Bioelectrochemistry 106 (2015) 335–342

Contents lists available at ScienceDirect

Bioelectrochemistry

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

Scaffold electrodes based on thioctic acid-capped gold nanoparticles coordinated Alcohol Dehydrogenase and Azure A films for high performance biosensor

C. Gómez-Anquela [a], T. García-Mendiola [a], José M. Abad [a], M. Pita [c], F. Pariente [a], E. Lorenzo [a,b,*]

[a] Department of Analytical Chemistry, Universidad Autónoma de Madrid, 28049, Spain
[b] IMDEA Nanociencia, Madrid, Spain
[c] Instituto de Catalisis y Petroleoquimica, CSIC. C/ Marie Curie, 2, L10., 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 28 March 2015
Received in revised form 19 June 2015
Accepted 21 June 2015
Available online 30 June 2015

Keywords:
Alcohol dehydrogenase
Biosensor
Gold nanoparticles
Electrografting

ABSTRACT

Nanometric size gold nanoparticles capped with thiotic acid are used to coordinate with the Zn (II) present in the catalytic center of Alcohol Dehydrogenase (ADH). In combination with the NADH oxidation molecular catalyst Azure A, electrografted onto carbon screen-printed electrodes, they are used as scaffold electrodes for the construction of a very efficient ethanol biosensor. The final biosensing device exhibits a highly efficient ethanol oxidation with low overpotential of −0.25 V besides a very good analytical performance with a detection limit of 0.14 ± 0.01 μM and a stable response for more than one month.

1. Introduction

Accurate and sensitive determination of ethanol in different samples is an important challenge in many diverse areas. In particular, in clinical and forensic chemistry the toxic effects caused by the presence of high levels of alcohol in blood result in several disorders that can lead to severe health damages, besides work and traffic accidents and generally loss of quality of life. On the other hand, in fermentation processes of wine, beer or in distillation of spirits control of ethanol concentration is essential to prevent damage in the process. Moreover, recently there has been an extraordinary interest, by the chemical industry, in the production of ethanol as an alternative fuel to other petroleum products. Thus, several analytical methodologies have been developed to determine ethanol either in biological samples, such as blood, saliva, urine, etc. or in samples from crude extracts obtained from a determined fermentation process. These methodologies, among others, include spectrophotometry [1], high performance liquid chromatography [2], gas chromatography [3,4] or refractometry [5]. Many of these methods provide accurate and precise determination of ethanol but present some disadvantages derived from large and tedious protocols of sample preparation that results in large analysis times. Moreover, most of these methodologies require an extensive and expensive instrumentation.

The importance of ethanol as analyte and the growing interest of determining the alcohol levels in situ, outside the laboratory, demand for new methods of analysis leading to portable devices capable to determine it in whole samples [6] in a fast, simple, and accurate form. Biosensors based on selective ethanol conversion enzymes, are a valuable alternative methodology for sensitive, accurate, selective, inexpensive and rapid determination of alcohol concentration in all types of samples. In this way, two kinds of enzymes, Alcohol Dehydrogenase (ADH) [7–9] and Alcohol Oxidase (AOx) have been used in the fabrication of alcohol biosensors [10,11]. In particular, the first one has been most widely used due to its high stability, which makes it more suitable to resist the immobilization process.

Yeast Alcohol Dehydrogenase I (ADH) belongs to a group of redox enzymes that are present in many organisms. This group of enzymes catalyzes the oxidation of alcohols to their corresponding aldehydes or ketones. The oxidized form of nicotinamide adenine dinucleotide cofactor (NAD^+) takes the electronic pair involved in the process and is transformed into the corresponding reduced form (NADH). In this case the cofactor acts as a second substrate, for this reason these enzymes are called dehydrogenases dependent on NAD^+. Yeast alcohol dehydrogenase (ADH) is a tetramer of four identical subunits [12] with 347 amino acid residues each and a calculated mass of

* Corresponding author at: Facultad de Ciencias. c/ Francisco Tomás y Valiente, 7. Departamento de Química Analítica y Análisis Instrumental. Universidad Autónoma de Madrid, 28049 Madrid, Spain.
E-mail addresses: cesar.anquela@uam.es (C. Gómez-Anquela), tania.garcia@uam.es (T. García-Mendiola), josemaria.abad@uam.es (J.M. Abad), marcospita@icp.csic.es (M. Pita), felix.pariente@uam.es (F. Pariente), encarnacion.lorenzo@uam.es (E. Lorenzo).

http://dx.doi.org/10.1016/j.bioelechem.2015.06.009
1567-5394/

147.396 Da (36.849 Da/subunit), each subunit contains two zinc (II) ions that are tetracoordinated [13]. The zinc ion located in the catalytic lobe is coordinated with two cysteines, one glutamic and one histidine. It is involved in the catalytic oxidation of ethanol. The other zinc ion is coordinated with four cysteines [12] and is located in a structural lobe. In the active center this enzyme has two binding domains: the first one binds the substrate (ethanol) and the second one is reserved for the oxidized form of the cofactor [14]. Electrochemical biosensors based on yeast ADH have the advantage of using a stable protein (compared to biosensors based on AOx) but also have the disadvantage of being dependent on the presence of NAD^+ into the bio-catalytic layer. To avoid this inconvenience, the cofactor should be added to the solution or immobilized along with the enzyme on the electrode surface. In addition, the reduced form of this cofactor (NADH) needs large overpotential for reoxidation [15–17], which increases the risk of interferences from substances present in the matrix. The large overvoltage intrinsic with the NADH oxidation onto solid electrodes is due to the formation of no electroactive oxidation products that rapidly adsorb on the electrode surface fouling it [15,16]. To overcome this problem, the electrode surface can be modified with substances capable to act as redox mediators reducing the overpotential [18–22]. Other approaches to prevent the electrode fouling are based on the modification of electrode surface with carbon nanotubes or graphene sheets [23–26]. These studies have demonstrated that carbon nanotubes show electrocatalytic activity towards NADH oxidation by themselves or by their antifouling capabilities [15,24]. The problem with these nanostructured electrodes is the poor solubility of the starting nanocarbon sources, which involve large protocols of suspension-solubilization and the lack of precise analytical tests to quantify the real electroactive area of the modified electrode.

Recently, we have modified screen-printed carbon electrodes (SPCE) with Azure A (AA) by electro-grafting of the corresponding diazonium salt generated in situ. Such modified electrodes show a potent and persistent electrocatalytic effect to the oxidation of NADH [18]. Moreover, it has been reported that electrodes modified with phenothiazine dyes show a strong affinity for proteins and DNA [27]. The binding strength have been estimated to be around 200 nN, equivalent to 10^2 C–C single bonds [28], suggesting that phenothiazine modified electrodes can be used in the construction of biosensor. In particular, we thought that AA modified SPEs can be a good choice for the development of dehydrogenases based biosensors for two reasons: i) the phenothiazine modified surface can act as an effective molecular adhesive for the enzyme and ii) it can be an efficient redox mediator for electroxidation of the NADH enzymatically generated. However, it is well known that the electronic coupling between the enzyme and the electrode is relatively weak. To overcome this problem, nanoparticle-enzyme hybrid systems has been employed [29–34]. Most of these approaches are based on a simple deposition of nanoparticles and enzymes on an electrode support [35–37]. In general, large nanoparticles have been used, and the enzyme has been immobilized on the nanoparticles used as an extension of the electrode surface [35,38]. However, the incorporation of a nanoparticle into the protein offers an interesting route to improve electron transfer. In this case, a suitable nanoparticle must be employed. For instance, previous works have demonstrated that the presence of a small (1.4 nm) gold nanoparticle can enhance exceptionally the turnover rate of metaloenzymes based bioelectrocatalysis by optimizing electrical connectivity [39,40].

Chemical derivatization of gold nanoparticles or direct coupling to biomolecules is further facilitated by the use of capping ligands containing, in addition to the thiol group, a functional group such as an amine or a carboxylic moiety that can be used for linking to proteins through either covalent or noncovalent interactions. In this sense, thioctic acid-capped nanoparticles show high stability in aqueous solutions and serve well as a scaffold for direct coupling of proteins retaining their biological function [41,42].

In the present work we have applied the concept to achieve both specific recognition of a metallic center and enhancement of the electrical connectivity to an electrode by using the coordination properties of the metal center of the enzyme for its specific recognition by ligands attached to a gold nanoparticle. The interconnection strategy followed is based on the introduction of a carboxylate-functionalized gold nanoparticle of suitable size inside the pocket of the enzyme to achieve direct coordination with its metal. Following this strategy we describe the construction and characterization of an improved ethanol biosensor based on the direct immobilization of alcohol dehydrogenase, coordinated through the catalytic zinc (II) to thioctic acid capped gold nanoparticles, on the surface of an electrografted phenothiazine (AA) layer, which have a dual function: bioadhesive and NADH oxidative catalyst. The prevention of enzyme leakage from the biosensing layer is also studied.

2. Experimental section

2.1. Chemicals and stock solutions

Azure A (AA), β-Nicotinamide adenine dinucleotide disodium salt hydrate, Alcohol dehydrogenase (ADH) from Saccharomyces cerevisiae (≥300 U/mg; EC 1.1.1.1), Thioctic acid (TO), Sodium tetrachloroaurate (III) hydrate ($HAuCl_4$), Tetraoctylammonium bromide (TOABr), Sodium borohydride ($NaBH_4$), polyethilenglycol (PEG), nafion, polyethylenimine (PEI) and Chitosan medium molecular weight (CHIT) were purchased from Sigma Chemical Co. and used as received. Acetic acid solution was obtained from Fluka. Sodium nitrite was purchased from Riedel-de-Haën and Absolute ethanol (EtOH) was obtained from Fischer Scientific. Other reagents used in this work were of analytical grade.

Stock solutions of ADH were prepared in 0.1 M pH 8.0 phosphate buffer solution (PBS) at a final concentration of 10.0 mg/mL (8.3 U/μL) and stored frozen at −20 °C. In these conditions the enzyme activity remains stable at least four weeks.

Ultrapure grade water (18.2 MΩ cm) from a Millipore Milli-Q system was used in the experiments.

2.2. Apparatus

Electrochemical experiments were carried out at room temperature using an Autolab PGSTAT 30 potentiostat from Eco-Chemie (KM Utrecht, The Netherlands) using the software package GPES 4.9 (General Purpose Elec. Experiments) and were performed in a homemade single compartment electrochemical cell. Integrated screen-printed carbon electrodes (4 mm diameter, SPCEs) from DropSens S.L (Oviedo, Spain) including a silver pseudoreference electrode and a carbon counter electrode were used. The electrodes were connected using a SPE connector (DropSens S. L.) as interface. O_2-free nitrogen was used to remove the oxygen from the solutions and a continuous flow of nitrogen was maintained during the voltammetric experiments.

Total reflection X-ray fluorescence (TXRF) analysis was performed by using a TXRF Bruker PicoFox S2 spectrometer (Bruker AXS Microanalysis GmbH, Berlin, Germany) equipped with an X-ray tube which included a Mo anode, a multilayer monochromator and a Si (SDD) detector with an active area of 30 mm^2 were used.

Transmission electron microscopy (TEM) measurements were carried out with a 200 KV JEOL 2100 TEM/STEM microscope. Coupled Energy-dispersive X-ray analyzer (EDX) from Oxford Instruments was used for the elemental surface analysis. High angular annular dark field scanning transmission electron microscopy (HAADF-STEM) was also used to collect z-contrast images.

UV–Vis spectra were recorded in a double beam PharmaSpec UV-1700 series (Shimadzu Co., Kyoto, Japan) by using a 1 cm optical path length quartz cells.