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Membrane Bound Enzyme Hosted in Liquid Crystalline Cubic Phase for Sensing and Fuel Cells

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ABSTRACT

Monoolein or phytantriol liquid crystalline cubic phase and carbon nanotubes were employed as the matrices for the immobilization of a membrane bound enzyme–fructose dehydrogenase (FDH). Entrapment within the cubic phase protects the guest protein from chemical and physical degradation, thereby facilitating retention of its native conformation and bioactivity. The mesophase environment was therefore found appropriate for retaining FDH close to the electrode surface. Phytantriol was used as the cubic phase component in case of measurements carried out in biological fluids containing hydrolyzing enzymes. Fructose dehydrogenase shows direct electron transfer when embedded in the matrix, thus does not require any mediators. The bioanode was employed as a biosensing device for fructose. Due to the resistance of fructose dehydrogenase to oxygen it was found useful for the construction of a membrane less biofuel cell. The enzymatic fuel cell based on FDH in the cubic phase film at the anode and laccase at the biocathode showed open circuit potential of 703 ± 10 mV in the presence of 40 mM fructose in buffer solution, and power density of 850 $\mu W\,cm^{-2}$ at 250 mV in solutions under conditions of continuous flow of dioxygen.

1. Introduction

Electron exchange between conducting surfaces and immobilized redox enzymes can be accomplished either by direct electron transfer (DET), or by a pathway involving redox-active mediating compounds (MET). The DET approach, specifically its application in bioelectrocatalysis, has recently received much attention. [1,2] Elimination of mediators should simplify the process and remove several difficulties, although to-date larger current densities have been achieved using the MET approach. D-Fructose dehydrogenase (FDH) from *Gluconobacter* was first obtained and characterized by Yamada et al. [3] Fructose dehydrogenase (FDH) is a membrane bound enzyme of molecular mass of ca. 140 kDa that containes flavin and heme c group and catalyses the oxidation of D-fructose to 5-keto-D-fructose. In DET, fructose oxidation and reduction of the flavin-containing subunit is followed by electron transfer to the heme c-containing subunit where DET occurs. [4] High selectivity toward D-fructose makes FDH applicable in enzymatic fructose detection in clinical or food analyses. Several amperometric biosensors based on FDH have been reported. [5–7] Fructose dehydrogenase is an oxygen insensitive enzyme and can also act as biocatalyst on an anode for biofuel cell development. [8,9]

DET transport was obtained for FDH adsorbed on Ketjen black (KB) particles [10] and on highly oriented pyrolytic graphite (HOPG), [11] Pt electrode modified with multi walled carbon nanotubes, or silver nanoparticles on polycrystalline gold electrodes. [12] Hydrophobic environment is essential to retain optimal activity and stability of the membrane-bound enzymes. Thus incorporation of FDH and sarcosine dehydrogenase (SDH) into the vesicles made of sorbitane monooleate (SPAN 80) or soybean lecithin increases the stability and activity of both enzymes. [13]

Ideally, membrane proteins would be optimally stabilized in a membrane-mimetic matrix that resembles the natural environment. Liquid-crystalline lipidic cubic phase (LCP) can be regarded as a potential matrix of this kind. Entrapment within the cubic phase protects the guest protein from chemical and physical degradation, thereby facilitating retention of its native conformation and bioactivity. LCP can incorporate relatively large soluble and membrane proteins and allow their spectroscopic investigation. [14] Lipids that form LCPs are typically non-toxic, biocompatible and biodegradable, rendering LCPs highly attractive for various

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applications. These include the crystallization of proteins, specifically membrane proteins, [15,16] drug delivery, [17,18] biosensing and biofuel cell construction. [19–23] For applications in biological milieus, which typically contain hydrolyzing enzymes, phytantriol and other isoprenoid-type lipids can be regarded as more stable building blocks of LCPs. [18,24] Redox proteins incorporated within LCPs can be readily addressed by mediators which shuttle electrons between the electrode surface and the protein. The mediator may be dissolved in the electrolyte solution, or be linked to the lipidic three-dimensional matrix similar to inserted electroactive amphiphiles.

2. Experimental

D-fructose dehydrogenase from *Gluconobacter sp.* (EC 1.1.99.11) was purchased from Toyobo (activity 90U/mg). Single Walled Carbon Nanotubes were supplied by Nanocyl. Monoolein (1-oleoyl-rac-glycerol) (MO), D-fructose, Triton X-100 were purchased from Sigma and were used as received. Phytantriol (PT) was obtained from DSM as a gift. Na_2HPO_4, KH_2PO_4 and citrate acid were from POCh (Polish Chemicals Co.). To preserve activity of the enzyme experiments were performed in the presence of 0.1% TritonX-100 in solution. All solutions were prepared using Milli Q water (18.2 MΩ·cm^{-1}), Millipore, Bedford, MA, USA. Apple juice Cappy and Apple mint juice Hortex were purchased at local stores. Prior to their analysis juices were diluted 40-100 times.

Cyclic voltammetry experiments were conducted using a CHI 700B bipotentiostat in a three-electrode arrangement with a silver/silver chloride reference electrode (Ag/AgCl) and a platinum sheet as the counter electrode. Working electrodes were prepared according to the procedure described below:

2.1. LCP preparation

The lipid was first melted in a glass vial at 38 °C in a water bath. Appropriate amount of the enzyme in aqueous solution was subsequently added thereto, the composition being based on the phase diagrams of the phytantriol-water or monoolein-water system. [24,25] Cubic phase of diamond symmetry (Pn3m) was employed throughout. The ratios for the MO/H_2O/FDH and the PT/H_2O/FDH LCPs were 63/36/1% (w/w) and 72/27/1% (w/w), respectively. To obtain homogenous, transparent and viscous LCPs, the samples were incubated at 38 °C in a water bath and then centrifuged for ca. 15 min in a MPW 56 centrifuge.

2.2. Preparation of the FDH modified electrode:

Prior to the modification of the GC anode with pristine carbon nanotubes, it's suspension in ethanol (4 mg ml^{-1}) was sonicated for 20 minutes. 20 μl of pristine carbon nanotubes suspension in ethanol was dropped onto the electrode surface and left to dry, and this procedure was repeated twice. Electrodes were subsequently covered with a thin layer of LCP.

All current densities were calculated using the geometrical area of the GCE (BAS) (A = 0.071 cm^2).

Biofuel cell parameters were examined in dioxygen saturated 0.15 M McIlvaine buffer solution, pH 5. Single walled carbon nanotubes with naphthalene group were applied for laccase immobilization. Biocathode was prepared according to our published procedure. [26] Potentials of each of the bioelectrodes vs. Ag/AgCl were measured under applied resistances. The cell voltage (V_{cell}) was measured under varying resistances in the range from 1 kΩ to 10 MΩ.

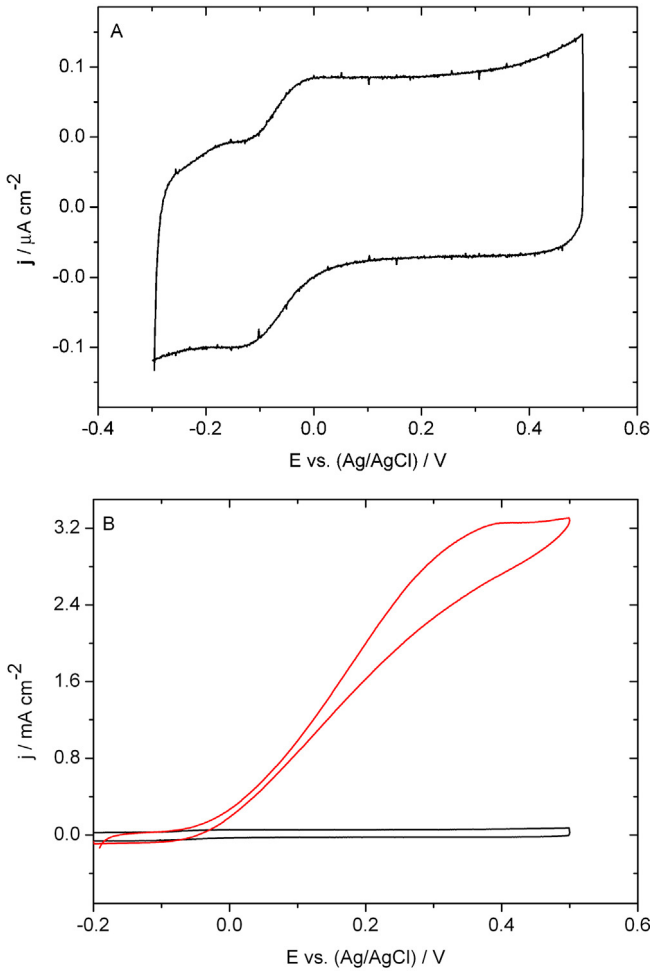

Fig. 1. Cyclic voltammograms of FDH in the absence (A, B-black trace) and presence (B-red trace) of 40 mM fructose in McIlvaine buffer pH 5.0, recorded using GC electrode modified with MO-based LCPs containing 1% (w/w) FDH. Scan rate: 1 mV s^{-1}.

3. Results and Discussion

Membrane-bound enzymes retain optimal activity and stability in a native-like hydrophobic environment. FDH isolated from *Gluconobacter sp.* was immobilized on an electrode surface decorated with a carbon nanotube network in LCP, and was used as a biocatalyst of the fructose oxidation process. Interplay between the lipids and protein can influence the cubic structure. Hydrophilic and medium size globular proteins could be incorporated into water channels without affecting lipidic phase transition. 1% (w/w) FDH was added to the monoolein/water mixture, and formation of the transparent and solid-like cubic phase was observed microscopically. Glassy carbon electrode decorated with single-walled carbon nanotubes covered with LCP provided the hydrophobic environment for the membrane protein. FDH experiments were performed in the McIlvaine buffer in the presence of 0.1% Triton X-100, the latter preserving the enzyme stability [8]. In the absence of fructose, a reversible signal at–0.075 V was observed which can be attributed to the redox potential of heme c since flavin potential is more negative [Fig. 1A]. In the presence of fructose in the buffer solution a sigmoidal catalytic wave is obtained, indicating that FDH works as an electrocatalyst for the oxidation of D-fructose without any electron transfer mediator [Fig. 1B]. In the absence of enzyme no catalytic wave was observed. It can be concluded that the flavin center is responsible for fructose oxidation. Electrons produced in this process are transferred to the heme c center, which can directly