

Development of Dehydrogenase-Based Bioanode Using Poly (Phenosafranin) -Functionalized SWCNT Nanocomposites and its Application to Ethanol Biosensor

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Abstract: A New type of dehydrogenase-based amperometric ethanol biosensor was constructed using alcohol dehydrogenase (ADH) which was immobilized on the edge-plane pyrolytic graphite (EP-PG) electrode modified with poly(phenosafranin)-functionalized single-walled carbon nanotube (PPS-SWCNT). The PPS-SWCNT modified EPPG electrode was prepared by electropolymerization of phenosafranin on the EPPG electrode which was previously coated with SWCNT. The performance of the ADH/PPS-SWCNT/EPPG electrode was evaluated using cyclic voltammetry and amperometry in the presence of ethanol. The fabricated ethanol biosensor provided a reasonable sensitivity of $2.0 \mu\text{A} \cdot \text{cm}^{-2} \cdot \text{mmol}^{-1} \cdot \text{L}$ and a low detection limit ($36 \mu\text{mol} \cdot \text{L}^{-1}$) for the electrocatalytic oxidation of ethanol with a linear concentration dependence upto $\sim 1.0 \text{mmol} \cdot \text{L}^{-1}$ at a detection potential of 0.2 V.

Key words: phenosafranin; electropolymerization; NADH; SWCNT; electrocatalysis

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1 Introduction

The quantitative measurement of alcohol is very important for clinical and forensic purposes in the analyses of human breath^[1]. Ethanol is an important compound in medicine, biotechnology and the food industry, etc., and it is often monitored for toxicological and psychological effects^[2]. In certain industrial fields, such as fermentation and distillation, the ethanol concentration can reach toxic levels, causing inflammation of the nasal mucous membrane and conjunctiva, irritation of the skin and, at high levels, e-

ven alcohol poisoning^[3]. Some analytical methods have been developed during the years for the determination of ethanol. An amperometric enzyme-based biosensor is one of the best choices for biochemical analysis due to their good selectivity, sensitivity, rapid response, miniature size, and reproducible results^[4]. The good selectivity is attributed to the specific catalysis by the enzyme.

In most cases, NAD^+ (the oxidized form of β -nicotinamide adenine dinucleotide (NADH))-dependent enzyme-based biosensors require NADH as a reagent for their functioning. Electrode modification

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with carbon nanotubes (CNTs) has been shown to produce an electrocatalysis for NADH electrooxidation, thus allowing the preferable construction of dehydrogenase enzyme based electrochemical biosensors^[5-7]. Concerning enzyme biosensors based on CNTs-composite electrodes, they have been found to possess improved analytical performance as a consequence of the unique properties of CNTs with the well-known advantages of composite electrode designs such as simple renewability and low background current^[8]. Many recent studies have aimed at the functionalization of CNTs with various molecules using covalent^[9-10] or non-covalent bond formation approaches^[11-12] in order to obtain desired properties. The covalent functionalization is based on the formation of chemical bonds between CNTs and functional molecules, while the non-covalent functionalization is based on adsorption, wrapping, π -stacking, or hydrophobic/ π - π interactions, etc. Among the many different strategies to functionalize CNTs, electrochemical functionalization can provide an efficient, clean, and more versatile alternative^[13]. Fabrication of CNTs/conducting polymer composites has become of great interest since the CNTs can improve the electrical and mechanical properties of polymers. It has been demonstrated that the CNTs/conducting polymer composites possess the original properties of the individual components with a synergistic function^[14-16]. For example, single-walled carbon nanotubes (SWCNTs)/poly(3-octylthiophene) composites have been shown to have good photovoltaic properties^[17], and CNTs/polyaniline composites have been found to be a good material in batteries with a high-charge-discharge capacity, low-charge voltage and high-discharge voltage^[18]. So far, several conducting polymers such as polypyrrole, polyaniline, polyphenothiazine, polythiophene and their derivatives have been used to fabricate CNTs/polymer composites^[19-23]. However, there are limited studies on the CNTs/poly(phenazine) dye composites^[24], although poly(phenazine) dyes are widely used as redox mediators in the construction of electrochemical sensors and bio-

sensors^[25-28].

The present work aims at developing dehydrogenase-based ethanol biosensor by immobilizing alcohol dehydrogenase (ADH) on the surface of the poly(phenosafranin) (PPS)-functionalized SWCNT nanocomposite. Previously we have reported the electrocatalytic oxidation of NADH by the basal-plane pyrolytic graphite (BPPG) modified with PPS film prepared by electropolymerization of phenosafranin (PS) which is an *N*-substituted phenazine with a half-wave potential of -0.458 V at pH 7.0^[29]. We have also reported an excellent electrocatalysis of PPS-functionalized SWCNT modified edge-plane pyrolytic graphite (EPPG) electrode for the NADH oxidation^[28]. Here, by immobilizing ADH onto the PPS-SWCNT nanocomposite film, dehydrogenase-based ethanol (ADH/PPS-SWCNT/EPPG) biosensor was fabricated. The obtained results showed that the ADH and PPS-SWCNT nanocomposite-based bioanode possesses an efficient electrocatalytic activity for ethanol oxidation.

2 Experimental

2.1 Chemicals and Reagents

Single-walled carbon nanotube (SWCNT) with a diameter = 1.2 ~ 1.5 nm and a length = 2 ~ 5 μm were purchased from Aldrich (Tokyo, Japan). Phenosafranin (PS) was purchased from Acros Organics (Geel, Belgium). Oxidized form of β -nicotinamide adenine dinucleotide (NAD^+ , Oriental Yeast Co. Ltd., Tokyo, Japan) was used as received. Alcohol dehydrogenase (ADH) from Baker's yeast, dehydrated ethanol and 25% glutaraldehyde (GA) solution in water were purchased from Wako (Japan). Bovine serum albumin (BSA) was purchased from Sigma (USA). Tris-HCl buffer solution (TBS; 0.05 mol \cdot L⁻¹, pH 8.2) was used as the supporting electrolyte for electrochemical experiments. The solutions throughout this work were always prepared using deionized water from a Milli-Q water system (Millipore, Japan). Edge-plane pyrolytic graphite (EPPG) (Bioanalytical Systems Inc. (BAS); 3 mm in diameter) disk was used as the working electrode. All of the other chemicals were of analytical grade and were

used without further purification.

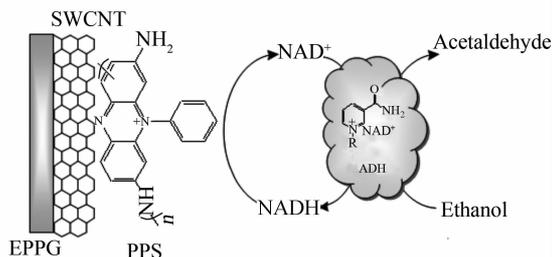
2. 2 Apparatus and Electrochemical Measurements

Cyclic voltammetric and amperometric measurements were carried out with an ALS CHI 750Cz electrochemical analyzer (Eco Chemie, Ultecht, The Netherlands) using a conventional two-compartment three-electrode system with the prepared bioanodes as working electrodes, a spiral Pt wire as counter electrode and a Ag|AgCl|KCl(sat.) as reference electrode. The amperometric measurements of the ethanol sensor fabricated were performed in $0.1 \text{ mol} \cdot \text{L}^{-1}$ PBS, stirred at ca. 300 r/min using a magnetic stirrer. A personal computer was used for data storage and processing. Electrolyte solutions were de-aerated by bubbling Ar gas (99.99%) for at least 30 min prior to electrochemical measurements. All the measurements were carried out at room temperature ($25 \pm 1 \text{ }^\circ\text{C}$).

2. 3 Electrode Preparation and Enzyme Immobilization

The EPPG electrodes were first carefully polished with emery paper and then transferred to $0.1 \text{ mol} \cdot \text{L}^{-1}$ H_2SO_4 solution and the potential scan was repeated between -0.2 and 1.4 V at $100 \text{ mV} \cdot \text{s}^{-1}$ until a stable cyclic voltammetric response was attained. 1 mg of the SWCNT was dispersed in 1 mL DMF to form a 1 mg/mL SWCNT suspension solution with the aid of ultrasonic agitation. The SWCNT modified EPPG (SWCNT/EPPG) electrode was prepared by casting 30 μL of the SWCNT suspension on the EPPG electrode surface and air-drying the casted suspension solution. A film of PPS was prepared by immersing the SWCNT/EPPG electrode in $0.2 \text{ mol} \cdot \text{L}^{-1}$ H_2SO_4 solution containing $0.5 \text{ mmol} \cdot \text{L}^{-1}$ PS for 10 min and then by repeating the potential scan between -0.5 and 1.3 V at $50 \text{ mV} \cdot \text{s}^{-1}$. The thus-fabricated PPS-SWCNT modified EPPG electrode is hereinafter abbreviated as PPS-SWCNT/EPPG electrode. Finally, alcohol dehydrogenase (ADH) was immobilized by a cross-linking reaction using glutaraldehyde (GA) in bovine serum albumin (BSA) ma-

trices. For attaching the enzyme layer, typically 4.5 μL of ADH-BSA-GA mixture (2 μL of 20 mg/mL ADH solution in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS, pH 8.2 + 1 μL 1% (by mass) BSA solution in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS, pH 8.2 + 1.5 μL 1% (by mass) GA (in water) was put on the PPS-SWCNT/EPPG electrode and air-dried at room temperature for at least 1 h. The electrode assembly fabricated (named as ADH/PPS-SWCNT/EPPG electrode) is schematically shown in Scheme 1.



Scheme 1 Scheme of the ADH-based composite electrode for the electrocatalytic oxidation of ethanol

3 Results and Discussion

3. 1 Electrocatalytic Characterization of the ADH/PPS-SWCNT/EPPG Electrode for Ethanol Oxidation

The response of the ADH/PPS-SWCNT/EPPG biosensor was electrochemically tested by cyclic voltammetry in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) containing $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ in the absence (----) and presence (—) of $20 \text{ mmol} \cdot \text{L}^{-1}$ ethanol (Fig. 1). No electrocatalytic response was observed at the ADH/PPS-SWCNT/EPPG electrode in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) containing $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ at a scan rate of a $5 \text{ mV} \cdot \text{s}^{-1}$ in the potential range of interest. Upon addition of $20 \text{ mmol} \cdot \text{L}^{-1}$ of ethanol, the cyclic voltammetric response was characterized by a large anodic peak with a decrease in the reduction current. The onset potential of ca. -0.2 V vs. Ag|AgCl|KCl(sat.) shows a reasonable relevance to that obtained for NADH oxidation at the PPS-SWCNT nanocomposite modified electrode. The result of NADH oxidation is shown in the inset of Fig. 1. Upon the addition of NADH, a dramatic enhancement in

the anodic peak current is observed which comes from the mediated oxidation of NADH to NAD^+ . These results confirmed the efficient electrocatalytic property of the biocomposite film electrode which is associated with the oxidation of ethanol via ADH catalysis. ADH catalyzes, in the presence of NAD^+ as a coenzyme, the oxidation of ethanol to acetaldehyde and simultaneously, NAD^+ is reduced to NADH. Thus, the anodic peak (solid line) in Fig. 1 corresponds to the electrooxidation of NADH.

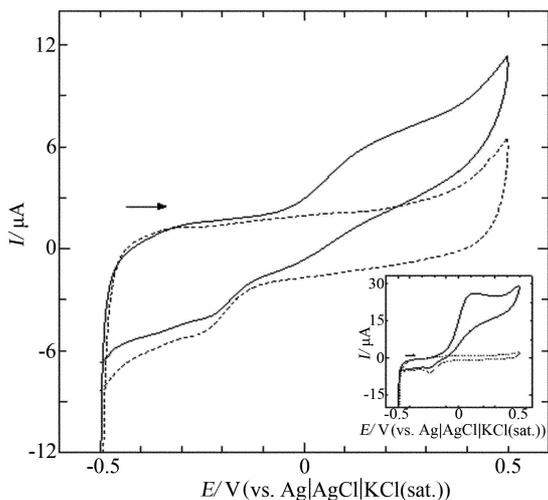


Fig. 1 CVs obtained at the ADH/PPS-SWCNT/EPPG electrode in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) containing $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ in the presence (—) and absence (---) of $20 \text{ mmol} \cdot \text{L}^{-1}$ ethanol

inset shows the voltammetric responses of $5 \text{ mmol} \cdot \text{L}^{-1}$ NADH at the bare EPPG (---) and PPS-SWCNT/EPPG (—) electrodes in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2)

scan rate: $5 \text{ mV} \cdot \text{s}^{-1}$

3.2 Optimization of Electrocatalysis for Ethanol Oxidation

The performance of the ADH/PPS-SWCNT/EPPG electrode towards the ethanol oxidation was found to be depended on the loading amounts of SWCNT and ADH, the NAD^+ concentration and pH (Fig. 2). As shown in Fig. 2a, the electrocatalytic current increases with the increasing loading amount of SWCNT and reaches a maximum value at $20 \mu\text{L}$ of SWCNT. When the loading amount of SWCNT is higher

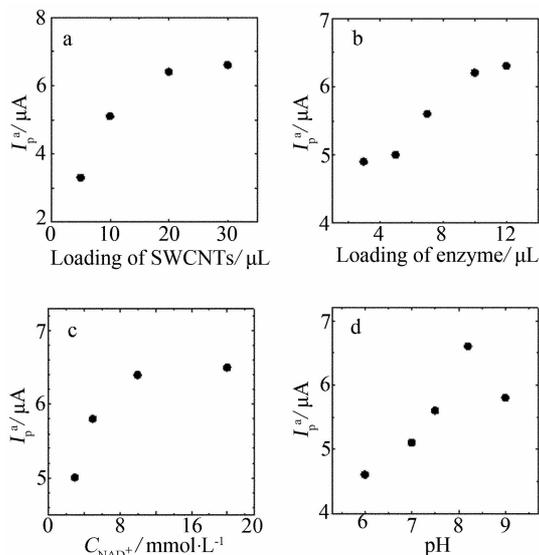


Fig. 2 Effects of experimental parameters on the electrocatalytic response of the ADH/PPS-SWCNT/EPPG electrode for the ethanol oxidation in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) in the presence of $20 \text{ mmol} \cdot \text{L}^{-1}$ ethanol

a. SWCNT loading, b. ADH loading, c. NAD^+ concentration, d. pH in above cases, the anodic peak current (I_p^a) values were estimated at $5 \text{ mV} \cdot \text{s}^{-1}$

than $20 \mu\text{L}$, the response remains same which means that this amount is effectively enough to observe a sufficient response for the electrocatalysis of ethanol.

The magnitude of the current response of the amperometric ethanol biosensor depends mainly on the enzyme kinetics which determines the rate at which NADH is generated enzymatically within the ADH/PPS-SWCNT biocomposite and the electrochemical reaction kinetics which controls the rate at which NADH can be converted to NAD^+ to generate the measured electric signal within the ADH/PPS-SWCNT biocomposite. Fig. 2b shows the effect of the amount of enzyme dropped on the electrode surface on the voltammetric response of the enzyme electrode. With increasing the loading of the ADH-BSA-GA mixture, the catalytic peak current increases and the current reaches an almost constant value at the loading of more than $10 \mu\text{L}$, indicating the presence of an adequate amount of enzyme in the PPS-SWCNT modified electrode. Thus $10 \mu\text{L}$ of the ADH-BSA-GA mixture was used for preparation of the ethanol biosensor.

The NAD^+ concentration in the ethanol solution is another important parameter for ethanol detection. The effect of the NAD^+ cofactor concentration on the response of the biosensor for $20 \text{ mmol} \cdot \text{L}^{-1}$ ethanol was investigated using the above-optimized enzyme loading. The NAD^+ concentrations in the solution ranged between 3 and $20 \text{ mmol} \cdot \text{L}^{-1}$, and the obtained results are displayed in Fig. 2c. The signal increases with the increase in the concentration of NAD^+ up to $10 \text{ mmol} \cdot \text{L}^{-1}$ and remains practically constant at the higher concentration.

As the enzyme activity is dependent upon the pH value of a buffer solution, the effect of the solution pH of $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS containing $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ on the current response of $20 \text{ mmol} \cdot \text{L}^{-1}$ ethanol at the ADH/PPS-SWCNT biocomposite film modified EPPG electrode was examined. A plot of the current response against pH is shown in Fig. 2d. The current response increases with increasing the pH value in the range of 6.0 to 8.2 , and reaches a maximum value at pH 8.2 . It decreases dramatically with an increase in pH value above 8.2 . Therefore, a solution of pH 8.2 and $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS were selected for examining the sensitivity of the ethanol biosensor.

3.3 Amperometric Determination of Ethanol

Based on the good electrocatalysis of the ADH/PPS-SWCNT/EPPG electrode for the ethanol oxidation, its amperometric response as ethanol sensor was examined in a stirred $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) (at 300 r/min). After stabilization of the baseline current, ethanol was injected into the buffer solution. ADH catalyzes the oxidation of ethanol and simultaneously the cofactor NAD^+ gets reduced to NADH according to the following enzymatic reaction.



According to this reaction, the signal resulting from the NADH oxidation increases with increasing the concentration of ethanol. Fig. 3 shows the current-time response to the successive addition of $250 \mu\text{mol}$

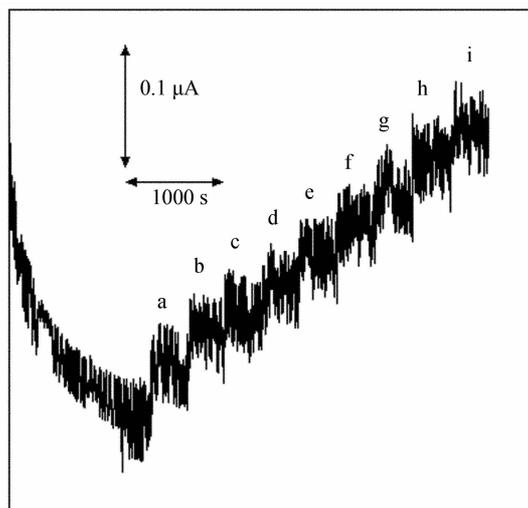


Fig. 3 Current ~ time response of the ADH/PPS-SWCNT/EPPG electrode for the successive addition of $250 \mu\text{mol} \cdot \text{L}^{-1}$ ethanol at 0.2 V in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) containing $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ $C_{\text{ethanol}}/\mu\text{mol} \cdot \text{L}^{-1}$: a. 250 , b. 500 , c. 750 , d. 1000 , e. 1250 , f. 1500 , g. 1750 , h. 2000 , i. 2250
the solution was stirred with a magnetic stirrer at 300 r/min

$\cdot \text{L}^{-1}$ ethanol to the solution. In this case, the electrode potential was kept at 0.2 V vs. $\text{Ag}|\text{AgCl}|\text{KCl}$ (sat.). Upon addition of an aliquot of ethanol, the current increased steeply to a stable value within 5 s , demonstrating that the electrocatalytic response is very fast. The plot of the current vs. ethanol concentration gave a good calibration graph as shown in Fig. 4. The linear response range is upto $\sim 1.0 \text{ mmol} \cdot \text{L}^{-1}$ which is found to be wider than the literature value ($10 \sim 425 \mu\text{mol} \cdot \text{L}^{-1}$)^[30]. From the slope of the linear portion, the sensitivity was calculated to be $2.0 \mu\text{A} \cdot \text{cm}^{-2} \cdot \text{mmol}^{-1} \cdot \text{L}$ and the limit of detection (LOD) was estimated as $36 \mu\text{mol} \cdot \text{L}^{-1}$. The LOD is lower than that obtained by immobilizing ADH on Au nanoparticles ($49 \mu\text{mol} \cdot \text{L}^{-1}$)^[31] and is much lower than $0.1 \text{ mmol} \cdot \text{L}^{-1}$ and $90 \mu\text{mol} \cdot \text{L}^{-1}$ reported for the sensors based on injection of the recognition element^[32] and the immobilization of ADH on SWCNT via polyelectrolyte of poly (dimethyldiallylammonium chloride)^[33], respectively.

The operational stability of the ADH/PPS-SWCNT/EPPG sensor was also examined by a continuous

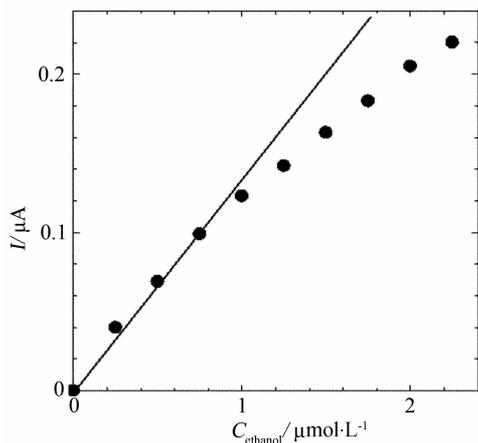


Fig. 4 The plot of the steady-state current against ethanol concentration using the data obtained from Fig. 3

measurement of $1.0 \text{ mmol} \cdot \text{L}^{-1}$ ethanol at the applied potential of 0.2 V over a period of ca. 45 min. Fig. 5 shows a considerably stable amperometric response with a less than 7% signal drift demonstrating a considerable resistance against the so-called electrode fouling. The observed stability of the PPS-SWCNT-based biosensor may be probably due to the surface coating of the three-dimensional network of SWCNT by the PPS which prevents the SWCNT from the direct adsorption of ethanol and its oxidation product,

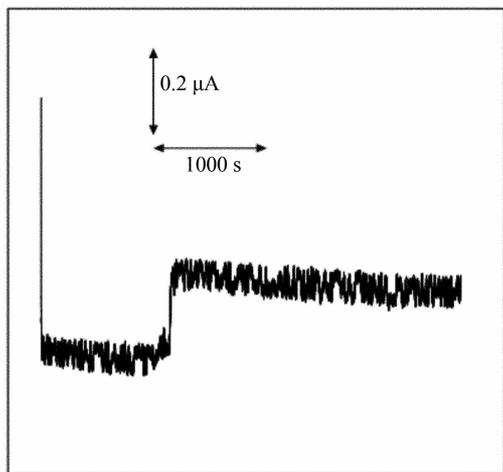


Fig. 5 A continuous recording of the current response of the ADH/PPS-SWCNT/EPPG electrode in $0.05 \text{ mol} \cdot \text{L}^{-1}$ TBS (pH 8.2) containing $10 \text{ mmol} \cdot \text{L}^{-1}$ NAD^+ and $1.0 \text{ mmol} \cdot \text{L}^{-1}$ ethanol at an operating potential of 0.2 V other experimental conditions are the same as in Fig. 3

i. e. , the electrode fouling. In addition, a large surface area of the SWCNT allows a large amount of ADH to be immobilized within the carbon nanotubes assembly, resulting in a CNTs-based enzyme reservoir.

4 Conclusions

A new kind of ethanol sensor based on the dehydrogenase enzyme immobilized onto PPS-functionalized SWCNT nanocomposite modified EPPG electrode was successfully constructed. The fabricated ethanol biosensor provided a reasonable sensitivity of $2.0 \mu\text{A} \cdot \text{cm}^{-2} \cdot \text{mmol}^{-1} \cdot \text{L}$ and a low detection limit ($36 \mu\text{mol} \cdot \text{L}^{-1}$) with a linear concentration dependence upto $\sim 1.0 \text{ mmol} \cdot \text{L}^{-1}$ at a detection potential of 0.2 V .

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聚酚藏花红功能碳纳米管生物阳极制备及其在乙醇传感器应用

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摘要: 应用电化学法聚合酚藏花红(PPS)功能化的单壁碳纳米管, 以其作为烟酰胺辅酶(NADH)氧化的电化学催化剂(电极), 构建基于乙醇脱氢酶的安培型乙醇生物电化学传感器. 该电极于 0.0 V 时, 对 NADH 具有很好的催化性能. 而单体酚藏花红则由于其电位过低(-0.48 V), 不能显示催化性能. 循环伏安和计时安培法测试表明: 该传感器的碳纳米管的载量, 固定化酶的量, NAD⁺ 的浓度以及溶液的 pH 都能直接影响它的性能. 经优化制备的乙醇传感器在 0.2 V 电位下, 对乙醇响应的灵敏度为 $2.0 \mu\text{A} \cdot \text{cm}^{-2} \cdot \text{mM}^{-1}$, 检测限为 $36 \mu\text{mol} \cdot \text{L}^{-1}$. 表现出很好的稳定性, 连续测定 45 min 后, 响应电流下降仅为起始值的 7%. 本文的研究为电化学生物传感器的创新开发提供了新的思路.

关键词: 酚藏花红(PS); 电化学聚合; 烟酰胺辅酶(NADH); 单壁碳纳米管(SWCNT); 电催化