stored in phosphate buffer at 4 °C while it is not used.

3. Results and discussion

3.1. Morphology

Fig. 1 shows the SEM images of the bare CFE (A), Ru/Rh-CFE (B) and GLUD-Ru/Rh-CFE coated with glutaraldehyde (C). A single carbon fiber of 8 μ m diameter (Fig. 1A) was used for the modification of metal particles. Electrochemical co-deposition of Ru and Rh onto the CFE is shown below to impart a substantial increase in electrocatalytic activity and to increase the effective electroactive surface area. Ultrafine metal nanoparticles (Ru and Rh) of size between 100 and 250 nm are observed over the entire surface of carbon fiber, leading to increase in the microscopic as well as geometric areas, indicated from increased diameter to 9 μ m (Fig. 1B). Deposition of individual metal particles over CFE (under similar conditions) shows quite diverse morphologies, Fig. S1 (see Supplemental information). A heterogeneous microstructure with aggregates of particles was observed when Rh alone was deposited onto the CFE, whereas a thin film like morphology was observed for Ru deposition. A different morphology is observed (Fig. 1C) following the enzyme immobilization and the glutaraldehyde coating, with thin layers of the enzyme and glutaraldehyde covering the Ru/Rh nanoparticles. Close examination reveals a “blanket” of the enzyme, connecting the metal aggregates. Such different microstructures are attributed to the effect of the enzyme upon the nucleation and growth of the metal deposits. The modification of Ru/Rh-CFE with GLUD and glutaraldehyde decreases the effective microscopic area, yet increases the fiber diameter further to 10 μ m. The presence of Ru/Rh was further confirmed by EDX analysis, Fig. S2 (see Supplemental information). The atomic percentage of metal particles was determined by EDX from multiple-spot EDX analysis of Ru and Rh emission intensities as 15.53% and 84.46%, respectively.

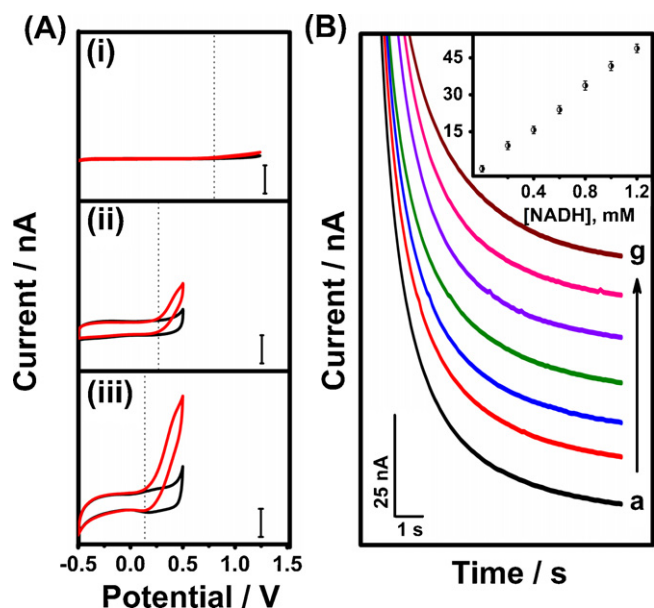


Fig. 2. (A) Cyclic voltammograms for the oxidation of 500 μM NADH at the bare (i), Ru (ii), and Ru/Rh (iii) CFEs; scale bar: 100 nA; scan rate: 50 mV s^{-1} . Black and Red solid lines correspond to the blank (phosphate buffer) and 500 μM NADH solutions, respectively. (B) Chronoamperograms obtained at Ru/Rh-CFE for various NADH concentrations (b $\rightarrow$ g: 200 μM increments to 1.2 mM, along with the blank phosphate buffer solution, a); step potential: +0.4 V. Inset shows the calibration curve (the currents were measured at a time of 10 s).

3.2. Electrocatalytic activity of Ru/Rh-CFE towards NADH

Cyclic voltammetric measurements were performed to evaluate the electrocatalytic activity of Ru/Rh-CFE towards the oxidation of NADH. Typical cyclic voltammograms obtained for 500 μM NADH in phosphate buffer (scan rate: 50 mV s^{-1}) at the bare (i), Ru-CFE (ii), and Ru/Rh-CFE (iii) are shown in Fig. 2A. At the bare CFE, the oxidation of NADH is observed above +0.8 V. On the other hand, Ru-CFE and Ru/Rh-CFE display an anodic response, starting at +0.3 V and +0.2 V, respectively. While the Ru/Rh coating enhances the electroactivity, the Rh-CFE did not show any electrocatalytic activity towards the oxidation of NADH (not shown). Apparently, the favorable response of the bi-metals coated CFE transducer reflects the synergic effect of the Ru and Rh co-deposits. The metal deposition on CFE also resulted in a substantial increase in the current response for the oxidation of NADH at Ru/Rh-CFE, ascribed to the large surface area provided by the metal nanoparticles.

Hydrodynamic voltammograms were further obtained at bare and Ru/Rh-CFE for the detection of 100 μM NADH in phosphate buffer, Fig. S3 (see Supplemental information). The NADH oxidation starts at +0.2 V, and a substantial rise in current was observed at potentials higher than +0.3 V. On the other hand, an applied voltage of more than +0.9 V is required to obtain a similar NADH response at the bare CFE. At such, +0.4 V was selected as a detection potential at the Ru/Rh-CFE for further measurements.

An inherent problem for the anodic NADH measurement is the surface fouling associated with the adsorption of oxidation products. The Ru/Rh coating over CFE greatly enhances the stability of the NADH anodic response. For example, Fig. S4 (see Supplemental information) compares the stability of the response for 35 repetitive chronoamperometric measurements of 100 μM NADH at bare and Ru/Rh-CFE. A highly stable response was observed at Ru/Rh-CFE over 35 repetitive measurements and retained more than 90% of its initial response. In contrast, the bare CFE displays a rapid loss of its activity (up to 55% decrease of the response) after

25 repetitive measurements. Fig. S5 (see Supplemental information) compares the amperometric response obtained at bare (A) and Ru/Rh-CFE (B) for 300 μM NADH in stirred phosphate buffer. The bare CFE shows a rapid loss of the signal (90% current depressions), indicating a complete passivation associated with adsorption of the reaction products. In contrast, the response of the Ru/Rh-CFE remains highly stable throughout the entire period of operation (RSD: 5.05%).

Chronoamperometry was performed at Ru/Rh-CFE (step potential: +0.4 V) for a series of NADH solutions over the range 0–1.2 mM in the phosphate buffer, Fig. 2B. The Ru/Rh-CFE responds favorably to the 200 μM increments in the NADH level. Current measurements were made after 10 s and the corresponding calibration plot is shown in inset. The current response displays a good linear relationship with the concentration of NADH with the sensitivity of $48 \pm 7 \mu\text{A M}^{-1}$ (R^2 : 0.9960; n = 3 and CV: 0.2). The limit of detection is estimated to be 10 μM (3σ). The electrocatalytic behavior and also the electrode preparation were highly reproducible, as reflected by a RSD of 5.9% for 3 different electrodes for the detection of 1 mM NADH.

3.3. Development of α -ketoglutarate enzyme microsensor

Following the development of effective NADH transducer, we focused on the optimization and characterization of the GLUD-Ru/Rh-CFE microsensor. The new biosensor is based on anodic detection of the depleted NADH at a GLUD-Ru/Rh-CFE (Eq. (1)). To optimize the enzyme loading and hence α -KG response, we evaluated the current response of the GLUD-Ru/Rh-CFE by varying the amount of GLUD loading. Six microelectrodes were fabricated using solutions containing different levels of GLUD in the range between 1 and 3.5 kU mL^{-1} . Chronoamperograms were then recorded in the phosphate buffer containing 1 mM NADH, 100 μM NH_4Cl and 500 μM α -KG. As indicated in Fig. S6 (see Supplemental information), the current response at GLUD-Ru/Rh-CFE was dependent on the amount of enzyme incorporated into the electrode. An increase in the current was observed up to 3 kU mL^{-1} , with no further increase in the response for higher enzyme loading. Hence, ensuing measurements were performed with this amount of GLUD. Besides, the NADH and NH_4Cl concentrations were optimized and 1 mM and 100 μM , respectively, yielded the most favorable α -KG response. After being optimized the conditions, chronoamperograms were recorded at GLUD-Ru/Rh-CFE for the detection of NADH between 0.2 and 1.2 mM in phosphate buffer. A linear calibration curve with a sensitivity of $32 \pm 3 \mu\text{A M}^{-1}$ (R^2 : 0.9865; n = 3 and CV: 0.25) was obtained over this concentration range. The decrease in the sensitivity (from $48 \pm 7 \mu\text{A M}^{-1}$) observed after the GLUD immobilization, reflects the hindered NADH transport through the enzyme layer.

The concentration dependence of α -KG was evaluated under the optimal experimental conditions by measuring the decreased NADH response in the presence of ammonia (Eq. (1)). Fig. 3A displays a typical amperogram recorded at GLUD-Ru/Rh-CFE for the successive additions of 100 μM α -KG in stirred phosphate buffer containing 100 μM NH_4Cl and 1 mM NADH. The GLUD-Ru/Rh-CFE responds rapidly to the α -KG additions, with steady-state current signals reached within 6 s. Such current changes lead to a highly linear response to the concentration of α -KG between 100 and 600 μM , indicate a detection limit of around 20 μM . The corresponding calibration plot (shown as inset) has a slope of $42 \mu\text{A M}^{-1}$ (R^2 : 0.999; n = 3 and CV: 0.1). Typical cellular concentrations of α -KG range between 100 and 300 μM , depending on the type of cells and its physiological state. Fig. 3B displays the high stability of the amperometric response of α -KG (100 μM) obtained at GLUD-Ru/Rh-CFE in a stirred phosphate buffer. No apparent sensitivity loss is observed for the detection of α -KG dur-

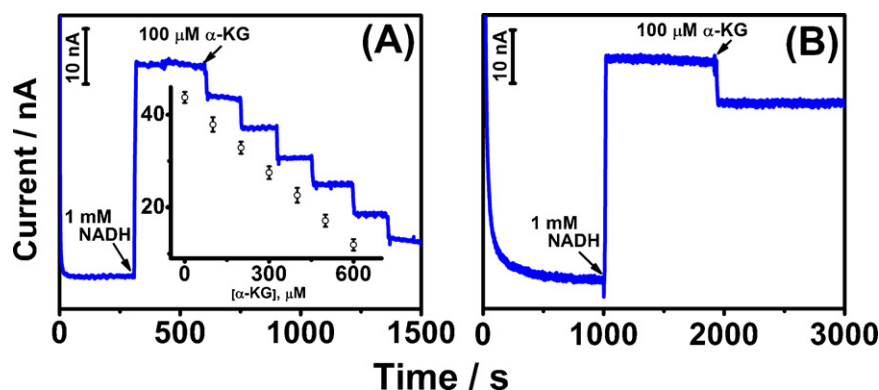


Fig. 3. (A) Amperogram obtained at GLUD-Ru/Rh-CFE for the successive additions of 100 μM $\alpha\text{-KG}$ in stirred phosphate buffer containing 100 μM NH_4Cl . Inset shows the calibration curve. (B) Amperogram showing the stability of the $\alpha\text{-KG}$ response at the GLUD-Ru/Rh-CFE. First arrow indicates the addition of 1 mM NADH and the second arrow indicates the addition of 100 μM $\alpha\text{-KG}$. $E = +0.4\text{ V}$; stirring rate: 300 rpm.

ing this extended period of operation ($\sim 1000\text{ s}$). Such operational stability of GLUD-Ru/Rh-CFE reflects the use of glutaraldehyde as a protective membrane, which improves the stability of the enzyme through the formation of enzyme-to-enzyme and enzyme-to-electrode covalent linkages.

We have examined the effect of electroactive interferences such as uric acid (UA) and ascorbic acid (AA) upon the anodic detection of NADH at GLUD-Ru/Rh-CFE. The presence of 0.2 mM UA has a minimal ($>1\%$) effect on the current of 1 mM NADH in phosphate buffer, while 0.2 mM AA yielded a large deviation in the NADH signal. As such, the performance of the biosensor was noticeably altered by the presence of physiological concentrations of AA. Accordingly, the interference from AA may need to be addressed before using this biosensor for biological applications. One possible way to eliminate the influence from AA is by using surface-confined ascorbate oxidase (Pariente et al., 1997). The reproducibility of the current response at the GLUD-Ru/Rh-CFE was also evaluated. A RSD value of 5.7% was estimated for three electrodes fabricated concurrently. Besides, the repeatability was evaluated using three electrodes and performing successive $\alpha\text{-KG}$ calibration curves for each electrode. These data yielded a RSD value of 7.3%.

4. Conclusions

In this proof of concept study, a $\alpha\text{-ketoglutarate}$ biosensor based on the co-deposition of Ru and Rh nanoparticles over CFE is presented. The co-deposition of metal nanoparticles on CFE resulted in the substantial improvement of analytical performance for the detection of NADH in terms of mitigating the overpotential, resistance to fouling, high sensitivity, stability and reproducibility providing significant advantages over bare CFE. The modification of Ru and Rh was found to be favorable environment of the immobilization of GLUD enzyme. The GLUD-Ru/Rh-CFE exhibited a sensitive and stable response for the detection of $\alpha\text{-KG}$ in phosphate buffer. In addition, the biosensor showed high reproducibility and stability. The new carbon fiber based biosensor holds considerable promise for various biomedical and biotechno-

logical applications involving real time electrochemical detection of $\alpha\text{-KG}$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2011.01.026](https://doi.org/10.1016/j.bios.2011.01.026).

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