

Autotolerant ceruloplasmin based biocathodes for implanted biological power sources

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ABSTRACT

High-performance autotolerant bioelectrodes should be ideally suited to design implantable bioelectronic devices. Because of its high redox potential and ability to reduce oxygen directly to water, human ceruloplasmin, HCp, the only blue multicopper oxidase present in human plasma, appears to be the ultimate biocatalyst for oxygen biosensors and also biocathodes in biological power sources. In comparison to fungal and plant blue multicopper oxidases, e.g. *Myrothecium verrucaria* bilirubin oxidase and *Rhus vernicifera* laccase, respectively, the inflammatory response to HCp in human blood is significantly reduced. Partial purification of HCp allowed to preserve the native conformation of the enzyme and its biocatalytic activity. Therefore, electrochemical studies were carried out with the partially purified enzyme immobilised on nanostructured graphite electrodes at physiological pH and temperature. Amperometric investigations revealed low reductive current densities, i.e. about $1.65 \mu\text{A cm}^{-2}$ in oxygenated electrolyte and in the absence of any mediator, demonstrating nevertheless direct electron transfer based O_2 bioelectroreduction by HCp for the first time. The reductive current density obtained in the mediated system was about $12 \mu\text{A cm}^{-2}$. Even though the inflammatory response of HCp is diminished in human blood, inadequate bioelectrocatalytic performance hinders its use as a cathodic bioelement in a biofuel cell.

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1. Introduction

Ceruloplasmin, Cp, belongs to a diverse family of blue multicopper oxidases, BMCOs, and it is the only BMCO present in human plasma. Similarly to all other BMCOs, Cp oxidises a wide range of structurally unrelated substrates with concomitant reduction of O_2 to H_2O , without liberating reactive oxygen species [1]. However, a ferroxidase activity has historically been considered as a primary role of Cp [2,3]. Cp has a molecular weight of some 132 kDa [4] and consists of six compact domains arranged in a triangular symmetry [5]. Cp, carrying six copper ions, is unique among other BMCOs which usually contain four copper ions. Hence, Cp has three coppers at the T1 sites, i.e. T1A, T1B, and T1C, and three coppers in a trinuclear cluster, consisting of the T2 and T3 sites (Fig. 1) [6]. Such a design was created by nature with the aim of efficient electron transfer from reducing substrates to oxygen without a formation of harmful intermediates. Electrons from the reducing substrates

are accepted one at a time at T1A-copper site, followed by the intramolecular electron transfer to the trinuclear cluster, which in its turn utilizes them for conversion of oxygen to water [2]. T1C-copper remains, though, permanently reduced due to the high redox potential (ca. 1 V) [5]. Since Cp accommodates five coppers in the reduced state, and reduction of O_2 to H_2O is a four-electron process, upon re-oxidation of the enzyme there is one extra electron that equilibrates between the T1A and T1B coppers [5]. This not only explains the biphasic re-oxidation kinetics of the fully reduced enzyme, where one of the phases is extremely fast and the other one is slow [7], but also the low values of kinetic constants measured at steady-state conditions [5,6].

Up till now only a few, essentially unsuccessful, attempts have been made to achieve mediator-less bioelectroreduction of O_2 catalysed by human ceruloplasmin, HCp, [8–10]. However, direct electron transfer between electrode and enzyme was observed [9]. It was suggested that the bioelectrocatalytic unresponsiveness of this complex multi-functional redox enzyme might be associated with a very complex mechanism of internal electron transfer involving kinetic trapping [9]. Bioelectrocatalytic reduction of O_2

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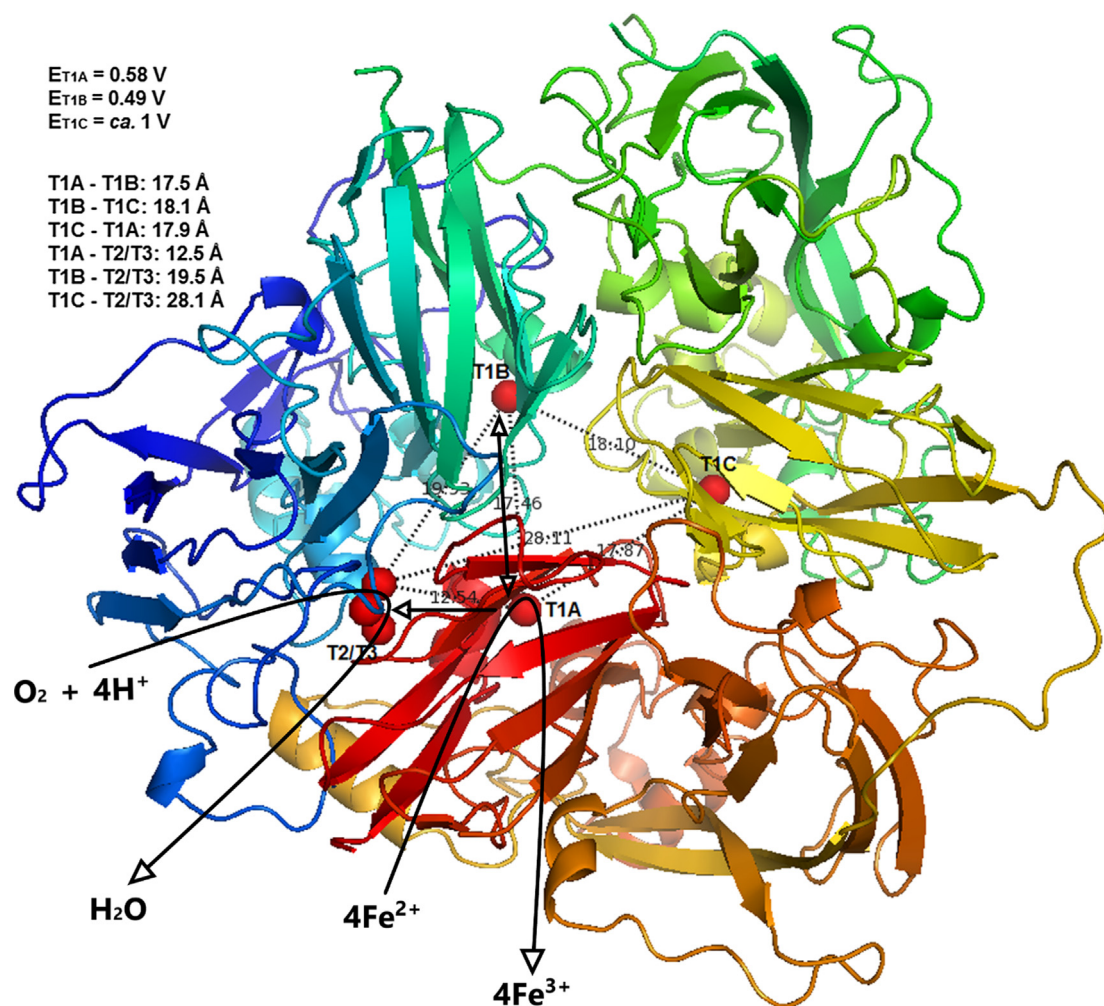


Fig. 1. The three-dimensional structure of Hcp visualised by PyMol program (PDB 1KCW). The six protein domains are colored as blue (1), yellow (2), light green (3), green (4), brown (5), red (6). Six copper ions are shown in red.

by Hcp is not only scientifically interesting but is also of practical importance. If bioelectroreduction of O_2 on Hcp modified electrodes were to be achieved, Hcp could be used as a bioelement for implantable bioelectronic devices, e.g. oxygen sensitive biosensors or biocathodes in implanted biological power sources. To that end, an anode based on the human enzyme sulfite oxidase has already been constructed [11], and hence the development of a matching cathode would allow formation of biofuel cells with attenuated inflammatory response and low cytotoxicity.

Hence, the hemocompatibility of Hcp by means of calorimetry and ELISA was investigated, comparing the results to fungal and plant enzymes, *Myrothecium verrucaria* bilirubin oxidase (*MvBOx*) and *Rhus vernicifera* laccase (*RvLc*), respectively. Moreover, the electrochemistry of Hcp was re-investigated, opting for either mediator-less or mediator based bioelectroreduction of O_2 , using a partially purified enzyme.

2. Materials and methods

2.1. Chemicals and materials

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, KH_2PO_4 , NaCl, KCl, $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, NaOH, L-ascorbic acid, $\text{K}_4[\text{Fe}(\text{CN})_6]$, $\text{K}_3[\text{Fe}(\text{CN})_6]$, CH_3COONa , acetic acid (99.9%), multiwalled carbon nanotubes (O.D. = 10–15 nm; I.D. = 2–6 nm; length = 0.1–10 μm ; > 90%) (MWCNTs), and Hank's bal-

anced salt solution (HBSS, 9.8 g l^{-1}) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sterile saline (NaCl 9 mg ml^{-1}) was obtained from Fresenius Kabi (Copenhagen, Denmark). $(\text{NH}_4)_2\text{SO}_4$ was acquired from Kebo Lab (Spånga, Sweden). Ethanol (95%) was obtained from Kemetyl AB (Haninge, Sweden). Ar, N_2 and O_2 gases were purchased from AGA Gas AB (Sundbyberg, Sweden). All chemicals were of analytical grade and used without further purification. All solutions were prepared using water purified with a PURELAB UHQ II system from ELGA Labwater (High Wycombe, UK).

2.2. Bio-preparations

Concentrations of all bio-preparations were determined by measuring the absorbance using a UV–VIS spectrophotometer UV-1700 Pharma Spec from Shimadzu Corporation (Kyoto, Japan). An example of a typical spectrum is shown in Fig. S1.

2.2.1. Commercial human ceruloplasmin (cHcp)

cHcp (lyophilized powder) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in 100 mM phosphate buffer (PB) pH 7.4 to a final concentration of 2 mg ml^{-1} (15 μM). The concentration of the enzyme was determined spectrophotometrically using $\epsilon = 1.06 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm [12]; the A_{610}/A_{280} ratio was equal to 0.023. The purity of the preparation was con-

firmed by SDS/PAGE analysis (Fig. S2). Further dilution to 1.5 mg ml^{-1} ($11 \text{ }\mu\text{M}$) was made for calorimetric experiments.

2.2.2. Partly purified human ceruloplasmin (pHCp)

200 ml venous blood was collected from apparently healthy volunteers into BD Vacutainer tubes (Plymouth, UK), and subsequently transferred into 4x50 ml conical centrifuge tubes. Blood donors, participating in this study, gave informed consent in accordance with the WMA Declaration of Helsinki. Then the blood was allowed to coagulate for ca. 40 min [3]. Next, it was centrifuged for 10 min at 1050g using a Universal 320 Hettich Zentrifugen from Andreas Hettich GmbH & Co (Tuttlingen, Germany). The supernatant was separated from the precipitated blood cells by a pipette and centrifuged once again (10 min, 1050g). Then, the serum (ca. 80 ml) was collected and the precipitate was discarded. A $(\text{NH}_4)_2\text{SO}_4$ precipitation procedure, as well as further steps of partial purification, were carried out according to the protocol described in [13], with some modifications. The target percentage of saturation were chosen to be 40% and 80%. The amount of $(\text{NH}_4)_2\text{SO}_4$ powder required, for 40% or 80% saturation, was gradually added under continuous stirring to the human serum. After the addition the mixture was stirred for ca. 30 min, and then left to stand overnight. The next day it was divided into Eppendorf tubes and centrifuged for 20 min at 16000g using Eppendorf centrifuge 5402 from Eppendorf-Netheler-Hintz GmbH (Hamburg, Germany). In case of the 40% alternative, the precipitate was discarded, and the supernatant was collected for another precipitation procedure, as described above. In case of the 80% run, the supernatant was discarded, and the precipitate was dissolved in a minimal amount of 50 mM PB pH 7.4. Subsequently, the dissolved sample was dialysed against ca. 2 L of 50 mM PB pH 7.4 overnight using a Da MW cut-off ca. 14,000 membrane from Sigma-Aldrich (St. Louis, MO, USA). The next day the dialysed fraction was added to an ion-exchange column ($V = 14.7 \text{ cm}^3$) packed with Toyopearl DEAE-650 M from Tosoh Corporation (Tokyo, Japan) and equilibrated with 50 mM PB pH 7.4. After the whole sample was applied, it was washed with one bed volume of starting buffer and then eluted with 150 mM PB pH 7.4. While the sample was eluting, a blue band of ceruloplasmin moved down the column. The blue enzyme fraction (ca. 3–4 ml) was collected. All the above procedures were carried out in a cold room at $4 \text{ }^\circ\text{C}$, unless otherwise specified.

The HCp fraction was transferred to an Amicon 8010 stirred ultrafiltration cell equipped with an ultrafiltration membrane, NMWL 30,000 Da, obtained from EMD Millipore Corporation (Bedford, MA, USA). The membrane was initially rinsed with ultra-pure water in accordance with the manual. First, the sample was diluted 3 times with ultra-pure water in order to achieve a buffer strength of 50 mM (as it was eluted with 150 mM PB from the column). Second, the ultrafiltration was carried out under continuous stirring at 3.8 bar (4 atm), N_2 operating pressure. The procedure was repeated 4 times using 50 mM PB pH 7.4 for dilutions. Third, ca. 1.5 ml of the concentrated blue fraction was collected, and the absorbance spectrum was recorded. The concentrations of HCp (usually 3–4 mg ml^{-1} , i.e. 23–30 μM) were determined using $\epsilon = 1.06 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm [12] (Fig. S1). After purification, the A_{610}/A_{280} value was typically 0.018. The SDS/PAGE analysis of the partially purified preparation is shown in Fig. S2. The sample was stored in a refrigerator at $4 \text{ }^\circ\text{C}$ and used for electrochemical investigations within 10 days.

2.2.3. Homogenous human ceruloplasmin (hHCp)

HCp was purified from human plasma ($A_{610}/A_{280} = 0.035$) using a two-stage method based on the interaction with neomycin, as described in [14]. The purity of the preparation was confirmed by SDS/PAGE analysis (Fig. S3).

2.2.4. Myrothecium verrucaria bilirubin oxidase (MvBOX)

MvBOX, 3.61 mg ml^{-1} ($60 \text{ }\mu\text{M}$) in 20 mM Tris buffer, 0.1 M Na_2SO_4 , pH 8.0, was kindly supplied by Novozymes A/S (Bagsværd, Denmark) and diluted with 100 mM PB pH 7.4 to a final concentration of 1.5 mg ml^{-1} ($25 \text{ }\mu\text{M}$).

2.2.5. Human serum albumin (HSA)

HSA, lyophilized powder, was purchased from Sigma-Aldrich (St. Louis, MO, USA), and diluted with 100 mM PB pH 7.4 to a final concentration of 1.5 mg ml^{-1} ($22 \text{ }\mu\text{M}$).

2.2.6. Rhus vernicifera laccase (RvLc)

RvLc, 9.54 mg ml^{-1} ($87 \text{ }\mu\text{M}$) in 200 mM Na_2HPO_4 , was obtained from A.N. Bach Institute of Biochemistry, RAS (Moscow, Russia). The enzyme solution was further diluted with 100 mM PB pH 7.4 to a final concentration of 1.5 mg ml^{-1} ($14 \text{ }\mu\text{M}$) for calorimetric studies and to 4.77 mg ml^{-1} ($44 \text{ }\mu\text{M}$) for electrochemical measurements.

2.2.7. Trametes hirsuta laccase (ThLc)

ThLc, 5.8 mg ml^{-1} ($93.5 \text{ }\mu\text{M}$) in 100 mM PB pH 6.5, was obtained from A.N. Bach Institute of Biochemistry, RAS (Moscow, Russia).

2.3. Calorimetric measurements

2.3.1. Biomodification of graphite electrodes (GE)

GEs, 3.05 mm in diameter, 38.10 mm long, from Alfa Aesar Ltd. (Karlruhe, Germany) were cut into ca. 4 mm long pieces and polished from both ends using a silicon carbide grinding paper, Grit 1000/P 2500 from Buehler (Lake Bluff, IL, USA), to achieve a weight of $50 \pm 0.5 \text{ mg}$. The GE pieces were rinsed thoroughly with ethanol, immersed in ethanol and left overnight. After drying in air, the GEs were immersed in 1.5 mg ml^{-1} solutions of MvBOX, cHCp, RvLc, and HSA, respectively, for 1 h. Finally, the biomodified electrodes were thoroughly rinsed with 10 mM phosphate buffered saline (PBS) pH 7.4, containing 137 mM NaCl and 2.7 mM KCl, and used for calorimetric measurements.

2.3.2. Calorimetric studies of immune response

For calorimetric investigations at $37 \text{ }^\circ\text{C}$, a four-channel isothermal heat conduction (ITC) micro-calorimeter TAM 2277 from Thermometric AB (Järfälla, Sweden) was used. Bio-modified GEs were placed in clean and dry 1.3 ml stainless steel ampoules and covered with 1 ml of heparinized whole blood, obtained from apparently healthy donors, previously diluted twofold with HBSS buffer. The ampoules were tightly closed with stainless steel lids by means of a teflon gasket. Ampoules were slowly inserted into the calorimeter preheater and equilibrated for 10 min at $37 \text{ }^\circ\text{C}$ before the ampoules were slowly lowered into the measuring chamber; the heat released was recorded using software, i.e. DigiTam, provided by Thermometric AB (Järfälla, Sweden). The device was previously electrically calibrated against a reference ampoule containing 1 ml of water. The signal was read after 60 min, when steady state heat output was achieved. Control measurements were also performed with only blood in order to obtain background values of resting state. For these experiments informed consent was obtained from blood donors in accordance with the WMA Declaration of Helsinki. Blood was collected into BD Vacutainer tubes (Plymouth, UK) containing lithium heparin, and used for the experiments within 3 h.

2.4. Enzyme linked immunosorbent assay (ELISA)

2.4.1. Whole blood incubations

Heparinized blood was obtained from three apparently healthy donors. Then, 900 μl of cHCp / MvBOX / RvLc / HSA solution in ster-

ile saline ($5 \mu\text{g ml}^{-1}$) was mixed with $200 \mu\text{l}$ of heparinized blood in Eppendorf tubes and incubated at 37°C for 6 h. After incubation, samples were resuspended and centrifuged for 5 min at 900g, Heraeus Pico 21 microcentrifuge from Thermo Fisher Scientific (Osterode am Harz, Germany). The plasma supernatants were collected and stored at -80°C until cytokine measurement. For these experiments informed consent was obtained from blood donors in accordance with the WMA Declaration of Helsinki. Blood was collected into BD Vacutainer tubes (Plymouth, UK) containing lithium heparin, and used for the experiments immediately upon collection.

2.4.2. Cytokine ELISA

Human IL-6 ELISA kit from Thermo Fisher Scientific (San Diego, CA, USA) was used for inflammatory response studies of proteins of fungal, plant and human origin, *i.e.*, MvBOX, RvLc, cHCp and HSA. Interleukin 6 (IL-6) was quantified according to the manufacturer's instructions. Assays were carried out in 96-well immunoplates from MaxiSorp (Wiesbaden, Germany). Samples containing MvBOX and RvLc were diluted 15 times with sterile saline solution prior to ELISA studies, whereas samples containing cHCp and HSA were used without dilution. The results were processed taking the dilution factor of MvBOX and RvLc samples into account. The absorbance was read at 450 nm, PowerWave XS Microplate Spectrophotometer from Bio-Tek Instruments Inc. (Highland Park, VT, USA). The experiments were repeated at least three times with determination of standard deviations.

2.5. Kinetic studies

The specific activity of HCp samples, as well as MvBOX, ThLc and RvLc, in homogenous solution was determined by measuring the initial O_2 consumption rates ($\Delta\text{C}_{\text{O}_2} / \Delta t$), *i.e.* measurable linear rates, using an Oxygraph Clark-type electrode from Hansatech Ltd. (Norfolk, England) at 37°C , under continuous stirring. Rates of O_2 uptake were assessed by the Oxygraph Plus software provided by Hansatech. At the substrate concentration significantly higher than K_M , as it was the case in this study, the reaction is independent on substrate concentration (zero-order kinetics), and enzyme is working at its maximum rate V_{max} , and therefore k_{cat} is following the formula $k_{\text{cat}} = V_{\text{max}} / E_0$, where E_0 is the total enzyme concentration. In order to define k_{cat} , V_{max} was determined from the linear part of the curve, and then divided by enzyme concentration present in the Clark cell [15,16].

Measurements were performed in air-saturated 10 mM PBS pH 7.4, containing 5 mM substrate, *i.e.* L-ascorbic acid or $\text{K}_4[\text{Fe}(\text{CN})_6]$; $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, 1 mM, was also used as a substrate. The $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ solution was freshly prepared in a buffer thoroughly purged with Ar, just before the experiments. When a stable baseline was established, $1 \mu\text{M}$ HCp was injected *via* a microsyringe into the airtight reaction cell. The total volume of the reaction mixture in the cell was $250 \mu\text{l}$.

Deactivation of HCp was performed using a VWR Digital Heat-block from Henry Trobmer LLC (Thorofare, NJ, USA) in order to confirm the origin of the biocatalytic signal. Hence, the enzyme solution was incubated in an Eppendorf tube for 10 min at 95°C , and then injected into the reaction.

The catalytic activity of MvBOX, ThLc, and RvLc was evaluated in the presence of 5 mM $\text{K}_4[\text{Fe}(\text{CN})_6]$. In case of MvBOX and RvLc measurements were performed in 10 mM PBS pH 7.4, however, in case of ThLc 50 mM acetate buffer pH 4.2 was used. The total volume of the reaction mixture in the cell was $250 \mu\text{l}$, enzyme concentration $0.05 \mu\text{M}$ (MvBOX, ThLc) and $0.3 \mu\text{M}$ (RvLc).

The catalytic activity of pHCP adsorbed on GE electrode was measured as follows: a piece of GE (2.5 mm long, $d = 3.05 \text{ mm}$) was immersed into pHCP solution for 20 min, then rinsed with PBS buffer and placed into the Clark-type electrode cell filled with

$225 \mu\text{l}$ of 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ at 37°C under continuous stirring, subsequently O_2 consumption rate was recorded. Analogous measurement was performed for the bare GE piece, *i.e.* not biomodified, in order to obtain background value.

2.6. Electrochemistry

2.6.1. Assembly of biomodified nanostructured graphite electrodes

GEs from Ringsdorff Werke GmbH (Bonn, Germany) with a geometrical area of 0.073 cm^2 were used as working electrodes in electrochemical measurements. GEs were polished on a wet silicon carbide grinding paper Grit 1000/P 2500 Buehler (Lake Bluff, IL, USA), rinsed thoroughly with ultrapure H_2O , and allowed to dry.

The nanotube modification was carried as follows: a suspension of MWCNTs (10 mg ml^{-1} in 5 mM PB pH 7.4) was sonicated using an ultrasonic cleaner from WVR International (Leuven, Belgium) for 10 min and then diluted with ethanol to a final concentration of 0.5 mg ml^{-1} . $10 \mu\text{l}$ of the suspension was placed on the polished and dry GE surface and left to evaporate [9]. Then, $10 \mu\text{l}$ of pHCP solution, *ca.* 4 mg ml^{-1} in 50 mM PB pH 7.4, was dropped on the MWCNT modified GE (GE/MWCNT) surface and allowed to adsorb for 20 min. Immobilisation time of 20 min is an optimised value, that gave best bioelectrocatalytic response, as other immobilisation times were also tested. Similarly, $10 \mu\text{l}$ of MvBOX, (3.61 mg ml^{-1}) and RvLc (4.77 mg ml^{-1}) were dropped on the GE/MWCNT and allowed to immobilise for 20 min.

2.6.2. Electrochemical measurements

Cyclic voltammetry (CV) and amperometry were performed in a thermostated electrochemical cell at 37°C , containing 25 ml of electrolyte solution, using a $\mu\text{Autolab Type III/FRA2}$ potentiostat/galvanostat from Metrohm Autolab B.V. (Utrecht, The Netherlands). $\text{Ag}|\text{AgCl}|3\text{M KCl}$ (0.21 V vs. NHE) was used as the reference electrode and a platinum mesh as a counter electrode. The electrolyte was composed of 10 mM PBS pH 7.4, and heparinized human blood. All potentials in this work are given vs. NHE.

Anaerobic conditions were established by purging the electrolyte solution with Ar for *ca.* 15 min before making measurements, and a stream of Ar was kept above the electrolyte solution during the measurements. Analogously, aerobic conditions were established using O_2 .

3. Results and discussion

3.1. Inflammatory response studies

The inflammatory response of human blood towards GEs biomodified with fungal, plant and human proteins, MvBOX, RvLc, cHCp and HSA, respectively, was measured using ITC. It was assumed that with a more intense inflammatory response, appropriately increased heat generation should be observed. The investigations were performed using blood from three different donors. Heat production induced by the response of blood cells towards biomodified GE chunks was measured at 37°C . Calorimetric response for whole blood was also recorded (Table 1), and val-

Table 1

Calorimetric responses for whole blood, and whole blood containing biomodified GE electrodes. Values are presented per 1 ml of whole blood.

	$P_{\text{blood 1}}, \mu\text{W ml}^{-1}$	$P_{\text{blood 2}}, \mu\text{W ml}^{-1}$	$P_{\text{blood 3}}, \mu\text{W ml}^{-1}$
GE/MvBOX	54.0	49.0	82.4
GE/RvLc	53.0	48.4	82.6
GE/cHCp	50.4	46.6	81.2
GE/HSA	48.8	47.0	66.6
Blood	45.6	41.8	51.4

ues are in agreement with earlier reported data [17]. In keeping with the initial premise, increased heat production was observed for GE modified with MvBOx and RvLc, compared to GE modification with cHCp and HSA (Table 1). Be that as it may, absolute values varied significantly depending on the number and types of cells in the particular blood sample [17], giving high standard deviations. Therefore, standard deviations were not provided for the calorimetric data. The results from the calorimetric measurements show a similar pattern in response to the differently biomodified GE although there are individual differences as can be seen from the resting state. Calorimetry is a sensitive method that will detect minute changes in response to biochemical, chemical or physical events. Hence, the signal might be a summation of the heat generated by different processes [18,19], and, therefore, the calorimetry data obtained are of moderate precision. Thus, it was decided to carry out a more specific inflammatory response such as release of IL-6, and quantitate it using ELISA.

The proinflammatory cytokine IL-6 is formed as a response to infections and injuries in human bodies, triggering the host defence through mediation of the acute phase response [20]. In this study human IL-6 ELISA was used to quantify the generation of IL-6 in human blood from three different donors, in response to fungal, plant and human proteins. For reference, (patho-) physiological concentration of HcP in human plasma is ca. 200–900 $\mu\text{g ml}^{-1}$ [21,22], and in the current ELISA assays the protein concentration was ca. 2 orders of magnitude lower. The results indicate that the amount of IL-6 formed in blood incubated with MvBOx and RvLc is 15–20 times higher than the amounts caused by cHCp and HSA. As it appears, the calorimetric data corresponds to the results obtained by the analysis of IL-6, but, as in the case of calorimetry, absolute values varied significantly in blood from different donors (Table 2). The experiments were repeated at least three times, giving standard deviations of less than 10%. However, it cannot be excluded that the release of IL-6 is otherwise triggered, e.g. by impurities present in the protein preparations, and further investigations are needed to substantiate the current claims.

Nevertheless, the above results verified our assumption of low response provoked by human proteins compared to enzymes of non-human origin. Hence, further electrochemical studies of HcP were carried out, attempting to realise a biocompatible cathode for implantable biofuel cells.

3.2. Measurements of biocatalytic activity using a Clark-type electrode

As HcP is mostly known for its ferroxidase activity, the initial kinetic measurements relied on ferrocyanide, $\text{K}_4\text{Fe}(\text{CN})_6$, as a reducing substrate. However, in our hands cHCp was catalytically inactive towards ferrocyanide, and quite low activity ($k_{\text{cat}} = 0.03 \text{ s}^{-1}$) was observed when pHcP was used. Similarly, the catalytic constant of hHCp was very close to that of pHcP (Table 3). For comparison, we have additionally measured catalytic constants for MvBOx, ThLc and RvLc towards ferrocyanide (Table 3), the biocatalysts most often used in biocathodes [23–25]. However, since both MvBOx and ThLc have been stored in the freezer for some period of time, k_{cat} values differ from the ones reported earlier in the lit-

Table 2
Concentrations of IL-6 produced by human blood in response to MvBOx, RvLc, cHCp and HSA.

	IL-6, pg ml ⁻¹ blood 1	IL-6, pg ml ⁻¹ blood 2	IL-6, pg ml ⁻¹ blood 3
MvBOx	1179 ± 38	1765 ± 81	788 ± 24
RvLc	1844 ± 110	2395 ± 239	766 ± 64
cHCp	58 ± 3	140 ± 7	34 ± 2
HSA	47 ± 1	123 ± 12	26 ± 1

Table 3
Apparent catalytic constants of O₂ reduction by HcP samples, as well as by MvBOx, ThLc and RvLc, using different electron donating substrates.

	Ascorbate	(NH ₄) ₂ Fe(SO ₄) ₂	K ₄ [Fe(CN) ₆]
k_{cat} , s ⁻¹ (pHCp)	0.42 ± 0.02	1.01 ± 0.08	0.03 ± 0.003
k_{cat} , s ⁻¹ (hHCp)	0.28 ± 0.01	–	0.03 ± 0.002
k_{cat} , s ⁻¹ (cHCp)	0.10 ± 0.006	0	0
k_{cat} , s ⁻¹ (MvBOx)	–	–	27 ± 2.0
k_{cat} , s ⁻¹ (ThLc), pH 4.2	–	–	20 ± 1.9
k_{cat} , s ⁻¹ (RvLc)	–	–	0.3 ± 0.01

erature [26,27]. Low k_{cat} number obtained for RvLc is due to the low redox potential of T1 site (ca. 0.42 V) [28], which is almost similar to the midpoint potential of ferri-/ferrocyanide couple obtained in our studies (ca. 0.43 V). From the Table 3, it follows that the values for those enzymes are several orders of magnitude higher compared to values obtained for HcP samples.

Next, another donor of Fe(II) ions, ferrous ammonium sulphate, (NH₄)₂Fe(SO₄)₂, was used to assess the biocatalytic capabilities of HcP; (NH₄)₂Fe(SO₄)₂ has been applied by other research groups to demonstrate ceruloplasmin's ferroxidase activity [3,29,30], but cHCp didn't exhibit catalytic activity towards this substrate either. However, experiments with pHcP resulted in a k_{cat} of 1.01 s⁻¹, with ferrous ammonium sulphate auto-oxidation rates subtracted. The k_{cat} value obtained is in good agreement with earlier published results [6,29,30].

Ascorbic acid was previously studied as a potential substrate for ceruloplasmin in humans [31,32], and hence it was used as an electron donor for HcP in our studies. The obtained data show that the cHCp activity was rather low at 0.10 s⁻¹, whereas the obtained turnover number for pHcP was 0.42 s⁻¹ (Fig. 2). Interestingly, the hHCp k_{cat} was lower at 0.28 s⁻¹. The values obtained were within the range of earlier reported data [31,33,34]. Lyophilization of HcP has been shown earlier to affect its structure and copper content as well as impair its oxidase activity [14], so the data presented here complies with previously reported investigations.

Thermal inhibition of enzyme samples was performed in order to confirm the biocatalytic origin of the signals obtained. After thermal treatment, no activity towards the reducing substrates mentioned above was detected.

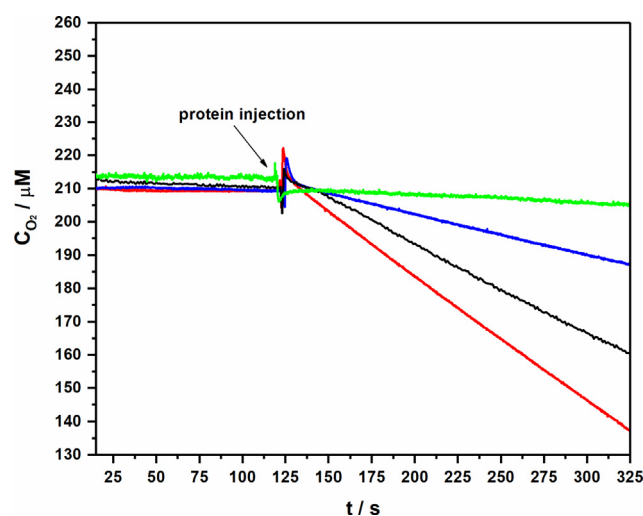


Fig. 2. O₂ consumption by different HcP samples measured using a Clark-type electrode in the presence of ascorbate as an electron donating substrate. Red line – partly purified HcP (pHCp), black line – homogenous HcP (hHCp), blue line – commercially available HcP (cHCp), green line – temperature deactivated pHcP. $C_{\text{protein}} = 1 \mu\text{M}$; 10 mM PBS pH 7.4, 5 mM ascorbate; 37 °C.

Apparently, partial purification of HCp preserved the native conformation of the enzyme and retained co-factors necessary for both biocatalytic and bioelectrocatalytic activities and, therefore, it was decided to use the partially purified enzyme for further electrochemical investigations.

In order to prove that pHcP is biocatalytically active while adsorbed on GE, O₂ consumption rate was recorded using Clark-type electrode when GE/pHcP piece was immersed into K₄[Fe(CN)₆] solution. The results show a weak catalytic activity of adsorbed pHcP towards K₄[Fe(CN)₆] in comparison to just bare GE (Fig. S4).

3.3. Electrochemical investigations

Since cHCp appeared to be catalytically inactive, it was decided to carry out electrochemical experiments with the partially purified enzyme, pHcP, which in terms of biocatalytic activity in

homogenous solution even outperformed hHCp. GE/MWCNT electrodes were used for biomodification in order to ensure higher enzyme loading, and possibly a more favourable biomolecule orientation on the electrodes.

Initially, cyclic voltammetry of GE/MWCNT/pHcP electrodes was performed in 10 mM PBS pH 7.4 at 37 °C under anaerobic and aerobic conditions. However, no bioelectroreduction of oxygen was detected. The capacitance of GE/MWCNT/pHcP electrode was reduced compared to non-biomodified GE/MWCNT (Fig. S5), which verifies the protein adsorption on the electrode surface. In previous studies of commercially available HCp immobilised on bare Au and graphite electrodes, CNT modified graphite and AuNP modified gold electrodes, and even covalently attached to thiol-modified Au electrodes, without a mediator in different electrolytes, no bioelectroreduction of oxygen was observed [5,6]. Generally, decreasing catalytic activity of enzymes in heterogenous systems, as compared to homogeneous systems, is not an unexpected outcome. The rationale might be diffusion limitations during heterogenous biocatalytic processes, or as in the case of HCp, a complex mechanism where the electrode is not well suited to replace a substrate for the enzyme, perhaps coupled to restriction of protein breathing motions after adsorption on the electrode surface. It was also shown earlier that absence of catalytic activity is not caused by denaturation of the enzyme [8,9,35,36]. However, upon addition of a mediator, ferricyanide, K₃[Fe(CN)₆], to the electrolyte, a bioelectrocatalytic current density of about 12 μA cm⁻² was observed (Fig. 3). Background CVs recorded with non-biomodified GE/MWCNT are presented in Fig. S6. Fig. 4 provides a comparison with well pronounced mediated bioelectrocatalysis of MvBOx (Fig. 4A) and RvLc (Fig. 4B) modified graphite electrodes, which starts at a higher redox potential and delivers at least one order of magnitude higher bioelectrocatalytic currents. Respective background CVs are shown in Fig. S7. Apparently, the biocatalytic activity of enzymes in homogenous solution complies with the outcomes of heterogenous bioelectrocatalysis, *i.e.* one cannot expect high bioelectrocatalytic currents from an enzyme that has low catalytic activity in homogenous solution.

Furthermore, amperometric investigations of GE/MWCNT/pHcP at 0.41 V vs. NHE were performed in 10 mM PBS, pH 7.4, at 37 °C. Remarkably, low reductive current densities, *i.e.* ca. 1.65 μA cm⁻² were observed in the absence of any kind of mediator (Fig. 5).

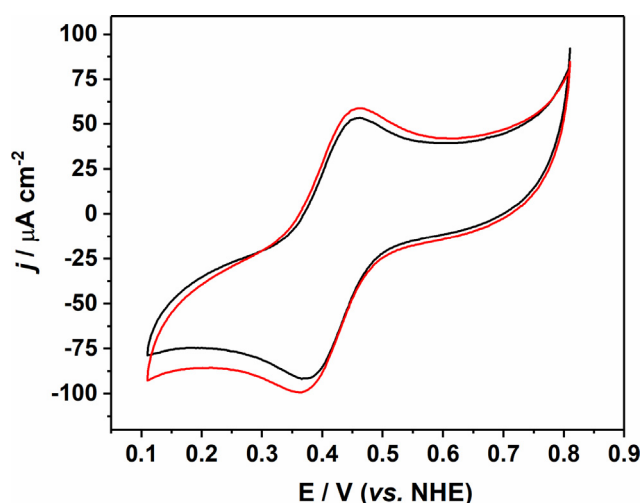


Fig. 3. CVs of GE/MWCNT electrode modified with partly purified HCp (pHcP) in the presence of a mediator in O₂ saturated electrolyte (red line) and Ar saturated electrolyte (black line), scan rate 25 mV s⁻¹. Conditions: 10 mM PBS pH 7.4, 0.5 mM K₃[Fe(CN)₆]; 37 °C.

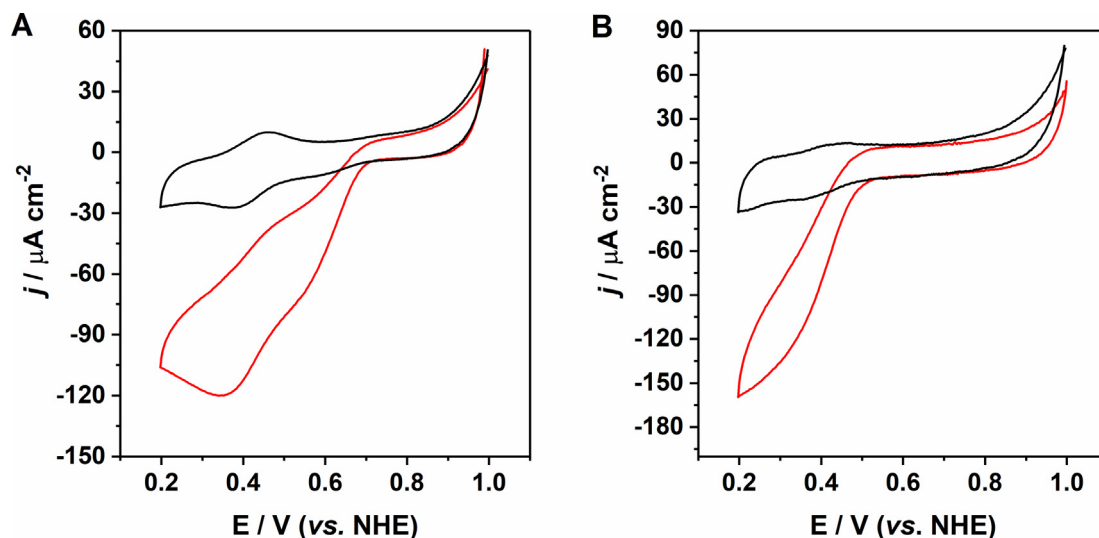


Fig. 4. CVs of GE electrodes modified with MvBOx and RvLc in the presence of a mediator, scan rate 25 mV s⁻¹. A. CVs of GE/MvBOx in O₂ saturated electrolyte (red line) and Ar saturated electrolyte (black line). B. CVs of GE/RvLc in O₂ saturated electrolyte (red line) and Ar saturated electrolyte (black line). Conditions: 10 mM PBS pH 7.4, 0.05 mM K₃[Fe(CN)₆]; 37 °C.

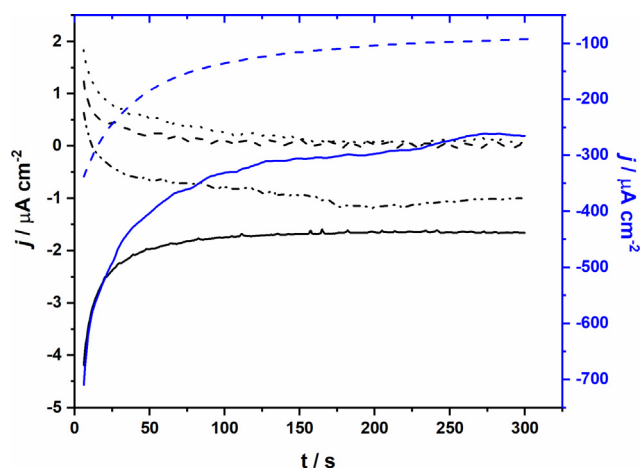


Fig. 5. Amperometric studies of GE/MWCNT electrodes modified with partly purified HcP (pHcP), MvBOx and RvLc performed at 0.41 V vs. NHE. Black (smooth) line – GE/MWCNT/pHcP in O₂ saturated electrolyte, black (dash dot dot) line – GE/MWCNT/pHcP in Ar saturated electrolyte, black (dash) line – GE/MWCNT in O₂ saturated electrolyte, black (dot) line – GE/MWCNT in Ar saturated electrolyte, blue line – GE/MWCNT/MvBOx in O₂ saturated electrolyte, blue (dash) line – GE/MWCNT/RvLc in O₂ saturated electrolyte. Conditions: 10 mM PBS pH 7.4, 37 °C.

Minor reductive currents, which were masked on CVs due to the capacitive current contribution, became obvious by applying a constant potential. However, compared to the response from the GE/MWCNT/MvBOx and GE/MWCNT/RvLc electrodes in an O₂ saturated electrolyte, the currents obtained with the pHcP modified electrode were negligible.

Electrochemical studies were also carried out in human blood, however, no bioelectroreduction of oxygen was observed.

4. Conclusions

Harvesting electrical power from the human body has recently attracted significant attention. Implanted devices for electric power generation based on biological catalysts have illustrated the significant importance of the concept, that also is considered to be the most biocompatible and eco-friendly [37]. Autotolerant biocathodes are truly required for implantable biological power sources. To the best of our knowledge, the only biocatalyst, existing in human blood, that may satisfy this criteria is HcP. Therefore, in this work we attempted to design an autotolerant biocathode based on HcP by investigating the hemocompatibility, the biocatalytic, and the bioelectrocatalytic capabilities of this bioelement. An anode, based on human sulfite oxidase, has already been realised [11], and a human enzyme based cathode would permit the construction a hemocompatible biofuel cell.

Hemocompatibility of HcP was confirmed by means of calorimetry and IL-6 ELISA. In comparison to fungal and plant enzymes, MvBOx and RvLc, respectively, the IL-6 amounts induced by HcP, in analogy to HSA, were *about* 20 times lower. However, we cannot exclude the possibility that the inflammatory response was provoked by other factors, e.g. impurities present in protein preparations. It must be noted, that studies of biocatalyst hemocompatibility have not been performed earlier and further investigations are required.

As earlier unsuccessful attempts to achieve O₂ bioelectroreduction by HcP drew on commercially available preparations, in this work electrochemical studies were carried out with partly purified HcP of higher biocatalytic activity. HcP was immobilised on nanostructured graphite electrodes, demonstrating direct electron transfer based minor O₂ bioelectroreduction for the first time. Additionally, electrochemical studies based on mediated electron

transfer showed only slightly higher bioelectrocatalytic currents, i.e. about 12 μA cm⁻². Obviously, the bioelectrocatalytic performance of HcP is, by far, second to enzymes typically used in biocathodic applications. However, due to restricted oxygen availability and diffusional limitations [38], the performance of implanted fuel cells is limited by the biocathode, and even the stifled output of an autotolerant HcP based cathode might be regarded as promising.

Notwithstanding the low inflammatory response in human blood, it is obvious that HcP exhibits low bioelectrocatalytic activity and hence it is a poor candidate for use as a cathodic bioelement in biofuel cells. One of the possible solutions to increase biocatalytic activity of HcP could be connected to directed mutagenesis of the enzyme, however, parallel investigations of inflammatory response of the mutated enzyme would be necessary. The present study provides further insight in terms of structural and catalytic properties of the most enigmatic blue copper oxidase, human ceruloplasmin. Also, the study gives incentives for future engineering of biocompatible biocatalysts and offers a straightforward methodology to evaluate inflammatory responses that can be triggered by implanted biological power sources.

Declaration of Competing Interest

The author declare that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioelechem.2021.107794>.

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