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# One-step fabrication of integrated disposable biosensor based on ADH/NAD$^+$/meldola's blue/graphitized mesoporous carbons/chitosan nanobiocomposite for ethanol detection

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ABSTRACT

A novel strategy to simplify the dehydrogenase-based electrochemical biosensor fabrication through one-step drop-coating nanobiocomposite on a screen printed electrode (SPE) was developed. The nanobiocomposite was prepared by successively adding graphitized mesoporous carbons (GMCs), meldola's blue (MDB), alcohol dehydrogenase (ADH) and cofactor nicotinamide adenine dinucleotide (NAD$^+$) in chitosan (CS) solution. MDB/GMCs/CS film was prepared. Cyclic voltammetry measurements demonstrated that MDB was strongly adsorbed on GMCs. After optimizing the concentration of MDB and the working potential, the MDB/GMCs/CS film presented a fast amperometric response (5 s), excellent sensitivity (10.36 nA μM$^{-1}$), wide linear range (10–410 μM) toward NADH and without any other interference signals (such as AA, UA, DA, $H_2O_2$ and metal ions). Furthermore, concentrations of ADH and NAD$^+$ in nanobiocomposite and the detection conditions (temperature and pH) were also optimized. The constructed disposable ethanol biosensor showed an excellent linear response ranged from 0.5 to 15 mM with high sensitivity (67.28 nA mM$^{-1}$) and a low limit of detection (80 μM) and a remarkable long-term stability (40 days). The intra-batch and inter-batch variation coefficients were both less than 5% ($n=5$). The ethanol recovery test demonstrated that the proposed biosensor offered a remarkable and accurate method for ethanol detection in the real blood samples.

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## 1. Introduction

The accurate determination of ethanol in complex specimens is very important in clinical and forensic medical [1]. Additionally, this is also a significant determinant in controlling the fermentation process and product quality in the beverage, food and other industries [2]. In the past several decades, numerous methodologies have been developed for such an analysis, including refractometry [3], liquid chromatography [4], gas chromatography [5] and spectrophotometer [6]. Recently, an electrochemiluminescence ethanol biosensor was also developed [7]. In spite of these the methodologies could accurately quantify the ethanol from complex samples, however, the characteristics, such as the time consuming process, complex to perform, laborious sample pre-treatment and expensive instrumentations [8], have, for the moment, become more difficult to meet the need of fast and *in-situ* measurements. The electrochemical biosensor has drawn tremendous attention with excellent properties, including a good sensitivity, easy adaptability for *in-situ* analysis, relatively inexpensive instrument [9] and less reagent consumption.

Actually, a great number of electrochemical biosensors based on alcohol oxidase (AOX) and alcohol dehydrogenase (ADH) for ethanol detection, have been reported in the past several decades [10–15]. ADH-based biosensor is superior to AOX-based compared with the natures of stability and specificity [16]. ADH catalyzes the conversion of ethanol to acetaldehyde in alkaline condition in the presence of cofactor NAD$^+$ (an oxidized form of nicotinamide adenine dinucleotide) and a reduced form of NADH generated, which would be detected on the electrode system. Considering constructing a reagent-less biosensor for rapid and accurate determination of ethanol in the complex samples, several major problems should be overcome: (1) the large overpotential of NADH on the traditional bare electrodes leads to the electrode surface fouling and ultimately results in an electrode system lacking sensitivity and stability [17]; (2) at the large overpotential, the electroactive molecules coexisting with NADH, such as ascorbic acid (AA), uric acid (UA) and dopamine (DA), are also readily oxidized, causing a very poor specificity of the

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biosensor [18]; (3) the leakage of $NAD^+$ modified on the columnar electrode renders the biosensor unstable when the measurement was performed. Considerable efforts have been devoted to synthesizing novel nanomaterials to decrease the electro-oxidation overpotential as well as to increase the sensitivity of the biosensor toward NADH in the past several decades [19,20]. Nonetheless, the potential is not decreased enough and the interference of AA is still existing [21,22]. Additionally, cofactor $NAD^+$ is used as a reagent mixed with samples [13,23,24]. Recently, several novel strategies and materials are also developed to overcome these problems in constructing a reagent-less dehydrogenase-based biosensor [9,25–27]. ADH and $NAD^+$ were immobilized on the Nafion/MWCNTs/Au NPs/Meldola's blue (MDB) composite film in our previous research and the high stability of the proposed biosensor was obtained [1]. These above studies present an outstanding performance for the real sample analysis. However, the fabrication of dehydrogenase-based biosensor often includes several step-by-step procedures for surface immobilization of enzyme and $NAD^+$ and electrocatalysts, which were technically complicated and time-consuming [28,29]. Multiple steps for biosensor fabrication inevitably make it difficult to minimize the biosensor-to-biosensor deviation [9]. Consequently, more efforts should be made to simplify the fabrication procedures of dehydrogenase-based biosensor.

The past few years have seen an explosion in the use of ordered mesoporous carbons (OMCs) to design novel biosensors [30–32]. This kind of material offers attractive features that can be exploited in electrochemistry, such as good electronic conductivity, high specific surface area, large pore volume and size, and wide open ordered structure [31]. Several studies have pointed out that OMCs can be used for adsorption of dyes [33,34], implying that the OMCs have great potential in developing mediator-based biosensor. Graphitized mesoporous carbons (GMCs), possessing the features of structural homogeneity with significant graphite-like domains and stacking heights, have also been identified as a promising material for use in high-performance components in electrochemical applications [35].

In this study, a novel strategy was developed to immobilize MDB on the electrode through GMCs adsorption. Electrochemical characters of MDB/GMCs/CS film toward NADH were investigated. Furthermore, the nanobiocomposite, including ADH, $NAD^+$, MDB, GMCs, and CS, was prepared for one-step fabrication of reagent-less dehydrogenase-based biosensor. In this system, CS, with its cationic nature, remarkable film-forming property and excellent biocompatibility, not only acted as a dispersing agent for GMCs, but also as a binding factor for ADH immobilization. Screen printed electrode (SPE) has been widely used in electrochemical biosensing due to the inherent superiority of its manufacturing process, which is inexpensive, rapid and capable of mass production [36]. Three-electrode system SPE was employed to develop disposable dehydrogenase-based biosensor. The performances of the integrated biosensor toward ethanol were investigated. Additionally, stability of MDB in different conditions was also evaluated. Compared to the existing methods for fabrication of dehydrogenase-based biosensor, the strategy demonstrated here was facile and efficient. The ability of the biosensor for real sample analysis was also evaluated.

## 2. Experimental

### 2.1. Reagents

ADH (EC. 1.1.1.1, 390 U $mg^{-1}$ protein, from *Saccharomyces cerevisiae*) and Meldola's blue were purchased from Sigma. β-nicotinamide adenine dinucleotide trihydrate (oxidized form, $NAD^+$, >97%), β-nicotinamide adenine dinucleotide reduced dipotassium salt (reduced form, NADH, 92%), L-ascorbic acid (AA, >99%), uric acid (UA, >99%), dopamine hydrochloride (DA, >99%), Tris and chitosan (CS, deacetyiation, 90–95%) were obtained from BBI. Graphitized mesoporous carbons (GMCs, particle size <500 nm) were obtained from Aldrich without further purification. All other reagents were of analytical grade and used as received. Water (resistivity, 18.2 MΩ) was purified using the Millipore-Q water purification system. The fresh and normal whole blood samples (EDTA-2K anticoagulation) utilized in this study were collected from the First Affiliated Hospital of Chongqing Medical University.

### 2.2. Apparatus and instrumentations

Amperometric and cyclic voltammetric measurements were performed on an electrochemical workstation (CHI660D, Chenhua Instrument Company of Shanghai, China). A disposable screen printed electrode (SPE, diameter, 3 mm; geometric area, 0.071 $cm^2$) consisting of a carbon working electrode, a carbon counter electrode and a silver pseudo-reference electrode was purchased from Delta-biotech (B1008153, China) and used as received. UV–vis absorbance spectroscopy was performed using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). All experiments were performed at room temperature (25 ± 2 °C) unless otherwise stated.

### 2.3. Preparation of NADH biosensors

2 mg GMCs were firstly dispersed in 1 ml CS (0.5% solution in 0.05 M HCl, the pH was adjusted to 6.0 using 1.0 M Tris aqueous solution) by ultrasonic for 15 min, then 10 μl 10 mM MDB aqueous solution was added and allowed to mix overnight at 4 °C. In control experiments, GMCs/CS and MDB/CS suspension were also prepared. 5 μl MDB/GMCs/CS, GMCs/CS and MDB/CS suspension were uniformly coated on the working electrode of the SPE and allowed to dry at 4 °C (approximately 6 h). In order to optimize the concentration of MDB, various volumes of MDB (1 μl, 4 μl, 7 μl, 10 μl and 13 μl) were added in the GMCs/CS suspension.

### 2.4. Preparation of nanobiocomposite and construction of ethanol biosensor

The following optimized procedures were used for the preparation of nanobiocomposite. MDB/GMCs/CS suspension was re-prepared with the previous procedures just doubling the concentration of GMCs, MDB and CS. 100 μl ADH (16 mg $ml^{-1}$ in Tris–HCl solution, 0.1 M, pH 6.0) and 100 μl $NAD^+$ (16 mM in Tris–HCl solution, 0.1 M, pH 6.0) were successively mixed with 200 μl re-prepared MDB/GMCs/CS suspension by gently shaking. After that, 5 μl nanobiocomposite was carefully coated on the naked SPE and allowed to dry at 4 °C in darkness (approximately 6 h).

### 2.5. Preparation of integrated biosensor for real sample analysis

In order to adjust the pH value of samples to 8.0 when measurements performed, a 10 μl drop of the Tris–HCl (0.1 M, pH 8.0) was placed onto the SPE near to (but not on) the working electrode and allowed to dry at room temperature. After nanobiocomposite was coated and dried, a hydrophilic membrane with a hole was placed over the ethanol biosensor with double-sided adhesive tape to control the volume of samples on the SPE [1] (shown in Scheme 1).

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