OLGA ALEKSEJEVA

BLUE COPPER PROTEINS AS BIOELEMENTS FOR BIOELECTRONIC DEVICES

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FOR BIOELECTRONIC DEVICES**

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**BLUE COPPER PROTEINS
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BIOELECTRONIC DEVICES**

Malmö University, 2019
Faculty of Health and Society
Department of Biomedical Science

To the science

"Through hardship to the stars."

"Per aspera ad astra."

"Через тернии к звездам."

Non est ad astra mollis e terris via, Lucius Annaeus Seneca

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ABSTRACT

This thesis is focused on bioelements for biological electric power sources, specifically, on blue copper proteins with and without an intrinsic biocatalytic activity, *i.e.* ability to reduce oxygen directly to water. These proteins, *viz.* different laccases, ceruloplasmin, and rusticyanin, were characterised in detail and employed for the construction of both self-charging and conventional biosupercapacitors. First, similarities and particularities of oxygen electroreduction *vs.* bioelectroreduction were reviewed. Moreover, being a promising candidate for the construction of autotolerant implantable biocathodes, the electrochemistry of human ceruloplasmin was revisited. For the first time, a clear bioelectrocatalytic reduction of oxygen on ceruloplasmin modified electrodes was shown. Second, computational design combined with directed evolution resulted in a high redox potential mutated laccase, GreeDo, with increased redox potential of the T1 site, increased activity towards high redox potential mediators, as well as enhanced stability. Third, GreeDo was electrochemically characterised in detail. The mutant exhibited higher open circuit potential values and onset potentials for oxygen bioelectroreduction compared to the parental laccase, OB-1. Moreover, the operational stability of GreeDo modified graphite electrodes was found to be more than 2 h in a decidedly acidic electrolyte, in agreement with the extended operational and storage stabilities of the enzyme in acidic solutions. Fourth, multi-cell single-electrolyte glucose/oxygen biodevices with adjustable open-circuit and operating voltages, which are independent on the difference in equilibrium redox potentials of the two redox couples, gluconolactone/glucose and oxygen/water, *viz.* 1.18 V, but dependent on the number of half-cells in the biodevice construction, were designed and tested. The biodevices were made from tubular graphite electrodes with electropolymerised poly(3,4-ethylenedioxythiophene) modified with *Trametes hirsuta* laccase and *Neurospora crassa* cellobiose dehydrogenase as

the cathodic and anodic biocatalysts, respectively. Due to the interplay between faradaic and non-faradaic electrochemical processes, as well as between ionic and electronic conductivities, the open-circuit voltage of the self-charged biodevice is extraordinarily high, reaching 3 V, when seven biosupercapacitors operating in a common electrolyte were connected in series. Moreover, glucose/oxygen biodevices could be externally discharged at an operating voltage exceeding the maximal limiting open-circuit value of 1.24 V for the complete glucose oxidation. Last but not least, a conventional biosupercapacitor, *i.e.* a biodevice lacking self-charging ability, was composed of *Acidithiobacillus ferrooxidans* rusticyanin modified gold electrodes. The complete biodevices as well as separate electrodes were thoroughly characterised electrochemically. The symmetrical biosupercapacitor based on two identical gold electrodes modified with rusticyanin is able to capacitively store electricity and deliver electric power, accumulated mostly in the form of biopseudocapacitance, when charged and discharged externally.

POPULÄRVETENSKAPLIG SAMMANFATTNING

I denna avhandling beskrivs framställning av s.k. biosuperkondensatorer, dvs. proteinbaserade elkraftkällor som kan lagra elektrisk energi kapacitativt. Den lagrade energin kan levereras antingen kontinuerligt, eller som energipulser. Fördelarna med att använda proteiner är att de är riskfria ur hälsosynpunkt, miljövänliga och har låga produktionskostnader. Inom ramen för detta arbete har olika proteiner, med och utan enzymatisk aktivitet, dvs. vissa lackaser, ceruloplasmin och rusticyanin, kartlagts elektrokemiskt och biokemiskt. Proteiner användes därefter för att tillverka biosuperkondensatorer.

Den ständigt ökande globala efterfrågan på elektrisk energi har accentuerat de problem som vidlåder produktion av elkraft och lagring av elektrisk energi. Produktionen, som hämmas av låg verkningsgrad i energiomvandlingssteget, medför ofta negativa miljökonsekvenser, samtidigt som lagring av elektrisk energi präglas av bristfällig teknik och avsevärda förluster. Elektrisk energi lagras vanligtvis i ineffektiva och kostsamma konventionella batterier eller kondensatorer. Sådana lagringsenheter är ofta baserade på miljöskadlig teknik och har fundamentala begränsningar, bl.a. hög självurladdning och otymplig storlek. Superkondensatorer och moderna uppladdningsbara batterier å andra sidan, har avsevärt högre laddningskapacitet och i denna avhandling beskrivs hur uppladdningsmekanismen hos en superkondensator omsätts i en biosuperkondensator. Biosuperkondensatorns aktiva beståndsdel, ett s.k. bioelement, utgörs av ett redoxprotein som saknar katalytisk aktivitet, vilket medför att den måste laddas via en extern strömkälla, och biosuperkondensatorns kapacitet beror av redoxproteinets kemiska egenskaper.

En ökande efterfrågan på elkraft framställd med fossila bränslen har medfört att ansträngningarna att identifiera hållbara bränslen, parat med effektivisering av energianvändningen, har intensifierats. Bränsleceller, som i ett steg

omvandlar kemisk energi till elektrisk energi, kan utnyttja en mängd olika bränslen och omvandlingsprocessen har mycket hög verkningsgrad. Biologiska bränsleceller kan anpassas till en mängd olika organiska kolbaserade bränslen och kan på så sätt bidra till en hållbar energianvändning. I denna avhandling presenteras en ny sorts biobränslecell, en självladdande biosuperkondensator, eller om man så vill, en laddningslagrande biobränslecell.

Självladdande biosuperkondensatorer, som kombinerar en enzymatisk biobränslecell och en elektrokemisk kondensator (superkondensator) i en för- enad konstruktion, och som kan utvinna elektrisk energi genom oxidation av biobränslen, ses i förstone som mikro- eller nanoströmkällor för implanter- bara biomedicinska sensorer. Enzymers substratspecificitet, och effektivitet under milda betingelser, medger att den naturliga blandningen av (bio)bränsle och (bio)oxidationsmedel kan förbli intakt, och att det inte finns behov av särskilda membran eller cellseparatorer. Den okomplicerade konstruktionen möjliggör långtgående miniaturisering, vilket ökar användbarheten i biomedicinska sammanhang. Beroende på tillämpningen, kan strömuttaget vara kon- tinuerligt, på en relativt låg nivå, eller alternativt korta strömpulser på en vä- sentligt högre nivå.

I ett kort perspektiv är strömförsörjning av mikroskaliga implantat eller s.k. transient bioelektronik viktiga tänkbara tillämpningsområden. Emellertid kan utvecklingen av biokompatibla och beständiga biokatalysatorer möjliggöra tillämpningar där långsiktig driftsstabilitet är en förutsättning.

LIST OF PUBLICATIONS

I. Sergey Shleev, Viktor Andoralov, Dmitry Pankratov, Magnus Falk, **Olga Aleksejeva**, and Zoltan Blum. Oxygen electroreduction versus bioelectroreduction: direct electron transfer approach. *Electroanalysis* 2016, 28, 1 – 19.

II. Ivan Mateljak, Emanuele Monza, Maria Fatima Lucas, Victor Guallar, **Olga Aleksejeva**, Roland Ludwig, Donal Leech, Sergey Shleev and Miguel Alcalde. Increasing redox potential, redox mediator activity, and stability in a fungal laccase by computer-guided mutagenesis and directed evolution. *ACS Catalysis* 2019, 9, 4561-4572.

III. **Olga Aleksejeva**, Ivan Mateljak, Emanuele Monza, Fatima Lucas, Victor Guallar, Roland Ludwig, Sergey Shleev, and Miguel Alcalde. Electrochemistry of a high redox potential laccase obtained by computer-guided mutagenesis combined with directed evolution. Submitted manuscript.

IV. **Olga Aleksejeva**, Elena Gonzalez-Arribas, Chiara Di Bari, Antonio L. De Lacey, Marcos Pita, Roland Ludwig, Victor Andoralov, Zoltan Blum, and Sergey Shleev. Membrane-free and mediator-less high voltage glucose/oxygen electric power biodevices. In manuscript.

V. Elena González-Arribas, Magnus Falk, **Olga Aleksejeva**, Sergey Bushnev, Paula Sebastián, Juan M. Feliu, Sergey Shleev. A conventional symmetric biosupercapacitor based on rusticyanin modified gold electrodes. *Journal of Electroanalytical Chemistry* 2018, 816, 253 – 258.

Contribution:

Paper I. Performed electrochemical investigations of human ceruloplasmin, purified from human blood, on nanostructured graphite electrodes. Took part in literature review, writing of the section 2.3 and preparation of graphical data.

Paper II. Performed redox titrations as well as relevant data fitting and calculations, carried out complementary electrochemical measurements. Prepared graphical data and tables.

Paper III. Performed all the experimental part. Took part in writing of manuscript, evaluation of results and preparation of graphical data.

Paper IV. Performed large part of the experimental work and data evaluation. Took part in manuscript writing and preparation of graphical data.

Paper V. Performed redox titrations as well as relevant data fitting and calculations, prepared graphical data.

Additional publications, not included in this thesis

Journal articles:

1. Elena González-Arribas, **Olga Aleksejeva**, Tim Bobrowski, Miguel Duarte Toscano, Lo Gorton, Wolfgang Schuhmann, and Sergey Shleev. Solar bio-supercapacitor. *Electrochemistry Communications* 2017, 74, 9-13.

Book chapters:

1. Sergey Shleev, **Olga Aleksejeva**, Magnus Falk, Zoltan Blum. Biodegradable electric power devices. In: *Bioelectrochemistry Design and Applications of Biomaterials*, Ed. Serge Cosnier (2019) Walter de Gruyter GmbH, Berlin/Boston.

THESIS AT A GLANCE

Paper	Objectives	Main findings / conclusions	Illustration
I. Oxygen electroreduction versus bioelectroreduction: direct electron transfer approach.	To compare oxygen electroreduction and bioelectroreduction.	The results show that theoretically blue multicopper oxidases outperform Pt catalyst by several orders of magnitude, having significantly lower overpotential for oxygen electroreduction. While oxygen electroreduction process does occur on enzyme modify electrodes operating in complex solutions, the performance of unprotected metal electrodes in complex electrolytes appeared to be inadequate.
II. Increasing redox potential, redox mediator activity, and stability in a fungal laccase by computer-guided mutagenesis and directed evolution.	To modify the redox potential of the T1 site of high redox potential laccase without compromising the enzyme's activity and stability.	Computational design combined with directed evolution resulted in a GreeDo variant of high redox potential laccase with increased potential of the T1 site and activity towards high-redox potential mediators, as well as enhanced thermal and acidic pH stability.
III. Electrochemistry of a high redox potential laccase obtained by computer-guided mutagenesis combined with directed evolution.	To perform comparative electrochemical characterisation of GreeDo variant of a high redox potential fungal laccase obtained by laboratory evolution together with computational-guiding mutagenesis, in comparison to its parental version, OB-1.	Both laccases, when immobilised on graphite electrodes, were capable of both direct and mediated electron transfer based bioelectroreduction of oxygen at low overpotentials. GreeDo, however, exhibited higher open circuit potential values and onset potentials compared to OB-1. Contrary to OB-1, GreeDo was stable in acidic electrolytes. Moreover, operational stability of GreeDo in adsorbed state was higher than in homogenous solution at acidic pH.

<p>IV. Membrane-free and mediator-less high voltage glucose/oxygen electric power biodevices.</p>	<p>To create a biodevice with adjustable open circuit potential values, depending on a number of half-cells in series, operating in one electrolyte.</p>	<p>Single-electrolyte based multi-cell glucose/oxygen biodevices with adjustable open circuit potentials and operating voltages were designed and tested. Due to the interplay between faradaic and non-faradaic electrochemical processes, as well as between ionic and electronic conductivities, the open-circuit voltage of the self-charged biodevice is extraordinarily high, reaching 3 V, when seven biosupercapacitors operating in one electrolyte are connected in series. Moreover, the glucose/oxygen biodevice can be externally discharged at an operating voltage exceeding the maximal limiting open-circuit value of 1.24 volts for the complete glucose oxidation. Our results demonstrate both potential and limitation of high voltage biological power sources utilising biofuels and biooxidants.</p>
<p>V. A conventional symmetric biosupercapacitor based on rusticyanin modified gold electrodes.</p>	<p>To design and test a new kind of bioelectronics device, conventional biosupercapacitor, based on the biopseudocapacitive properties of a redox protein, rusticyanin.</p>	<p>A conventional biosupercapacitor, <i>i.e.</i> a biodevice without self-charging ability that has to be charged externally, was built of gold electrodes modified with a copper containing redox protein, rusticyanin. The symmetrical biodevice was capable to capacitively store charge and deliver electric power derived mostly from the biopseudocapacitance.</p>

ABBREVIATIONS

AuNP – gold nanoparticle
BMCO – blue multicopper oxidase
CDh – cellobiose dehydrogenase
CE – counter electrode
CV – cyclic voltammogram
CYT – cytochrome domain
DET – direct electron transfer
DH – dehydrogenase domain
ECC – electrochemical capacitor
EFC – enzymatic fuel cell
EIS – electrochemical impedance spectroscopy
ESC – enzymatic supercapacitor
ET – electron transfer
FAD – flavin adenine dinucleotide
FC – fuel cell
GE – graphite electrode
HCp – human ceruloplasmin
IET – intramolecular electron transfer
IMD – implantable medical device
Lc – laccase
MET – mediated electron transfer
Nc – *Neurospora crassa*
NHE – normal hydrogen electrode
OCP – open circuit potential
OCV – open circuit voltage
Ox – oxidised species
PEDOT – poly(3,4-ethylenedioxythiophene)

Rc – rusticyanin
RE – reference electrode
Red – reduced species
SAM – self-assembled monolayer
T1 – type 1 copper
T2 – type 2 copper
T3 – type 3 copper
Th – *Trametes hirsuta*
TNC – trinuclear copper cluster
WE – working electrode

INTRODUCTION

The unification of electronic components and biological systems has led to a development of novel devices with extraordinary properties and functionalities. The devices have attracted considerable research efforts, owing to fundamental scientific issues and potential practical applications. A widely used term such as “bioelectronics”, implies functional integration of two different fields of science and engineering – biology and electronics, resulting in a new subclass of biotechnology [1-3]. The most challenging achievements in bioelectronics are related to biomedical practices, in particular promoting the direct connection of electronic devices with biological systems. The successful integration of electronics with living organisms requires energy sources capable of harvesting electrical power directly from physiological processes to provide energy for the electronic and biological parts of the system [3, 4].

Biodevices used for electrical power production utilising interfacial electron transfer processes, *i.e.* redox reactions, are among the most biocompatible and promising. Bioelectrochemical systems, typically biofuel cells, based on bioelements, such as enzymes, interfaced with electrodes are of particular importance [3, 5-8]. Biofuel cells are utilising the biocatalytic activity of enzymes to harvest electrical energy by oxidation of biomolecules and have been viewed as micro-scale energy sources for powering implantable bioelectronic devices [3, 9]. Integration of enzymatic biofuel cells with supercapacitors resulted in biodevices with improved characteristics, *i.e.* biosupercapacitors, allowing high power output in pulse mode [10-12]. Despite the fact that biofuel cells for *in vivo* applications were suggested a long time ago, very few practical realisations have been achieved up to date [9, 13-19]. Thereby, there is a space for improvement in terms of current-voltage output, stability, biocompatibility, *etc.*

This thesis is focused on bioelements for biological electric power sources, *i.e.* blue copper proteins having and lacking intrinsic biocatalytic activity. These proteins, *viz.* different laccases, ceruloplasmin, and rusticyanin, were thoroughly characterised electrochemically and biochemically. They were also employed for the construction of both self-charging and conventional bio-supercapacitors.

BIOLOGICAL POWER SOURCES

Biological power sources can be classified on the basis of the bioelement used, *i.e.* protein, organelle, and cell-based devices. Additionally, biological power sources can be further differentiated into two large groups: biofuel cells and biosupercapacitors. Biosupercapacitors can be subdivided into conventional and self-charging biodevices, whereas biofuel cells can be described as either conventional or charge-storing biodevices [11]. Even though this thesis is focused on protein based biosupercapacitors, self-charging as well as conventional biodevices, a short description of enzymatic fuel cells is in order.

Enzymatic fuel cells

Enzymatic fuel cells (EFCs) are a class of fuel cells (FCs), in which enzymes are used as catalysts to accelerate the oxidation of biofuel and/or reduction of oxidant for conversion of chemical energy into electrical energy [20]. The functioning principle of an enzymatic fuel cell is similar to that of a conventional fuel cell, *i.e.* the biofuel is oxidised at the bio-anode and the electrons derived are then travelling through an external circuit to be released at the bio-cathode, where the bio-oxidant is reduced [21]. Depending on the anodic biocatalyst, different types of fuels can be oxidised at the bio-anode, *e.g.* carbohydrates, alcohols, and amino acids. At the bio-cathode the bio-oxidant, *e.g.* molecular oxygen (O_2), hydrogen peroxide, or organic peroxides, is reduced by the cathodic biocatalyst [21]. A schematic representation of an EFC, based on cellobiose dehydrogenase (CDh) as anodic biocatalyst and laccase (Lc) as cathodic biocatalyst, is shown in Fig. 1.

Figure 1. Schematic representation of an enzymatic fuel cell, where oxygen reduction is catalysed by laccase at the bio-cathode and glucose is oxidised at the cellobiose dehydrogenase based bio-anode.

Enzymes benefit from notable advantages over conventional catalysts in terms of biocompatibility, higher efficiency, higher activity under mild conditions, and selectivity. The latter aspect is very important in the design of biofuel cells, since the fuel and oxidant can be introduced as a mixture in a common electrolyte, without any membranes [22-24]. This also provides an opportunity for miniaturisation of biological power sources.

Enzymatic bioelectrocatalysis

Bioelectrocatalysis is the acceleration of an electrochemical reaction by means of biocatalysts. Electron transfer (ET) to/from an electrode is executed by enzymes catalysing redox reactions, *i.e.* oxidoreductases. The redox reactions, catalysed by enzymes, consist of two half reactions – reduction and oxidation (Fig. 2 A). Hence, bioelectrocatalysis implies the substitution of one of the coupled redox reactions by the electrochemical one (Fig. 2 B). Direct ET (DET) based bioelectrocatalysis (Fig. 2 B) implies an absence of any freely diffusing or immobilised mediators, which means that electrons are transferred directly to/from protein from/to the electrode. This configuration results in less potential losses due to required difference between redox potential of en-

zymes and mediators to drive mediated ET (MET) based bioelectrocatalysis (Fig. 2 C) at significant rates [20, 25].

Figure 2. Schematic representation of the functioning principle of multi-centre redox enzymes (A) as well as of direct (B) and mediated (C) cathodic bioelectrocatalysis.

The main physiological function of enzymes is to accelerate rates of chemical reactions in order to comply with the requirements of the organism. The enzyme kinetics can be described by Michaelis-Menten equation (Eq. 1):

$$(1) \quad V = V_{max} \frac{[S]}{[S] + K_M}$$

where $[S]$ is the substrate concentration, K_M – the Michaelis constant, V_{max} – maximum rate of the reaction [26].

In enzymatic bioelectrocatalysis, when it comes to cathodic reactions catalysed by multi-centre redox enzymes, the electron transport from electrode to the substrate consists of the following steps: ET from the electrode to the redox centre of the enzyme, intramolecular ET (IET) from the reduced redox centre to the active site, where reduction of the substrate (typically O_2) occurs, diffusion of the substrate to the active site and its conversion into the product (Fig. 3) [27, 28].

Figure 3. Schematic representation of steps involved in cathodic bioelectrocatalysis by means of multi-centre redox enzymes.

The very first electrochemical investigations of O_2 electroreduction by proteins were performed by Scheller and co-authors as early as in 1970's [29-31]. In these pioneer studies Cyt P-450 was used as a cathodic protein. Later other proteins, such as multicopper oxidases [32, 33], nitrite reductases [34] and peroxidases [35], were exploited as cathodic biocatalysts.

Self-charging biosupercapacitors / Charge-storing enzymatic fuel cells

An enzymatic supercapacitor (ESC), *i.e.* a self-charging biosupercapacitor, which depending on the mode of usage can be called a charge-storing enzymatic fuel cell, is a combination of electrochemical capacitor (ECC), also referred to as a supercapacitor, and an enzymatic fuel cell (EFC). ESCs are conjoined by design, consisting of both capacitive and charging components, which perform simultaneously as an ECC and an EFC [10]. Such devices can be miniaturised down to nm size, and can be used in continuous mode, when long-time low current is required, as well as in pulse mode where short-time high current is relevant [10]. However, the intrinsic ESC, that is a biodevice

which cannot function as conventional EFC, would be capable to deliver power only in pulse mode [36].

Similar to a conventional capacitor, an electrochemical capacitor stores energy by charge separation on two electrodes. However, contrary to a conventional capacitor, the ECC stores charge in form of a double layer, which is created at the electrode-electrolyte interface [37]. Owing to high charge and discharge rates, ECCs are suitable for applications where high power is required [38]. ECCs, however, may utilise not solely a double layer capacitance in their function. There is also another type of capacitance, pseudocapacitance, which is based on faradaic processes at the electrode surface. The fast and reversible faradaic processes in combination with the non-faradaic charging of the double layer taking place at the same electrode allow ECCs to store much more energy [38, 39]. Hence, the total capacitance (C_{total}) of an electrode obeys the equation (Eq. 2):

$$(2) \quad C_{total} = C_{dl} + C_{\phi},$$

where C_{dl} is the double layer capacitance and C_{ϕ} is pseudocapacitance [39].

In Fig. 4 given below a typical ESC, which is composed of charging (ECC) and capacitive parts (EFC), is schematically presented. The capacitive site is built out of nanomaterials, typically nanotubes or nanoparticles, along with conductive polymers [39], exploiting both double-layer capacitance and pseudocapacitance; the charging part is made of biocatalyst modified electrodes (CDh on the anodic side – left, Lc on the cathodic side – right).

Figure 4. Schematic representation of an enzymatic supercapacitor, where the capacitive site is typically made of nanomaterials along with conducting polymers, and the charging site is composed of biocatalyst modified electrodes (here laccase at the bio-cathode and cellobiose dehydrogenase at the bio-anode).

The reported figures of merit for biosupercapacitors as well as for EFCs typically are the open circuit voltage (OCV) and the power output. However, for practical applications operational and storage stability is essential. Theoretically, the OCV of a biodevice is given by the difference in redox potentials of the biofuel oxidised at the anode and the bio-oxidant reduced at the cathode. However, in practice, each of these reactions proceeds with a certain overpotential (η), determined by biocatalyst and experimental parameters, which finally affects the OCV of a biodevice [22, 40].

When bioelectrodes are working in DET mode, electrons are directly transferred between the electrodes and the enzyme redox centres, and therefore the OCV is defined by the potential differences between the electrochemical control centres of immobilised enzymes [21, 40]. In the case of multi-centre redox enzymes in general, and blue multicopper oxidases in particular, the electrochemical control redox centre (the redox site with fast and reversible electron exchange with the electrode) [41] can be changed depending on different factors, like pH, presence of inhibitors, and activators, *etc.* For instance, at pH 8, the control centre of bilirubin oxidase is the type 1 copper (T1), whereas at acidic pH, the T1 site does not define the potential of bioelectrocatalysis [42]. In MET based systems the use of a mediator is required to shuttle the elec-

trons between the protein and current collector (electrode) [20, 43]. Consequently, in MET based EFCs the OCV is determined as the difference between the redox potentials of the mediators [40, 44]. Moreover, mediators are often toxic, may leak during continuous operation, and can even cause crossover, necessitating separation of the half-cells [45]. Therefore, DET based electric power biodevices are preferable, offering advantages such as simplicity of fabrication, possibility for miniaturisation, non-toxicity, compartment-less/membraneless design [21].

DET based self-charging biosupercapacitors are discussed in **Paper IV**.

Conventional biosupercapacitors

In conventional biosupercapacitors, redox proteins lacking biocatalytic activity, *i.e.* without the ability to charge a biodevice, are employed as bioelements (Fig. 5). In such devices, the biopseudocapacitance of redox proteins is exploited, *i.e.* redox proteins serve as the pseudocapacitive component defining the total capacitance of a biodevice. As those devices are lacking self-charge capability, they have to be charged externally [46].

Figure 5. Schematic representation of a conventional biosupercapacitor, *i.e.* a biodevice lacking self-charging ability, made of non-catalytically active redox protein (here rusticyanin) modified nanostructured electrodes.

In **Paper V** a complete functional conventional supercapacitor based on two gold electrodes modified with a copper containing redox protein, rusticyanin, is presented.

Design of bioelectrodes

Bioelements

1. *Blue copper proteins*

T1 or blue copper is a mononuclear metal centre participating in single electron transfer processes. Blue copper sites are widely distributed in nature and can be found in large enzymes ($Mw \geq 60$ kDa), as well as in small non-catalytic proteins mediating ET reactions. These biological species are known as blue copper proteins or cupredoxins. Presence of the T1 copper gives a rise to the absorption band around 600 nm, which is assumed to appear from charge transfer between the copper ion and the sulphur atom of a cysteine ligand [47]. Blue copper proteins, used for the construction of separate biocathodes and complete biodevices within the scope of this thesis, are discussed below.

1.1 *Blue multicopper oxidases / Cathodic enzymes*

Blue multicopper oxidases (BMCOs) belong to a widespread family of enzymes found in numerous organisms with characterised examples from archaea, bacteria and eukaryotes, including mammals. Their functioning principle implies oxidation, typically by single ET, a variety of substrates ranging from polyphenols, lignin and ascorbate, to transitional metal ions. Simultaneously, O_2 is being reduced releasing two H_2O without production of any intermediates [48, 49]:

The catalytic centre of BMCOs typically consists of four copper ions: the substrate is oxidised at T1 site, which is *ca.* 13 Å away from the trinuclear cluster (TNC), where reduction of O_2 is taking place (Fig. 6). The TNC consists of a pair of type-3 copper ions (T3) and a type-2 copper ion (T2), arranged in a unique triangular fashion [50, 51].

Figure 6. The general structure of the trinuclear cluster and T1 site of blue multicopper oxidases as well as the electron transfer pathways [50].

BMCOs are of a great interest for different applications. Owing to O_2 reduction to H_2O , fabrication of bio-cathodes for biofuel cells and biosupercapacitors, that could potentially power implantable medical devices, is of particular significance [51].

The BMCOs used in this thesis were of fungal and mammal origins, *i.e.* *Trametes hirsuta* laccase (*ThLc*), mutants of PM1 laccase (OB-1 and GreeDo) – fungal, and human ceruloplasmin (HCp) – human. Basic biochemical, kinetic and structural properties of these enzymes are discussed in detail below.

Laccases

Laccases, (p-diphenol:dioxygen oxidoreductase, EC 1.10.3.2) are known to be of fungal, plant, bacterial and insect origin [52, 53]. This type of enzymes are capable of oxidising different types of compounds, such as methoxy-, mono-, di- and poly- phenols, aromatic and aliphatic amines, hydroxyindoles, benzenethiols, carbohydrates, and inorganic/organic metal compounds [53-56], with concomitant reduction of O_2 to H_2O . Due to the broad substrate range, laccases (Lcs) are being evaluated for various biotechnological applications [55]. Lcs with a high redox potential of the T1 site, *i.e.* 0.73-0.79 V [33, 57], are of a special interest in a field of biotechnology, in particular in designing biological electric power sources [52, 58].

Trametes hirsuta laccase

Even though the first discovered Lc came from tree sap, enzymes of fungal origin are subjects of extensive investigations. Fungal Lcs are involved in both intra- and extra cellular processes, such as delignification, morphogenesis, pigmentation and pathogenesis [53, 59-61]. Presently, white-rot fungi is the main source of Lcs, expressed both in natural and recombinant ways [62]. *Trametes hirsuta* Lc (*Th*Lc) with molecular weight of *ca.* 70 kDa and pH optimum of 3.5-4.5 is a typical representative of fungal Lcs (Fig. 7) [33]. The low pH optima of fungal enzymes can be attributed to their growth under acidic conditions, *i.e.* the variations in optimal pH ranges between plant and fungal Lcs are due to their physiological differences [63]. Similarly, bioelectrochemical investigations show that the peak activity for *Th*Lc lies in the acidic region of pH [33, 64]. As for all BMCOs, the important characteristic of this enzyme is the redox potential of the T1 site. A value of 0.78 V *vs.* NHE was obtained for T1 centre of *Th*Lc by potentiometric titrations at pH 6.5 due to the higher stability of the protein at this particular pH [65, 66]. *Th*Lc has been extensively used for construction of biological electric power sources due to its high redox potential and relatively good stability [67-70], however, the application at physiological pH is a limiting factor.

Figure 7. Tertiary structure of *Trametes hirsuta* laccase (PDB file: 3FPX). Copper atoms are shown as brown spheres.

In this thesis *Th*Lc based electrodes were electrochemically characterised and used for the construction of ESCs in **Paper IV**.

Laccase from basidiomycete PM1 and its mutants

PM1Lc (Fig. 8) belongs to a group of high redox potential Lcs isolated from the western Mediterranean area and is known for its high thermostability. In addition, it is highly stable in the pH interval from 3 to 9, and has a redox potential 0.76 V *vs.* NHE at pH 6.5 [71]. The OB-1 mutant with a molecular weight of *ca.* 60 kDa, obtained by laboratory evolution of PM1Lc, appeared to be tolerant to the presence of organic co-solvents and retained 90 % of its activity in the pH range 3-9 after 4 h of incubation [72]. The OB-1 variant was tailored further by computer-guided mutagenesis and directed evolution to yield the GreeDo mutant, increasing the E_{T1} value from 0.74 V to 0.79 V *vs.* NHE. In parallel, thermal and acidic pH stabilities were improved, as well as activity towards high redox potential mediators. In the scope of this thesis both variants, OB-1 and GreeDo, were characterised biochemically, electrochemically and spectroelectrochemically (**Papers II-III**).

Figure 8. Tertiary structure of PM1 laccase (PDB file: 5ANH). Copper atoms are shown as red spheres.

Human ceruloplasmin

Human ceruloplasmin (HCp) is the only member of BMCOs present in human plasma. This multi-functional enzyme is known to oxidise ferrous ions to ferric ions, facilitate conversion of Cu (I) into Cu (II), as well as induce the oxidation of catechols (*L*-adrenaline, *L*-noradrenaline, *etc.*) and synthetic amines (*p*-phenylenediamine, *p*-aminophenol, *etc.*). Moreover, it exhibits the activity of ascorbate oxidase, NO-oxidase, glutathione-linked peroxidase and superoxide dismutase [73, 74]. Hence, the main role of HCp in human body still remains unresolved. Nevertheless, a ferroxidase activity has historically been considered as a primary role of the enzyme [75, 76].

HCp consists of one subunit with a molecular weight of 132 kDa, which is folded into six domains, arranged into a triangular matrix (Fig. 9). Contrary to other BMCOs, it contains six copper ions and three of them are bound to the T1-binding sites, found in domains 2, 4 and 6, whilst three other copper ions situated at the interface between domains 1 and 6 constitute the TNC [77]. Electrons from the reducing substrates are accepted one at a time at the T1 site, followed by the IET to the TNC, which in its turn utilises them for conversion of O₂ to H₂O [75]. Three T1 sites are separated between each other by a distance of *ca.* 18 Å. Two copper ions located in domains 4 and 6, T1B and T1A, respectively, share a similar ligand structure [77]. Redox potentials of the T1A and T1B centres are known to be 0.58 V and 0.49 V, as determined by potentiometric titrations at pH 5.5 [78]. The third T1 centre, T1C, located in domain 2, has a different ligand structure, and is known to be permanently reduced having a redox potential of at least 1 V [77, 79]. T1A is the closest to the T2/T3 cluster, separated by a distance of *ca.* 13 Å, and has been identified as a primary electron acceptor from reducing substrates [80, 81].

Up till now only a few, unsuccessful, attempts have been made to achieve mediator-less bioelectroreduction of O₂ catalysed by HCp [82-84], though DET between the electrode and the enzyme has been observed [83]. It was suggested that the bioelectrocatalytic inertness of this multi-functional redox enzyme might be associated with a very complex mechanism of IET involving a kinetic trapping behavior. HCp could be a very attractive candidate for fabrication of low immunoresponse biocathodes, if bioelectroreduction of O₂ is achieved.

Figure 9. Tertiary structure of human ceruloplasmin (PDB file: 1KCW). Copper atoms are shown as red spheres.

In the scope of this thesis, the bioelectrocatalytic capabilities of HCp were investigated in **Paper I**.

1.2 Rusticyanin

Rusticyanin (Rc), a small copper containing protein with a molecular weight of *ca.* 16 kDa (Fig. 10), has its origin from the periplasm of the gram-negative bacterium *Acidithiobacillus ferrooxidans* where it is taking part in the respiratory oxidation of Fe^{2+} to Fe^{3+} . It is a highly acid stable member of the family of T1 containing blue copper proteins and shows optimal redox activity at pH 2.0. It contains only one copper ion (T1) and has a redox potential of 0.68 V at pH 2.0 [47, 85, 86].

Only a couple of studies regarding electrochemistry of Rc were reported so far, and only one paper covers electrochemistry of the immobilised protein [87, 88]. In this thesis Rc was studied in **Paper V**, where it was electrochemically characterised and used for assembly of a conventional biosupercapacitor based on protein modified gold electrodes.

Figure 10. Tertiary structure of rusticyanin (PDB file: 1RCY). The copper atom is shown as a brown sphere.

2. Anodic enzymes

Cellobiose dehydrogenase

Cellobiose dehydrogenase (EC 1.1.99.18) is an extracellular flavocytochrome constituting a significant fraction of the lignocellulotic enzymes secreted by white and brown rot, plant-pathogenic and composting fungi from the dicarotic phyla Basidiomycota and Ascomycota [89, 90]. This widely spread enzyme plays an important role in wood decomposition, participating in degradation and modification of polymers such as cellulose, hemicellulose and lignin by generating hydroxyl radicals. Cellobiose dehydrogenase (CDh) consists of two separate domains of different structures interconnected by a polypeptide linker region (Fig. 11). The larger domain flavodehydrogenase (DH), with a molecular weight of *ca.* 65 kDa is catalytically active, while the smaller cytochrome domain (CYT), with a mass of *ca.* 25 kDa, contains haem B as a cofactor and functions as an ET protein [90, 91]. Typically, basidiomycete CDhs evolve under pH values below 4.5 and exhibit a strong preference towards cellobiose and cello-oligosaccharides, while glucose and other monosaccharides are quite poor substrates. Ascomycete CDh producers are capable to degrade wood under extreme environmental conditions, *i.e.* elevated pH and temperature, unsuitable for most basidiomycetes [92-94]. In the oxidative half-reaction of the catalytic cycle of CDh, two electrons from the carbohydrate substrate are relayed by the flavin adenine dinucleotide (FAD) cofactor

of the DH domain to two- or one-electron acceptors, quinones or complex metal ions, respectively, or by internal ET, to the haem B (*cytochrome c*) in the CYT domain [89]. The actual pH is affecting the distance between the DH and CYT domains and consequently the internal ET. By increasing the pH, the domains separate owing to electrostatic repulsion, thus attenuating internal ET. Regarding bioelectrochemical applications, the CYT domain is capable of acting as a natural mediator allowing DET between the biomolecule and the electrode [91, 95]. The electrochemically determined redox potential of the *haem b* cofactor of the CYT domain is *ca.* 0.1 V *vs.* NHE at neutral pH [94, 96]. Due to the capability of DET to the electrode surface and high activity at physiological pH, CDh is an attractive candidate for construction of “third generation” biosensors, whose functioning principle is based on DET between proteins and the electrode [97], as well as bioanodes for EFCs [90, 96]. In a scope of this thesis the *Neurospora crassa* CDh (*NcCDh*) of ascomycetous origin (Fig. 11) was used as the anodic bioelement in the construction of ESCs (Paper IV).

Figure 11. Tertiary structure of *Neurospora crassa* cellobiose dehydrogenase (PDB file: 4QI7). Haem is shown with red sticks, FAD with blue sticks.

Electrode materials

The main parameters to take into account when selecting an electrode material are the electrical conductivity and hardness of the material. Thus, electrodes are typically made of solid supports, such as gold and platinum (foil and rod), or carbon in form of paper, rod, paste, metallised carbon, glassy carbon, carbon fibre, nanotube film [98]. Large surface area is an important factor to consider in the design of enzymatic electrodes, since it allows higher enzyme loading, ensuring larger biocatalytic currents, and enhances the capacitance of the electrode – important parameters in the construction of biological power sources [21, 99, 100]. A variety of nanomaterials and nanostructuring techniques have been employed in the development of efficient biodevices. Nanomaterials of comparable size to, or smaller than, the size of enzyme molecules may significantly improve communication between the enzyme and the electrode, thus enhancing the bio-catalytic performance [101, 102].

Carbon based materials with unique structure and attractive properties have been extensively used in fabrication of enzymatic electrodes due to good stability, low cost, and availability [99, 103, 104]. Spectrographic graphite is naturally highly porous, ensuring high enzyme loading, and one of the handiest electrode materials to work with. Modification of electrodes with carbon nanomaterials, such as carbon nanotubes, nanoparticles, carbon black, and graphene, are contributing to higher roughness factor/enzyme loading, and depending on modifier size, even facilitating DET between biocatalyst and the electrode surface [99, 101, 102, 105]. In this thesis, carbon nanotubes modified graphite electrodes (GEs) were used for investigation of the bioelectrocatalytic capabilities of HCp (**Paper I**).

Excluding carbon, gold is the most frequently used electrode material in bioelectrochemical studies [106]. However, bare gold is rarely used due to deactivation of enzymes on the metal surfaces [107]. In order to maintain the physiological activity of biocatalysts on a gold surface, self-assembled monolayers (SAMs) – a well-known strategy for immobilisation, orientation, and organisation of biomolecules at interfaces – have been widely used [108, 109]. Owing to the thermodynamically favoured Au-S bond, the most often used methods is chemisorption of sulphur derivatives, *i.e.* thiols, on gold surfaces [109-112]. SAM properties can be further modified, *e.g.* by alkanethiols terminated with functional groups that can be positively or negatively charged depending on the pH, thus promoting electrostatic interactions [112]. In **Pa-**

per IV SAM modified gold electrodes were used for the assembly of a conventional biosupercapacitor.

Nanoscale size metal and metal related materials, such as metal oxides and metal salts with good conductivity, are often used in bioelectronics to promote DET and increase the real surface area of electrodes [101, 102, 104]. Au-based nanomaterials, such as nanoparticles, nanorods, nanoporous gold *etc.*, are widely used as electrode support in the construction of bioelectrodes [10, 102, 105, 113-115]. A variety of methods have been developed for preparation of nanoparticles of controlled shape and size [116]. In this thesis gold nanoparticles (AuNPs) were used in **Paper III** for enhancement of DET between graphite electrodes and mutant laccases.

Conducting polymers and related nanocomposites present a wide array of novel electrochemical properties. Pseudocapacitors based on conducting polymers are of low cost and lightweight, allowing high specific energy and power together with high conductivity and flexibility. Conducting polymers manage charge storage through reduction and oxidation events, *i.e.* faradaic processes, resulting in high energy densities compared to ECCs whilst ensuring better power performance and longer lifetime relative to batteries [117-120]. Furthermore, conductive polymers can be biocompatible, biodegradable, and porous, their electrical, physical, and chemical properties can be adjusted to the specific requirements of a particular application, as well as altered and controlled through stimulation by electricity, light, pH, *etc.* [121-123]. Their conductivity is coming from the ability of electrons to jump within and between the chains of the polymer, due to the conjugated backbone formed by a series of alternating single and double bonds leading to the availability of delocalised electrons [124]. Dopants also contribute to the polymer conductivity, stabilising the backbone, as well as introducing additional charge carriers by removing/adding electrons and localising them as polarons or bipolarons [124-126]. Polyheterocycles is a family of conductive polymers with high stability and conductance, including such representatives as polypyrrole, polyaniline, and polythiophenes [123, 127-129]. Poly(3,4-ethylenedioxythiophene) (PEDOT) has been used to enhance the capacitance of biodevices described in **Paper IV**.

Immobilisation techniques

There are a number of techniques for bioelement immobilisation onto supports ranging from physical adsorption and ionic linkages based on weak physical bonding, to irreversible and stable covalent attachment. It is essential to realise that chemical and physical properties of the protein may undergo changes upon the immobilisation.

In this work the following immobilisation methods were used:

- Physical adsorption – the immobilisation strategy widely used to develop enzymatic bio-electrodes. It requires soaking of the support in a solution and incubating for certain time. Another way is allow the enzyme solution to dry on the electrode surface, and thereafter rinse away the non-adsorbed biomolecules. Enzymes are adsorbed on a supporting matrix by means of weak non-specific forces such as hydrogen bonding, Van der Waals, or hydrophobic interactions. Obviously, these relatively weak non-specific forces may lead to reversible processes and enzyme leakage from the matrix can take place [130, 131].
- Covalent binding is one of the most extensively used immobilisation methods, forming stable covalent bonds between functional groups of the enzyme molecules and the support matrix. The functional groups of enzyme, necessary for the formation of covalent bonding, should not be important for the biocatalytic activity, and typically involve binding through the side chains of lysine (amino group), cysteine (thiol group), aspartic and glutamic acids (carboxylic group, imidazole and phenolic group). Covalent immobilisation ensures strong bonds between the enzyme and electrode support, therefore only insignificant leakage of enzyme can take place [130, 131].
- Cross-linking is an irreversible immobilisation technique based on the formation of covalent cross-linkages among the enzyme molecules. It is typically performed by using multidentate reagents, which interconnects biomolecules creating a three dimensional network [131].
- Covalent attachment and crosslinking were used to immobilise biomolecules onto the electrodes in **Papers III** and **V**, respectively. In **Papers I – IV** physical adsorption was used as an immobilisation method.

ELECTROCHEMICAL TECHNIQUES

As the scope of this thesis lies in a field of bioelectrochemistry, which is an interdisciplinary branch of scientific research studying biological phenomena by means of experimental and theoretical electrochemical methods [132], the concept of electrochemistry and electrochemical techniques used in the present work will be discussed below.

Intro to the electrochemistry

Electrochemistry is studying processes taking place at the electrode-electrolyte interface, where transfer of charged species is of the main interest [133, 134]. Electrode reactions are of a heterogeneous character and occur in the region of junction between the electric conductor (electrode) and ionic conductor (electrolyte), where the charge distribution differs from the bulk solution. The electrode processes are influenced by the structure of this region, as well as by the nature of electric and ionic conductors. Charge separation can be represented by capacitance and resistance impeding the charge transfer [133].

Electrochemical cell

The potential of an electrochemical cell can be defined from the redox potentials of the respective half-reactions. Conventionally, the left half reaction is considered to be an oxidation (anodic), and the right one – a reduction (cathodic). Consequently, the cell potential is equal to the potential difference of cathodic (E_c) and anodic (E_a) reactions, which are determined by thermodynamics [133, 134]:

$$(4) \quad E_{cell} = E_c - E_a$$

In experimental procedures normally the cell response is determined by the single electrode, *i.e.* working electrode (WE). This can be fulfilled in a two-electrode system, whereas the second electrode does not influence the response of WE. Typically, the second electrode is the reference electrode (RE), and the cell current is low. However, generally a three-electrode cell and a potentiostat are being used. In this case, the current is flowing between WE and counter electrode (CE), which has a significantly larger surface area compared to WE, and the potential of WE is controlled *vs.* RE [134].

Non-faradaic processes

Processes of adsorption and desorption occurring on the electrode surface, which can be affected by changing the potential of the electrode or electrolyte composition, are called non-faradaic processes. Even though charge doesn't cross the electrode-electrolyte interface, the external currents can flow, whilst the applied potential or solution composition are being altered.

As charge transfer is not taking place, when the potential perturbation is applied, the electrode-electrolyte interface behaviour is similar to a capacitor and can be described by equation (Eq. 5):

$$(5) \quad C = Q / E$$

where Q is the charge stored at the capacitor (C), E – potential across the capacitor (V), and C – the capacitance (F). In order for the capacitor to be charged, the potential is applied until the charge on the metal plates is satisfying the above-mentioned equation. Throughout the charging process, a current, which is called charging or capacitive current, will flow [135].

The distribution of charged species and oriented solvent molecules at the electrode-electrolyte interface is called the electrical double layer. The closest layer to the electrode is composed of solvent molecules (oriented dipoles) as well as of other ions or molecules, which are called “specifically adsorbed”. This inner layer is named the Helmholtz or Stern layer [135].

Figure 12. Proposed model of electrical double layer formed on a negatively charged electrode.

The loci of centres of specifically adsorbed ions is called inner Helmholtz plane (IHP). Loci of centres of solvated ions, non-specifically adsorbed, are forming the outer Helmholtz plane (OHP) (Fig. 12). The non-specifically adsorbed ions are spread in a three dimensional space comprising the diffuse layer, which extends into the bulk electrolyte [135].

In electrochemical experiments presence of double-layer capacitance and existence of charging currents cannot be ignored. Especially, when low concentrations of redox species are involved in the studies, the charging current can be much more significant compared to the current resulting from charge transfer reactions (faradaic current) [135].

Faradaic processes

Transfer of charge across the electrode-electrolyte interface causes the oxidation or reduction to happen. Since this process follows Faraday's law, it is called faradaic [135].

The simple ET reaction, taking place at the electrode:

implies a series of steps for the transformation of oxidised species (*Ox*) into the reduced ones (*Red*). If the ET is fast, the electrochemical reaction is reversible. Consequently, concentrations of *Ox* and *Red* are in equilibrium with the electrode potential (*E*), and the process is governed by the Nernst equation:

$$(7) \quad E = E^{\circ'} + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]}$$

where $E^{\circ'}$ - formal potential of redox reaction (V), *i.e.* the redox potential at the particular set of conditions, R - universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T - temperature (K), F - Faraday constant (96485 C mol^{-1}), n - number of electrons involved in the redox reaction [135].

The applicability of the Nernst equation, and consequently the reversibility of the system, is also dependent on time given for the electrode reaction to reach the equilibrium [133]. In case of a reversible reaction, the electrode reaction kinetics is significantly faster than mass transport of the redox species from bulk electrolyte to the electrode surface. In case of an irreversible reaction, the electrode reaction cannot proceed in the opposite direction. Extra energy, (η), has to be applied to the system in order to overcome the kinetic barrier. Quasi-reversible systems reveal the transitional behaviour between reversible and irreversible reactions. The required η is of relatively small value, so that such reactions can proceed in the reverse direction [133]. When the applied potential differs from the equilibrium potential

$$(8) \quad \eta = E - E^{\circ'},$$

the current (i) can be described by equation:

$$(9) \quad i = F A k^0 ([Ox] e^{-\alpha f \eta} - [Red] e^{(1-\alpha) f \eta})$$

where k^0 is the standard rate constant, α is the transfer coefficient related to the symmetry of the energy barrier of the redox reaction, A - surface area, and $f = F/RT$. This relation or variations obtained from it can be used to describe problems related to the heterogeneous kinetics in electrochemistry, and it is known as the Butler -Volmer equation.

At equilibrium conditions the oxidative/anodic current (i_a) and the reductive/cathodic current (i_c) are equal, and consequently the net current is zero. The electrode adopts the potential dictated by the bulk concentration of *Ox*

and *Red* species following the Nernst equation, *i.e.* the open circuit potential (OCP). The balanced faradaic activity, called the exchange current i_0 , which is equal either to i_c or i_a , can be expressed by formula:

$$(10) \quad i_0 = F A k^0 [Ox]^{(1-\alpha)} [Red]^\alpha ,$$

whereas in case of $[Ox] = [Red]$, $i_0 = F A k^0 C$ [135].

In enzymatic bioelectrocatalysis, the electron transport from electrode to the substrate consists of several steps (Fig. 3). If IET is not the limiting step in O_2 bioelectroreduction, the bioelectrocatalytic current (i_{cat}) can be calculated using the electrochemical expression of Michaelis-Menten equation [27, 28, 136]:

$$(11) \quad i_{cat} = n F A_{real} \Gamma k_{cat} C_{O_2} / (C_{O_2} + K_M)$$

where k_{cat} is the apparent rate constant for the bioelectrocatalytic process, K_M – the Michaelis constant, Γ – surface concentration of the enzyme, F – Faraday constant, n – number of electrons involved in the redox process, A_{real} – real surface area of the electrode, C_{O_2} – concentration of O_2 .

However, the total current produced by the bioelectrocatalytic reaction (i) consists of several determinants and can be described by equation:

$$(12) \quad 1/i = 1/i_{ET} + 1/i_{cat} + 1/i_{diff}$$

where i_{ET} is the heterogeneous ET limiting current, i_{diff} is the limiting diffusion current [27, 136].

Electrochemical techniques

Since in this thesis (Papers I-V) electrodes modified with biologically derived materials were employed, electrochemical responses were studied taking into account surface confined electroactive species.

Spectroelectrochemistry

A combination of two rather different techniques, electrochemistry and spectroscopy, is being widely used to study redox-transformations of inorganic and organic compounds, as well as biological species [137]. The redox states of molecules are changed electrochemically by addition or withdrawal of electrons at the electrode-solution interface with concomitant spectral measurements. Such techniques are typically used to obtain spectra and redox potentials, as well as to monitor the subsequent chemical reactions of the studied redox species.

The most common optical technique used in spectroelectrochemistry is absorption spectroscopy in the UV, visible, or infrared regions [137]. Large biological molecules, such as proteins and nucleic acids, tend to absorb light in the UV-Vis range of the spectrum [138]. Therefore, absorption spectroscopy in combination with electrochemistry is often used to study the ET processes and determine redox potentials of proteins.

Thin layer electrochemistry allows to control the redox state of species in a very small volume of solution with concomitant absorption (*A*) measurements. In this work (**Papers II and V**) a specially designed spectroelectrochemical cell with a volume of *ca.* 1 μ l and optical path length of 1 cm [139] has been used for determination of redox potentials of blue copper proteins. The potential of the system is set by applying the potential in accordance with the Nernst equation (Eq. 7) for reversible reaction.

The redox species are converted from one oxidation state to another by a range of applied potentials, and each respective value of $[Ox]/[Red]$ is registered spectrally. Each potential is maintained until the Nernst equilibrium is reached. Often the redox centres of biological species, such as proteins, are buried deeply in the structure and therefore the ET between the electrode and bio-component might be sluggish. In such cases, another redox substance, a mediator, is typically added to enhance the ET between the electrode and redox centres of proteins. Small mediator molecules can easily diffuse deep into the protein matrix and shuttle the electrons between the electrode and redox centres of proteins (Fig. 13) [137].

Figure 13. Schematic representation of mediated electron transfer in a spectroelectrochemical cell in case of reductive titration.

Values of $\log[A/(A_0-A)]$, *i.e.* $\log[\text{Ox}/\text{Red}]$, are then plotted *vs.* corresponding values of E , giving a linear dependence known as a Nernst plot. Herein, the formal potential (E°), which may be denoted the midpoint potential (E_m), can be obtained from the horizontal axis intercept and number of electrons (n) from the slope of the linear fit [137].

One can determine E_m and n from the plot of the absorbance measured at certain wavelength *vs.* the applied potential, and fit this dependence using non-linear least-squares procedure to the following equation based on Nernstian dependence [139]:

$$(13) \quad A = \epsilon_{red} l C_t + l(\epsilon_{ox} - \epsilon_{red}) \frac{C_t \exp\left[\frac{(E-E_m)nF}{RT}\right]}{1 + \exp\left[\frac{(E-E_m)nF}{RT}\right]}$$

where l is the length of the optical path of the cell. It is not crucial to know the values of the specific absorption coefficients of the oxidised and reduced form of the redox couple ϵ_{ox} and ϵ_{red} , or the total concentration of the enzyme, C_t , in order to estimate n and E_m [139]. Below are shown examples of absorbance spectra from redox titration of a protein, linear least square regression analysis (Nernst plot, Fig. 14 B) and direct data fitting of the potentiometric titration curves (Fig. 14 A) [70].

Figure 14. Spectroelectrochemical studies of bilirubin oxidase. A) Potentiometric titration curves, and parameters obtained from non-linear data fit. B) Spectra from the titrations, and Nernst plot showing linear dependence of $\log [Ox/Red]$ vs. E and obtained parameters [70].

Cyclic voltammetry

Potential sweep methods are the most widely used among the methods available for studying electrode processes. Their essence is the application of the continuous time-varying potential to the working electrode. These techniques allow to determine the mechanism of electrochemical reaction, identify species present in solution as well as to analyse reaction rates.

There are two forms of potential sweep electrochemical techniques, linear sweep voltammetry and cyclic voltammetry, in the latter the sweep direction is reversed at certain potential. In linear sweep voltammetry the potential is scanned with a certain scan rate in only one direction, with a stop at some particular value of E . In cyclic voltammetry the potential is swept until E_{max} and then inverted, and swept until the E_{min} value is reached [133]. So, under control of experimenter are such parameters as potential limits, scan rate and number of scans [134]. The dependence of the potential on time is shown on Fig. 15 A [133].

Figure 15. Cyclic voltammetry. A) Variation of applied potential with time. B) Cyclic voltammogram of electroactive species adsorbed on the electrode.

The important information that one would like to obtain from cyclic voltammograms (CVs) is the number of peaks on forward and reverse scans, shape of the peaks, peak potentials, peak current densities, charges associated with peaks, peak separation, as well as differences between cycles. And of a particular interest is how these features change with potential scan rates and potential limits [134].

Due to the electrochemical reaction at the electrode-electrolyte interface, faradaic currents (I_f) can be observed in the particular zone of applied potential. However, there is also a contribution of capacitive current (I_c), because on sweeping the potential the charge of double layer is changing, and the capacitive contribution is increasing with increasing scan rate. Therefore, the total current of the system equals:

$$(14) \quad I = I_c + I_f$$

In Fig. 15 B a CV of a surface bound redox species with fast (reversible) electron transfer is presented, giving identical cathodic and anodic waves on forward and reverse scans. In case of an ideal reversible system, the anodic peak potential (E_{pa}) should be equal to the cathodic peak potential (E_{pc}), with a total width at half-height ($\Delta E_{p1/2}$) of the cathodic/anodic wave:

$$(15) \quad \Delta E_{p1/2} = 3.53RT/nF = 90.6/n \quad (25^\circ \text{C})$$

The position of E_p with regards to E^0 is dependent on the relative strength of adsorption. The peak current at each point of the CV is proportional to the scan rate. The area under the wave represents the charge needed for the full reduction/oxidation of the layer, and the peak current (I_p) is given by equation:

$$(16) \quad I_p = \frac{n^2 F^2 \nu A \Gamma}{4 RT}$$

where Γ is the surface concentration of adsorbed species, A – surface area, ν – scan rate, F – Faraday constant, n – number of electrons, R – gas constant.

For irreversible systems $Ox + e \rightarrow Red$, no inverse peaks appear in the CV upon reversing the scan direction [133]. The electrochemical reaction becomes irreversible when the rate of ET is insufficient to sustain the equilibrium at the surface. The peaks become broader and lower, because slow ET reactions require additional energy, η , *i.e.* more energy than that determined by thermodynamics [134]. In case of quasi-reversible systems, the kinetics of oxidation and reduction processes must be taken into account contemporaneously. As the sweep rate increases, the extent of irreversibility increases as well, *i.e.* there is an increase in separation of cathodic and anodic peaks, as well as decrease in peak currents compared to the reversible case [133].

In bioelectrochemistry cyclic voltammetry is a widely used technique to characterise protein modified electrodes and complete biodevices. This electroanalytical method is used to determine the redox potentials of enzymes or co-enzymes, as well as for defining η of the system, which is equivalent to efficiency. The method can provide detailed information about the catalytic cycle of the system, *i.e.* ET kinetics, reaction mechanisms, current densities, and redox potentials [140, 141]. Moreover, cyclic voltammetry has been often used to determine the capacitance of bioelectrodes and complete biodevices, and to differentiate between double layer and pseudocapacitance. The area-specific capacitance can be calculated by using the following equation:

$$(17) \quad C_a = \frac{Q}{A \Delta E}$$

where C_a is the area specific capacitance, Q is the charge obtained from the integral area of CV, E is the applied potential window [142, 143].

In this thesis cyclic voltammetry was employed for characterisation of bioelectrodes in **Papers I, III, IV, V**.

Amperometry

Amperometric technique implies measurement of current response as a function of time, while applying a constant potential [135]. The experimentally observed current has a contribution of both faradaic and capacitive processes, and the latter distorts the short time responses. Consequently, the amperometric experiment is limited by charging currents at short times and by mass transfer at longer times [134].

When the potential is stepped from the initial value, the total charge at the time (t) is:

$$(18) \quad Q = nFA \Gamma + Q_{dl}, \text{ whereas}$$

$$(19) \quad Q = It$$

where Γ is the concentration of surface confined species, A – surface area, Q – electric charge, Q_{dl} – charge of double layer. In **Papers III and IV** amperometry was used for characterisation of electrodes and biodevices.

As an example of application of an amperometric technique in practical setups, an O_2 electrode, which in its most widely used form was first described by Clark in 1956, can be considered. Since then there have been many alterations of Clark's design, but the functioning principle remained the same. Essentially, it is an electrochemical cell, which consists of a pair of wires, one of platinum and another much larger one of silver. A potential of *ca.* -0.6 V is applied to the platinum electrode with reference to the silver. As a result, the current flowing through the cell is linearly dependent on the O_2 partial pressure in the solution hosting the electrodes. In order to prevent poisoning, the electrodes can be coated with an O_2 permeable membrane, thus isolating them chemically and electrically from the test solution except of O_2 , which can penetrate the membrane and give the same O_2 partial pressure at the electrode-solution interface as in the bulk matrix [144]. The typical Clark-type electrode set-up without the reaction vessel is shown in Fig. 16.

Figure 16. Typical Clark-type electrode set-up.

In this work O_2 concentration measurements for determination of the biocatalytic activity of the enzymes in homogenous solution by using Clark-type electrode were performed in **Papers I and III**.

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) implies application of a small perturbation, typically of applied potential or current in case of stationary electrodes, so that the system is not perturbed far from equilibrium. The advantage of the small disturbance is that linear forms of mathematical equations can be applied for the description of the system.

The sinusoidal response from the applied constraint may vary in both phase and amplitude from the imposed signal. Differences in phase and amplitude allow conclusions to be drawn about the electrode processes, such as diffusion, kinetics, double layer, coupled reactions, *etc.* [133].

Commonly in alternating current impedance a sinusoidal potential with a small amplitude E_0

$$(20) \quad E_t = E_0 \sin (wt)$$

is applied upon the direct current potential, typically the equilibrium potential, and the current response (I_t) is registered. E_t is the potential at time t , and

$\omega = 2\pi f$ is the angular frequency. The current response will be also sinusoidal, however, different in amplitude (I_0) and phase from the applied sinusoidal potential:

$$(21) \quad I_t = I_0 \sin(\omega t + \varphi)$$

where φ is a phase angle between current output and potential perturbation [134].

The phase shift takes place due to the behaviour of electrochemical systems in accordance with electrical circuit that includes both capacitances and resistances.

The impedance of the system (Z) can be presented by expression analogous to Ohm's law [134]:

$$(22) \quad Z = E_t / I_t = \frac{E_0 \sin(\omega t)}{I_0 \sin(\omega t + \varphi)} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \varphi)}$$

The plot of impedance in the complex plane (Nyquist plot) deals with the variation of total impedance and combines both high and low frequency regions. The measured total impedance of the cell is the combination of the series of resistances and capacitances. These two components are the real and imaginary components of Z , respectively, and therefore $Z_{Re} = R$ and $Z_{Im} = 1/\omega C$. The Nyquist plot and equivalent circuit for the simple charge transfer reaction – Randles cell, one of the simplest and most common cell models, are shown below (Fig. 17) [135].

Figure 17. Nyquist plot and equivalent circuit for a simple charge transfer reaction [135].

The imaginary component of the plot above comes purely from the double layer capacitance (C_d), however, at high frequencies its impact goes to zero - all the current is charging current and the only impedance it meets is R_Ω (solution resistance). As the frequency decreases, a finite value of C_d appears on Z_{Im} . Going even lower in frequency C_d has a high impedance, and current flows mostly through R_{ct} (charge transfer resistance) and R_Ω . At low frequencies the electrode process often becomes diffusion controlled and the linear correlation between Z_{Im} and Z_{Re} , *i.e.* Warburg impedance, rises [135].

In the real systems both high and low frequency regions will be combined (Fig. 18). The important feature is the relation between R_{ct} and Warburg impedance. If the system has slow kinetics, it will have a large R_{ct} value (charge transfer controlled), and limited region, where the mass transfer is an important factor. In the opposite case, R_{ct} can be negligible in relation to R_Ω and Warburg impedance. In that case charge transfer is very facile and mass transfer starts to play a significant role (system is under diffusion control), therefore, the semicircular region is not properly defined [135].

Figure 18. Nyquist plot for an electrochemical system, where regions of mass-transfer and kinetic control are found at low and high frequencies, accordingly [135].

EIS studies were carried out in **Papers IV and V** for characterisation of bio-devices and separate bioelectrodes.

Application of electrochemical techniques for characterisation of biosupercapacitors

Since a self-charging biosupercapacitor/charge-storing EFC is a combination of an EFC and an ECC, normally its performance is evaluated in terms of OCV, power output, polarisation response, capacitance, and charge-discharge cycling [10, 22, 38, 40], *i.e.* taking into account features of charging and capacitive components. Theoretically, the OCV of a biodevice is given by the difference in formal potentials of biofuel oxidised at the anode and bio-oxidant reduced at the cathode. However, in practice, each of these reactions proceeds with certain η , determined by biocatalyst and experimental parameters, which finally affects the OCV of a biodevice [22, 40]. Current-voltage characteristics, *i.e.* polarisation response and power output ($P = IE$), can be obtained using discharge of a device with calibrated resistor upon reaching a stable OCV (**Paper V**) [39], and by applying potential steps starting from OCV and registering the steady state response at each step (**Paper IV**) [10, 145]. Linear sweep voltammetry at low scan rate [146] can be also used to obtain current-voltage characteristics, as a near steady-state current response is registered upon the change of potential. While recording current-voltage responses, one of the electrodes of an electric power biodevice serves as WE and another as RE and CE. The typical power and polarisation curves, obtained by applying potential steps, are presented in Fig. 19 A. Power curve consists of two voltage extremes, *i.e.* at one extreme the maximum voltage is provided at zero current (open circuit), at another extreme no useful electrical work is done resulting in a so called short circuit current. Useful power is obtained when the current lies between the open and short circuit voltage [22, 23], and the maximal power together with operating voltage can be extracted from the maximum on the power curve. Due to the naturally fast mechanism of releasing and storing the charge, electrochemical capacitors are capable to deliver high power in pulse mode [38]. Therefore, the operational stability of electrochemical capacitors, as well as of biosupercapacitors, is evaluated on the basis of charge-discharge cycle lifetime. In Fig. 19 B the charge/discharge cycling of an ESC is demonstrated, and the power output is calculated from the steady-state voltage and applied resistance values.

Figure 19. Characterisation of self-charging biosupercapacitors. A) Polarisation (dashed line) and power output (solid line). B) Charge-discharge curves [10].

Furthermore, the overall capacitance of the biodevice and bioelectrodes can be calculated from charge/discharge curves using the integral area of the discharging cycle, the known value of resistance, and Ohm's law (Eq. 23) [143]:

$$(23) \quad E = IR$$

RESULTS AND DISCUSSION

The aim of this thesis was to assess bioelectrocatalytic capabilities of bioelements for construction of biological electric power sources, *i.e.* blue copper proteins, as well as to present and evaluate the performance of electric power biodevices made thereof. For biodevice construction proteins having and lacking intrinsic catalytic activity were employed.

Biochemical and electrochemical studies of cathodic bioelements

In the review (**Paper I**), O₂ electroreduction and bioelectroreduction are compared. The calculations presented therein show that theoretically BMCOs outperform Pt catalyst by several orders of magnitude. The direct experimental results indicate that η for O₂ electroreduction on BMCO modified electrodes is significantly lower compared to the noble metal catalyst, even in acidic electrolytes, where Pt is highly active. Besides, in complex solutions the performance of unprotected metal electrodes appeared to be inadequate. Furthermore, in this review electrochemistry of a promising candidate for the construction of autotolerant implantable biocathodes, HCp, was revisited. For the first time, a clear MET based bioelectrocatalytic reduction of O₂ on HCp modified nanostructured graphite electrodes in the microampere range was shown.

Another candidate for construction of biocathodes, the GreeDo variant of the high redox potential basidiomycetous Lc, obtained by laboratory evolution together with computational-guiding mutagenesis, was assessed biochemically and electrochemically (**Papers II – III**). Lc mutant libraries were examined using synthetic high redox potential mediators, whereas computer-guided evolution investigations were carried in parallel to conduct mutagenesis without affecting the protein stability. As a result, E_{T1} of the evolved enzyme was enhanced from 0.74

V to 0.79 V, with simultaneous improvement of thermal and acidic pH stabilities as well as activity towards high redox potential mediators that constitutes the uniqueness of this mutant, because earlier attempts to improve E_{T1} values in Lcs resulted in sacrifice of either stability or activity. The investigation also showed that the hydrophobic environment around the T1 copper is one of the main structural criteria in modulating the redox potential in fungal Lcs. Electrochemical studies of this mutant revealed the increase of onset potential of DET based O_2 bioelectroreduction and OCP values compared to its parental version, OB-1. The operational stability of GreeDo in acidic electrolyte was investigated in the adsorbed state, as well as in homogenous solution. The results of stability tests demonstrated that the enzyme was more stable in the adsorbed state.

Blue copper proteins in a construction of biosupercapacitors

Biosupercapacitors are supercapacitors manufactured using biological materials [11]. ESC combines ECC and EFC, and the capacitive part is based on highly porous materials, storing charges in a electrical double layer [147]. In addition to irregular and textured materials, the capacitive part can be also built from abiotic and biotic organic materials [46, 148, 149], and conducting organic polymers [12, 39], storing charges through both non-faradaic and faradaic charge separation events. In case of conventional biosupercapacitors, the bioelement functions as pseudocapacitive component, while in ESCs it also serves as the biocatalyst [11].

In **Paper IV** another blue copper protein, *ThLc*, was employed as a cathodic biocatalyst in a construction of multi-cell ESCs made of biomodified PEDOT/GEs. It is a well-known fact that voltage increase is impossible by connecting several electrochemical cells in series in one electrolyte, but in this paper single-electrolyte based multi-cell glucose/oxygen biodevices with adjustable OCVs and operating voltages were designed and tested. The voltages appeared to be independent on the difference in equilibrium redox potentials of the two redox couples, gluconolactone/glucose and O_2/H_2O , *viz.* 1.18 V, but dependent on the number of half-cells in the biodevice construction. The biodevices were made from tubular GEs with electropolymerised PEDOT modified with *ThLc* and *NcCDh* as the cathodic and anodic biocatalysts, respectively. Due to the interplays between faradaic and non-faradaic electrochemical processes, as well as between ionic and electric conductivities, the biodevice composed of three cells was capable to provide a remarkable and stable open-circuit voltage of *ca.* 1.8

V. The voltage increase was proportional to the number of ESCs in the system, thus it could be further tuned by varying the number of cells. However, a biodevice composed of seven cells, was able to provide a voltage of only 3.0 V, which was lower than a value predicted by Kirchoff's law, due to either instability of individual ESCs or H_2O electrolysis. The ESCx3 system delivered *ca.* 2 μA of current at an operating voltage of 1.25 V, which exceeds the maximal limiting open-circuit value of 1.24 V for the complete glucose oxidation. EIS investigations of the system revealed a decrease of both charge transfer and solution resistances upon the electrical connection of all three cells due to the shift of the charge flow pathway.

In **Paper V** an entirely new kind of bioelectronic device – a conventional biosupercapacitor, which was built from a copper containing redox protein, was reported. This conventional biosupercapacitor, *i.e.* a biodevice without self-charging ability that has to be charged externally, was made of Rc modified gold electrodes. Before the biodevice was assembled, detailed spectroelectrochemical investigations of Rc, in solution and immobilised state, were carried out, including estimation of the redox potential of the T1 site (0.62 V *vs.* NHE), protein midpoint potential when adsorbed on a self-assembled monolayer (0.34 V *vs.* NHE), as well as biocapacitance of Rc modified gold electrodes (115 $\mu\text{F cm}^{-2}$). The symmetrical biosupercapacitor based on two identical Rc modified gold electrodes was capable to capacitively store charge and deliver electric power derived mostly from the biopseudocapacitance, when charged and discharged externally. While charged during just 5 s, the biodevice with a total capacitance of about 73 $\mu\text{F cm}^{-2}$ supplied a maximum of 4 $\mu\text{A cm}^{-2}$ peak current at 0.40 V. The biodevice, which was subjected to at least 50 charge/discharge cycles without a significant loss of ability to store electric energy, had a leakage current of less than 50 nA cm^{-2} .

OUTLOOK

The global electric energy requirements are constantly increasing all over the world. Due to the growing demand of fuel based electric power, significant efforts have been dedicated towards development of sustainable energy sources as well as rational consumption of the energy. FCs are devices that transform the chemical energy of fuels into electrical energy, whereas biofuel cells have been designed to employ a broad range of carbon sources as fuels, thus obtaining clean energy from renewable sources [150].

This thesis is focused on bioelements for construction of biological electric power sources, namely protein based biosupercapacitors, capable of energy transformation, capacitive storage and delivery either in pulse or continuous modes. Proteins as well as their reaction products, in case of biocatalysts, are considered to be safe and environmentally friendly. Moreover, production of proteins is quite inexpensive [21]. ESCs, which unite EFC and ECC in a single indivisible module, capable of extracting electrical energy by oxidising biofuels, are mainly viewed as micro- or even nano-power sources for implantable biomedical devices. Due to the selectivity of enzymes and their activity under mild conditions, the mixture of fuel and oxidant can be introduced in the same physiological matrix, eliminating the need for separators and providing opportunities for miniaturisation, thus making this type of power sources attractive for biomedical applications, *viz.*, for powering implantable medical devices (IMDs) [22].

The power demand for IMDs may vary depending on their type and application [151]. Power requirements for different types of medical devices are shown in Fig. 20.

Figure 20. Examples of implantable medical devices, their power requirements and battery life time [152].

Even though potentially implantable EFCs/ESCs have been widely debated [153], the real trials were mostly carried out in model solutions [154–156] or *in vitro* in human blood or serum [154, 157, 158]. However, in the last decade several studies on biological power sources implanted in living organisms have been reported (Fig. 21). Noteworthy, first *ex vivo* investigations in human blood were carried out in our research group. The EFC was tested in flow-through of human blood during 10 min. and was able to power a low voltage display [159].

Figure 21. Development of implanted enzymatic fuel cells/enzymatic supercapacitors functioning in vivo in living organisms [153].

As can be seen from the IMD power requirements (Fig. 20) and the latest achievements in the field, biological power sources are situated at the low end of the power spectrum, having typical power outputs in the range of microwatts to milliwatts, thereby limiting their use in a wide range of applications [160]. Taking into account an interplay between the size of the biocatalysts, their intrinsic activities, and the standard heterogeneous electron transfer constants, along with electrode depolarisation, catalyst poisoning and mass transfer limitations neither OCV values even close to 1 V or power densities higher than few tens of $\mu\text{W cm}^{-2}$ can be expected [11].

Nevertheless, one of the ways to overcome the low voltage limitation would be to assemble EFCs/ESCs in series [8, 161], thereby increasing the total output voltage proportionally to the number of cells [8, 18, 162-164]. Several attempts have been made to implement this approach in living organisms, however, it was never successful in the same living creature [9, 17, 18, 165]. Herein (Paper IV) we demonstrated three-cell based self-charging biosupercapacitor operating in a common electrolyte in a flow system, allowing the precise triplication of voltage upon the electrical connection. The results show proof-of-principle operation of a high-voltage biological power source, which might give an idea for future design considerations.

The EFCs/ESCs assembled in series used for activating a pacemaker or analogous devices can operate for hours, in the best case for several days, and apparently in terms of operational stability they cannot compete with the conventional batteries capable of providing electrical power supply for bioelectronic devices for at least 10 years [8, 9, 166]. Obviously, the stability challenge has been a study subject for many research groups, and EFCs implanted in animals have been reported to remain functional during several months [19, 167].

Thence, the long-term stability of the implanted biofuel cells, which implies the stability of individual bio-electrodes in physiological fluids, is the critical issue for their future application in a field of biomedicine [8, 22]. Thus, development of stable autotolerant biocatalysts contributing to higher OCV values and operational stability in a physiological environment would be of a great advantage. **Paper II** introduces a high redox potential Lc, obtained by laboratory evolution in combination with computer-guided mutagenesis, with high thermostability, catalytic efficiency, and activity extended to the alkaline pH range, thus realising a good candidate for a bio-cathode functioning in physiological fluids [72, 168, 169]. Therefore, its bioelectrocatalytic capabilities and operational stability were investigated in **Paper III**.

As discussed above, biological power sources appear to produce low power and have relatively poor operational stability. The question is where to go? What could be the niche application of such devices in nearest future? Powering of temporary microscale implants and transient bioelectronics could be the potential application for these environment friendly power sources in the closest perspective. However, engineering of biocompatible highly stable biocatalysts may open wider doors to the applications where long-term operational stability is a critical requirement.

The recent bodyNET concept, which implies a network of sensors, screens and smart devices, integrates wearable and implantable bioelectronic devices into the system, that will extend our perceptions and allow people to sense and interact with each other and the surrounding environment on a new advanced level [170]. Research on biological power sources is in the scope of priorities contributing to the fulfilment of this concept, to which the present work had some impact.

This thesis can be viewed as a tiny step towards investigating and overcoming some of the challenges related to protein based biological power. Hopefully, the obtained results can be useful for future development of biocompatible, stable and efficient biological power sources.

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I

Oxygen Electoreduction versus Bioelectroreduction: Direct Electron Transfer Approach

Sergey Shleev,^[a, b] Viktor Andoralov,^[c] Dmitry Pankratov,^[a, b] Magnus Falk,^[a, d] Olga Aleksejeva,^[a] and Zoltan Blum^[a]

Dedicated to the 60th birthday of Professor Doctor Wolfgang Schuhmann

Abstract: Being inspired by a very recent review entitled: “Electrocatalysis and bioelectrocatalysis – Distinction without a difference” and following the general approach employed by Prof. Dr. Schuhmann, in the current work we point to the similarities and differences between oxygen electroreduction and bioelectroreduction processes. To additionally distinct our paper from the recent review we touch on different bioelements, such as redox proteins and living cells, but we still keep a special emphasis on oxidoreductases, biocatalysts par excellence. Moreover, we also exclusively focus on oxygen electroreduction based on direct electron transfer reactions. On the one hand, we corroborate the previously made con-

clusion regarding intrinsically high activity of the active sites of biological catalysts, especially redox enzymes, which results in mass transfer and heterogeneous electron transfer limited currents from oxygen reducing bioelectrodes. On the other hand, we disagree with the statements regarding the exceptionality of precious metal catalysts, and the notion of a huge trade-off between high activity and stability of non-precious metal catalysts and bioelements. We show that the activity and stability of noble metal based cathodes is very far from perfect, especially when these electrodes operate in complex electrolytes, such as physiological fluids, e.g. human blood.

Keywords: Electroreduction • Oxygen • Noble metal • Bioelectroreduction • Biocatalyst

1 Introduction

Electroreduction and electrooxidation can be commonly referred to as electrocatalysis. Electroreduction processes occur in a variety of electrochemical devices, including sensors and electrolyzers, as well as fuel cells and some supercapacitors. These devices are used for analytical and synthetic purposes, as well as for energy conversion and storage, respectively. A variety of simple and more complex compounds can be used as oxidants, including hydronium ion (H_3O^+), organic and inorganic peroxides (ROOR'), nitric oxide (NO), nitrite (NO_2^-), and molecular oxygen (O_2).

Among different reductive processes of different oxidants, reduction of O_2 is the key reaction not only in many natural and artificial systems, but also in many living organisms, i.e. aerobes. As the oxygen partial pressure in the atmosphere increased, aerobic organisms came to dominate the planet's species [1]. They, including humans, use the aerobic oxidation of different biofuels in order to extract as much energy as possible. Simultaneously, partial reduction of O_2 , occurring during aerobic respiration, will generate harmful reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide and hydroxyl radicals, which need to be safely disposed of by the organism. In many electrochemical devices similar problems exist, i.e. the complete direct four-electron O_2 reduction to the innocuous product H_2O is not fully realized, and H_2O_2 will be produced, which should be addi-

tionally utilized either by chemical decomposition or further two-electron electroreduction to H_2O .

This review paper is devoted to O_2 (bio)electroreduction, as it is the most common cathodic reaction in (bio)electrochemical systems.

2 O_2 Electroreduction

O_2 is the most useful compound among the possible oxidants because of its availability (about 0.25 mM in pure H_2O at 25 °C [2]) and the high redox equilibrium potentials of the $\text{O}_2/\text{H}_2\text{O}_2$ and especially the $\text{O}_2/\text{H}_2\text{O}$ couples (+0.27 V and +0.82 V vs. NHE at 25 °C, pH 7, Eqs. 1 and 2, respectively [3]):

- [a] S. Shleev, D. Pankratov, M. Falk, O. Aleksejeva, Z. Blum
Department of Biomedical Science, Faculty of Health and Society, Malmö University, Skåne
20506 Malmö, Sweden
*e-mail: sergey.shleev@mah.se
- [b] S. Shleev, D. Pankratov
Kurchatov NBICS Centre, National Research Centre
“Kurchatov Institute”, 123182 Moscow, Russia
- [c] V. Andoralov
KEMET Electronics AB, Skiftesvägen 16, 563 31 Gränna,
Sweden
- [d] M. Falk
NanoFlex Limited, iTac, Daresbury Laboratory, Sci-Tech
Daresbury, Keckwick Lane, Daresbury, WA4 4AD, United
Kingdom

2.1 Thermodynamics and Kinetics of O₂ and H₂O₂ Electroreduction

The thermodynamic parameters for the overall reaction of O₂ and H₂O₂ reductions can be expressed through the well-known equation:

$$\Delta G_T^0 = -nFE_T^0 \quad (3)$$

where ΔG_T^0 – the change in standard Gibbs free energy, n – the number of electrons transferred in the half-reaction, F – the Faraday constant, and E_T^0 – the standard electrode potential of the half-reaction.

For the two half-reactions of O₂ reduction (Eqs. 1 and 2) standard potentials (*i.e.* at 25 °C but pH 0) were measured experimentally and found to be +0.68 and +1.23 V, respectively [4]. Indeed, ΔG^0 for O₂ reduction to H₂O₂ and especially H₂O is very low, *viz.* –1.36 eV and –4.92 eV, respectively.

The standard potential of the H₂O₂/H₂O couple (Eq. 4) is also very high (+1.77 V) [4]. Indeed, ΔG^0 for H₂O₂ reduction to H₂O is also low, *viz.* –3.54 eV.

Sergey Shleev received his PhD in biochemistry from A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, in 2001.

After that, he held various positions at Swedish Universities (Lund and Malmö) until 2011, when he became an Associate Professor in biomedical technology at Malmö University, Sweden. During the same year he also habilitated at the Institute of Biochemistry, Russia. From 2013 he is a Professor in biomedical technology at Malmö University. His current main scientific and technological interests are studies of multi-center redox enzymes and their applications in bioelectronics operating *in vivo* and *ex vivo*.

Magnus Falk was born in 1983 in Åtvidaberg, Sweden. He received his MSc in Biochemical engineering from the Department of Physics, Chemistry and Biology at Linköping University. In 2014, he defended his PhD at the Department of Biomedical Science at Malmö University, Sweden, supervised by Prof. Sergey Shleev. He is currently employed at NanoFlex Limited, working on the development of nanoband electrodes for a variety of sensor applications.

Viktor Andorlov received his PhD in electrochemistry from A.N. Frumkin Institute of Electrochemistry, Russian Academy of Sciences, Moscow, in 2009. From 2010 till 2012 he was a postdoctoral researcher at Malmö University, Sweden, working on the development of bioelectronic devices based on the redox enzymes. From 2012 he is an Engineer Electrochemist at KEMET Electronics Corporation, Sweden. Currently, his research interests include electrolytic capacitors and their components.

Olga Aleksejeva is a PhD student at the Department of Biomedical Science, Malmö University, Sweden, under the supervision of Prof. Sergey Shleev. In 2014 she received her MSc Degree in Analytical Chemistry from Lund University, Sweden. Her current research is focused on enzymatic fuel cells and biosupercapacitors.

Dmitry Pankratov is a PhD student at the Department of Biomedical Science, Malmö University, Sweden, under the supervision of Prof. Sergey Shleev. In 2008 he received his MSc degree in Physical Chemistry from Bryansk State University, Russia. From 2009 till 2012 he was a lecturer at the Moscow State University of Mechanical Engineering, Russia. His current research interests include nanostructured enzymatic electrodes and their application in bioelectronic devices.

Zoltan Blum received his PhD in organic chemistry from Lund University, Sweden, in 1981. After a short spell in the pharmaceutical industry he returned to Lund University and became an Associate Professor in 1990. From 1998 he has held various academic and administrative positions at Malmö University. Currently, his research interests include bioelectronics and its applications.

Review

ELECTROANALYSIS

For practical applications, however, processes should not only be energetically favorable, but they should progress at reasonable rates, thus thermodynamics and also kinetics need to be considered.

The rate of O_2 and H_2O_2 reduction is defined by the kinetics of the two main stages: (1) reactant diffusion or the mass transfer stage, and (2) the reactant ionization stage. In turn, the ionization process can be divided into three sub-stages, such as (2.1) reactant adsorption, (2.2) electron transfer (ET), and (2.3) product desorption. The adsorption of O_2 or H_2O_2 on the electrode surface and product desorption (H_2O_2 or H_2O) to the solution have a huge impact on the actual charge transfer event, which usually is a very slow step, determining the rate of the overall reduction processes.

O_2 and H_2O_2 reductions in real systems can occur in several ways as schematically illustrated in Figure 1.

Since direct ET (DET) between the electrode support and non-biological catalysts for O_2 electroreduction (surface and molecular) is an extremely fast process, there are only two major limiting steps in the overall cathode performance, *i.e.* diffusion (step 1, Figure 2), and catalytic (step 2, Figure 2) limitations. The diffusion limiting currents for cathodes (Figure 2, step 1; i_{diff}) strongly depend on the device design. For submerged cathodes, i_{diff} values are usually not so high and they can be calculated from the Levich equation (Eq. 5), when rotating electrodes are used:

$$i_{\text{diff}} = 0.62nFD^{2/3}A_{\text{geometric}}C_{\text{oxidant}}\nu^{-1/6}\omega^{1/2} \quad (5)$$

Here, i_{diff} is the diffusion limiting current, D is the diffusion coefficient of the oxidant, about $2.0 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for $\text{O}_2(\text{aq})$ and about $1.6 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for $\text{H}_2\text{O}_2(\text{aq})$, A is the geometric area of the electrode, C_{oxidant} is the bulk concentration of O_2 and H_2O_2 ($2.5 \cdot 10^{-7} \text{ mol cm}^{-3}$ and $1.2 \cdot 10^{-6} \text{ mol cm}^{-3}$ of O_2 in air- and oxygen-saturated solutions, respectively), ν is the kinematic viscosity of the solution ($0.01 \text{ cm}^2 \text{ s}^{-1}$ at 25°C , a typical value for aqueous solutions), and ω is the angular frequency (in rads^{-1}) of the cathode.

Fig. 1. A schematic representation of different pathways during O_2 and H_2O_2 electroreduction. According to Sethuraman *et al.* (2009) [5] with some additions and changes. Notes: k_1 is the rate constant of the direct four-electron reaction; k_2 and k'_2 are rate constants of the O_2 reduction reaction to H_2O_2 and H_2O_2 oxidation, respectively; k_3 is the rate constant of H_2O_2 reaction to H_2O ; k_4 is the rate constant of the catalytic decomposition of the intermediate product, $k_5(k'_5)$ and $k_6(k'_6)$ are the rate constants of the adsorption (desorption) of O_2 and H_2O_2 , respectively; k_7 and k_8 are the constants for O_2 and H_2O_2 diffusion, respectively.

Fig. 2. A schematic representation of the mechanism of an electrocatalytic reaction in a non-biological catalyst based cathode.

For a very efficient gas-diffusion electrode (GDE) the current densities obtained are much higher compared to devices that are fully immersed into electrolytes. To a first approximation, the i_{diff} value for a GDE can be evaluated using Eq. 6 and it is related to the O_2 partial pressure at the catalyst layer and the O_2 partial pressure in the bulk solution [6].

$$i_{\text{diff}} = \frac{p_{\text{O}_2}^{\text{bulk}} i}{p_{\text{O}_2}^{\text{bulk}} - p_{\text{O}_2}^{\text{diff, layer}}} \quad (6)$$

The catalytic limiting current for cathodes (both GDE and immersed electrodes) can be calculated from the Butler-Volmer equation (Eq. 7):

$$i_{\text{cat}} = nFC_{\text{sur}}A_{\text{real}}k_0 \exp\left(\frac{\alpha n_L F(E^0 - E)}{RT}\right) \quad (7)$$

Here, i_{cat} is the steady state catalytic current, C_{sur} is the surface concentration of O_2 or H_2O_2 , n_L is the number of electrons in the limiting step of the cathodic process; A_{real} is the real electrode area, k_0 is the standard heterogeneous ET rate constant, α is the charge transfer coefficient, E^0 and E are the stationary and applied potentials, respectively (in the ideal case E^0 is equal to E^{red}).

In a mixed kinetic regime the total current from the cathode can be described by Eq. 8, which is analogous to an expression giving the resultant conductance of a group of resistors connected in series, where i is the obtained steady-state current:

$$1/i = 1/i_{\text{cat}} + 1/i_{\text{diff}} \quad (8)$$

2.2 Non-Biological Electrocatalysts

To a first approximation, all electrocatalysts can be divided into two major groups, *viz.* conventional molecular and surface electrocatalysts. The former comprise metal complexes including composite nanomaterials (*vide infra*)

and thus ET between a particular catalyst and an electrode surface is a very fast process. The latter comprise materials such as metals and alloys, or conducting metal oxides and sulfides. Thus, there is no heterogeneous ET *per se* for surface electrocatalysts, since the particular conducting support and catalyst pair is just the same piece of material, or the junction between them, if present, is highly conducting.

2.3 O₂ Electroreduction on Metal Catalysts

The fundamental basis of the mechanism of the O₂ electroreduction reaction (ORR) on different electrodes have been studied extensively: Pt [7], Fe [8], Au [9], Ag [10], Hg [11], and Pd [11]. Among metallic electrodes Pt is the best catalysts, since four-electron O₂ electroreduction (Eq. 2) is usually observed on Pt group metals, whereas two-electron reduction of O₂ (Eq. 1) is well-known for other metal-based electrodes.

The kinetics of ORR on Pt is well described by the Butler-Volmer equation (Eq. 7) for the cathodic half-reaction [12]. On a pure Pt surface the Tafel slope ($dE/d\log(i)$) is equal to 120 mV/decade at sufficiently high overpotential (η) and the first ET to the adsorbed protonated O₂ is the rate determining step. At low η O₂ coverage on the Pt surface is higher and the rate determining step is a pseudo two-electron process, decreasing the slope to ca. 60 mV/decade, *i.e.* indicating a different mechanism on the Pt/PtO surface compared to pure Pt [13]. The slope of the half-wave potential vs. pH (dE/dpH) is equal to 59 mV/pH (at 25 °C). The transfer of the following three electrons are each fast processes, and the Tafel slope is not affected. Two Tafel slopes are in fact usually obtained for ORR, 60 mV/decade and 120 mV/decade, respectively, depending on the electrode materials used and on the potential range.

In order to improve Pt-based catalysts further, different approaches are exploited nowadays, including usage of nanomaterials. On one hand, the usage of nano-dispersed Pt based systems significantly decreases the costs of the catalyst. On the other hand, the specific activity ($\mu A/cm^2_{Pt}$) of nano-catalysts is ~10 times lower compared to a bulk polycrystalline Pt electrode [14]. The changes in surface properties effect the adsorption of oxygen containing species (OH_{ads}) and, concomitantly, the kinetics of O₂ reduction, related to an increase of low-index facets when decreasing the particle size [15].

By alloying Pt with suitable noble metals or *d*-block transition metals, multi-component systems and core-shell-like structures with higher specific activities compared to mono-component Pt-based catalysts can be created [16,17]. The formation of alloys between Pt and different transition metals results in the improvement of the basic parameters of active centers for O₂ reduction, such as the lattice parameter and the center of *d*-bond energy [18]. The superior activity primarily arises from reduced OH adsorption to the Pt atoms, the oxygen species are instead adsorbed mostly on the second metal, allowing O₂

to be reduced on Pt in a four-electron process. As an example of already reported structures, FePt/C catalysts have demonstrated roughly a four-fold increase in catalytic current compared to commercially available Pt/C (Figure 3), with the better performance and stability observed for face centered tetragonal (fct)-FePt NPs compared to face centered cubic (fcc) structures [19].

Core-shell-like structures were originally developed to minimize the amount of Pt in catalysts for O₂ electroreduction [20]. Theoretical calculations and experimental data have shown that Pd is the best carrier for the layer of Pt atoms [21,22]. In the case of alloy systems, core-shell-like structures can be built on the segregation of Pt atoms [18] and on dissolution of non-Pt components [23], which may result in a skeletal form on the surface of Pt nanoparticles [24].

Besides the particle size there are other factors affecting the catalytic activity, so it is essential to know the details about the alloy formation. Earlier investigations [25] showed that the Pt-Pt nearest-neighbor distance was the main factor influencing the catalytic activity for ORR. Later the alloying effect was studied by using catalysts of similar particle size in order to eliminate the particle size effect. The heat treatment temperature and consequently the degree of alloying defines the Pt-Pt nearest-neighbor distance of the catalysts. The specific activity was found to increase with decreasing Pt-Pt distance in all studied cases. This can be explained by adsorption of O₂ on Pt surfaces and the electronic state of Pt. O₂ is adsorbed on Pt in a dual site mode, and consequently the Pt-Pt nearest-neighbor distance is of main importance in determin-

Fig. 3. ORR current densities from commercial Pt/C, fcc-FePt/C, and fct-FePt/C catalysts in O₂ saturated 0.5 M H₂SO₄ (A) and schematic illustration of (B) chemically disordered fcc-FePt/C and (C) chemically ordered fct-FePt/C. Reprinted from [19], copyright (2010), with permission from The American Chemical Society.

ing the adsorption behavior. Apart from the heat-treatment temperature, the nature of the transition metal used for alloy formation affects the Pt–Pt distance as well [26].

It is well known that adsorbed anions from the electrolyte, have a significant effect on the chemical reactions taking place on the electrode surface [27]. The ORR rate is affected by alterations in the Pt surface structure, and this is often due to anion adsorption or oxidation [28,29]. In the presence of specifically adsorbed anions, the nanoparticles become more spherical, and thus the average Pt–Pt bond weakens near the surface. Adsorption of anions from the electrolyte changes the electronic and/or geometric properties of Pt nanoparticles and the available adsorption sites are blocked, thus inhibiting the ORR. Nevertheless, the effect of alloying, as well as the particle morphology, appear to have a considerable impact on the strength of the anion adsorption [30].

The catalytic film thickness also impacts the ORR; a catalytic layer consisting of a Pt submonolayer electrodeposited on an Au surface exhibits higher activity compared to bulk Pt, whereas ORR inhibition was observed for Pt multi-layers [31]. Such a behavior can be explained by changes in the electronic structure, and should be taken into account regarding the further development of effective O₂ reducing electrodes.

Two reaction steps can be considered as sluggish (potential-determining) during ORR, *viz.* initial formation of an adsorbed HOO* intermediate (for materials that bind O₂ intermediates too weakly) and final removal of adsorbed HO* (or O*) groups from the surface (for materials that bind O₂ intermediates too strongly) [32]. Adsorption energies of these species are strongly correlated and cannot be independently varied by changing the catalyst, which results in so-called scaling relations [33]. The constant difference between ΔG_{HOO^*} and ΔG_{HO^*} of 3.2 eV (at 0 V) determines a minimal $-0.4 \text{ V } \eta$ to make all reaction steps downhill in free energy and to achieve the highest ORR activity [34]. Despite the fact that the universal character of scaling relations imposes significant restrictions on the development of highly effective catalysts for ORR, these limitations may be bypassed by introducing an alternative O₂ reduction mechanism related to a change of the active site structure [35], or by using enzyme molecules, which are able to catalyze ORR at high specific rates (*vide infra*) [36]. Stabilities of HOO*, HO*, and O* intermediates roughly correlate with the stability of adsorbed O₂ (oxygen binding energy, ΔE_{O}), therefore this parameter is a good descriptor for the catalytic activity of different materials in ORR [36]. According to the volcano plot presented in Figure 4, Pd, being a relatively inexpensive catalyst, is the second most active metal for O₂ reduction reaction.

However, a major problem of Pd-based cathodes is their insufficient stability. To stabilize Pd, as well as to improve its kinetic characteristics towards O₂ electroreduction, a number of bimetallic systems based on transition elements were characterized, *i.e.* PdAu [38] and PdFe [39,40], as well as PdCr, PdCo, and PdNi [41]. These rela-

Fig. 4. Trends in oxygen reduction activity plotted as a function of the oxygen binding energy. Reprinted from Norskov et al. [37], copyright (2004), with permission from The American Chemical Society.

tively stable catalysts however reduce O₂ with H₂O₂ formation, and H₂O₂ may accumulate in the electrolyte, which leads to degradation of the electrochemical devices [40,42]. This problem can be overcome by increasing the catalyst selectivity, as well as by increasing the ability to catalyze immediate chemical decomposition of H₂O₂, which is done by formation of core-shell-like structures containing low amounts of Pt. Figure 5 shows the principle of the activation and stabilization of nanoparticles based on a PdCoPt/C multi-system [23].

2.4 Mathematical Modeling of O₂ Electroreduction on Noble Metals

Typical modeled voltammograms of O₂ electrocatalytic reduction on polycrystalline Pt and Au electrodes immersed in acidic solutions are presented in Figure 6 below.

The curves were modeled based on the standard heterogeneous ET rate constants (k_0) per single metal atom (Pt: $1.2 \cdot 10^{-5} \text{ s}^{-1}$; Au: $2.4 \cdot 10^{-9} \text{ s}^{-1}$ and $2.4 \cdot 10^{-11} \text{ s}^{-1}$ for O₂ and H₂O₂ electroreduction processes, respectively), which were calculated from experimentally obtained exchange current densities (Pt $\sim 10^{-6} \text{ A cm}^{-2}$; Au $\sim 10^{-10}$ and $10^{-12} \text{ A cm}^{-2}$) [43] using Eq. 9.

$$j_0 = nFk'_0C_{\text{bulk}} \quad (9)$$

Here, j_0 is the exchange current density, k'_0 is the standard heterogeneous ET rate constant for the electrode surface (in cm s^{-1}), and C_{bulk} is the bulk concentration of O₂ or H₂O₂.

The following information can be extracted from the model curves in Figure 6. First, the maximal current density for non-GDE devices is highly dependent on convection and does not exceed 10 mA cm^{-2} or 0.5 mA cm^{-2} for very high rpm rotating electrodes, and almost stationary

Fig. 5. Dependence of the first derivative of i/i_0 (i_0 – initial current) on the number of potential cycles for different catalysts. Conditions: 0.5 M H_2SO_4 , cycling in the range from 0.6 up to 1.2 V vs. NHE, PdCoPt/C was synthesized according to [23], Pt/C from ETEK (40 wt.% Pt).

Fig. 6. Typical voltammograms of O_2 electrocatalytic reduction on polycrystalline Pt (solid curves) and Au (dashed curves) electrodes. η – overpotential equal to $E_{\text{appl.}} - E^0_{\text{O}_2}$. The curves were modeled using Eqs. 5 and 7. O_2 concentration was assumed to be $1.2 \cdot 10^{-6} \text{ mol cm}^{-3}$. All other parameters used for modeling are presented in the text below the equations.

devices, respectively. Second, the voltammograms can be divided into three regions as marked for a Pt electrode on the curve in Figure 6: (i) limited by the catalytic kinetics (η is in the range from -0.3 up to 0 V; Eq. 7), (ii) mixed kinetic region (η is in the range from -0.5 up to -0.3 V; Eq. 8), and (iii) mass transfer limited region (η lower than -0.5 V; Eq. 5). Finally, half-wave potentials of ORR depend strongly on k_0 -values of electrocatalysts.

2.5 O_2 Electroreduction on other Catalysts

Since the usage of noble metal based catalysts is economically untenable, significant attention is paid to study the reaction of O_2 electroreduction using other types of catalyst. From this perspective several different types of mate-

rial are considered as possible alternatives, such as metal- N_4 pyro-polymers [44,45], metal chalcogenides [46] including metal oxides [47,48], metal nitrides and oxynitrides [49–51], metal carbonitrides [52], as well as metal free catalysts, e.g. heteroatom-doped carbon-based materials [53].

Since many biocatalysts rely on heterocyclic complexes in their active sites, this review describes only one type of alternative catalysts, which is based on pyro-polymers. Development of metal- N_4 pyro-polymers, formed by pyrolysis of carbon supports, with an adsorbed transition metal forming a metal- N_4 active site core (Figure 7), in electrocatalysis for O_2 reduction reactions represents a good example of a biomimetic approach. Studies of natural catalysts gave rise to the idea of using an active center motif, e.g. a metal-porphyrin complex, as biomimetic catalysts for O_2 electroreduction (Figure 7).

The mechanism of O_2 reduction using metal-porphyrin systems has been intensively studied [54,55]. The total number of electrons in ORR is less than 4 but higher than 2, as only a portion of O_2 is reduced directly to H_2O via a four-ET pathway. The Tafel slope is similar to the typical Pt slope, which indicates a similarity in the mechanism with an attenuated electrochemical stage for these catalysts, viz. the transfer of the first electron to the adsorbed O_2 molecule. For cobaltous pyro-polymers, however, intricate reaction pathways of O_2 electroreduction were shown [54].

Despite obvious advances in the development of new types of non-biological catalyst, they are still quite expensive systems due to the use of metal-porphyrin complexes as precursors. In addition, they have much lower activity compared to catalysts based on precious metals, especially Pt. Thus, the search for renewable and inexpensive, but

Fig. 7. A biomimetic inspiration in the development of cathodic catalysts for fuel cells.

still highly efficient and stable catalysts continues. One of the options is biological catalysis and bioelectroreduction.

3 O₂ Bioelectroreduction

As biocatalyst, proteins, enzymes and their combinations, or whole microorganisms can be used. More recently, a new group of O₂ reducing electrodes have emerged, organelle based cathodes, which lies somehow in between these two and exploits mitochondria or chloroplasts as natural bioelements [56]. However, the latter will not be detailed in the current work because of the limited number of currently available papers on this topic. The remaining part of this review is devoted to DET-based bioelectrodes (with just a few exceptions), exploiting the phenomenon of bioelectrocatalysis, and a direct comparison with Pt-based cathodes. However, before that, one should emphasize one of the general obstacles in the development of efficient biocathodes, *i.e.* lack of, or at least poor, electric communication between the bioelement and the electron conducting material of the electrode. This problem does not exist in the case of non-biological catalysts based cathodes. Thus, it is important to clearly divide possible mechanisms to electrically couple biocatalytic and electrochemical reactions.

To the first approximation, bioelectronic devices can be classified as either first, second, or third generation bioelectronics [57,58]. Briefly, first generation bioelectronics rely on the electroactivity of the enzyme substrate or product; the second generation draws on the usage of redox mediators, and the third generation is based on DET reactions. With respect to cathodic reactions, exemplified by O₂ reduction to H₂O (Eq. 2) using blue multi-copper oxidases (BMCOs) as biocatalysts, this classification is schematically presented in Figure 8.

The left part of the scheme (Figure 8) reflects a very old but still widely used approach based on an O₂ sensitive electrode developed by Clark as early as in 1953 (Figure 8A) [59]. In this approach, O₂ is reduced to H₂O during the biocatalytic reaction and its concentration change is monitored by the electrochemical reaction of

O₂ reduction on a Pt electrode. Thus, in the first generation bioelectronic devices redox enzymes are electrically isolated from the electrode surface and, instead of bioelectrochemical process, electrochemical reduction of O₂ on the surface of the electrocatalyst occurs. In the middle part of the scheme (Figure 8) a bioelectrochemical system is shown based on a mediated ET (MET) reaction, which couples biocatalytic and electrochemical processes resulting in bioelectrochemical reduction of O₂ (Figure 8B). In the second generation bioelectronic devices BMCOs are electrically connected to the electrode via artificial or natural redox mediators, which are either solubilized in the electrolyte or immobilized on the electrode surface. The right part reflects a DET based approach, where electrons can be directly transferred to the active site of the BMCOs and a “real” bioelectrocatalytic reduction of O₂ to H₂O occurs (Figure 8C). In the third generation bioelectronic devices a redox enzyme is electrically wired to the electrode surface, in the absence of mediators.

3.1 History of Bioelectrocatalysis: Discovery and Enterprise

The history of bioelectrocatalysis is a quite intriguing subject because it clearly shows the general trend of spiral-like development in science. The very first papers on combining redox enzymes and electrodes (enzyme-based amperometric biosensors or “enzyme electrodes”) appeared as early as in the nineteen sixties [60,61]. However, the first reports on DET with a redox active protein were published many years later, in 1977, when Eddowes with Hill, and Yeh with Kuwana, independently showed that cytochrome c (Cyt c) immobilized on different electrodes showed virtually reversible electrochemistry, as revealed by cyclic voltammetry [62,63]. Cyt c is a small redox protein, which is active in biological ET chains but has no intrinsic catalytic activity. Thus, no one was bothered to exploit the scientific finding of DET for certain applications at that time. Shortly after, the phenomenon of bioelectrocatalysis was discovered and studied in detail by Russian scientists. Already in 1978 it was shown that DET was also possible for a much larger redox protein with intrinsic enzymatic activity—laccase (Lc) [64]. For several years Russian electrochemists were intensively studying bioelectrocatalytic reactions of different enzymes, such as Lc, peroxidase, and hydrogenase [65–70]. However, in most cases bioelectrochemical results from the Russian electrochemical school were presented as real polarization curves (not voltammograms, as widely used by others) with positive cathodic currents and potentials expressed vs. a real hydrogen electrode immersed in the buffer systems used (without recalculation to commonly used reference electrodes), which caused some difficulties for other scientists to directly compare newly gathered results with data already available.

Later, DET properties have been shown for many different oxidoreductases as reviewed in Refs. [71–78]. At present, enzymatic bioelectrocatalysis is one of the most

Fig. 8. A schematic representation of three possible mechanisms to electrically couple biocatalytic and electrochemical reactions.

popular subjects of bioelectrochemistry; nowadays oriented towards fabrication of mediator-less and *a priori* less toxic and more stable bioelectronic devices (when compared to mediated biodevices), taking cues from nanotechnological achievements [78–81].

3.2 O₂ Electroreduction on Redox Protein Modified Electrodes

Drawing on the general spiral-like turn of events of the scientific interest regarding bioelectrocatalysis, yet another turn of the spiral can be observed in this field, *viz.* the usage of small heme-containing redox proteins, e.g. myoglobin and hemoglobin, as bioelements for DET-based O₂ reducing electrodes (see a comprehensive review on this topic [82]). However, since redox proteins in most cases have no intrinsic biocatalytic activity, the η needed for O₂ reduction on these biodevices is very high. Moreover, O₂ is reduced to H₂O₂ (Eq. 1) instead of H₂O (Eq. 2). Interestingly, bioelectroreduction of H₂O₂ to H₂O on heme-containing redox protein based electrodes also occurs [82–84]. As such, the reduction of H₂O₂ to H₂O (Eq. 4) is quite interesting for biocathode applications due to the extraordinarily high equilibrium potential of the H₂O₂/H₂O couple (+1.34 V vs. NHE at pH 7.4 and 25 °C [3]). However, because of the absence of intrinsic biocatalytic activity, η -values needed for H₂O₂ reduction to H₂O on these biodevices are also very high. Indeed, protein based systems are used nowadays for biosensor development [85], whereas DET based peroxidase modified electrodes are claimed to be high potential enzymatic cathodes [86,87].

3.3 O₂ Electroreduction on Microorganism Modified Electrodes

In contrast to discrete protein based electrodes, intact microorganisms can also efficiently catalyze the electroreduction of O₂. Even though this review is focused on DET based enzymatic electrodes, a very short description of ORR by microbes is in order. A wide diversity of microorganisms has the ability to electronically communicate with electrodes, with a broad range of practical applications, where instead of enzymes intact microorganisms are utilized to catalyze the reaction. However, most microbial bioelectrodes need the addition of external mediators to operate, but exceptions can be found. Some microorganisms can electrochemically interact with electrodes, directly donating or accepting electrons from electrode surfaces without artificial mediators. This can be facilitated either by ET *via* microbially produced soluble redox-active molecules, short-range DET between redox-active molecules on the outer cell surface and the electrode; or long-range electron transport through conductive biofilms [88]. Per definition, several microbial electrodes can still be classified as mediator-less.

Microbial bioelectrodes show great promise as cheap and self-regenerating parts in electrolysis cells and micro-

bial fuel cells (MFCs), to replace the expensive Pt catalysts typically used [89–93]. Although little is known about the mechanism of electron uptake by bacteria, microbes themselves could e.g. produce natural mediators or have *c*-type cytochromes incorporated in the membrane to help facilitate electronic coupling to the electrode material [94]. Improved cathodic reaction rates impact the power output of MFCs and represent a major challenge for research and development, where bacteria can facilitate sustainable and cost-effective cathode catalysis for O₂. To mention some examples, the presence of a seawater biofilm on the cathode surface led to efficient catalysis of oxygen reduction [95], employing a cathodic microbial community leading to a strong decrease in activation losses for O₂ reduction [96], growth of the acidophile bacterium directly on a graphite electrodes significantly reduced the η needed for ORR [97], and microbial catalysis of ORR has also been discussed in a recent review [98]. Studies of microbe-cathode interactions have provided insights to the field, with cathode design for highly efficient chemical conversion from the improved ET [92,93], and several different microorganisms for extracellular ET have been extensively studied [88,99].

3.4 Redox Enzymes as Bioelements for DET Based O₂ Reducing Bioelectrodes

Nature has mainly chosen two metals to build active centers of redox enzymes responsible for O₂ reduction, *viz.* iron and copper. Consequently, all biocatalysts widely used in biocathodes at present can be divided into three groups, *viz.* iron-, copper-, and iron/copper-containing redox enzymes. One should emphasize that among the 1654 oxidoreductases known to date (www.enzymedatabase.org) only about 100 enzymes unequivocally display DET reactions [74,76–78,100–104]. Interestingly, many metallo-oxidases (catalyzing reduction of O₂ to H₂O) display well-pronounced bioelectrocatalysis and almost all of them show DET, whereas flavo-enzymes (catalyzing reduction of O₂ to H₂O₂), like native glucose oxidase, does not undergo direct electron transfer [105]. The reasons for the presence or absence of DET for redox proteins were considered by Guo and Hill, when the authors identified all oxidoreductases as intrinsic or extrinsic enzymes [106]. In addition to the enzyme structure, which is the key parameter in the Guo and Hill classification, the mechanism of biocatalysis seems to be another very important factor for DET and especially bioelectrocatalytic properties of the enzymes, as described below.

3.4.1 Iron-Containing Enzymes

Non-elemental iron is the key part of Fe_xS_x-clusters and heme-groups of many oxidoreductases. One of the most interesting groups of heme-containing redox enzymes is the Cyt P450 super-family. These enzymes are able to catalyze O₂ reduction directly to H₂O, *viz.* Cyt P450 oxidases catalyze a monooxygenase reaction with quite complicat-

ed biocatalytic cycle mechanisms, *i.e.* insertion of one atom of oxygen into an organic substrate (RH), while the other oxygen atom is reduced to H₂O (Eq. 10):

To interface these enzymes to electrode surfaces and to electrochemically drive the catalytic cycle has proven to be a very difficult task. Omission of redox partners needed to simplify ET pathways, generally resulted in high heterogeneous ET rates but without success in terms of detectable substrate turnover [103]. To the contrary, addition of substrates to the Cyt P450/electrode electrochemical system increases the catalytic rate (k_{cat}) of O₂ electroreduction, shifts the potential in the anodic direction, but the total cathodic current drops [107]. Instead of a four-electron reduction of O₂ (Eq. 2), when immobilized on the electrode surface, cyt P450 catalyzes a two-electron reduction process (Eq. 1) [108,109]. Indeed, intermediates formed during electrocatalysis do not follow the natural biocatalytic cycle of the enzymes, and thus, efficient bioelectrocatalysis, needed to construct efficient biocathodes, could not be achieved.

3.4.2 Iron/Copper-Containing Enzymes

In addition to oxidoreductases containing a single metal ion and a single cofactor, there are complex enzymes with several metal ions and redox centers in their structures. An example of this is the unique iron/copper-containing super-family of proteins, terminal oxidases (TOxs). The enzymes are large transmembrane protein complexes found in bacteria and the mitochondria of eukaryotes, which catalyze the four-electron reduction of O₂ to H₂O (Eq. 2) coupled to generation of a proton electrochemical gradient across the membranes in which they are embedded. The simplified overall biocatalytic reaction is presented in Eq. 11.

Here, S_{red} and S_{ox} are reduced and oxidized substrates, respectively. For two main classes of TOxs, *viz.* Cyt *c* oxidase (Cyt *c* Ox, EC 1.9.3.1) and quinol oxidase (QOx, EC 1.10.3.11) oxidases), S_{red} is ferro-cyt *c* and quinol, respectively. Different types of TOxs, such as *aa3*, *ba3*, *bo3*, have been previously investigated electrochemically in detail [110–115]. Well-pronounced non-turnover signals for Cyt *c* Ox adsorbed on different electrodes were registered. The reported heterogeneous ET constants vary from 0.002 s^{−1} up to 277 s^{−1} depending on the first electron acceptor of the enzyme; heme *a*, heme *a*₃, or Cu_A [110–112].

In spite of the efficient electric connection between enzyme and electrode, and in certain cases, similar ET pathways for homogeneous and heterogeneous systems alike (in its native environment, *i.e.* the inner mitochondrial membrane, electrons from the natural electron

donor are transferred to the Cu_A center and further on to the other centers, *i.e.* heme *a*, heme *a*₃, and Cu_B site) [111], DET based bioelectrocatalytic reduction of O₂ on Cyt *c* Ox-modified electrodes was often not registered. Cyt *c* Ox is a special enzyme and its main physiological role is not O₂ reduction (Eq. 2), but rather the translocation of H⁺ against the proton electrochemical gradient, driven by the potential difference between Cyt *c* and O₂ (Eq. 13). Indeed, Cyt *c* Ox catalyzes bioelectroreduction of O₂ at high η [116], *viz.* from 0.4 V up to 0.8 V in the case of Cyt *c* Ox [113] and QOx [114], respectively, since the potentials of the redox centers of the enzyme are optimized to accept electrons from ferri-cyt *c*/ferro-cyt *c* and ubiquinone/ubiquinol couples ($E^{\text{th}} = 0.25$ V and 0.10 V vs. NHE at pH 7, respectively [3]). To conclude, TOx is not an optimal biocatalyst for O₂ electroreduction per se.

3.4.3 Copper-Containing Enzymes

The last group of enzymes, which indeed might be the most suitable bioelements in the design of efficient O₂ reducing enzymatic electrodes, is copper-containing enzymes, which can be simply classified based on the structure of their active sites as Cu-T2 (galactose oxidase), Cu-T3 (tyrosinase), and Cu-T1-T23 oxidoreductases [77]. DET electrochemistry of copper containing enzymes have been described in several reviews [76,77].

Galactose oxidase (*d*-galactose:oxygen 6-oxidoreductase, EC1.1.3.9) is a T2 copper-containing enzyme, which catalyzes the oxidation of *D*-galactose to *D*-galacto-hexodialdose with concomitant reduction of O₂ to H₂O₂. Despite the fact that the enzyme catalyzes O₂ reduction in accordance with Eq. 1, and the two-electron process seems to be easier realized compared to four-electron O₂ bioelectroreduction, attempts to obtain cathodic bioelectrocatalytic currents from galactose oxidase modified electrodes were unsuccessful [77]; apparently, the biocatalytic cycle requirement, *i.e.* formation of a special intermediate galactose adduct in order for O₂ to access the active site of the enzyme (see Scheme 1 in Ref. [117]) cannot be met upon immobilization.

Tyrosinase (monophenol, dihydroxyphenylalanine: dioxygen oxidoreductase, EC 1.14.18.1) is a copper oxidase, which carries a coupled binuclear Cu active site (the T3 site) [118]. This enzyme catalyzes the ortho-hydroxylation of monophenols (cresolase activity) and the two-electron oxidation of *ortho*-diphenols to *ortho*-quinones (catecholase activity). Both biochemical reactions are coupled to O₂ reduction to H₂O. However, acceleration of O₂ electroreduction to H₂O was never observed on tyrosinase modified electrodes, whereas DET reactions were clearly shown [119,120]. The absence of bioelectrocatalytic reduction of O₂, in all likelihood, reflects the complicated mechanism of the enzymatic process, where mono- or diphenol binding to the Cu-T3 center is critical for proper biocatalysis to occur (see Figure 10 in Ref. [118]).

Cu-T1-T23 oxidoreductases, or, in other words, blue multicopper oxidases (BMCOs), belong to a diverse

group of enzymes containing three copper sites, Cu-T1, Cu-T2, and Cu-T3, catalyzing the oxidation of organic and inorganic compounds with concomitant reduction of O_2 directly to H_2O , without formation of highly reactive oxygen species, see Eq. 12.

Most BMCOs (e.g. bilirubin oxidases (BOx) [121,122], plant and fungal [119], as well as bacterial [123,124] Lcs), when adsorbed on bare or modified electrodes, efficiently catalyze DET-based O_2 electroreduction to H_2O , however with a few exceptions. For instance, non-turnover signals for another BMCO, ceruloplasmin (Cp), were clearly observed indicating efficient DET, but pronounced bioelectrocatalytic currents for the enzyme were not registered [119]. Based on detailed investigations of the enzyme it was suggested that the absence of bioelectrocatalysis seems to be related to the ordered mechanism of Cp function [125]. Moreover, only very recently even MET based bioelectrocatalytic activity of this enzyme has been shown (Figure 9A) [126]. The authors attributed the presence of the ferrooxidative bioelectrocatalytic signal to the specific assembly of the paramagnetic human protein at electrode surfaces covered with ferromagnetic nanoparticles (Au/Fe@CNps electrodes) in the presence of a magnetic field. Our recent studies, however, showed much higher bioelectrocatalytic activity of human Cp, which was adsorbed on non-magnetic carbon nanotube modified electrodes (GR/CNTs electrodes) in the absence of a magnetic field (cf. Figures 9A and 9B).

Nevertheless, even when nanostructured electrodes and low redox potential mediators are used, the obtained current densities are only in the $\mu A cm^{-2}$ range. Thus, further investigations are needed to understand the electrocatalytic inertness of this interesting but by far the most complex blue oxidase, which, contrary to all other BMCOs, contains six copper ions because of three T1 Cu sites instead of just one.

The k_0 -values for BMCOs immobilized on different electrodes were calculated from experimental data, using both non-turnover and bioelectrocatalytic signals. The obtained heterogeneous ET constants vary significantly; from $0.4 s^{-1}$ for k_0 measured under anaerobic conditions [127] or in the presence of enzyme inhibitors [128] up to $3000 s^{-1}$ in the presence of an enzyme substrate, O_2 [129].

3.5 Mechanism of O_2 Bioelectroreduction by BMCOs

The mechanism of O_2 bioelectrocatalytic reduction by different BMCOs, e.g. BOx, plant, fungal, and bacterial Lc, was the subject of investigation in many studies [77,119,122,125,127,130–135]. The ET mechanism is schematically presented in Figure 10 using one of the most studied BMCOs, fungal *Trametes* Lc, as an example.

Briefly, the electrons are transferred from the electrode to the first electron acceptor, the T1 Cu site of the adsorbed BMCO [77,130,135,136]. The shortest distance

Fig. 9. CVs of different human Cp modified nanostructured electrodes immersed in $K_3[Fe(CN)_6]$ containing phosphate buffer in the absence (black curve) and presence (red curve) of O_2 . (A) Au/Fe@CNps; background-subtracted CVs recorded in the presence of a magnetic field (40 mT), (B) GR/CNTs; CVs recorded in the absence of a magnetic field.

Fig. 10. A schematic representation of bioelectrocatalysis at BMCO modified electrodes.

between the surface of the enzyme and the Cu-T1 site is less than 14 Å [137–140], and if properly oriented on the electrode surface, such a distance will allow efficient electron tunneling (Figure 10). The electron is then transferred through an intramolecular ET mechanism (IET) to the Cu-T23 cluster (the distance is *ca.* 13 Å for all BMCOs; Figure 10), where O_2 is reduced to H_2O in accordance with the half-reaction presented in Eq. 2.

The above mechanism of ET during bioelectroreduction of O_2 by BMCOs immobilized on different electrodes is widely accepted by the scientific community [77, 122, 130, 131, 133, 135]. However, other ET pathways for BMCO based heterogeneous systems were also proposed, *e.g.* the possibility to directly electrically address the Cu-T23 copper cluster of some enzymes [125, 127, 130, 141, 142]. In spite of the fact that this possibility is theoretically confirmed in recent crystallographic and electrochemical studies of a fungal BOx (both the T1 Cu site and Cu-T23 cluster lie sufficiently close (< 14 Å) to the surface of the enzyme for DET at biologically relevant rates) [140], additional electrochemical investigations with T1 Cu-depleted protein samples are obviously needed to prove or rule out this interesting hypothesis.

3.6 Mathematical Analysis of DET Based O_2 Bioelectroreduction

One of the best ways to mathematically analyze biocatalytic O_2 electroreductions is based on direct comparison of bioelectrocatalytic and electrocatalytic processes. It is appropriate to thoroughly discuss the similarities and differences in this context, starting from similarities and ending with significant differences. Logically following the electroreduction part of the review, we will begin the bioelectroreduction section with thermodynamics, briefly discussing certain peculiarities of biocatalysts used for ORR and H_2O electrooxidation.

A true catalyst has no influence on the direction of the process and should catalyze forward and reverse reactions equally well. Despite a huge size compared to conventional molecular and especially surface electrocatalysts, many oxidoreductases are excellent electrocatalysts, facilitating chemical transformations under almost reversible conditions [143]. Many complex, multicenter redox enzymes, *e.g.* hydrogenases [144] and dehydrogenases [145], show fully reversible electrochemistry. Even though most electrocatalysts, including biomolecules, operate preferentially in one direction because of secondary effects beyond the elementary catalytic cycle [143], O_2 reducing bioelectrocatalysts and especially BMCOs, seem to have a too steep “catalytic bias”, possibly due to an exceptionally complex catalytic cycle, which proceeds through the formation of many different intermediates to achieve four-electron reduction of O_2 [146]. Firstly, only one biocatalyst, as was shown just two years ago, is able to catalyze H_2O electrooxidation without assistance from sunlight, a BMCO-Lc [147]. Secondly, the O_2/H_2O electrochemical transformation catalyzed by Lc is far from being

reversible because of different mechanisms of ET during O_2 bioelectroreduction and H_2O bioelectrooxidation. Finally, despite an old report on diode-like behavior of succinate dehydrogenase [148] and old hypothesis of a possible occurrence of transistor-like behavior in redox enzymes [149], modulation of the activity of an enzyme by means of an electrical potential, *i.e.* transistor-like behavior, has been only recently shown using an O_2 reducing multi-center redox enzyme; not surprisingly this enzyme was Lc [141]. Because of the dedication of this work, the best way to conclude the “thermodynamic part” is to repeat the title of *Prof. Schuhmann's* recent review with just a small modification: “ O_2/H_2O electrocatalysis and bioelectrocatalysis – Distinction without a difference”.

As for the kinetics, the first and actually the last complete resemblance between “bio” and “non-bio” processes is the diffusion limited regime, when the maximal i_{diff} values are similar (step 1 in Figures 2 and 11).

In this case theoretical current densities can be calculated from Eq. 5 and the maximal values highly depend on the rotation of the (bio)devices (Figure 12).

Some convection of the solution at motionless (bio)-cathodes still occurs, due to natural convection originating from the build up of density gradients and building vibrations (which has a major frequency usually below 1 Hz). Nevertheless, maximal i_{diff} values are close or below 0.1 mA cm^{-2} in air saturated electrolytes, and they will never exceed 0.5 mA cm^{-2} , even when O_2 saturated solutions are used. These theoretical calculations are in good agreement with experimentally obtained current densities from motionless Pt and BMCO modified electrodes in the O_2 mass transfer limitation regime (*cf.*

Fig. 11. Schematic representation of the mechanism of a bioelectrocatalytic reaction at a biocathode.

Fig. 12. Dependences of the limiting diffusion currents on the square root of electrode rotation in inverse coordinates.

Figures 14B and 14B, *vide infra*). For practical applications, rotation of biocathodes is in general a nonsensical idea, whereas biodevices based on GDE [150–154] with experimentally obtained current densities up to 10 mA cm⁻² are more suitable.

The first significant difference, or, following the recent review [155], the first significant distinction between “bio” and “non-bio” processes, reflects the electron exchange properties for both systems. Even the quite low k_0 -values calculated for certain biocatalysts, e.g. a few s⁻¹, are still many orders of magnitude higher than those for inorganic and organic catalysts (*vide supra*). Moreover, most oxidoreductases are highly active; almost 40 years ago it was shown that k_0 -values for the majority of the enzymes lies in the range of 10–1000 s⁻¹, when enzymatic activities were assayed in homogeneous solutions [156]. The quite low constants often arrived at in electrochemical systems might just reflect conformational changes of surface confined proteins, with partial or even complete deactivation of the biocatalysts. For instance, it was shown that the main reason for the deactivation of BMCO-modified Au electrodes is enzyme flattening on the bare Au surface [157], which results in relatively low apparent k_{cat} -values (in the case of BOx) or complete loss of enzymatic activity (in the case of Lc). However, when Lc was immobilized on specifically covered surfaces in order to protect the protein from the metal, high k_0 -values of up to 3000 s⁻¹ were realized [129,158]. Indeed, theoretical i_0 values calculated for a planar Pt ($k_0 = 1.2 \cdot 10^{-5}$ s⁻¹) cathode are more than three orders of magnitude lower (ca. 1 μA cm⁻²) compared to enzyme based devices (up to 3 mA cm⁻²), assuming a monolayer coverage of an average enzyme with MW about 60 kDa and Γ around 5 pmol cm⁻² with k_0 -value equal to 3000 s⁻¹. Thus, high redox potential BMCOs are theoretically much more efficient catalysts for ORR than Pt. This is in good agreement with the previously made conclusion that “the efficiency of bioelectrocatalytic reactions is more often limited not by the intrinsic activity of the active sites but

by their inaccessibility for electric coupling to the electrode and for mass transport” [155].

To avoid any confusion one should immediately emphasize a second principal difference between “bio” and “non-bio” processes. While a k_0 -value during bioelectroreduction describes a heterogeneous ET rate between biocatalyst and electrode surface, but not bioelectrocatalysis as such, k_0 is a direct measure of the electrocatalytic activity of an electrocatalyst, *vide supra* (also cf. Figures 2 and 11 and note the absence of step 3 in Figure 2). Thus, when it comes to simple electrocatalytic systems for O₂ reduction, the theoretical i_{cat} values are usually calculated based on the Butler-Volmer equation, in which k_{cat} does not appear, being “substituted” with k_0 (Eq. 7). It was also shown that DET reactions for simple redox proteins could be well-described by both Butler-Volmer theory, using an equation adjusted to bioelectrochemical systems (Eqs. 13 and 14), and Marcus ET theory [159]. However, ET in bioelectrocatalytic systems based on complex redox enzymes, which contain several redox centers (marked as A and B in Figure 11), is a very complicated process (cf. Figures 2 and 11), and, since the Butler-Volmer model is more empirical than Marcus theory, the latter should be used for k_{ET} calculations (Eqs. 13 and 15),

$$i_{ET} = nFA_{real}\Gamma k_{ET} \quad (13)$$

Here i_{ET} is the ET limiting current; A_{real} is the real electrode area, Γ is the surface concentration of adsorbed biocatalysts, k_{ET} is the rate of ET (Eqs. 14 and 15),

$$k_{ET} = k_o \exp\left(\frac{an_L F(E^0 - E)}{RT}\right) \quad (14)$$

or

$$k_{ET} = \sum_i \frac{1}{\exp(\pm(E - E_i)F/RT) + 1} \cdot \frac{4\pi^2 V_0^2 e^{-\beta r}}{N_A h} \cdot \frac{\exp\left(-\frac{(\pm F(E_i - E^0) + \lambda)^2}{4\lambda RT}\right)}{\sqrt{4\pi\lambda RT}}, \quad (15)$$

where E – the applied potential, E^0 – the formal reduction potential of the adsorbed couple, E_i – the potential of a specific Fermi level, N_A – Avogadro number, h – Plank constant, V_0 – the degree of electronic coupling, β – the decay coefficient, r – the distance between centers, and λ – the reorganization energy.

Third, for O₂ biocatalysts i_{cat} might reflect the “true” catalytic activity of the enzyme, mathematically expressed via the Michaelis-Menten kinetics adjusted to electrochemical systems (Eq. 16).

$$i_{cat} = nFA_{real}\Gamma k_{cat} C_{O_2} / (C_{O_2} + K_M) \quad (16)$$

Here, i_{cat} is the catalytic limiting current, C_{O_2} is the bulk concentration of O₂, and K_M is the Michaelis constant of the enzyme with respect to the biooxidant.

Last but not least, if i_{cat} limits the overall bioelectrocatalytic output (step 2, Figure 11), limiting currents will be potential independent. However, very often in enzymatic bioelectrocatalysis, when significant diffusion limitations are overcome by rotating the electrode, CVs might not reach a plateau and the bioelectrocatalytic currents strongly depend on the potential applied to enzyme-modified electrodes (cf. slopes of CVs in Figures 13A and 14A, *vide infra*). This behavior reflects heterogeneous ET limitations in bioelectrocatalytic systems in a broad potential range because of dispersion of interfacial ET rates due to random enzyme orientation on the electrode surface [160].

In summary, to the first approximation, the general mechanism of a bioelectrocatalytic reaction can be divided into three main steps, *viz.* the ET reaction (in this case the catalytic current will be equal to i_{ET} , Eq. 13), the biocatalytic reaction within the enzyme (i_{cat} , Eq. 16), and the diffusion of the substrate to the electrode surface (i_{diff} , Eq. 5, if a rotating disk electrode is used). Under steady state conditions, such a mechanism gives rise to Eq. 17, which is analogous to an expression giving the resultant conductance of three resistors connected in series (cf. Eqs. 8 and 17) [145].

$$1/i = 1/i_{ET} + 1/i_{cat} + 1/i_{diff} \quad (17)$$

One should note, however, that up to date no realistic model indicates that the current can actually be written as the sum of the reciprocal of i_{ET} , i_{cat} and i_{diff} [143,161,162].

3.7 Direct Experimental Comparison of Pt and BMCO Based Cathodes

To the best of our knowledge the only direct comparison of a Pt electrode and a DET-based oxidoreductase-modified cathode was carried out using just one redox enzyme, BOx [135]. Nevertheless, in a single, direct experiment it was substantiated that the minimum η required for O_2 reduction by the enzyme is significantly smaller than that required at Pt. Below we provide direct experimental comparison of Pt and different BMCO-modified electrodes, arriving at a just slightly different conclusion.

Typical CVs of ORR on Pt and BMCO-modified disk electrodes are presented in Figures 13 and 14, respectively. As one can see from Figure 13, Pt is an excellent catalyst for ORR in acidic media, whereas at neutral pH O_2 electroreduction starts at around 0.5 V vs. NHE only, *i.e.* η for ORR is higher than 0.3 V. It should be also noted that η -values for O_2 bioelectroreduction strongly depend on the type of biocatalyst used (Figure 14 and Table 1).

Thus, low redox potential BMCOs (for details concerning redox potentials of BMCOs see Refs. [130,163]) in general, and plant Lcs in particular (Figure 14), decrease η much less efficiently compared to Pt and especially high redox potential enzymes, such as fungal Lc and BOx (Table 1). The redox centers of low potential BMCOs

Fig. 13. CVs of a Pt electrode in air saturated 0.1 M universal buffer at different pH values: 3.0 (black curve), 5.0 (red curve), 7.0 (blue curve), 9.0 (purple curve). A and B – electrodes rotated at 200 rad s^{-1} and motionless electrodes, respectively. Scan rate – 5 mV s^{-1} .

(plant and bacterial Lc) responsible for substrate oxidation, which are also the first electron acceptors during bioelectrocatalysis (*vide supra*), are optimized to accept electrons from quite low redox potential compounds [164–166], since these enzymes participate in lignin transformation and pigment syntheses [118]. In contrast, high potential BMCOs (fungal Lc and BOx) are built as strong oxidizers to participate in complicated natural processes, *e.g.* lignin biodegradation [167] and bilirubin oxidation [146]. Thus, it is not surprising that high potential BMCOs reduce O_2 at high redox potentials.

On the other hand, one can clearly see that a plant Lc (RvLc) has the highest difference between measured OCP values and E_{T1} (Table 1). In other words, in spite of the low value of the redox potential of the first electron acceptor compared to other enzymes (Figure 14), the plant Lc still polarizes the electrode in a surprisingly efficient way, even though the final OCP is slightly lower than that for Pt.

Contrary to the Cu-T1 centers of BMCOs, which are built to provide substrate specificity for these enzymes [164,166], the Cu-T23 cluster of both low and high redox potential BMCOs is designed to efficiently reduce O_2 , the only known electron acceptor for these enzymes so far

Fig. 14. CVs of BMCO-modified carbon electrodes. Green solid curves – *Rhus vernicifera* plant Lc (RvLc); Red curves – *Myrothecium verrucaria* fungal BOx (MvBOx); Blue curves – *Trametes hirsuta* fungal Lc (ThLc). A and B – electrodes rotated at 200 rad s⁻¹ and motionless electrodes, respectively. Conditions: air saturated 0.1 M universal buffer pH 5.0, scan rate – 5 mV s⁻¹, CVs were recorded starting from OCP potentials of enzyme-modified electrodes; potentials are given vs. NHE; black dotted curves – bare electrodes with ultrathin layers of a carbon material (about 1 μm) fabricated in accordance to Ref. [168].

[118,169]. Indeed, the structure of this Cu center is conserved among all MCOs: 8 histidines acting as ligands for three Cu ions (two from Cu-T3 and one from Cu-T2 sites) combine into the unique Cu cluster [137–140]. One can assume that at least some intermediates of the Cu-T23 cluster have high redox potentials in all BMCOs [146]. Thus, one can hypothesize that for low redox potential enzymes, like plant Lcs, E_{T1} might be much higher compared to E_{T1} , ensuring a high OCP despite quite low

E_{T1} . Nevertheless, high redox potential BMCOs seem to be the best cathodic bioelements for the design of high performance DET based enzymatic electrodes. Of course, when taking into account huge differences in the roughness factor (two orders of magnitude) between a polished Pt disc electrode (Figure 13) and high surface area carbon electrodes with immobilized BMCO (Figure 14), one understands that electrocatalytic current densities from bioelectrodes are much smaller compared to the signal from a metal catalyst based cathode. However, η for ORR on Pt is still much higher, especially in neutral and acidic electrolytes. Moreover, in complex (containing different anions, which inhibit the ORR (*vide supra*), and also low potential electrochemically active compounds, like ascorbate, urate, etc.) non-acidic electrolytes, e.g. human blood, BOx based biocathodes clearly outperform a Pt electrode (Figure 15). Thus, at least for certain applications, e.g. devices operating in human physiological fluids, carbon electrodes modified with high redox potential BMCOs might be the only option, especially if metal protection with a membrane is technologically challenging.

Fig. 15. CVs (first scan) of a Pt electrode (black curve) and a BOx modified graphite electrode (red curve) submerged in air saturated human blood (scan rate – 50 mV s⁻¹). The current density was calculated per electrochemically active surface area taking into account electrode porosity.

4 Conclusions and Perspectives

In this review, the importance of the ORR has been highlighted, as it is the key reaction not only in many natural

Table 1. Comparison between open circuit potentials (OCP) of Pt and BMCO modified electrodes at pH 5, redox potentials of the appropriate Cu-T1 site (E_{T1}), and overpotentials of O₂ (bio)electroreduction (η).

Catalyst	OCP	E_{T1}	Δ (OCP- E_{T1})	η
Pt (pH 5)	720	–	–	210
RvLc	700	470	230	230
MvBOx	850	710	140	80
ThLc	870	800	70	60

Note: all potentials are given in mV vs. NHE.

and artificial systems, but also in many living organisms. The overall complexity of O_2 reduction has inspired a significant amount of research, allowing us to get a better understanding on how it proceeds under different conditions. O_2 electroreduction in artificial systems is accelerated by simple and complex, inorganic and organic, non-biological and biological catalysts. A widely-held notion in the “ORR community” is that among all possible catalysts, Pt is the best one. Our calculations clearly show that certain biocatalysts, e.g. BMCO, at least theoretically, outperform Pt by several orders of magnitude. Moreover, our direct experimental results, as well as available data in the literature, also show that η for ORR on BMCO-modified electrodes is lower compared to Pt, even under conditions, which are quite optimal for the metal catalyst, i.e. acidic electrolytes. Furthermore, our direct measurements show that in complex solutions, like human physiological fluids, the performance of an unprotected Pt electrode is inadequate, whereas an unprotected redox enzyme based electrode catalyzes O_2 electroreduction at low η , generating respectable current densities. We also find that both processes, electroreduction and bioelectroreduction, are restricted by mass transfer limitations, when high performance (bio)electrodes are used. O_2 is inexpensive and readily available, but its concentration in solutions is relatively low. In fact, even when relying on GDEs, the O_2 concentration in air is insufficient, when compared to the maximal concentrations of other possible oxidants in the accompanying electrolytes.

Detailed knowledge of the interrelationships linking electroreduction and bioelectroreduction of O_2 “is essential for enabling a deeper understanding of nature’s bioelectrochemical reactions, and for insightful design of functional catalysts inspired by models from living nature” [155]. During operation, O_2 reducing catalysts “are constrained under thermodynamic non-equilibrium” [155]. Thus, they are “inherently and unavoidably unstable during operation conditions” [155]. Hence, here is another widely-held notion that complex organic and especially biological catalysts are intrinsically unstable. Even though this paper only slightly touches stability issues, being mostly focused on the activity of biological catalysts, while working on the review we realized that seriously limited intrinsic operational stability of biocatalysts might be just fictitious. Microbial electrodes are stable for years because of cell proliferation and growth. Moreover, our literature searches revealed a complete absence of reports, when the active life time of a redox enzyme was investigated, under conditions to address the total possible turnover numbers of a biocatalyst. In other words, previous studies regarding operational stabilities of enzymatic cathodes in general and BMCO-modified electrodes in particular (cf. one among several comprehensive studies in the field, i.e. [170]) were done under conditions, when other factors seriously affecting the operational life-time, such as time dependent deactivation of a surface confined biocatalyst (usually explained in a trivial way using an ambiguous wording such as “conformational changes”),

proteolysis, and many more, could not be disregarded. Apparently, no one precisely knows for how long a redox enzyme will be catalytically active under ideal conditions, which is the maximal number of catalytic cycles (if this number actually exists) for a particular enzyme, etc. We hope that this review will be the launching point of more sophisticated investigations of biological catalysts, including but not limited to O_2 electroreduction, to answer the scientifically intriguing and technologically important questions emphasized above.

Abbreviations

η	overpotential
BMCO	blue multicopper oxidase
Box	bilirubin oxidase
CNT	carbon nanotube
Cp	ceruloplasmin
CV	cyclic voltammogram
Cyt	cytochrome
Cyt c Ox	cytochrome c oxidase
DET	direct electron transfer
ET	electron transfer
GDE	gas-diffusion electrodes
GR	graphite rod
IET	intramolecular electron transfer
k_0	standard heterogeneous electron transfer rate constant
k_{cat}	catalytic constant
Lc	laccase
MFC	microbial fuel cell
MET	mediated electron transfer
NHE	normal hydrogen electrode
NP	nanoparticle
ORR	oxygen reduction reaction
QOx	quinol oxidase
TOx	terminal oxidase

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III

Increasing Redox Potential, Redox Mediator Activity, and Stability in a Fungal Laccase by Computer-Guided Mutagenesis and Directed Evolution

Ivan Mateljak,[†] Emanuele Monza,^{‡,§} Maria Fatima Lucas,^{‡,§} Victor Guallar,^{*,‡,||} Olga Aleksejeva,[‡] Roland Ludwig,[#] Donal Leech,[∇] Sergey Shleev,[‡] and Miguel Alcalde^{*,†,||}

[†]Department of Biocatalysis, Institute of Catalysis, CSIC, Cantoblanco, 28094 Madrid, Spain

[‡]Barcelona Supercomputing Center, Jordi Girona 29, 08034 Barcelona, Spain

[§]Zymvol, C/Almogavars 165, 08018 Barcelona, Spain

^{||}ICREA: Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys 23, 08010 Barcelona, Spain

[‡]Biomedical Sciences, Health and Society, Malmö University, 20560 Malmö, Sweden

[#]Department of Food Sciences and Technology, VIBT—Vienna Institute of Biotechnology, BOKU—University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria

[∇]Department of Chemistry, National University of Ireland, Galway University Road, SW4 794 Galway, Ireland

Supporting Information

ABSTRACT: Fungal high-redox-potential laccases (HRPLs) are multicopper oxidases with a relaxed substrate specificity that is highly dependent on their binding affinity and redox potential of the T1Cu site (E_{T1}). In this study, we combined computational design with directed evolution to tailor an HRPL variant with increased E_{T1} and activity toward high-redox-potential mediators as well as enhanced stability. Laccase mutant libraries were screened *in vitro* using synthetic high-redox-potential mediators with different oxidation routes and chemical natures, while computer-aided evolution experiments were run in parallel to guide benchtop mutagenesis, without compromising protein stability. Through this strategy, the E_{T1} of the evolved HRPL increased from 740 to 790 mV, with a concomitant improvement in thermal and acidic pH stability. The kinetic constants for high-redox-potential mediators were markedly improved and were then successfully tested within laccase mediator systems (LMSs). Two hydrophobic substitutions surrounding the T1Cu site appeared to underlie these effects, and they were rationalized at the atomic level. Together, this study represents a proof-of-concept of the joint elevation of the E_{T1} , redox mediator activity, and stability in an HRPL, making this versatile biocatalyst a promising candidate for future LMS applications and for the development of bioelectrochemical devices.

KEYWORDS: laccase, redox potential, redox mediator, stability, directed evolution, computer-aided design

INTRODUCTION

Laccases (E.C. 1.10.3.2) are multicopper oxidases that catalyze the one-electron oxidation of a broad range of compounds, a process coupled to the four-electron reduction of molecular oxygen to water. The enzyme is organized into two catalytic copper centers: the T1Cu site, where the substrate is oxidized, and the trinuclear Cu cluster—consisting of one T2 and two T3 coppers—where the reduction of molecular oxygen takes place.^{1,2} Predominantly distributed in higher plants, bacteria and fungi, laccases are classified for bioelectrochemical applications as low-, medium-, and high-redox-potential enzymes. This classification is based on the equilibrium potentiometric titrations of the redox potential at the T1Cu site (E_{T1}), ranging roughly from 0.43 to 0.79 V in reference to

the NHE (normal hydrogen electrode).^{3–5} The T1Cu site has a three coordinated trigonal planar structure in proteins with axial noncoordinating residues Phe or Leu (medium- and high-redox-potential laccases) and a four coordinated trigonal elongated tetrahedral geometry in proteins with axial coordinating Met (low-redox-potential laccases from bacteria and plants). Moreover, this mononuclear copper site is wired to the T2/T3 Cu cluster by a His–Cys–His tripeptide at a distance of ca. 12 Å.^{1,6–8}

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High-redox-potential laccases (HRPLs) secreted by basidiomycete white rot fungi are particularly relevant in a biotechnological context given their capacity to oxidize compounds with a higher redox potential that cannot be transformed by their medium- and low-redox-potential counterparts.^{2,9} In addition, HRPLs can be combined with redox mediators that act as diffusible electron shuttles between the enzyme and the oxidizable compound in the so-called laccase mediator systems (LMSs), thereby expanding the laccase substrate portfolio.^{10–12} As such, HRPLs have applications in bioremediation, synthetic chemistry, pulp-biobleaching, food, and textile industries.^{9,13–15} In addition, a high E_{T1} offers an attractive set of advantages for engineering biodevices (biosensors and high-redox-potential enzymatic cathodes of biofuel cells), allowing the laccase to accept electrons directly from the cathode, to generate higher current densities, and to lower the overpotential for O_2 reduction.^{4,16} Generally, electron transfer from the substrate to the T1Cu is the rate-limiting step in laccase catalysis, highlighting the difference between the redox potential of the substrate and the T1Cu as the main driving force for the reaction, together with the substrate binding affinity.^{6,17–22}

The last couple of decades have witnessed an intense race to discover the factors responsible for the striking differences in E_{T1} between laccases, mainly by focusing rational engineering enterprises on the coordination sphere of the T1Cu and its surroundings.² Modification of the E_{T1} without endangering the enzyme's activity and stability has been pursued intensively by many laboratories around the world (including ours), albeit with little success. For example, mutagenesis studies were carried out to elucidate the effect of the axial ligand on E_{T1} . Accordingly, the mutations Phe463Met in *Trametes villosa* laccase and Leu499Met in *Botrytis aclada* laccase were shown to provoke a decrease in the redox potential of approximately 0.1 V.^{19,23} By contrast, the Met502Leu substitution in the bacterial CoT₁ laccase increased the E_{T1} by 0.1 V, albeit at the expense of thermodynamic stability and poorer catalytic rates for all substrates tested.⁸ Alternatively, mutating the axial ligand from Leu to Phe in the low-redox-potential *Myceliophthora thermophila* laccase (MtL) did not produce significant changes in its enzymatic properties, including its redox potential, meaning that the nature of the axial ligand is not the only factor that affects E_{T1} .⁶ The highly conserved tripeptide preceding the axial ligand Leu–Glu–Ala/Val–Ser–Gly (for high- and low-redox-potential laccases, respectively) was also switched between high- and low-redox-potential laccases in the same study. While this modification significantly altered the laccases' enzymatic properties, the E_{T1} remained unaffected. We later studied the complete VSG tripeptide in MtL by combinatorial saturation mutagenesis, and again the E_{T1} was not modified.²⁴ Laccase's E_{T1} is regulated by many factors including the hydrophobicity in the milieu around the T1Cu site and its particular coordinating geometry as well as hydrogen bonding, stacking and electrostatic interactions in the protein backbone, and restrictions in protein folding, such that any subtle modification of the E_{T1} can disrupt the protein's normal functioning.^{1,25–28} Indeed, laccase-based catalysis is not fully understood yet since the process is complex and includes not only substrate oxidation at the T1Cu site but also oxygen reduction, along with intramolecular electron transfer (IET) between the T1Cu site and T2/T3 cluster. As the target value of E_{T1} may also influence the rate of IET, a further increase in the E_{T1} may

result in a sluggish uphill IET, jeopardizing the overall biocatalytic reaction. Therefore, understanding how to modify the physicochemical features that influence the E_{T1} of HRPLs without compromising their enzymatic activity and stability while defining the structural determinants behind such alterations remains a challenge, a holy grail for laccase engineers.

Here, we have increased the E_{T1} of an HRPL, its activity toward high-redox-potential mediators, and its stability, by running both computer-aided evolution and *in vitro* directed evolution campaigns in parallel. A reliable high-throughput screening assay that combined two high-redox-potential mediators was adapted to screen *in vitro* mutant libraries toward a higher E_{T1} , while maintaining high activity and stability. After several rounds of laboratory evolution assisted by computationally guided mutagenesis, the final HRPL variant was biochemically, spectroelectrochemically, and computationally characterized at the atomic level. These findings are, to the best of our knowledge, the first experimental evidence of an enhancement in the E_{T1} of an HRPL in conjunction with an improvement in its activity on high-redox-potential mediators and of its stability to temperature and acidic pHs.

■ RESULTS AND DISCUSSION

Computationally Guided Mutagenesis and Directed Evolution. The departure point in this study was an evolved laccase from basidiomycete PM1 that was isolated in the western Mediterranean region and that belongs to a group of HRPLs with strong thermostability. We previously submitted this HRPL to directed evolution to improve its functional expression in yeast, yielding the OB-1 variant used in the present study²⁹ (Figure S1). In this new directed evolution campaign, a high-throughput assay was developed on the basis of the oxidation of an inorganic transition metal complex, the redox mediator potassium octacyanomolybdate IV ($K_4Mo(CN)_8$, $E = 0.78$ V versus NHE), to screen OB-1 mutant libraries for a higher E_{T1} . To avoid a substrate-dependent bias during screening, the assay was complemented with two colorimetric methods based on the oxidation of other synthetic mediators that acted as positive and negative selection controls, respectively: violuric acid, $E = 0.92$ V versus NHE; and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), $E = 0.69$ V versus NHE.³⁰ It is worth noting that violuric acid is a N–OH mediator that follows the HAT (hydrogen atom transfer) mechanism, whereas $K_4Mo(CN)_8$, like ABTS, follows the ET (electron transfer) route yet without forming radicals since its electron exchange is centered on the metallic atom of the complex that contributes to high stability in both oxidation states.¹² Screening assays were validated by establishing a coefficient of variance (16%) and a reliable response, with high linearity from yeast supernatants produced in microcultures (i.e., 96-well plate format; Figure S2). Two consecutive rescreens were set up to limit the selection of false positives.

The whole evolution strategy is summarized in Figure S3. After applying distinct mutational loads and using polymerases with different biases (Figure S4), three independent mutant libraries were constructed and explored (~2400 clones were screened). From this round of evolution, the L3G3 mutant (with Ala162Pro substitution) was identified that displayed a 1.7- and 1.3-fold improvement over the parental OB-1 for

Figure 1. Spectroelectrochemical characterization. (A, B) Potentiometric titration curves: 1, reductive titration; 2, oxidative titration; for the parental OB-1 and the GreeDo mutant, respectively. (C, D) Spectra from the reductive titration, corresponding to the oxidized enzyme (1.0 V), the partly oxidized protein (0.8 and 0.75 V), and the fully reduced laccase (0.5 V), for the parental OB-1 and GreeDo mutant, respectively. Inset: typical Nernst plot of the dependence of the applied potential on the absorbance at 600 nm and the average parameters calculated from the titrations. E_{m6} , midpoint redox potential; b , slope of the titration curve, determined from linear fit; n , number of electrons involved in redox reactions.

$K_4Mo(CN)_8$ and violuric acid, respectively, while its activity on ABTS was reduced by ~20%.

With the aim of retrieving cooperative mutations, in parallel to the *in vitro* evolution experiment, we ran a new theoretical aided evolution approach. This computational method is based on ranking sequence positions instead of point mutations, ordering them according to the frequency of beneficial mutations. False positives are not filtered out since a position is chosen rather than a given mutation. We run this protocol based on violuric acid docking on the laccase model. For this *in silico* round, over 40 positions, all within 10 Å from the substrate, were screened by molecular modeling, employing PELE (Protein Energy Landscape Exploration), a Monte Carlo algorithm capable of effectively sampling the protein–ligand conformational space.³¹ The screening process was based on identifying those positions that (after mutation) improved the protein–ligand interaction energy when using an oxidized substrate as a probe, in our case oxidized violuric acid (Figure S5). Thus, by enhancing the protein–product interaction, we aim to improve the thermodynamics of the reaction by helping to reduce the electron transfer transition state barrier (following Hammond's postulate). As a result, nine positions were identified. Importantly, one of the top 5 positions agreed with position 162 (ranking 3), which had been identified by the experimental parallel study, in an L3G3 variant (with Ala162Pro mutation). In addition, we selected position 458 (ranking 1) that had more beneficial (stabilizing) substitutions.

These two positions (Ala162 and Ala458) were then subjected to experimental combinatorial saturation muta-

genesis, which generated a double mutant, Ala162Val–Ala458Leu (referred to as GreeDo) with a 2.5- and 4.8-fold improvement in activity toward $K_4Mo(CN)_8$ and violuric acid, respectively, and only a minor decrease in its activity on ABTS (~0.8-fold). Other double variants were identified in the same screening with slightly milder improvements in activity, for example, Ala162Asn–Ala458Arg with 2.3-, 1.5-, and 0.9-fold improvements in activity on $K_4Mo(CN)_8$, violuric acid, and ABTS, respectively; and Ala162Ile–Ala458Leu with 1.8-, 3.7-, and 0.7-fold improvements, respectively, although they were not further characterized given their low stability (Figure S3). Given that GreeDo outperformed these counterpart double mutants in terms of activities and stabilities, we centered the ensuing evolution on this template. First, we constructed independent saturation mutagenesis libraries on Pro160 and Ile452. Position 160 (ranking 4), belonging to the loop 159–164 (Figures S3 and S5), was identified by PELE. By contrast, Ile452 was not unveiled by PELE, but it was selected for mutagenesis due to its proximity to the T1Cu (3.5 Å) and to the sulfur atom of the coordinating Cys450 (4.0 Å). Unfortunately, after exploring both positions, we did not find any positive variant. Thereafter, we applied focused random mutagenesis and DNA recombination by MORPHING within a specific protein segment in the vicinity of the T1Cu (Leu383–Thr461).³² The Leu383–Thr461 segment contains loop 388–389 identified through *in silico* experiments (Figure S5) and also includes an α helix in the vicinity of T1Cu where the 456Leu–Glu–Ala458 tripeptide is located, which is a highly conserved motif of HRPLs. However, this set of

experiments did not yield any further improvement. Accordingly, after screening ~6000 clones in several rounds of evolution, the parental OB-1 and the GreeDo mutant were produced, purified to homogeneity, and characterized thoroughly.

Spectroelectrochemical, Biochemical, and Computational Characterization. *Spectroelectrochemical Studies.* To assess the change in the redox potential produced by directed evolution, the parental OB-1 and the GreeDo laccase variant were characterized electrochemically by open-circuit potential measurements combined with mediated spectroelectrochemical redox titrations, using the protocol developed previously.^{33,34} First, open-circuit potentials (OCPs) of laccase-modified graphite electrodes were measured (Table S1 and Figure S6). The OCP of the GreeDo mutant increased over 30 mV compared to that of OB-1 laccase (from 0.76 up to 0.79 V). Taking into account the equilibrium redox potential of the oxygen/water couple equal to 0.87 V at pH 6.0, 25 °C, the OCP value of 0.79 V is extraordinarily high since it implies only a 0.08 V overpotential (η) for the oxygen reduction reaction. Moreover, OCP and η values of GreeDo-modified graphite electrodes are identical to or even higher than these potentials measured for laccase-modified graphite electrodes using enzymes with the highest E_{T1} reported to date.⁴ Second, E_{T1} values of both enzymes were accurately determined. The E_{T1} of the GreeDo mutant increased over 50 mV compared to that of OB-1 laccase (from 0.74 to 0.79 V; Figure 1A–D, Table S2). The engineering deployed in our studies allowed us to artificially match the highest redox potential of the T1 Cu site, i.e., close to 0.8 V versus NHE at pH 5–7, which does exist in some native laccases.¹⁹ On one hand, this “natural” value seems to stand as a limit to the enzyme evolution, when it comes to E_{T1} . On the other hand, taking into account the equilibrium redox potential of the oxygen/water couple, viz. 0.88 V at pH 6 with 59 mV/pH dependence and only a slight pH dependence of E_{T1} , i.e., 10–25 mV/pH, a further enhancement of E_{T1} of multicopper oxidases (MCOs) might still be doable.^{35–37} Furthermore, encouraging results related to azurin, a single copper protein, were reported recently, where the authors achieved fine-tuning of E that spanned the entire range of physiological E from +0.970 to –0.954 V versus NHE, exceeding the E of natural mononuclear cupredoxin by ~0.3 V, opening the possibility that these results could be translated in multicopper proteins.³⁸ Nevertheless, to the best of our knowledge, this is the first time that the E_{T1} in an HRPL is enhanced while the absence of mutation at the axial ligand, the most common strategy used to modify E_{T1} , supports the assumption that other structural determinants underlie the modification in laccase redox potential.^{6,8,19,23}

Biochemical Characterization. Kinetic constants were assessed with the redox mediators used during screening ($K_4Mo(CN)_8$, violuric acid, and ABTS). Moreover, 2,6-dimethoxy phenol (DMP, $E = 0.58$ mV versus NHE) was included in the measurements as a standard phenolic laccase substrate.³⁹ The catalytic efficiency (k_{cat}/K_m) of GreeDo was higher than that of the parental OB-1 for both high-redox-potential mediators tested, indicating that the increased E_{T1} is at least partially responsible for the improved kinetics with high-redox-potential mediators (Table 1). Indeed, the enhanced kinetic constants of GreeDo for $K_4Mo(CN)_8$ were striking, with a 7.5-fold improvement in catalytic efficiency. The very high redox potential of violuric acid makes oxidation by laccase thermodynamically unfavorable, and thus, the

Table 1. Kinetic Constants^a of OB-1 and GreeDo Variants at pH 4.0

substrate	kinetics constants	OB-1	GreeDo
$K_4Mo(CN)_8$	K_m (mM)	1.02 ± 0.11	0.20 ± 0.01
	k_{cat} (s^{-1})	143 ± 28	209 ± 11
	k_{cat}/K_m ($mM^{-1} s^{-1}$)	140	1045
violuric acid	K_m (mM)	16.3 ± 2.9	18.0 ± 4.5
	k_{cat} (s^{-1})	20.3 ± 0.9	35.1 ± 2.6
	k_{cat}/K_m ($mM^{-1} s^{-1}$)	1.25	1.95
ABTS	K_m (mM)	0.014 ± 0.001	0.027 ± 0.003
	k_{cat} (s^{-1})	455 ± 10	384 ± 11
	k_{cat}/K_m ($mM^{-1} s^{-1}$)	32 734	14 222
DMP	K_m (mM)	0.205 ± 0.010	0.141 ± 0.008
	k_{cat} (s^{-1})	250 ± 5	172 ± 2
	k_{cat}/K_m ($mM^{-1} s^{-1}$)	1250	1229

^aReactions were performed in triplicate, and substrate oxidations were followed through spectrophotometric changes. To calculate the K_m and k_{cat} values, the average V_{max} was represented against substrate concentration and fitted to a single rectangular hyperbolic function with SigmaPlot 10.0.

kinetic parameters are several orders of magnitude lower than those of $K_4Mo(CN)_8$. Nevertheless, GreeDo showed a 1.7-fold increased k_{cat} for violuric acid, although during screening we observed a 4.8-fold activity improvement. Leaving aside possible differences in the activity values between purified enzymes and supernatants (whose measurements can be affected by the media background, cell growth, and secretion levels), the screening assay for violuric acid was performed at pH 4.0 over 20 h. Hence, we suspected that these discrepancies in activity could also be explained by a higher pH stability at the acidic range for GreeDo (see the *Biochemical Characterization* section below). The k_{cat}/K_m for DMP was maintained, whereas there was a 2.3-fold drop for ABTS. This decrease probably reflects unfavorable binding of the substrate, given that the introduction of two more hydrophobic residues into the binding pocket might hinder the accommodation of the bulky ABTS molecule (see the computational analysis below).

The majority of high-redox-potential mediators are poor laccase substrates because the reaction is not thermodynamically favorable, particularly due to the differences between the redox potential of the mediator and the T1Cu site.^{11,12} Accordingly, the activity of the GreeDo mutant on high-redox-potential mediators was further tested by preparing several LMSs for the oxidation—decolorization—of two recalcitrant high-redox-potential dyes, reactive black 5 (RB5, $E = 0.92$ V versus NHE) and poly R-478.^{40,41} The general basis of the LMS is the combination of laccase with low-molecular-weight compounds which act as diffusible electron carriers; i.e., once oxidized by the laccase, they can oxidize higher-redox-potential compounds that otherwise are hardly oxidized by laccase alone. Along with $K_4Mo(CN)_8$ and violuric acid, in this experiment we included 1-hydroxybenzotriazole (HBT, $E = 1.08$ V versus NHE), another high-redox-potential mediator. The LMS formed by $K_4Mo(CN)_8$ and the GreeDo mutant gave rise to higher rates of decolorization when compared to the parental OB-1 enzyme (Figure 2A). The same effect was observed for the LMS with violuric acid and HBT, although to a lesser extent, as the laccase activity on such mediators was weaker (Figure 2B,C). Indeed, there was a strong correlation between

Figure 2. Dye decolorization by the LMS. The parental OB-1 and the GreeDo variant were coupled to LMS and tested for the decolorization of recalcitrant dyes. Gray bars, reactive black 5 (RB5); white bars, poly R-478. (A) LMS with $K_4Mo(CN)_8$. (B) LMS with HBT. (C) LMS with violuric acid. Decolorization was measured after a 24 h incubation at room temperature in a reaction mixture containing 100 mM sodium phosphate/citrate buffer, pH 4.0, 0.016 μ M purified laccase (for $K_4Mo(CN)_8$ and HBT) or 0.016 μ M laccase (for violuric acid) and the 1 mM redox mediator. The initial dye concentrations were 0.0075% and 0.015% for RB5 and poly R-478, respectively. Decolorization was monitored at 598 and 520 nm for RB5 and poly R-478, respectively. Each point represents the mean and standard deviation of three independent measurements.

Figure 3. Acidic pH stability. The parental OB-1 (black bars) and the GreeDo variant (gray bars) were incubated for 0, 4, 24, 48, 72, and 96 h at different pHs: (A) pH 2; (B) pH 3; (C) pH 4; (D) pH 5. Residual activity was measured with 3 mM ABTS in 100 mM sodium phosphate/citrate buffer pH 4.0, and the laccase activity was normalized to the activity at time 0 for each pH. Each point represents the mean and standard deviation of three independent measurements.

the kinetic constants given in Table 1 and differences in the mediator's redox potential.

The kinetic thermostability of the GreeDo mutant was studied by measuring its half-life ($t_{1/2}$), the time required by the enzyme to lose 50% of its initial activity at room temperature when incubated at 70 °C. Unlike previous studies in which slight modifications of the laccase redox potential came at the cost of compromising stability, the enhanced E_{T1} of GreeDo was accompanied with a strong increase in thermostability, with the $t_{1/2}$ of OB-1 increasing from 23 to 60 min for the GreeDo variant (Figure S7). This noticeable improvement in thermostability in an already thermostable laccase underlines the structural robustness gained through the mutations that enhanced the E_{T1} . The pH stability was

measured in the pH range from 2.0 to 9.0. After a 96 h incubation at pH 6.0–9.0, both laccases retained their full activity, as expected at such a pH range (Figure S8A–D). It is well-known that the stability of fungal laccases is pH-dependent such that they are tolerant of neutral–alkaline pHs, but they become rapidly inactive at an acidic pH. This is certainly an intriguing enigma, whereby fungal laccases can only work under acidic conditions in which they are quite labile, yet while they are very stable at neutral–alkaline pH, the inhibitory effect provoked by the attachment of OH^- to the T2/T3 cluster inactivates them.⁴² When stability at acidic pHs was measured, the GreeDo variant retained roughly 60% of its residual activity after a 96 h incubation at pH 3.0 whereas the activity of OB-1 was negligible. Indeed, the GreeDo laccase

Figure 4. Location of the mutations. Representation of (A, C) the parental OB-1 and (B, D) GreeDo mutant in cartoon and surface mode. The Ala162Val and Ala458Leu mutations (depicted as red sticks) are in the vicinity of the T1Cu site closing the catalytic pocket. The surface representation highlights the shrinkage of the catalytic pocket by the hydrophobic Val162 and Leu458, reducing solvent accessibility to the nearby T1Cu site. The copper atoms are shown as blue spheres and are coordinated by 10 His residues and 1 Cys, depicted as sticks. The His–Cys–His tripeptide responsible for electron transfer is highlighted in cyan, and the axial ligand Phe460 is indicated as black sticks. Asp205, Leu456, and Glu457 are highlighted in cartoon mode as sticks. The model is based on *Trametes trogii* laccase, PDB 2HRG (96% sequence identity).

even conserved $\sim 20\%$ of its activity after 96 h at pH 2.0 (Figure 3A–D). These values mean a roughly 11- and 18-fold higher tolerance of GreeDo at pH 3.0 and 2.0, respectively, relative to OB-1. The improvement in the stability of GreeDo relative to the parental OB-1 was enhanced by the gradual increase in acidity (Figure S9) with the activity profile of the GreeDo variant slightly shifted toward acidic pH (Figure S10).

Structural and Computational Analysis. It is thought that placing the T1Cu of laccase, and that of other blue copper proteins, in a more hydrophobic environment may reduce solvent accessibility, better stabilizing the less charged Cu (I) ion and, hence, elevating the redox potential.^{27,43,44} Indeed, our findings are consistent with these observations. The Ala162Val and Ala458Leu mutations of GreeDo were mapped onto the OB-1 laccase model, and both mutations were seen to be located in the vicinity of the T1Cu coordination sphere, collapsing the catalytic cavity when the hydrophobicity at this

site augments (Figure 4). To know whether the combination of such substitutions could be found in any other HRPL, we ran a multiple sequence alignment (MSA) using basidiomycete laccases, which addressed that, indeed, there is no natural laccase reported that brings together both Ala162 and Leu458 (Figure S11). Ala458Leu lies in the α helix of the highly represented Leu–Glu–Ala tripeptide of HRPLs⁶ (456Leu–Glu–Ala458, using the PM1 laccase numbering), and the MSA indicates that this residue is also highly conserved, with only two laccases (from *Antridiella faginea*⁴⁵ and *Cyathus bulleri*⁴⁶) also presenting Leu at this position. Conversely, the mutation Ala162Val—located in the flexible loop participating in the substrate binding cavity—is not conserved among basidiomycete laccases. It is worth noting that the PM1 wild type laccase originally harbored Val162, but in the course of evolution toward functional expression, it was substituted by Ala, together with six more mutations (His208Tyr–Ser224Gly–

Ala239Pro–Asp281Glu–Ser426Asn–Ala461Thr) that conformed a unique mutational backbone for activity, heterologous functional expression, and stability.²⁹ By reverting Ala162 through a stepwise mutagenesis process, the redox potential and other important biochemical characteristics were notably improved within this particular mutational context.

According to our model, the Ala162Val and Ala458Val mutations shield the coordinating His455, reducing its exposure to the solvent (Figure 4). The side chains of Val162 and Leu458 lie close to one another, and they are orientated in such a way that they could displace any water molecules between them, which could contribute to the elevated E_{T1} . Moreover, enhanced hydrophobicity is proven to be important for protein stability.⁴⁷ As such, it is reasonable to think that the hydrophobic shell in GreeDo is both producing an energetically favorable effect to stabilize the metal center while positively influencing temperature and pH stability. In this regard, rusticyanin, a member of the cupredoxin family with the highest redox potential of its clade (680 mV versus NHE—roughly twice that of any other cupredoxin), has a characteristic broad network of hydrophobic interactions near the copper site. The limited exposure of the copper atom to water and its extreme acid stability are derived from the stabilization and rigidity of the copper binding site.⁴⁸ A similar behavior has also been shown for azurin, another cupredoxin protein, whose redox potential was increased first by enhancing hydrophobicity of the axial ligand and through hydrogen bonding.²⁵ After that, a further enhancement of redox potential (~ 0.3 V) was accomplished introducing two hydrophobic residues in the secondary coordination sphere of the T1 Cu, which also agrees with our results.³⁸ Although HRPLs are more complex enzymes than cupredoxins, with several redox centers and an intramolecular electron transfer pathway between them, analogous laws seem to govern their E_{T1} , highlighting enhanced hydrophobicity as the main driving force to increase the redox potential while conserving stability.

As mentioned above, when the difference in redox potential between the T1Cu site of the laccase and the reducing substrate is larger, the catalytic efficiency is better.^{17–19} This is paramount in terms of expanding the laccase substrate scope, where the E_{T1} may define the limits of their application. However, there are examples where low-redox-potential laccases can oxidize some low-redox-potential substrates with higher efficiency than their HRPL counterparts, suggesting that, in addition to the E_{T1} , binding events are also important in the overall oxidation process.^{21,22} To analyze the substrate binding pattern in the evolved GreeDo mutant, we turned again to the computational analysis. Alanine at positions 162 and 458 in the parental OB-1 leaves the pocket open, establishing a shallow binding site, which spreads beyond the catalytic residues. By contrast, substituting these alanines with a larger valine and leucine concentrates the pocket around His455 and Asp205, the first electron and proton acceptor, respectively. This is clearly seen when comparing the binding site pockets, as analyzed with SiteMap, for the parental OB-1 (Figure S12A) and the GreeDo mutant (Figure S12B) models. This change produces the varied effects on the different substrates studied in this work. In the case of ABTS, it hinders the formation of the most favorable binding mode in favor of a weaker one, consistent with the lower catalytic efficiency of GreeDo for this substrate. Conversely, the extended binding pocket in the parental OB-1 bound violuric acid and HBT unproductively (top docked poses with little or no spin

density), the latter failing to produce catalytic contacts with His455 and Asp205. In the GreeDo mutant, the shrinkage of the pocket forces violuric acid and HBT to contact these two residues, enhancing its oxidation (Figure S12C). The improved kinetic constants for $K_4\text{Mo}(\text{CN})_8$ oxidation were not rationalizable by the docking results since both variants displayed virtually the same binding mode, coming into close contact with His455 and nearby Asp205. However, further analysis by molecular dynamics simulations indicated that the root-mean-square deviation (RMSD) distribution is more favorable for the GreeDo mutant, which forms more reactive contacts with this redox mediator (Figure S13).

Together, these results highlight how both the E_{T1} and the features controlling substrate binding should be taken into account when assessing the catalytic performance of laccases. Moreover, hydrophobicity in the proximity of the T1Cu can exert beneficial effects on distinct enzymatic properties: redox potential, catalytic performance for high-redox-potential mediators, acidic pH stability, and kinetic thermostability.

■ CONCLUSIONS AND OUTLOOK

By oxidizing a wide range of high-redox-potential substrates, harnessing redox mediators to expand their substrate scope, or simply just transferring electrons directly from cathodic compartments, HRPLs emerge as true “wildcard biocatalysts” for dozens of biotechnological applications. Here, we have combined computational methods and directed evolution for the first time to design an HRPL variant with elevated E_{T1} , strong activity for high-redox-potential mediators, and improved stability in terms of temperature and pH. Understanding precisely how E_{T1} is regulated and why it varies significantly among structurally similar laccases still remains a challenge. Nevertheless, our findings point to the hydrophobic environment of T1Cu as one of the major structural determinants in modulating redox potential in fungal laccases. Accordingly, further engineering efforts should focus on this region to better understand modulations of redox potential over a wider range. We hypothesize that introducing an even more extensive hydrophobic network in the surroundings of the T1Cu site could raise the redox potential, yet special attention should be paid to not fully enclose the catalytic pocket as substrate binding could otherwise be impeded. Conversely, accessibility to the T1Cu site is not crucial from an electrochemical point of view since an HRPL properly connected to a cathodic compartment can accept electrons in a direct manner.

Considering all the improved properties, where an increase in the redox potential along with an enhancement in the activity for the high-redox-potential mediator stands out, this highly stable evolved HRPL could represent a suitable candidate to be used in future LMS programs (including bioremediation, pulp-biobleaching, and other harsh industrial processes) as well as biodevice engineering applications (biosensors and biofuel cells). Finally, the present study highlights the potential of combining *in silico* and experimental enzyme evolution. In particular, current modeling techniques seem mature enough to quickly provide specific regions of the enzyme where to focus more robust experimental evolution techniques.

■ MATERIAL AND METHODS

Materials. The expression shuttle vector pJRoC30 containing a uracil auxotrophy and ampicillin marker for selection came from California Institute of Technology (CALTECH). The yeast transformation kit, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DMP (2,6-dimethoxyphenol), violuric acid, HBT (1-hydroxybenzotriazole), $K_4Mo(CN)_6$, reactive black 5, poly R-478, Antifoam 204, and Taq DNA polymerase were from Sigma-Aldrich/Merck (Darmstadt, Germany). The PfuUltra was purchased from Agilent. *Saccharomyces cerevisiae* BJ5465 was acquired from LGC Promochem (Barcelona, Spain). *Escherichia coli* XL2-Blue was purchased from Stratagene (La Jolla, CA). PTM1 trace salts, Zeocin, *Pichia pastoris* strain X-33, and its expression vector pPICZ B were from Invitrogen (Carlsbad, CA). The Zymoprep yeast plasmid miniprep kit and Zymoclean Gel DNA recovery kit were from Zymo Research (Orange, CA). The NucleoSpin Plasmid kit was purchased from Macherey-Nagel (Düren, Germany). The restriction enzymes (*Bam*HI, *Xho*I, *Bst*I, *Xba*I, *Sac*I), the Antarctic phosphatase, and the DNA ligation kit were bought from New England Biolabs (Hertfordshire, UK). Culture media was prepared with the ingredients and recipes described elsewhere.⁴⁹

Laboratory Evolution. The parent OB-1 was obtained as described elsewhere.²⁹ All PCR products were loaded onto a preparative agarose gel and purified by use of the Zymoclean Gel DNA recovery kit. The recovered DNA fragments were cloned under the control of the *GAL1* promoter of the pJRoC30 expression shuttle vector, with use of *Bam*HI and *Xho*I to linearize the plasmid and to remove the parent gene. The linearized vector was loaded onto a preparative agarose gel and purified with the Zymoclean Gel DNA recovery kit.

Error-Prone PCR (epPCR). The whole parental type gene was subjected to epPCR with (i) Taq DNA polymerase in the presence of $MnCl_2$ (mutational load: 1–3 mutations/Kb); (ii) Mutazyme II with low mutational frequencies—M1—(0–4.5 mutations/Kb) and Mutazyme II with high mutational frequencies—M2—(4.5–9 mutations/Kb). (i) Mutagenic PCR was prepared in a final volume of 50 μ L containing 3% DMSO, 90 nM sense (5'-CCTCTATACTTTAACGTCAAAGG-3') and RMLC antisense (5'-GCTTACATTCACGCCCTCCC-3'), 0.3 mM dNTPs (0.075 mM each), 0.01 mM $MnCl_2$, 0.1 ng/ μ L template, 1.5 mM $MgCl_2$, and 0.05 U/ μ L Taq DNA polymerase. (ii) Mutagenic PCR was prepared in a final volume of 50 μ L containing 1X buffer Mutazyme II (10 $\times$), 3% DMSO, 370 nM sense (5'-CCTCTATACTTTAACGTCAAAGG-3') and RMLC antisense (5'-GCTTACATTCACGCCCTCCC-3'), 0.8 mM dNTPs (0.200 mM each), 40 ng/ μ L template, and 0.05 U/ μ L Mutazyme II polymerase. epPCRs were performed on a gradient thermocycler (Mycycler, BioRad) using the following parameters: 95 °C for 2 min (1 cycle); 94 °C for 45 s, 50 °C for 30 s, 72 °C for 90 s (30 cycles in the case of Taq polymerase and Mutazyme II with high mutational frequencies, 20 cycles in the case of Mutazyme II with low mutational frequencies); and 72 °C for 10 min (1 cycle). A 200 ng portion of epPCR product was mixed with 100 ng of the linearized vector and transformed into *S. cerevisiae* competent cells using the yeast transformation kit. Inserts and linearized plasmid shared 40 bp of homology to allow recombination and the *in vivo* cloning by the yeast. Transformed cells were plated on SC drop-out plates

being incubated for 3 days at 30 °C. A total of 2400 clones were screened (1000 clones for Taq/ $MnCl_2$, 1000 clones for the M1 library, and 400 clones for the M2 library).

Combinatorial Saturation Mutagenesis (Ala162, Ala458). Three PCR reactions were carried out in a final volume of 50 μ L containing 3% DMSO, 0.3 mM dNTPs (0.075 mM each), 0.05 U/ μ L PfuUltra DNA polymerase, and 2 ng/ μ L template, but each of them with different primers. For PCR 1, 0.25 μ M RMLN was used as forward primer and three reversed primers in the same reaction with concentrations according to the protocol of Kille and co-workers:⁵⁰ (i) AHN162R (5'-TTGATGCTGCGACCGAGGCCGTTGATAAGAGTAGCATCGGCAGTCGGAHNCGCCGGCCGACTT-3'), (ii) CDB162R (5'-TTGATGCTGCGACCGAGGCCGTTGATAAGAGTAGCATCGGCAGTCGCGDCBCGCCGGCCGACTT-3'), and (iii) CCA162R (5'-TTGATGCTGCGACCGAGGCCGTTGATAAGAGTAGCATCGGCAGTCGCGCA-CGCCGGCCGACTT-3'). For PCR 2, 0.25 μ M of CZF was used as forward primer (5'-GATGCTACTCTTATCAACGGCCTCGGTCGAGCATCAA-3') and 0.25 μ M of CZR as reverse primer (5'-GGAAGTCGATGTGGCAGTGG-AGGAACCACGGGCCGGG-3'). For PCR 3, three forward primers were used in same reaction with concentrations according to Kille et al.:⁵⁰ (i) NDT458F (5'-CCCCG-GCCCGTGGTTCCTCCACTGCCACATCGACTTCCACCTTGAGNDTGGGTTCCAGTGCCTG-3'), (ii) VH458F (5'-CCCCGGCCCGTGGTTCCTCCACTGCCACATCGACTTCCACCTTGAGVHGGGGTTCACGGTTCGTC-3'), and (iii) TGG458F (5'-CCCCGGCCCGTGGTTCCTCCACTGCCACATCGACTTCCACCTTGAGTGGG-GGTTACGGTTCGTC-3') and 0.25 μ M RMLC as reverse primer. Codon substitutions, where N = A/T/C/G, D = no C, H = no G, B = no A, and V = no T, are shown in italics. PCR reactions were performed using the following program: 95 °C for 2 min (1 cycle); 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s (28 cycles); and 72 °C for 10 min (1 cycle). PCR products were transformed together with linearized plasmid into yeast by IVOE,⁵¹ and 1550 clones were screened.

Saturation Mutagenesis at Pro160 and Ile452. Saturation mutagenesis was carried out using degenerated NNS codons (N = A/T/C/G; S = G/C), creating two libraries. For library P160, 50 μ L was used containing 3% DMSO, 0.3 mM dNTPs (0.075 mM each), 0.05 U/ μ L PfuUltra DNA polymerase, and 2 ng/ μ L template (GreeDo) with 0.25 μ M RMLN and 0.25 μ M P160-R (5'-ATCGGCAGTCGGTACCGCSNNGCCGACTTTGGCAGCCAAGTG-3') or 0.25 μ M RMLC and P160-F (5'-CACTTGGCTGCCAAAGTCGGCNSNGCGGTACCGACTGCCGAT-3'). For library I452, 50 μ L was used containing 3% DMSO, 0.3 mM dNTPs (0.075 mM each), 0.05 U/ μ L PfuUltra DNA polymerase, and 2 ng/ μ L template (GreeDo) with 0.25 μ M RMLN and 0.25 μ M of I452-R (5'-GTGAACCCTAACTCAAGGTGGAAGTCSNNGTGGCA-GTGGAGGAACC-3') or 0.25 μ M RMLC and I452-F (5'-GGTTCCTCCACTGCCACNNSGACTTCCACCTTGAGTTAGGGTTAC-3'). PCR reactions were performed as follows: 95 °C for 2 min (1 cycle); 95 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s (28 cycles); and 72 °C for 10 min (1 cycle). PCR products were transformed together with linearized plasmid into yeast by IVOE.⁵¹ For each library, 500 clones were screened.

Focused Evolution at the Leu383–Thr461 Segment. The region between Leu383–Thr461 was subjected to MORPH-ING (mutagenic organized recombination process by homol-

ogous *in vivo* grouping).³² Mutagenic PCR was prepared in a final volume of 50 μL containing 3% DMSO, 90 nM GreeDo-epPCR-F (5'-CCTCTGGAAGTGTA TACTCGTCCC-3'), 90 nM 9C1-epPCR-R (5'-GGGACCGGGTTCGTAGCGGC-GACGTC-3'), 0.3 mM dNTPs (0.075 mM each), 0.1 MnCl_2 , 1.5 mM MgCl_2 , 0.05 $\text{U}/\mu\text{L}$ Taq DNA polymerase, and 1 ng/ μL template. The amplification parameters were 94 $^\circ\text{C}$ for 2 min (1 cycle); 94 $^\circ\text{C}$ for 45 s, 50 $^\circ\text{C}$ for 30 s, and 72 $^\circ\text{C}$ for 90 s (28 cycles); and 72 $^\circ\text{C}$ for 10 min (1 cycle). The remaining portions of the whole GreeDo gene were amplified by high-fidelity PCR in a final volume of 50 μL containing 3% DMSO, 0.5 μM HF9C1-F (5'-GTCGTCATGGCCGAGGACAT-TCCC-3'), and 0.5 μM RMLC or 0.5 μM HF9C1-R (5'-GGAGATCTCAATGTCCGCGTTGCA-3') for the other high-fidelity fragment, 1 mM dNTPs (0.25 mM each), 0.05 $\text{U}/\mu\text{L}$ PfuUltra DNA polymerase, and 2 ng/ μL template. High-fidelity PCR was carried out with the following program: 95 $^\circ\text{C}$ for 2 min (1 cycle); 95 $^\circ\text{C}$ for 45 s, 50 $^\circ\text{C}$ for 30 s, and 72 $^\circ\text{C}$ for 90 s (28 cycles); and 72 $^\circ\text{C}$ for 10 min (1 cycle). PCR products were transformed together with linearized plasmid into yeast by IVOE,⁵¹ and 1000 clones were screened.

High-Throughput Screening. Individual clones were picked and inoculated in sterile 96-well plates (Greiner Bio-One, GmbH), referred to as master plates, containing 200 μL of SEM per well. In each plate, column number 6 was inoculated with the parent type, and one well (H1-control) was inoculated with *S. cerevisiae* transformed with pJRoC30-MtL plasmid (laccase without activity). Plates were sealed with Parafilm to prevent evaporation and incubated at 30 $^\circ\text{C}$, 220 rpm, and 80% relative humidity in a humidity shaker (Minitron, Infors) for 5 days. The master plates were centrifuged (Eppendorf 5810R centrifuge) for 10 min at 2500g and 4 $^\circ\text{C}$. Aliquots of the supernatants (20 μL) were transferred from the master plates to three replica plates with the aid of a liquid handler robotic station Freedom EVO (Tecan). A 180 μL portion of a corresponding reaction mixture [100 mM sodium phosphate/citrate buffer pH 4.0 (3 mM ABTS or 2 mM $\text{K}_4\text{Mo}(\text{CN})_8$ or 20 mM violuric acid)] was added to each plate with the help of a pipetting robot (Multidrop Combi Reagent Dispenser, Thermo Scientific). The plates were briefly stirred and absorbance was measured at 418 nm for ABTS ($\epsilon_{\text{ABTS}}^{\bullet\bullet} = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$), at 388 nm for $\text{K}_4\text{Mo}(\text{CN})_8$ ($\epsilon_{\text{K}_4\text{Mo}(\text{CN})_8} = 1460\text{ M}^{-1}\text{ cm}^{-1}$), and at 515 nm for violuric acid ($\epsilon = 98\text{ M}^{-1}\text{ cm}^{-1}$). The ABTS measurement was performed in kinetic mode, while $\text{K}_4\text{Mo}(\text{CN})_8$ and violuric acid were in end point mode with a plate reader (SPECTRAMax Plus 384, Molecular Devices). The values were normalized against the parent type of the corresponding plate, and selected variants came from the activity improvements for $\text{K}_4\text{Mo}(\text{CN})_8$ and violuric acid. To rule out the selection of false positives, two rescreenings were carried out as described elsewhere.²⁹

DNA Sequencing. Laccase genes were sequenced by GATC-Biotech. The primers used were RMLN, PM1FS (5'-ACGACTTCCAGGTCCCTGACCAAGC-3'), PM1RS (5'-TCAATGTCCGCGTTCGCAGGGA-3'), and RMLC.

Biochemical Characterization. The production and purification were adapted from previous protocols with minor modifications.^{52,53} ABTS, DMP, $\text{K}_4\text{Mo}(\text{CN})_8$, and violuric acid kinetic constants for laccase variants were estimated in sodium phosphate/citrate buffer (pH 4.0, 100 mM). Reactions were performed in triplicate, and substrate

oxidations were followed through spectrophotometric changes ($\epsilon_{418}(\text{ABTS})^{\bullet\bullet} = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{469}(\text{DMP}) = 27\,500\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{388}(\text{K}_4\text{Mo}(\text{CN})_8) = 1460\text{ M}^{-1}\text{ cm}^{-1}$; $\epsilon_{515}(\text{violuric acid}) = 98\text{ M}^{-1}\text{ cm}^{-1}$). To calculate the K_m and k_{cat} values, the average V_{max} was represented against substrate concentration and fitted to a single rectangular hyperbolic function with SigmaPlot 10.0. Kinetic thermostability and pH stability were performed as reported previously.⁴⁹

Spectroelectrochemical Characterization. All spectroelectrochemical characterization was performed with enzymes produced in *P. pastoris*.

Enzyme Preparation. Laccase variants produced in *S. cerevisiae* do not yield a sufficient amount of protein required for spectroelectrochemical studies. We turned to *Pichia pastoris* as an alternative host due to its ability to grow at very high cell densities under the control of strong promoters and to secrete high amounts of protein while maintaining similar biochemical properties.⁵² Both OB-1 and GreeDo variants were cloned in *P. pastoris* under the inducible PAOX1 promoter, produced in the MBR 7 L bioreactor, and purified to homogeneity as reported previously.⁵²

Electrode Preparation. Two types of working electrodes were used in this study, viz. graphite electrodes (GEs) from Alfa Aesar (Ward Hill, MA) with a geometrical area of 0.073 cm^2 and a homemade capillary gold electrode (*vide infra*). GEs were polished on a silicon carbide grinding paper (Grit 1000/P 2500, Buehler), rinsed thoroughly with Milli-Q H_2O , and let dry. For biomodification, 10 μL of either OB-1 or GreeDo solution was dropped onto the GE surface and allowed to adsorb for 20 min.

Open-Circuit Potential Measurements. Open-circuit potentials of biomodified electrodes were measured using a $\mu\text{Autolab Type III/FRA2}$ potentiostat/galvanostat from Metrohm Autolab B. V. (Utrecht, The Netherlands) in an electrochemical cell with a total volume of 40 mL thermostated at 25 $^\circ\text{C}$. Ag/AgCl(sat. KCl (0.199 V versus NHE)) was used as a reference electrode and biomodified graphite electrode as a working electrode. The experiments were carried out in O_2 -saturated 0.1 M phosphate buffer pH 6.0.

Redox Titration. The redox potential of the T1 site of laccases was defined by mediated spectroelectrochemical redox titration according to the procedure reported earlier.^{33,34} The home-built spectroelectrochemical cell consisted of a 1 cm long capillary Au electrode with an inner diameter of 350 μm , operating both as the working electrode and as a cuvette with a total volume of just 1 μL . The input and output optical fibers (FCB-UV 400/050-2 and FCUV 200, respectively) were obtained from Ocean Optics (Dunedin, FL) and attached to the ends of the capillary. The system comprised a DH-2000 light source and an HR4000CG-UV-NIR spectrometer from Ocean Optics. The spectra were recorded with Ocean Optics Spectra Suite software. The potential of the Au capillary electrode was controlled using a three-electrode potentiostat (Zata Electronics, Lund, Sweden). A platinum wire was used as a counter electrode, and a Ag/AgCl(3 M NaCl) electrode, separated from the enzyme solution by two ceramic frits and a buffer solution salt bridge, served as a reference electrode. The working capillary Au electrode was cleaned for ca. 24 h in freshly prepared piranha solution (3:1 (v:v) 96% sulfuric acid:30% hydrogen peroxide solution). For improvement of the electrochemical communication between the electrode and the enzyme molecules, the reduced forms of three redox mediators, $\text{K}_4[\text{Fe}(\text{CN})_6]$, $\text{K}_4[\text{W}(\text{CN})_8]$, and $\text{K}_4[\text{Mo}(\text{CN})_8]$,

were used. The final concentration of each mediator in the cell was 2 mM, and their midpoint redox potentials were 0.43, 0.52, and 0.78 V versus NHE.⁵⁴ All the reported potentials in this work are given versus NHE.

Modeling and Computer-Aided Evolution. The OB-1 model was built with Schrodinger homology modeling software, using PDB 2HRG (96% sequence identity) as a template.^{55,56} Protonation states of titratable residues were optimized with PROPKA simulating pH 4.0 (experimental conditions) and double-checked with the H++ server.^{57,58} The T1 pocket was characterized with SiteMap, and violuric acid was docked with Glide SP.^{59,60} To do so, substrate electrostatic potential (ESP) charges were obtained optimizing its geometry at the M06/6-31G* level of theory, modeling the solvent with the Poisson–Boltzmann model, using Jaguar.⁶¹ The five most favorable poses of the OB-1/substrate complex were scored with quantum mechanics/molecular mechanics (QM/MM) spin density calculations, a theoretical tool that proved its utility in laccases' reactivity rationalization and design.^{21,62,63} The conformation that, among those that display catalytic contacts with His455 and Asp205, had the highest substrate spin density was selected for enzyme evolution.

OB-1 redesign was carried out with PELE.^{64,65} Importantly, violuric acid was modeled in its oxidized state, aiming at pushing the electron transfer driving force by stabilizing the substrate binding after losing one electron. Every residue within 10 Å of the substrate was mutated, one by one, to all the possible remaining 19 amino acids. For each enzyme variant, the substrate was perturbed with 0.5 Å translations and 0.1 radians rotations while the protein backbone was subjected to small anisotropic network model (ANM) displacements. The simulation of each mutant (and the reference OB-1) lasted 1 h, accumulating ~30 Monte Carlo steps. The enzyme–substrate interaction energy was then used to score each mutant. A residue position was considered to be promising if at least two mutations led to a decrease in interaction energy greater than 3 kcal/mol (compared to the results from the reference OB-1). Such a severe threshold was intended to select only a few positions for further analysis.

Analysis of Mediator Binding Mode. The GreeDo mutant was built and refined (side chain repacking) on top of the OB-1 model with Prime, and the T1 cavities of both enzymes were characterized with SiteMap.^{55,60} The detected pockets were then used to dock VA, HBT, $K_4Mo(CN)_8$, and ABTS with Glide SP.⁵⁹ Ligand charges were obtained as mentioned above. The five most favorable poses were scored with QM/MM spin density calculations.²¹

Molecular Dynamics Analysis. The parental/ $K_4Mo(CN)_8$ and GreeDo/ $K_4Mo(CN)_8$ systems were solvated with a 10 Å buffer of waters in an orthorhombic box, neutralized, and 0.15 M NaCl was added. After equilibration (default settings), 20 × 3 ns production runs were performed with Desmond at 300 K. The OPLS-2005 force-field and the SPC explicit water model were used.^{66,67} The temperature was regulated with the Nosé–Hoover chain thermostat with a relaxation time of 1.0 ps, and the pressure was controlled with the Martyna–Tobias–Klein barostat with isotropic coupling and a relaxation time of 2.0 ps.^{68,69} The RESPA integrator was employed with bonded, near, and far time steps of 2.0, 2.0, and 6.0 fs, respectively.⁷⁰ A 9 Å cutoff was used for non-bonded interactions together with the smooth particle mesh Ewald method.⁷¹

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.9b00531.

Additional data and figures including structures, UV–vis spectrum, linearity of the $K_4Mo(CN)_8$ assay, determination of the coefficient of variance, schematics, relative activity vs number of clones, potentiograms, residual activity vs time, improvement in residual activity, pH activity profile, and molecular dynamics simulations (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: victor.guallar@bsc.es.

*E-mail: malcalde@icp.csic.es.

ORCID

Roland Ludwig: 0000-0002-5058-5874

Miguel Alcalde: 0000-0001-6780-7616

Notes

The authors declare no competing financial interest.

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III

Electrochemistry of a high redox potential laccase obtained by computer-guided mutagenesis combined with directed evolution

Olga Aleksejeva^a, Ivan Mateljak^b, Roland Ludwig^c, Miguel Alcalde^b, and Sergey Shleev^{a*}

^a Department of Biomedical Science, Health and Society, Malmö University, 20560 Malmö, Sweden

^b Department of Biocatalysis, Institute of Catalysis, CSIC, Cantoblanco 28094 Madrid

^c Department of Food Sciences and Technology, VIBT – Vienna Institute of Biotechnology BOKU – University of Natural Resources and Life Sciences, A-1190 Vienna, Austria

*Corresponding author: Sergey Shleev, Department of Biomedical Science, Health and Society, Malmö University, 20560 Malmö, Sweden. E-mail: sergey.shleev@mau.se.

Abstract

Electrochemical characterization of the GreeDo variant of a high redox potential fungal laccase obtained by laboratory evolution together with computer-guided mutagenesis, in comparison to its parental variety (the OB-1 mutant), is presented. Both laccases, when immobilized on graphite electrodes either by direct physical adsorption or covalently attached *via* gold nanoparticles, were capable of both non-mediated and mediator-based bioelectroreduction of molecular oxygen at low overpotentials. GreeDo exhibited higher open circuit potential values and onset potentials for oxygen bioelectroreduction compared to OB-1. However, even though in homogeneous catalysis GreeDo outperforms OB-1 in terms of turnover numbers and catalytic efficiency, when exposed to high redox potential substrates, direct electron transfer based bioelectrocatalytic currents of GreeDo and OB-1 modified electrodes were similar. High operational stability of freely diffusing GreeDo and also the immobilized enzyme in the acidic electrolyte was registered, in agreement with high storage stability of GreeDo in acidic solutions.

Keywords: Laccase, Oxygen bioelectroreduction, Direct electron transfer, Graphite electrode, Gold nanoparticles; T1 site redox potential

1. Introduction

Laccase (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2) is a multi-copper enzyme, facilitating the oxidation of different organic and inorganic compounds with concomitant reduction of molecular oxygen (O_2) to water (H_2O) [1]. Since the enzyme catalyzes the oxidation of a large variety of substrates, it has become the subject of significant interest in many fields of research, such as development of bioelectronic devices (biosensors, and biocathodes for biological power sources), biodegradation of xenobiotics, bioremediation, food processing, and organic synthesis [2-4].

Laccases (Lcs) contain four copper ions of three types, defined as T1, T2 and T3. The substrate oxidation takes place at the T1 site, whereas O_2 is reduced at the T2/T3 cluster formed by T2 and T3 copper ions [1, 5]. An important characteristic of Lcs is the standard redox potential of the T1 site (E_{T1}), which values that lie between 0.43 V and 0.79 V vs. NHE for different Lcs [6]. The catalytic efficiency, *i.e.* the k_{cat}/K_M value, for certain reduced substrates is known to be dependent on E_{T1} [7, 8]. Therefore, Lcs with high E_{T1} values, *i.e.* ca. 0.73-0.79 V [9], are of particular interest in some specialized branches of biotechnology, *e.g.*, bleaching and bioremediation processes [2]. Moreover, high redox potential Lcs are usually capable of direct electron transfer (DET) reactions, which offer several advantages for fabrication of enzyme-based bioelectronic devices, first of all, biodevices which electroreduce O_2 at low overpotentials. Specifically, the lowest overpotential for O_2 reduction reaction, *viz.* 0.06 V, was reported for the high redox potential Lc from the basidiomycete *Trametes hirsuta* [10].

Very recently, we reported the GreeDo mutant, a laboratory evolved laccase with increased E_{T1} and improved pH and thermal stability, which was engineered from an already high-redox potential laccase mutant (the OB-1 variant, originally evolved for heterologous functional expression in yeast [11]) [12]. The GreeDo mutant was obtained by combining computational design and directed evolution methods, and represents the first and so far the only experimental evidence on the enhancement of $E_{T1}^{0'}$ with concomitant improvement of activity and stability in high redox potential Lcs. Below, a comparative electrochemical characterization of the GreeDo mutant and Lc OB-1 mutant, is presented.

2. Materials and methods

2.1. Chemicals and materials

BF_4Bu_4N , 4-nitrobenzenediazonium tetrafluoroborate, sodium nitrite, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), 2,2'-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium acetate, hydrochloric acid, acetic acid, N-hydroxysuccinimide (NHS), 6-mercapto-1-hexanol (MH), $NaIO_4$, NaF, KCl, NaOH, $HAuCl_4$, and morpholino-ethanesulphonic acid (MES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetrakis(hydroxymethyl) phosphonium chloride (THPC) was obtained from Fluka Chemie AG (Buchs, Germany). Absolute ethanol (EtOH) was obtained from CCS Healthcare AB (Borlänge, Sweden). Acetonitrile (HPLC grade) was purchased from RdH Laborchemikalien GmbH & Co. (Seelze, Germany). $NaClO_4$ was obtained from Merck (Darmstadt, Germany). All aqueous solutions were prepared using water purified with the PURELAB UHQ II system from ELGA Labwater (High Wycombe, United Kingdom). Argon (Ar) and oxygen (O_2) gases were supplied by AGA Gas AB (Sundbyberg, Sweden). Spectrographic graphite electrodes (GES) of 3.05 mm in diameter were obtained from Alfa Aesar (Ward Hill, MA, USA).

2.2. Enzymes

Two variants of basidiomycete Lc PM1, GreeDo mutant (9 mg mL⁻¹), and parental OB-1 mutant (10 mg mL⁻¹), in 20 mM bis-tris buffer pH 6.0 were used. The Lc from basidiomycete PM1 with high thermostability was isolated from the western Mediterranean region and submitted to directed evolution for functional expression in yeast, yielding the parental OB-1 variant [11]. A new mutant version, GreeDo, was obtained from OB-1 by combined computational and directed evolution to improve E_{T1} . Both enzymes were purified to homogeneity and thoroughly characterized, as described in Ref. [12].

2.3. Biomodification of GEs with Lcs by physical adsorption

GEs were polished on a silicon carbide grinding paper (Grit 1000/P 2500, Buehler, USA), rinsed thoroughly with ultrapure H₂O, and dried. For biomodification, 10 µl of either GreeDo or OB-1 solution were dropped onto the GE surface and allowed to adsorb for 20 min.

2.4. Covalent attachment of Lcs onto GEs modified with gold nanoparticles

Gold nanoparticles (AuNPs) were synthesized for electrode modification. 5 ± 3 nm AuNPs freshly prepared in aqueous media by addition of 1 mM HAuCl₄ to 60 mM NaOH solution containing 1 mM THPC under vigorous stirring [13]. The resulting AuNPs were filtered with a PTFE 0.200 µm pore size filter from Corning Incorporated (Kaiserslautern, Germany). The AuNPs size was determined by UV–Vis spectroscopy using a numerical approximation method [14].

Prior to surface modification, GEs were polished on a silicon carbide grinding paper (Grit 1000/P 2500, Buehler, USA), sonicated for 5 min in ultrapure water, and dried [15]. Thereafter, polished and cleaned GEs were modified with 4-aminophenyl groups, as described in Ref. [15]. Then, the modified electrodes were immersed during 2 h in a 14.5 mM NaNO₂ and 0.5 M HCl solution in order to convert the aromatic amino groups on the electrode surface into diazonium groups. Subsequently, the electrodes were incubated into an aqueous dispersion of AuNPs during 3–72 h. Afterwards, two cyclic voltammograms (CVs) were recorded between 0.6 V and –0.6 V at 0.2 V s⁻¹ with the GE/AuNPs in 50 mM acetate buffer, pH 4.2, containing 100 mM NaClO₄ at room temperature [16].

The GE/AuNP electrodes then were immersed in an acetonitrile solution containing 2 mM p-nitrophenyldiazonium tetrafluoroborate and 100 mM BF₄Bu₄N. One CV from 0.6 V to –0.6 V with 0.2 V s⁻¹ scan rate was recorded. Afterward, the electrodes were taken into a previously deoxygenized 9:1 H₂O/EtOH, 0.1 M KCl solution, and electrochemical reduction of nitro groups on the GE/AuNP surface was carried out by running two CVs from 0 V to –1.4 V at 0.2 V s⁻¹ scan rate. The amino-terminated GE/AuNP electrodes were then immersed overnight in a water solution containing 1 mM MH [16].

Five microliters of either GreeDo (9 mg mL⁻¹) or OB-1 (10 mg mL⁻¹) solution were placed into 55 µL of 47 mM NaIO₄ solution for 30 min. Afterward, 90 µL of 100 mM Na₂HPO₄ was added to the mixture. The modified GE/AuNP electrodes were then incubated in either GreeDo or OB-1 solution for 90 min. The GE/AuNP/GreeDo or GE/AuNP/OB-1 electrodes were rinsed with 10 mM MES buffer, pH 6.0. Finally, the electrodes' surface was covered with 10 µL of 10 mM MES, pH 6.0, buffer solution containing 36 mM EDC and 17 mM NHS, and covered to avoid evaporation, letting the reaction take place for 2 h [16].

2.5. Electrochemical measurements

Electrochemical experiments were performed using a µAutolab Type III/FRA2 potentiostat/galvanostat from Metrohm Autolab B. V. (Utrecht, The Netherlands) in an

electrochemical cell with a total volume of 40 mL thermostated at 25 °C. Ag|AgCl| KCl_{sat} (0.199 V vs. NHE), Pt wire, and biomodified GEs were used as a reference, counter, and working electrodes respectively. Three different electrolytes were used, *viz.* 0.1 M phosphate buffer pH 8.0, 0.1 M phosphate buffer pH 6.0, and 0.1 M acetate buffer pH 4.0 containing 100 mM NaClO₄. Cyclic voltammograms (CVs) were recorded in O₂ saturated electrolytes in a potential window from 1.0 V to 0.2 V vs. NHE with a scan rate of 0.025 V s⁻¹. All the reported potentials are given vs. NHE.

2.6. Operational stability of GreeDo in homogenous solution

GreeDo was incubated in the solution of 10 mM K₄Mo(CN)₈ in 0.1 M acetate buffer, pH 4.0 at a concentration of 0.1 mg ml⁻¹ under continuous stirring. 10 µl of enzyme was taken from the solution and injected into an Oxygraph Clark-type electrode from Hansatech Ltd. (Norfolk, England), containing 0.25 mL of 10 mM K₄Fe(CN)₆ in air saturated 0.1 M acetate buffer, pH 4.0. Rates of O₂ uptake, measured at 25 °C under continuous stirring, were assessed by Oxygraph Plus software provided by Hansatech Ltd. (Norfolk, England).

3. Results and Discussion

Initially, the two Lcs were physisorbed on GEs, following a simple method for physical adsorption of blue multicopper oxidases (MCOs) on porous spectroscopic graphite electrodes [9, 17]. CVs of GreeDo/GEs and OB-1/GEs were recorded in acidic and alkaline electrolytes, revealing well-pronounced bioelectrocatalysis at pH 4.0, but indicating complete absence of bioelectrocatalytic reduction of O₂, at least at low overpotentials, at pH 8.0 (Fig. 1). These results are in good agreement with data obtained for homogeneous biocatalysis, where the absence of enzymatic activity for both Lcs at pH 8 was observed [12]. The obtained catalytic current densities were about 230 µA cm⁻² (here and below the bioelectrocatalytic currents were measured at an applied potential of 0.2 V, *i.e.* at the highest overpotential without non-enzymatic O₂ electroreduction to H₂O₂) for both enzymes (*cf.* dotted and solid lines in Figs. 1A). Despite the well-pronounced bioelectrocatalytic process on biomodified GEs, the recorded CVs were far from ideal. No sigmoidal shape was observed, whereas a well-pronounced potential dependence of current outputs was registered, suggesting a sluggish DET (DET-rate limited process in which there is a superposition of different electrochemical rate constants [18]) along with a random orientation of both enzymes on the electrode surface. Thus, for an efficient DET communication between graphite electrodes and Lcs, a previously developed nanotechnological approach was tried. Specifically, instead of random adsorption, oriented covalent binding of both enzymes to small gold nanoparticles was tested. Porous GEs allow covering a large surface area with AuNPs, which can be tailored by surface chemistry to improve biomolecule orientation and therefore enhance DET [16, 19]. Moreover, contrary to large NPs [20], AuNPs of a size comparable to the dimensions of biomolecules are known to perform as electronic bridges between electrodes and enzyme redox centers, thus enhancing the rate of DET reactions [16, 21, 22].

The CVs of AuNPs decorated GEs with adsorbed Lcs demonstrated bioelectrocatalytic reductive current in the absence of a redox mediator (Fig. 2). The onset potential in the case of GreeDo/AuNPs/GEs was higher compared to OB-1/AuNPs/GEs, *i.e.* 0.83 V vs. 0.79 V, in agreement with the 0.05 V E°_{T1} difference of the enzymes (Table 1). However, contrary to bare GEs, the obtained catalytic current densities were *ca.* 5 times lower. Moreover, the bioelectrocatalytic current output from GreeDo/AuNPs/GEs was lesser compared to OB-1/AuNPs/GEs, *i.e.* 40 and 50 µA cm⁻², respectively. Admixture of a mediator, *i.e.* ABTS, did enhance the bioelectrocatalysis, *i.e.* the current density values reached 60 and 90 µA cm⁻². These

results are in agreement with data from homogeneous assays using ABTS as an enzyme substrate, where higher catalytic constants and lower Michaelis constants were determined for the parental enzymes [12]. However, in homogeneous catalysis in the case of high redox potential electron donors, *i.e.* violuric acid and molybdenum cyanide, the biocatalytic process is strictly limited by the oxidation of the reduced substrate at the T1 site and it is highly dependent on E_{T1} . Indeed, for these substrates, catalytic constants and efficiency of the mutated Lc were substantially higher compared to the parental type [12]. The significant difference between DET and mediated ET current outputs (Figs. 2B and 2D), as well as a strong potential dependence of current outputs (Figs. 2A and 2C), still pointed to a random orientation of both Lcs on the electrode surface despite surface nanostructuration and covalent attachment of enzymes. The electrocatalytic signals were absent upon addition of NaF to the electrolyte, confirming the bioelectrocatalytic origin of the currents obtained. Fluoride ions are well-known inhibitors of bio(electro)catalytic reduction of O_2 , because of their binding to the T2/T3 cluster of MCOs [23], preventing the intramolecular electron transfer (IET) between the T1 site and the T2/T3 cluster [24, 25].

Since neither DET enhancement was achieved, nor were bioelectrocatalytic current outputs improved by complex enzyme immobilization procedure and advanced electrode modification, additional studies were done, again using simple bare GEs. Taking into account the fact that electrocatalysis is not actually related to current increase but rather to a decrease of the O_2 electroreduction overpotential, open circuit potential (OCP) measurements of GreeDo and OB-1 modified electrodes were performed. OCP values of GreeDo/GEs and OB-1/GEs were measured at pH 4.0, 6.0, and 8.0, giving on average 0.03 – 0.06 V higher values for the mutant variant compared to the parental enzyme and being in agreement with $E_{T1}^{0'}$ difference, *i.e.* 0.05 V (Table 1). The OCP values decreased with increasing pH, following the trend of *ca.* 0.04 V/pH, which is precisely in between the T1 site and T2/T3 cluster pH dependencies (about 0.02 and 0.06 V/pH [26]). Moreover, OCP values obtained at pH 6.0 were almost identical to the $E_{T1}^{0'}$ of both Lcs (Table 1). Similar results (the dependence of OCP on E_{T1} , as well as identical OCP and E_{T1} values at pH 6.0) were noticed previously for other high redox potential fungal Lcs [9]. Furthermore, quite low overpotentials for O_2 bioelectroreduction were obtained in all electrolytes, but with zero difference between the initial OCP of GreeDo/GEs and the equilibrium redox potential of the O_2/H_2O couple. Last but not least, the addition of 50 mM NaF to the pH 8.0 electrolyte resulted in 16% OCP drop. The only report in the literature regarding O_2 electroreduction by a high redox potential laccase at $pH > 7$, revealed bioelectrocatalysis at high overpotential and low current (less than 50 $\mu A\ cm^{-2}$ below 0.35 V vs. NHE) [27]. Thus, just abovementioned experimental results, in addition to small bioelectrocatalytic currents at high overpotentials registered at GreeDo/GEs (*cf.* solid and dashed curves in Fig. 1B at applied potentials below 0.2 V), pointed to the fact of bioelectrocatalytic reduction of O_2 by the enzyme in alkaline electrolyte with zero overpotential.

To prove or disprove such a strong statement, *i.e.* Lc based bioelectrocatalysis at pH 8.0, with zero overpotential on the top, additional OCP and amperometric measurements were performed at pH 4.0 and 8.0. Contrary to the pH 4.0 circumstances, the OCP of GreeDo/GE was decreasing with time at pH 8.0 owing to OH^- inhibition of the enzyme (*cf.* Fig. 3A and 3B). Moreover, the adsorbed enzyme was fully reduced in just 15 sec when a potential of 0.3 V was applied, and close to zero currents were registered after that. Furthermore, the initially observed OCP value of 0.76 V was never again achieved. Thus, as evident from the results presented in Fig. 3, a bioelectrocatalytic process at pH 8.0 was not observed and the obtained results could be explained, at least to a certain extent, using the "control center" designation in the electrochemistry of MCOs [28]. At pH 4.0 the IET is fast and the T1 site is not the control center. Thus, E_{T1} influences but does not define the potential of bioelectrocatalysis. Indeed,

high OCP values reflect a high redox potential of the T2/T3 cluster, rather than the E_{T1} . At pH 6.0, the IET is the rate determining slow step of the overall bioelectrocatalytic reaction and the T1 is the control center, which defines the potential of bioelectrocatalysis. At pH 8.0, the IET rate between the T1 site and the T2/T3 cluster is close to zero, and the measured OCP values reflect the potential of the T1 site. Additional confirmation comes from the fact that the potential dependence between pH 4.0 and pH 6.0 was 0.05 V/pH (close to the T2/T3 potential/pH dependence), whereas between pH 6.0 and pH 8.0 it was 0.02 V/pH (close to the T1 site). One can also speculate that the OCP decrease in the case of fluoride inhibition at pH 8.0 relies on a T2/T3 $\rightarrow$ T1 IET process, or in other words, reduction of the T1 site by a low redox potential *pro tem* T2/T3 cluster formed by the attachment of OH⁻ and F⁻ ligands.

Finally, the operational stability of GreeDo in solution and in adsorbed state was investigated in the acidic electrolyte, pH 4.0, using an aliquot of the enzyme containing solution and by single-potential amperometry, respectively. The enzyme operating in solution containing the high redox potential substrate was relatively stable, whereas GreeDo/GEs were even more stable, retaining *ca.* 60% of the initial activity after 2 h of continuous operation (*cf.* dotted and solid curves in Fig. 4). These results are in agreement with the behavior of the enzyme in homogeneous solutions. It is well known that the stability of fungal laccases is pH-dependent such that they are tolerant of neutral-alkaline pH, but rapidly become inactive at acidic pH. When the storage stability of GreeDo at acidic pH was measured, the mutant retained roughly 80% of its initial activity after a 96 h incubation at pH 4.0, whereas the activity of OB-1 was almost negligible [12].

4. Conclusions

The biocatalytic and bioelectrocatalytic properties of GreeDo and OB-1 Lcs are in good agreement. Both enzymes were capable of reducing O₂ at low overpotentials in the presence and absence of the redox mediator, ABTS. The onset and open circuit potentials for DET based O₂ bioelectroreduction was slightly higher for GreeDo Lc, in agreement with the E_{T1} values of the enzymes. MET based bioelectrocatalytic currents were higher for OB-1 modified electrodes compared to GreeDo bioelectrodes, following the trend for homogeneous catalysis in the case of substrates with redox potentials below the E_{T1} . High operational stability of GreeDo was registered in 0.1 M acetate buffer, pH 4.0, in agreement with the high storage stability of the enzyme in acidic solutions.

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Table 1. The T1 site redox potentials and open circuit potential values for graphite electrodes modified with GreeDo mutant and OB-1 parental type Lcs in O₂ saturated electrolytes, as well as the equilibrium redox potentials of O₂/H₂O couple at the same conditions.

	E_{T1}, pH 6 (V)	OCP, pH 4.0 (V)	OCP, pH 6.0 (V)	OCP, pH 8.0 (V)
GreeDo	0.79	0.89	0.79	0.76
OB-1	0.74	0.86	0.75	0.70
O₂/H₂O	-	0.99	0.88	0.76

Figure legends

Fig. 1. CVs of biomodified GEs in O₂ saturated (solid lines) and Ar saturated (dashed lines) electrolytes recorded at a scan rate of 0.025 V s⁻¹. (A) GreeDo (red curves) and OB-1 (black curves) modified electrodes in 100 mM acetate buffer, pH 4.0, containing 150 mM Na₂SO₄. (B) GreeDo (red curves) and OB-1 (black curves) modified electrodes in 100 mM phosphate buffer, pH 8.0, containing 150 mM Na₂SO₄.

Fig. 2. CVs of biomodified AuNP/GEs in O₂ saturated 50 mM acetate buffer, pH 4.0, containing 100 mM NaClO₄ recorded at a scan rate of 0.025 V s⁻¹. (A) GreeDo modified electrodes in the absence (solid line) and presence (dashed line) of 50 mM NaF. (B) GreeDo modified electrodes in the absence (solid line), presence (dotted line) of 0.1 mM ABTS without NaF, and after the addition of 50 mM NaF (dashed line). (C) OB-1 modified electrodes in the absence (solid line) and presence (dashed line) of 50 mM NaF. (D) OB-1 modified electrodes in the absence (solid line) and presence (dotted line) of 0.1 mM ABTS without NaF, as well as after the addition of 50 mM NaF (dashed line).

Fig. 3. Open circuit potential measurements of GreeDo/GEs in O₂ saturated electrolytes before (dashed lines) and after (solid lines) applied potential of 0.3 V. (A) pH 4.0. (B) pH 8.0. Inserts: Amperometric responses recorded at 0.3 V.

Fig. 4. Operational stability of GreeDo in solution (dotted line with squares) and GreeDo immobilized on GEs (solid line) measured at pH 4.0.

Figure 1

Figure 2

Figure 3

Figure 4

IV

Membrane-free and mediator-less high voltage glucose/oxygen electric power biodevices

**Olga Aleksejeva ^a, Chiara Di Bari ^b, Elena Gonzalez-Arribas ^a, Antonio L. De Lacey ^b,
Marcos Pita ^b, Roland Ludwig ^d, Victor Andoralov ^e, and Sergey Shleev ^{a,f,*}**

^a *Biomedical Science, Malmö University, 20560 Malmö, Sweden*

^b *Instituto de Catálisis y Petroleoquímica, 28049 Madrid, Spain*

^c *Medical Imaging and Physiology, Skåne University Hospital, 205 06 Malmö, Sweden*

^d *BOKU–University of Natural Resources and Applied Life Sciences, A-1190 Wien, Austria*

^e *KEMET Electronics AB, 563 31 Gränna, Sweden*

^f *A.N. Bach Institute of Biochemistry, 119071 Moscow, Russia*

* Corresponding author.

E-mail address: sergey.shleev@mau.se (S. Shleev).

The fabrication of biological electrochemical power sources has seen an increasing interest due to their many niche applications, e.g., for powering portable and implantable electronic devices. Hybrid devices, so-called bio-supercapacitors, typically consist of an electrochemical capacitor and a biological fuel cell. In contrast to conventional biological fuel cells, which deliver low currents operating continuously, self-charging bio-supercapacitors can provide high electric currents in a pulsed mode. However, one of the major obstacles that has prevented a more widespread application of biological electrochemical power sources in general and enzymatic supercapacitors in particular, is their low operating voltage of less than 0.5 V. Here, we report on a multi-cell single-electrolyte glucose/oxygen enzymatic supercapacitor with adjustable open-circuit and operating voltages that do not depend on the difference in equilibrium redox potentials of the two redox couples, gluconolactone/glucose and oxygen/water (1.18 V), but on the number of half-cells connected in series. With seven bio-supercapacitors operating in one electrolyte and connected in series, we achieved an operating voltage of 3 V, more than twice the thermodynamic limit. Moreover, we show that the glucose/oxygen biodevice can provide electrical power at an operating voltage exceeding the maximal limiting open-circuit value of 1.24 V in the case of complete glucose oxidation. We anticipate our work to serve as a starting point for new designs of simple and low cost biological power sources that are capable of reaching the necessary operating voltage and power output to supply portable electronic devices without the need for step-up converters or energy managing systems. Given the market size of 3V electronic devices, this could truly lead to significant advancements in our continued strive for emissionless and renewable energy sources.

Fuel cell (FC) technology relies on the direct conversion of chemical energy into electrical energy and has gained much attention as an alternative power generator in our continued strive for emissionless and renewable energy sources ¹. Enzymatic FCs (EFCs) utilise biocatalytic features of “green” catalysts, *viz.* enzymes, instead of abiotic catalysts ²⁻⁴. To a first approximation, they function just like conventional FCs in that a fuel is oxidised at the anode, the electrooxidatively released electrons flow through an outer electrical circuit to the cathode where they combine with an oxidant ^{5,6}. Most EFCs utilise glucose as biofuel and molecular oxygen (O₂) as biooxidant. The glucose oxidation reaction by O₂ is well-known (Eq. 1), since it is the same reaction that sustains aerobic life:

Voltage is one of the main characteristics of an EFC, specifically, its open-circuit voltage (OCV) and its operating voltage at maximal power output (OV). It follows from thermodynamics that the maximal OCV of a single electrochemical cell, including an EFC, cannot exceed the value established by the standard Gibbs free energy of a spontaneous redox reaction. For glucose/O₂ devices this is 1.24 V (Eq. 1) ⁷. Moreover, for two-electron glucose oxidation the maximal OCV of an EFC is even lower, 1.18 V (Eqs. 2 and 3), which results from the difference between the equilibrium redox potentials of the two redox pairs (pH 7.0): -0.36 V for gluconolactone/glucose and 0.82 V for O₂/H₂O ⁸.

It is common knowledge that the output voltage cannot be increased by simply connecting several electrochemical cells in series in a single electrolyte as they would short-circuit. Nevertheless, a recent pioneering study presented a biodevice consisting of two glucose/O₂ EFCs connected in series and operating in the same electrolyte with an OCV equal to 1.35 V, which is above the thermodynamic limit (*vide supra*)⁹. However, the study neither presented a possible mechanism to explain the observed voltage increase nor did the authors explain more than a doubling of the OCV. Moreover, the authors did not discuss the possible limitations of their biodevices but claimed that their design could be used to stack an unlimited amount of EFCs to reach any voltage and power necessary to operate portable electronic devices⁹. Their findings are even more surprising considering that they used cellulose membranes along with a mediator on the anodic side. The use of such redox mediators typically results in low voltages because of significant differences between the redox potentials of half-reactions and mediators and the additional overpotentials needed to drive the electrochemical processes at significant rates⁶.

Here, we present a novel glucose/O₂ multi-cell biodevice that can achieve high OCVs and OVVs without the need for mediators or membranes. These biodevices are built from several enzymatic supercapacitors (ESCs) that combine the capacitive and charging components in a single module and are thus able to combine the advantages of both an EFC and an electrochemical capacitor¹⁰. By performing a detailed electrochemical analysis of both, the individual enzymatic electrodes and the fully assembled self-charging biodevice, we are able to explain and visualize (Fig. 1, Supporting Movie S1) the mechanism behind the voltage and current increases when several devices are connected in series in the same electrolyte.

Manufacturing highly capacitive bioelectrodes

Recently, EFCs have been integrated with supercapacitors to form hybrid biodevices¹¹, *i.e.*, supercapacitive biological FCs/self-charging bio-supercapacitors^{12,13}. The capacitive part of these biodevices can be based on highly porous materials that store charges in a form of electrical double layer capacitance¹². Alternatively, the capacitive part can also be built from abiotic or biotic organic materials that store the charges through both non-faradaic and faradaic redox reactions; examples include redox proteins¹⁴⁻¹⁶, Os-complex based redox polymers¹⁷⁻¹⁹, and conducting organic polymers^{13,20}. To obtain highly capacitive bioelectrodes that possess both a pseudo- and double-layer capacitance, we fabricated tubular graphite electrodes (TGEs) by cutting graphite rods into 1 cm pieces. The TGEs were modified with poly(3,4-ethylenedioxythiophene) (PEDOT), a conducting organic polymer, as described in the Materials and Methods section (Fig. 2). From scanning electron microscopy (SEM) images we were able to determine the thickness of the resulting conductive polymer layer as *ca.* 12 μm (Fig. 2c). Axial through holes of 1 mm were drilled through both the PEDOT/TGEs and low capacitive bare TGEs that are used for control measurements.

Both the low and high capacity TGEs were biomodified with the same biocatalysts, namely *Neurospora crassa* cellobiose dehydrogenase (CDh) for the anode and *Trametes hirsuta* laccase (Lc) for the cathode. We connected three CDh/PEDOT/TGEs and three Lc/PEDOT/TGEs via plastic tubes through which a fuel-rich (50 mM glucose) electrolyte (50 mM acetate buffer, pH 4.2) with oxidising agent (1.2 mM O_2) was being pumped at a flow rate of 0.09 ml s^{-1} causing the electrodes to charge. Measurements of the OCV between the outermost electrodes of our membrane-free and mediator-less ESCx3 based biodevices yielded an average value of 0.60 V $\pm$ 0.05 V (Figs. 1a and 3a). Once the devices were electrically connected in series OCV increased to 1.78 V (Figs. 1b and 3a). Despite the use of a single electrolyte, the obtained OCV for the chain obeyed Kirchhoff's second law and resulted in a

tripling of the individual OCVs. With all three ESCs connected in series, we applied a potential of 1.25 V and recorded the amperometric response. The initial current was 2.3 μA but current output decreased with time (Fig. 3a), resembling the properties of supercapacitors rather than FCs. Electrochemical impedance spectroscopy (EIS) was performed by applying potentials to the outermost electrodes equal to the previously measured OCVs, *i.e.*, 0.60 V when disconnected and 1.78 V once all three cells were electrically connected in series. The respective Nyquist plots show that the apparent solution resistance decreased once all intermediate ESCs were connected, while the fits confirmed the decrease in both charge transfer and solution resistance (Fig. 3b). The tubular configuration of the biodevice along with the low ionic strength electrolyte resulted in a high internal resistance of several hundred $\text{k}\Omega$, which was significantly reduced upon connecting the two inner ESCs. The capacitance of the ESCx3 biodevice should follow Kirchoff's law for capacitors that are connected in series, namely $1/C_{(\text{ESCx3})} = 3/C_{(\text{ESCx1})}$. From EIS measurements we obtained $C_{(\text{ESCx1})} = 66.3 \mu\text{F}$ which should yield $C_{(\text{ESCx3})} = 22.1 \mu\text{F}$. Our measurements yielded $C_{(\text{ESCx3})} = 29.9 \mu\text{F}$ which is close to the theoretical prediction (Fig. 3b, embedded table).

We used the same flow system to investigate CDh/PEDOT/TGEs and Lc/PEDOT/TGEs separately, using a tubular silver rod as an Ag/AgCl reference electrode (Supporting Fig. S1). Measurements of the OCVs at the bioanodes and cathodes yielded $-0.02 \pm 0.02 \text{ V}$ and $+0.61 \pm 0.02 \text{ V}$, respectively. This suggests that the OCV previously obtained values at the outermost electrodes of the ESCx1 and ESCx3 based biodevices of about 0.60 V were reasonable. Both, the resistance and capacitance values of the separate bioelectrodes agree with the numbers obtained for ESCx1 (*cf.* table insets in Figs. 3b and S1).

Comparative potentiometric investigations at zero current were performed for ESCx3s consisting of CDh/PEDOT/TGEs and Lc/PEDOT/TGEs, as well as for EFCx3s made of CDh/TGEs and Lc/TGEs in order to evaluate the impact of the conductive polymer on the

output of the biodevice. The response was initially recorded at the outermost electrodes of the tubular system. Once the inner cathode/anode pairs were electrically connected, OCV increased rapidly and reached a value of 1.78 V. While this value remained relatively stable in the ESCx3s (Fig. 4a and Supporting Fig. S2), in the EFCx3s the initial OCV increase was followed by an almost immediate decrease to 0.85 V (*cf.* red and black curves in Fig. 4a).

By performing a similar electrochemical analysis of ESCx3 in a conventional open cell filled with the stagnant electrolyte, we found that upon connecting the inner cells, there was only a minor increase of OCV of *ca.* 0.05 V (Fig. 4a). EIS of the ESCx3s indicated a decrease of the total resistance upon connecting the inner electrodes. Upon connection, the capacitance increased significantly because both the inner and outer parts of the electrodes were immersed in the electrolyte (Fig. 4b).

The maximal electric power generated by the ESCx1 was 1.6 μ W at a voltage of 0.40 V (Fig. 5a). EFCx1, *i.e.*, the biodevice without conductive polymer layer, delivered 0.95 μ W of maximal power output at a voltage of 0.31 V. Thus, the ESC achieved a 1.6 times higher maximal power output compared to the EFC, which highlights the importance of the conductive polymer for the performance of biodevices.

In order to determine the maximal voltage of multi-cell ESC based glucose/O₂ biodevices, we recorded the potentiometric responses for a biodevice consisting of seven ESCs connected in series. Immediately after electrically connecting the six inner anode/cathode pairs, the voltage reached about 3.0 V (Fig. 5b). Hence, for this ESCx7 based biodevice the total voltage did not quite reach the sum of the individual voltages. Moreover, the initial value of 3.0 V could not be maintained but decreased to about 2.5 V within about 15 min (Fig. 5b), mostly due to water electrolysis which occurs when bioelectrodes are subject to high voltages.

Conclusions

This study presented glucose/O₂ biodevices composed of enzymatic supercapacitors (ESCs) operating in series in the same electrolyte. When applying an external voltage, they were capable of generating a μW electric power at voltages that exceeded the thermodynamic limits of 1.18 V for the glucose/O₂ pair, in the case of two-electron glucose oxidation, and even 1.24 V for the complete glucose oxidation. Owing to redox enzymes, biocatalysts *par excellence*, individual bio-supercapacitors operating in a single electrolyte through the bioelectrocatalytic oxidation of glucose at the bioanodes and the reduction of O₂ at the biocathodes (Supporting Movie M1), yielding an OCV increase from 0 V up to 0.60 V (Fig. 1a). By connecting three cells in series, the OCV value increased from 0.60 V to 1.78 V, *i.e.*, it reached the sum of all three contributing cells. Although the electrochemical cells were connected and short-circuit currents (i_{sc}) flowed between the bioanodes and -cathodes (Fig. 1b), a depolarisation of the inner ESC electrodes did not occur. This was due, in part, to the high capacitance of the bioelectrodes (*ca.* 60 μF), and also because of the high solution resistance (*ca.* 60 k Ω) that kept the resulting i_{sc} small enough (at *ca.* 10 μA) so it could be compensated, at least in part, by the on-going bioelectrocatalytic reactions (Eqs. 2 and 3 for the bioanodes and cathodes, respectively) which yielded current outputs of about 7 μA . The EFCx3 based biodevice could deliver an initial electrical power of 2.9 μW at an operational voltage of 1.25 V (Fig. 1c). However, the current output steadily decreased because the self-charged ESCs were discharging with time, both via i_{sc} and the load current, i_{lc} (Fig. 3a).

Depending on the fuels and oxidants used, several niche applications can be envisaged for these new biodevices, *e.g.*, for powering portable and implantable electronic devices. A similar approach can be used to design high voltage biodevices operating in aqueous solutions

containing different alcohols, lactate, ascorbate, or other energy-rich compounds. The potential of such high-voltage multi-cell biodevices in combination with these biofuels cannot be overestimated since they have an equilibrium redox potential that is significantly lower than with glucose, which would mean lower OCVs and OV_s from single-cell ESCs. A common limitation for all high voltage biological power sources, irrespective of the type of biofuel used, is the particular voltage for the electrolysis of water which depends on the electrocatalytic properties of the biodevice's electrodes and the density of the enzymatic layers after biomodification. When using biofuels that are electrocatalytically active, *e.g.*, ascorbate, additional limitations may be imposed by the electrooxidation of these biofuels at the cathodes, which results in electrode depolarisation and a decrease in the OCV. Nevertheless, we believe that membrane-free and mediator-less high voltage electric power biodevices based on ESCs hold great promise for the future.

Methods

Chemicals and materials. Na₂HPO₄·2H₂O, KH₂PO₄, NaCl, KCl, L-ascorbic acid, uric acid, sodium L-lactate, xanthan gum, LiClO₄, aniline (99.5 %), H₂SO₄ (99.9 %), PSS (poly(4-styrene sulphonic acid)), PEG (poly(ethylene glycol)), glycerol (99.5 %), CH₃COONa, acetic acid (99.9 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-glucose was obtained from KEBO AB (Stockholm, Sweden). Argon (Ar) and oxygen (O₂) gases were supplied by AGA Gas AB (Sundbyberg, Sweden). All chemicals were of analytical grade and used without further purification. All solutions were prepared using water purified with the PURELAB UHQ II system from ELGA Labwater (High Wycombe, UK). Graphite rods (3.05 mm in diameter, 38.10 mm in length) were purchased from Alfa Aesar Ltd. (Karlsruhe, Germany). Silver rods (Ø 3 mm, 99.997% purity, 10.0 ± 0.5 mm length) was purchased from Goodfellow Cambridge

Ltd. (Huntingdon, England). Plastic tubes (3.0 mm in diameter) were obtained from Becton, Dickinson Co. (Franklin Lakes, NJ, USA).

Redox enzymes. *Trametes hirsuta* Lc with a protein concentration of 5.8 mg mL⁻¹ and a specific activity of 290 U mg⁻¹ (measured at pH 5.0 with ferrocyanide as electron acceptor) from basidiomycete (strain *T. hirsuta* 56 obtained from the laboratory collection of the Moscow State University of Engineering Ecology, Russia) was purified and isolated following the procedure previously reported ²¹. For the electrode biomodification, the enzyme preparation was diluted with 100 mM phosphate buffer (pH 6.5) to a final concentration of 3 mg mL⁻¹. *Neurospora crassa cellobiose* CDH with a protein concentration of 12 mg mL⁻¹ and a volumetric activity of 63 U mL⁻¹ (measured at pH 5.5 with cytochrome c as electron acceptor) was recombinantly expressed in the *P. pastoris* strain X-33 under control of the AOX promotor and purified to homogeneity following the procedure previously reported ²². Prior to biomodification, the enzyme was diluted with 50 mM acetate buffer (pH 4.2) to a final concentration of 3 mg mL⁻¹.

Fabrication, characterisation, and biomodification of electrodes. PEDOT was electrochemically synthesised on graphite rods using cyclic voltammetry prior to drilling, resulting in PEDOT modified TGEs (PEDOT/TGE) (Fig. 2). The electropolymerisation procedure was carried out as described previously ²³. In summary, the electropolymerisation solution consisted of 20 mM EDOT monomer, 1 mM PEG, and 0.1 M LiClO₄ in water. PEG was added to ensure better solubility of EDOT. The potential was scanned from 0.01 V to 1.51 V vs. NHE with a scan rate of 0.05 V s⁻¹. After electropolymerisation, the rods were carefully rinsed with water and dried. SEM images of PEDOT/TGEs were obtained using an EVO LS10 scanning electron microscope from Zeiss (Jena, Germany) equipped with a LaB₆ filament.

Imaging was done in high vacuum mode using a secondary electron detector set to 15 kV accelerating voltage and 50 pA probe current.

For fabrication of the biocathodes and bioanodes, 20 μ l of Lc and CDh solutions were injected into the tubular electrodes, TGE and TGE/PEDOT, and left for 30 min.

Biodevice construction and operation. To design ESCs, freshly prepared biomodified PEDOT/TGEs were incorporated into plastic tubes from Becton, Dickinson Co. (Franklin Lakes, NJ, USA), which were connected to a flask with the acidic electrolyte. The electrolyte was pumped through the system using a peristaltic pump from Minipuls 2 Instrument AB Lambda (Stockholm, Sweden). Electrodes were connected to a μ Autolab Type III/FRA2 potentiostat/galvanostat from MetrohmAutolab B.V. (Utrecht, The Netherlands) using a conductive foil Element14 from Farnell (Leeds, England). Cathodes (Lc/PEDOT/TGEs) served as working electrodes and anodes (CDh/PEDOT/TGEs) as reference/counter electrodes. When three (ESCx3) or seven ESCs (ESCx7) were placed in series, the electrical connection/disconnection of the inner cells was achieved via a tumbler switch from Kjell & Company (Sundsvall, Sweden).

To design EFCs, freshly prepared biomodified TGEs were incorporated into plastic tubes, which were connected to the flask with the electrolyte. Electrodes were connected to the potentiostat using a conductive foil. The cathode (Lc/TGE) served as the working and the anode (CDh/TGE) as a reference/counter electrode. When three complete EFCs were placed in series (EFCx3), the inner cells were electrically connected/disconnected via a tumbler switch from Kjell & Company.

The characterisation of biodevices and EDOT electropolymerisation were performed in a 50 ml electrochemical cell, carrying an Ag|AgCl|3M NaCl (0.21 V vs. NHE) reference electrode from Bioanalytical Systems (West Lafayette, IN, USA) and a platinum wire counter electrode.

Characterisation of biomodified PANI/TGEs was performed using a tubular custom built Ag|AgCl combined reference/counter electrode of 10.0 ± 0.5 mm length (0.075 V vs. NHE), made by drilling 1 mm holes into the silver rod. The internal surface of the Ag tubes was electrochemically covered with a layer of AgCl following the procedure described previously.

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OCVs of EBSCx3 and EBSCx7 were first measured at the edges of the tubular system, and only once the voltage had stabilised did we electrically connect the inner cells. For the measurements, the signal was let to stabilise before disconnecting the inner cells again.

Electrochemical impedance spectroscopy (EIS) was performed from 0.01 Hz to 10000 Hz with a 0.01 V amplitude. EIS spectra were registered at the edges of the biodevices both without and with connecting the inner cells.

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Author contributions

O.A., E.G.-A., and C.D.B. carried out all the experiments. R.L. isolated, purified, and characterised redox enzymes. V.A. performed the EIS modelling studies. A.L.D.L., M.P., V.A., and S.S. discussed the results, analysed the data, and formulated the conclusions. O.A. and S.S. prepared the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at

Figures and Figure Legends

Figure 1 | Schematic representation of the operating mechanism of the glucose/O₂ ESCx3 based biodevice. a, OCV measurement with disconnected inner cells. **b**, OCV measurement with connected inner cells. **c**, Amperometric measurement with an applied potential of 1.25 V, while inner cells are connected. i_{lc} – load current, i_{sc} – short-circuit current.

Figure 3 | Potentiometric and amperometric responses, as well as EIS spectra of the glucose/O₂ biodevices. **a**, Typical potentiometric response from ESCx3 (black curve, left ordinate axis) with the arrow indicating the time when the inner cells were electrically connected. The amperometric response (red curve, right ordinate) at an applied potential of 1.25 V, while inner cells are connected. **b**, Nyquist plots of ESCx1 and ESCx3 recorded in the flow system with connected and disconnected inner cells. Inserts: Schematic representations of the biodevice connections, equivalent circuits, and values obtained from data fitting.

Figure 4 | Typical potentiograms and EIS spectra with flowing and stagnant electrolyte.

a, Typical potentiograms for ESCx3 and EFCx3, both with flowing electrolyte, and for ESCx3 in an open cell with a stagnant electrolyte (*). Arrows indicate the times when the inner cells were electrically connected (solid arrow stem) and disconnected (dotted stem). **b**, Nyquist plots for ESCx1 and ESCx3 recorded in the open cell with stagnant electrolyte (*) in the connected and disconnected states. Inserts: schematic representation of the tests, equivalent circuits, and values obtained from data fitting.

Figure 5 | Typical electrical outputs for a single cell biodevice and a typical potentiometric response for a 7-cell biodevice. a, Typical electrical outputs from ESCx1 and EFCx1. **b,** Typical potentiogram for ESCx7. Arrows indicate when inner cells were electrically connected (solid arrow stem) and disconnected (dotted stem).

Supporting Information

Membrane-free and mediator-less high voltage glucose/oxygen electric power biodevices

**Olga Aleksejeva ^a, Chiara Di Bari ^b, Elena Gonzalez-Arribas ^a, Antonio L. De Lacey ^b,
Marcos Pita ^b, Roland Ludwig ^d, Victor Andoralov ^e, and Sergey Shleev ^{a,f,*}**

^a *Biomedical Science, Malmö University, 20560 Malmö, Sweden*

^b *Instituto de Catálisis y Petroleoquímica, 28049 Madrid, Spain*

^c *Medical Imaging and Physiology, Skåne University Hospital, 205 06 Malmö, Sweden*

^d *BOKU—University of Natural Resources and Applied Life Sciences, A-1190 Wien, Austria*

^e *KEMET Electronics AB, 563 31 Gränna, Sweden*

^f *A.N. Bach Institute of Biochemistry, 119071 Moscow, Russia*

Supporting Figure S1 | EIS studies of separate bioelectrodes. Nyquist plots of CDh/PEDOT/TGEs and Lc/PEDOT/TGEs.

Supporting Figure S2 | Stability test of ESCx3. Arrow indicates the moment when inner cells were electrically connected.

Supporting Movie M1 | Dynamical visualisation of the operating mechanism of a glucose/oxygen ESCx3 based biodevice.

V

A conventional symmetric biosupercapacitor based on rusticyanin modified gold electrodes

Elena González-Arribas^a, Magnus Falk^a, Olga Aleksejeva^a, Sergey Bushnev^{b,d}, Paula Sebastián^c, Juan M. Feliu^c, Sergey Shleev^{a,b,d,*}

^a Malmö University, 20506 Malmö, Sweden

^b National Research Center “Kurchatov Institute”, 123 182 Moscow, Russia

^c Institute of Electrochemistry, University of Alicante, 03080 Alicante, Spain

^d A. N. Bach Institute of Biochemistry, 119071 Moscow, Russia

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ABSTRACT

Here we report on an entirely new kind of bioelectronic device – a conventional biosupercapacitor, which is built from copper containing redox proteins. Prior to biodevice fabrication, detailed spectroelectrochemical studies of the protein, viz. *Acidithiobacillus ferrooxidans* rusticyanin, in solution and in adsorbed state, were performed, including estimation of the redox potential of the T1 site (0.62 V vs. NHE), protein midpoint potential when adsorbed on a self-assembled monolayer (0.34 V vs. NHE), as well as biocapacitance of rusticyanin modified gold electrodes ($115 \mu\text{F cm}^{-2}$). The symmetrical biosupercapacitor based on two identical gold electrodes modified with rusticyanin is able to capacitively store electricity and deliver electric power accumulated mostly in the form of biopseudocapacitance, when charged and discharged externally. When charged during just 5 s, the biosupercapacitor with a total capacitance of about $73 \mu\text{F cm}^{-2}$ provided a maximum of $4 \mu\text{A cm}^{-2}$ peak current at 0.40 V. The biodevice, which can be charged and discharged at least 50 times without a significant loss of ability to store electric energy, had a low leakage current below 50 nA cm^{-2} .

1. Introduction

Recently an entire new class of bioelectronics devices, viz. self-charging biosupercapacitors, has been disclosed [1]. These hybrid biodevices are able to convert different types of energies into electric energy and simultaneously store the electric power in the same volume used for conversion. Nowadays chemical [2–7] and solar [8,9] biodevices are known. In chemical biosupercapacitors redox active proteins with catalytic activities (i.e. redox enzymes), electronically wired to the electrode surface, are used to accelerate energy transformation, whereas their potential biosupercapacitive properties do not play an actual role in biodevice function. Even though biosupercapacitive properties of redox proteins, i.e. faradaic contribution to the total electrode capacitance due to their intrinsic redox activities [10] attributed to quasi-reversible or even reversible electrochemistry of surface confined biomolecules [11,12], are well-known [1], a conventional biosupercapacitor, i.e. incapable of self-charging, in which the biopseudocapacitance is exploited (i.e. redox proteins serve as the pseudocapacitive component determining the total capacitance of the biodevice) has not been fabricated and characterized so far, as illustrated

below chronologically.

The fundamental concept of a conventional biosupercapacitor was demonstrated as early as 2012 by Malvankar and co-workers, using a bioanode which was able to store electrical charges in the cytochrome c network of a bacterial biofilm [13]. Moreover, due to the special design of the electrode, which consisted of two gold anodes separated by a non-conducting gap, successful galvanostatic charge/discharge measurements of the biodevice were performed. However, a biofilm with catalytic activities and thus ability for self-charging was exploited to store electrical charges. Moreover, the term “biosupercapacitors” was actually not used in this study, which nevertheless should be highly valued and considered as the pioneering report in the field. The term “biosupercapacitor” was used one year later by Sattarahmady with co-workers to emphasize the biocompatibility of a double-layer supercapacitor based on electrodes coated with a redox inactive protein – albumin [14]. Nowadays, because of significant interest in implantable bioelectronics in general, and implantable biosupercapacitors in particular [15], this technological approach is widely used to design biocompatible supercapacitors using different proteins including redox active molecules, like myoglobin, hemoglobin, and cytochrome c

* Corresponding author at: Malmö University, 20506 Malmö, Sweden.
E-mail address: sergey.shleev@mah.se (S. Shleev).

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[16,17]. In addition to biocompatibility, biodevices based on protein modified electrodes often show superior properties in term of total capacitance [16,17], unexpectedly, even when redox inactive proteins, like albumin and fibronin, are used [14,18]. Of course, redox activity of proteins containing only amino acids is known for decades [19,20]. Moreover, it has recently been shown that redox active proteins are surprisingly good electric conductors, resembling solid-state semiconductors [21,22]. Nevertheless, it is commonly accepted that proteins, when adsorbed on electrode surfaces, significantly decrease total capacitance due to an insulating layer formed, when redox inactive proteins are used or when direct electron transfer reactions between redox active proteins and electrodes are not established [1,23]. Thus, despite the fact that several highly capacitive protein modified electrodes have already been disclosed, conventional biosupercapacitors (*i.e.* based on the biosupercapacitive properties of redox proteins) have not yet been realized. Here we demonstrate a complete functional conventional biosupercapacitor based on gold electrodes modified with a copper containing redox protein, rusticyanin (Rc). Whereas there are many papers regarding the electrochemistry of cupredoxins, *e.g.* azurin [24,25] and plastocyanin [26], as well as pseudoazurin, umecyanin, stellacyanin, and plantacyanin [27], only two electrochemical studies of Rc are reported in the literature, *i.e.* Refs. [28, 29]. While Alcaraz with co-workers carried out electrochemical investigations of the protein in solution [28], the only paper regarding electrochemistry of immobilized Rc, *viz.* Ref. [29], contains several contentious issues, which raise some doubts regarding the direct electric wiring of the protein to the electrode surface (*vide infra*). Thus, we begin our paper with detailed spectroelectrochemical studies of free and immobilized Rc, as the fundamental basis for its practical applications in bioelectronics.

2. Experimental

2.1. Chemicals and materials

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, KH_2PO_4 , NaCl, H_2SO_4 , $(\text{NH}_4)_2\text{S}_2\text{O}_8$, Whatman® cellulose DE52 and CM52, glutaraldehyde (grade II), mercaptopropionic acid (MPA), HAuCl_4 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, and indium tin oxide (ITO) conductive glasses ($15\text{--}25\ \Omega\ \text{sq}^{-1}$) were purchased from Sigma Aldrich (Steinheim, Germany). 99.5% ethanol was acquired from CCS Healthcare AB (Malmö, Sweden). Argon (Ar) and oxygen (O_2) were supplied by AGA Gas AB (Sundbyberg, Sweden). All chemicals were of analytical grade and used without further purification. All solutions were prepared using water (H_2O) purified with the PURELAB UHQ II system from ELGA Labwater (High Wycombe, United Kingdom). Polycrystalline gold (Au) disk electrodes with a geometric area of $0.02\ \text{cm}^2$ were from Bioanalytical Systems (Indiana, USA).

2.2. Redox protein

Acidithiobacillus ferrooxidans Rc was isolated and purified according to a previously described protocol [30], modified by replacing the cation exchange resin cellulose CM52 with CM32. The highly purified preparation stored at -23°C contained > 95% of Rc with a molecular mass of 17 kDa with a few minor protein impurities, as determined by SDS/PAGE (Supporting Fig. S1). The protein concentration equal to $16\ \text{mg mL}^{-1}$ was determined using two independent methods, *viz.* in accordance with methodology presented in Ref. [31], as well as using extinction coefficients provided in Ref. [30].

2.3. Electrode preparation

Gold disk electrodes (Au electrodes) were used as working electrodes in fundamental studies. Prior to surface modification, the electrodes were polished with $0.3\ \mu\text{m}$ agglomerated alpha alumina suspension from Struers (Copenhagen, Denmark), thoroughly cleaned with

ethanol and ultrapure H_2O , followed by sonication using the XB2 ultrasonic cleaner. After polishing, the Au electrodes were cleaned electrochemically by performing cyclic voltammetry (CV) for 25 cycles from $-0.2\ \text{V}$ to $1.7\ \text{V}$ vs. $\text{Ag}|\text{AgCl}||3\ \text{M NaCl}$ in $0.5\ \text{M H}_2\text{SO}_4$.

For surface modification, MPA was used to form a self-assembled monolayer (SAM). The cleaned Au electrodes were immersed overnight in $10\ \text{mM}$ MPA ethanol solution. The Au electrodes were then rinsed with ethanol and ultrapure H_2O . $1\ \mu\text{L}$ of glutaraldehyde solution (25% in H_2O) was drop casted onto the chemically modified electrode surface followed by $2\ \mu\text{L}$ of Rc solution. The Au electrodes were covered to prevent evaporation, left for 2 h at room temperature, and finally gently rinsed with ultrapure H_2O .

Au nanoparticles (AuNPs) modified ITO based electrodes were used to demonstrate the functionality of biosupercapacitors. To fabricate nanostructured electrodes, ITO conductive glasses were cut into chips of $10\ \text{mm} \times 25\ \text{mm} \times 1.1\ \text{mm}$. The following cleaning procedure involved sonication in ethanol and subsequently in methylene chloride, 15 min each, and additionally storing in ultrapure water for 20 min. 20 nm AuNPs were synthesized, concentrated, and characterized as previously described [32]. The dispersion of AuNPs was immersed in an ultrasonic bath for 20 min for an efficient homogenization. Afterwards, $5\ \mu\text{L}$ of the dispersion were dropped on the ITO chips and dried in air. Rc was immobilized on the nanostructured Au surface as mentioned just above. The geometric area of AuNPs modified ITO electrode (ITO-AuNPs) with immobilized protein (ITO-AuNPs-SAM-Rc) was measured to be ca. $0.07\ \text{cm}^2$.

The electrochemically active (real) area of AuNP/Au was calculated from the experimentally measured charge (q_{real}) associated with the gold oxide reduction process performed by running CV in H_2SO_4 . The theoretical charge density (σ_0) associated with the reduction of the gold oxide is $390 \pm 10\ \mu\text{C cm}^{-2}$ [33]. The electrochemically active (real) area of ITO-AuNPs was calculated to be $2.47\ \text{cm}^2$ (Supporting Fig. 2).

2.4. Redox titration

The redox potential of the T1 site of Rc was determined by mediated spectroelectrochemical redox titration following the procedure reported previously [34,35]. The home-built spectroelectrochemical cell consisted of a 1 cm long capillary Au electrode with an inner diameter of $350\ \mu\text{m}$, serving both as the working electrode and as a cuvette with a total volume of just $1\ \mu\text{L}$. The input and output optical fibers (FCB-UV 400/050-2 and FCUV 200, respectively) were purchased from Ocean Optics (Dunedin, Florida, USA) and attached to the ends of the capillary. The system comprised a DH-2000 light source and an HR4000CG-UV-NIR spectrometer from Ocean Optics. The spectra were recorded with the Ocean Optics Spectra Suite software. The potential of the Au capillary electrode was controlled using a three-electrode potentiostat (Zata Electronics, Lund, Sweden). A platinum wire served as a counter electrode, and a $\text{Ag}|\text{AgCl}||3\ \text{M NaCl}$ electrode, separated from the enzyme solution by two ceramic frits and a buffer solution salt bridge, was used as a reference electrode. The working capillary Au electrode was cleaned for ca. 24 h in freshly prepared 3:1 (v:v) 96% sulfuric acid: 30% hydrogen peroxide solution (piranha). For enhancement of the electrochemical communication between the electrode and the enzyme molecules, the reduced forms of three redox mediators, $\text{K}_4[\text{Fe}(\text{CN})_6]$, $\text{K}_4[\text{W}(\text{CN})_6]$ and $\text{K}_4[\text{Mo}(\text{CN})_6]$, were used. The final concentration of each compound in the cell was $2\ \text{mM}$, and their midpoint redox potentials were 0.43, 0.52 and $0.78\ \text{V}$ vs. NHE [36].

2.5. Electrochemical measurements and characterization

Electrochemical impedance spectroscopy (EIS), CV, and chronoamperometric charge/discharge procedures were performed using a $\mu\text{Autolab Type III/FRA2}$ potentiostat/galvanostat from Metrohm Autolab B.V. (Utrecht, The Netherlands). The reference and counter electrodes were a $\text{Ag}|\text{AgCl}||3\ \text{M NaCl}$ and a Pt wire, respectively. All

electrochemical measurements were performed in 50 mM phosphate buffer (PB) pH 7.4, purged with Ar in order to remove O_2 . EIS spectra of bare Au electrodes (Au), MPA modified Au electrodes (Au-SAM), and Rc modified Au electrodes with immobilized MPA (Au-SAM-Rc) were recorded in 50 mM PB, 0.1 M KCl, pH 7.4, in the presence and absence of 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) redox couple from 0.1 Hz to 70 kHz with 10 mV amplitude at 0.43 V (the formal potential of $[Fe(CN)_6]^{3-/4-}$ redox couple) and 0.15 V (the redox potential of Rc), using ZView software from Scribner Associates Inc. (North Carolina, USA) to fit the data. Potentiostatic charge-discharge cycles of Au-SAM-Rc based bio-supercapacitors were performed using amperometry and two symmetric electrodes, one as the working electrode and the other as a combined reference/counter electrode. The potential steps used were 0 V and 0.4 V. Different times between pulses were applied, *i.e.* 5 and 40 s, respectively.

A single potential amperometry was employed to charge a biosupercapacitor based on two ITO-AuNPs-SAM-Rc electrodes at a constant potential of 0.4 V during 60 s. Chronopotentiometry was used to monitor the OCV of the biosupercapacitor at zero current after charging and while discharging, when connecting a resistor from Velleman Inc. (Forth Worth, TX, USA) or a low voltage liquid crystal display from RISE Acreo (Kista, Sweden) to the circuit.

3. Results and discussion

Electrochemical capacitors, also known as supercapacitors, are used for rapid power delivery and recharging (*i.e.*, high power density) [37]. Following this definition, as well as taking into account the presence of self-charging biosupercapacitors (*vide supra*), the “conventional biosupercapacitors” term should be used for biodevices with high biocapacitance and low double-layer capacitance, and which can be rapidly charged and discharged externally. Below a conventional symmetric biosupercapacitor based on Rc modified gold electrodes is presented.

Rc is a rather small (*ca.* 16 kDa) blue copper (Cu) containing protein. Thus, by immobilizing the protein at the electrode surface, *e.g.* modified Au, the Cu ion should be directly accessed electronically (Fig. 1). In this case, Rc can be utilized as the biocapacitive component of the conventional biosupercapacitor, since the charge will be

primarily stored at the T1 Cu site (faradaic term, additional details can be found in Ref. [10]), with protonation/deprotonation of accessible residues (non-faradaic term [37]) contributing to a lesser extent to the overall capacitance. However, as mentioned above, there is only one report in the literature regarding electrochemistry of immobilized Rc [29]. In that report, quantum dots modified electrodes were used to obtain a direct quasi-reversible electrochemical reaction of surface confined protein. Below a direct quasi-reversible electrochemistry of Rc on planar (*i.e.* without additional nanostructuration) Au electrodes is described.

The Au-SAM-Rc electrodes were first characterized individually using CV and EIS, and the results were compared with the data using MPA modified electrodes. Fig. 2 shows typical cyclic voltammograms of Au, Au-SAM, and Au-SAM-Rc electrodes. As expected, upon surface modification with MPA the capacitance is reduced from $50 \mu F cm^{-2}$ down to $14 \mu F cm^{-2}$. Contrary to previous studies of the immobilized protein, in which cathodic peaks of bare electrodes (Au and ZnS quantum dots modified Au electrodes without Rc) were observed with peak potentials and currents close to the cathodic peak of the immobilized protein (Fig. 4a in [29]), no pronounced electrochemical signals on non-biomodified electrodes were registered by us (Fig. 2). However, after modification with Rc, well-pronounced redox peaks from the T1 Cu site of the protein are observed. Also, in contrast to the previous study of the immobilized protein [29], in which huge peak-to-peak separation (*ca.* 0.35 V) was observed along with completely different shapes and incommensurable areas of the cathodic and anodic peaks, a quasi-reversible direct electron transfer based electrochemistry of Rc (peak-to-peak separation of about 0.08 V only) were registered in the present work (Fig. 2).

A surface coverage of $0.73 nmol/cm^2$ could be estimated from integration of the area of the reduction peak, corresponding to a complete densely packed multi-layer of electronically addressable Rc on the electrode surface. Modification with Rc dramatically increased the total capacitance of MPA modified Au electrodes, from $14 \mu F cm^{-2}$ to $146 \mu F cm^{-2}$, where a majority of the capacitance can be attributed to the faradaic process related to the redox transformation of Rc ($115 \mu F cm^{-2}$, Fig. 2b). Thus, modification with the redox protein increased the capacitance of the MPA-Au system more than ten-fold.

Fig. 1. Schematic representation of conventional symmetric biosupercapacitor based on Rc modified gold electrodes. The structure of Rc is visualized using known crystal structure of the proteins (PDB file 1RCY). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Cyclic voltammograms of Au electrodes submerged in 50 mM PB pH 7.4 recorded with a scan rate of 20 mV s^{-1} , showing the contribution of pseudo (blue) and double-layer (grey) capacitances into the total capacitance of bio-modified electrodes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In previous studies, the midpoint potential of the immobilized protein was found to be 0.88 V vs. NHE at pH 6.86 [29], i.e. $0.20\text{--}0.26 \text{ V}$ higher compared to the redox potential of Rc in solution ($0.62\text{--}0.68 \text{ V}$) [28,38]. Such a high value is unexpected, considering especially the equilibrium redox potential of $\text{O}_2/\text{H}_2\text{O}$ couple, 0.82 V , under these conditions. Thus, the anodic peak reported in Ref. [29] could be attributed to H_2O electrooxidation on biomodified electrode surfaces rather than to the electrooxidation of the T1 site of the protein. This suggestion regarding the different origin of the cathodic and anodic processes could be also used to explain the different temperature dependency of peak potentials observed by the authors: while the potential of the cathodic peak was highly dependent on temperature, the anodic peak remained unchanged when the temperature was increased from 18°C to 43°C [29]. Contrary to previous studies, in present work the potential of the T1 Cu site of surface confined Rc has been estimated to be 0.34 V vs. NHE , which is significantly lower than values previously reported for Rc in solution.

One could speculate that the immobilization of the protein destabilizes the coordination to the Cu ion. Indeed, for unfolded Rc one of the histidine ligands to the copper center has been observed to detach, lowering the redox potential by 0.1 V [28]. Thus, the protein's coordination to the carboxylated SAM along with covalent linkage of the protein on the electrode surface may result in a lowering of the redox

Fig. 3. Mediated redox titration of Rc. a) Potentiometric titration curve. Insert: Nernst plot of the dependence of the applied potential on the absorbance at 597 nm and parameters calculated from the titration. b) Spectra from the titration, corresponding to oxidized Rc (0.90 V), partly oxidized protein (0.65 V and 0.60 V), and fully reduced Rc (0.45 V).

potential. To confirm the fact that a significant shift of redox potential of Rc is solely attributed to the protein immobilization, spectro-electrochemical studies of Rc in solution were performed, as described below.

The redox potential of the T1 Cu site of Rc was accurately measured by mediated redox titration following a previously developed procedure [34]. Least-squares linear regression analysis of the 597-nm Nernst plots provided a midpoint potential (E_m) value of 0.63 V vs. NHE and a slope of 0.064 V (Fig. 3A, insert), which implies a number of electrons (n) equal to 0.92 . The linear correlation coefficient was high, 0.998 . In addition to the least-squares regression analysis, the titration curves were also analyzed by direct data fitting. In this case, even better results were obtained, as presented in Fig. 3. Thus, the measured potential of Rc in solution coincide with previously reported values; indeed, ca. 0.3 V shift is attributed to a conformational change of the protein due to its immobilization on the electrode surface.

The Au-SAM-Rc electrodes were further characterized by EIS, an effective method to monitor the changes in double layer capacitance (C_{dl}) and charge transfer resistance (R_{ct}) induced by the electrode modification. Fig. 4b shows the impedance responses of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox couple in 50 mM PB , 0.1 M KCl pH 7.4 on bare electrode (Au), MPA modified electrode (Au-SAM), and MPA modified

Fig. 4. EIS studies of Au electrodes in 50 mM PB containing 0.1 M KCl, pH 7.4, in the presence of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ couple. a) Equivalent circuit model used to perform theoretical fits for the EIS data. b) Nyquist plot of different electrodes at the midpoint potential of the redox couple. The inset shows the expansion of the high frequency range.

electrode with immobilized Rc (Au-SAM-Rc). The impedance spectra were fitted in the equivalent circuit shown in Fig. 4a. Supporting Table S1a shows the numeric values obtained from the fitting of each spectrum. As evident from the data, modification of Au electrodes with MPA did not affect solution resistance (R_s), whereas this parameter was slightly affected by Rc immobilization. The formation of the SAM decreased R_{ct} from 288 down to 172 Ω , which can be attributed to the presence of $-\text{OH}$ groups on the bare Au surface that repelled the negatively charged redox couple. After Rc immobilization, R_{ct} increased up to 8500 Ω , since Rc forms an insulating layer on the electrode surface. A slightly higher C_{dl} value was obtained for bare Au electrode since the SAM seems to insulate the electrode surface as well, which is in good agreement with the results obtained by CV (Fig. 2a). For the electrode with immobilized Rc, the highest C_{dl} value was obtained. The Warburg impedance (Z_w) increased significantly, viz. from 8295 for Au to 10,105 for Au-SAM and to 37,751 $\Omega \text{ s}^{1/2}$ for Au-SAM-Rc, as the resistance for the diffusion of the redox species from the bulk electrolyte to the electrode surface increased.

The Au-SAM-Rc electrodes were additionally characterized by EIS in the absence of the redox mediator (Supporting Fig. S3). R_{ct} , which is inversely proportional to the rate of electron transfer, notably differ for Au-SAM and Au-SAM-Rc (Supporting Table S1b), viz. the value obtained for Au-SAM is more than 10 times higher than for Au-SAM-Rc electrode. Since EIS measurements were performed at the redox potential of Rc, the lower R_{ct} value reflects a higher rate of electron transfer, as expected. As for C_{dl} , a higher value is obtained for the electrode modified with Rc in accordance with the capacitive feature of the Au-SAM-Rc electrodes due to the contribution of the T1 copper center of Rc to the faradaic charge storage.

After detailed (spectro)electrochemical characterizations, two Au-SAM-Rc electrodes were combined to assemble a symmetric conventional biosupercapacitor. The biodevice was investigated using chronoamperometric charge/discharge procedure (Fig. 5). The biosupercapacitor was charged at 0.4 V to allow the complete oxidation/reduction of the accessible Cu ions, and displayed a rapid charge/discharge

Fig. 5. Chronoamperometric studies of the conventional symmetric biosupercapacitor based on two identical Au-SAM-Rc electrodes. Potential pulses at 0.4 V and 0, every 40 s (a) and every 5 s (b).

profile with good reproducibility with a low leakage current below 50 nA cm^{-2} . Initial currents of $5 \mu\text{A cm}^{-2}$ and even higher were obtained, when the conventional biosupercapacitor was tested every 40 s (Fig. 5a). The biodevice also showed quite good operational stability with no substantial decrease in the performance after 50 charge/discharge cycles (Fig. 5b). A total capacitance equal to $61 \mu\text{F cm}^{-2}$ was derived from the discharge curves, in good agreement with the CV measurements, since the total capacitance of an assembled capacitor is determined by the series connection of the two electrode and theoretically should be half of $146 \mu\text{F cm}^{-2}$, i.e. $73 \mu\text{F cm}^{-2}$. No conductive additives were used and the biodevice was almost solely based on the capacitive properties of the immobilized Rc, enabling a ten-fold increase in capacitance compared with an electrochemical capacitor without redox protein. On the one hand, examination of basic supercapacitor/rechargeable battery features of the designed electric power device [37] revealed that the biodevice has some typical characteristics of a “rechargeable battery”, e.g. a peak-shaped CV profile (Fig. 2). On the other hand, a setup with two identical electrodes (Fig. 1), the fast charging time and a well-pronounced current decrease during potentiostatic discharging (Fig. 5) substantiate the “supercapacitor” moniker for the biodevice.

Finally, to demonstrate the functionality of biosupercapacitors, high surface area nanostructured electrodes based on AuNPs were exploited

Fig. 6. Charge (black) and discharge (blue) curves for the biosupercapacitor based on two ITO-AuNPs-SAM-Rc. The first discharge curve was obtained using 500 k Ω external resistor and the second curve by connecting the biodevice to the flexible low voltage display. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Supporting Fig. 2). The biodevice was built from two ITO-AuNPs-SAM-Rc. The total charge (Q) equal to 39.2 μC , accumulated in the biosupercapacitors, was calculated by integration of the area contained in the discharge curve (Supporting Fig. S4). Fig. 6 shows the amperometric charge curve, demonstrating a fast charging process of the biosupercapacitors, and a discharge process, when connecting 500 k Ω resistor to the circuit. Another fast charging curve is also shown, followed by the second discharge curve, when connecting the liquid crystal display instead of the resistor (Fig. 6). Supporting Fig. S5 demonstrates the ability of the designed conventional biosupercapacitor to power real electronic device.

4. Conclusions

In summary, detailed spectroelectrochemical studies of MPA modified Au electrodes with immobilized Rc were performed. Quasi-reversible direct electron transfer based electrochemistry of immobilized protein was shown with the midpoint potential of the redox center of the protein significantly shifted from the potential of the T1 Cu site of Rc in solution. A pseudobiocapacitance/double layer capacitance ratio equal to 3.7 for Rc modified electrodes was achieved. Based on fundamental studies a conventional symmetric biosupercapacitors was realized. The biodevice, which could be externally charged and discharged at least 50 times with initial current densities as high as 5 $\mu\text{A cm}^{-2}$ without significant loss of electrochemical activity, had a low leakage current below 50 nA cm^{-2} . Employment of redox active proteins, i.e. *renewable and biodegradable molecules*, which can be potentially produced at *low costs*, to design biomaterials with superior properties, like “on-off” characteristics, high capacitance and *good biocompatibility*, allows creation of new bioelectronic devices, e.g. protein based memory devices [39] or conventional biosupercapacitors, as the one reported herein.

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

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