

Electrical wiring of *Shewanella oneidensis* MR-1 with
osmium polymers of different redox potentials

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Master thesis in Bio-Analytical Chemistry

Department of Biochemistry and Structural Biology

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Abstract

The results of studies have shown that the several applications such as microbial electrochemical system (MES), bio-electrochemical systems (BESs) and production of beneficial chemicals are due to electrochemical connection among electrodes and microorganisms. The following two factors are important in choosing the microbe mediated electrochemical biosensors: I) Binding of bacterial cells on the electrode, II) Efficient electron transfer (EET) between the electrode and the cells by a mediator.

In this study, gram-negative bacterium *Shewanella oneidensis* MR-1 was used in MES due to its unique characteristic features such as metal ion-reducing bacterium and direct ET capability. Osmium redox polymers (ORPs) were acted as efficient electron transfer (EET) mediators to direct the electrochemical flow of biomaterials to electrodes.

In the present study, the effect of the formal potential (E^0) of three flexible osmium redox polymers was investigated: $[\text{Os}(\text{4,4'-dimethoxy-2,2'-bipyridine})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMOPVI]); $[\text{Os}(\text{4,4'-dimethyl-2,2'-bipyridine})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMPVI]); and $[\text{Os}(\text{4,4'-dichloro-2,2'-bipyridine})_2(\text{PVI})\text{Cl}]^+$ ([Os-DCPVI]). The electron donating or withdrawing ability of the functional group on the bipyridyl ligands coordinated to the osmium redox center can greatly affect the Os (II/III) redox potential.

Our results demonstrated that out of the three polymers, Os-DMPVI with E^0 of +121 mV vs $\text{Ag}|\text{AgCl}|\text{KCl}(\text{sat.})$ had the greatest ability to produce electrical energy in the presence of lactate as a substrate because of the methyl functional group. Os-DMPVI polymer modified graphite electrode connected with *S. oneidensis* MR-1 exhibited a current density of $13.62 \mu\text{A cm}^2$ with a significant decrease in the start-up time for electrocatalysis.

1. Introduction

Microbial electrosynthesis is a form of microbial electrocatalysis in which electrons are supplied to living microorganisms. For example, by applying an electric current, various species of bacteria can respire with the help of an insoluble substrate via a cathode in an electrochemical cell. Two such examples of microbes are *Geobacter sulfurreducens* strain GSU1501 and *Shewanella oneidensis* strain MR-1 (MR-1). Subsequently, the electrons can be exploited by the bacterial species to reduce carbon dioxide to yield industrially relevant products^{1,2}.

A microbial fuel cell (MFC) work by metabolizing the substrate by microbion, which transfer the gained electrons from microbial electrocatalysis to the anode. This can occur either directly through the membrane or via mobile redox shuttles on the bacterium. MFC converts energy which is available in a bio-convertible substrate into electricity, which can be achieved when bacteria switch from the natural electron acceptor to an insoluble acceptor, such as the MFC anode (**Fig. 1**). Such electron transfer (ET) can occur in two manners; via membrane-associated components or when the soluble electron shuttles. The electrons so generated subsequently flow through a resistor to a cathode, where the electron acceptor is reduced (**Fig. 1**). In this manner, MFC can create electrical current and produce carbon dioxide similar to an anaerobic respiration.

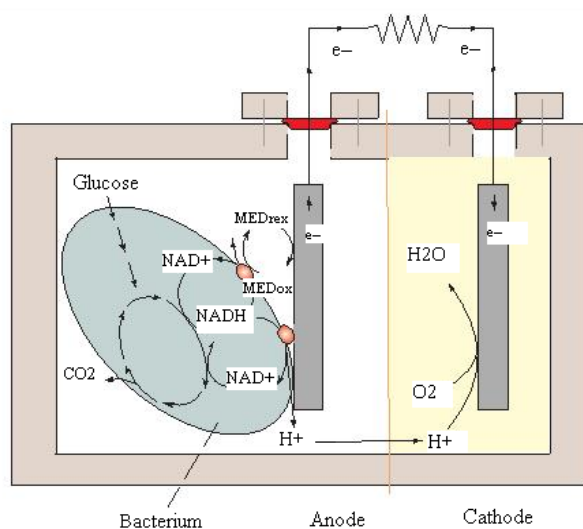


Fig. 1 A schematic representation of a microbial fuel cell. Substrate is metabolized by bacteria, which transfer the gained electrons to the anode. This can occur either directly through the membrane or via mobile redox shuttles. MED, redox mediator; Red oval, terminal electron shuttle in or on the bacterium³.

Microbial bioelectrochemical systems (BESs) interconvert electrical and chemical energy. The key component of a BES is a bioelectrode colonized by microorganisms that function as living “catalysts” for ET resulting from the oxidation of compounds to an anode in order to generate an electric current. Such extracellular ET by microbial BESs occurs by different mechanisms. ET may take place directly via outer membrane cytochromes and conductive pili, which are widely known as nanowires. Alternatively, ET may also be mediated via exogenous or endogenous electron shuttles^{4,5,82}.

The applications of microbial BESs have increased exponentially in recent times due to the production and uses of the possible electrical current driven biochemical/fuel cells⁶. However, lack of sufficient ET from bacteria to electrodes restricted the performance of microbial BESs⁷. In order to enhance the ET rates to electrodes, there have been constant efforts to modify and improve upon not only the microbial genetic, but also the synthesis and development of new electrode materials⁸². In this process, the electrode surface has been modified and new polymeric electrode materials have been developed, which resulted in overcoming limitations of the slow ET⁸.

New strategies have been developed in order to overcome the limitations of the slow ET by constructing a conductive artificial biofilm on an anode in MFC, wherein bacteria were immobilized in the conductive polypyrrole matrix, resulting in an enhanced current generation⁹. In addition, the use of osmium (Os) redox polymers has recently gained attention for biosensor and biofuel cell applications because of their efficient electron shuttling properties, reversibility and polymeric nature. More specifically, the development of enzyme-based fuel cells enhanced the application of Os redox polymers in establishing electrochemical communication between microbiome used in MFC⁸.

Considering the efficient and enhanced ET properties of the Os-polymers^{8,10}, the objective of this study was to investigate the ability of modified Os-polymers in the improvement of the ET from the exoelectrogen *Shewanella oneidensis* MR-1 to the electrodes. Therefore, new class of Os-polymers with different redox potentials were synthesized in order to extract electrons from the microbes, which facilitated ET to electrodes similar to redox polymers used earlier^{8,10}.

2. Aim

This study was performed to investigate the natural electrogen growth at a polymer-modified anode to improve the current generated by the microbe *Shewanella oneidensis* MR-1. In addition, comparing the ability of Os-based redox polymers in the electron transfer (ET) from the microbial cell, *S. oneidensis*, to the solid electrodes and determining the mechanism of action of the extracellular ET to solid acceptors were also important goals of this work. We expected that the procedure of natural growth or artificial immobilization of electrogens like *S. oneidensis* at Os-based redox modified polymers covered electrode could enhance many-fold the electrocatalytic current generation. In this work, we compared three modified Os-polymers with different redox potentials: $[\text{Os}(4,4'\text{-dimethoxy-2,2'-bipyridine})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMOPVI]), $E^{0'} = -59 \text{ mV}$, $[\text{Os}(4,4'\text{-dimethyl-2,2'-bipyridine})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMPVI]), $E^{0'} = +121 \text{ mV}$ and $[\text{Os}(4,4'\text{-dichloro-2,2'-bipyridine})_2(\text{PVI})\text{Cl}]^+$ ([Os-DCPVI]), $E^{0'} = +290 \text{ mV}$ vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat.})}$ which were synthesized (**Table 1**) in order to find out the most efficient ET in these cells as also reported earlier¹¹⁻¹⁵.

3. State of knowledge

3.1 Microbial fuel cells (MFCs)

3.1.1 History/ Introduction

Microbial fuel cells (MFC) convert a bio-convertible substrate into electricity. This can be carried out by bacteria which move from the natural electron acceptor to an insoluble acceptor on the MFC anode (**Fig. 1**). In this process the substrate is metabolized by bacteria, which in turn transfer the gained electrons to the anode. Such transfer of electrons occur in two ways either directly through the bacterial membrane or via redox shuttles³.

A MFC consists of two electrodes that are always in contact with an electrolyte solution. However, in the anode compartment, fuel is oxidised by the bacteria. The electrons attained from oxidation of the fuel are later transferred (ET) to the anode. This flow of ET from the anode to the cathode via an external circuit can be exploited to generate the electrical work. However at the cathode, in a reduction reaction, the electrons can be transferred to an electron acceptor which is usually O₂. Alternatively, this can also be done with the help of bacteria. The cathode and anode compartments are separated via a semipermeable membrane which prevents direct exothermic reaction but can transport positively charged ions from anode to cathode³.

3.1.2 Advantages of microbial fuel cells

Microbial fuel cells (MFCs) have advantages over other kinds of BESs such as enzyme based ones. This makes them attractive as alternative sources in such a system. For example MFCs can directly convert substrate energy to electricity which enables a high conversion efficiency. Also, MFCs operate efficiently at ambient or at low temperatures contrary to all current bio-energy processes. An MFC neither require gas treatment nor energy input for aeration in case the cathode is passively aerated¹⁶. Most importantly MFCs have longer lifetime which is up to five years as compared to the enzymatic biofuel cells which work typically 7 to 10 days¹⁷. Therefore, MFCs have a potential for applications especially in areas where electrical infrastructure lacks.

3.1.3. Electron transfer in microbial fuel cells

3.1.3.1. Direct electron transfer

Direct electron transfer (DET) in a MFC occurs by a physical contact between the bacterial cell membrane and the fuel cell anode, without diffusing redox compounds which participate in the removal of the electrons from the cell to the electrode.

I. Membrane-bound cytochromes

Membrane-bound cytochromes such as c-type cytochromes and multi-heme proteins are known to evolve with sediment inhabiting metal reducing microorganisms. *Shewanella*, being one of them, often relies on solid terminal electron acceptors like iron(III) oxides as described before^{18,19}. In such a system the MFC anode can function as the solid electron acceptor (**Fig. 2A**). In addition the DET via outer membrane cytochromes requires a physical contact with the bacterial cell. In another sense the contact between the cytochrome and the fuel cell anode is vital since bacteria in the first monolayer at the anode surface will be electrochemically active²⁰. Thus the MFC performance can be limited by the cell density of the bacterial monolayer or film.

II. Electronically conducting nanowires

It has recently been demonstrated in literature that *Shewanella* can evolve itself electronically while conducting molecular pili also known as “nanowires” which allow it to reach and utilize more distant solid electron acceptors as described before^{4,21}. These nanowires allow the *Shewanella* to use an electrode which is not in direct contact with the cell as its sole electron acceptor as shown in (**Fig. 2**). As obvious the pili are connected to the membrane bound cytochromes in bacteria, through which the ET takes place to the outside of the cell. The formation of such connection in the form of nanowires allow the development of thicker electroactive biofilms which enable them higher anode performances.

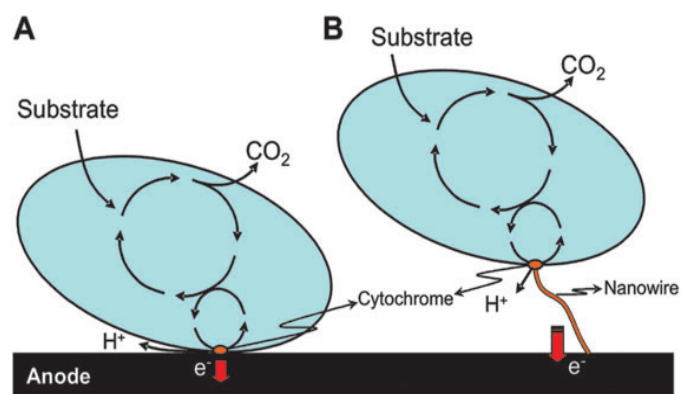


Fig. 2 DET by (A) membrane-bound cytochromes, (B) electronically conducting nanowires (Schröder, 2007).

3.1.3.2. Mediated electron transfer (MET)

Another form of ET is mediated electron transfer (MET) which represent an effective means to wire the bacterial metabolism to a fuel cell anode. However different approaches have been suggested and different kind of MFCs can thus be classified based on the nature of the mediating redox species in the substrate²².

I. MET via exogenous (artificial) redox mediators

Cohen described production of a minute current by anaerobically grown bacterial cultures which may exhibit a strongly negative potential²³. He explained this low generation of the current to a lack of electromotively active oxidation–reduction products in an MFC. He further proposed that the introduction of inorganic or organic substances such as benzoquinone or potassium ferricyanide facilitate the ET from cultures to the immersed electrodes.

II. MET via secondary metabolites

The mediator in MFCs serves as a reversible terminal electron acceptor in the sense that ET can happen from the bacterial cell either to the anode or to the aerobic layers of the bacterial film. At this place it becomes re-oxidized and thus is again available for subsequent redox reactions. In this way one molecule can be used for thousands of redox cycles. Subsequently, the production of small amounts of these compounds on the anodic bacterial film help the microbes to dispose of electrons at high rates. However, in batch cultures, such redox mediators facilitate the ET and enhance current generation^{3,24}.

III. MET via primary metabolites

In comparison with secondary metabolites, the oxidative substrate is closely related with the substrate degradation. The quantity of reduction equivalents produced in this manner matches with the amount of oxidized metabolites. In order to be as negative as possible the redox potential should be accessible for electrochemical oxidation under MFC conditions. There are two types of anaerobic metabolic pathways, anaerobic respiration and fermentation, that can lead to the formation of reduced metabolites which will be suitable for MFC utilization²².

3.1.4. Metabolism in microbial fuel cells

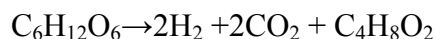
In order to determine the bacterial electricity generation, various metabolic pathways involved in microbial electron and proton flows should be studied. Substrate also influence the potential of the anode in the bacterial metabolism^{25,26}. It is known that the increased MFC current decreases the potential of the anode, forcing the microbes to deliver the electrons through relatively reduced complexes. The potential of the anode therefore determines the redox potential of the bacterial electron shuttle, and hence the metabolism. The microbes employed in MFCs ranges from aerobes, facultative anaerobes or strict anaerobes.

Bacteria can use the respiratory chain in an oxidative metabolism at high anode potentials. Electrons and protons can be transported through the well established NADH dehydrogenase, ubiquinone, coenzyme Q or cytochrome^{27,28}. The use of this canonical pathway was lately investigated by Kim et al. (2004). The electron transport system which they used in MFC involved NADH dehydrogenase, Fe/S proteins and quinones as a platform for electron carriers. Oxidative phosphorylation have usually been observed in almost all kinds of MFCs which yield high energy efficiencies²⁶.

Possible reactions during fermentation of glucose can be as follows²⁹:



or



Accordingly a maximum of 1/3 of a hexose substrate electrons can be used to generate current in theory however 2/3 of substrate give rise to products such as acetate and butyrate³⁰. Therefore the 1/3 of the total electrons are possibly available for electricity generation [18] and are available from outside by mobile electron chain³¹. As already known, this type of metabolic can imply production of high amount of acetate or butyrate. Further, this pathway is substantiated by significant hydrogen production which was observed when the microbial cultures were incubated in a separate fermentation test anaerobically²⁴.

Such metabolic variation, in association with the observed redox potential data generally provides new insight into microbial electrodynamics. An MFC operated at low external resistance can initially generate low current and therefore can have a high anode potential but

low MFC cell potential. With constant growth of the culture, the metabolic turnover rate will increase and hence the current. In case a high resistance is used, the anode potential becomes low which is applicable even at small levels of current. In such cases, low redox facultative anaerobes can be selected.

3.2 *Shewanella oneidensis* MR-1

3.2.1. Introduction

Two of the best studied model systems in MFC are based on the ability of the bacteria to respire insoluble substrates. These organisms are *Geobacter sulfurreducens* strain GSU1501 and *Shewanella oneidensis* strain MR-1 (MR-1)^{1,2}. While both of them use a variety of multi heme *c*-type cytochromes, it is only *Shewanella* which is able to respire insoluble substrates without a direct contact^{32,33}. In any case, both are known to produce conductive “nanowires” that can facilitate respiration of the insoluble substrates as described before^{4,21}. However, these “nanowires” alone cannot elucidate the potential ability of *Shewanella* to reduce the insoluble substrates even at a distance.

Shewanella oneidensis MR-1 (**Fig. 3**) is taken as a model organism for this study. In addition, the electrode to be used in MFCs will be graphite with Os-polymer modified for constant growth or attachment of *Shewanella oneidensis*. After growth, *Shewanella oneidensis* MR-1 will be fixed onto electrode surfaces. Whole-cell biocatalytic organisms growth will inspire microbe-electrode communications and a current generation will be recorded.

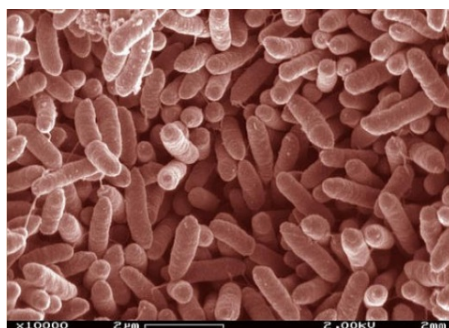


Fig. 3 Bacterial nanowires created by *Shewanella oneidensis* MR-1 on the graphite fingered electrode of a microbial fuel cell³⁴.

Shewanella is gram negative γ -Proteobacteria abundantly found in sedimentary and chemically-

stratified aquatic environments³⁵. Such diverse distribution of this species is possible because of its versatile respiratory capacity. It can respire about 20 organic and inorganic compounds under anoerobic conditions including many insoluble or toxic metals^{35,36}. Extracellular respiration takes place in *Shewanella* and by this process electrons are transferred (ET) to an insoluble acceptor by a mechanism that enables the delivery of electrons from the cytosol to the extracellular substrate. ET is accomplished using specialized membrane-bound and periplasmic electron carriers including quinones and c-type cytochromes that span the outer membrane, inner membrane and periplasm. This respiratory versatility of *Shewanella* is supported by over 40 c-type cytochromes genes found within the *Shewanella* genome^{36,37}.

One such species, known as *Shewanella oneidensis* MR-1 is the most extensively studied strain in this genus and pathways involved in its extracellular respiration have well been studied. With advanced biological techniques and a sequenced genome, *Shewanella oneidensis* has now become one of the most studied model organism in biotechnological applications³⁷⁻³⁹.

3.2.2. Cell wall structure

Gram staining

Bacteria can broadly be classified into two categories: Gram-positive and Gram-negative based on differences between their cell wall structure. Both types differ in the external structure but are similar in the internal. Gram staining is still favoured as one of the best techniques to determine whether a bacterium is Gram-positive or Gram-negative in diagnostic microbiology.

The crystal violet-iodine complex is retained by a Gram-positive bacterial species used in the Gram stain. On the other hand a Gram-negative species will not retain it and thereby they can be distinguished based on their color. Under the microscope Gram-negative appears pink and Gram-positive appears purple due to retention of the crystal violet-iodine complex so formed. The differences in the chemical and physical properties of the cell wall of the both species which are responsible for whether the crystal violet-iodine complex is retained or not are described below (Fig. 4).

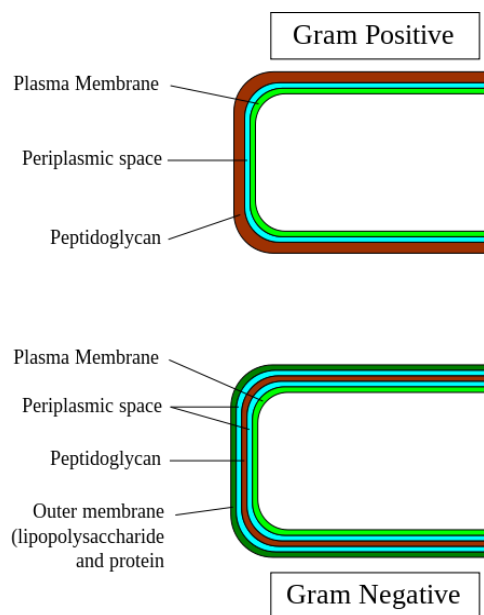


Fig. 4 Showing gram-positive and gram-negative bacteria cell wall structure⁴⁰.

Porin

The most important characteristic of Gram-negative bacteria structure is the lipopolysaccharide (LPS) outer membrane. The LPS layer contains spaces known as porins, which permit inactive diffusion of specific molecules into the periplasmic space. Porins function as channels specific to various forms of molecules in which particles can diffuse through. Porins are different from other membrane proteins in the sense that they are big enough to allow passive diffusion. The channel made up of porin is partially blocked by a loop, known as loopole. As shown in (**Fig. 5**), a loopole is covered completely with charged amino acids arranged on the contrasting sides of the channel responsible in generating a transversal electric field through the pores.

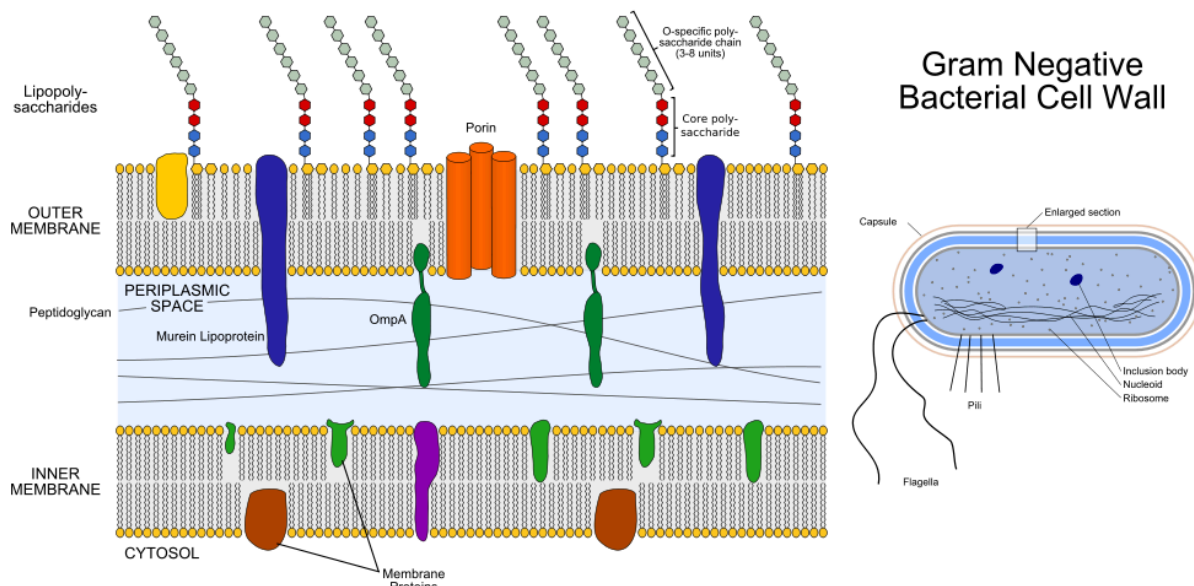


Fig. 5 Cell wall structure⁴¹.

3.2.3. Exogenic electron transfer of *Shewanella*

The earliest form of known process of respiration on earth is the extracellular electron transfer for respiration of insoluble oxide minerals by microbes⁴². In nature, microbes catalyze the breakdown of organic matter, which is coupled to the reduction of a terminal electron acceptor. Some of the well known electron acceptors in sediment and soil environments are insoluble Fe(III) oxide minerals which are reduced to Fe(II) by dissimilatory metal-reducing bacteria under anaerobic environments. The resulting Fe(II) oxide minerals are soluble and can thereby diffuse to the anoxic/oxic interface which can either be assimilated or reoxidized⁴³.

Three different mechanisms of extracellular ET are known, which explain how dissimilatory metal-reducing bacteria can respire insoluble substrates. There are either by direct contact or with the help of nanowires or by electron shuttling. Two of the best-studied model systems for how bacteria respire insoluble substrates are *Shewanella oneidensis* strain MR-1 and *Geobacter sulfurreducens* strain GSU1501^{1,2}.

3.3. Osmium redox polymers (ORPs)

Redox hydrogels are cross-linked polymer structural networks, which are capable of swelling in water. Such hydrogels represent an electron-conducting phase in which water soluble molecules can diffuse and dissolve⁴⁴. Redox hydrogels have been commonly used as electron shuttling diffusion Electron Transfer mediators in membrane less biofuel cells⁴⁵. Redox hydrogels are based on a mechanism where the electrons are conducted by self-exchange reactions through collisions between rapidly reduced and rapidly oxidized centers which are bound to the backbone of cross-linked networks of polymer⁴⁶. Therefore Redox centers are required to come close to the electrode so that electrons can cross the distance as define by the Marcus theory³¹. The rate of ET increases or decreases based on the concentration of redox center concentrations. Therefore the ET becomes maximum when the hydrogels are placed in a close proximity of their redox potential as shown before⁴⁷. On the other hand the rate of ET decreases exponentially with an increased distance between the redox centers as shown by a previous study⁴⁰.

Redox hydrogels comprising Os^{2+/3+} are often referred to as Osmium redox polymers (ORPs). The redox potential of hydrogel depends mainly on concentrations of chloride which influence their E^0 – value. Incorporation of flexible long polymers like Os^{2+/3+} upon covalently binding the redox centers allows them to swing and exchange electrons. On the other hand, the electron diffusion coefficient (De) increases as the length of the tether becomes longer and vice a versa. The ORPs are highly polycationic polymers and thus avoids partial phase separation with enzymes and bacterial cells, which are usually polyanionic at physiological pH. Synthesis and structure of ORPs in the field of bioelectrochemistry has been greatly elucidated by Heller et al previously (2006).

In a pioneer study, Heller et al, used ORPs to electrically “wire” the redox centers of enzymes with electrodes for efficient ET between enzymes and electrodes^{48,49}. He demonstrated that there were strong interactions between polycationic ORPs and polyanionic enzymes, which reduced the ET distance between the redox centers and electrodes. In order to increase the ET, following parameter of the ORPs are presumed important:

- ORPs should be hydrophobic and water soluble, charged or hydrogen binding domains should be available to bind to the enzymes.

- A small part of the ORPs bind with the electrode and the other major part is available to bind to the enzymes which is possible when the ORPs are water soluble and with a high molecular weight.
- In order to achieve a long-term stability of ORPs, which are stabilized by the redox hydrogels initially through electrostatic interactions, they should be incorporated with a great number of enzyme molecules by cross-linker, e.g., poly-(ethylene glycol)-diglycidyl ether (PEGDGE).

4. Materials and methods

4.1 Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich/Merck. The water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). The electrochemical studies were performed under sterilized and anoxic conditions. The piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES buffer; pH = 7) and minimal medium (M1) containing 5 mM sodium L-lactate (Sigma-Aldrich, St. Louis, MO, USA), LB medium and tryptic soy agar were prepared and used for all studies as described before^{50,51}. Polymers $[\text{Os}(4,4'\text{-dimethoxy-2,2'\text{-bipyridine}})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMOPVI]), $E^0 = -59 \text{ mV}$, $[\text{Os}(4,4'\text{-dimethyl-2,2'\text{-bipyridine}})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMPVI]), $E^0 = +121 \text{ mV}$ and $[\text{Os}(4,4'\text{-dichloro-2,2'\text{-bipyridine}})_2(\text{PVI})\text{Cl}]^+$ ([Os-DCPVI]), $E^0 = +290 \text{ mV}$ vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$ were synthesized in the same ways as reported previously¹¹⁻¹⁵.

4.2 Equipment

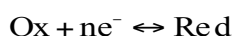
All measurements were accompanied either by AUTOLAB PGSTAT 30 (Eco Chemie, Utrecht, The Netherlands) or by PalmSens (Palm Instruments BV, The Netherlands) with a conventional three electrode set-up. In both, a modified graphite electrode (GE), an $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$ electrode (+0.197 V vs. NHE, Sensortechnik Meinsberg, Germany), served as the working, reference and auxiliary electrodes. The sealed, water-jacketed glass vessels connected to a thermostat (Paratherm U2 electronic, Julabo, Schwarzwald, Germany) were employed as electrochemical cells that maintained temperature control. All the experiments were performed under anoxic conditions, achieved by sparging argon for 20 min in the buffer solution/growth medium before use.

4.3 Experimental

4.3.1 Electrochemical techniques

Amperometric techniques involving measurement of current by a constant applied potential that involve measuring the current as a function of the applied potential were mostly used in this work. Electrochemical techniques deal with interplay between electricity and chemicals. There are

several electrochemical techniques and approaches to study electrochemical reactions as described before⁵². Cyclic voltammetry, linear sweep voltammetry, chronoamperometry, and amperometry are a few of them and they differ in the outcome of the electrochemical signal used for the measurement. A typical electrochemical reaction consists of the charge transfer between the electrode and a species in solution. Several factors, such as the applied potential at the electrode, the nature and reactivity of the species, and the structure of the region at interface affect the reaction. An electrochemically reversible redox reaction follows the following scheme at the electrode surface:



Where Ox and Red are the oxidized and reduced species, respectively, and $n\text{e}^-$ are the number of electrons involved in the reaction. The potential of the electrode is related to the concentration of the redox species involved in the electrochemical process, as described by the Nernst equation:

$$E = E^{0'} + \frac{RT}{nF} \ln \frac{[\text{Ox}]}{[\text{Red}]}$$

Where

$E^{0'}$ = formal potential of the redox reaction,

R = universal gas constant,

T = absolute temperature in Kelvin,

N = number of electrons involved in the reactions,

F = is the Faraday constant, and

[Ox] and [Red] = concentrations of the oxidized and reduced species.

4.3.2. Measurements with the chronoamperometric technique

One of the well known techniques to measure electrochemical detections is Amperometry. In this process, the electrons, once collected from the oxidation of the substrate, are transferred to a working electrode and in this process generates a measurable catalytic current. For this a constant potential is applied at the electrode and the current generated by a redox reaction on the surface of the electrode is measured. Three types of electrodes are used; Reference electrode (RE) made up

of Ag|AgCl, Working electrode (WE) made of various materials e.g. graphite or noble metals (Pt, Au), and the Auxiliary electrode (AE) made from Pt wire.

Similar to Amperometry is chronoamperometric detection which is defined as the overall measurement of the current between auxiliary and working electrode while taking into consideration the reference electrode (e.g., Ag|AgCl|KCl_(sat)). In this process a redox analyte is reduced at cathode and is oxidised at anode. On the other hand a redox analyte can also be reduced by applying an analyte-specific electrochemical potential on the working electrode, which constantly remains in the analyte solution. Thus, the measured current correlates to the concentration of redox analyte.

Various factors affect the rate of oxidation and reduction in all electrochemical reactions. They can be summarize as:

- 1) The first one is the mass transport of material, which is to and from the electrode interface
- 2) The second is the kinetics of electron transfer (ET) at the electrode, which further depends on:
 - a) Electrode surface characteristics
 - b) Reaction characteristics
 - c) Temperature during reaction

Diffusion is a mass transfer and can be defined as the movement of substance under the influence of a concentration gradient or difference in a system. The electro-active species depends on diffusion from the bulk solution to the electrode or vice a versa. However, a concentration gradient can improve in case a reaction depletes (or produces) some special particles at the electrode surface. On the other hand, it was shown earlier that when the solution around the electrode is stirred, for example in a flow cell, a stable diffusion profile can be established at the surface of the electrode resulting in establishment of a steady constant incurrent⁵³. Thus, mass transfer of substance to the electrode surface can be manipulated in two manners. Either by moving the solution with respect to the electrode surface or by moving the electrode in the solution with the help of rotating disk electrode or rotating ring-disk electrode respectively⁵⁴.

In stable solution and under non-equilibrium conditions however in case of a planar electrode placed without stirring, the solution contains extra electrolyte and a limited amount of electroactive substance. The application of a suitable potential, at $t = 0$, the concentration (C) of the electroactive component at the electrode surface decrease to zero ($[C] = 0$) instantly. Thus, the material will flow from the solution to the electrode surface (towards concentration gradient), and the gradient will become stabilized. As a consequence, the diffusion layer grows further into the bulk solution with the passage of time. In this manner, a non-steady state current may change the slope of the diffusion gradient. On the other hand the strength of electric field to the electrode surface changes in a particular area as a function of time. This has previously been explained by the Cottrell equation⁵⁵:

$$I = nFACD^{1/2}\pi^{-1/2}t^{-1/2}$$

I = Current [Ampere],

n = Number of electrons transferred/molecule,

F = Faraday's constant [$96,500 \text{ C mol}^{-1}$],

A = Electrode area [cm^2],

D = Diffusion coefficient [$\text{cm}^2 \text{ s}^{-1}$], and

C = Concentration [mol cm^{-3}]

Taking into account the potentials applied in the chronoamperometry technique, the current response to a positive potential is calculated as the current towards positive amounts proceeding by a decrease in time. However at the negative potentials, the current 'sharp point' to more negative values with a 'reverse' decay to more positive values. In any case the diffusion is controlled by one of the electrodes and current 'decays' towards zero amperes with the passage of time. As described the Cottrell equation is used for the determination of the electroactive surface area in chronoamperometry⁵⁶.

4.3.3. Cyclic voltammetry

Cyclic voltammetry, CV, is a technique widely used for investigating how an electrode or electrolyte behaves. The basic idea is to record the amount of current that passes through the working electrode, when sweeping the potential over the working electrode and the counter electrode (**Fig. 6**). E_i is the starting potential, E_λ is the most positive potential that is of interest,

and E_f is the least positive potential of interest. The reference electrode is used to make sure that the potential over the electrodes have correct readings.

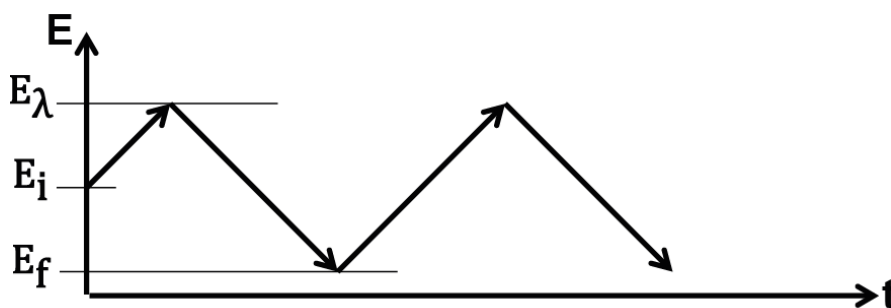
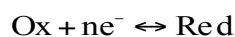


Fig. 6 How the applied voltage changes with time

When cycling the voltage over an electrode, one will reduce and oxidize it according to:



The result obtained using cyclic voltammetry is a diagram looking relatively similar to (Fig. 7). Where E_p^a is the anodic peak potential as well as the corresponding anodic (i_p^a) peak current and E_p^c is also the cathodic peak potential as well as the corresponding cathodic (i_p^c) peak current.

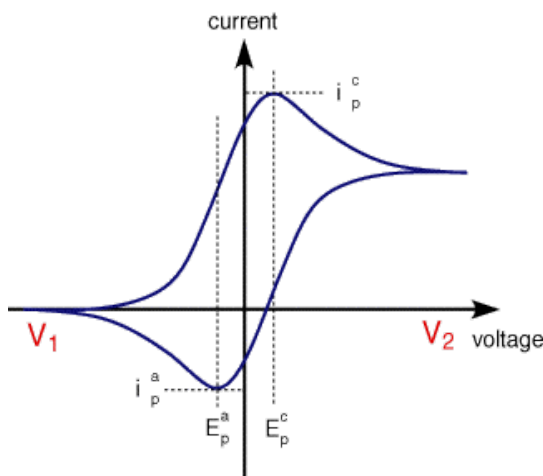


Fig. 7 Typical cyclic voltammogram for a reversible redox process⁵⁷.

4.3.4. Microbial growth conditions and inoculum preparation

Shewanella oneidensis MR-1 (LMG 19005/ATCC 700550) were collected from Belgian Coordinated Collections of Microorganisms/ Laboratorium Microbiology (BCCM™/LMG, Ghent University, Belgium). Bacteria cells were grown and maintained on tryptone soya agar. For

inoculum preparation, a single well-isolated colony was transferred to 15 mL of LB broth in a 50-mL tubes and incubated aerobically at 30 ± 2 °C for 24 h, while shaking at 150 rpm in an incubator shaker (Universal shaker SM 30 A, Edmund Bühler GmbH, Germany). The cells grown in LB were thereafter collected by centrifuging at 4000 rpm for 10 min. The cells were subsequently washed first with 50 mM PIPES buffer (pH 7.0) and then with minimal medium (M1) by centrifuging as described before⁸.

Consequently, the cells were transferred to 20 mL of minimal growth medium containing PIPES buffer (15.1 g L^{-1}); NaOH (3.0 g L^{-1}); NH_4Cl (1.5 g L^{-1}); KCl (0.1 g L^{-1}); $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.6 g L^{-1}); NaCl (5.8 g L^{-1}); mineral solution (10 mL L^{-1}); vitamin solution (10 mL L^{-1}), and amino acid solution (10 mL L^{-1}) as described before^{50,51}.

4.3.5. Working electrode preparation

Spectrographic graphite rods (Ringsdorff Werke, GmbH, Bonn, Germany) were used for preparing the working electrodes. The procedure described by Coman et al. (2009), was followed to fabricate the osmium (Os) polymer modified working electrodes. $[\text{Os}(4,4'\text{-dimethoxy-2,2'\text{-bipyridine}})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMOPVI]), $E^0 = -59 \text{ mV}$, $[\text{Os}(4,4'\text{-dimethyl-2,2'\text{-bipyridine}})_2(\text{PVI})\text{Cl}]^+$ ([Os-DMPVI]), $E^0 = +121 \text{ mV}$ and $[\text{Os}(4,4'\text{-dichloro-2,2'\text{-bipyridine}})_2(\text{PVI})\text{Cl}]^+$ ([Os-DCPVI]), $E^0 = +290 \text{ mV}$ vs. $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$, were synthesized¹¹⁻¹⁵.

The end of the graphite rod was polished on fine emery SiC paper (Tufbak Durite, P1200) and carefully washed with Milli-Q water and then dried. 20 μL of Os polymer solution (10 mg mL^{-1} in Milli-Q water) was subsequently spread on the active surface of the electrode (projected surface area; 0.282 cm^2). The electrode was dried at the room temperature for 10-15 min and used for further bioelectrochemical experiments. For comparison, the control experiments were carried out with unmodified graphite electrodes. All experiments were repeated at least three times and the cyclic voltammetry was recorded at a scan rate of 10 mV s^{-1} .

5. Results and discussion

5.1 Characterisation of *Shewanella oneidensis* MR-1

With genetic manipulation techniques available and a sequenced genome, *Shewanella oneidensis* MR-1, a gram negative bacteria has become an important model organism in biotechnological applications³⁹. In addition, it is one of the microbes, which can utilize membrane bound cytochrome to conduct a direct electron transfer⁵. In addition *Shewanella oneidensis* MR-1 can transfer electrons over a cell length of about 50 μm to a known insoluble electron acceptor. Therefore, *Shewanella oneidensis* MR-1 was selected in this study as a model organism. In this study, *Shewanella oneidensis* MR-1 was incubated at $30\pm 2^\circ\text{C}$ for about 72 h as reported and then the cells were harvested to be used as an inoculum in potentiostatic half-cell set ups hosting a similar minimal growth medium. For every experiment, bacterial inoculum were taken after washing with PIPES solution, which were subsequently used in the Potentiostatic half-cell setup in M1 medium in order to measure the electric current at controlled temperature of 26°C .

5.2 Lactate metabolism

Shewanella oneidensis MR-1 is a well known Gram-negative anaerobic bacterium that can produce energy by connecting the oxidation of organic reagents to reduction of many types of electron acceptors under in vitro conditions. Such metabolic abilities of *Shewanella* make it useful to many types and number of electron acceptors. For example *Shewanella oneidensis* MR-1 can produce energy, which is required for fermenting pyruvate. In brief, *Shewanella* anaerobically produces acetate when allowed to grow on lactate as a single source of energy⁵⁸. Under anoxic conditions pyruvate dehydrogenase in *Shewanella* is not involved in the lactate metabolism. On the other hand it is essential for anaerobic conditions. The pyruvate fermentation by *Shewanella oneidensis* MR-1 involves a group of reactions and respiration steps. The basic enzyme in anaerobic glycolysis is lactate dehydrogenase (LDH) that catalyzes the switch between pyruvate and lactate and plays an essential role in energy metabolism⁵⁹. This process is known as anaerobic fermentation and is a key pathway to renew Nicotinamide adenine dinucleotide (NAD^+) to allow glycolysis to continue⁶⁰. NAD^+/NADH is the cofactor for this reaction and act as a hydrogen acceptor or donor according to the reaction pathway shown below⁶¹ (Fig. 8).

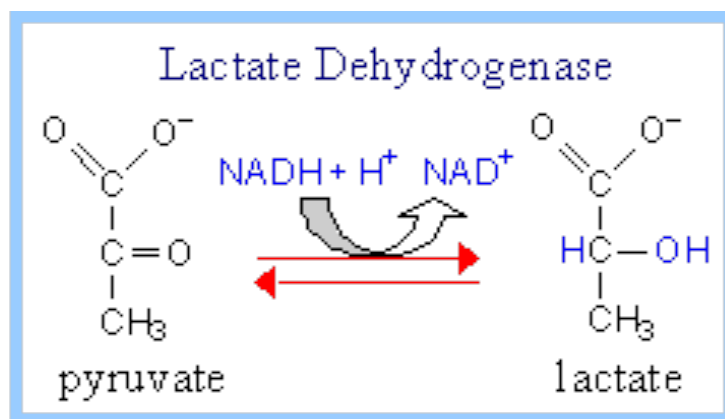


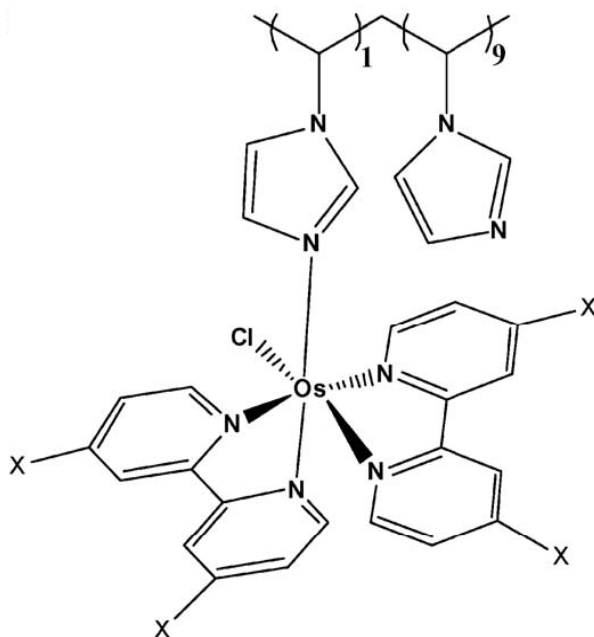
Fig. 8 Lactate Dehydrogenase, act as a catalyze for reducing ketone group to hydroxyl, yielding lactate, as NADH is oxidized to NAD^+ ⁵⁹.

5.2 Current generation by the Os-polymers without *Shewanella oneidensis*

In order to investigate the influence of the formal potential (E°) of the Os-complexes bound to the polymeric backbone on the efficiency of mediation of the ET from *Shewanella oneidensis* MR-1 to the electrode, three different osmium redox polymers were studied which span a potential range between -59 and +290 mV vs. $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$. Characteristics of the three Os-polymers are summarized in Table 1 (Column 1 and 2) and the general chemical structure of the synthesized redox Os-polymers are also shown in Scheme 1. By altering the coordinating ligands as shown in Scheme 1, the osmium derived complexes can provide a redox complex of lower potential, resulting in the production of high current densities by the electrodes⁶². In our experiments, we showed that the replacement of the 4,4'- dimethyl functional group on both bipyridines by a more electron-donating methoxy- functional group and by a more electron-withdrawing chloro- functional group on the bipyridine ligands coordinated to the osmium redox center, can shift the Os(II/III) redox potential from +121 mV vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$ to a lower (-59 mV) and a higher (+290 mV) values, respectively (Table 1, Column 3).

Table 1. Characteristics of the three Os-polymers

Os-Polymer	Structure of Os-Polymers	$E^{0'}$ vs Ag AgCl KCl_{sat}
Polymer a		
Os-DMOPVI (Os-Polymer-2)	Os-DMOPVI [Os(4,4'-dimethoxy-2,2'-bipyridine) ₂ (PVI)Cl] ⁺ (X = OCH ₃)(1:9)	-59 mV
Polymer b		
Os-DMPVI (Os-Polymer-3)	Os-DMPVI [Os(4,4'-dimethyl-2,2'-bipyridine) ₂ (PVI)Cl] ⁺ (X = CH ₃)(1:9)	+121 mV
Polymer c		
Os-DCPVI (Os-Polymer-8)	Os-DCPVI [Os(4, 4'-dichloro-2, 2'-bipyridine) ₂ (PVI)Cl] ⁺ (X = Cl), (1:11)	+290 mV



Scheme .1 General chemical structure of the osmium redox polymer: **Os-DMOPVI** $[\text{Os}(4,4'\text{-dimethoxy-2,2'-bipyridine})_2(\text{PVI})_{10}\text{Cl}]^{+/2+}$, (**X** = **OCH₃**) (Os-(dmobpy)PVI (1:9), $E^0 = -59$ mV vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$; **Os-DMPVI** $[\text{Os}(4,4'\text{-dimethyl-2,2'-bipyridine})_2(\text{PVI})_{10}\text{Cl}]^{+/2+}$, (**X** = **CH₃**) (Os-(dmbpy)PVI (**1:9**), $E^0 = +121$ mV vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$; **Os-DCPVI** $[\text{Os}(4,4'\text{-dichloro-2,2'-bipyridine})_2(\text{PVI})_{10}\text{Cl}]^{+/2+}$, (**X** = **Cl**) (Os-(dc(bpy)PVI (1:9), $E^0 = +290$ mV vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$.

(**Fig. 9**) of this study shows a typical cyclic voltammograms (CVs) of the different osmium redox polymers at the surface of modified graphite electrodes without bacterial cells in 50 mM PIPES at pH 7.0 and the scan rate 10 mV s^{-1} which act as a control. As can be seen in the CV, Os-DMOPVI, Os-DMPVI and Os-DCPVI displayed a pair of quasi-reversible redox peaks for Os redox centre in the polymer with a formal potential ($E^{\circ'} = 1/2(E_{\text{pa}} + E_{\text{pc}})$ the mean value of the anodic, E_{pa} , and cathodic peak, E_{pc} , potentials) equal to -40 mV, +80 mV and +110 mV vs. $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat})}$ respectively for these electrodes, which were in agreement with previously published data for these polymers⁶³.

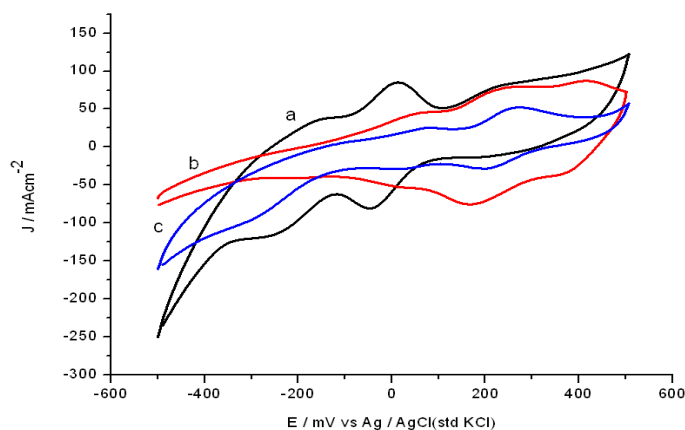


Fig. 9 Cyclic voltammograms (CVs) of (a) Os-DMOPVI, black line (b) Os-DMPVI, red line (c) Os-DCPVI, blue line modified graphite electrode. Conditions: 50 mM PIPES at pH 7.0; scan rate of 10 mV s^{-1} in the absence of *Shewanella oneidensis* MR-1.

5.3 Combination of the Os-polymers with *Shewanella oneidensis*

Osmium redox polymers are cationic in their chemical structure and are known to form strong electrostatic interactions with the anionic bacterial cell membranes. Factors such as polymer molar mass, solubility of the polymer, and swelling affects the variances in film thickness density and apparent electron diffusivity of the final modified polymer⁶³. The recorded CVs of the three types of osmium redox polymers during 10 cycles at a scan rate of 10 mVs^{-1} indicated a good stability of such modified electrodes⁶³.

Finally, these modified electrodes were examined after immobilization of the living Gram-negative bacteria *Shewanella oneidensis* MR-1 to the electrode surfaces. In general, the generated current can be attributed to the natural extracellular ET abilities of the *Shewanella oneidensis* via flavins, cell bound cytochromes, and/or nanowire⁶. However, the enhanced current generation in the case of modified anode can reasonably be attributed to the Os-redox centres embedded in the polymer matrix. Similarly, we showed improved ET to the cell bound cytochromes at the cell–electrode and the cell–polymer–electrode boundary as shown in (**Fig. 10**) . Furthermore, the advanced ET can be related to the creation/existence of a long-distance electron channelling/self-exchange by Os-redox centers that mediate the ET from distant cells to electrodes (**Fig. 10**). An improved cell support and therefore biofilm creation on the polymer-modified anodes might also have contributed to the current generation and also aided the polymer to stay intact and steady on the electrode surface⁸. In a similar way an Os polymer

modified electrode was prepared and thereafter submerged in Gram-negative bacteria *Shewanella oneidensis* MR-1 solution in order to calculate the generated current attributed to the *Shewanella* natural extracellular ET abilities.

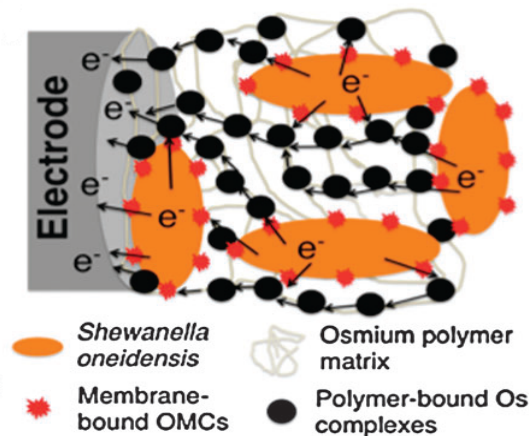


Fig. 10 Proposed schematic electron transfer (ET) from *Shewanella oneidensis* MR-1 to the osmium polymer modified electrode⁸.

5.4 Current generation by the Os-polymers with *Shewanella oneidensis*

The ultimate objective of the current project was to study and compare the capability of Os-based redox polymers to improve ET from the exoelectrogen *Shewanella oneidensis* MR-1 to the electrodes. With three polymer-modified anodes, the bacteria were added to the growth medium and natural electrochemical growth of electrogens at the anode was studied. The amount of Os polymer that was necessary to modify the electrode and the stability of the bacteria were optimized previously by Patil et al (2012). During this study, we have carried out chronoamperometric (CA) experiments in semi-batch with modified electrodes with osmium polymer with a variable start-up time with 18 mM sodium lactate substrate as shown in (**Fig. 11**, Graph B, C and D) and compared the results with unmodified graphite electrodes with osmium polymer (**Fig. 11**, Graph A).

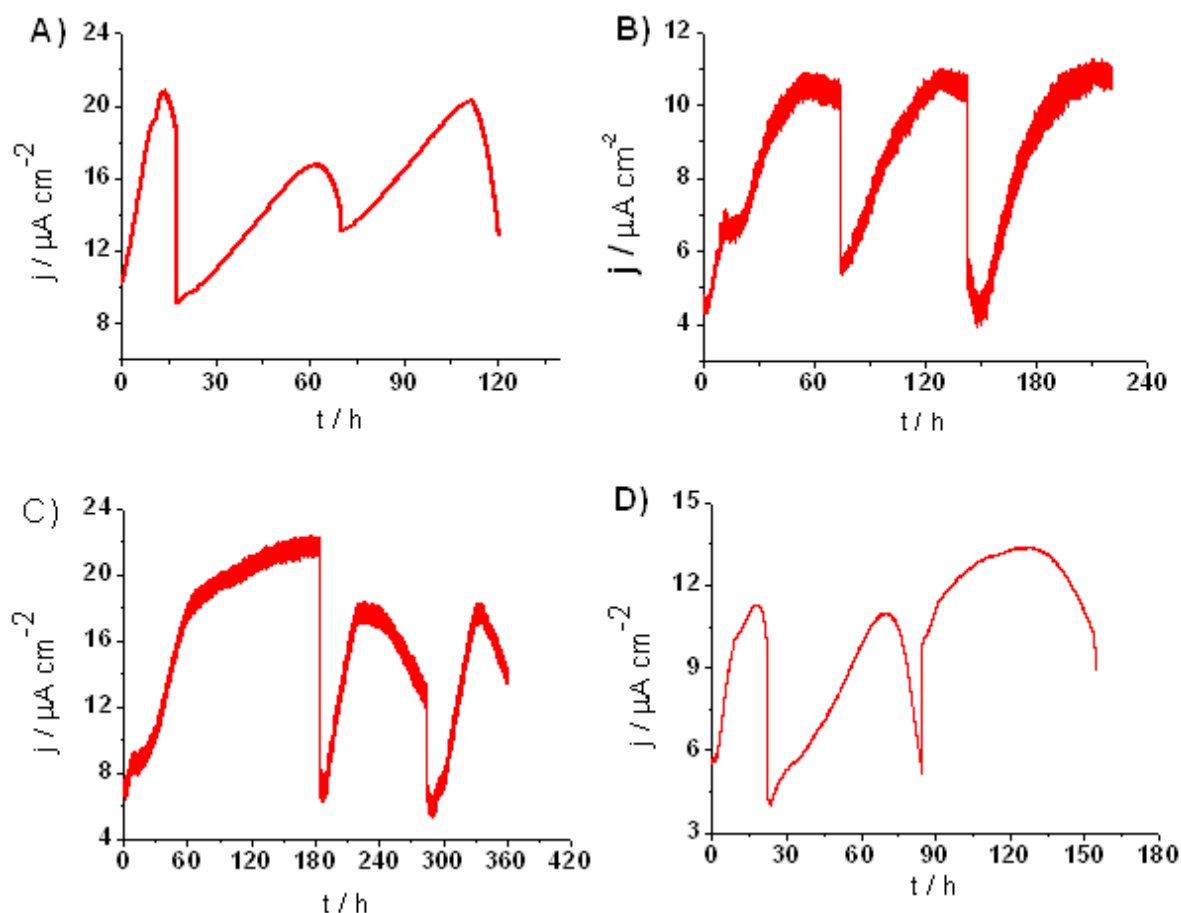


Fig. 11 Representative chronoamperometric measurements showing bioelectrocatalytic current generation (A) at unmodified graphite electrode with the start-up time 27 hours (B) Os-DMOPVI/GE the start-up time of 3.5 hours (C) Os-DMPVI/GE with the start-up time of 8 hours (D) Os-DCPVI/GE; the start up time 2 hours by *Shewanella oneidensis* MR-1. The substrate was 18 mM sodium lactate.

A gradual growing in the present generation with lactate as a substrate was detected after a initial time period with both polymer-modified and unmodified anodes (Control, Graph A). The start-up time of current generation with the best polymer-modified anode was shorter than that of the unmodified anode (Graph B, C and D). The decrease in start-up time for current generation is attributed to the contribution of the Os-redox centres embedded in the polymer matrix which facilitated ET from bacterial cells to the electrode. Current generation with Os-DMOPVI/GE, Os-DMPVI/GE, Os-DCPVI/GE and unmodified graphite electrode (GE) exhibited 6.46, 13.62, 5.66 and 8.53 $\mu\text{A cm}^{-2}$ respectively as represented in (**Fig. 12**). During this study, the maximum

biocatalytic current for all the osmium polymers was found to be in 18 mM of sodium lactate concentration. It was shown that Os-DMPVI ($E^{0'} = +121$ mV) (**Fig.12**, lane 3) generates a maximum bioelectrocatalytic current ($13.62 \mu\text{A cm}^{-2}$) which was a 1.6-fold increase in the maximum current density as compared to the unmodified anode under a similar set of experimental conditions. This could be attributed to a greater flexibility and better accessibility to extract electrons from the inner membrane of the bacterial cells which is composed of lipid bilayer, thereby making Os-DMPVI, the best electron mediator in our experiments. Moreover, the redox potential of this polymer was found to be $E^{0'} = +121$ mV vs $\text{Ag}|\text{AgCl}|\text{KCl}_{(\text{sat.})}$ that falls in the compatible range of the common respiratory chain molecules.

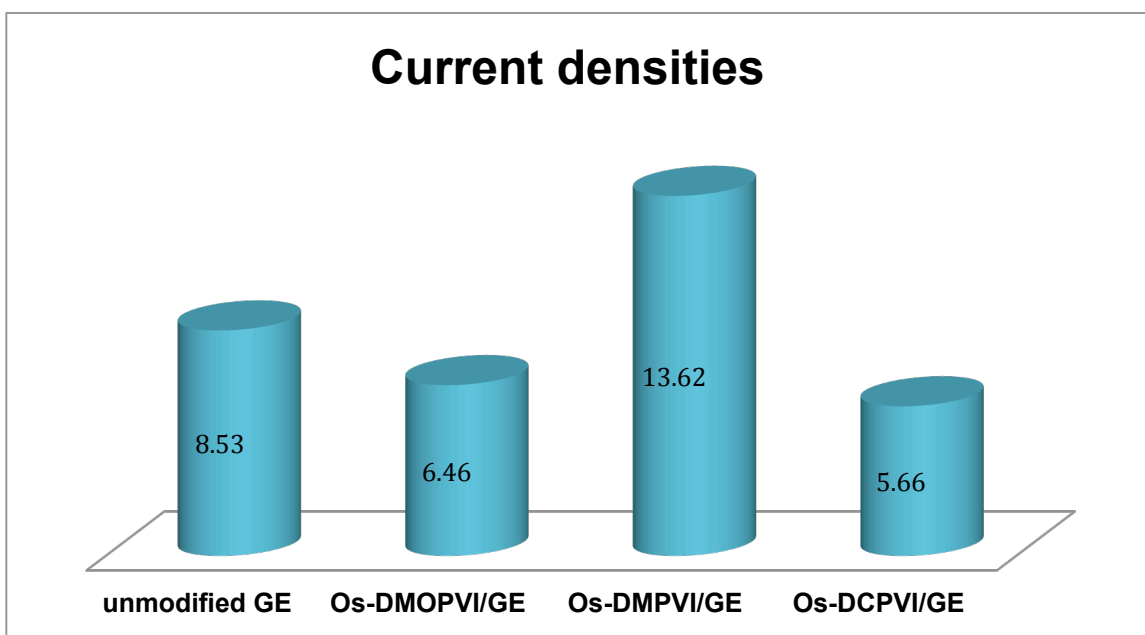


Fig. 12 Steady state maximum current densities ($\mu\text{A cm}^{-2}$) derived from Fig 11. All current densities appear maximum at the relative standard deviation less than 10%.

6. Conclusion

In this study, we have demonstrated the possibility to “wire” *Shewanella oneidensis* MR-1 to graphite electrodes with the help of three different Os redox polymers **Os-DMOPVI** [Os(4,4'-dimethoxy-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺²⁺, (**X** = **OCH₃**) (Os-(dmobpy)PVI (1:9); **Os-DMPVI** [Os(4,4'-dimethyl-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺²⁺, (**X** = **CH₃**) (Os-(dmbpy)PVI (1:9); **Os-DCPVI** [Os(4,4'-dichloro-2,2'-bipyridine)₂(PVI)₁₀Cl]⁺²⁺, (**X** = **Cl**) (Os-(dc(bpy)PVI (1:9). Of these investigated Os-redox polymers, Os-DMPVI with $E^{0'} = +121$ mV vs Ag|AgCl|KCl_(sat) yields a significant current generation of 13.62 $\mu\text{A cm}^{-2}$. The reason could be because of the greater accessibility of this polymers (Os-DMPVI) $E^{0'} = +121$ mV to the *Shewanella oneidensis* MR-1 membrane. The Os-DMPVI apparently increased the diffusion of electrons and caused a more effective electron transfer (ET) from the reduced nicotinamide adenine dinucleotide reaction centers of the bacteria to the electrode. Our results showed that *Shewanella oneidensis* MR-1 presents a good option to be wired to the osmium redox polymers over the graphite electrode surface.

Studies of the interactions between osmium redox systems and micro-organisms like *Shewanella oneidensis* MR-1 open new possibilities for basic bioelectrochemical studies. Such system can be used for reagentless biosensing and can result in the construction of more robust and improved performance of MFCs. On the basis of published literature and this work, we anticipate that the use of osmium redox polymers as electron shuttles can enable the possibility of exploration of a wide range of microbial catalysts for applications as both bioanodes and biocathodes in BESs. Several advantages are associated with these systems, such as ease of immobilization, high synthetic flexibility, excellent redox conductivity, possibility of forming multilayer scaffolds and strong electrostatic interactions with microbial cell surfaces. All these factors can be of great help in order to construct MFCs with osmium redox systems with micro-organisms for their promising use in the microbial bioelectrochemical system (BES) research.

This study can be effectively used in other related areas such as development of conjugated polymer-gold nanoparticles or development of a new modified anode with immobilization of electrogens like *Shewanella oneidensis* MR-1 in order to increase the electrocatalysis many fold.

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List of abbreviations

Ag AgCl	silver/silver chloride
BESs	bioelectrochemical systems
BFCs	biofuel cells
cm ⁻²	square centimetre
CV	cyclic voltammetry
CA	chronoamperometry
DET	direct electron transfer
ET	electron transfer
E^0	formal potential
e ⁻	electron
E_i	starting potential
E_g	most positive potential
E_f	least positive potential
E^c	cathodic potential
E^a	anodic potential
Fig	figure
Fe	ferric
F	faraday constant

g	gram
GE	graphite electrode
h	hour
i^c	cathodic current
i^a	anodic current
KCl	potassium chloride
LPS	Lipo-polysaccahrade
MFC(s)	microbial fuel cell(s)
MED	mediator
MET	mediated electron transfer
mM	milimolar
mL	mililiter
mg	miligram
min	minute
μA	microampere
n	number
NaOH	sodium hydroxide
NaCl	sodium Chloride
NADH	nicotinamide adenine dinucleotide
Os	osmium
Os-DCPVI	[Os(4,4'-dichloro-2,2'-bipyridine) ₂ (poly-

	vinylimidazole) ₁₀ Cl] ⁺²⁺
Os-DMPVI	[Os(4,4'-dimethyl-2,2'-bipyridine) ₂ (poly-vinylimidazole) ₁₀ Cl] ⁺²⁺
Os-DMOPVI	[Os(4,4'-dimethoxy-2,2'-bipyridine) ₂ (poly-vinylimidazole) ₁₀ Cl] ⁺²⁺
Ox	oxidised
pH	Negative logarithm of the hydrogen ion concentration
Pt	platinum
rpm	rotate per minute
R	universal gas constant
Red	reduced
RE	reference electrode
sat	saturated
T	absolute temperature
V	volt
vs	versus
WE	working electrode

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