Full Paper

Detection of NADH and Ethanol at Titanium Containing MCM-41 with Low Overpotential

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Abstract
Titanium-containing MCM-41 (Ti-MCM-41) modified glassy carbon electrode (GCE) can exhibit an excellent electrocatalytic activity towards the oxidation of β-Nicotinamide adenine dinucleotide (NADH). A dramatic decrease in the overvoltage of NADH oxidation reaction is observed at 0.28 V vs. SCE. The application in the amperometric biosensing of ethanol using alcohol dehydrogenase enzyme (ADH) also has been demonstrated with this material. The proposed sensor shows a highly sensitivity, an acceptable reproducibility and a good stability. The linear range of ethanol is 25 – 1000 μM and the detection limit is 8.0 μM. Ti-MCM-41 modified electrode not only can be used to detect the concentration of NADH in biochemical reaction, but also as the potential matrix for the construction of dehydrogenases sensor.

Keywords: NADH, Ti-MCM-41, Electrocatalytic, Sensors, ADH, Ethanol

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1. Introduction

β-Nicotinamide adenine dinucleotide (NADH) is involved as a cofactor in several hundred enzymatic reactions of NAD^+/NADH-dependent dehydrogenases. The dehydrogenase enzymes catalyze the oxidation of a variety of families, such as alcohol, lactate, glucose, aldehyde, and carbohydrate, that are of immense interest from the analytical point of view. The electrochemical oxidation of NADH has been the subject of numerous studies related to the development of amperometric biosensors [1, 2]. Problems inherent to such anodic detection are the large overvoltage encountered for NADH oxidation at ordinary electrodes [3], which limits the selectivity of the determination in a real sample [4]. Furthermore, the reaction at a high overpotential involves radical intermediates that cause electrode fouling [5] and the loss of analytical sensitivity, reproducibility, and operational lifetime [6]. Consequently, considerable effort has been devoted toward the goal of identifying new electrode materials and new methods that will reduce the overpotential for NADH oxidation and minimize surface passivation effects [7, 8]. More traditional methodology incorporates the use of mediators to catalyze the NADH reaction. Several mediators have been studied to date including *ortho*- and *para*-quinones, phenylenediamines, phenoxazines, alkylphenazines, phenothiazines, neutral red and phenothiazine dyes [9 – 13]. But the fast electron transfer between the electrode and the mediators and between the mediators and NADH and the stability and toxicity of the mediators limit their in vivo applications. Furthermore, some new materials such as carbon nanotube [7], carbon fiber [14, 15] and nanotube chitosan [16] et al. and some immobilized methods [17, 18] have been used to decrease the overvoltage of NADH and used to detect NADH concentration, which promote the biosensor development greatly.

Recently, the unique structural and catalytic properties of mesoporous molecular sieves for structuring an electrochemical/electron transfer environment and resistance to biodegradation have attracted considerable attention [19]. Mesoporous molecular sieves have a large specific surface area, high mechanical, thermal, and chemical stability, good adsorption and penetrability. With the appropriate dimensions and functionalizations, it can act as current nanocollectors and as electron relays to an electrode [20] which brings new capabilities to electrochemical devices and has many potential sensing applications [21 – 23]. However, to the best of our knowledge, up to now, there is no report on reducing the overpotential for NADH oxidation on mesoporous materials except for our preliminary communication [24]. Here we report the oxidation of NADH at glassy carbon electrodes (GCE) modified with titanium-containing MCM-41 (Ti-MCM-41) coating and the application in NADH biosensor. Ti-MCM-41 modified GCE offers a marked decrease in the overvoltage for NADH oxidation reaction (compared to bare GCE) to be observed at 0.28 V (vs. SCE; pH 6.2) and to circumvent NADH surface fouling effects. Subsequently, the modified electrode is used to sense

ethanol using alcohol dehydrogenase (ADH) enzyme and NAD^+. The sensor exhibits a good electrocatalytic behavior and a good stability to NADH and ethanol.

2. Experimental

2.1. Materials and Reagents

NADH, ADH, and NAD^+ were purchased from Sigma and used as received. Polyvinyl alcohol (PVA) (average degree of polymerization, 1800 ± 100) was purchased from Shanghai Laize Factory of Fine Chemicals. Tetrabutyl titanate was purchased from Shanghai Reagent Factory. All other chemicals were of analytical grade and were used without further purification. All solutions were made up with doubly distilled water. The solution of NADH was prepared freshly before each experiment. Phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with H_3PO_4 or NaOH.

2.2. Electrode Modification

Ti-MCM-41 was prepared following a recipe similar to that reported by Tatsumi [25]. 30 mg Ti-MCM-41 was dispersed into 10 mL doubly distilled water and stirred for 3 hours to obtain a 3 mg/mL Ti-MCM-41 suspension. The obtained suspension of 100 μL was then mixed with 5 μL 3% PVA solution of ethanol/water ($V:V$ 1:1) to produce Ti-MCM-41/PVA colloid that was used for the following work.

The GCE (3 mm in diameter) were polished to a mirror-like finish with 1.0, 0.3 and 0.05 μm alumina slurry (Bühler) followed by rinsing thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then allowed to dry at room temperature. 3 μL Ti-MCM-41/PVA colloid was dropped on the pretreated GCE surface and allowed to dry under ambient condition for 3 hours. After the modified electrode was rinsed with doubly distilled water twice or thrice, Ti-MCM-41 modified GCE was obtained. When not in use the electrode was stored in 0.1 M pH 6.2 PBS at 4 °C.

2.3. Apparatus and Measurements

UV-visible spectra were recorded on a Hitachi 340 spectrometer. Nitrogen adsorption measurements were performed on a Belsorp 28SA analyzer. Energy dispersive spectroscopy (EDS) was performed on Thermo Noran analyzer. Scanning electron micrograph (SEM) was obtained using a JEOL JSM-5610LV Scanning Electron Microscope. Cyclic voltammetric and amperometric measurements were performed on CHI 660 electrochemical workstation (CH Instruments, USA). All electrochemical experiments were carried out in a cell containing 5.0 mL 0.1 M pH 6.2 PBS and using a platinum wire as auxiliary, a saturated calomel electrode as reference and the Ti-MCM-41 modified GCE as working electrodes. Two pumps of Luminescence Analyzer (IFFM-D) were used for flow injection analysis (FIA) to deliver flow streams. The flow rate was optimized at 1.20 mL/min. In amperometric measurements, ADH (2 mg) and NAD^+ (4 mg) were dissolved in 0.1 M pH 6.2 PBS, prior the addition of the ethanol. After the background current reached to a steady state value, a successive addition of ethanol was added to a continuously stirred PBS. The sensor responses were measured as the difference between total and residual currents. All experiments were carried out at (25 ± 2 °C).

3. Results and Discussion

3.1. The Characterization of Ti-MCM-41 and Ti-MCM-41 Modified Electrode

The characterization of the prepared Ti-MCM-41 has been done with UV-visible spectra, EDS and N_2 adsorption isotherms. In the UV-visible spectra of Ti-MCM-41, the band at 220 nm which has been assigned to Ti in tetrahedral coordination [26] is observed indicating Ti is incorporated into the framework of MCM-41 (Fig. 1A). N_2 adsorption isotherm shows the mesopore diameter is about 2.81 nm and the specific surface area is about 1050 m^2/g (Fig. 1B). EDS shows that the atom ratio of Si to Ti is about 1:0.01. The parameters of Ti-MCM-41 are listed in Table 1. In order to know how the distribution of Ti in the material, TEM

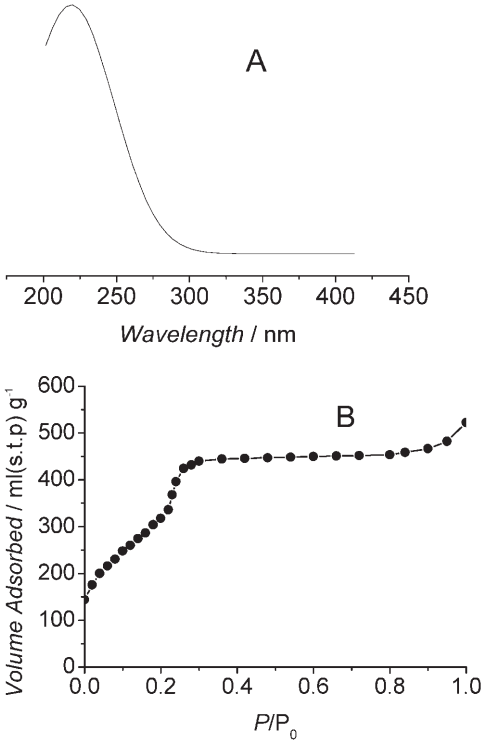

Fig. 1. UV spectrum (A) and adsorption isotherm of N_2 at 77 K (B) of Ti-MCM-41.

Table 1. Pore Characterization of Ti-MCM-41. A_{BET}, specific surface area; d_{100}, XRDd_{100} space; a_0, lattice parameter; D, mesopore diameter; L, wall thickness; Si:Ti, the atom ratio of Si to Ti.

A_{BET} (m^2/g)	d_{100} (nm)	a_0 (nm)	D (nm)	L (nm)	Si : Ti
1050	3.84	4.43	2.81	1.62	1 : 0.01

analysis has been done. However, it is difficult to distinguish Si and Ti because there is virtually no difference in TEM and this work needs further study.

The morphologies of Ti-MCM-41 modified electrode surface was characterized by SEM. The aggregates of the Ti-MCM-41 matrix on modified electrode surface displayed a chemically clean uniform structure and were well distributed while bare PVA film did not display any distinct feature.

3.2. Electrochemical Oxidation of NADH at a Ti-MCM-41 Modified Electrode

The main objective of the present investigation was whether Ti-MCM-41 modified GCE improved the oxidation of NADH. It was found that no peak was observed at a bare GCE. Upon addition of NADH, the oxidation resulted in a peak with the potential of + 0.70 V (Figures not shown). No peak was observed at Ti-MCM-41 modified GCE without the addition of NADH (curve a in Fig. 2), which showed Ti-MCM-41 was electroinactive in the potential window. Upon addition of NADH, Ti-MCM-41 modified GCE exhibited a dramatic enhancement in the anodic peak current at 0.28 V (curve b in Fig. 2). The enhanced anodic current in the presence of NADH came from the oxidation of NADH to NAD^+ and the value of anodic peak potential of NADH

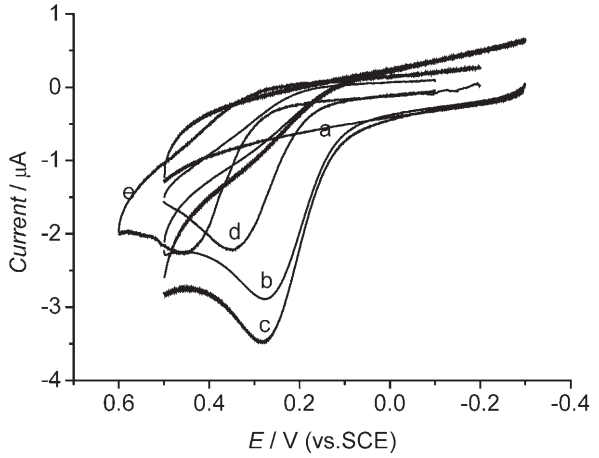

Fig. 2. Cyclic voltammograms of Ti-MCM-41 modified GCE in the absence (a), the presence of 0.10 (b), 0.15 mM (c) NADH and amorphous silica gels doped with TiO_2 (d) and MCM-41 (e) modified GCEs in the presence of 0.10 mM NADH in 0.1 M pH 6.2 PBS at 0.050 V/s.

oxidation of 0.28 V obtained at present case was more negative than 0.33 and 0.36 V at multiwall CNTs and singlewall CNTs modified GCE, respectively [7]. These results indicated that Ti-MCM-41 was more effective for electron transfer from NADH. From Figure 2, it was also found the magnitude of the catalytic current was proportional to the solution concentration of NADH, which covered the values of a great relevance in biosensor design and application.

The presence of regular porous structure has already showed its benefits on the analytical performance and has been used to improve NADH oxidation or to reduce the overpotential [27 – 29]. In the present case, the decrease of the potential of NADH oxidation might be related to the higher hydrophilicity induced by Ti-MCM-41 at the modified electrode. The hydration water of Ti-MCM-41 also proffered to the electrode surface a better contact with the solution containing NADH and consequently could participate in increasing the electron transfer rate. Furthermore, regular mesoporous structure has much larger specific surface area (1050 m^2/g) than amorphous silica gels (300 m^2/g) which leads to the increase of the signal response and can be expressed by the lowing of the overvoltage for NADH oxidation. For comparison, cyclic voltammograms of amorphous silica gels doped with TiO_2 with addition of NADH is shown in curve d Figure 2. The anodic potential of NADH oxidation is 0.35 V under the same experimental conditions which is the same value as that of amorphous silica gels doped with Nb_2O_5 [30].

The oxidation of NADH on MCM-41 modified GCE was also shown in curve e Figure 2. It was obviously that upon addition of NADH to PBS, the anodic peak also appeared, but it appeared at 0.45 V which was more positive than 0.28 V of Ti-MCM-41 modified GCE (curve b in Fig. 2). The substantial decrease in potential observed at Ti-MCM-41 was attributed to acceleration of the proton-transfer step by the metal oxide component of the composite [31].

The effect of scan rate on electrocatalytic properties of the Ti-MCM-41 modified GCE to NADH was also evaluated. At 0.01 – 0.20 V/s, the anodic peak currents were proportional to the square root of scan rate, $v^{1/2}$, indicating the currents were limited by the diffusion of NADH in solution.

The electrocatalytical properties of the Ti-MCM-41 modified GCE to NADH showed a strong dependence on solution pH. It was found that both the anodic peak potential and anodic peak current were pH-dependent. The anodic peak potential shifted to negative direction with the increasing of solution pH. Figure 3A shows plot of the anodic peak potential vs. pH (from 4.0 to 9.0). It produces a line with the slope of – 26.75 mV/pH which is close to the theoretical value of 59/2 mV/pH indicating the oxidation of NADH is a two-electron, one-proton reaction. Figure 3B shows the dependence of the anodic peak currents on the solution pH. It can be seen that the largest anodic current is obtained at pH 6.2. The anodic current increases with the increasing of the solution pH when the solution pH is lower than 6.2 and decreases when the solution pH is higher than

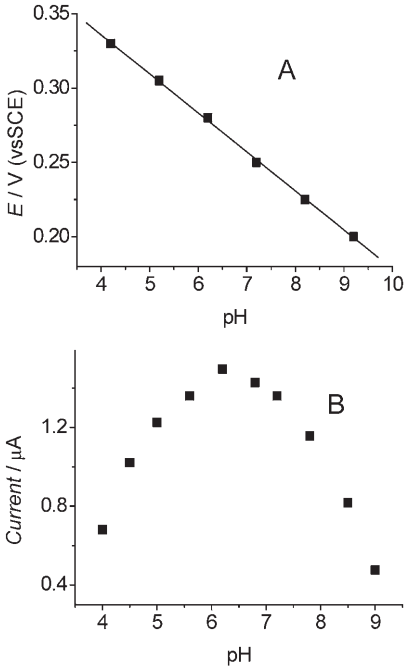

Fig. 3. Dependence of anodic peak potentials (A) and peak currents (B) for the oxidation of 0.10 mM NADH on the solution pH (0.1 M PBS) at Ti-MCM-41 modified GCE. Scan rate: 0.05 V/s.

6.2. Hence, the solution pH of 6.2 is used in the following experiments.

3.3. Flow Injection Analysis for NADH Determination

NADH determination was studied by establishing a FIA system with a thin-layer cell. An optimal flow rate of 1.2 mL/min was obtained by evaluating the analytical performance of the peak width and the measurement reproducibility of the sensor. Typical flow injection response for NADH at Ti-MCM-41 modified electrode with an applying potential of 0.28 V was shown in Figure 4. With an injection time of 10 s and a period of 30 s, the sensor gave a steady base-line. The linear response range of the sensor to NADH concentration was from 10 to 1200 μM with a correlation coefficient of 0.9992 (inset in Fig. 4). The linear range was wider than that of 3 – 50 μM from GCEs modified with transition metal complexes containing 1, 10-Phenanthroline-5, 6-dione ligands [32] and 10^{-4} – 10^{-2} M from enzyme modified by electropolymerization of aminobenzene isomer and PPQ on electrode [33]. The detection limit of 8.0 μM were obtained at a signal to noise ratio of 3 which was lower than 5×10^{-4} from electrode modified by PPQ [33] since the large specific surface area and high enzyme loading on the porous materials.

The influences of foreign species were investigated by analyzing a standard solution of 1.0 mM NADH, to which interfering species were added. 0.1 mM uric acid, 0.1 mM *p*-acetaminophenol did not cause any observable interference to the sensor response to glucose, and only ascorbic acid at

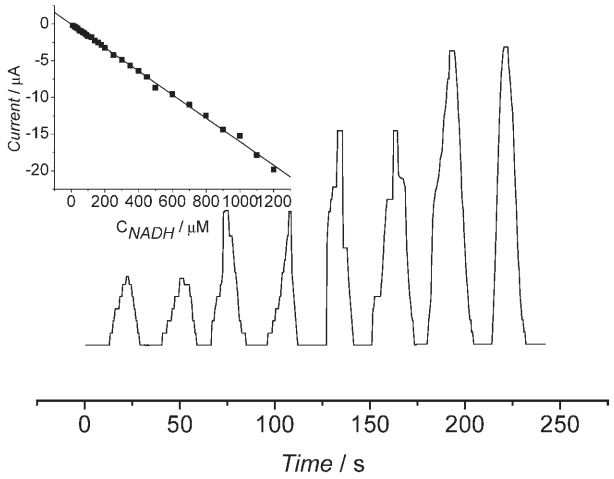

Fig. 4. FIA for 200, 400, 600 and 800 μM NADH at the enzyme electrode at 0.28 V. Carrier solutions: 0.1 M pH 6.2 PBS with a flow rate of 1.2 mL/min. Inset: Plot of electrocatalytic currents vs. NADH concentration.

the concentration of 0.1 mM produced the relative response of about 5.0%, indicating these species coexisting in the sample matrix did not affect the determination of glucose.

The effect of drying time (from dropping Ti-MCM-41/PVA colloid to the electrode surface to the electrode surface drying completely) on the operational stability of the Ti-MCM-41 modified GCE to NADH was evaluated. The drying time only needed about 20 minutes. The operational stabilities were examined by checking their relative response currents (the ratios of the catalytic currents detected at different drying times to the current at drying time for 3 h) in 0.1 M pH 6.2 PBS containing 0.1 mM NADH (Fig. 5). It was noted the operational stability of drying time for 20 minutes was slightly lower than that for 3 h, which indicated prolonging drying time increased the stability. But the sensor also exhibited a good performance by using drying time for 20 minutes.

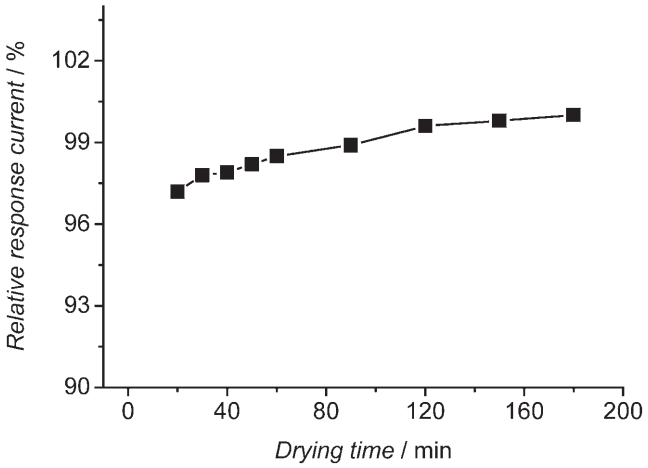

Fig. 5. Effect of drying time on the operational stability in 0.1 M pH 6.2 PBS containing 0.1 mM NADH at Ti-MCM-41 modified GCE. Scan rate: 0.05 V/s.

3.4. Amperometric Response and Calibration Curve for Ethanol

ADH enzyme catalyzes the oxidation of ethanol, and simultaneously the cofactor NAD^+ gets reduced to NADH. By sensing the amount of NADH produced in the system, it is possible to quantify ethanol. In the present studies, the Ti-MCM-41 modified electrode is used along with yeast ADH for the amperometric sensing of ethanol. The enzyme (2 mg) and NAD^+ (4 mg) were dissolved in the 0.1 M PBS (pH 6.2) prior to the addition of the analyte. Figure 6 shows the steady-state response for different additions of ethanol at a potential of 0.28 V. The response of the sensor is rather fast, and the enzyme electrode achieved 95% of the steady-state-current in less than 20 seconds which can be used as an efficient sensor for ethanol detection. The calibration curve of the sensor under the optimized experimental conditions was shown in inset in Figure 6. The calibration range of ethanol was done from 25 to 1200 μM and the linear response range of the sensor to ethanol concentration was from 25 to 1000 μM with a correlation coefficient of 0.9989. The detection limit of 10.0 μM were obtained at a signal to noise ratio of 3 which was lower than 0.1 mM with the SIRE biosensor P100 [34], 49 μM on Au nanoparticles [35] and 0.5 mM on mercaptopyrimidine and thiocytosine monolayer-modified electrodes [36].

3.5. The Stability and Reproducibility of Ethanol Sensor

The detection reproducibility of the ethanol sensor was studied by a FIA system with a thin-layer cell. The reproducibility was ascertained by monitoring the current response for ten replicate injections of 0.10 mM ethanol with an applying potential of 0.28 V. The relative standard deviation (*RSD*) was 4.2%, indicating a good reproducibility of the sensor for FIA. Thus the sensor could repeatedly be used for FIA or on-line determination of ethanol. The fabrication reproducibility of six electrodes, made independently, showed an acceptable reproducibility with a *RSD* of 7.2% for the current determined at 0.10 mM ethanol.

In addition to good reproducibility, Ti-MCM-41 imparts ethanol sensor a good long-term stability. The stability is quite good because it is not related to the activity of enzyme since ADH is added in solution and it can be applied in-situ determination.

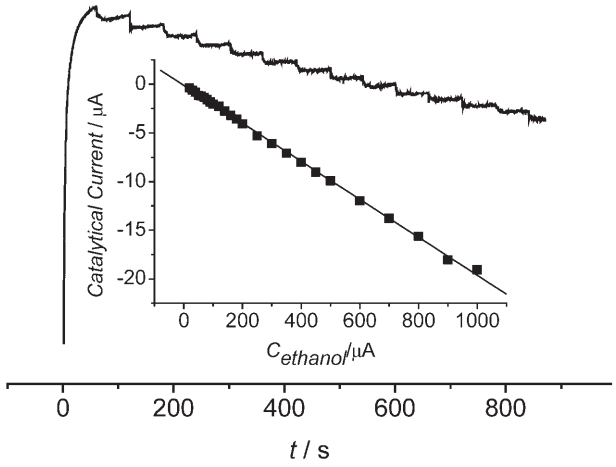

Fig. 6. Amperometric responses of Ti-MCM-41 modified GCE upon successive additions of 5 μL 0.1 M ethanol to 5.0 mL 0.1 M pH 6.2 PBS at 0.28 V. Inset: Plot of electrocatalytic currents vs. ethanol concentration.

4. Conclusions

In summary, we present a new material for NADH and ethanol detection. Ti-MCM-41 modified GCE can exhibit an excellent electrocatalytic activity towards the oxidation of NADH. It can reduce the overvoltage of NADH oxidation reaction dramatically and can circumvent NADH surface fouling effects. The new application described to sensor development has been demonstrated by the construction of a very simple ethanol sensor. The sensor exhibits a good performance with low cost, convenient preparation, and sensitive and reproducible detection to ethanol. Ti-MCM-41 modified electrode can be used in sensors to study the electrocatalytic reaction of biological important systems because the method of preparation is simple and the Ti-MCM-41 can present a good matrix for efficient redox reactions of biomolecules.

5. Acknowledgements

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