Journal of Electroanalytical Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Electroanalytical Chemistry

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

Pore size effect of MgO-templated carbon on enzymatic H_2 oxidation by the hyperthermophilic hydrogenase from *Aquifex aeolicus*

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ARTICLE INFO

Keywords:
Hydrogenase
MgO-templated carbon
Biocatalysis
Direct electron transfer
Pore size

ABSTRACT

Hydrogenase from the hyperthermophilic bacterium *Aquifex aeolicus* was immobilized in MgO-templated carbon (MgOC). Two different pore sizes were investigated, large pore size of 150 nm ($MgOC_{150}$) and smaller pore size of 35 nm ($MgOC_{35}$). Direct H_2 oxidation proceeded in both $MgOC_{150}$ and $MgOC_{35}$. Hydrogenase embedded in the carbon material exhibited the expected properties in terms of onset for H_2 oxidation and kinetics of formation of the inactive state at high potentials, whatever the size of the pores. Pore size much larger than the size of the enzyme favored the loading of the enzyme, yielding to high catalytic current reported to the capacitance. Pore size closer to the enzyme diameter, as determined by DLS, enhanced the stability of the enzyme at high temperature.

1. Introduction

Metalloenzymes are very attractive catalysts for use in biodevices such as biosensors, bioreactors and biofuel cells. Although their activity and specificity tend to overpass those of chemical catalysts, two of the main issues are their wiring to electrodes and their stability. Electrode nanostructuration through use of high surface/volume carbon materials has been demonstrated not only to enhance the electron transfer rate and the catalytic current, but also the stability with time of the bioelectrodes [1,2]. These features are linked to the combined effect of efficient wiring of a high loading of enzyme and protection against conformational changes thanks to a suitable nanoenvironment. Hierarchic porosity is researched for both high enzyme loading and efficient mass transport of product and substrate. One relevant question is the optimal pore size of the carbon material which allows the best compromise between high activity and good stability of the enzyme. A recent simulation study of direct electrocatalysis of hydrogenase (Hase) for H_2 oxidation and bilirubin oxidase (BOD) for O_2 reduction suggested that the catalysis is the most efficient when the pore size approaches the diameter of the enzyme [3]. In that way, a control of the pore size of the carbon material is especially required. We reported the use of MgO-templated carbon (MgOC) for efficient immobilization of various enzymes. Actually, MgO is a suitable template because it is thermally and structurally stable during carbonization and it is easy to remove from the resulting carbon in dilute acid solution [4]. It is furthermore easily tunable in pore size and the interconnected mesopores provide a high surface area. Glucose dehydrogenase [5], fructose dehydrogenase [6] and bilirubin oxidase [7,8] were efficiently immobilized in such carbon materials. In agreement with other reported works [9], it was suggested that enhanced contact area between enzyme and carbon of pore size close to enzyme size increased bioelectrode stability by preventing denaturation and aggregation of enzymes.

On the other hand, we recently reported a highly performant H_2/O_2 enzymatic fuel cell based on two thermostable enzymes, i.e. *Aquifex aeolicus* [NiFe] hydrogenase (*Aa* Hase) and *Bacillus pumilus* BOD both entrapped in carbon nanotube-modified carbon felts [10]. In the case of *Aa* Hase, very high currents approaching 1 A/mg of enzyme were reached thanks to the suitable functionalization of the carbon nanotube walls. Although the carbon felt post-functionalized by carbon nanotubes was demonstrated to develop mesoporosity suitable for enzyme immobilization, no strict control of the pore size was achieved. In this work, we report for the first time the study of *Aa* Hase immobilization in two MgOC with different pore sizes. We show how the magnitude of the catalytic current for H_2 oxidation and its stability depends on the pore size of the material.

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https://doi.org/10.1016/j.jelechem.2017.12.041
Received 27 October 2017; Received in revised form 13 December 2017; Accepted 15 December 2017

2. Experimental

2.1. Chemicals and materials

N-methyl-2-pyrrolidone (NMP) and Methylene Blue (MB) were purchased from Sigma-Aldrich. 0.05 M 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), pH 7, was used as buffer for hydrogenase activity assays. 0.1 M sodium phosphate buffer, pH 7, was used for electrochemical studies.

The MgOCs with large pore size of 150 nm ($MgOC_{150}$) and smaller pore size of 35 nm ($MgOC_{35}$), were obtained from Toyo Tanso (Osaka, Japan) [6]. MgOC dispersions were prepared by sonication of 1 mg/mL MgOC in NMP for 5 h. The dispersions were than let for 1 day to allow undispersed particles to precipitate and the supernatant was used for electrode modification.

Membrane-bound [NiFe]-hydrogenase from *Aquifex aeolicus* (*Aa* Hase) was purified as described elsewhere [11].

2.2. Hydrogenase activity assay

Catalytic activity of *Aa* Hase for hydrogen oxidation was measured spectrophotometrically at 30 °C in a 1 cm cuvette containing 0.05 M Hepes pH 7 and 0.2 mM MB as an electron acceptor. The solution was saturated by hydrogen for 10 min prior to the measurements. The reaction was started by addition of a defined *Aa* Hase amount and the decrease of the solution adsorption was followed at 550 nm as a result of MB reduction. The hydrogen atmosphere was kept during all the measurement in the cuvette.

2.3. Dynamic light scattering

Dynamic light scattering (DLS) was used to determine the hydrodynamic diameter (D_h) of *Aa* Hase using a Zetasizer Nano ZS (Malvern Instruments) with a scattering angle of 173°. To determine the particle size, the provided software uses the Stokes-Einstein relation to obtain the intensity averaged size distribution from the raw correlation data. For the *Aa* Hase analysis, the size distribution assays were performed at 25 °C. For each assay three measurements were performed, each one consisting in 10–15 runs of 10 s. In addition, a temperature trend assay from 25 to 70 °C with 5 °C intervals was used to calculate the aggregation point of the enzyme. We displayed our results using the correlograms, Z-average (mean intensity size of sample) and Dv50 mean volume size using the Zetaziser software 7.12. *Aa* Hase was prepared at 75 nM in 100 mM phosphate buffer pH 7. The refractive index and viscosity of the buffer containing sample was measured using an Abbemat and DMA machine by Anton Paar. A refractive index of 1.335 and viscosity 0.9520 cp was used to change the size distribution from intensity based to a volume based.

2.4. Electrochemistry measurements

Cyclic voltammetry (CV) and chronoamperometry were performed using a potentiostat from Autolab PGSTAT30 analyzer controlled by Nova software (Eco Chemie). The sealed electrochemical cell was equipped with three electrodes, a platinum wire as an auxiliary electrode and an Ag/AgCl electrode (KCl saturated) as a reference electrode. All potentials are quoted vs Ag/AgCl reference electrode. During all the measurements the electrode was rotating with rate 2000 rpm and hydrogen was constantly bubbled through electrolyte solution.

2.5. Electrode modification

Pyrolytic graphite electrode (PG, geometric surface area 0.07 cm^2) was used as support for MgOC modification. 2 μL of $MgOC_{35}$ or 5 μL of $MgOC_{150}$ dispersion was dropped on the PG surface and dried in the vacuum chamber until a pressure less than 0.1 mbar. Smaller amount of $MgOC_{35}$ was deposited in order to account for slightly higher concentration and electroactive surface area of the latter. Unlike most of the works, a particular attention was paid to avoid any mass-transfer limitations inside of MgOC film. This means that only small amount of MgOC was initially deposited on the PG electrode to ensure that enzyme and hydrogen can diffuse rapidly inside porous structure of MgOC deposit within a time-frame of the experiment. Since only small amount of MgO was used, the formed adsorbate adhered strong enough to the electrode surface (due to the carbon-carbon adhesion). When the membrane was used, it played an additional protective role. We intentionally avoided the use of any binder since we believe that it may clog the pores and make them inaccessible for the enzyme.

Aa Hase adsorption on the MgOC-modified electrodes was performed in the potentiostatic conditions by adding the defined amount of the enzyme to the electrochemical cell and following the catalytic current of H_2 oxidation at − 0.3 V. The concentration of *Aa* Hase in the cell (75 nM) was chosen taking into account our previous study showing this concentration is close enough to a saturating one for carbon-based nanomaterials [10].

For the chronoamperometric stability experiments, a dialysis membrane (cut off 12–14 kDa) was put on the electrode after enzyme adsorption to ensure the absence of additional enzyme desorption/adsorption from the solution during the experiment.

2.6. Microscopy

The carbon structures of $MgOC_{35}$ and $MgOC_{150}$ were observed by field emission scanning electron microscopy (FE-SEM, SU-8020, Hitachi, Japan).

3. Results and discussion

3.1. Hydrodynamic diameter of Aa Hase and MgOC morphology

To verify the compatibility of the *Aa* Hase diameter and MgOC pore diameters, we performed DLS on the enzyme. Beforehand, we calculated the relative mass diameter of the *Aa* Hase enzyme using the amino acid composition of the two subunits of the enzyme. We obtained a diameter of 6.39 nm. The theoretical hydrodynamic diameter for a globular *Aa* Hase is D_h 8.88 nm, taking into account the ionic layer surrounding the enzyme. By DLS we obtained a hydrodynamic diameter of D_h 17.7 ± 1.3 nm for *Aa* Hase at 25 °C (Fig. 1A). The DLS value of the hydrodynamic diameter can be explained either by the presence of the transmembrane helix yielding a non-globular enzyme, or the presence of detergent molecules surrounding the enzyme. When the *Aa* Hase sample was heated using a temperature trend from 25 °C to 70 °C, no aggregation point was observed. This is expected given that *Aa* Hase is purified from a hyperthermophilic bacterium. Nevertheless, we did observe a difference in size depending on the temperature. This is clearly shown in Fig. 1 where the major peak shifts to the left and narrows as the temperature increases, suggesting that *Aa* Hase is the most stable at 50 °C with a hydrodynamic diameter of 13.5 ± 0.7 nm.

FE-SEM images of the carbons used in this study with MgO powder having the particle size of about 35 and 150 nm are shown in Fig. 1B. The pores in the carbon are disordered and replicate the cubic morphology and the size of MgO crystals.

3.2. Influence of MgOC pore size on H_2 oxidation catalytic current

Aa Hase was immobilized on deposits of MgOC presenting two different pore sizes, which were chosen on the basis of the DLS measurement of *Aa* Hase diameter. The smallest pore size (35 nm, denoted $MgOC_{35}$) is close to *Aa* Hase hydrodynamic diameter and would favor a tight contact between the enzyme and the material, while the largest pore size (150 nm, denoted $MgOC_{150}$) is expected to enhance the loading of the enzyme and the diffusion of substrate. The efficiency of