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Electrical Wiring of Live, Metabolically Enhanced *Bacillus subtilis* Cells with Flexible Osmium-Redox Polymers

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Abstract: The present study explores genetic engineering of the respiratory chain and the application of two different flexible osmium redox polymers to achieve efficient electric communication between the gram-positive organism *Bacillus subtilis* and an electrode. Poly(1-vinylimidazole)$_{12}$-[Os-(4,4′-dimethyl-2,2′-bipyridyl)$_2$Cl$_2$]$^{+/2+}$ (osmium redox polymer I) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (osmium redox polymer II) were investigated for efficient electrical "wiring" of viable gram-positive bacterial cells to electrodes. Using a *B. subtilis* strain that overproduces succinate/quinone oxidoreductase (respiratory complex II), we were able to improve the current response several fold using succinate as substrate, in both batch and flow analysis modes, and using gold and graphite electrodes. The efficiency of the osmium redox polymer, working as electron transfer mediator between the cells and the electrode, was compared with that of a soluble mediator (hexacyanoferrate). The results demonstrated that mediators did not have to pass the cytosolic membrane to bring about an efficient electronic communication between bacterial cells with a thick cell wall and electrodes.

Introduction

Electrochemical communication between viable bacterial cells and electrodes has been in focus for the past few years,[1−6] exploring different electron transfer pathways from the organism to an inert electrode material. This includes using various electron transfer mediators (produced by the living cell or artificial), through direct electron transfer via cytochrome rich membranes,[6] or electrically conductive pili produced by the bacteria.[3] In the case of added soluble mediators, both 1 e^- no H^+ acceptors (e.g., hexacyanoferrate) and 2 e^-, H^+ acceptors (e.g., thionine and neutral red) have been successfully used.[7−9] Some microorganisms self-mediate the electron transfer by producing their own mediators, for example, *Pseudomonas aeruginosa* was shown to produce pyocyanin,[10,11] *Escherichia coli* K-12 a "non-identified" self-mediator,[12] and *Shewanella* sp. various flavins.[13] Polymeric redox active films grown on metallic surfaces such as gold or platinum have also been used in microbial fuel cells with increased efficiency, for example, polyaniline and fluorinated polyanilines on platinum.[14−16] However, there is no general agreement on the mechanism on how mediators interact with the living cell, for example, whether the mediator needs to penetrate the membrane to communicate with the living cell or not.

Since the first applications of flexible osmium redox polymers for enzyme based, reagentless mediated biosensing were described,[17,18] polymeric mediators still attract attention due to the efficient electron shuttling properties combined with the polymeric structure, promoting a stable adsorption, as well as a possibility for multiple layers of both immobilized enzymes[19−21] and microbial cells[22−25] on the electrode surface.

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Artificial mediators offer the possibility for an enhanced electron transfer between microbial cells and electrodes. The important role of these electron shuttles in whole cell biosensors and microbial fuel cells is to replace the natural final electron acceptor (e.g., oxygen in the case of aerobic bacteria, Fe(III) oxides/complexes in the case of anaerobic organisms), thus preventing the problem of limiting concentrations of the electron acceptors. It has been shown that, for single enzymes, the rate constants for osmium polymer mediation varies with the mediator redox potential, demonstrating that the mediator-enzyme overpotential should be optimized for maximum power output.[26]

In the present study, two different flexible osmium redox polymers: poly(1-vinylimidazole)$_{12}$-[Os-(4,4′-dimethyl-2,2′-bipyridyl)$_2$Cl$_2$]$^{+/2+}$ (osmium redox polymer I) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (osmium redox polymer II) were investigated for efficient electrical wiring of the gram-positive bacterium *Bacillus subtilis*. The two Os-polymers were chosen because they differ greatly in both redox potential and the length of the side chains, where the $Os^{2+/3+}$ functionalities are located at their ends. Furthermore, their efficiency in wiring gram-negative bacteria was previously investigated.[22−25] It was concluded that, due to the high molecular weight of the Os-polymers, mediation could be brought about without penetration of the inner cytoplasmic membrane.[22−25] The current research is thus a logical continuation of previous work from our group, involving the electrical connection between viable bacterial cells and electrodes using osmium polymers. Our initial studies[22] in this field were made with the structurally rather simple gram-negative *Gluconobacter oxidans*,[27] where we addressed redox enzymes from the cytoplasmic membrane yielding response for glucose, fructose, ethanol, and glycerol. In further studies focus was on the structurally more complex gram-negative *Pseudomonas putida* and *Pseudomonas fluorescens*,[23,24] where response currents could be obtained both for substrates being metabolized in the cytoplasmic membrane (glucose) as well as in the cytosol of the cell (phenol). Recently we have also showed that introduction of a cytochrome to the cytoplasmic membrane of *E. coli* greatly facilitated the communication between these gram-negative bacterial cells and the osmium polymers.[25] In the current study we use the gram-positive model organism *B. subtilis*, with a substantially thicker peptidoglycan cell wall, which at a first glance could be expected to be more difficult to permeate by the osmium polymeric mediators. In *B. subtilis* the cell wall has a diameter ≈35 nm.[28] It constitutes a multilayered structure composed mainly of peptidoglycan and teichoic acids. The polyelectrolytic properties of the peptidoglycan and teichoic acids provide a continuum of anionic charge between the cytoplasmic membrane and the environment.[29] These properties of the cell wall may instead facilitate the connection between the cells and the polycationic Os-polymer and further to the electrode. We believe that the approach taken in this work adds to the understanding of how gram-positive cells may communicate with their surroundings through electron conductive structures present in the layers of peptidoglycan/teichoic acids. This is also in line with the recent hypothesis raised by Ehrlich[30] on that electron conducting structures are present in the periplasm of gram-positive bacteria (peptidoglycan, teichoic acids), which must be responsible for conveying electrons from the cytoplasmic membrane to the outer surface of the cell wall. Another recent publication that support such a theory is the work by Marshall and May,[31] who show that gram-positive *Thermincola ferriacetica* strain Z-0001 readily can grow onto a graphite electrode and exhibit direct electron transfer communication.

To improve the electron efficacy further, both by adding transmembrane cytochrome like in previous work with *E. coli*,[25] but also to enhance the metabolic turnover, we used the *B. subtilis* strain 3G18/pBSD-1200, which overproduces succinate:quinone oxidoreductase (SQR) from a plasmid about 5-fold compared to wild type bacteria. SQR is also referred to as respiratory chain complex II, with EC 1.3.5.1. SQR is present in the respiratory chain of mitochondria and in many microorganisms.[32] SQR and quinol:fumarate reductase (QFR) comprise a family of enzymes that have a conserved general structure in both pro- and eukaryotes. SQR catalyzes a two electron succinate oxidation, transferring the electrons via flavin and FeS clusters to the quinone binding site(s) for concomitant two electron reduction of quinone to quinol. The structures of *E. coli* SQR, which contains one heme,[33] and of two QFRs, the QFR from *E. coli* (which lacks heme) and the QFR from *Wolinella succinogenes* (which contains two hemes) are known.[34,35] The latter enzyme closely resembles *B. subtilis* SQR (see Figure 1) that has been overproduced in the 3G18/pBSD-1200 strain, used in the current study. Spectroelectrochemical (redox mediator potentiometry) measurements of the redox potentials of the two hemes in *Bacillus subtilis* complex II was found to be around + 65 mV for heme$_H$ and −95 mV for heme$_L$ when working with solubilized SQR.[36−38] When the complex is situated in the membrane, the redox potentials of the two hemes were found to be 50 mV lower (+16 and −132 mV).[36,37] The succinate/fumarate redox couple has a pH dependent midpoint redox potential and is about +25 mV at pH 7.0.[39]

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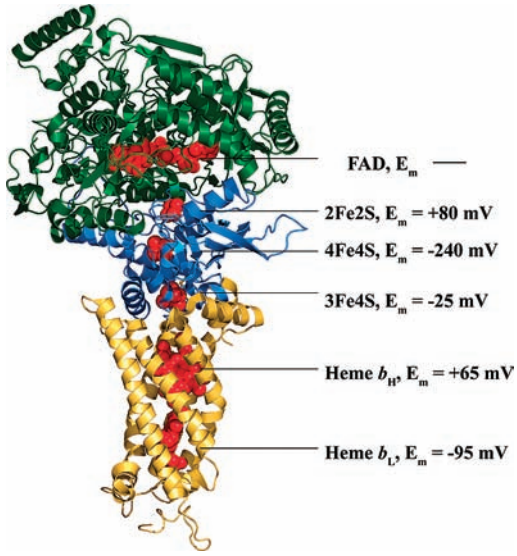

Figure 1. High resolution structure of *W. succinogenes* QFR (PDB 1QLA) solved at 2.2 Å resolution.[35] The enzyme comprises three protein subunits: subunit A, colored in green, with a covalently bound FAD; subunit B, colored in blue, containing three Fe−S clusters; subunit C, coured in yellow, containing two heme *b* groups. All six prosthetic groups are presented as red colored spheres, and the midpoint potentials correspond to *B. subtilis* SQR.

First, the performance of the redox polymers as electron acceptors directly active with the living gram-positive cell was investigated. The influence of the presence of oxygen in the buffer on the current response was evaluated. The electron transfer efficiency of the osmium redox polymer was compared with that of a soluble mediator, hexacyanoferrate. The current response characteristics of the bacterial cell modified electrodes were evaluated for succinate oxidation in both batch analysis and flow analysis mode, using gold and graphite electrodes. The viability of the "wired" cells was carefully considered and checked throughout the experiments.

Experimental Section

Organism and Culture Conditions. *Bacillus subtilis* 3G18/pBSD-1200 contains the entire *sdhCAB* operon on a low copy (5−10 copies per cell) number plasmid.[36] The strain was kept on Tryptose blood agar base medium (TBAB) with 5 μg/mL chloramphenicol. For SQR production the bacterial cells were grown in nutrient sporulation medium with phosphate (NSMP), containing 5 μg/mL chloramphenicol.[40] Batches of 100 mL were incubated in 1 L baffled E-flasks, aerated at 200 rpm at 37 °C. The cells were harvested in the early stationary growth phase, where the SQR content is maximal.[41] The cells were harvested by centrifugation at 12000 × g for 15 min, washed once in 50 mM potassium phosphate buffer at pH 8.0, centrifuged again as before, resuspended in 20 mM MOPS buffer at pH 7.4 to a concentration of 1 g/mL (wet weight), and used immediately for electrochemical measurements.

For some experiments the cytoplasmic membrane was isolated and purified according to a previously published protocol.[36,42]

Chemicals. Poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-bipyridyl)$_2$Cl$_2$]$^{+/2+}$ (osmium redox polymer I) was generously donated by TheraSense (Alameda, CA, http://www.therasense.com/). Poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (osmium redox polymer II) was synthesized according to a previously published protocol.[43] Succinic acid, D(+)glucose, potassium chloride, MOPS (3-(*N*-morpholino)propanesulfonic acid sodium salt) and aldrithiol-4 (4,4′-dipyridyl disulfide) were purchased from Sigma-Aldrich Chemicals (Steinheim, Germany). All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using water purified and deionized (18 MΩ) with a Milli-Q system (Millipore, Bedford, MA).

Preparation of the Electrodes Modified with Bacteria. Gold electrodes (BAS, West Lafayette, IN, $A = 0.02$ cm^2) were electrochemically cleaned by cycling in 0.1 M NaOH between 0 and −1000 mV versus NHE, followed by mechanical polishing on Microcloth (Buehler, Lake Bluff, IL) in an aqueous alumina FF slurry (1 and 0.1 μm, Struers, Copenhagen, Denmark). The electrodes were rinsed with water, ultrasonicated for 5 min in Milli-Q water, followed by cycling in 0.5 M H_2SO_4 between 0 and +1950 mV vs NHE, and finally rinsed with Milli-Q water. Formation of a self-assembled monolayer (SAM) of aldrithiol at the electrode surface was done by immersing the electrode in a saturated aqueous solution of the thiol for 2 h (at room temperature). In the following step, a 5 μL portion of osmium redox polymer solution (10 mg/mL in Milli-Q water) was spread over the surface of the modified gold electrode and water was allowed to evaporate at room temperature for 30 min. Finally, a 5 μL portion of *B. subtilis* suspension in 20 mM MOPS buffer (pH 7.4) was dropped on the modified electrode surface. The droplet was allowed to gently dry for 1 h at room temperature and then a dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA, molecular mass cutoff: 6000−8000) was used to trap the bacterial cells onto the surface to prepare a permselective membrane electrode.[44] The dialysis membrane (presoaked in buffer) was pressed onto the electrode and fixed tightly to the electrode with a rubber O-ring and Parafilm.

When using graphite electrodes, the material was spectrographic graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity, http://www.sglcarbon.com/). The electrode was cut and polished on wet emery paper (Tufbak, Durite, P1200) and afterward carefully rinsed with Milli-Q water and dried. Then 5 μL of osmium polymer solution was spread onto the entire active surface of the electrode (0.0731 cm^2). The electrode was dried at room temperature for 10 min and then a 5 μL of the cell suspension was spread onto the surface and further dried until a layer was formed on the surface. The modified electrode was mounted in a flow through electrochemical cell and the required potential was applied until a stable background current was obtained before any substrate injection into the flow system.[45]

Measurement Setup. All measurements were performed at room temperature: 22 ± 1 °C. Cyclic voltammetry was performed using an AUTOLAB PGSTAT 30 (Eco Chemie, Utrecht, The Netherlands) equipped with GPES 4.9 software. A standard three-electrode configuration was used with an Ag|AgCl (sai'd KCl) reference electrode (Radiometer, Copenhagen, Denmark) and a counter electrode made of a platinum foil.

Amperometric measurements were done in both batch and flow modes. Batch mode experiments were performed using the same electrodes as for cyclic voltammetry. In flow mode, a standard three electrode flow through amperometric wall jet cell was used,[45] containing the working electrode (gold or graphite electrodes modified with cells), an Ag|AgCl (0.1 M KCl) reference electrode (Beta Sensor AB, Södra Sandby, Sweden, http://betasensor.com), and a counter electrode made of a platinum wire, all connected to the AUTOLAB PGSTAT 30. The modified electrode was press fitted into a Teflon holder and inserted into the wall jet cell and kept at a constant distance (ca. 1 mm) from the inlet nozzle. A

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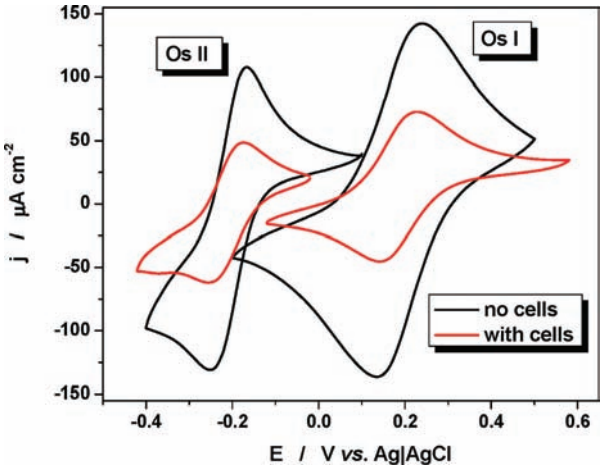

Figure 2. Cyclic voltammograms of the osmium redox polymers (types **I** and **II**) adsorbed at a thiol modified gold electrode, in the absence and presence of bacterial cells. Experiments were performed in a 20 mM MOPS buffer (0.1 M KCl) at pH 7.4. Scan rates are 10 mV/s.

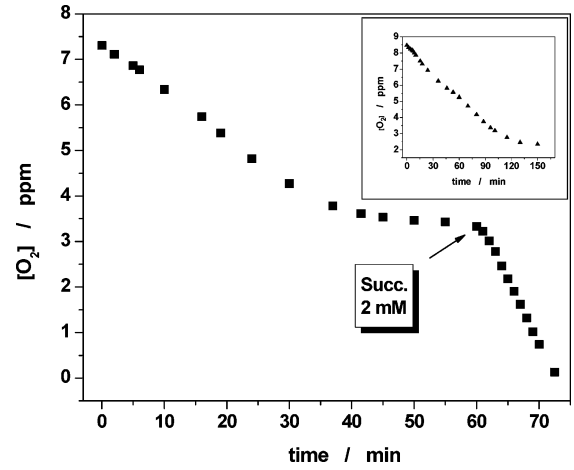

Figure 3. Oxygen level measurements for a suspension of *B. subtilis* cells in buffered solution; the arrow indicates the addition of substrate. The graph inset shows the oxygen consumption for the bacterial cells mixed with the osmium polymer type **I**.

peristaltic pump Minipuls 2 (Gilson, Villiers-le-Bel, France) maintained the flow of the solutions at 0.5 mL/min.

For the flow injection experiments a small potentiostat (Zäta Elektronik, Höör, Sweden) was used. The response currents were recorded on a strip chart recorder (Kipp & Zonen, Delft, The Netherlands). The injector was an electrically controlled six-port valve (Rheodyne, Cotati, CA), and the injection loop volume was 50 μL. The MOPS buffer (20 mM MOPS (pH 7.4), 0.1 M KCl), used as electrolyte, was carefully degassed under vacuum prior to experiments. In the cases where an inert atmosphere was needed, the solutions were instantly degassed with argon and during measurements argon was blown over the solution.

Oxygen consumption measurements of cells and of cells mixed with an Os-polymer suspended in 20 mM MOPS at pH 7.4 were done using an HI 2400 dissolved oxygen bench meter (Hanna Instruments Inc., Woonsocket, RI) with PC communication.

Results and Discussion

The two osmium polymers (poly(1-vinylimidazole)$_{12}$-[Os-(4,4′-dimethyl-2,2′-bipyridyl)$_2$Cl$_2$]$^{+/2+}$ (**I**) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (**II**)) used in this study to "wire" viable gram-positive cells, the structures of which have been shown previously,[20,23,43] have different redox potentials (around +350 mV vs NHE for polymer **I** and 0 mV vs NHE for polymer **II**) and different electron transfer efficiencies due to different lengths of the side chains.[43] The cyclic voltammetric responses for Au electrodes modified with the two polymers in the absence and presence of bacterial cells are depicted in Figure 2. Given the same concentration (10 mg/mL) of the polymers in aqueous solution and the same amount of cells used for both electrodes the current responses are comparable for the two mediators. The decrease in the current response in the presence of bacterial cells is reasonable considering that the Os-polymers will form strong electrostatic complexes with the anionic peptidoglycan coating of the living cells. As a consequence, the mobility of the individual redox units is reduced and the current response lowered, which is expected, from previous investigations with redox enzymes.[19] However, as can be seen in Figure 2, the cells do not influence the redox potentials of the two polymers.

The viability of the cells trapped at the electrode surface is a critical issue. A way to check whether the cells are metabolically active is to measure the oxygen consumption of a suspension of the cells, because *B. subtilis* cells primarily use oxygen as the final electron acceptor. The cells were mixed with either of the osmium polymers and the respiratory rate was measured, to check for any possible toxicity to the cells. The oxygen consumption was not influenced by the presence of the Os polymer (see Figure 3, inset). After washing the *B. subtilis* cells and suspending them in the plain MOPS buffer, they still contain residual nutrients and consequently, the oxygen level is decreased to a constant level until the food reserves are depleted (Figure 3). Then when succinate is added to the solution, the cells start to respire almost instantly, and the oxygen level drops linearly with time.

Cyclic voltammetry using moderate scan rates (5−10 mV/s) exhibited some electrocatalytic efficiency in the presence of succinate for the two osmium polymers and bacterial cells in dearated solutions (results not shown), which is similar to what was found previously for gram-negative cells in the presence of various substrates.[22−25] The generated current density was somewhere in the order of several μA/cm^2 for both Os mediators. Because, in this case, cyclic voltammetry offered only qualitative information, the next step was to use amperometry at a stationary potential for more accurate, quantitative evaluation of the electron transfer efficiency. The amperometric measurements were performed in flow mode using a pump with a flow rate of 0.5 mL/min. For Os polymer **I** the applied potential was chosen to be +500 mV versus NHE (a potential value sufficiently more positive than the $E^{0\prime}$-value of the polymer, to guarantee a potential independent response). Once a stable baseline was obtained, 1 mM succinate was added to the buffer solution, and this succinate containing buffer was continuously pumped toward the modified gold electrode (Figure 4). When a visible, stable steady state current response was observed the concentration of succinate in the flow buffer was increased in 1 mM steps up to 4 mM. When the concentration of succinate in the running buffer reaches a value around 4 mM, the current response signal starts to decrease, a phenomenon observed when using both mediators. The decrease in the signal could be attributed either to product inhibition or pH changes inside the cell. It cannot be due to an accumulation of succinate over time inside the cells because the response decreases rapidly when starting the experiments directly with a 4 mM succinate solution (for details, see Supporting Information, Figure S1). The maximum current density response is somewhere around 5 μA/cm^2.

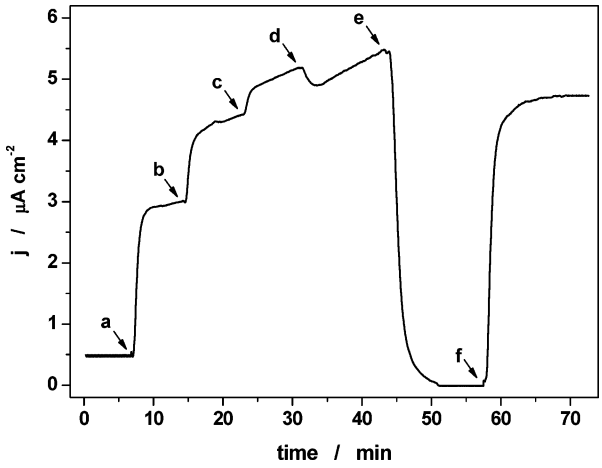

Figure 4. Current density response of intact *B. subtilis* (3G18/pBSD-1200) cells mediated with the osmium redox polymer **I** (E_{app} = +500 mV vs NHE) on an aldrithiol-modified gold electrode. The arrows indicate the successive addition of 1 mM succinate (**a**−**d**), washing with MOPS buffer (**e**), and addition of 3 mM succinate (**f**) in the buffer solution (20 mM MOPS, 0.1 M KCl, pH 7.4). The measurements were performed in a continuous flow mode using a peristaltic pump (0.5 mL/min).

In the case of Os polymer **II**, a similar approach as above was used and the applied potential (+200 mV vs NHE) was also here substantially more positive than the $E^{0\prime}$ value to guarantee a potential independent response. Various concentrations of succinate were added stepwise as above to the running buffer solution and the steady state current response was measured. The same inhibition phenomenon as above could be observed at succinate concentrations higher than 4 mM. The maximum current density response in this case is around 1.5 μA/cm^2 (see Supporting Information, Figure S2). The explanation for this difference between the two mediators will be further discussed below when discussing the effect of the presence of oxygen in the running buffer solution.

During the process of aerobic respiration oxygen is used as the final electron acceptor. For cell-electrode communication purposes, the electrons should preferably be donated to the electrode using the artificial mediator. In this case, a competition for the reduced equivalents generated inside the cells arises between molecular oxygen and the artificial electron acceptor system. Thus, the effect of completely removing oxygen (using argon) from the buffer solution was investigated. The results obtained were markedly different for the two osmium polymers (see Figure 5). When using the high potential (type **I**) polymer, the current response generated remained insensitive to changes in the oxygen concentration. In contrast, the response for the cells wired with the low potential polymer, increased drastically (more than 10×) when O_2 was removed from the solution and returned to a low level once air was bubbled through the solution. The most likely explanation for this difference is related to the superior thermodynamic driving force in the case of Os-polymer **I**, having an $E^{0\prime}$ value of +350 mV vs NHE. Thus, Os-polymer **I** efficiently competes with O_2 as electron acceptor, whereas Os-polymer II, because of its low $E^{0\prime}$ value (0 mV vs NHE), cannot efficiently compete with O_2 as electron acceptor. The response to glucose was also investigated. As can be seen in Figure 5 and Figure S3, in the presence of 2 mM succinate, an addition of 2 mM glucose does not result in a further increase in the response current, reflecting that the overproduced SQR is mostly responsible for the generated electrocatalytic current.

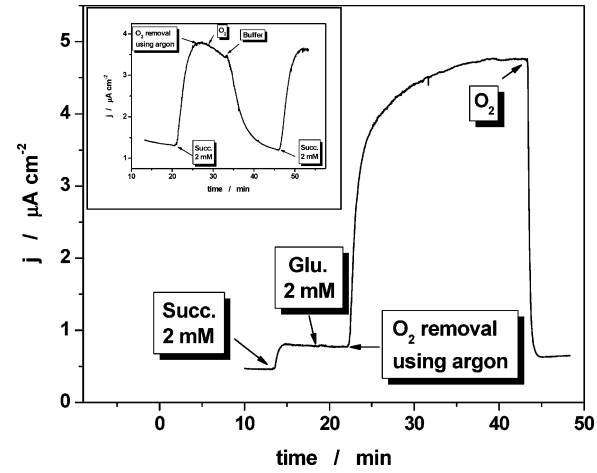

Figure 5. The impact of oxygen removal on the current density response generated by the presence of succinate at an osmium polymer **II** mediated *B. subtilis* (3G18/pBSD-1200) cells modified gold electrode. The graph inset shows the effect of oxygen on the Os polymer **I** mediated cells.

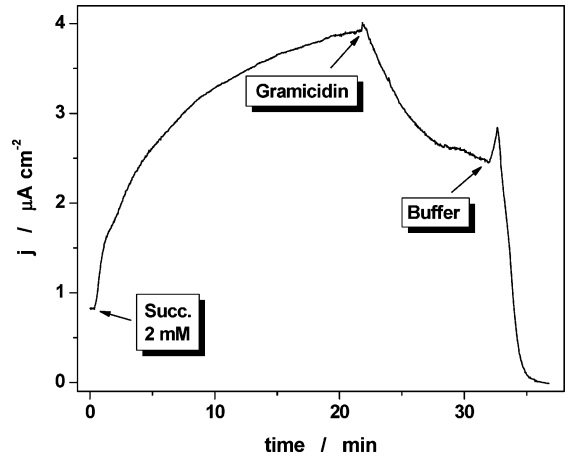

Figure 6. Addition of an uncoupler affects on the current density generated by succinate addition at a gold electrode modified with osmium redox polymer **II** mediated *B. subtilis* (3G18/pBSD-1200) cells. The measurements conditions are the same as in the previous figures.

To test the competition theory, the efficiency of the osmium polymers was also compared to that of a freely diffusing mediator, hexacyanoferrate (**III**). In this case, amperometry, when the system was polarized at a potential of +500 mV versus NHE ($E^{0\prime}$ of hexacyanoferrate is around +400 mV vs NHE) was used for the Os polymer **II** and the results were compared in the presence of oxygen (see Supporting Information, Figure S3). The low molecular weight and freely diffusing monomeric hexacyanoferrate (**III**) proves to be a better competitor for oxygen compared with the Os-polymer **II**, the current increasing approximately four times when using 2 mM succinate as substrate.

In *B. subtilis*, an electrochemical potential across the membrane ($\Delta\mu_H^+$) is required for succinate uptake into the cells.[46,47] When gramicidin was added to the flow buffer, dissipating the $\Delta\mu_H^+$, the current density response decreased with time (Figure 6). Gramicidin is an antimicrobial peptide that abolishes the electrochemical potential across the membrane by forming

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(47) Ghei, O. K.; Kay, W. W. *Biochim. Biophys. Acta, Biomembr.* **1975**, *401*, 440–57.

transmembrane pores that drastically increase the membrane's permeability to cations, eventually causing cell death.[48] Thus, the observed response to gramicidin further supports that the "wired" *B. subtilis* cells are fully intact and alive during the experiments described above. Control experiments with the isolated cytoplasmic membranes containing SQR in 2 mM succinate using either of the osmium polymers exhibited a response current, in agreement with the work by Jeuken et al.[42] However, when adding gramicidin no change in current density was shown revealing that in this instance there was no $\Delta\mu_H^+$ across the membrane initially.

Stability tests were also performed, following the current response of the system over long-term periods. This was done by continuously pumping a flow buffer containing a constant concentration of succinate for several hours through the flow cell housing the working electrode with the *B. subtilis* cells. The response slightly decreased with time and reached approximately 73% of the initial response after 6 h (see Supporting Information, Figure S4). When the pump is stopped and, thus, also the continuous steady state supply of succinate to the cells, the succinate consumption at the electrode surface can be followed with time by recording the decrease in current response. This shows that the turnover rate of succinate inside the cells is very high.

To check whether SQR is the main component responsible for the current generation when adding succinate, the response generated from an equivalent amount of wild type *B. subtilis* (strain 3G18) was compared with that of the cells of the 3G18/pBSD-1200 strain. Having the same amount of cells (mg/mL), using the low potential osmium polymer as mediator and removing oxygen from the solution, it is clearly seen (see Supporting Information, Figure S5) that the average response for, for example, 2 mM succinate is 9 times lower for the wild-type *B. subtilis*, confirming the utility and importance of the overproduced SQR in generating a high current response. This is of particular importance in microbial fuel cells, where the use of genetically modified bacteria could improve the power output of such devices.

The possibility of using graphite as electrode material was also evaluated. In this case, no membrane was used to entrap the cells and the polymer together on the electrode surface, but simple physical adsorption. Graphite electrodes were previously shown to be good electrode supports for a variety of gram-negative bacterial cells that show DET contact properties with electrodes.[49] As mentioned above in a very recent paper,[31] it is now also shown one example of a gram-positive bacterial cell can thrive onto graphite with DET characteristics. Graphite is a less hydrophobic material than the noble metals and it is anticipated that bacterial cells may be strongly adsorbed onto graphite with retained viability as was shown previously.[49] With this type of modified electrode the technique used to measure the response was flow injection analysis. The same method had previously failed to give satisfactory results in the case of the permselective *B. subtilis* gold-modified electrodes due to mass transfer restrictions causing long response and equilibration times. However, for both *Gluconobacter* and *Pseudomonas* cells physically entrapped behind a dialysis membrane onto a thiol-modified gold electrode, it was shown in the presence of the osmium polymers that the response is quick enough, allowing the flow injection mode to be used to test for response linearity and stability.[22,23] This shows that, for the assembly of gram-positive *B. subtilis* cells with osmium polymers on thiol-modified gold electrodes and a dialysis membrane, there is a higher mass transfer resistance than for the gram-negative *Gluconobacter* and *Pseudomonas* cells. Using graphite, a calibration curve (current density vs substrate concentration) was possible to be obtained. From Figure 7, one can also observe the substrate inhibition at high succinate concentrations (~4 mM) and also a linear dependence between the response and the concentration in the μM (5−200) range.

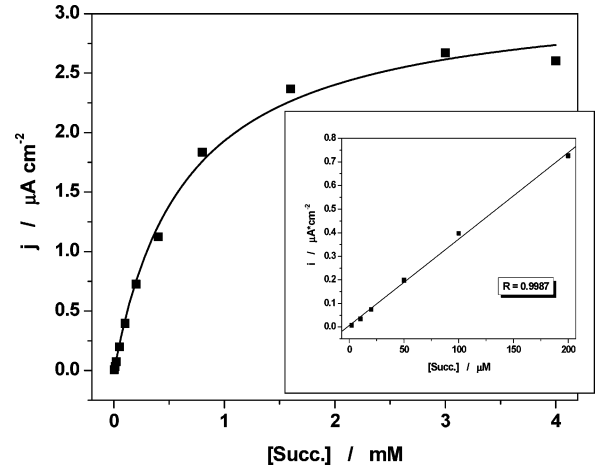

Figure 7. Calibration curve (current density vs substrate concentration) for succinate at a graphite electrode modified with osmium redox polymer **II** mediated *B. subtilis* (3G18/pBSD-1200) cells. Measurements were performed in flow injection analysis mode. The graph inset represents a linear dependence of current density with the concentration of substrate in the μM range.

Conclusion

High molecular weight osmium redox polymers proved to be effective in "wiring" viable gram-positive *B. subtilis* cells to electrodes. The generated current depended on the substrate concentration, with a linear dependence in the μM range. In the case of the low potential polymer (**II**), the removal of oxygen from the solution had a great impact on the current response, a phenomenon not observed in the case of the high potential polymer (**I**). Addition of gramicidin generated a decrease in the current response by obliterating the membrane potential needed for succinate uptake by the living cells. Wild type *B. subtilis* cells generate a much lower current response for succinate compared to the cells that overproduced SQR. This is expected, assuming that the source of the response is the oxidation of succinate by SQR, and demonstrates that such metabolic enhancement can greatly improve the efficacy of electrochemical communication between bacterial cells and electrodes.

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Supporting Information Available: Additional information including figures for substrate inhibition, current density response for Os-polymer **II**, a comparison with the soluble mediator hexacyanoferrate (**III**), stability characteristics, a comparison between the amperometric response for wild type *B. subtilis* cells, and cells overproducing SQR and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

JA905442A

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