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Bacterial-biofilm enhanced design for improved electrocatalytic reduction of oxygen in neutral medium

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ABSTRACT

The specific reactivity and ability of biofilms to form stable polymer-like hydrogel aggregates of microorganisms adhering to common solid (including the glassy carbon electrode) surfaces have been explored here to form systems analogous to electrocatalytic redox-polymer modified electrodes. Growth of biofilms has been demonstrated with use of *Yersinia enterocolitica*, a robust Gram-negative rod-shaped bacteria known to be resistive to pH changes (4-10) and temperature variations (0–40 °C). Charge distribution and propagation within the biofilm have been enhanced by introduction of multi-walled carbon nanotubes. The fact that carbon nanotubes are derivatized with the carboxyl-group containing 4-(pyrrole-l-yl) benzoic acid has facilitated the hybrid material integrity and stability, namely through electrostatic attractive interactions between anionic carboxyl sites and positively charged domains of bacterial aggregates. In neutral media, the biofilm-based composite (hybrid) matrices have exhibited themselves electrocatalytic activity during electroreductions of oxygen and hydrogen peroxide (with possibility of its sensing in a broad range of concentrations). By immobilizing additional catalytic (cobalt porphyrin) sites, a truly bifunctional redox-polymer-like electrocatalytic system capable of significantly enhancing oxygen reduction currents has been produced. Apparently, the reduction of oxygen (to hydrogen peroxide) is initiated at cobalt porphyrin centers, and the second step (decomposition of hydrogen peroxide intermediate to water) is pursued at reactive sites (perhaps c-cytochrome) existing within biofilm matrix. Comparative measurements have been performed with the biofilm-supported platinum nanoparticles as well as with such a model catalytic system as platinized carbon nanotubes. The proposed electrode designs are relevant to biosensing and to the development of alternate cathode materials for biofuel cells or biobatteries.

1. Introduction

There has been growing interest in fabrication of electrocatalytic systems for oxygen and hydrogen peroxide reductions that would be useful in biological media, i.e. neutral solutions. While stable bioelectrocatalytic systems for efficient (four-electron and preferably with low-overpotential) oxygen reduction are of importance to such technologies as biofuel cells and biobatteries, the well-behaved catalytic electrodes for accurate and reliable voltammetric or amperometric detection and determination of hydrogen peroxide are of interest to the development of both biomedical and environmental sensors.

Oxygen biocathodes utilizing fungal laccases or bilirubin oxidases are highly specific and often perform better at ambient temperatures than noble metal (e.g. platinum) based catalysts for the oxygen electroreduction in neutral media. The enzymes belong to a group of proteins with the copper active centers, and they can lower the oxygen reduction reaction overpotential both in the absence [1,2] and presence of mediators [3]. Despite significant progress in their practical utilization, high cost and the complex proteic structure of those enzymes often result in lack of stability and poor reproducibility of operation [4].

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Attractive new concepts of designs for alternate bioelectrocatalytic systems originate from recent advances in microbial fuel cell research. Bacteria have been demonstrated to catalyze oxidations of a wide array of organic compounds via a few, direct or indirect, mechanisms of electron transfer between the electrode and microbial cell. More precisely, the microbe-electrode interactions involve excretion of soluble redox mediators or secondary metabolites [5], electron transfer through membrane-bound cytochromes, or extracellular electron transport occurring via conductive pili (so-called electron conducting nanowires) [6–9]. Mixed cultures of bacteria growing at cathodes of microbial fuel cells have been demonstrated to significantly reduce the overpotential for oxygen reduction presumably by inducing the four-electron reduction mechanism to water [10–15] rather than less effective two-electron reduction of oxygen to hydrogen peroxide. The oxygen activation mechanisms [16] and the charge distribution dynamics within the bacterial based systems are still object of discussion.

In the present work, we consider *Yersinia enterocolitica* as Gram-negative bacterium [17] capable of growing stable biofilms in the 0 to 45 °C temperature range. Among important features for electrocatalytic application is the system's resistivity to pH changes what is crucial when it comes to studying redox processes involving protons (e.g. oxygen reduction). *Y. enterocolitica* is known to exist at pH's from 4 to 10; and it often survives even under truly acidic [18] or harsh environmental conditions [19,20]. *Yersinia* genome contains genes associated with environmental stress modulations. Consequently, *Y. enterocolitica* survives diverse environmental insults such as high temperature, hydrogen peroxide (which appears as the undesirable oxygen reduction intermediate), osmolarity and low pH's [21]. We show here that this bacterium exhibits itself electrocatalytic properties and, by analogy to electroactive bacteria [22,23], it is capable of mediating or inducing the oxygen reduction.

Macrocyclic N_4-complexes are known to catalyze redox reactions involving such simple diatomic inorganic molecules as O_2, H_2, and N_2. Representative examples include enzymatic processes with sulfite reductase [24], nitrate reductase, cytochrome c oxidase [25], blue copper oxidases, pseudo-catalase [26], photosystem II [27], nitrogenase and hydrogenase [28]. Many macrocyclic N_4-complexes of cobalt and iron have often been considered as the reduction electrocatalysts [29], particularly toward the reduction of oxygen [30].

To improve dynamics of charge propagation within three-dimensional biofilms (i.e. having thicknesses on the micrometer level), we have introduced multi-walled carbon nanotubes (CNTs) [30–34] that are characterized by good electronic conductivity and mechanical stability. Literature examples included electrocatalytic reductions of hydrogen peroxide, oxygen and carbon dioxide [35–37]. When combined with metalloporphyrins, the CNT-containing biocatalytic layers exhibited reasonable activity during reduction of oxygen. Nevertheless, the H_2O_2 intermediate, rather than H_2O, was predominantly produced as final product under such conditions [35,38–40].

In this study, to produce a hybrid biofilm-based bioelectrocatalytic film, we have utilized derivatized or functionalized multi-walled carbon nanotubes (CNTs) [41–43], namely CNTs modified with ultra-thin layers of organic, 4-(pyrrole-1-yl) benzoic acid (PyBA), anionic adsorbates [43] capable of exhibiting attractive interactions with positively charged domains of the biological matrix. The fact that our hybrid system has also contained Co-porphyrin centers (capable of inducing electroreduction of oxygen), in addition to presence of reactive sites (possibly c-type cytochromes) within the biofilm matrix (capable of catalyzing reductive decomposition of the hydrogen peroxide intermediate) has led to enhancement effect in terms of both increasing of the oxygen reduction current densities (important in biosensing) and shifting potentials toward more positive values (important in bioenergetics). In other words, we have extended our previous concept of fabricating the bifunctional bioelectrocatalytic system for oxygen reduction [43] to a new design in which, instead of enzyme, biofilm is used. Importance of the biofilm based matrix toward hydrogen peroxide reduction is also evident from kinetic analysis based on measurements of steady-state currents at different concentrations. Finally, comparative diagnostic experiments have been performed model catalytic Pt nanoparticles.

2. Experimental

2.1. Chemicals and reagents

All chemicals were obtained from Sigma–Aldrich and are of highest available purity. Co(III) protoporphyrin IX was from Frontier Scientific; Nafion, multi-walled carbon nanotubes (CNTs) and 4-(pyrrole-1-yl) benzoic acid (PyBA) were from Aldrich. Platinum black was obtained from Alfa Aesar. CNTs were purified as reported elsewhere [44] by exposing them to 12 mol dm^{-3} HCl solution for 1 h, followed by treatment with 3 mol dm^{-3} HNO_3 for 8 h under reflux conditions. Later, the CNT samples were washed with large amounts of water until pH ≈ 7 was reached.

Solutions were prepared using triply-distilled subsequently-deionized (Millipore Milli-Q) water. They were de-aerated (using pre-purified argon), or saturated with oxygen, for at least 10 min prior to the experiments. Argon was also used to keep air-free atmosphere over the solution during measurements. Experiments were conducted at room temperature (20 ± 0.5 °C).

2.2. Bacterial culture and biofilm formation

Yersinia enterocolitica (Ye9; wild-type, bacterial strain pYV^+, serotype O:9) was obtained from the Applied Microbiology Facility, Faculty of Biology, University of Warsaw. The *Y. enterocolitica* strain Ye9 was originally from the collection existing in National Institute of Public Health – National Institute of Hygiene (NIPH – NIH), Poland. Identification was also confirmed by phenotypic and genotypic methods based on the analysis of the highly conserved 16 S rRNA (rrs) gene and other biomarkers (virulence genes).

Bacteria were cultivated strictly aerobically at 25 °C in a standard Luria-Bertani (LB) medium, which was a mixture composed of 10 g of peptone, 5 g of yeast extract and 10 g of sodium chloride per liter. After growing under aerobic conditions (with shaking for 24 h), the bacterial culture appearing in stationary phase constituted the diluted culture of *Y. enterocolitica*. Then the suspension was diluted (1:50) in the LB medium (until the optical density OD600 = 0.1). Thus prepared cell-suspension was poured over the glassy carbon plate and incubated for 48 hours at 25 °C. Later, the resulting films were subjected to drying under argon atmosphere followed by over-coating with Nafion (as described later).

Thickness of the biofilm based catalytic layers was estimated to be on the level 3 μm (±0.6 μm; based on standard deviation of 8 independent experiments) by using an Alpha-step profilometer (Tencor Corp.). To obtain boundary between the bare and modified substrate during electrodeposition, the glassy carbon slide (electrode substrate) was only partially covered with the diluted cell suspension. The thickness value was approximate because it was difficult to distinguish bare and modified portions at the planar electrode substrate.