



Regeneration of the nicotinamide cofactor using a mediator-free electrochemical method with a tin oxide electrode

Yang Hee Kim, Young Je Yoo*

School of Chemical and Biological Engineering, Seoul National University, Seoul 151-744, Republic of Korea

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ABSTRACT

Tin (IV) oxide was made using an anodization and annealing method and was used as a working electrode in an electrochemical cofactor regeneration reaction. This material was formed with a large surface area, and by changing the preparation conditions, it was possible to control the morphology. Tin oxide has redox properties similar to those of frequently used mediators required for electron transfer between cofactors and an electrode. Therefore, by using tin oxide as a novel electrode, mediator-free electrochemical cofactor regeneration may be possible. Oxidation and reduction of the nicotinamide cofactors, NAD(P)H and NAD(P)⁺, were carried out under various reaction conditions. The results showed a high efficiency for oxidizing NADH over a broad range of pH and temperatures. The oxidation tendency of NADPH was also observed, and it demonstrated a similar reaction tendency as NADH. When using a tin oxide electrode, NAD⁺ was readily reduced to NADH, though the efficiency of this reaction was lower than for NADH oxidation. Oxidation of 2-propanol to acetone was used as a model system using alcohol dehydrogenase and the cofactor regeneration system suggested in this study. The electroenzymatic reaction showed efficient regeneration of NADP⁺ without a mediator.

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1. Introduction

Enzymes are very attractive catalysts for chemical production in industrial applications [1,2]. Among various kinds of enzymes, oxidoreductases are valuable enzymes that can synthesize many kinds of products used in pharmaceutical applications, as food additives, and so on [3–5]. These enzymes also have various applications including the construction of biosensors [6–8], degradation of pollutants [9], and fuel generation [10]. They often require a nicotinamide cofactor, such as NAD(H)⁺ or NAD(P)H, for their activity. However, these cofactors are too expensive to be used in stoichiometric amounts. The high cost of these cofactors is one of the bottlenecks that increases the processing cost in oxidoreductase reactions. Therefore, it is very important to efficiently regenerate these cofactors.

There are many methods for the regeneration of these cofactors, including enzymatic, chemical, electrochemical, and photochemical methods [11]. The enzymatic method is commonly used in lab-scale to pilot-scale reactions for chemical synthesis. To establish the reaction, a second enzyme and a co-substrate are used for cofactor regeneration. Various enzymes have been used to regener-

ate cofactors, such as formate dehydrogenase [12,13], NADH oxidase [14], alcohol dehydrogenase [15], phosphite dehydrogenase [16], glucose dehydrogenase [17], glutamate dehydrogenase [18], and so on. Among these enzymes, formate dehydrogenase is most frequently used. It demonstrates high regeneration efficiency and there is no need to separate out by-products because it produces only carbon dioxide as a by-product. Enzymes that participate in these reactions can also be used for the regeneration of cofactors in enzyme-coupled reactions. However, enzymatic cofactor regeneration has some disadvantages, such as high enzyme cost, enzyme instability, and restricted reaction conditions.

Electrochemical cofactor regeneration [19,20] is a simple and cost effective process even though it requires an electrochemical apparatus. Cheap electricity can be used instead of an enzyme for the cofactor regeneration. Also, no by-product is produced in this system, which facilitates the recovery of the desired product as it does not require a co-substrate. In many cases, mediators such as an artificial electron transferring agent were used in a conventional electrochemical cofactor regeneration system to allow for efficient electron transfer, to reduce overpotential, and to prevent electrode fouling [21–26]. In spite of these advantages, mediated electrochemical systems have some problems. For example, some kinds of mediators are known to be toxic to enzymes, resulting in enzyme deactivation. Furthermore, the stability of the mediator can affect the performance and stability of the entire reaction process.

* Corresponding author. Tel.: +82 2 880 7411; fax: +82 2 887 1659.
E-mail address: yjyoo@snu.ac.kr (Y.J. Yoo).

In this study, we present a new electrochemical cofactor regeneration method using metal oxide electrode which does not require an electron transferring agent, mediator. A novel electrode was prepared, characterized, and its performance was tested under various reaction conditions. Subsequently, it was applied to an enzyme reaction that required cofactor regeneration.

2. Materials and methods

2.1. Chemicals and reagents

High purity tin (99.9%) and aluminum (>99%) were purchased from Nilaco (Tokyo, Japan). All chemicals were purchased from Sigma–Aldrich (St. Louis, USA) or Junsei (Tokyo, Japan) and used without further purification. The aqueous solutions were prepared using deionized water from a Milli-Q water purification system (Millipore, France).

2.2. Preparation of electrode

To prepare the tin oxide electrode, anodization was performed using high purity tin as an anode. Aluminum was used as a cathode material. Prior to the anodization, tin and aluminum sheets were cleaned by sonication in trichloroethylene for 2 h. For the anodization, the reaction volume was 50 mL and the distance between the anode and cathode was 20 mm. A constant voltage was supplied to the tin sheet using a PowerPac HC™ power supply (BioRad, Hercules, USA) for 2 min at room temperature. Anodization was carried out in 0.5 M oxalic acid without stirring. Tin (II) oxide (SnO), which has a black color, could be obtained by anodization. Tin (IV) oxide (SnO₂) was prepared by the annealing of Tin (II) oxide (SnO) for 3 h at 500 °C [27].

2.3. Characterization of tin oxide electrode

To confirm whether the tin oxide was properly formed on the tin substrate, X'Pert PRO X-ray Diffractometry (XRD; PANalytical, Almelo, Netherlands) and FT-IR 200 Fourier Transform Infrared Spectroscopy (FT-IR; Jasco, Tokyo, Japan) were used. The surface morphologies of the prepared tin oxide were analyzed by JSM-6330F Field Emission Scanning Electron Microscope (FE-SEM; JEOL, Tokyo, Japan) and the surface area was measured using an ASAP 2010 Brunauer–Emmett–Teller apparatus (BET; Micromeritics, Norcross, USA).

Electroanalytical measurements were taken using the Autolab PGSTAT 302N potentiostat/galvanostat (Eco Chemie, Utrecht, Netherlands). This instrument was run by GPES (Eco Chemie). The cyclic voltammetry experiment was carried out in 20 mL of potassium phosphate buffer (pH 7.5, 100 mM) in a 25 mL reactor at room temperature. A conventional three-electrode system was used. Prepared tin oxide was used as a working electrode, platinum wire as a counter electrode, and RE-5B Ag/AgCl (Bioanalytical Systems Inc., W. Lafayette, USA) as a reference electrode.

2.4. Determination of cofactor concentration

The concentration of NAD(P)H was determined based upon the absorbance value at 340 nm using a UVIKON 930 UV–vis spectrophotometer (KONTRON Instrument, Zurich, Switzerland).

The concentrations of electrolyzed cofactors were also monitored via a YoungLin HPLC (Young Lin Instrument Co. Ltd., Anyang, Korea). The HPLC was utilized with a BAKERBOND™ (J.T. BAKER, Phillipsburg, USA) 4.6 mm × 250 mm RP column. The mobile phase was 96% 0.15 M citrate-phosphate buffer (pH 6.8) containing 1 mM EDTA and 4% methanol and measured by spectrophotometric detector at 260 nm [28]. The flow rate was 0.3 mL min⁻¹ at a temperature of 30 °C. Retention times were found to be 11.41 min and 12.67 min for NADH and NAD⁺, respectively.

2.5. Electrochemical oxidation of NAD(P)H and reduction of NAD⁺

Using tin oxide as a working electrode, oxidation of NAD(P)H was performed. The electrochemical oxidation of NAD(P)H was carried out in 100 mM potassium phosphate buffer at the given pH and temperature using a potentiostat. Platinum wire and Ag/AgCl were used as a counter and reference electrode, respectively. The reaction volume was 20 mL. The reaction mixture was stirred continuously and the reaction temperature was maintained at the given temperature.

2.6. Electroenzymatic oxidation of 2-propanol

Alcohol dehydrogenase from *Thermoanaerobium brockii* was used to oxidize 2-propanol to acetone in the presence of NADP⁺. The electroenzymatic oxidation of 2-propanol was carried out in a one compartment electrochemical reactor with 20 mL operation volume. Tin oxide was used as a working electrode. Platinum wire and Ag/AgCl were used as a counter and reference electrode, respectively. Oxidation potential (−0.5V vs. Ag/AgCl) was constantly applied for oxidation of

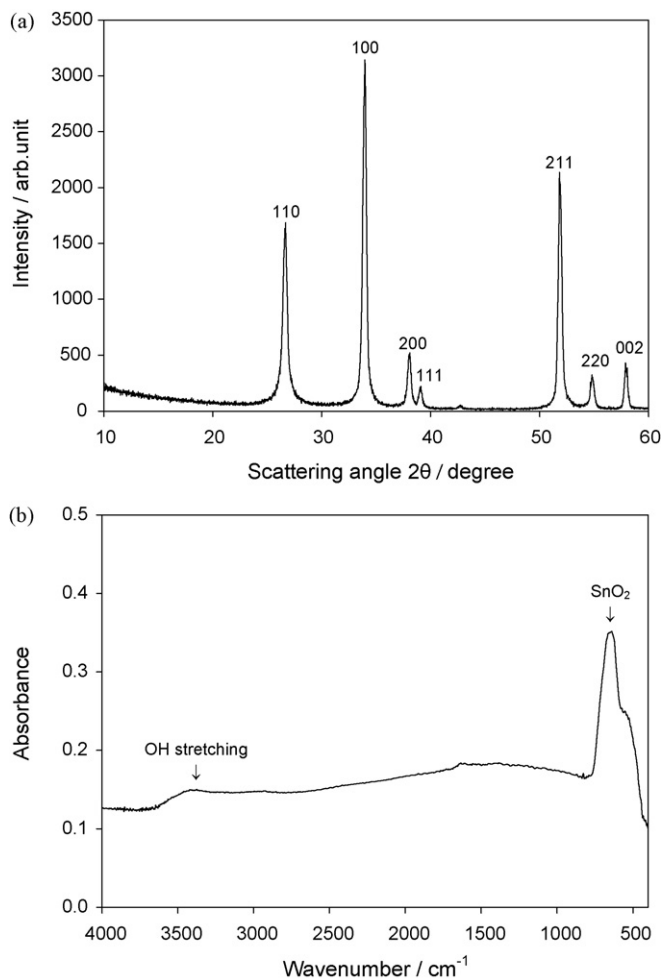


Fig. 1. Qualitative analysis of tin oxide: (a) XRD and (b) FT-IR.

2-propanol using a potentiostat. The electroenzymatic reaction was performed in a temperature-controlled oven and the mixture was gently stirred.

Samples of 2-propanol and acetone were analyzed by HP 5890 series II Gas Chromatography (Agilent, Palo Alto, USA) using a HP-INNOWAX (cross-linked PEG, 15 m × 0.25 mm × 0.5 μm) column with a flame ionization detector. The oven temperature was kept at 45 °C, and the temperature of the injector and detector were 250 °C and 275 °C, respectively. Helium was used as a carrier gas. The retention time of 2-propanol and acetone were 5.4 min and 2.7 min, respectively.

3. Results and discussions

3.1. Preparation and characterization of tin oxide

The new electrode material was prepared with a large surface area, was not toxic to the enzyme, and was readily used for the covalent immobilization of the enzyme. We chose metal oxide as an electrode material because it satisfied these requirements. Porous metal oxide electrodes can be easily prepared via several methods, and it has been shown that metal oxides are biocompatible with the enzyme [29,30]. Moreover, it is easy to immobilize enzymes on the metal oxide because of the presence of surface hydroxyl groups. Tin oxide was selected as an electrode material and was formed by anodization and annealing. Prepared tin oxide was observed by XRD and FT-IR. The sharp peak in Fig. 1(a) indicates prepared tin oxide. The prepared material showed the same peak position as tin oxide, as referenced in the database. Fig. 1(b) showed the spectrum of tin oxide (SnO₂) by FT-IR. It was observed near 400–800 cm⁻¹. This position is the same as in previously reported results [31]. Tin

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