Int. J. Electrochem. Sci., 10 **(2015)** 947 - 955

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

The Fabrication of Screen Printed Electrode Mixed Ferrocenemethanol and Thionin for β-hydroxybutyrate Biosensor

Bijun Feng and You-Nian Liu[*]

College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, P. R. China
[*]E-mail: liuyounian@csu.edu.cn

Received: 8 October 2014 / *Accepted:* 7 November 2014 / *Published:* 2 December 2014

In this paper, the screen printed electrode mixed ferrocenemethanol(Fc-OH) and thionin was prepared. Fc-OH acted as the oxidant and thionin as the electron transfer, the electrochemical oxidized behavior of β-hydroxybutyrate at the screen printed electrode was investigated in the present of β-hydroxybutyrate dehydrogenase and nicotinamide adenine dinucleotide (NAD). Moreover, we built a new method to detect β-hydroxybutyrate. The linear relationship of oxidized current at potential of 0.3 V with the concentration of β-hydroxybutyrate in the range of 50 μM to 5 mM was obtained by chronoamperometry (I-t), and the detection limit was 16 μM. The electrode was successfully applied to detect β-hydroxybutyrate in real samples without interference from AA and ACP.The preparation of screen printed electrode was simple, good reproducibility.

Keywords: Ferrocenemethanol; thionin; screen printed electrode; β-hydroxybutyrate; biosensor

1. INTRODUCTION

Diabetic ketoacidosis (DKA) is an acute complication of diabetes mellitus and often occurs in type 1 diabetes. The main reason is a serious shortage of insulin caused by the rapid rise of blood sugar. Ketone bodies such as β-hydroxybutyrate and acetoacetate decomposite abnormally in the blood. β-hydroxybutyrate, one of the main blood ketone bodies, has been considered as a critical indicator for diagnosis of diabetic ketoacidosis[1,2]. The determinations of β-hydroxybutyrate levels in biological samples is very desirable in type 1 diabetes, DKA, pregnancy complicated by diabetes, and managing toxic ketoacidoses and other medical conditions, it can provide the potential to avoid the DKA and is a signal of early diagnosis diabetes and ketonaemia[3,4].

Up to now, there are many methods employed to detect the concentration of β-hydroxybutyrate. Sorensen et al. developed hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC–MS/MS) to detemine β-hydroxybutyrate and β-hydroxy-β-methylbutyrate in whole blood. The β-hydroxybutyrate was separated from isobaric interferences through chromatography, the method did not require chemical modification of the analytes[5]. Kimura et al. used 3-hydroxybutyrate dehydrogenase coupled with acetoacetate decarboxylase to convert β-hydroxybutyrate to acetone, then the acetone was detected by head-space gas chromatography[6]. However, these spectrophotometric assays often need the special equipment and reagents, multiple processes, and the professional operator[7].

Since Clark and Lyons proposed the first glucose enzyme electrode in 1962, there has been a lot of attention paid to develope novel biosensors for the fast, stable, reproducible, sensitive and selective quantification of glucose, especially for disposable electrochemical sensors based on screen-printed carbon electrode (SPCE) technology[8]. Such sensors are usually made by screen-printed electrode with suitable conducting pastes (e.g., carbon, Ag/AgCl, etc.) on a substrate and then immobilizing a bioreagent layer on the electrode surface[9,10]. Compared to the common electrode, the screen-printed electrode, due to its low cost, easy to prepare and high reproducibility, have attracted extensive interest in various fields. Several reports described the β-hydroxybutyrate detection using electrochemical methods[11-13]. Li et al. developed a disposable amperometric biosensor immobilized with β-hydroxybutyrate dehydrogenase, potassium ferricyanide, nicotinamide adenine dinucleotide (NAD) for monitoring the blood ketones[14]. Khorsand et al used carbon nanotube-modified screen printed electrode to reduce the oxidation potential with the low detection of 9 μM [15]. As for the low concentration of β-hydroxybutyrate in normal serum levels, and the results affected by the electroactive interferences in plasma, such as ascorbic acid (AA), uric acid (UA) and acetaminophen (ACP), we employed ferrocenemethanol(Fc-OH) as the oxidant and thionin as the electron transfer and studied the electrochemical behavior of β-hydroxybutyrate at the screen printed electrode modified with β-hydroxybutyrate dehydrogenase and NAD. Spectrometric measurement of the β-hydroxybutyrate was compared to the electrochemical method in the real whole blood sample test.

2. EXPERIMENTAL

2.1 Materials

Ferrocenemethanol(Fc-OH), thionin, β-hydroxybutyrate, glutaraldehyde, nicotinamide adenine dinucleotide (NAD), β-hydroxybutyrate dehydrogenase, uric acid (UA), ascorbic acid (AA), ascorbic acid oxidase, urate oxidase, hydroxyethyl cellulose(HEC), chitosan, acetaminophen (ACP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbon ink and insulation ink were acquired from JUJO (Tokyo,Japan). The hydrophilic film and double sided adhesive tape were from 3M China Co., Ltd (Shenzhen, China). Silver chloride ink was purchased from Cambridge Nano-Tech. Ltd

(Xuzhou, China). Aqueous solutions were prepared using Millipore water (Simplicity Model, Billerica, MA, USA).

2.2 Electrode Preparation

We prepared the screen-printed carbon electrode mixed with 5% Fc-OH referenced to the literature[16]. 1 mg hydroxyethyl cellulose (HEC) and 1 mg chitosan were dissolved into phosphate buffer solution (pH=7.0) and hydrated 2 h at room temperature, and 2 ku D-3-hydroxybutyrate dehydrogenase, 2 ku ascorbic acid oxidase, 1 ku urate oxidase, 2 mg nicotinamide adenine dinucleotide (NAD), 4 mg thionin were added. Then 50 μL of 10% glutaraldehyde was dropped into enzyme solution and reacted 0.5 h. Acquired 10 μL above solution dropped into the working electrode, dried at 45 ^{o}C for 0.5 h, For fabrication of the stable sample reaction cell, the hydrophilic film and double sided adhesive tape was covered the react area.

2.3 Detection of β-hydroxybutyrate at screen printed electrode

All electrochemical measurements were carried out with a model CHI660B Electrochemical Workstation (CH Instruments, Austin, TX, USA). For measured the electrochemical behaviors of the screen printed electrode with differential pulse voltammetry (DPV), the screen-printed carbon electrode mixed with 5% Fc-OH as the working electrode and the silver chloride electrode as the reference electrode, the supporting electrolyte was 10 mM $KClO_4$ in PB solution (pH=7.0). For amperometric detection of β-hydroxybutyrate at electrochemical β-hydroxybutyrate biosensor, the applied potential was set at 0.3 V. The value of the current was acquired at 20 s at amperometric i-t curve. After amperometric measurements of β-hydroxybutyrate in PB solution at intervals, the electrodes were kept dry at room temperature. All experiments were carried out at room temperature unless otherwise stated.

2.4 Preparation and analysis of real samples

Aliquots (5.0 mL) of blood samples without any treatment were accessed to the anticoagulation tube contained 10 μL heparin. For measurement of the β-hydroxybutyrate concentration in blood sample using amperometric i-t method, the results were compared to those determined with automatic biochemical analyzer (Beckman Instruments, Inc., California, USA).

For the stability of electrodes, we chose a bath of electrodes containing 50 strips to a sealed packaging. After each measurement, the rest of electrodes were maintained in sealed packaging at room temperature to keep clean and dry.

3. RESULTS AND DISCUSSION

3.1 Electrochemical analysis of screen printed electrode

DPV was employed to study the behavior of screen-printed carbon electrode (SPCE) mixed with Fc-OH or the mixture of Fc-OH and thionin. As shown in Fig 1, there was an obvious oxidation peak at 0.24 V,which was suggested to the oxidized process of Fc-OH at the screen printed electrode. When the thionin was added to the screen printed electrode mixed with Fc-OH, the oxidized current was higher than that acquired from the screen printed electrode mixed with Fc-OH. The result revealed thionin had good electron transfer ability which can accelerate the electron transfer between Fc-OH and electrode.

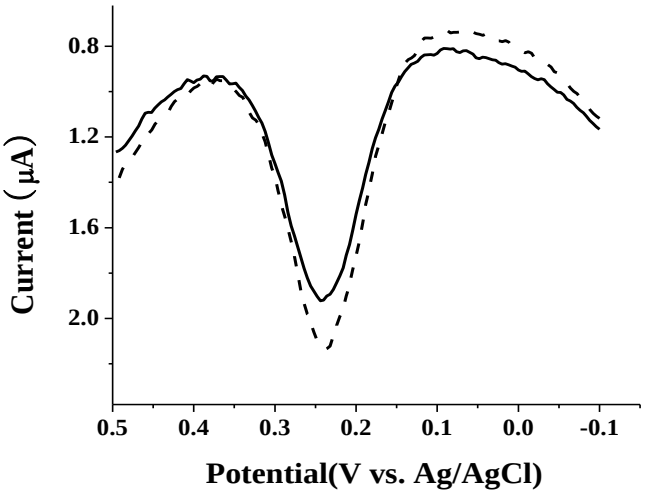

Figure 1. Differential pulse voltammetry(DPV) of PB solution at screen printed electrode mixed with Fc-OH (solid line) or the mixture of Fc-OH and thionin(dashed line)

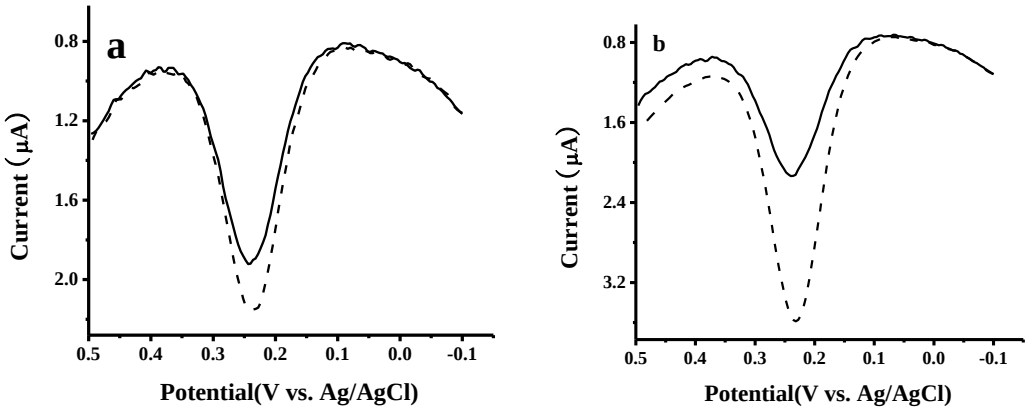

Figure 2. Cyclic voltammograms of PBS solution (dashed line) and 0.3 mM β-hydroxybutyrate (solid line) at screen printed electrode mixed Fc-OH (a) or the mixture of Fc-OH and thionin(b).

When 0.3 mM β-hydroxybutyrate was added to the PB solution, the current at two different screen printed electrodes mixed with Fc-OH(cf. Fig 2a) or Fc-OH and thionine(cf. Fig 2b) increased which was ascribe to the catalytical oxidized of β-hydroxybutyrate by Fc-OH. However, the increased current at screen printed electrode mixed with Fc-OH and thionine was 1.1 μA was significantly higher than the screen printed electrode mixed with Fc-OH (0.4 μA). The results indicated thionine can accelerate the electron transfer between the solution and the electrode surface. Furthermore, the screen printed electrode mixed with Fc-OH and thionin had a catalytic oxidized ability of β-hydroxybutyrate.

3.2 Detection of β-hydroxybutyrate at β-hydroxybutyrate biosensor

The amperometric I-t method was employed to determine β-hydroxybutyrate according to measure the oxidized current at β-hydroxybutyrate biosensor of the screen printed electrode mixed with Fc-OH and thionine. When the oxidized potential was set at 0.3 V, the relationship of oxidized current at β-hydroxybutyrate biosensor with various concentrations of β-hydroxybutyrate was shown in Fig 3. The oxidized current was increased with the added concentation of β-hydroxybutyrate. There is a good linear relationship between the oxidized current accquried from I-t curve at 20 s and the concentration of β-hydroxybutyrate from 50 μM to 5 mM. The calibration curve was I (μA) =0.68[β-hydroxybutyrate] (mM) +1.32 and the correlation coefficient was 0.99. From the variations of the baseline signals, we estimated the detection limited was 16 μM. Li et al. developed a disposable amperometric biosensor for monitoring the β-hydroxybutyrate. hydrophilic gel CMC was printed on the screen-printed carbon electrodes, then the β-Hydroxybutyrate dehydrogenase, cofactor NAD and potassium ferricyanide were immobilized on the electrode surface by adsorption. The response time of the sensor is 50 s. and the linearity in the range 1.5–550 mg L^{-1}（14.4μM -5.28mM）[14]. Compared these results, our method is more simple and has shorter response time.

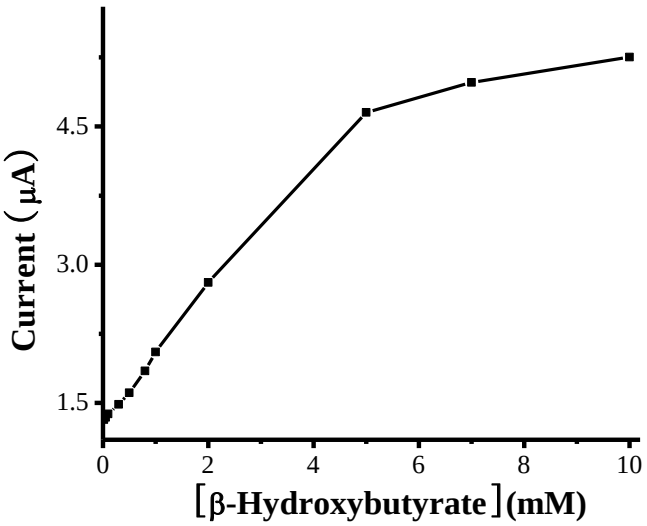

Figure 3. The relationship of oxidized currents with various β-hydroxybutyrate concentrations from 0 to 10 mM at screen printed electrode with the potential of 0.3 V.

3.3 The influnce of pH on β-hydroxybutyrate biosensor

We investigated the response current of β-hydroxybutyrate biosensor at various pH values from 6.0 to 8.0. β-hydroxybutyrate was dissolved in various pH solutions and prepared various concentrations from 0.5 mM to 4 mM.The response currents at various pH with β-hydroxybutyrate were shown in Fig. 4. The oxidized currents was increased with the pH increased at low concentration of β-hydroxybutyrate due to the best catalytical activity of β-hydroxybutyrate dehydrogenase at 8.0. However, when the concentration of β-hydroxybutyrate arrived to 4 mM, the oxidized current did not increase with the increase of pH, which was mainly affected by the electron transfer between Fc-OH and thionine. So we selected pH 7.0 in the experimental.

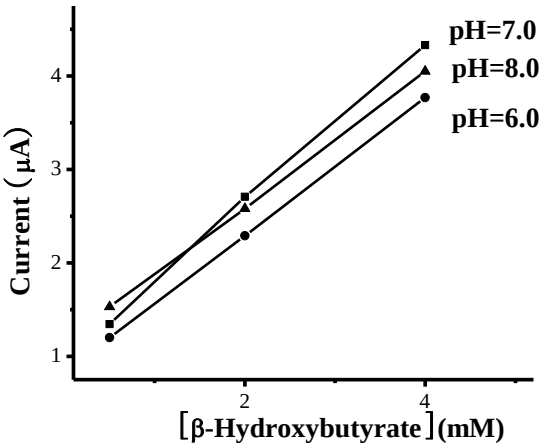

Figure 4. The current response of β-hydroxybutyrate at various pH.

3.4 Stability and reproducibility of β-hydroxybutyrate biosensor

The stability and reproducibility of the β-hydroxybutyrate biosensor towards β-hydroxybutyrate detection was estimated by assaying the response of 300 μM β-hydroxybutyrate in PB solution. The screen printed electrode kept stable and the catalytical oxidized current still retained 96.4% of the initial current after 140 days. After 10 times continuous measurements for the same bath of electrochemical strips, the results revealed the intra-assay and inter-assay coefficient of variation of the electrode was 3.57 % and 4.03 %. The above results indicated that the β-hydroxybutyrate biosenso had good stability and reproducibility which suggested the β-hydroxybutyrate biosensor could be used as the commercial electrode in real application.

3.5 The anti-interference ability of β-hydroxybutyrate biosensor

The electrochemical signal is usually affected by the electroactive interferences in plasma, such as ascorbic acid (AA), uric acid (UA) and acetaminophen (ACP) in the process of electrochemical determination of real blood samples, these interferences have the similar redox potential. Therein, the anti-interference ability of β-hydroxybutyrate biosensor was considered in the physiological

conditions. When the potential of β-hydroxybutyrate biosensor was set at 0.3 V, there were obvious electrochemical response signals in addition of AA and UA at β-hydroxybutyrate biosensor unmodified with ascorbic acid oxidase and urate oxidase. The signals were due to the oxidized reaction of AA and UA at the β-hydroxybutyrate biosensor surface (cf. Table 1). When β-hydroxybutyrate biosensor mixed with ascorbic acid oxidase and urate oxidase, AA and UA can be catalytical oxidized rapidly and be eliminated. Furthermore, when addition 5 mM ACP in the solution, the oxidized current at β-hydroxybutyrate biosensor was basically unchanged, therein, we concluded the β-hydroxybutyrate biosensor had the good anti-interference ability.

Table 1. The current of 300 μM β-hydroxybutyrate solution with addition of various interferences at β-hydroxybutyrate biosensor mixed or unmixed with ascorbic acid oxidase and urate oxidase.

Electrodes	AA(5 mM)	UA(0.5 mM)	ACP (5 mM)
Oxidized current at β-hydroxybutyrate biosensor mixed with ascorbic acid oxidase and urate oxidase(μA)	1.42	1.45	1.47
Oxidized current at β-hydroxybutyrate biosensor unmixed with ascorbic acid oxidase and urate oxidase(μA)	3.17	2.59	1.43

3.6 Real sample measurement

Aliquots (5.0 mL) of blood samples without any treatment were accessed to two anticoagulation tubes contained 10 μL heparin. Take one whole blood to centrifuge 10 min at the speed of 3500 rpm and acquired the plasma. Then the sample was measured using automatic biochemical analyzer. The method of β-hydroxybutyrate determined by hospital used the continuous monitored enzyme kinetics, according to detect the reduced rate of nicotinamide adenine dinucleotide (NAD) at 340 nm, which was directly proportional to the concentration of β-hydroxybutyrate. Another whole blood was measured with amperometric i-t method, 2 μL sample was added to the screen printed electrode, then we got the oxidized current at 20 s at I-t curve, then the concentration was calculated according to the calibration curve. As shown in Table 2, the results based on our method were in excellent agreement with those obtained with the commercial automatic biochemical analyzer, it also suggested that the β-hydroxybutyrate biosensor can effectively be used to determine the β-hydroxybutyrate in whole blood samples. Shimomura et al. developed amperometric β-hydroxybutyrate biosensor based on a screen-printed carbon electrode containing Meldola's Blue, NAD and 3-β-hydroxybutyrate dehydrogenase immobilized on mesoporous silica, the sensor required the sample volume of 10 μL for the measuremen and maintained >90 % of its initial response after being stored for over 6 months[11]. Compared these methods, our methods is more suitable for commercial purposes.

Table 2. The comparation of β-hydroxybutyrate determined by hospital and our method.

Samples	Results of hospital (μM)	This method (μM)
1	151	147
2	276	261
3	502	487
4	831	816

4. CONCLUSIONS

We designed a new method of β-hydroxybutyrate sensor successfully. Ferrocenemethanol(Fc-OH) was employed as the oxidant and thionin as the electron transfer, the electrochemical oxidized behavior of β-hydroxybutyrate at the screen printed electrode was investigated in the present of β-hydroxybutyrate dehydrogenase and nicotinamide adenine dinucleotide (NAD) was investigated. There was good relationship between the electrocatalytic oxidized current and the β-hydroxybutyrate concentration from 50 μM to 5.0 mM with the limited detection was 16μM. Furthermore, the electrode had good stability and reproducibility, and can effectively eliminate the interference such as ascorbic acid (AA), uric acid (AA) and acetaminophen (ACP). In addition, this method was also successfully employed for β-hydroxybutyrate detection in blood real samples. We therefore conclude that the rapid and sensitive β-hydroxybutyrate integrated to portable devices can apply in the field of clinical analysis.

References

1. L. Laffel, *Diabetes Metab. Res. Rev.*, 15 (1999) 412.
2. R. C. H. Kwan, P. Y. T. Hon, W. C. Mak, L. Y. Law, J. Hu and R. Renneberg, *Biosen. Bioelectron.*, 21 (2006) 1101.
3. T. M. Wallace and D. R. Matthews, *QJM*, 97 (2004) 773.
4. F. Khorsand, S. Riahi, S. E. Fard, S. Kashanian, A. Naeemy, B. Larijani and K. Omidfar, *Nanobiotech. IET*, 7 (2013) 1.
5. L. K. Sorensen, N. F. Rittig, E. F. Holmquist, E. F. Holmquist, K. A. Jorgensen, J. O. L. Jorgensen, N. Moller and M. Johannsen, *Clin. Biochem.*, 46 (2013) 1877.
6. M. Kimura, K. Kobayashi, A. Matsuoka, K. Hayashi and Y. Kimura, *Clin. Chem.*, 31(1985) 596.
7. S. Felby and E. Nielsen, *Forensic Sci. Int.*, 64 (1994) 83.
8. L.C. Clark and C. Lyons, *Ann. N.Y. Acad. Sci.*, 102 (1962) 29.
9. J. Wang, *Biosens. Bioelectron.* 21 (2006) 1887.
10. V. Gubala, L.F. Harris, A.J. Ricco, M.X. Tan and D.E. Williams, *Anal. Chem.*, 84 (2012) 487.
11. T. Shimomura, T. Sumiya, M. Ono, T. Ito andTaka-aki Hanaoka. *Anal. Bioanal. Chem.*, 405 (2013) 297.
12. N. J. Forrow, G. S. Sanghera, S. J. Walters and J. L. Watkin, *Biosens. Bioelectron.* 20 (2005) 1617.
13. L. Fang, S. H. Wang and C. C. Liu, *Sens. Actuators B, Chem.*, 129(2008) 818.
14. G. Li, N. Z. Ma and Y, *Sens. Actuators B, Chem.*, 109 (2005) 285.

15. F. Khorsand, M. D. Azizi, A. Naeemy, B. Larijani and K. Omidfar, *Mol. Boil. Rep.*, 40 (2013) 2327.
16. P. Chen, Y. Peng, M. He, X.C. Yan, Y. Zhang and Y.-N. Liu, *Int. J. Electrochem. Sci.*, 8 (2013) 8931 .