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Performance of enzymatic fuel cell in cell culture

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ABSTRACT

Here we present the very first study of an enzymatic fuel cell (EFC) in a cell culture. An EFC with *Corynascus thermophilus* cellobiose dehydrogenase (CDH) based bioanode and *Myrothecium verrucaria* bilirubin oxidase (BOx) based biocathode was constructed at the bottom of a medusa cell culture plate. The constructed EFC had a power density of up to 25 μW cm^{-2} at 0.5 V potential in simple buffer solution and in cell culturing medium. L929 murine fibroblast cells were seeded on top of the EFC and possible effects of the EFC on the cells and *vice versa* were studied. It was shown that on average the power of the EFC drops by about 70% under a nearly confluent layer of cells. The EFC appeared to have a toxic effect on the L929 cell line. It was concluded that the bioanode, consisting of CDH, produced hydrogen peroxide at toxic concentrations. However, the toxic effect was circumvented by co-immobilizing catalase on the bioanode.

1. Introduction

Biofuel cells (BFCs) can be defined as fuel cells that are based on biological catalysts such as enzymes and living cells (Bullen et al., 2006). In an investigation of the decomposition of organic compounds by *Escherichia coli* in 1911 the first BFC was essentially constructed (Potter, 1911). Since then BFCs have been developed into more compact systems utilizing enzymes, organelles or microbes (Bullen et al., 2006). The enzymatic FCs (EFCs) based on three-dimensional (3D) nanostructures loaded with enzymes represent one of the most compact BFC designs (Wang et al., 2009). 3D nanostructures are usually obtained by modifying a surface with nanomaterials, such as carbon nanotubes (CNTs), metallic nanoparticles (MNPs), growing porous or hierarchical materials, *etc.* The main advantages of EFCs are the possibility to fuel the devices with renewable substrates, *e.g.* carbohydrates, and a relatively easy realization of implantable design. The biocompatibility of BFCs is studied; however, these investigations are considered to be undertaken more intensively in the future (Barton et al., 2004). In this context, it should be noted, that there are two types of EFCs, specifically those in which enzymes and electron conducting electrode structures are wired using mediators and those that are based on direct electron transfer (DET) reactions. Some mediator systems are soluble and might infer additional biocompatibility threat by their leakage. To achieve DET the enzymes are, usually, adsorbed on the clean electrode surface directly or by pre-coating the electrode with surface-active molecules, such as polymers or thiols (Cusmà et al., 2007; Jena and Raj, 2006; Li et al., 2010; Zhang et al., 2007). Additionally, DET based EFCs do not require any membranes (Falk et al., 2012b), which is an advantage in terms of miniaturization and simplicity of the construction.

The compact structures of these DET-based EFCs occupy small volumes and can be fitted in areas with small dimensions. Thus, nanostructured DET based EFCs have been suggested as implantable devices (Falk et al., 2013; Rasouli and Phee, 2010; Wei and Liu, 2008) and their performances in animals has been studied recently (Castorena-Gonzalez et al., 2013; Falk et al., 2013; Halamkova et al., 2012; Zebda et al., 2013). However, demonstrating the performance of EFCs in implanted situations rarely address their biocompatibility issue down to cellular level. It is known that, *e.g.* NPs, which are used in EFC design (Falk et al., 2012a; Wang et al., 2011), might be toxic to eukaryotic cells (Buzea et al., 2007; Hussain et al., 2005; Murphy et al., 2008). Of course, the biocompatibility of EFC is much more complex than the biocompatibility of NPs.

In this work, performance of DET based EFC was investigated in cell culture. The assessment of biocompatibility of EFCs *in vitro* using cell cultures is a good alternative to animal studies since it might simplify pre-clinical trials and give additional information

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concerning the function of eukaryotic cells subjected to 3D biomodified nanostructured electrodes. Thus, we investigated DET based biocathode, bioanode, as well as complete EFC in cell culture platforms to address the simplest and essential questions on viability of eukaryotic cells at BFC electrodes. The current work describes the performance of EFC in cell culture platform and summarizes viability response of fibroblast L929 cells to the functional enzymatic carbohydrate/oxygen BFC deposited on the bottom of cell culture plates. To the best of our knowledge this is the first attempt to design and study a BFC in a cell culture platform.

2. Materials and method

2.1. Nanomaterials

Gold nanoparticles (AuNPs) were synthesized by reducing Au salt, $HAuCl_4$ (Sigma-Aldrich, St. Louis, USA) according to Turkevich's method (Turkevich et al., 1951). Briefly, 50 mL of 1 mM $HAuCl_4$ (aq) solution was heated to 90 °C. Then 5 mL of 1% (w/w) sodium citrate (Sigma-Aldrich, St. Louis, USA) was added under vigorous stirring. After 15 min the reaction was continued without stirring for further 10 min. The concentration of AuNPs in the dispersion was determined to be $\sim 6 \times 10^{-9}$ M by comparing (overlapping) the optical absorption spectra against the extinction efficiency *vs*. wavelength generated by MiePlot software. The particles had a diameter of approximately 15 nm according to atomic force microscopy (AFM) measurements. A commercial AFM (MultiMode 8SPM with a NanoScope V control unit, Bruker AXS, Santa Barbara, USA) was set to tapping mode in air and settings were continuously adjusted by the Scan Asystauto control software. The AuNPs dispersion was concentrated by centrifugation at 10,000*g* for 30 min in 2.0 mL eppendorf tubes. Then, about 90% of supernatant was removed. To improve homogeneity of the resulting AuNP slurry it was ultrasonicated for 7 min using Transsonic 460/H from Elma GmbH&KG sonicator (Singen, Germany). The final concentration of AuNPs was approximately 50 times higher than in the original dispersion. The AuNP slurry was kept at +4 °C before usage.

The stability of EFCs are usually reported for 24 h or more with mixing of the solution at the BFC electrodes (Lim and Palmore, 2007). The mixing is, however, impossible to realize in cell culture plates, especially when eukaryotic cells are grown on the top of electrodes. Nevertheless, we recorded the apparent stability at quiescent conditions of the EFC electrodes in medusa plates. As can be seen (Fig. 1b and c) the current density decays to about 30 μA cm^{-2} at biocathode and 10 μA cm^{-2} at bioanode. If the medusa plate is shaken gently, after a 24 h measurement the current increases considerably (Supplementary materials, Fig. S3). This indicates that the diffusion of fuel (lactose) and oxidant (oxygen) through a stagnant solution layer at the electrodes is one of the major factors resulting in observed decrease of electrode current and thus apparent limitation of stability. Additionally, stability of complete EFC was assessed in PBS buffer by measuring power density (Supplemetary materials, Fig. S4). Without a load of EFC its power decreased by about 30% over a 24 h period. This indicates that enzyme inactivation is also one of the major reasons for power loss by the EFC. In summary, the stability of BFC in complex cell medium seems to be similar as in simple buffer solution.

2.2. Chemicals

Phosphate buffered saline (PBS, 50 mM phosphate, 140 mM NaCl, pH 7) was prepared as known in the arts. A 100 mM lactose solution was made in PBS buffer using α-lactose (Sigma-Aldrich). Polyethyleneimine (PEI), CAS number: 9002-98-6 and hexadecyltrimethylammonium bromide (CTAB) was purchased from Sigma-Aldrich. N-(6 mercapto) hexylpyridinium (MHP) was kindly provided by the Center for Physical Sciences and Techology, Lithuania. All chemicals were of analytical grade and all aqueous solutions were prepared using deionized water (18 MΩ cm) prepared by Purelab Flex II system from ElgaWaterlab (High Wycombe, UK). The reagents for CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS-assay) were from Promega (Madison, USA).

2.3. Enzymes

Cellobiose dehydrogenase (CDH) from *Corynascus thermophilus* was isolated and purified to homogeneity as described previously (Harreither et al., 2012). Bilirubin oxidase (BOx) from *Myrothecium verrucaria* was a kind gift from Amano Enzyme Inc. (Nagoya, Japan). The BOx preparation was additionally purified by Dr. Olga V. Morozova (A.N. Bach Institute of Biochemistry, Moscow, Russia) in the frame of "3D-nanobiodevice" EU project. Catalase (Cat) from bovine liver was purchased from Sigma-Aldrich and used without further purification.

2.4. Cell culturing

Minimum essential medium (MEM; No. 0820234DJ) was purchased from Life Technologies (Carlsbad, California). A 5 mL mixture of Penicillin (10,000 U mL^{-1}) and Streptomycin (10,000 μg mL^{-1}), 5 mL L-glutamine (1 M), 5 mL HEPES (1%) and 50 mL Foetal Bovine Serum (Life Technologies, Carlsbad, USA) were added to 500 mL MEM. The murine fibroblast cells (L929, ATCC LGC Standards, Teddington, UK: passage no: 20-30) were passaged 2–3 times per week and incubated at 5.0% CO_2, 37 °C.

2.5. Deposition of EFC at the bottom of the cell culture plate

Eight-welled cell culture plates (8W2x1E) from Applied Biophysics Inc. (Troy, USA), called medusa plates (Supplementary materials, Fig. S1a), were used. Each well contained two gold electrodes of 0.25 mm diameter. In this work, the electrodes were pre-coated by pipetting 2 μL of 1% (w/w) PEI solution on their surface. This step prevented the droplets with EFC components, as specified below, from sliding out of the electrode position. The biocathode and bioanode were modified with a total of three and five layers of AuNP, respectively. For each AuNP layer 2 μL concentrated slurry of AuNPs was pipetted on top of the PEI-modified gold electrode. After drying deposited AuNPs, BOx solution (2 μL, 1 mg mL^{-1}) was placed directly on the biocathode and left to dry in air, followed by three subsequent washing steps by PBS buffer. The bioanode was coated with a monolayer of MHP by depositing 2 μl of 1% (w/w) MHP solution for 2 h, after which the electrode was carefully rinsed with PBS buffer. CDH alone in a concentration of 1.0 mg mL^{-1} or CDH and Cat mixture at a 6:4 ratio (0.6 mg mL^{-1} CDH and 0.4 mg mL^{-1} Cat) were dropped on the AuNP–MHP-modified electrodes and left for 1 h to dry. The bioanode and biocathode electrodes were used in cell culture experiments immediately after three washing steps with PBS buffer.

2.6. Evaluation of the performance of EFC in cell culture plate

The performance, *i.e.*, current-potential dependences and stability of the EFC on medusa plate, was first tested in PBS buffer and in cell culture medium containing 5 mM lactose. After checking the background currents with cyclic voltammetry (CV) the bioanode and biocathode (Supplementary materials, Fig. S2a) were