Bioelectrochemistry 85 (2012) 1–6

Contents lists available at SciVerse ScienceDirect

Bioelectrochemistry

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

Bioelectrochemistry of non-covalent immobilized alcohol dehydrogenase on oxidized diamond nanoparticles

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ARTICLE INFO

Article history:
Received 12 July 2011
Received in revised form 7 October 2011
Accepted 11 November 2011
Available online 20 November 2011

Keywords:
Adsorption
Alcohol dehydrogenase
Nanodiamonds
Bioelectrochemistry

ABSTRACT

Diamond nanoparticles are considered a biocompatible material mainly due to their non-cytotoxicity and remarkable cellular uptake. Model proteins such as cytochrome *c* and lysozyme have been physically adsorbed onto diamond nanoparticles, proving it to be a suitable surface for high protein loading. Herein, we explore the non-covalent immobilization of the redox enzyme alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* (E.C.1.1.1.1) onto oxidized diamond nanoparticles for bioelectrochemical applications. Diamond nanoparticles were first oxidized and physically characterized by X-ray diffraction (XRD), FT-IR and TEM. Langmuir isotherms were constructed to investigate the ADH adsorption onto the diamond nanoparticles as a function of pH. It was found that a higher packing density is achieved at the isoelectric point of the enzyme. Moreover, the relative activity of the immobilized enzyme on diamond nanoparticles was addressed under optimum pH conditions able to retain up to 70% of its initial activity. Thereafter, an ethanol bioelectrochemical cell was constructed by employing the immobilized alcohol dehydrogenase onto diamond nanoparticles, this being able to provide a current increment of 72% when compared to the blank solution. The results of this investigation suggest that this technology may be useful for the construction of alcohol biosensors or biofuel cells in the near future.

1. Introduction

Protein interaction with solid surfaces is believed to play a key role in the development of future nanotechnologies, biomaterials, and biotechnological processes [1]. The immobilization of proteins onto nanomaterials widely opens the door for fundamental and applied research [2].

The use of nanodiamonds holds an interesting and bright future for the development of a variety of biological and analytical platforms. This material has been investigated during the last years due to its properties: hardness, dopability, and biocompatibility [3,4]. In fact, the immobilization of different biomacromolecules (e.g. DNA [5], proteins [6], and cells [7]) onto diamond nanoparticles has shown the feasibility of using these platforms for biological and biomedical applications [5,7–12]. For instance, it has been demonstrated that diamond nanoparticles are more compatible than multi-walled and single-walled carbon nanotubes for different cell types [13].

However, since it is particularly important to understand the immobilization behavior of these biomacromolecules, research has been conducted to assess the adsorption capacities of certain proteins on nanodiamonds. In 2004, Chang et al. reported on the noncovalent immobilization of cytochrome *c* (12,000 g/mol) onto nanodiamonds of different sizes [14]. This investigation revealed the nanodiamond size effect on the adsorption of the protein, revealing a packing density of 8.0×10^{12} molecules/cm^2 and 3.0×10^{12} molecules/cm^2 for 100 nm and 5 nm diamond particles (mostly agglomerates). In addition to the nanodiamond size affecting the adsorption behavior of proteins on nanodiamonds, the solution pH has been also considered in another investigation conducted in 2007. In this research, the hen egg white lysozyme (14,000 g/mol) was noncovalently immobilized on 100 nm diamond particles with pH variations [9,15]. The results demonstrated that besides hydrogen bonding, electrostatic interactions play a key role in the adsorption behavior of the selected protein onto the nanodiamonds. In fact, the packing density of the protein was dependent on the solution pH. Also, insulin (5,800 g/mol) has been physically immobilized onto nanodiamonds as a strategy for protein delivery, by using pH changes as the trigger to release the protein [16]. The latter is another example of the outstanding adsorption capacity of nanodiamonds and their applicability in the nanomedicine field.

Accordingly, it has been well established the tremendous capability of nanodiamonds to be used for biological and biomedical applications [17]. For instance, these nanodiamond platforms are suitable to be used as protein supports for bioanalytical applications.

Due to this, and to further extend the current knowledge in the area of nanodiamonds for analytical applications here we present

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doi:10.1016/j.bioelechem.2011.11.002

the noncovalent immobilization of the enzyme alcohol dehydrogenase (141,000 g/mol) onto diamond nanoparticles. The alcohol dehydrogenase-nanodiamond (ADH-ND's) bioconjugates were characterized for the adsorption variation with pH, and relative activity, as well as physical characterization.

Furthermore, the ADH-ND's bioconjugates were utilized as a bioelectrochemical cell for the proof-of-concept of a biosensor or biofuel cell. Therefore, in this work we explored the utilization of nanodiamonds "beyond" the above-mentioned biological applications to extend its utilization as a support for redox enzymes in bioelectrochemical applications.

2. Experimental section

2.1. Diamond nanoparticle oxidation

Alcohol dehydrogenase from *Saccharomyces cerevisiae* (ADH) (E.C.1.1.1.1, initial activity of 300 units/mg protein) was purchased from Sigma Aldrich (St. Louis, MO), stored at −20 °C, and used without further purification. NAD^+ and NADH were purchased from Sigma Aldrich and used as received. All other chemicals in this work were from Sigma Aldrich and of ACS grade.

Synthetic diamond powder (high purity UDD, 2–50 nm particle size) from Alit Co. (Kiev, Ukraine) was cleaned and carboxylated/oxidized as reported [5,14]. The carboxylation/oxidation procedure was performed by heating 2 g of diamond powder at 70 °C for 3 d in a reflux system in a 9:1 (v/v) mixture of H_2SO_4 and HNO_3, followed by heating at 85 °C for 2 h in 0.1 M NaOH and 0.1 M HCl solution, respectively. The resulting diamond nanoparticles were washed several times with nanopure water (18.3 MΩ, Barnstead) until the supernatant reached neutral pH, and subsequently dried at 115 °C for 24 h. X-ray diffraction of as received and oxidized DNP's were done with a Rigaku, Ultima III X-ray diffractometer system using Ni filtered CuK radiation. The X-ray source was operated at 40 kV and 44 mA. Powder samples were mounted on a silicon zero background material. The XRD spectra were obtained using a high precision and high resolution parallel beam geometry in the step scanning mode with a counting time of 11 s per 0.02°. Scans were recorded in the 2 θ range of 0° to 140°. Lattice parameters and crystallite sizes were calculated using Jade 7 Plus software (Rigaku). Monocrystal sizes were determined from the Scherrer equation using the Pearson-VII profile function. The Scherrer equation was applied to the peaks at 42.9°, 75.0° and 92.0°.

2.2. Non-covalent immobilization of ADH on nanoparticles

For the ADH immobilization, 2.5 mg of the oxidized diamond nanoparticles were sonicated in 1000 μL of 10 mM phosphate buffer solution of different pH (e.g. 4.4, 5.5 and 8.8) overnight in plastic Eppendorf vials to create a suspension and minimize agglomeration. ADH solutions were added to these vials to achieve final concentrations ranging from 0.1 to 4.0 mg/mL while keeping a constant volume. These solutions were thoroughly mixed for 3 h using an orbital shaker at 170 rpm. After the 3 h period, samples were centrifuged at 13,000 rpm for 10 min and supernatants were withdrawn with 2 mL needle syringes to measure the concentration of non-adsorbed enzyme [15]. For the second wash, diamond nanoparticles with adsorbed protein were resuspended in phosphate buffer and submitted to 10 min of shaking at 170 rpm. After the second wash, no protein was detected in the supernatant. Protein concentrations were determined from the absorbance at 280 nm using a calibration curve made with ADH standard samples.

IR spectra were recorded at room temperature using a FT-IR Microscope (Thermo Nicolet, NEXUS 870) in ATR mode after the samples were lyophilized for 24 h (water remotion) to assess information related to protein characteristic IR peaks. Transmission electron microscopy (FEI Tecnai F20 200 kV TEM) images were also taken for the oxidized DNP's, and for the ADH-ND's bioconjugates. For the oxidized sample, a plasma treatment was used to remove any solvent present in the samples. However, the ADH-ND's samples were stored and dried under vacuum before measurements.

2.3. Relative activity determination of ADH-ND's bioconjugates

Relative activity of the protein, once immobilized (ADH-DNP's), was determined as described by Kägi et al. with some minor variations [18]. In brief, after the immobilization process was performed, the ADH-ND's bioconjugates were resuspended in 8.5 mL of 50 mM sodium pyrophosphate buffer at pH 8.8 under slow constant stirring speed. Thereafter, 500 μL of 12 mM NAD^+ were added as a co-factor followed by the addition of 1000 μL of 1 M ethanol to start the reaction. Then, aliquots of 100 μL from the reaction mixture were periodically withdrawn and poured in 100 μL of 1 M sodium carbonate to quench the reaction. Then, 300 μL of 50 mM sodium pyrophosphate were added to the solution in order to have a final volume of 500 μL. Thereafter, the NADH produced was measured with a Shimadzu 2450 UV–vis spectrophotometer at 340 nm. In such sense, the catalytic activity of the protein was defined as the increment in product concentration relative to the amount of protein used. Also, the same procedure was followed to determine the reference catalytic activity, which is the activity of the as-received protein from the vendor.

2.4. Bioelectrochemical cell experiment

An in-house two-electrode Teflon cell was built for the bioelectrochemical cell experiments. In brief, the cathode electrode was prepared by paint-brushing a platinum black ink solution (i.e., 85% metal, 15% Nafion) to a final loading of 4 mg/cm^2. In the other hand, the anode side was made of poly-methylene green carbon cloth (p-MG/CC) [19]. The anode was prepared by cycling the potential 12 times between −0.3 V and 1.3 V vs. Ag/AgCl in a solution containing 0.4 mM methylene green, 10 mM sodium tetraborate, and 0.1 mM sodium nitrate. Afterwards, the electrode was thoroughly washed with deionized water, dried overnight, and stored in a desiccator until further use. On both anode and cathode electrodes, platinum gauze was used as an electrical contact.

For the actual bioelectrochemical cell experiment, the sample with the highest % relative activity was used as a criterion to maximize the current output. In brief, 5 mL of the ADH-DNP's bioconjugates were poured in the electrochemical cell followed by the addition of 666 μL of 6 mM NAD^+ and 464 μL of 1.4 M ethanol to initiate the reaction. It is important to point out that before adding the ethanol the cell was allowed to equilibrate for 1 h. Thereafter, the enzymatic reaction was allowed to undergo for 10 min after the linear potential polarization experiment was performed. Therefore, note that potentials are versus O_2/unsupported Pt/C electrode.

3. Results and discussion

After the nanodiamond oxidation via the strong acid procedure was performed it was of interest to have details on the physical state of the material. Therefore, a routine X-ray diffraction was executed to examine the material integrity after the oxidation process. Fig. 1 shows the XRD pattern of the as-received and oxidized sample. It can be observed an increment in crystallinity at the 111 facet appearing at 42.9°, and a significant signal decrease in the amorphous carbon region (0°–20°) after oxidation. Furthermore, the diameters of the diamond nanoparticles were calculated from the XRD data resulting in 5.0 nm and 5.1 nm for the as received and oxidized diamond nanoparticles, respectively. Now, this oxidation route was chosen due to the fact that is able to remove most of the amorphous carbon from the material. However, other routes for nanodiamond modification and functionalization have been reported, although employing milder conditions [20].