Forensic Science International 207 (2011) 177–182

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

A novel microassay for measuring blood alcohol concentration using a disposable biosensor strip

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ARTICLE INFO

Article history:
Received 11 July 2010
Received in revised form 26 September 2010
Accepted 1 October 2010
Available online 28 October 2010

Keywords:
Alcohol
Biosensor
Multi-wall carbon nanotubes
Gold nanoparticles
Screen-printed electrodes

ABSTRACT

A novel disposable amperometric biosensor strip for determination of blood alcohol concentration (BAC) with small volume of sample has been constructed using screen-printed electrodes (SPE), nanocomposite film and alcohol dehydrogenase (ADH). Firstly, the GNPs–MWCNT–Nafion nanocomposite film modified on a working electrode was made of Nafion-117, multi-wall carbon nanotubes (MWCNT) and gold nanoparticles (GNPs). After that Meldola's blue (MB) acted as an electron transfer mediator and the mixed solution of ADH, nicotinamide adenine dinucleotide (NAD^+) were modified in order on the nanocomposite film. At last, a hydrophilic membrane which had an eyehole in center was placed at the outermost of the working area to make a reaction tank of 5 μL, then the hydrophilic membrane/ADH–NAD^+/GNPs–MWCNT–MB–Nafion/SPE was prepared. The detection of BAC can be accomplished with 5 μL of blood sample obtained precisely by siphonage. Optimum conditions of the biosensor were experimentally determined by varying several important parameters: working potential, solution pH value, environmental temperature and interferences. Experimental results indicated that the biosensor possessed a good accuracy and stability, the linear response range was 2.0×10^{-4} to 25×10^{-3} mol/L and the detection limit of the biosensor was 5.0×10^{-5} mol/L (S/N = 3). In the measurement of blood samples, the proposed biosensor had excellent detection performance for measuring BAC and showed a good correlation with gas chromatography. The prepared biosensor strip can be valid for the analysis of BAC.

1. Introduction

Alcohol is the most common poisonous substance related to clinical medicine and forensic medicine, and it leads to a variety of health damages [1,2] and traffic accidents [3–5] frequently. Actually, more and more people died of alcohol abuse. As is known to all, measurement of blood alcohol concentration (BAC) is seen as the most convincing evidence to judge drunk driving [6]. So, the accurate measurement of BAC is very important for forensic medicine and lawsuit. Some analytical methodologies have been developed for such purpose, including spectrophotometry [7], high performance liquid chromatography [8], and gas chromatography [9]. These methods, however, have some disadvantages such as requiring expensive instrument, long time analysis and sample pre-treatment.

A biosensor based on selective alcohol conversion enzymes is utilized as a valuable alternative way for the sensitive, precise, inexpensive and rapid determining BAC. Especially the disposable amperometric biosensor is widely used to detect alcohol. Generally, two kinds of enzymes, alcohol dehydrogenase (ADH) [10,11] and alcohol oxidase (AOD) [12] were immobilized for the construction of alcohol biosensors. The former one is more steady than the type of AOD biosensor. ADH catalyzes alcohol to acetaldehyde in the presence of coenzyme–nicotinamide adenine dinucleotide (NAD^+) and then NAD^+ is reduced to NADH which can be oxidized through the displacement of electron and proton. The whole enzymatic reaction can be measured according to the following reaction schemes:

$$\text{Alcohol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + \text{NADH} + \text{H}^+$$
$$\text{NADH} \rightarrow \text{NAD}^+ + \text{H}^+ + 2e^-$$

However, the electro-oxidation of NADH requires a high overpotential in the detection system owing to the charge transfer kinetics characteristics [13] which will involve many side effects. In order to electrochemically oxidate NADH at low overpotential, some methods have been used to fabricate the alcohol biosensor. Meldola's blue (MB), acting as an electron transfer mediator can accelerate electron transfer between enzyme and electrode and decrease the deactivation of ADH [14,15], so the purpose of

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doi:10.1016/j.forsciint.2010.10.002

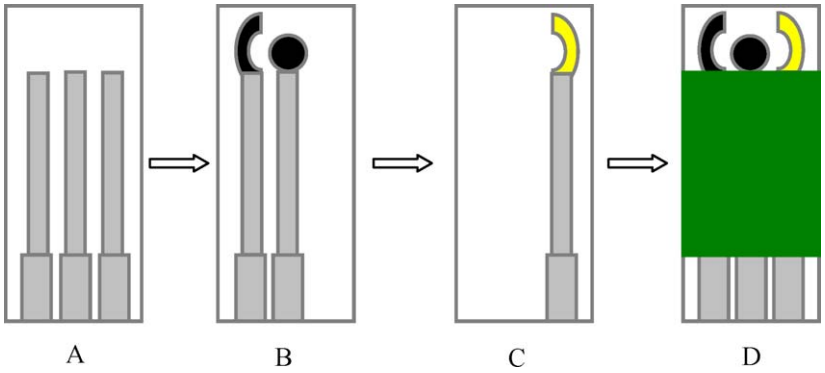

Fig. 1. The production steps for screen-printed electrodes: print conductive sliver strip (A), carbonic working and counter electrodes (B), Ag/AgCl reference electrode (C) and insulating layer (D) in order.

oxidizing NADH at low overpotential is easy to be implemented. Nafion, a kind of organic polymer, can form membrane and absorb MB firmly through ion exchange [16]. Multi-wall carbon nanotubes (MWCNT) represent a new type of carbon material [17] with the unique merits of promoting electron transfer reactions, large specific surface area, improving electric current signal, superordinary mechanical and structural properties have been used to modify biosensor extensively [18–20]. Gold nanoparticles (GNPs) are a kind of metal nano-material receiving much interest due to their good stability, large surface area to mass ratio, high catalytic activities and excellent biocompatibility. So they play a key role in various applications such as biosensors and electrochemical sensors [21,22]. Screen-printed electrodes (SPE) have enabled the production of modern sensors which can be incorporated in portable systems as an important requirement of analytical methods for direct analysis of a sample without pre-treatment [23]. Meanwhile, this technology can detect biological specimens quickly, conveniently and with low cost [24]. In recent years, Luo et al. [16,25] fabricated alcohol biosensors using ADH and SPE to measure alcohol concentration precisely, but he did not use the nanocomposites consisting of MWCNT and other nanomaterials which can enlarge the signal of detection amperometric current to improve the measurement performance of the biosensor. Although SPE was used in his study, but only carbonic working electrode was printed on one polyvinyl chloride (PVC) basal lamina, counter and reference electrodes were also a part of the electrochemical work station, so the measurement of BAC was completed in big volume reaction system.

The aim of this work presented here was to develop a disposable whole blood alcohol biosensor which was fabricated as hydrophilic membrane/ADH–NAD^+/GNPs–MWCNT–MB–Nafion/SPE. Hydrophilic membrane was placed at the outermost of the three electrodes to make a reaction tank of 5 μL, so small volume of sample could be measured. The electroanalytical nanobiocomposite film which could absorb MB was prepared by mixing Nafion, MWCNT and GNPs. MWCNT and GNPs acted as efficient conduits with synergistic effect for electron transfer. Experimental results indicated that the biosensor had satisfactory performance and could be used to measure the whole blood alcohol.

2. Materials and methods

2.1. Reagents

Alcohol dehydrogenase (ADH, EC. 1.1.1.1, A3263-15KU), nicotinamide adenine dinucleotide (NAD^+), Meldola's blue (MB), Nafion-117 solution (5%, m/V) and chlorauric acid ($HAuCl_4$, EC No. 2409484) were purchased from Sigma. Bovine serum albumin (BSA) was purchased from Roche. Sodium citrate was analytical grade. Carboxylation multi-wall carbon nanotubes (MWCNT, purity larger than 95%) were purchased from Chengdu Organic Chemicals Company Ltd. of Chinese Academy of Sciences. Carbon ink (423SS), silver ink (427SS) and chloride silver were purchased from Acheson. Phosphate buffer ($NaH_2PO_4{\cdot}2H_2O$–$Na_2HPO_4{\cdot}12H_2O$, PBS, 0.1 mol/L, pH 7.5) and absolute alcohol (analytical grade). The samples of whole blood alcohol were collected from the Forensic Identification Department of Chongqing Medical University and the First Affiliated Hospital of Chongqing Medical University. All other chemicals were of analytical grade and deionized water was purified with a Millipore Milli-Q system.

2.2. Apparatus

Electrochemical measurements were performed with a μAutolab III electrochemical work station (Ecochemie, The Netherlands). Manual screen-printer (Shengjiang, GuangDong, China) and SC-2000 gas chromatograph (Chongqing Sichuan Instrument Analyzer Company) were employed in this study.

2.3. Preparation of screen-printed electrodes

The simple production steps of the electrode are shown schematically in Fig. 1.

Firstly, three sliver strips were printed on polyvinyl chloride (PVC) basal lamina. Then carbonic working electrode, carbonic counter electrode and Ag/AgCl reference electrode were completed in order. At last, the surface layer was printed with the insulator. The working area was a disc with a diameter of 3 mm. Fig. 2 is the photograph of the screen-printed electrodes.

2.4. Preparation of the GNPs–MWCNT–Nafion hybrid composites

Colloidal gold nanoparticles (GNPs) were prepared according to the procedures mentioned before [26] by adding 2.5 mL 1% (w/w) of sodium citrate solution to 100 mL of boiling aqueous solution containing 1 mL of 1% (w/w) $HAuCl_4$ and stirring until the solution discloses claret-red. Preparations were stored in brown glass bottles at 4 °C. The diameter of the resulting gold nanoparticles measured by scanning electron microscopy was about 20 nm.

200 μL of 5% (m/V) Nafion-117 solution was added into 800 μL obtained GNPs with ultrasonic agitation for 10 min, and then 1 mg MWCNT was dispersed in the prepared GNPs–Nafion suspension by ultrasonic 40 min. A homogeneous, well distributed 1 mg/mL solution of GNPs–MWCNT–Nafion hybrid composites was obtained.

2.5. Fabrication of the nanocomposites modified alcohol biosensor

Prior to modifying enzyme, 3 μL of the GNPs–MWCNT–Nafion nanocomposites was cast onto the working electrode and dried in air at room temperature. The nanocomposites modified SPE was dip into 1 mmol/L MB (prepared by PBS, 0.1 mol/L, pH 7.5) at cyclic voltammetry scan to absorb MB until saturated, then GNPs–MWCNT–MB–Nafion/SPE was obtained.

2 mg ADH, 2 mg NAD^+ and 8 mg BSA were added into 200 μL PBS (0.1 mol/L, pH 7.5) to obtain an enzyme solution. The ADH–NAD^+/GNPs–MWCNT–MB–Nafion/SPE was prepared by casting 3 μL of the enzyme solution on the modified working electrode. Hydrophilic membrane with an eyehole in center was placed on the outermost of the working area to make a reaction tank of 5 μL. An integrity alcohol biosensor was fabricated eventually.

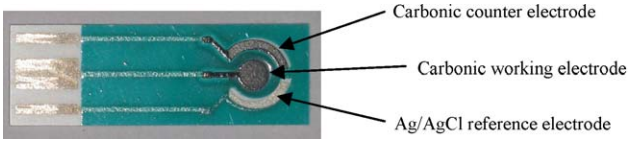

Fig. 2. Photograph of the screen-printed electrodes.

2.6. Measurement methods

In this study, cyclic voltammetry (CV) and chronoamperometry were used in the detection system. Standard curves were made by using the biosensor to measure 5 μL of different concentrations of standard whole blood alcohol–PBS solution. Real blood samples were measured by the biosensor.

3. Results and discussion

3.1. Electrochemical characteristics of the GNPs–MWCNT–MB–Nafion modified SPE

Cyclic voltammetry was used to represent the modified electrode. The effects of the scan rates on the peak current were investigated and the results are shown in Fig. 3. As could be seen in Fig. 3A, the scan patterns were symmetry well, both anodic peak currents (i_{pa}) and cathodic peak currents (i_{pc}) of the GNPs–MWCNT–MB–Nafion modified SPE rise gradually along with the scan rates (from 20 mV/s to 120 mV/s), $i_{pa}/i_{pc} \approx 1$. Meanwhile, the anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) did not shift basically. As shown in Fig. 3B, the i_{pa} and i_{pc} were proportional to the square root of different scan rates. It exhibited a good linearity and the correlation coefficient (r) was 0.99960 and −0.99909, respectively. Consequently, the peak current of the GNPs–MWCNT–MB–Nafion/SPE was controlled by the surface diffusion controlled process in solution. The results revealed that MB could be absorbed by GNPs–MWCNT–Nafion hybrid composites owing to some factors. Firstly, Nafion is a kind of organic polymer which can absorb MB fastness through ion exchange [16]. Secondly, MB can be firmly assembled on the surface of carbon nanotubes by π–π non-covalent stacking to design a new special nanocomposite interface to stimulate electron transfer [15]. In addition, MWCNT and GNPs can be dispersed in Nafion uniformly to obtain a stable and homogeneous solution.

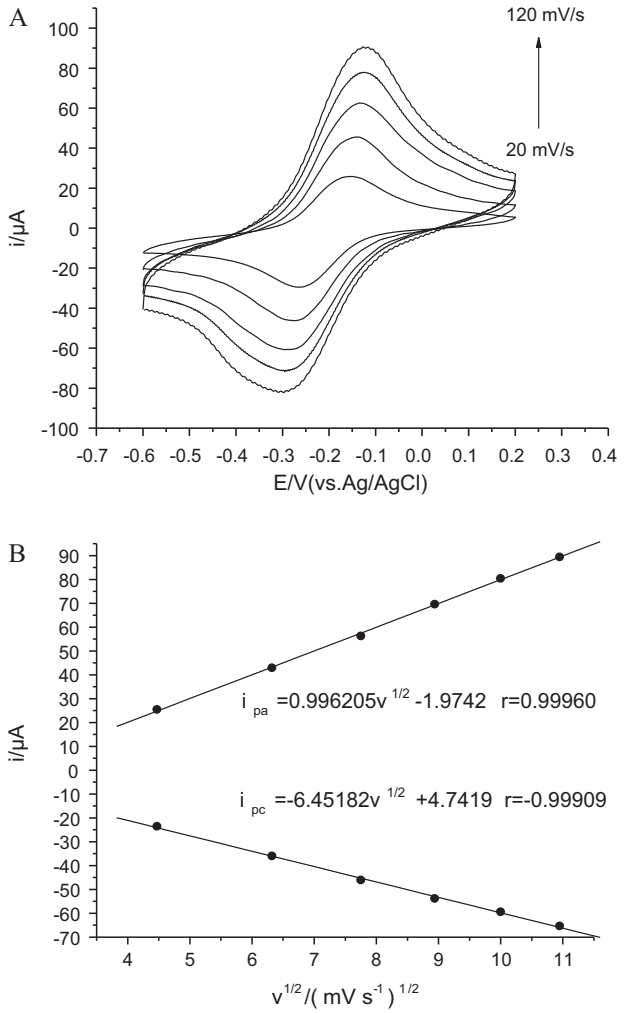

Fig. 3. (A) Cyclic voltammograms of GNPs–MWCNT–MB–Nafion/SPE at different scan rates: 20, 40, 60, 80, 100, 120 mV/s in 0.1 mol/L PBS (pH 7.5). (B) Plot of peak current vs. the square root of various scan rates.

3.2. Electrochemical behaviors of different modified SPE

Carbonic and metallic nano-materials have larger electroactive surface area that means higher electrocatalytic current. Fig. 4 represents the CVs of various prepared SPE: (a) bare SPE, (b) MB–Nafion/SPE, (c) MWCNT–MB–Nafion/SPE, (d) GNPs–MWCNT–MB–Nafion/SPE in 0.1 mol/L PBS (pH 7.5). As shown in the graph, good anodic peak and cathodic peak of MB were observed, and MB could transfer electron very well in different modified SPE. Compared with MB–Nafion/SPE, the i_{pa} of MWCNT–MB–Nafion/SPE and GNPs–MWCNT–MB–Nafion/SPE all increased, and they were 3.94, 4.67 times as large as MB–Nafion/SPE, respectively. Simultaneously, the i_{pa} of GNPs–MWCNT–MB–Nafion/SPE was maximal in all modified SPE and was 1.19 times as large as MWCNT–MB–Nafion/SPE. It can be seen from these experimental data that MWCNT and GNPs all have effects on amplifying currents, which are attributed to the superior electrical conductivity and higher electroactive surface area of them [19,22].

3.3. Electrochemical characteristics of the biosensor for alcohol

The cyclic voltammograms of the biosensor measurement in blank (a) and 5 mmol/L alcohol in 0.1 mol/L PBS (pH 7.5) (b) are shown in Fig. 5. The anodic peak and cathodic peak were symmetry and reversible, and the anodic peak current of the biosensor in 5 mmol/L alcohol was evidently larger than that in blank solution. In addition, the anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) did not shift on the whole. So, the modified ADH on the SPE could catalysis oxidation alcohol excellently and the electron could be transferred very well among ADH, MB and working electrode.

3.4. Determination of working potential

Electrochemical catalytic reaction controls the rate at which NADH can be oxidized to NAD^+ to generate the measure signal. Therefore, with the purpose of enhancing the sensitivity of the

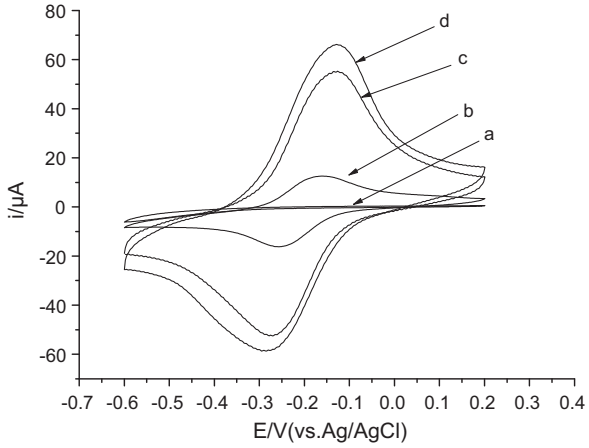

Fig. 4. Cyclic voltammograms of different modified SPE in 0.1 mol/L PBS (pH 7.5): (a) bare SPE; (b) MB–Nafion/SPE; (c) MWCNT–MB–Nafion/SPE; (d) GNPs–MWCNT–MB–Nafion/SPE. Scan rate: 50 mV/s.

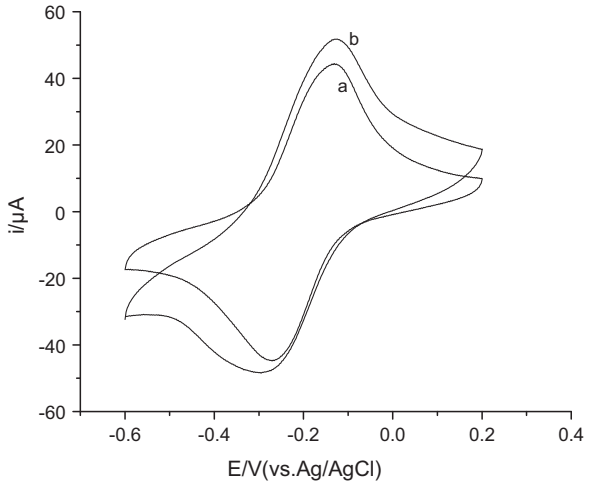

Fig. 5. Cyclic voltammograms of the modified biosensor to alcohol: (a) blank solution (b) 5 mmol/L alcohol in 0.1 mol/L PBS (pH 7.5). Scan rate: 50 mV/s.

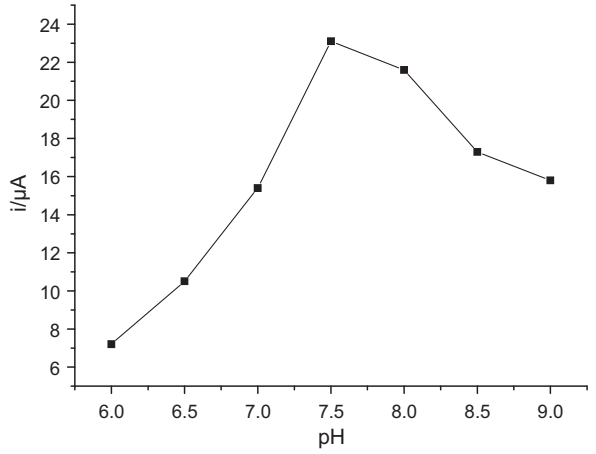

Fig. 7. Effects of solution pH values on current response of the modified biosensor in 0.1 mol/L PBS containing 5 mmol/L alcohol. Operating potential: −0.13 V vs. Ag/AgCl.

prepared alcohol biosensor, the working potential depending on the modified SPE was explored. Fig. 6 shows the amperometric current plot of the biosensor in different working potentials from −0.6 V to −0.05 V. As shown in the curve, the current increased continuously with the working potential increased from −0.6 V to −0.13 V vs. Ag/AgCl, reached a maximum value at −0.13 V, and then decreased over the rang of −0.13 V to −0.05 V. Consequently, the optimal working potential was selected at −0.13 V in order to obtain the most high response current and low interference during the determination.

3.5. Effect of solution pH value

In the enzymatic reactions, protons are transferred from one chemical substance to another, so the performance of prepared alcohol biosensor is influenced considerably by the pH value. The effect of solution pH value at the biosensor was investigated over the range of pH 6.0–9.0 in 0.1 mol/L PBS containing 5 mmol/L alcohol with an operating potential of −0.13 V vs. Ag/AgCl. A plot of the current response in different pH values is shown in Fig. 7. As could be seen that the current increased sharply with the increase of solution pH value at the pH value lower than 7.5, and reached a maximum value at pH 7.5. Then the response current decreased following with the pH value higher than 7.5. Hence, the pH 7.5 of PBS buffer was selected as the most appropriate buffer for the measurement of alcohol.

3.6. Effect of environmental temperature

The activities of enzyme can be greatly influenced commonly by temperature. Fig. 8 shows the amperometric current of the biosensor under different temperatures over the range of 10–40 °C in 0.1 mol/L PBS containing 5 mmol/L alcohol with an operating potential of −0.13 V vs. Ag/AgCl. As shown in the graph, the response current increased from 10 °C to 30 °C, and then decreased between 30 °C and 40 °C slowly. In the range of 25–30 °C, the current intensity changed a little, but the maximum current value appeared at 30 °C. So, the optimum temperature was selected at 30 °C in this study.

3.7. Chronoamperometric response of the biosensor for alcohol

Chronoamperometry (CA) was selected to analyze the detection performance of the alcohol biosensor. 90 μL PBS and 10 μL whole blood alcohol were taken in turn and mixed them immediately to obtain a whole blood alcohol–PBS solution. Standard whole blood alcohol–PBS solutions (0, 5, 10, 15, 20, 25 mmol/L) were prepared according to this. Fig. 9(A) is the amperometric current plots of the biosensor in different standard whole blood alcohol–PBS solutions

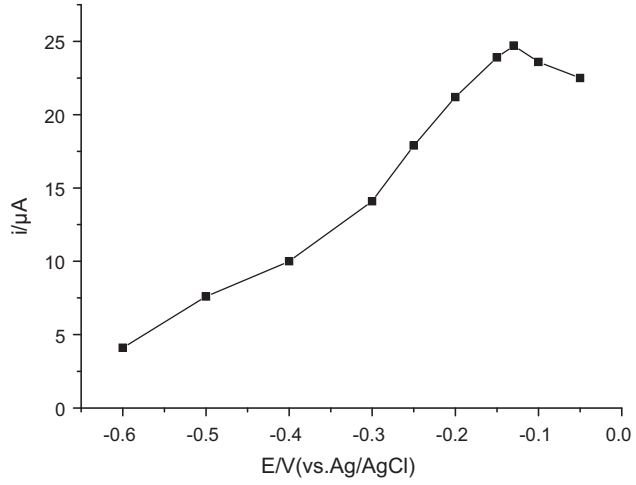

Fig. 6. An amperometric current plot of the biosensor at different working potentials from −0.6 V to −0.05 V in 0.1 mol/L PBS containing 5 mmol/L alcohol.

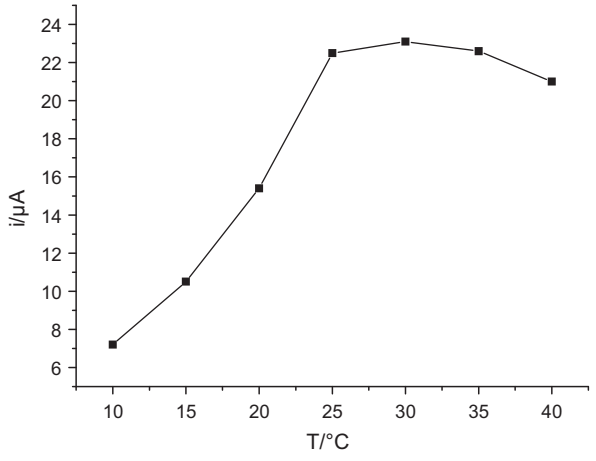

Fig. 8. The influence of temperature on the alcohol biosensor in 0.1 mol/L PBS containing 5 mmol/L alcohol with an operating potential of −0.13 V vs. Ag/AgCl.

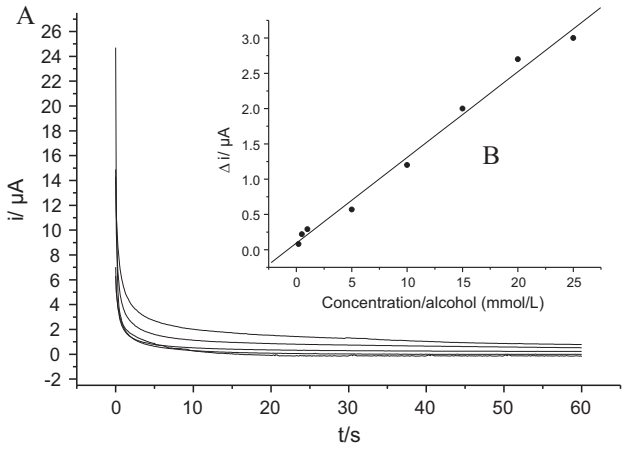

Fig. 9. (A) Chronoamperograms of the biosensor to different whole blood alcohol concentrations (0, 5, 10, 15, 20, 25 mmol/L) in 0.1 mol/L PBS. (B) Standard linear curve of the steady state response currents versus different alcohol concentrations. Working potential: −0.13 V vs. Ag/AgCl.

at the working potential of −0.13 V vs. Ag/AgCl. It could be seen that the biosensor had a steadily detection performance on alcohol. The amperometric current increased with the increasing of whole blood alcohol concentration and the response time was less than 15 s. Fig. 9(B) is the standard linear curve of the steady state response current versus different alcohol concentrations. As shown in the graph, the response currents were linear with the alcohol concentrations, and the linear equation was Δi (μA) = 0.09347 + 0.12141C (mmol/L), r = 0.99498. Additional, the linearity range of the amperometric current to alcohol concentration was 2.0×10^{-4} to 25×10^{-3} mol/L and the lowest detectable limit of the biosensor was 5.0×10^{-5} mol/L (S/N = 3). The prepared biosensor displayed excellent electrocatalysis to alcohol.

The apparent Michaelis–Menten constant (K_m) can evaluate the catalysis performance of enzyme. In this work, the K_m of modified ADH was 5.3 mmol/L which was calculated according to the Lineweaver–Burk equation: $I/I_{ss} = I/I_{max} + K_m/I_{max}\,C$ (I_{ss} is the current intensity after adding substrate, I_{max} is the maximum current value at fixation substrate concentration, C is the concentration of substrate).

3.8. Interference test and stability

During the measurement of BAC, some interferents in blood will be electrocatalysis at the applied working potential, so the current signal can be influenced by them. Table 1 is the influence of 20 mmol/L different interferents to the biosensor in 20 mmol/L whole blood alcohol–PBS solution with an operating potential of −0.13 V vs. Ag/AgCl. The interference rate was calculated by the equation: $(i_b - i_a)/i_a \times 100\%$ (i_a is the current value before adding interferent; i_b is the current value of after adding interferent). As shown in the table, the interference rates of various interferents were little and had not great inference to the measuring results, so BAC could be measured by the biosensor steadily. Low applied working potential was the most important factor for the excellent anti-interference ability. In addition, another factor was use small volume of sample in detection and specific ADH could catalysis alcohol quickly to generate a steadily response current.

Table 1
The influences of 20 mmol/L different interferents to the biosensor in 0.1 mol/L PBS contain 20 mmol/L whole blood alcohol with an operating potential of −0.13 V vs. Ag/AgCl.

Interference	$(i_b - i_a)/i_a \times 100\%$	Interference	$(i_b - i_a)/i_a \times 100\%$
Glucose	3.31%	Methanol	6.41%
Lactic acid	4.52%	Acetone	−4.92%
Ascorbic acid	−4.35%	*n*-Propanol	5.82%
Isobutyl alcohol	4.97%	*n*-Butanol	5.16%
Isopropanol	5.63%		

Also, the stability of the biosensor was examined using chronoamperometry among different storage days. The results showed that stored at 4 °C for 20 and 30 days, the response current of the biosensor in 0.1 mol/L PBS containing 5 mmol/L whole blood alcohol decreased 3.6% and 5.8% compared with initial current, respectively. The reproducibility of the alcohol biosensor was analyzed in 0.1 mol/L PBS containing 5 mmol/L whole blood–alcohol. At the same patch, 10 prepared biosensors were selected to measure alcohol, the intra coefficient variations was 4.1%. Taken out 3 prepared biosensors respectively from 4 different patches to measure alcohol, the inter coefficient variations was 4.7%. This prepared biosensor revealed favourable stability toward measuring alcohol.

3.9. Determination of alcohol in real blood samples

The practical application of the prepared alcohol biosensor was tested by the measurement of alcohol in authentic blood samples under optimum conditions. 60 blood samples of drunken driving drivers (50 cases of living and 10 cases of deceased) from Forensic Identification Department of Chongqing Medical University and the First Affiliated Hospital of Chongqing Medical University were selected, then 90 μL PBS and 10 μL whole blood sample were taken in turn and mixed them immediately to obtain a whole blood alcohol–PBS solution. 5 μL of mixture was taken, respectively, from each sample and measured alcohol directly using the biosensor. Meanwhile, the blood samples were tested in parallel using the SC-2000 gas chromatograph (Measuring conditions: GDX-102 column, column temperature 165 °C, hydrogenous flame detector temperature 200 °C, vaporizer temperature 190 °C, nitrogen gas flow rate 30–40 mL/min, hydrogen gas flow rate 30 mL/min, air flow rate 120 mL/min). Fig. 10 shows the linear correlation analysis of the biosensor versus the gas chromatography. Experimental data had shown that the proposed biosensor had a good detection performance of measuring alcohol and showed a good correlation with gas chromatography. The analytical curve was calibrated by the linear correlation equation: $Y = 1.03986X - 0.2644$, r = 0.99621. The prepared biosensor can be valid for the analysis of alcohol in blood.

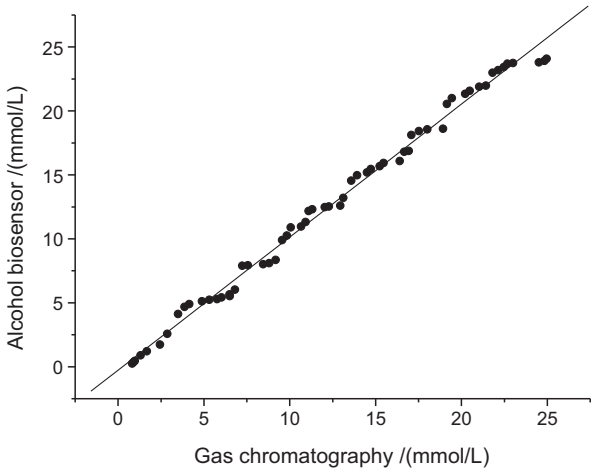

Fig. 10. Scatterplot of correlation analysis about the methods of the biosensor and gas chromatofraphy in measuring BAC of samples.

4. Conclusion

In summary, an alcohol biosensor is prepared by the combination of modified screen-printed electrodes with nano-materials and ADH to fabricate a novel detection strip. The nano-materials MWCNT and GNPs can accelerate electron transfer and enlarge the response current to increase the detection sensitivity. Screen-printed electrodes have the merits of manufacture facility and lower cost, therefore they can be extensively utilized in fabrication of the disposable biosensor. Sticking the hydrophilic membrane at the outermost of the three electrodes to make a reaction tank of 5 μL achieves the purpose of microassay for the determination of BAC and eliminates the necessity of pre-treatment of blood samples. The developed alcohol biosensor exhibits high sensitivity, wide linear range and good stability, and can be potentially applied to measure blood alcohol.

Acknowledgement

This work has been supported by the Scientific and Technological Project of Chongqing City, China (CSTC 2009AC5145).

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