Electrochemical properties of pH-dependent flavocytochrome c3 from Shewanella putrefaciens adsorbed onto unmodified and catechol-modified edge plane pyrolytic graphite electrode

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Electrochemical properties of pH-dependent flavocytochrome c_3 from *Shewanella putrefaciens* adsorbed onto unmodified and catechol-modified edge plane pyrolytic graphite electrode

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Abstract

The electroactivity of adsorbed flavocytochrome c_3, a tetraheme FAD-containing flavoenzyme isolated from the bacterium *Shewanella putrefaciens*, is investigated by cyclic voltammetry at an edge plane pyrolytic graphite electrode before and after modification with grafted catechol serving as an efficient pH sensor based on a redox readout. Flavocytochrome c_3 adsorption onto the unmodified or modified electrode surface is successfully achieved by cyclic voltammetry (100 consecutive cycles) in a flavocytochrome c_3 solution containing polymyxin as co-adsorbate. The immobilized flavocytochrome c_3 retains its electrochemical activity and its catalytic fumarate reductase activity. The redox activity of the protein arises from its FAD and four hemes cofactors. The experiments evidence that the hemes' redox potential of flavocytochrome c_3 from *Shewanella putrefaciens*, for which no crystal structure is yet available, depend on pH which is at variance with data from the other strains *Shewanella frigidimarina* or *Shewanella oneidensis*.

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1. Introduction

A century after the demonstration that electrons from microbial metabolism can be harvested with electrodes,[1] electron transfer processes in biofilms of microorganisms performing respiration on extracellular solids are now being studied in order to increase the performance of microbial electrochemical technologies such as microbial fuel cells.[2, 3] Extracellular electron transfer occurring at the microorganism/electrode interface stimulated a wide range of fundamental and applied studies because of important implications in biotechnology, bioenergetics and bioremediation.[4-13] Most studies focus on two Gram-negative mesophilic bacteria able to transfer electrons from their respiratory metabolism to extracellular solids, namely *Shewanella oneidensis* MR-1 and *Geobacter sulfurreducens*.[14] In these model electroactive microorganisms, multiheme *c*-type cytochromes transfer electrons from cytoplasmic and inner-membrane oxidizing enzymes towards cell surface redox proteins that are responsible for the reduction of solid phase electron acceptors.[15, 16] It is also known that electron transfer is usually coupled to proton transfer which causes acidification in the anodic biofilms and in the anolyte with deleterious consequences for the biofilm metabolism and stability.[3, 17, 18] The fundamental understanding of these processes that involve large proteins (>70 kDa) often with complex redox properties (with ≥10 heme centers in some multihemes cytochromes) is still in its infancy and precludes the optimization of microbial electrochemical technologies. In Gram-negative electroactive bacteria such as *Shewanella*, several redox proteins have pH-dependent potentials,[19] such as flavocytochrome c_3 (M_r = 63.8 kDa) which is one of the most abundant proteins in the periplasm and the only functional fumarate reductase in this microorganism.[20-22]

Our research focuses on the design and the development of an efficient and versatile electrochemical platform able to probe charge (electron/proton) transfer properties of

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proteins from Gram-negative electroactive bacteria on modified carbon electrodes.[23] The electrodes are first modified with pH-responsive electrophores such as quinone units and then proteins are immobilized for electrochemical studies. If relevant, the protein electroactivity can be probed directly at the modified electrode while proton transport, if any, performed by the protein can be detected thanks to the grafted pH sensor.[23] The aim of the present work is to demonstrate the electrochemical detection of a pH-dependent redox protein from electroactive bacteria at an electrode modified to act additionally as an efficient pH sensor based on a redox readout. The pH sensing electrode was previously designed and showed to allow the immobilization and study of pH-independent and redox active cytochrome *c*.[23] Here we extend this work to flavocytochrome c_3, a tetraheme FAD-containing periplasmic flavoenzyme isolated from the bacterium *Shewanella putrefaciens*,[20, 24-28] taken as a model pH-dependent redox protein from electroactive bacteria (Figure 1). The modification of the electrode surface with the pH sensing modifier (catechol) stems from our previous experience in the tailoring of bio-interfaces of carbon electrodes with covalent electrografting.[12, 13, 23, 29-33] The purification process and electrochemical properties of the flavocytochrome c_3 enzyme from *Shewanella frigidimarina* NCIMB400 and *Shewanella oneidensis* MR-1 strains have been previously reported.[20, 24-28]

Enzyme adsorption onto edge plane pyrolytic graphite surface (suitable to the study of this enzyme)[20, 26, 34, 35] is successfully performed by cyclic voltammetry in a flavocytochrome c_3 solution containing polymyxin B sulfate salt as co-adsorbate. The resulting modified electrode is studied by cyclic voltammetry in a protein-free phosphate buffer aqueous electrolyte at different pH values where its native fumarate reductase activity is evaluated. The ability of the polymyxin promoter to improve the interaction of the enzyme with the electrode is assigned to ternary salt bridges between complementary charged regions of the protein and the electrode surface.[36] The covalent grafting of catechol onto the edge plane pyrolytic graphite electrode is achieved by the one-pot/three steps

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electrochemical procedure consisting in the cathodic reduction of 4-nitrocatechol in an acidic solution containing sodium nitrite.[23]

The electrochemical properties of the adsorbed pH-dependent flavocytochrome c_3 enzyme from *Shewanella putrefaciens* are evaluated here for the first time at a carbon electrode either unmodified or modified by catechol allowing the simultaneous probing of pH change at the electrode surface, a property of high relevance for monitoring the extracellular electron transfer activity of electroactive biofilms growing on the surface of an electrode.

A   B
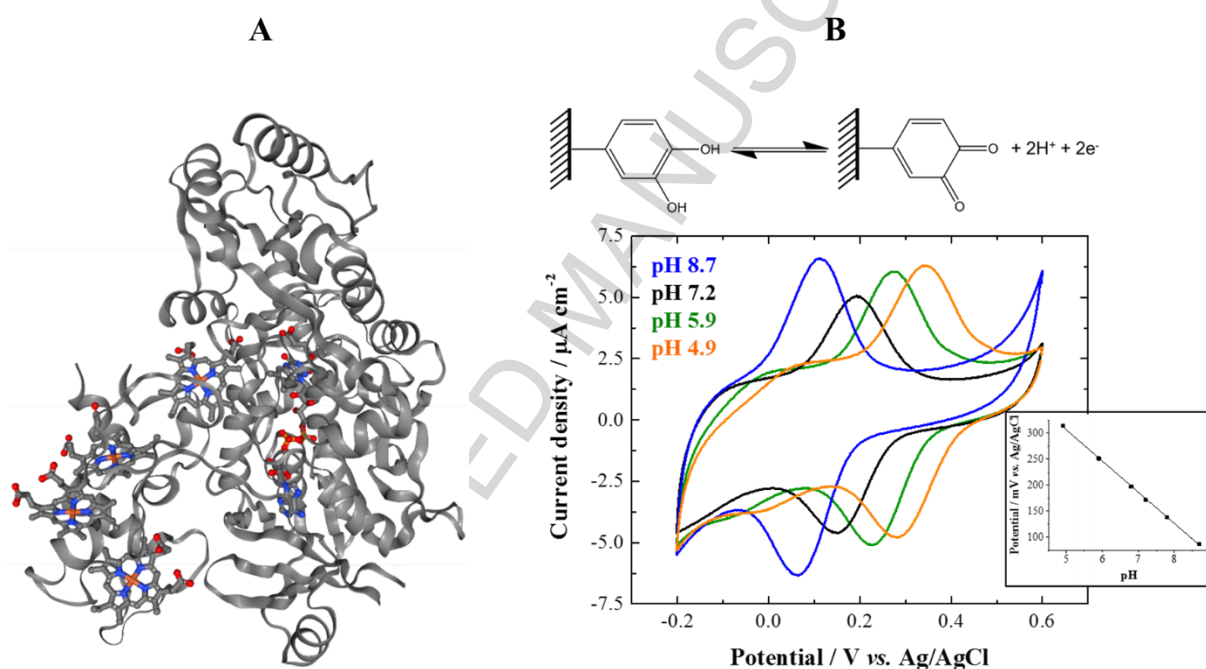
Figure 1. (A) Structure of flavocytochrome c_3 from *Shewanella oneidensis* strain MR-1 (Protein Data Bank file 1D4D)[37] and (B) electrochemical properties of the grafted quinone/hydroquinone redox couple as grafted pH sensor (reproduced from reference [23]).

2.   Material and methods

2.1.   Reagents

All solutions were prepared with Milli-Q water (resistivity 18.2 MΩ cm). Polymyxin B sulfate salt was obtained from Sigma Aldrich and stored at 4°C. Sodium hydrogen phosphate

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anhydrous ACS (99.0% min) and potassium dihydrogen phosphate ACS (99.0% min) from Alfa Aesar were used to prepare the 0.1 M aqueous phosphate buffer pH 7.2. Concentrated hydrochloric acid (12 M, 37%, ACS Reagent) and sodium hydroxide pellets (ACS) were obtained from VWR and were used to adjust the pH of the phosphate buffer solution. Sodium nitrite (>99%) and fumaric acid (≥98%) were obtained from Sigma Aldrich and 4-nitrocatechol (>98%) was obtained from Alfa Aesar.

2.2. Production and purification of flavocytochrome c_3 from *Shewanella putrefaciens*

Cells from *S. putrefaciens* DSM 9451 were purchased from DSMZ. These cells were grown under aerobic conditions in a batch culture at 30 ºC and 180 rpm using Terrific Broth medium. Cells were allowed to grow for 24 hours before harvesting by centrifugation (10,000 x g, 15 min, 4 ºC). The cell pellet was re-suspended in 20 mM Tris-HCl buffer, pH 7.6 containing a protease inhibitor cocktail and DNase. Cell disruption was obtained by two passages through a French Press at a pressure of 1000 psi. Cell debris were removed by centrifugation at 10,000 x g for 15 min at 4 ºC and the supernatant was ultracentrifuged at 200,000 x g for 1 hour at 4 ºC. The supernatant containing the soluble protein fraction was loaded onto a diethylaminoethyl column (GE Healthcare) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.6). A salt gradient from 0 to 1 M NaCl in the same buffer was applied and the fraction containing flavocytochrome c_3 (Fcc$_3$) was eluted at 200 mM NaCl. This fraction was dialyzed, concentrated and loaded onto a Q-Sepharose column (GE Healthcare), equilibrated previously with 20 mM Tris-HCl buffer (pH 7.6). The fraction containing Fcc$_3$ was eluted at 200 mM NaCl, using a salt gradient from 0 to 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.6). As a final purification step, the fraction containing Fcc$_3$ was dialyzed, concentrated and loaded onto a hydroxylapatite column (Bio-Rad Laboratories), pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.6). A potassium phosphate buffer (pH 7.6) gradient from 0.01 to 1 M was applied and the fraction containing Fcc$_3$ was eluted at 150 mM. The chromatographic fractions were routinely analyzed by SDS-PAGE

and UV–visible spectroscopy to select those containing the protein of interest. The purity of the protein was confirmed by a single band on SDS-PAGE. Pure samples had a typical absorbance ratio, $A_{SoretPeak}/A_{280nm}$, of approximately 4.5.

2.3. Spectrophotometry measurements

UV-visible absorption spectra were recorded using an UV-Visible spectrophotometer SHIMADZU UV-1605 and 1 cm optical length square cuvettes. The purified protein solution was diluted in 0.1 M phosphate buffer aqueous solution (Figure S1A) and in a methanol/ethanol/water mixture (1 mL of each solvent) used as blank (Figure S1B). Ten electrodes with immobilized protein on their surface were washed with the same solvents mixture (1 mL methanol + 1 mL ethanol + 1 mL ultra-pure water) and the presence of flavocytochrome c_3 was checked by UV-visible spectroscopy in this washing solution (Figure S1B).

2.4. Electrochemical measurements

Cyclic voltammetry was performed in a three-electrode cell with an edge plane pyrolytic graphite disk electrode (3 mm diameter) obtained from IJ Cambria Scientific Ltd as the working electrode. All potentials (±3 mV) are reported versus an Ag/AgCl, KCl 3 M reference electrode. A platinum wire was used as a counter electrode. Electrochemical experiments were performed at room temperature (19 ± 2 °C) with an Autolab PGSTAT204 potentiostat/galvanostat (Eco Chemie B.V., the Netherlands) using Nova as the electrochemical software (Metrohm). All solutions were deaerated by bubbling argon for 20 minutes before each measurement.

2.5. Surface modification procedures

The catechol grafting on pyrolytic graphite surfaces was performed by cyclic voltammetry in aqueous acidic solution (0.1 M HCl) containing 1 mM 4-nitrocatechol and 3 mM $NaNO_2$ by recording 3 cycles between +0.1 V and -0.9 V versus Ag/AgCl, KCl 3 M at a

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scan rate of 50 mV s^{-1} (Figure S2A).[23] Then, the catechol-modified pyrolytic graphite electrode was rinsed with ultra-pure water and studied by cyclic voltammetry in phosphate buffer solution at different pH values (Figure S2B). To immobilize flavocytochrome c_3, the electrode was immersed in a 2×10^{-5} mol L^{-1} flavocytochrome c_3 solution containing 9×10^{-4} mol L^{-1} polymyxin B sulfate salt as co-adsorbate and cycled (100 times) between +0.45 and -0.75 V versus Ag/AgCl, KCl 3 M at a scan rate of 100 mV s^{-1}. Then, the electrode was gently washed with phosphate buffer solution and immediately transferred to a protein-free phosphate buffer electrolyte for further cyclic voltammetry experiments.

3.   Results and Discussion

3.1.   Flavocytochrome c_3 electrochemical study and adsorption

In this section we discuss the electrochemical study and adsorption of flavocytochrome c_3 (Fcc$_3$) at an unmodified and catechol-modified edge plane pyrolytic graphite electrodes (Figure 2). The one-pot/three steps electrochemical reduction grafting procedure (Figure S2A) previously reported [23, 38, 39] is used for electrode modification with catechol. The protein adsorption was successfully performed at the unmodified (Figure 2A) and catechol-modified electrode (Figure 2B) by consecutive cyclic voltammetry (100 cycles) in an Fcc$_3$ solution containing polymyxin as co-adsorbate.
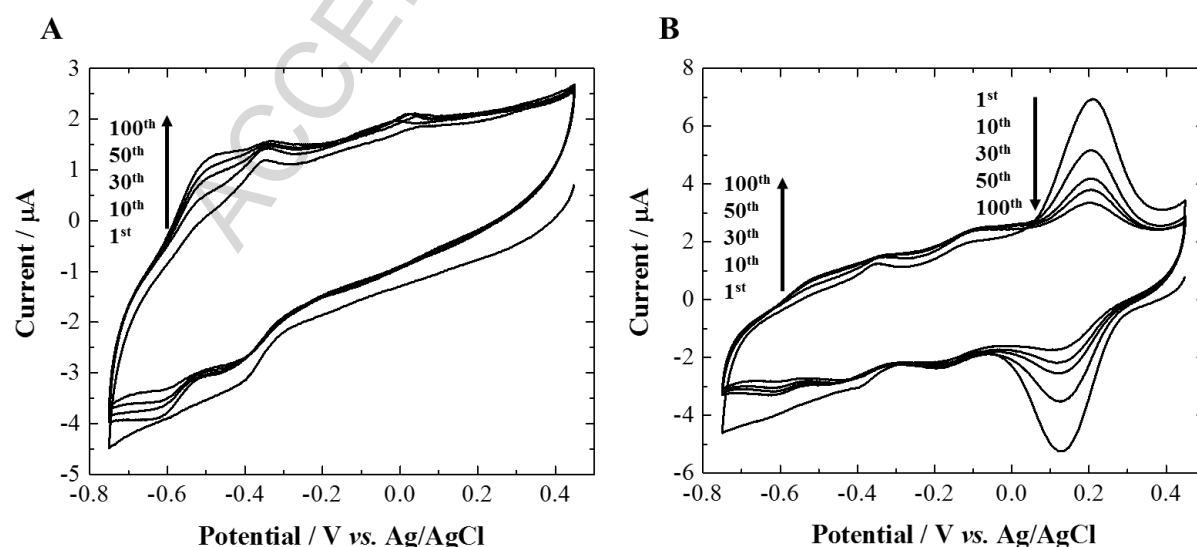
Figure 2. Cyclic voltammograms recorded at 100 mV s^{-1} on (A) bare and (B) catechol-modified edge plane pyrolytic graphite electrodes in 20 µM flavocytochrome c_3 solution 0.15 M phosphate buffer aqueous electrolyte at pH 7.6 with 0.9 mM polymyxin B sulfate salt under inert atmosphere (Ar).

Redox enzymes may be immobilized and electrochemically studied by protein film voltammetry.[20, 34, 35] The protein film is usually formed by depositing a concentrated solution on the electrode or by performing repeated cyclic voltammetry in a dilute solution (slow adsorption).[34, 35] To facilitate and to stabilize the enzyme adsorption onto carbon surface, adding a co-adsorbate in solution such as polymyxin is straightforward and efficient.[20, 35] Based on this protein adsorption method and combined with a technique previously used to immobilize cytochrome c,[23, 40] Fcc$_3$ is adsorbed by performing consecutive cyclic voltammograms (100 cycles here) in a Fcc$_3$ solution containing polymyxin B sulfate salt until a steady state current is reached (Figure 2). Cyclic voltammograms recorded on bare edge plane pyrolytic graphite electrode (Figure 2A) display two chemically reversible systems, one located at -0.380 V and assigned to the 2e$^-$/2H$^+$ FAD/FADH$_2$ redox couple,[20] and a broader system at -0.565 V that includes the contribution of the 4 heme groups (one electron per heme group).[19] An additional oxidation peak is observed at +0.03 V in the anodic scan and is related to the presence of protein in solution. As shown on Figure 2A, the Fcc$_3$ redox peaks current intensity increases during the successive cyclic voltammetry recorded at a bare pyrolytic graphite electrode in a solution of the protein until a steady state current is reached after 100 cycles. This suggests that the pyrolytic graphite electrode is saturated with surface-confined Fcc$_3$ captured from solution.

This experiment has been repeated at a catechol-modified electrode (Figure 2B). Concurrent with the expected increase of the Fcc$_3$ redox peaks current (at -0.380 V and -0.565 V) and assigned to the enzyme accumulation onto the electrode surface, we observe a decrease of the peak current of the main chemically reversible redox system of grafted catechol (located at +0.170 V, Figure 2B). A minor reversible redox system is also detected

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at -0.135 V (Figure 2B) and assigned to grafted catechol groups with a different linkage likely to involve a C-N bond.[41] After 100 consecutive cyclic voltammograms, a steady state current is also reached for the grafted catechol electroactivity and assigned to desorption of adsorbed catechol species non-covalently linked to the carbon surface. Importantly, the presence of the grafted catechol onto the pyrolytic graphite surface does not alter the Fcc_3 electrochemical response and its adsorption onto the modified electrode. Furthermore, the redox activity of Fcc_3 detected at -0.380 V and -0.565 V does not overlap with that of the grafted catechol detected at a significantly more anodic potential (+0.170 V). The large potential range and difference between the electrochemical activity of the redox protein and that of the surface bound redox pH sensor allows the facile identification of each electrochemical contribution in the cyclic voltammogram. This confirms that the grafted quinone/hydroquinone redox couple selected as the electrochemical pH sensor is fully compatible with the study of the Fcc_3 enzyme. These results demonstrate that Fcc_3 electrochemical study and adsorption can be easily carried out by cyclic voltammetry at both unmodified and catechol-modified edge plane pyrolytic graphite electrode in an electrolyte containing the protein and polymyxin as co-adsorbate. In addition, the electrochemical behavior of the adsorbed protein is unaffected when recorded at the modified electrode.

3.2.   Adsorbed flavocytochrome c_3 electrochemical properties

3.2.1. pH-dependent flavocytochrome c_3 redox system

In this section we report the pH-dependent electrochemical properties of flavocytochrome c_3 (Fcc_3) adsorbed onto unmodified and catechol-modified edge plane pyrolytic graphite electrodes. The enzyme-modified and catechol/enzyme-modified pyrolytic graphite electrodes were studied by cyclic voltammetry in a protein-free phosphate buffer aqueous electrolyte at different pH values (Figure 3).
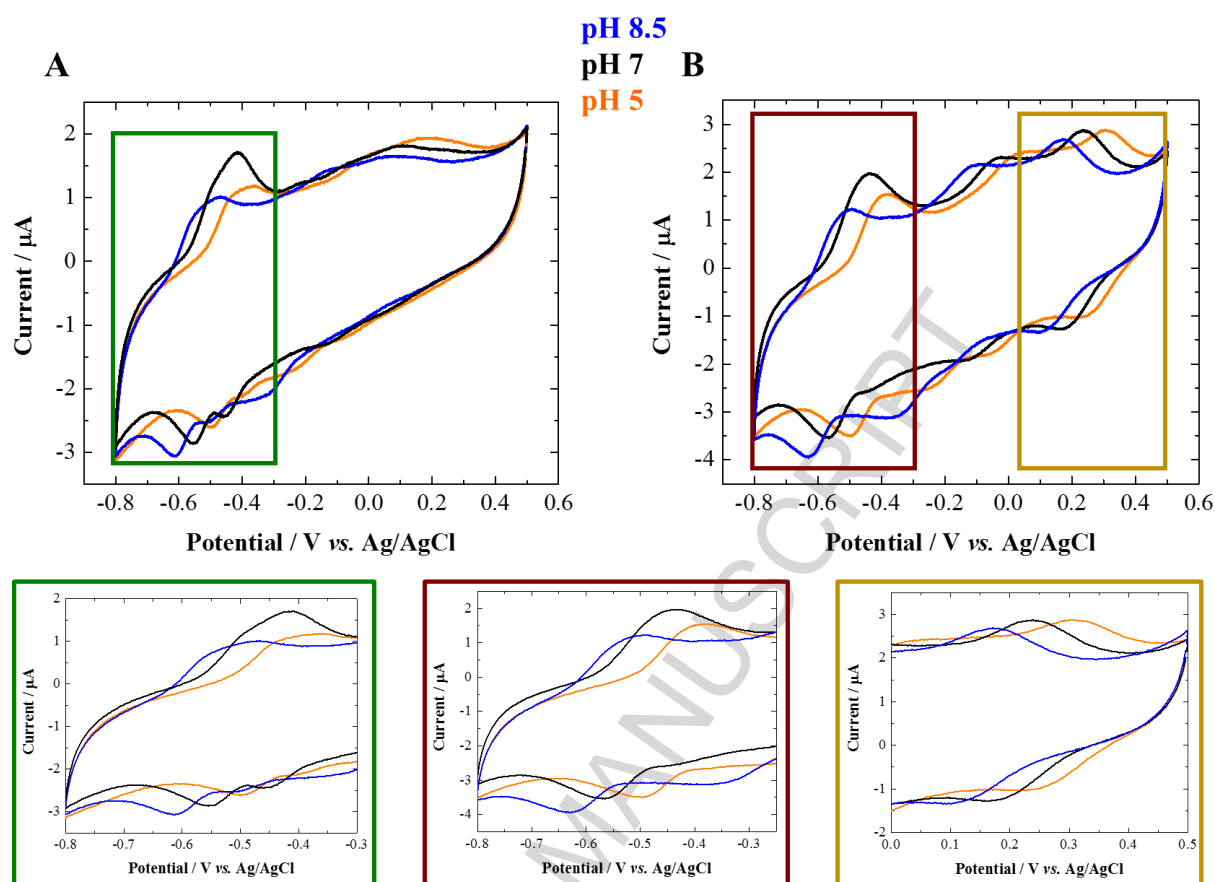
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Figure 3. **Top:** Cyclic voltammograms recorded at 100 mV s^{-1} on (A) Fcc$_3$-modified and (B) catechol/Fcc$_3$-modified pyrolytic graphite electrodes in 0.1 M phosphate buffer aqueous electrolyte successively recorded at pH 7 (black), pH 8.5 (blue) and pH 5 (orange) under inert atmosphere (Ar). **Bottom:** Focus on portions of figures A and B.

Figure 3A shows the electrochemical response of an edge plane pyrolytic graphite with adsorbed Fcc$_3$ recorded in a protein-free aqueous electrolyte. The more cathodic and chemically reversible redox system found around -0.43 V (pH 5), -0.49 V (pH 7) or -0.55 V (pH 8.5) is broad and includes both the FAD and the four hemes electrochemical contributions arising from the immobilized enzyme.[19, 20, 26] Looking more closely at this broad cathodic system, the two contributions of the FAD and hemes can be identified as well separated peaks on the cathodic scan and as partially overlapping peaks on the broader reverse anodic scan (Table 1).

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Table 1. Redox potentials of adsorbed Fcc3 from Figure 3A.

	pH 5	pH 7	pH 8.5
E_{pa} (FAD) / mV	-362	-415	-468
E_{pc} (FAD) / mV	-403	-459	-506
$E°'$ (FAD) / mV	-383	-437	-487
E_{pa} (Hemes) / mV	-443	-491	-558
E_{pc} (Hemes) / mV	-496	-554	-612
$E°'$ (Hemes) / mV	-470	-523	-585

A -59 mV cathodic shift dependence is expected in principle for the $2e^-/2H^+$ FAD/FADH$_2$ redox per increase of one pH unit at 298 K. However, as previously observed for flavocytochrome c_3 from *S. frigidimarina,*[20] we find a ca. -30 mV shift which indicates that only one proton is exchanged in the pH range tested (Figure S3A). Interestingly, we note that the hemes' redox response also shifts cathodically by ca. -32 mV per increase of one pH unit (Figure S3A). Although pH-dependence of heme's redox potentials is not uncommon,[42] the pH response of the hemes redox potential in Fcc$_3$ from *S. putrefaciens* is at variance with previous reports on Fcc$_3$ from *S. oneidensis* MR-1 and *S. frigidimarina*.[20] The surface coverage of the immobilized pH-dependent electroactive Fcc$_3$ estimated from faradaic charge integration of the cyclic voltammogram, by assuming that the process involves six electrons per protein (two electrons for FAD and four electrons for four hemes) and based on the geometrical area of the electrode (0.0707 cm^2), is ca. $1.0 \pm 0.3 \times 10^{-10}$ mol cm^{-2}.[34] This value is at least ten times higher than typical Fcc$_3$ enzyme coverages previously reported on pyrolytic graphite electrode,[20, 26, 28] showing that protein adsorption by consecutive cyclic voltammetry in a Fcc$_3$ solution is efficient and leads to a more stable deposit than that

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obtained by direct deposition of the concentrated protein solution. In addition, the linear relationship between the oxidative peak current (located at -0.450 V) and the scan rate confirms that the protein is confined to the electrode surface (Figure S4A).[43] These results show that Fcc$_3$ adsorption is easily and efficiently carried out by cyclic voltammetry at an edge plane pyrolytic graphite electrode in a solution containing the protein and polymyxin as a co-adsorbate. The electrochemical properties of the pH-dependent redox protein Fcc$_3$ from *S. putrefaciens* are kept intact in these conditions and hint to a pH-dependence of both the FAD and the hemes' redox systems by ca. -30 mV and -32 mV respectively per increase of one pH unit.

Following the same procedure detailed above, the experiment was repeated on the catechol/Fcc$_3$-modified pyrolytic graphite electrode in order to evaluate the influence of these two species immobilized on the same electrode surface on their individual and native electrochemical behavior. This modified electrode was transferred and studied by cyclic voltammetry in phosphate buffer aqueous electrolyte at various pH values (Figure 3B). The cyclic voltammograms recorded in phosphate buffer aqueous electrolyte at pH 7 show three main redox systems with an apparent normal potential located at -0.500 V for the adsorbed Fcc$_3$ electrochemical signal (assigned to the partially overlapping four hemes and FAD redox responses) and at -0.080 V and +0.200 V for the grafted catechol redox responses (Figure 3B). The apparent normal potential of the main redox system of the grafted catechol located at +0.200 V shifts cathodically by -37 mV per increase of pH unit (Figure S3B) suggesting that only one proton is exchanged per two electrons. Interestingly, this potential shift value per increase of pH unit is similar to that observed (-42 mV) for catechol grafted on glassy carbon electrode modified by lipid deposit and cytochrome *c*.[23] In contrast, the grafted catechol in the absence of the protein displays a -60 mV slope in the linear relationship of the potential to the pH (Figure S2B) as expected for the 2e$^-$/2H$^+$ quinone/hydroquinone redox couple.[23] This observation reveals a similar redox behavior of the catechol pH sensor in the presence of two different immobilized proteins on the electrode surface and suggests that

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this behavior may be generalized to modification by other proteins immobilized by different methods (co-adsorbate, integration in lipid deposits, etc.).

The apparent normal potential of the adsorbed Fcc_3 onto catechol/Fcc_3-modified electrode (Figure 3B) shifts by ca. -35 mV/pH unit (Figure S3B) as previously observed onto Fcc_3-modified electrode (Figure S3A). In addition, the variation of the anodic peak current of both adsorbed Fcc_3 (located at -0.490 V) and grafted catechol (located at +0.220 V) are linearly proportional to the scan rate (Figure S4B), confirming that these species are surface-confined at the electrode. Furthermore, the presence of the redox protein in the methanol/ethanol/water washing solution of modified electrodes has been confirmed by UV-visible spectroscopy measurements (Figure S1B) suggesting that the presence of the grafted catechol pH probe does not alter the protein adsorption onto electrode surface. These results show that catechol and flavocytochrome c_3 can be electrochemically immobilized and detected at the same pyrolytic graphite electrode without alteration of the enzyme pH-dependent redox properties. In addition, the pH-dependent redox properties of the grafted catechol in the presence of the immobilized enzyme suggest a similar electrochemical behavior of the catechol pH probe when modified by different proteins and different methods.

3.2.2. Fumarate reductase catalytic activity

In this section we investigate by cyclic voltammetry the fumarate reductase catalytic activity of flavocytochrome c_3 (Fcc_3) adsorbed onto unmodified and catechol-modified edge plane pyrolytic graphite electrodes in a phosphate buffer aqueous electrolyte in the absence and in the presence of fumarate in solution (Figure 4).
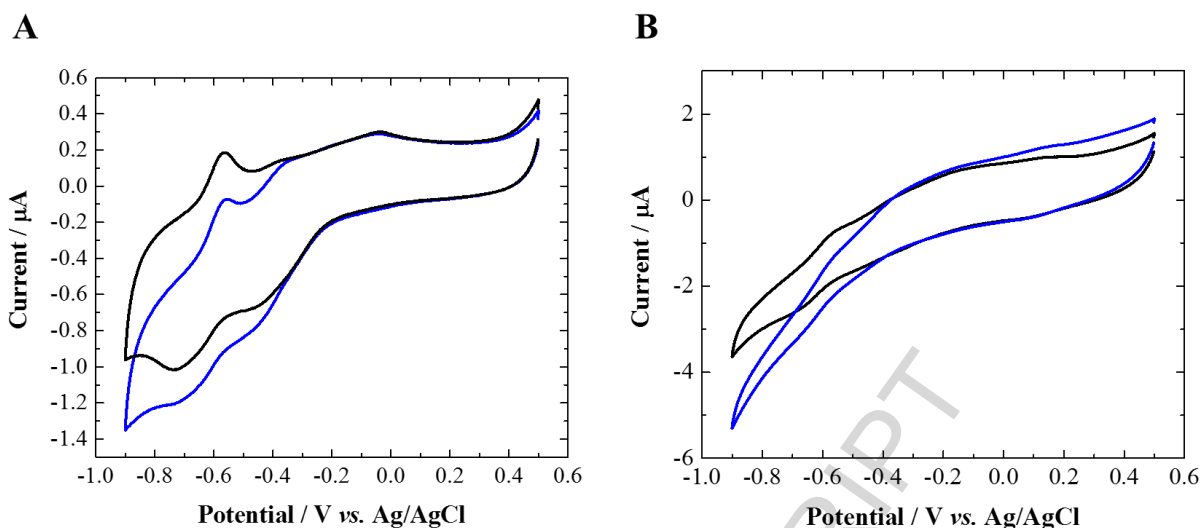
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A

B

Figure 4. Cyclic voltammograms (1st cycle shown) recorded at 20 mV s^{-1} on (A) Fcc$_3$-modified and (B) catechol/Fcc$_3$-modified pyrolytic graphite electrodes in 0.1 M phosphate buffer aqueous electrolyte at pH 8.3 under inert atmosphere (Ar) in the absence (black) and in the presence (blue) of 1 mM fumarate in solution.

In order to evaluate the native fumarate reduction catalysis activity of the adsorbed Fcc$_3$, cyclic voltammograms were recorded at 20 mV s^{-1} in phosphate buffer aqueous electrolyte in the presence and in the absence of 1 mM fumaric acid in solution (Figure 3).[20, 26, 28] In these conditions, the catalytic fumarate reduction activity of the adsorbed enzyme is evidenced at the Fcc$_3$-modified pyrolytic graphite electrode with a current enhancement of about 40% at -0.6 V in the presence of fumarate in solution (Figure 4A). This catalytic property of the adsorbed protein is kept intact at the catechol/Fcc$_3$-modified pyrolytic graphite electrode with a current enhancement of about 45% (at -0.6 V) in the voltammogram recorded in the presence of fumarate (Figure 4B). Furthermore, the presence of fumarate in solution and the catalytic activity associated to the adsorbed enzyme does not affect the grafted catechol reversible redox signal detected at +0.2 V in the cyclic voltammograms presented in Figure 4B. This demonstrates that both grafted catechol and adsorbed flavocytochrome c_3 can be easily immobilized and detected at the same pyrolytic

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graphite electrode while keeping intact the native catalytic properties of the protein and without alteration of the electrochemical response of the grafted catechol pH probe.

4. Conclusions

In summary, the electrochemical detection of adsorbed flavocytochrome c_3 onto edge plane pyrolytic graphite electrode has been achieved before and after surface grafting of a catechol pH probe. The periplasmic flavocytochrome c_3 redox enzyme from *Shewanella putrefaciens* has been successfully immobilized onto pyrolytic graphite electrode with a high surface coverage, following a simple and efficient strategy consisting of consecutive cyclic voltammetry in a flavocytochrome c_3 solution containing polymyxin as co-adsorbate until a steady state current is reached. The electrochemical detection of both grafted catechol and adsorbed flavocytochrome c_3 redox systems has been achieved while maintaining intact their native electrochemical properties such as the pH-dependent electroactivity of the redox enzyme and its fumarate reductase activity. The pH-dependent redox properties of the grafted catechol have been evaluated in the presence of the immobilized enzyme onto electrode and demonstrated the effectiveness of the catechol pH sensor in the presence of the immobilized protein. This work opens the opportunity to investigate larger and more complex redox proteins from electroactive bacteria with not only pH-dependent redox potentials but also extracellular proton transport ability, which is essential for viability of electroactive microorganisms and biofilms. Finally, the present study of flavocytochrome c_3 from *Shewanella putrefaciens* points to a pH dependence of the hemes redox potential which is at variance with data from other strains. As no crystal structure is yet available for flavocytochrome c_3 from *Shewanella putrefaciens* this work will fuel future spectroscopic and structural studies on this enzyme.

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Acknowledgements

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Table of Content Graphical Abstract
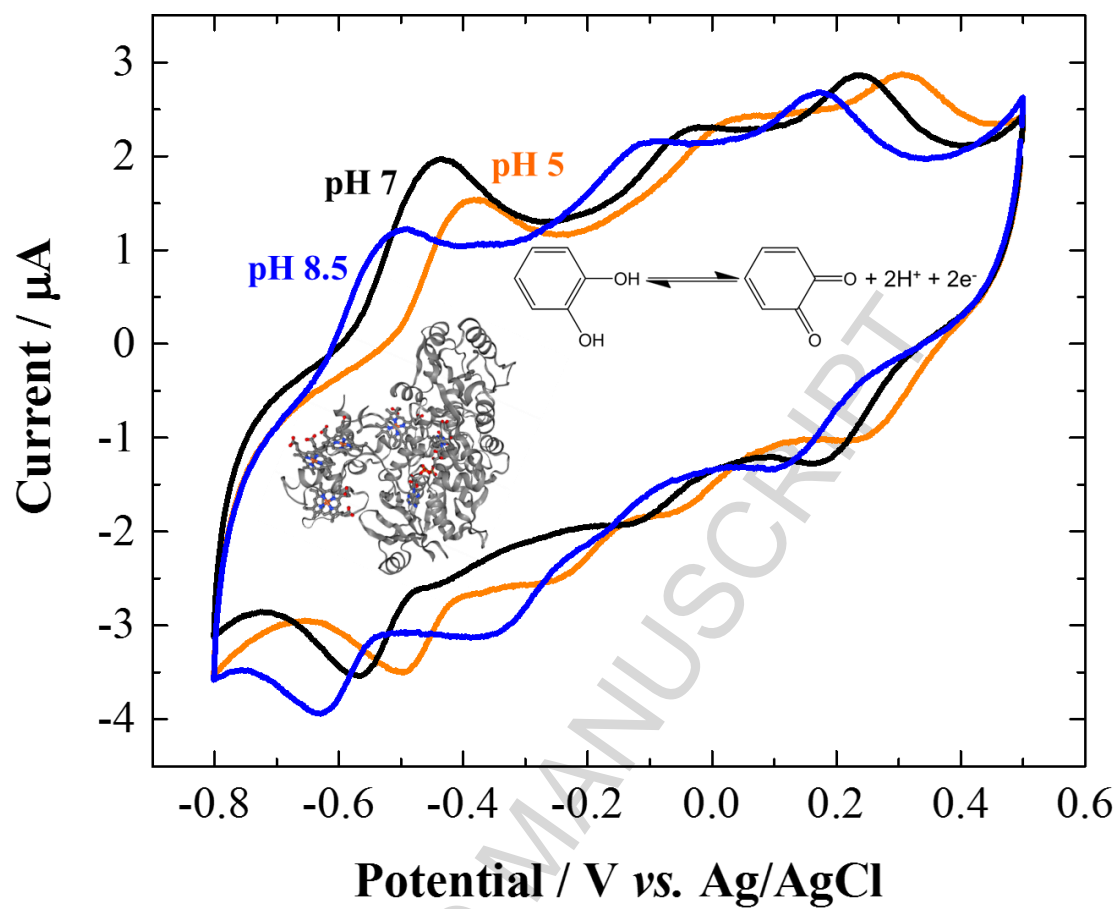
HIGHLIGHTS

- Electrochemistry of flavocytochrome c_3 from *Shewanella putrefaciens* is reported.

- Flavocytochrome c_3 is adsorbed on edge plane pyrolytic graphite electrode.

- The protein redox properties are evaluated at a catechol-modified electrode.

- The enzyme redox/catalytic properties are keep intact onto the pH sensor electrode.

- The hemes' redox potential is pH dependent contrary to data from other strains.