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Capsule-bound Pyomelanin as an Electrical Conduit in *Shewanella oneidensis* MR-1

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Abstract

Pyomelanin, a polyphenol derived from homogentisic acid and common in bacteria, has a yet poorly understood physiological role. Many functions have been proposed for pyomelanin, the most common being anti-oxidant or UV-shield. Pyomelanin from cultures of *Shewanella oneidensis* MR-1 (a Gram-negative γ-Proteobacteria) was extracted, purified and deposited in thin layers on InSn-coated conductive glass surfaces. I-V measurements at physiological relevant potentials have shown that thin layers of pyomelanin have non-linear conductive properties. The highest abundance of cell's pyomelanin (> 99 %) occurred in the cell envelope fraction. We hypothesize that one of the roles of the capsule-bound pyomelanin is to form a low solubility grid exchanging electrons with the exterior.

Keywords: Pyomelanin; Capsule; *Shewanella*; Semiconductor; Conduction.

Introduction

Many questions remain to be addressed about the functioning of microorganisms in microbial fuel cells [1-3]. Proposed mechanisms for electron transfer toward solid surfaces include: outer surface cytochromes, conductive pili or nanowires, conductive matrices with loosely bound cytochromes, various other proteins associated with the outer membrane, electron shuttles and complex electron conduits [4-7]. One less explored material in this regard is pyomelanin, a polymer with semiconductor properties often associated with proteins and concentrated in the outer cell layers [8]. When an electrical potential exists between a cell and a nearby solid surface, for example an electrode, capsule-bound pyomelanin may act similarly to an insoluble electron conductor. In nature, such situations would be encountered when surface-attached microbes transfer electrons toward insoluble oxides of iron, manganese or uranium [9-11]. Conversely, insoluble phases (*e.g.* pyrite, chalcocite) may also act as sources of electrons [12-13].

Pyomelanin is a negatively-charged hydrophobic polymer of imprecise structure and size, derived from homogentisic acid (HGA). It is common in fungi [14-16], and was also reported in many genera of bacteria, including *Pseudomonas* [17-18], *Legionella* [19] and *Shewanella* [20]. Upon elimination from cells the HGA auto-oxidizes and polymerizes into chains of pyomelanin [16-17, 21-22]. Based on chemical properties and measurements, the largest concentration of cell-bound pyomelanin can be found in the outer cell envelopes, lipopolysaccharide (LPS) layer and capsule [8].

The main function of pyomelanin remains debated. Melanins alter the electrical charge of a cell, especially when the polysaccharide capsule is small or absent [14]. Correlation between pyomelanin and virulence was found in *Cryptococcus* [23]. In *Legionella* pyomelanin increased resistance to light [24]. Antioxidant role was often proposed and shown in *Burkholderia* [25-26] and *Methylococcus thermophilus* [27]. Pyomelanin confers *Legionella* cells ferric reductase capabilities [19] and may help cells reduce, immobilize and chelate metals [19-20,28]. Pyomelanin may also be used to bind and recycle soluble electron shuttles such as riboflavin and to transfer electrons toward solid phases [29-30].

The capacity of melanins to transfer electrons is derived from its ability to change the basic state of its monomers from quinone state to hydroquinone state [31-32] (Figure 1). Because melanins have broad energy absorbing properties, their electron transfer capabilities are influenced by various energy sources, including ionizing radiation, UV, visible light and heat [33]. It was theorized that pyomelanin may act as a terminal electron acceptor in *Shewanella* [20], as a soluble electron shuttle [18, 34], or as a conduit for electrons [32]. In this report we present a method to study the validity of this latest hypothesis.

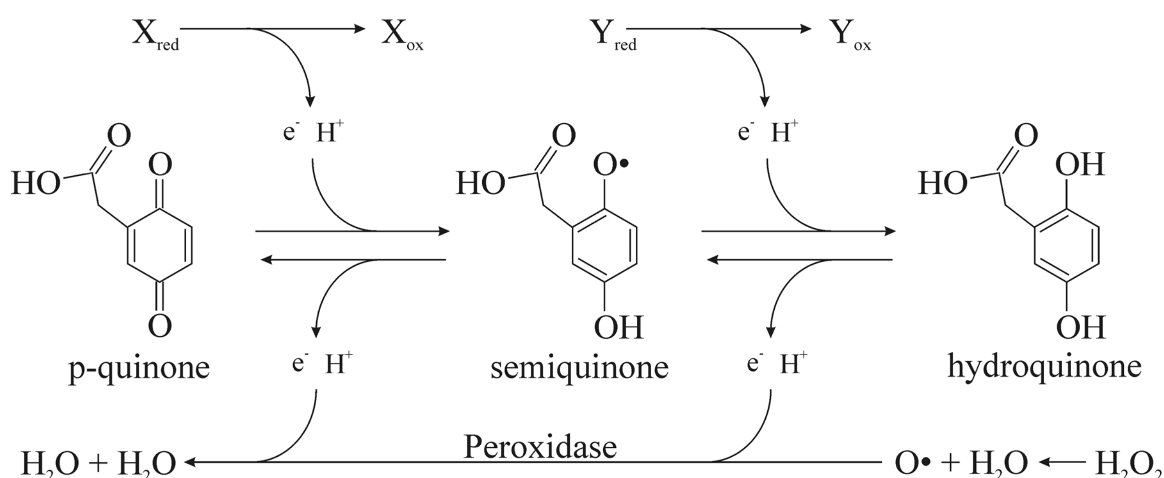

Figure 1. Example of how pyomelanin functions as an electron intermediary between electron donors (X_{red} and Y_{red}) and an electron acceptor (H_2O_2). In this example the hydroquinone is an antioxidant in the presence of H_2O_2. The figure only shows changes of state occurring in one HGA monomer of pyomelanin. X_{red} = chemical X in reduced state. X_{ox} = chemical X in oxidized state. Y_{red} = chemical Y in reduced state. Y_{ox} = chemical Y in oxidized state.

In *Shewanella*, electron shuttling cannot be the sole, or even the main function, of pyomelanin because even femtogram quantities of pyomelanin per cell would be sufficient to significantly increase Fe(III)-reduction rate, while *Shewanella* cultures may in some cases increase their pyomelanin content from < 1 fg to 115 fg per cell [8, 20, 35]. The hypothesis that the main function of pyomelanin is that of a terminal electron acceptor is also questionable, because the energy needed to produce pyomelanin is much larger than the energy associated with the electrons exchanged during redox reactions. We evaluated two potential roles for the capsule-bound insoluble pyomelanin (CBI-pyomelanin).

- CBI-pyomelanin may be part of an insoluble electron conduit network, helping surface-attached cells exchange electrons directly with a solid phase; and
- CBI-pyomelanin may be a temporary electron storage system, buffering fluctuations in the availability of redox chemicals, or in the access of cells to electron conductive surfaces.

Melanin is known to be a p-type semiconductor [31-32, 36-37]. Although many methods to produce thin films of melanin exist [38-43] most measurements were done on thick melanin layers and at relatively high voltages. No I-V measurements were yet made on thin layers of pyomelanin, comparable in thickness with the cell capsule (*e.g.* about 30-50 nm) and electrical potentials germane to living cells (*i.e.* tens to hundreds of mV). We extracted CBI-pyomelanin from Gram negative cells of *Shewanella oneidensis* MR-1. We present a simple method to produce thin layers of pyomelanin and to perform I-V measurements.

2. Materials and Methods

Production and extraction of pyomelanin

All chemicals were reagent grade. The procedure for extracting and quantifying pyomelanin is a modification from earlier reports [8, 35, 44]. Cells of *Shewanella oneidensis* MR-1 were grown in Luria Bertani broth (LBB) and LB agar (LBA) in aerobic conditions. Over-expression of pyomelanin was obtained by adding 11 mM L-tyrosine (Sigma) to the initial medium. To extract sufficient amounts of CBI-pyomelanin the Tyr-treated cells were grown in LBB for two weeks until late stationary phase. Before extraction, cell density was measured by absorption at 600 nm (Abs_{600}) and calculated relative to a standard of serial diluted *Shewanella* cells counted by microscopy. Cells were harvested by centrifugation (30 min., 4,500 rpm, 4 ºC). We collected both the supernatants and pellets and pellets were washed with a saline solution (5 g/L NaCl). Supernatants and washed pellets were frozen at -40 ºC for 15-30 min.

To rupture the cells, frozen pellets were rapidly thawed in a hot water bath (about 99-100 ºC) for 5 minutes, and this freeze-thaw treatment was repeated three times. Then, the pellets were vortexed vigorously for 1 min. and sonicated in an ice-water bath for 10 min. To each tube, 2 ml of 5 g/L NaCl washing solution was added and vortexed vigorously for another 1 min. The ruptured cells were centrifuged (10 min., 13,000 rpm, 20 ºC) and supernatants (containing the intracellular pyomelanin fraction) were also collected. The pellets were treated with 1 ml of chloroform (Fisher) do dissolve the lipid fraction and to help pyomelanin dissolution. After centrifugation (5 min., 13,000 rpm, 20 ºC) the chloroform was discarded. The excess chloroform was evaporated in a laminar hood for 10 min. Each pellet (representing insoluble cell envelopes) was washed with 5 g/L NaCl, vortexed for 1 min., centrifuged (5 min., 13,000 rpm, 20 ºC) and the supernatant was eliminated in order to remove the soluble pyomelanin from the cell envelopes fraction and to only retain the CBI-pyomelanin in the pellet. The pellets were incubated for 48 hours in a 1 M NaOH solution, in the dark at room temperature, to dissolve the CBI-pyomelanin. After centrifugation (5 min., 13,000 rpm, 20 ºC), aliquots of the supernatant were acidified (200 µL of 6 N HCl for each 1 mL supernatant) and allowed to precipitate the CBI-pyomelanin for 24 hours in the dark at 20 ºC. After another centrifugation (10 min., 13,000, 20 ºC) the supernatant was discarded and the pyomelanin-rich pellets were collected, and washed three times in 5 g/L NaCl solution to remove the excess of acid and other soluble molecules. To each pellet we added 1 ml of 1 M NaOH solution and incubated in the dark at 20 ºC for 48 hours to obtain a purified CBI-pyomelanin solution. The pyomelanin from supernatant was obtained by acidification to pH ≈ 1, allowed to precipitate for 48 hours and centrifuged as shown above. Pellets were washed and dissolved in 1 M NaOH. Eventually, three pyomelanin fractions were obtained from each culture: dissolved intracellular pyomelanin, dissolved extracellular pyomelanin and insoluble pyomelanin associated with cell envelopes (*i.e.* CBI-pyomelanin). To verify whether the concentration of pyomelanin may be a Tyr-induced artifact, we also grew cells for two weeks until stationary phase was reached in the absence of added Tyr and the extraction procedure shown above was repeated.

We produced a natural pyomelanin standard by extracting CBI-pyomelanin from a culture of *Shewanella oneidensis* MR1 grown for 2 weeks at 30 ºC in 11 mM initial tyrosine. After extraction, the pyomelanin pellet was stored for about two months in 6 N HCl to separate the pyomelanin from its protein complexes [37] and the melanin-rich pellet was dissolved in 1 M NaOH solution for 24 hours. The supernatant (5 min., 13,000 rmp, 20 ºC) was precipitated by acidification for 24 hours and centrifuged (5 min., 13,000 rmp, 20 ºC). The pyomelanin pellet was washed in water, dried, weighed on a CAHN electrobalance with +/-1 µg accuracy and re-dissolved in a known volume of 1 M NaOH. This solution was used to produce an Abs_{400} of natural pyomelanin standard and then to calculate the absolute concentration of the various pyomelanin solutions produced for this study. We also made comparisons with a commercial melanin standard (Sigma) dissolved in 1 M NaOH [20]. This method is well established ad was used for producing high quality pyomelanin [20,37].

In *Shewanella algae* the extracellular pyomelanin molecules range between approximately 12,000 and 14,000 MW [35]. Because HGA has a molecular weight of 191, each pyomelanin contains about 63-73 HGA monomers. Hence, each molecule of pyomelanin may be charged with about 120 to 150 electrons between the quinone state and the hydroxyquinone state (Figure 1). Pyomelanin does not have a unique structure or a uniform pattern throughout its structure. Assuming that a pyomelanin molecule with 13,000 MW is in B-configuration [32] its total length would be approximately 20 nm.

Construction of measurement cell and deposition of pyomelanin

To produce thin layers of pyomelanin and for I-V measurements we used: 50 mM iodine (Sigma) in ethylene glycol (Sigma-Aldrich), 50 mM KI (JT Baker) in ethylene glycol and InSn-coated Low E conductive glass about 30 Ohms (Pilkington). During I-V measurements currents were applied and changed with a DC regulated power supply instrument (Keithley). As measurement and recording instruments we used a model M-3890D multimeter (Metex). In experiments, the pyomelanin was deposited directly on the conductive glass (the deposition procedure is explained in the Results section). The I_2/KI solution used in the measurement cell contained 8.3 g KI, 75 ml of ethylene glycol, 1.26 g of I_2 (0.05 M), adjusted to 100 ml with H_2O.

We made I-V measurements of the thin layers of pyomelanin using the general principle of a Grätzel cell [45]. The conductive glass was cut in slides. We spread NaOH solution (pH 12) containing pyomelanin (about 0.32 mg pyomelanin per ml) over the conductive glass slides 2.5/7.5 cm). After evaporation at room temperature the glass slides were stored in the dark for 48 hours. Crystals of sodium hydroxide formed on the surface were washed by dipping the slides gently for about 5 min. into a liquid bath containing a pH 3.5 HNO_3 solution, and then in another bath of distilled water (dH_2O). Acid washing is important to limit the loss of pyomelanin by dissolution, which would occur in alkaline conditions. The glass slides were dried at room temperature, in the dark, overnight. Other glass slides were produced by depositing a graphite layer on the conductive side. Each measurement cells consisted of one pyomelanin-coated slide on the anode side and one graphite-coated slide on the cathode side (Figure 2). The anode and cathode slides were installed vertically in a glass slide holder with the conductive sides facing each other at approximately 1 mm distance and I_2/KI solution was added to cover approximately 12.5 cm^2 of the melanin coated surface.

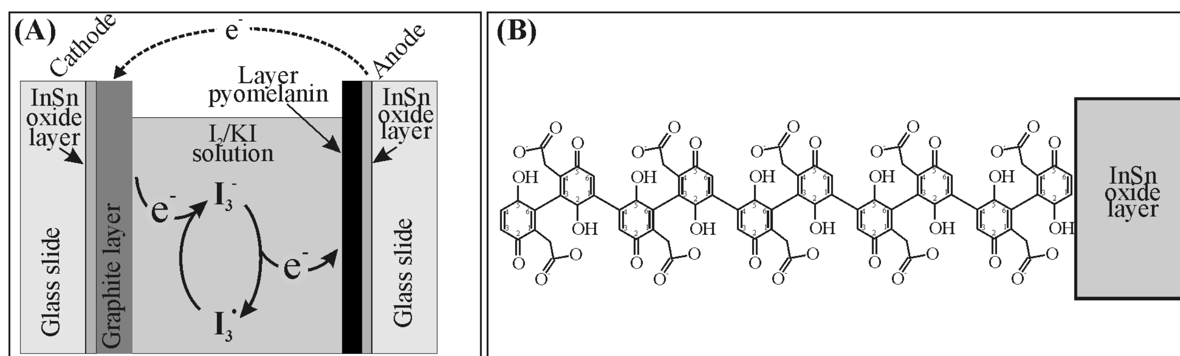

Figure 2. (A) Setup containing a thin layer of pyomelanin deposited on an InSn-coated glass, used to analyze the I-V properties of pyomelanin. (B) Diagram of part of a molecule of pyomelanin in B-configuration and hydroquinone state consisting of 10 HGA monomers deposited on an InSn-coated conductive surface. A typical 13,000 MW pyomelanin molecule contains about 70 HGA monomers.

3. Results and Discussions

I-V measurements

Figure 3 shows the result of a typical I-V measurement. Based on the amount of pyomelanin in the source solution, the glass surface load was less than 5.12 $\mu g/cm^2$. Electricity generation was seen if the pyomelanin-coated glass was exposed to light even briefly prior to measurements. This is likely caused by the photoactivation of pyomelanin. Hence, before all measurements the fully assembled cells were kept in the dark for at least 24 hours and the current production was monitored until the current readings have stabilized to a low level. Without this dark phase incubation, I-V profiles showed a consistent downward lopsided profile at low potentials, and widely dissimilar profiles were seen between replicate measurements. For I-V measurements we applied a small current in the 0-15 μA range, changed every 30 seconds toward a higher value and recorded the voltage afterward. The voltage readings have generally stabilized within approximately 20 seconds. The exponential I-V behavior shown in Figure 3 supports earlier results that pyomelanin has semiconductor properties [32]. As a semiconductor, pyomelanin may function as a charge regulator, protecting cells from overcharge and maintaining better uniformity across a broad range of electrical potentials.

Conductivity model

Turick *et al*. [8] found that depending on the level of expression, cells of *Shewanella algae* may contain between about 0 and about 116 fg pyomelanin. We found that in the presence of 11 mM Tyr and after two weeks of incubation a culture of *S. oneidensis* MR-1 showed higher concentration of pyomelanin in the CBI-pyomelanin reservoir than in all other reservoirs (namely pyomelanin from the extracellular solution and soluble intracellular pyomelanin) (Table 1). Unlike earlier measurements [8] we found that pyomelanin was also formed when Tyr was not added to the culture medium (Table 1). Apart from differences in the duration of incubation between different *Shewanella* strains, and between methods of extraction, this result may also be due to the fact that LB is a complex medium, with sufficiently abundant Tyr and phenylalanine to induce the formation of pyomelanin.

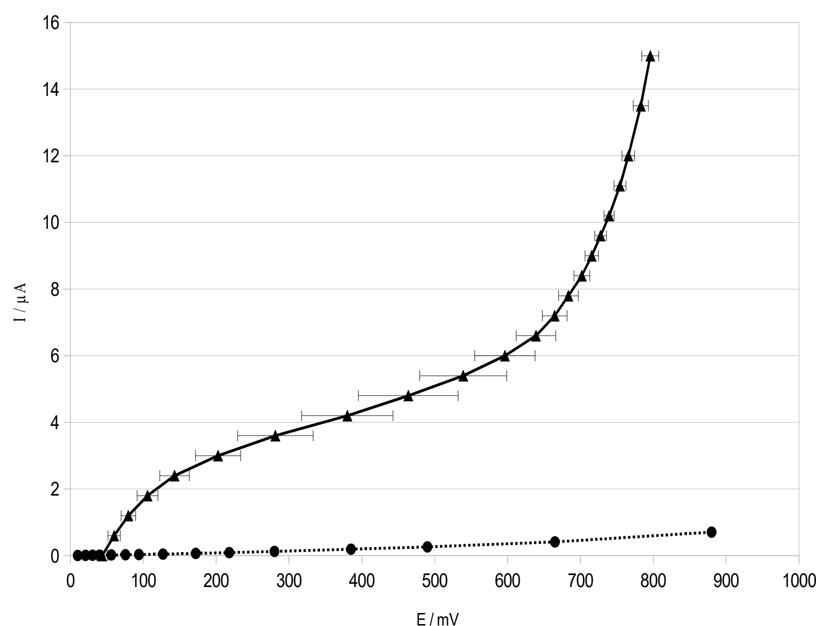

Figure 3. Typical profile of I-V measurements made in Grätzel cells with thin layers of pyomelanin deposited on conductive glass surfaces. ● Conductive glass. ▲ Conductive glass with pyomelanin. The following currents were applied (0; 0.6; 1.2; 1.8; 2.4; 3; 3.6; 4.2; 4.8; 5.4; 6; 6.6; 7.2; 7.8; 8.4; 9; 9.6; 10.2; 11.1; 12; 13.5 and 15 µA) at 30 seconds intervals. Each data point in the graph is the average of 8 series of voltage readings, and error bars are one standard deviation.

Because capsules contain most of the cellular pyomelanin, while representing but a fraction of the total cell volume, the abundance of the CBI-pyomelanin we have found (Table 1) underestimates its actual concentration in the capsule. The cell capsule in *Shewanella* cells varies in thickness between 20 and 130 nm [46-47]. Henceforth, in a cell with a volume of 2 μm^3 (excluding the capsule) a 20 nm thick capsule would represent about 10 % of the total cell+capsule volume. In this case the amount of CBI-pyomelanin we have found in *S. oneidensis* MR-1 was about 0.2 to 0.4 fg/cell, and pyomelanin was about 130-200 times more concentrated in the cell capsule than in the extracellular fluid. This difference in concentration supports that pyomelanin is more important to the cell as a capsule-resident insoluble polymer than as an extracellular solute. Arguably, the soluble extracellular pyomelanin is not released "deliberately" by the cells, and may represent pyomelanin "lost" by cells in the environment. Based on these results, we propose that it is more likely for the primary function of pyomelanin to be that of an insoluble electron conductor, rather than a soluble electron shuttle.

Table 1. The abundance of pyomelanin in three main reservoirs from cultures of *S. oneidensis* MR-1 after two weeks of incubation. Assuming that a cell has a volume of about 2 μm^3, a concentration of 100 µg/ml of cell is equivalent with 0.2 fg/cell.

	LB medium with no added Tyr	LB medium with 11 mM initial Tyr
Soluble extracellular pyomelanin	5.76 +/-0.04 (µg/ml)	14.86 +/- 0.04 (µg/ml)
Soluble intracellular pyomelanin	0 (µg/ml)	0.18 +/-0.04 (µg/ml)
CBI-pyomelanin	115.94 +/-0.04 (µg/ml)	185.93 +/-0.04 (µg/ml)

A *Shewanella* cell with a volume of 2 μm^3 and 1 fg of pyomelanin contains about $4.6 \cdot 10^4$ molecules of pyomelanin. One molecule of CBI-pyomelanin with 13,000 MW may be as

long as 20 nm, sufficient to stretch across a 20 nm thick capsule. In monolayers, the density of CBI-pyomelanin would be about $2.3 \cdot 10^4$ molecules per μm^2. Assuming an approximate diameter of 0.4 nm for each pyomelanin molecule in B-configuration (Figure 2), pyomelanin will cover about 0.3 % of the active surface in a cell containing 1 fg of pyomelanin and 30 % in a cell containing 100 fg pyomelanin. This means that when cells are in contact with solid surfaces, or with each other, numerous CBI-pyomelanin based conductive contacts will exist, and thus CBI-pyomelanin may act as a conductive network, even when contact area are small. This may explain why cells are producing more melanin than necessary to explain their metal oxidation/reduction rates [8, 20, 35].

Situations will exist when cells become detached from the cathode's surface in a solution with limited availability of soluble electron shuttles (*e.g.* flavins, small quinones, soluble pyomelanin, AQDS). Alternatively, cells can be detached from insoluble electron accepting particles (such as metal oxides or DMSO), in a solution still containing electron donors [10, 48-50]. The respiration rate of *Shewanella* varies between $6.7 \cdot 10^4$ and $3 \cdot 10^6$ electrons sec^{-1} $cell^{-1}$ [9, 51]. One fg of CBI-pyomelanin per cell is a reservoir of electrons (or electrons sink) that may support an electrical flow equivalent with that of cellular respiration for up to 95 seconds. This underestimates the amount of time cells may be able to "hold their breath" using the CBI-pyomelanin reservoir. In certain situations, the respiration rates of *Shewanella* cells drops from $6 \cdot 10^5$ electrons sec^{-1} $cell^{-1}$ to about $6 \cdot 10^3$ electrons sec^{-1} $cell^{-1}$ [52], and the concentration of pyomelanin per cell reaches 115 fg [8, 20, 35]. A *Shewanella* cell with 1 fg pyomelanin should contain sufficient electron reserves (or electron sink potential) in its CBI-pyomelanin reservoir to maintain an electron flow of $6 \cdot 10^3$ electrons sec^{-1} $cell^{-1}$ for about15 minutes should electron donors or electron acceptors become temporarily unavailable.

Based on the relative abundance of CBI-pyomelanin in different reservoirs of a cell culture, and on the pyomelanin conductive properties we propose that the main function of CBI-pyomelanin is to transport electrons between the outer membrane and solid surfaces the cells are attached to (Figure 4). Not discussed in Figure 4 is the possibility for the CBI-pyomelanin to work in conjunction with electron transfer proteins. Although oxidoreductases involved in electron exchange are present in the outer cell layers [6, 7, 53, 54] and the ability of melanins to bind proteins is well recognized [55-57], this possibility cannot be yet evaluated because too little is known about pyomelano-protein complexes [27, 58, 59].

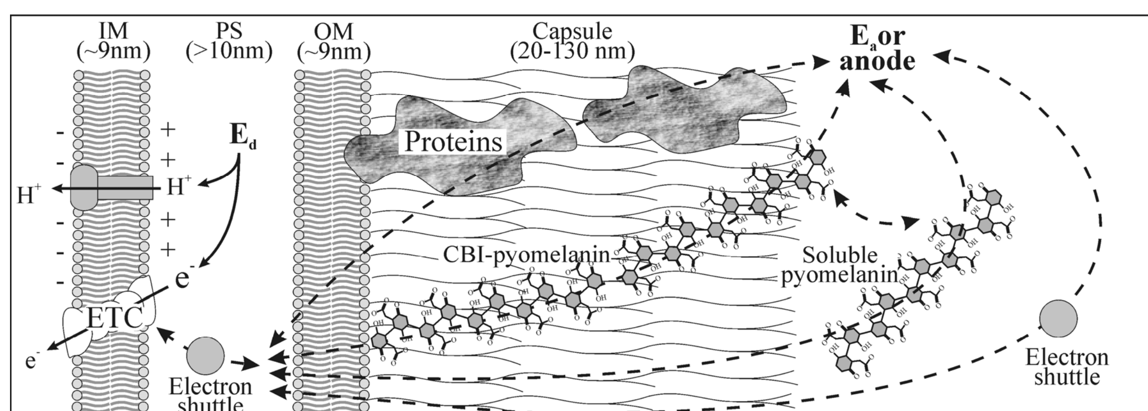

Figure 4. Proposed model for the place of CBI-pyomelanin as an electron conductor located in the cell capsule, relative to other potential routes for electron transfer between cells and solid conductive surfaces. CBI-pyomelanin = Capsule-bound insoluble pyomelanin. E_d = Electron donor. E_a = Terminal electron acceptor. IM = Inner membrane. PM = Periplasmic space. OM = Outer membrane. ETC = Electron transport chain.

4. Conclusions

We present a simple method to extract CBI-pyomelanin from a Gram negative bacterium (*S. oneidensis* MR-1), to deposit pyomelanin on thin layers and to measure its conductive properties at cell relevant potentials. Our measurements showed that thin layered pyomelanin have non linear conductor properties in the in the 40-800 mV range. We propose that CBI-pyomelanin forms an insoluble network in a cell's capsule, regulating the electrical current through the cell's circuitry. We also propose that CBI-pyomelanin is involved in direct conductive electron exchange between cells and solid conductive surfaces the cells are attached to. The significantly larger concentration of insoluble pyomelanin in the cell capsule relative to the soluble pyomelanin from the aqueous environment indicates that, with regard to electron transport, the primary role of pyomelanin is that of an insoluble conduit rather than a soluble shuttle. Cells with high expression of CBI-pyomelanin may also use this polymer as an easily accessible reservoir of electrons or temporary electron sink. One fg of CBI-pyomelanin per cell contains sufficient electron storage capacity to support the electrical flow needed by a cell from a couple to 16 min. when electron donors or electron acceptors become temporarily unavailable, and respiration rate is low.

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