Electronic Supplementary Material (ESI) for RSC Advances.

Supplementary Information

Ex vivo Electric Power Generation in Human Blood Using an Enzymatic Fuel Cell in a Vein Replica

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Movies M1 to M3

Movie M1. Two-dimensional ultrasound capture of the blood flow in the superficial vein. A video clip showing a two-dimensional ultrasound capture of the colour-Doppler flow in the median cubital vein of the right arm of the volunteer.

Movie M2. Tubular enzymatic fuel cell operating *ex vivo*. An animation clip demonstrating the performance of the tubular membrane-less and mediator-free enzymatic fuel cell in a human blood stream.

Movie M3. Proof-of-principle demonstration of electric power generation from human blood under homeostatic conditions. A video clip showing a proof-of-principle demonstration of *ex vivo* electric power generation in human blood using a biocompatible material based low-cost mediator-free and membrane-less enzymatic fuel cell, which supplies enough electricity to turn on a flexible low voltage display in less than 1 min.

1. Materials and Methods

1.1. Chemicals, enzymes, and buffers used

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solutions were prepared using water purified with a PURELAB flex 3 system from ELGA LabWater (High Wycombe, UK). Argon (99.9999% purity) was purchased from AGA Gas AB (Sundbyberg, Sweden).

Highly concentrated homogeneous preparations of *Corynascus thermophilus* cellobiose dehydrogenase and *Myrothecium verrucaria* bilirubin oxidase were kindly provided by Dr. Roland Ludwig from BOKU University (Vienna, Austria), and Dr. Miguel Duarte Toscano from Novozymes A/S (Bagsvaerd, Denmark), as gifts from the university and company, respectively.

The main buffer during *in vitro* studies was phosphate buffered saline (PBS) adjusted to pH 7.40, consisting of 5.9258 g of NaCl, 0.3019 g of KCl, 3.1643 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.6935 g of $NaH_2PO_4 \cdot 2H_2O$ in 1 L of water solution, with and without redox active biological compounds and viscosity adjusting agents (*vide infra*), prepared to be consistent with the concentration of blood electrolytes, as determined during analyses of the volunteers' blood, with or without 6.4 mM glucose, dissolved in the buffer a few hours before use to achieve mutarotational equilibrium. In some measurements redox active biological compounds (low redox potential bio-fuels) at physiologically relevant concentrations, *viz.* 1.92 mM lactate, 0.425 mM urate, and 0.045 mM ascorbate, were also used (*vide infra*). Relying on previous experimental investigations of viscosity values of blood analogues,[1] glycerol 25.3% (*v*/*v*) and xanthan gum 0.032% (*w*/*v*) were added to adjust the viscosity of the buffer to the physiological value (4.28 mPa s, *vide infra*). Oxygen concentration in buffers, equal to 0.082 mM, was established by mixing argon and air saturated electrolytes. To double-check the oxygen concentration, measurements using an Oxygraph Clark oxygen electrode from Hansatech Ltd. (Norfolk, England) at 37 °C with constant stirring, were performed.

1.2. Volunteer

A 40 years old, apparently healthy, male volunteer was participating in all measurements and procedures described herein. Prior to all studies the volunteer was carefully introduced to the background, objectives, and methodology of these studies. He was also informed that no monetary compensation would be offered, before he signed the information sheet for research participants, which was prepared based on recommendations given by the Swedish Research Council and the Council for International Organizations of Medical Sciences. The treatment of personal data was done in accordance with the provisions of the Personal Swedish Data Act (1998:204), based on Directive 95/46/EC, which aims to prevent the violation of personal integrity in the processing of personal data. The approval for the studies (document registration number ETIK 2009/180) was obtained in advance from the Lund/Malmoe Local Ethical Committee (Sweden).

1.3. Ultrasound investigations

Ultrasound measurements of the median cubital vein of the volunteer's right arm and images of the vessel were performed and acquired using an Acuson S2000™

ultrasound system equipped with the 9L4 probe from Siemens Medical Solutions (Mountain View, CA, USA). The vessel inner diameter was obtained from the two-dimensional images by measuring the distance from leading edge to leading edge. Pulsed Doppler registrations were acquired with a sample volume size just covering the whole vessel diameter.

The volunteer in the study likely to having a suitable vein (after ocular and palpative examination) was chosen after giving informed consent. The demands for suitability was that the subcutaneous visible vessel of interest would have a consistency in inner diameter close to 6 mm with a visible length of 1 cm to ensure an appropriate placement of a peripheral vein catheter, BD Venflon Pro Safety from Becton Dickinson Infusion Therapy AB (Helsingborg, Sweden). An appropriately chosen vein would also allow calculations of the limiting (*i.e*. maximal) power of implanted biodevices (because of the diameter consistency and blood laminar flow) and also closely resemble the tubular electrodes used for proper *ex vivo* electrochemical studies (*vide infra*). The suitability was confirmed during the ultrasound examination. The mean inner diameter of the vessel, *i.e.* the median cubital vein of the right arm, and volume blood flow rate (blood flow) were measured to be 4.9 ± 0.4 mm and 0.33 ± 0.07 mL s^{-1}, respectively (Fig. 2, Movie S1).

1.4. Fabrication of electrodes

Anodes and cathodes were made from rods of spectrographic graphite (type RW001, 3.05 mm in diameter) purchased from Ringsdorff Werke GmbH (Bonn, Germany), in which axial through holes (1.00 ± 0.07 mm in diameter) were drilled. The thus prepared graphite tubes were cut to lengths of 10.0 ± 0.5 mm, thoroughly rinsed with water, and dried. For biomodification, the graphite tubes were filled with enzyme solutions with a protein concentration of 10 mg mL^{-1} and left for incubation for 30 min. Shorter incubation time, *e.g.* 20 min as in our previous studies, in which disk spectrographic electrodes were used [2, 3], resulted in halving of the current outputs. Afterwards the electrodes were rinsed with PBS and used for electrochemical studies. Ag|AgCl combined reference/counter electrodes (10.0 ± 0.5 mm length) were made from silver rods, ∅ 3 mm, 99.997% purity, purchased from Goodfellow Cambridge Ltd. (Huntingdon, England), in which through holes were drilled as described above for graphite electrodes. The internal surface of the Ag tubes was electrochemically covered with a layer of AgCl using a previously described protocol.[4]

1.5. *Ex vivo* studies

The peripheral vein catheter (1.1 x 3.2 mm) was placed in the right arm median cubital vein of the subject according to standard medical practice (Fig. 3A) and fixed with a medical tape IV3000 from Smith & Nephew Medical Ltd. (Hull, England). The fixed catheter was connected to a 10 cm long Luer Lock tube from Discofix®C, (Melsungen, Germany), with inner and outer diameters of 3 and 6 mm, respectively, ending with a three-way stopcock (Fig. 3B). One of the openings of the three-way stopcock was used for blood sampling for clinical blood analysis during *ex vivo* studies (*vide infra*). A second opening the three-way stopcock was connected to a freshly prepared enzymatic anode and cathode combination (1 cm length each), incorporated into plastic tubes, 30 cm long in total (Fig. 3C), from Becton, Dickinson Co. (Franklin Lakes, NJ, USA), inserted into a 250 mL graduated glass cylinder from United Scientific Supplies (Waukegan, IL,

USA). The volumetric blood flow rate during the continuous bleeding of the volunteer was calculated from the total blood volume (200-400 mL per measurement) and time, measured using a digital timer 34-2362 from Clas Ohlson (London, UK). The blood volume velocity during *ex vivo* studies was measured to be 0.33 ± 0.03 mL s^{-1}. *Ex vivo* studies were done with at least one month intervals. Prior to experiments, the blood status of the volunteer was checked. Specifically, hematocrit values ($50 \pm 4\%$) and hemoglobin contents (164 ± 12 g L^{-1}), were controlled before each test ensuring that the values obtained were in accordance with the values expected for the investigated subject.

During electrochemical measurements, the bioanode and biocathode were connected to a μAutolab Type III/FRA2 potentiostat/galvanostat from Metrohm Autolab B.V. (Utrecht, The Netherlands) (Fig. 3D). The enzymatic fuel cell performance was evaluated by linear sweep voltametry using a scan rate of 1 mV s^{-1} from the open-circuit voltage to lower voltage regions, while recording the current in the circuit. The low scan rate eliminated charging currents of the double layer. During *ex vivo* tests of tubular enzymatic fuel cells the following average characteristics were registered: 0.31 ± 0.02 V open-circuit voltage and 0.74 ± 0.06 μW maximal electric power at a cell voltage of 0.16 ± 0.02 V (Fig. 4A, red curve).

For proof-of-principle demonstration of electric power generation, the bioanode and biocathode were connected to a low voltage flexible display from Acreo AB (Linköping, Sweden) (Movie S3). The volumetric blood flow rate during the continuous bleeding of the volunteer was calculated from the total blood volume (200-400 mL per measurement) and time, measured using a digital timer 34-2362 from Clas Ohlson (London, UK). The blood volume velocity during *ex vivo* studies was measured to be 0.33 ± 0.03 mL s^{-1}. After collection, blood was utilised following standard handling routines for biological hazards applied in hospitals. Briefly, the content of the graduated glass cylinder was autoclaved and the remains of the blood were sent to destruction, following regulations and recommendations existing at the Department of Biomedical Science, Malmö University.

1.6. *In vitro* studies

A tubular system, consisting of plastic tubes and tubular electrodes as described above, were hermetically connected to a 500 mL closed buffer flask from United Scientific Supplies, with the oxygen concentration appropriately adjusted (*vide supra*). The flask was thermostated using an Assistent WTE var 3185 thermostat from Glaswarenfabrik Karl Hecht GmbH (Sondheim v.d. Rhön, Germany). The electrolytes were pumped through the system using a peristaltic pump U1/4-8R from Alitea Instruments (Medina, WA, USA). Separate bioelectrodes, as well as complete enzymatic fuel cells, were connected to a μAutolab Type III/FRA2 potentiostat/galvanostat and evaluated by linear sweep voltametry with a scan rate of 1 mV s^{-1}. As a complementary method to characterize the biofuel cell performance, a few measurements were carried out with different resistors, 1–100 MΩ, from Velleman Inc. (Forth Worth, TX, USA), which were calibrated using the μAutolab.

1.7. Biomedical blood analysis

Blood samples from the donor were drawn anaerobically into 1 mL syringes Plastipak™ from Becton Dickinson (Madrid, Spain). In the case of *ex vivo*

electrochemical studies (Fig. 3D) they were directly examined using an ABL80 blood gas analyzer from Radiometer (Copenhagen, Denmark) regarding oxygen partial pressure (pO_2), glucose, pH, and electrolytes (K^+, Na^+, Cl^-), Averaged values were measured at 0.082 ± 0.012 mM, 6.4 ± 0.06 mM, 7.40 ± 0.02, and 4.05 ± 0.08 mM K^+, 141.4 ± 0.4 mM Na^+, 105.4 ± 0.1 mM Cl^-, respectively (non-fasting conditions). During blood storage under air saturated conditions oxygen concentration increased from 0.082 mM to 0.209 ± 0.009 mM, whereas glucose concentration and blood pH dropped in 10 min from 6.4 and 7.40 down to 4.8 ± 0.09 mM and 7.34 ± 0.02, respectively.

In the case of separate studies regarding possible changes of the main parameters during blood storage at different temperatures (Fig. 1), samples were transferred into heparinized (240 IU mL^{-1} blood Sodium Heparine) 85 µL plastic capillaries (safe clinitubes) using an ABL77/ABL80 FLEX capillary adapters, and finally sealed using capillary sealers, all from Radiometer, to minimize air contact. The sealed capillaries were stored on ice at 0 °C, at room temperature, 25 °C, or in a metal block at 37 °C heated using a Hotplate Stirrer Model L-81 from Labinco BV (Breda, The Netherlands). The blood was examined using the ABL80 blood gas analyzer. Consecutive measurements were carried out using 5 min intervals until 90 min post sampling.

Blood plasma redox active compounds, *viz.* lactate, urate, and ascorbate, were assayed spectrophotometrically during *ex vivo* measurements using a Pharmaspec UV-1700 spectrophotometer from Shimadzu (Kyoto, Japan). All measurements were performed following the instructions from manufacturers using specific kits from Randox Laboratories Ltd. (Crumlin, UK) for lactate and urate, as well as from BioAssay Systems (Hayward, CA, USA) in the case of ascorbate. Averaged values were found to be 0.045 ± 0.002 mM, 0.425 ± 0.010 mM, and 1.92 ± 0.03 mM for ascorbate, urate, and lactate, respectively.

Whole blood (dynamic) viscosity at a shear rate of 208 s^{-1} was calculated from hematocrit and total plasma protein values, *viz.* 44.4 ± 1.1% and 64 ± 2 g L^{-1}, respectively, as described previously.[5] Hematocrit values were obtained from a Sysmex automatic hematological analyzer XS-1000i from Sysmex Cooperation (Kobe, Japan) and total protein was determined spectrophotometrically using a Pierce BCA protein assay kit from Thermo Scientific (Rockford, IL, USA). The volunteer's blood density was measured gravimetrically using a graduated glass cylinder from United Scientific Supplies and a PC 2000 balance from Mettler Toledo (Stockholm, Sweden). The whole blood viscosity and density values were measured to be 4.28 ± 0.02 mPa s and 1.061 ± 0.002 kg L^{-1}, respectively, which are typical values for a white, apparently healthy and normal weight male.[5]

2. Supplementary Text

2.1. Catalyst selection

Among many possible non-biogenic and biogenic compounds, which are able to accelerate chemical reactions, oxidoreductases (redox enzymes) in general are exceptional biocatalysts,[6] reaching catalytic turnover numbers of 10^7 s^{-1}, *i.e.* close to the diffusion-controlled rates of redox reactions even in homogeneous systems. Thus, at least in theory, redox enzymes could be used to create the most powerful fuel cells, compared to all other devices based on both non-biogenic and biogenic compounds.[7] Moreover, oxidoreductases are natural renewable catalysts, which can be produced at low costs.

Additionally, the unparalleled selectivity makes enzyme utilization in different applications highly advantageous not only technologically, but also scientifically and commercially, by eliminating problems regarding cross-reactions and catalyst poisoning. This is pivotal in the design of biodevices, since it allows fabrication of membrane-less, single compartment biofuel cells, removing not only voltage losses, but also mitigating technical challenges that otherwise transpire.[8] Employing a direct electron transfer based design allows for significant simplifications and improvements in the construction of biodevices; mediator induced voltage losses can be altogether avoided and potentially toxic mediator compounds can be excluded.[9] Finally, many oxidoreductases are highly active at close to neutral pH values and at room temperature, *i.e.* conditions at which an implanted device would be expected to operate.

2.2. Analysis of biodevice performance

In spite of the seemingly identical artificial and natural systems, *i.e.* identical outer diameters of the tubes, flow rate, and composition of electrolytes (glucose and oxygen concentrations, as well as pH), measured voltages during the initial *in vitro* tests were impressively high compared to the *ex vivo* performance of the fuel cell, *i.e.*, 0.6 ± 0.03 V open-circuit voltage and 1.6 ± 0.1 μW maximal electric power at a cell voltage as high as 0.5 ± 0.01 V (*cf.* red and blue curves in Fig. 4A). Air saturated (as high as 0.22 mM oxygen) 5 mM glucose in PBS, pH 7.4, is usually considered in electrochemistry as an acceptable blood reference buffer to study potentially implantable fuel cells. Our results demonstrate that the performance of a glucose/oxygen biofuel cell in such buffers will be seriously exaggerated (even at significantly lower oxygen concentration) compared to biodevice operation *ex vivo* (*cf.* blue and red curves in Fig. 4A), indicating the necessity to use a more complex buffer. Based on literature data,[10, 11] as well as our own previous work concerning the operation of direct electron transfer based biofuel cells in different human physiological fluids, such as serum, plasma, saliva, sweat, and tears,[3, 9, 12, 13] we assumed that the presence of low redox potential bio-fuels reduce the performance of the bilirubin oxidase based biocathode, which becomes the limiting electrode during electric power generation. Thus, we determined the average concentrations of ascorbate, urate, and lactate in the volunteer's blood (*vide infra*) and performed additional *in vitro* studies in an electrolyte, which more closely mimics human blood (Fig. 4A).

Contrary to previous studies performed in oxygen-saturated chloride containing buffers,[10, 11] no deactivation of bilirubin oxidase by 0.425 mM urate was observed during both homogeneous and heterogeneous biocatalysis, whereas strong decrease in bio(electro)catalytic activity of the enzyme was detected in the presence of well-known enzyme inhibitors, such as F^- and CN^-. Hence we were led to infer that the significantly reduced biocathode performance is attributed to cathode depolarization, caused by parasitic oxidative processes occurring on exposed patches of the graphite electrode, *viz.* bare graphite (*cf.* curves in Fig. 4B). *Nota bene*, these direct non-enzymatic electrochemical reactions also occur on the bioanode surface, but they contribute to the overall current, though at significantly higher overpotentials compared to the bioelectrocatalytic oxidation of glucose (*cf.* curves in Fig. 4C). However, even in the presence of low potential redox active compounds the obtained electric power output from the enzymatic fuel cell was still slightly higher *in vitro* compared to *ex vivo*, *viz.* 0.83 ± 0.04 μW and 0.74 ± 0.06 μW, respectively. Thus, we proposed that the last

unaccounted factor in our studies and previous investigations, negatively affecting current output from the biodevice, was the high blood viscosity value, compared to the artificial electrolyte, essentially water, *i.e.* $4.28 \cdot 10^{-3}$ and $0.69 \cdot 10^{-3}$ Pa s, respectively.

Viscosity adjustment to the physiological level (*vide infra*) resulted in a close to authentic (complete) blood mimicking buffer, in which the performance of the tubular biofuel cell was almost identical to that *ex vivo* (*cf.* red and green curves in Fig. 4A). Specifically, during final *in vitro* tests of tubular enzymatic fuel cells in the complete blood mimicking buffer the following average characteristics were registered: 0.31 ± 0.02 V open-circuit voltage and 0.78 ± 0.06 μW maximal electric power at a cell voltage of 0.15 ± 0.02 V.

2.3. Calculations

2.3.1. Flow and velocity

Linear flow rates (v) of blood and buffers inside tubular graphite electrodes and the vein of interest, were calculated using Supplementary Equation S1.

$$v = \frac{4Q}{\pi D^2} \qquad \text{Supplementary Eq. 1}$$

where Q is the volumetric flow rate ($m^3\ s^{-1}$) and D is a tube diameter (m).

In our studies Q and D were measured to be $0.33 \cdot 10^{-6}$ $m^3\ s^{-1}$ and $4.9 \cdot 10^{-3}$ m, respectively. Thus, v values inside tubular graphite electrode and the vein were calculated to be 0.4204 m s^{-1} and 0.0175 m s^{-1}, respectively. Note that in human physiology volumetric and linear flow rates of blood are abbreviated as blood flow and blood velocity, respectively.

Reynolds numbers (R_e) were calculated using Supplementary Equation S2.

$$R_e = \frac{\rho V D}{\mu} \qquad \text{Supplementary Eq. 2}$$

where ρ is the fluid density (kg m^{-3}) and μ is the whole (dynamic) viscosity (Pa s).

In our studies ρ and μ were measured to be 1061 kg m^{-3} and $4.28 \cdot 10^{-3}$ Pa s, respectively. Thus, R_e values inside tubular electrodes and in the vein were calculated to be 104.22 and 21.26, respectively.

2.3.2. Limiting current and power outputs

Based on previous studies of the effective diffusivity of oxygen in whole blood[14] the diffusion coefficient of the bio-oxidant was assumed to be $1.38 \cdot 10^{-5}$ $cm^2\ s^{-1}$, taking into account the gas concentration in blood, 0.082 mM. The diffusion coefficient of glucose in blood at 37 °C was calculated using an approximate dependence of the diffusion coefficient on temperature in liquids, *i.e.* the Stokes–Einstein equation (Supplementary Equation S3):

$$\frac{D_{T_1}}{D_{T_2}} = \frac{T_1 \mu_{T_2}}{T_2 \mu_{T_1}} \qquad \text{Supplementary Eq. 3}$$

where D_T is the diffusion coefficient at a particular temperature T, T is the absolute temperature (K), and μ (Pa s) is the dynamic viscosity of the solvent at that temperature.

The calculation was done using a known value of the diffusion coefficient of the bio-fuel in water, *i.e.* $9.15 \cdot 10^{-6}$ cm^2 s^{-1} at 310.15 K,[15] assuming the viscosity of water being equal to $0.69 \cdot 10^{-3}$ Pa s.

Taking into account the main parameters of the tubular electrode system used in our studies, the maximal theoretical limiting current outputs (i_{Lim}) from bioelectrodes were calculated using Supplementary Equation S4.[16]

$$i_{Lim} = 5.43nFC_{bio}D^{2/3}x^{2/3}V_f^{1/3} \qquad \text{Supplementary Eq. 4}$$

where n is the number of electrons transferred, C_{bio} is the concentration of bio-fuel or bio-oxidant in blood (glucose 6.4 μmol cm^{-3} and oxygen 0.082 μmol cm^{-3}, respectively), D is the diffusion coefficients in whole blood at 37 °C ($1.48 \cdot 10^{-6}$ cm^2 s^{-1} and $1.38 \cdot 10^{-5}$ cm^2 s^{-1} for glucose and oxygen, respectively), x is the electrode length (1 cm), and v is the volumetric flow rate (0.33 cm^3 s^{-1}).

The maximal theoretical electric power outputs (P) from an implanted 1 cm long fuel cell were calculated singularly based on cathodic i_{Lim} using Supplementary Equation S5.

$$P = i_{Lim} \times V_{\max} \qquad \text{Supplementary Eq. 5}$$

where $V_{\max}$ is the maximal voltage of a glucose/oxygen fuel cell equal to 1.18 V, *i.e.* the potential difference between the standard redox potentials of the two half-cells, *i.e.* redox equilibrium potentials of gluconolactone/glucose and oxygen/water redox couples (Fig. 1, Supplementary Equation 6 and 7).

$$Glucono-1{,}5-lactone + 2H^+ + 2e^- \rightarrow Glucose \quad (E^0 = +0.05 \text{ V}) \qquad \text{Supplementary Eq. 6}$$

$$O_2 + 4H^+ + 4e^- \rightarrow H_2O \quad (E^0 = +1.23 \text{ V}) \qquad \text{Supplementary Eq. 7}$$

A broad physiological range of blood flow in superficial veins was assumed, *i.e.* 0.05-5 mL s^{-1}, taking into account variations in vessel sizes and locations, level of physiological activity, environmental conditions, etc.[17, 18] A broad range of blood glucose levels, from 2.2 mM for individuals with sever hypoglycemia up to 22 mM for individuals with hyperglycemia, has been reported,[19] and was taken into account in our calculations. A reported broad range of dissolved oxygen in venous blood, from 0.051 mM up to 0.086 mM[20] was also taken into account in our calculations. Since bioanode current outputs are significantly higher compared to biocathode currents, even at very low glucose concentrations, the maximal theoretical electric power outputs from an implanted 1 cm long fuel cell were calculated singularly based on cathodic limiting currents (Fig. 4E), taking into account the maximal theoretical voltage of the device equal to 1.18 V. Maximal (at maximal oxygen concentration equal to 0.086 mM and maximal blood flow equal to 5 mL s^{-1}) theoretical power output from 1 cm tubular fuel cell was calculated to be as high as 21 mW, whereas maximal glucose and oxygen mass transport limited current outputs were calculated to be equal to 5.119 and 0.177 mA, from the bioanode and the biocathode respectively.

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