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Surface modifications for enhanced enzyme immobilization and improved electron transfer of PQQ-dependent glucose dehydrogenase anodes

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ABSTRACT

Pyrroloquinoline quinone dependent soluble glucose dehydrogenase (PQQ-sGDH) enzymatic MWCNT electrodes were p roduced using 1-pyrenecarboxylic acid (PCA) activated through carbodiimide functionalization and 1-Pyrenebutyric acid N-hydroxysuccinimide ester (PBSE) as tethering agents. At 600 mV potential, the current density generated by the activated-PCA tethered PQQ-sGDH anode was significantly greater than the current density generated by the untethered PQQ-sGDH and PBSE tethered anodes, and performance was nearly identical to the performance of a covalently bound PQQ-sGDH anode. A technique for covalently bonding heme-b (hemin), a natural quinohemoprotein porphyrin redox cofactor, to carbon nanotubes modified with arylamine groups is reported. The resulting performance of the covalently bound hemin PQQ-sGDH anode is considerably higher than that of any other PQQ-sGDH anodes tested.

1. Introduction

Enzymatic electrodes, in which a polarized support material acts as either an electron donor or acceptor, take advantage of the fact that in the absence of a natural substrate some oxidoreductases are capable of direct electron transfer (DET) from the enzymatic active center to the electrode surface or vice versa [1]. However, unlike the well-choreographed interactions between redox enzymes and their natural substrates, the communication between an enzyme and a solid electrode like multi-walled carbon nanotubes (MWCNTs) [2] is hampered by relatively long electron tunneling distances [3]. Subsequently, the performance of biofuel cells or bio-sensors is restricted by, among other factors, the rate of interfacial electron transfer between the enzyme and the surface of the electrode [4,5]. Some of the enzymes capable of DET include multi-copper oxidases [6–12] and pyrroloquinoline quinone (PQQ) containing enzymes (i.e. quinoproteins and quinohemoproteins) [13,14], which do not require external mediators or diffusion of coenzymes and can interact electronically directly with the electrode support. With these enzymes it is has been demonstrated that a decrease of the electron tunneling distance between the enzyme and the electrode interface can be achieved by immobilizing the enzyme on the electrode surface covalently or non-covalently via bi-functional tethering agents [15–20]. 1-Pyrenebutyric acid N-hydroxysuccinimide ester (PBSE) is one of the most commonly used tethering agents and has been shown to preserve enzymatic activity and improve the electrocatalytic response of the biocatalyst [21–23]. Another approach for improving DET efficiency is to selectively orient the active site of the enzyme towards the electrode and in doing so further reduce the distance the electrons have to tunnel to or from the electrode [24–27].

In certain enzymes, such as NAD-dependent dehydrogenases, the coenzyme plays the role of a mediator providing the electron transfer by accepting electron(s) from the enzyme and shuttling them to the terminal electron acceptor, becoming oxidized and then returning to the enzyme to start the cycle over again in a process known as mediated electron transfer (MET) [28]. The advantage to the MET mechanism with regard to electrode development is that the large electron tunneling distance between the enzyme active site and the electrode is not as important of a factor as it is in DET. This is due to the mobility of the electron carriers, where the rate of the electron transfer is proportional to the concentration of the mediator, its diffusion, the mediator turnover rate from the enzyme, and the facility of the mediator-electrode electron transfer [29]. Enzymes that do not rely on specific external cofactors are not exempted from benefitting from MET enhancement, and

Abbreviations: DET, direct electron transfer; MET, mediated electron transfer; MWCNT, multi-walled carbon nanotube; MWBP, multi-walled buckypaper; PQQ, pyrroloquinoline quinone; PQQ-sGDH, pyrroloquinoline quinone dependent soluble glucose dehydrogenase; PBSE, 1-Pyrenebutyric acid N-hydroxysuccinimide ester; PCA, 1-pyrenecarboxylic acid; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; Sulfo-NHS, N-hydroxysulfosuccinimide sodium salt; PCA (EDC), PCA activated with EDC; Am, arylamine; AmHeme, hemin covalently bound to arylamine; AmPQQ, PQQ covalently bound to arylamine; AmIX, protoporphyrin IX covalently bound to arylamine; AdHeme, physically adsorbed hemin.

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engineering approaches to address the ohmic losses associated with the long tunneling distance of DET have resulted in utilization of mediators such as ferrocene [30] or its derivatives to help bridge the interfacial electron transfer distance and improve the electron transfer rate. However, mediators dissolved in aqueous electrolyte tend to diffuse or be transported away from the interfacial region meaning that a high concentration of these compounds throughout the electrolyte is necessary for sustained electron transport. Techniques for counteracting loss of the mediator from the interfacial active region have been developed, such as entrapping the mediator in a membrane [31] or otherwise binding it to the electrode support material [32,33]. Additionally, "wiring" of the electrode surface with PQQ and then reconstituting the enzyme onto the immobilized cofactor has been shown to improve the electron transfer from PQQ-dependent dehydrogenases to the electrode [34–37]. In quinohemoproteins, the PQQ cofactor is involved in the oxidation of the external electron source and is the first molecule in a subsequent chain of internal electron transfer steps involving one or more heme molecules before the reduction of the enzyme terminal electron acceptor. One type of heme found in many quinohemoproteins is heme-b, Fe(III) protoporphyrin (IX) chloride, available synthetically in the form of hemin. The macrocyclic structure of hemin allows it to be immobilized on an electrode surface via physical adsorption and has been shown to have the ability to participate in electron transfer reactions such as in reducing dissolved oxygen [38].

Towards the goal of expanding on the above techniques for enhancing electron transfer of quinoproteins, this study summarizes the results of non-covalent enzyme attachment with another type of tethering agent, activated 1-pyrenecarboxylic acid (PCA), in an attempt to compare the performance of PCA tethered PQQ-dependent soluble glucose dehydrogenase (PQQ-sGDH) anodes and anodes tethered with the well-studied PBSE. This work also reports on a technique for covalently immobilizing hemin onto MWCNT via deposition of arylamine groups and amide bond formation through carbodiimide reactions. The immobilized hemin was shown to have a positive effect on the electrochemical output of a PQQ-sGDH anode, which can be used in the development of enzymatic fuel cells for energy harvesting or explored for improving the design of glucose biosensors.

2. Materials and methods

2.1. Materials

Pyrenecarboxylic acid (PCA), 1-Pyrenebutyric acid N-hydroxysuccinimide ester (PBSE), Ethanol (EtOH), isopropyl alcohol (IPA), dimethyl sulfoxide (DMSO), 3-(N-morpholino) propanesulfonic acid (MOPS), $CaCl_2$, KCl, p-phenylenediamine (PPD), $NaNO_2$, 1N HCl, hemin (iron(III) protoporphyrin (IX) chloride) from Porcine, N-hydroxysuccinimide (NHS), protoporphyrin IX, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS), phenazine methosulfate (PMS), nitrotetrazolium blue (NTB), and D-glucose were obtained from Sigma (Sigma Aldrich, St. Louis, MO). 60gsm MWCNT buckypaper (MWBP) was obtained from Buckeye Composites (NanoTechLabs, Inc., Kettering, OH). PQQ-sGDH was obtained from Toyobo Enzyme (Toyobo U.S.A., Inc., New York, NY). Deionized (DI) water was HPLC grade. All materials were used as received unless indicated.

2.2. Anode preparation

2.2.1. Enzyme tethering

A 0.5 cm diameter circular disk of MWBP was submerged in a 10 mM solution of PCA or PBSE in EtOH or DMSO, respectively for 1 h. The MWBP disk was then rinsed three times in DI water and transferred to a 10 mg ml^{-1} solution of PQQ-sGDH in 20 mM MOPS buffer (pH 6.0) containing 6 mM $CaCl_2$ and 10 mM KCl. The MWBP was left in the enzymatic solution for 12 h (unless indicated otherwise) at 4 °C to allow enzyme immobilization on the disk to occur. After this step the disk was rinsed in MOPS buffer to remove any enzyme that wasn't attached to the MWBP.

2.2.2. Arylamine deposition

A 10 cm^2 MWBP sheet was pre-treated through submersion in IPA for 10 min. A solution of 1 mM PPD and 10 mM $NaNO_2$ in 0.5 M HCl was prepared in an ice bath, placed in the dark, and purged with nitrogen for 5 min. The solution was then transferred to an EC cell and the pre-treated MWBP sheet was placed in the solution and connected to a potentiostat as the working electrode. 3 M (non-saturated) Ag/AgCl reference electrode and stainless steel mesh counter electrodes were used to perform the electrochemical grafting in three-electrode mode. Consecutive CV passes were run from 0.4 V to −0.2 V at a scan rate of 100 mV s^{-1}.

2.2.3. Hemin physical adsorption

Physically adsorbed hemin samples were prepared by submerging a 0.5 cm diameter unmodified MWBP disk in a 10 mM solution of hemin in DMSO for 1 h after which the disk was rinsed three times in DI water and further exposed to the enzyme as necessary.

2.2.4. Hemin, PQQ, and protoporphyrin IX attachment

For covalent bonding of hemin, PQQ, and protoporphyrin IX, the arylamine modified MWBP sheet, prepared as described above, was incubated in a solution of 10 mM hemin, PQQ, or protoporphyrin IX in DMSO. NHS and EDC were added to the solutions to final concentrations of 20 μM and 40 μM, respectively. The mixture with the immersed MWBP was then stored at ambient temperature in a sealed container for 1 h. After this time the MWBP sheet was removed from the mixture, washed with DI water, and dried overnight. 0.5 cm diameter disks were then cut out of the modified MWBP sheet and PQQ-sGDH was attached to it as the experiment required.

2.2.5. Enzyme attachment through activated-PCA

The carboxylic groups of PCA were activated by EDC/Sulfo-NHS and amide bonds with the amine groups of PQQ-sGDH were formed. The process was initiated by first immobilizing the tether on a MWBP disk as described above and then submerging the disk in a 10 mg ml^{-1} PQQ-sGDH MOPS solution, followed by the addition of Sulfo-NHS and EDC to final concentrations of 20 μM and 40 μM, respectively. The disk was incubated in the solution for 2 h at 4 °C and then rinsed three times in MOPS buffer.

2.3. Time resolved ultraviolet–visible (UV–Vis) absorption characterization

UV–Vis absorption characterization of PQQ-sGDH in solution was performed according to the procedure described by the manufacturer (Toyobo Enzyme) using a reaction mixture of 0.9 ml 1 M D-glucose solution, 25.5 ml MOPS buffer (pH 6.0), 2.0 ml 3 mM PMS solution and 1.0 ml 6.6 mM NTB solution. The immobilized PQQ-sGDH samples required modification of the procedure to accommodate the inclusion of the MWBP disks which were prepared as described above, rinsed, and then pressed against the wall of an individual well of a 96-well plate. Samples were tested using a Spectramax M5 well-plate reader (Molecular Devices, Sunnyvale, CA) and in all cases after the sample was inserted in to the reader, the temperature was raised to 37 °C before the D-glucose/PMS/NTB stock solution was added. The sample absorbance at 570 nm was measured against a blank composed of MOPS buffer. The absorbances of the immobilized PQQ-sGDH samples (A_{imm}), were normalized to the