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Chlorhexidine digluconate exerts bactericidal activity vs. gram positive Staphylococci with bioelectrocatalytic compatibility: High level disinfection for implantable biofuel cells

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Abstract

Implanted devices destined for contact with sterile body tissues, vasculature or fluids should be free of any microbial contamination that could lead to disease transmission. The disinfection and sterilisation of implantable biofuel cells is a challenging and largely overlooked subject due to the incompatibility of fragile biocatalytic components with classical treatments. Here we report the development of a convenient “soft” chemical treatment based on immersion of enzymatic bioelectrodes and biofuel cells in dilute aqueous chlorhexidine digluconate (CHx). We show that immersion treatment in a 0.5% solution of CHx for 5 min is sufficient to remove 10^{-6} log colony forming units of *Staphylococcus hominis* after 26 h while shorter treatments are less effective. Treatments with 0.2% CHx solutions were ineffective. Bioelectrocatalytic half-cell voltammetry revealed no loss in activity at the bioanode after the bactericidal treatment, while the cathode was less tolerant. A maximum power output loss of *ca.* 10% for the glucose/O₂ biofuel cell was observed following the 5 min CHx treatment, while the dialysis bag had a significant negative impact on the power output. Finally, we report a proof-of-concept *in vivo* operation for 4 days of a CHx-treated biofuel cell with a 3D printed holder and additional porous surgical tissue interface. Further assessments are necessary to rigorously validate sterilisation, biocompatibility and tissue response performance.

Keywords: Implantable medical device • Enzymatic glucose biosensor • antibacterial treatment
• Sterilisation • Bioelectrocatalysis • *in vivo* biofuel cell

1. Introduction

Battery-powered implantable medical devices (IMDs) such as cardiac pacemakers, drug delivery pumps, and glucose biosensors, are revolutionising healthcare monitoring and management. Enzymatic biofuel cells that convert chemical energy into electrical energy from natural substrates in the body via enzymatic electrodes are an eco-friendly energy-harvesting alternative to miniature battery chemistries^[1]. Recent years have seen the emergence of glucose/O₂ enzymatic biofuel cells as promising implantable power sources for low-power electronics, despite major issues to address including limited power output, lifetime, biocompatibility and sterilisation^[2,3]. Since glucose and oxygen are abundant in the human body, IMDs could be powered from the body's resources in an autonomous way. Significant breakthroughs have been made in the area of *in vivo* biofuel cells since 2010 when our team reported the first example of an enzymatic biofuel cell implanted in an animal (the retroperitoneal space of a Wister rat) that produced power from glucose and oxygen^[4]. Katz and coworkers, and others, have made numerous important developments on *in vivo* biofuel cells that operate in invertebrates and other mammals^[5-11], while Lee et al. recently expanded *in vivo* biofuel cells to implantation in birds^[12]. The possibility to power electronic devices *in vivo* or in artificial biofluids, with appropriate power management, has been demonstrated for powering devices such as an LED, pacemaker, temperature sensor and neurostimulator^[8,11-13]. The power outputs are typically on the order of 10s to 100s of μ Ws and far from the several mWs that can be achieved with biofuel cells operating outside of the body. The limited performance *in vivo* is due to factors including limited substrate availability, biofouling, foreign body response and electrode-enzyme deactivation and/or inhibition. The latest developments on implanted biofuel cells include advanced power management and wireless systems to monitor and control biofuel cell activity^[6,8,12,14], and *in vivo* demonstration for 1 to 2 months^[6,15]. Recent advances have been made on the development of biocompatible antifouling interfaces, for example, based on polyvinyl alcohol (PVA), cross-linked chitosan, and zwitterionic matrices^[6,15,16]. To the best of our knowledge, only Cinquin and coworkers have explored the use of a "sterilisation" (or bactericidal) treatment for *in vivo* biofuel cells^[6,17]. It is important to recognise that a sterilisation process requires, and therefore implies, process validation, and as such, one must be prudent when using the term sterilisation.

Bacterial infection is a major problem associated with implantable devices during the surgical process and everyday use, and therefore, a subject of significance for implantable biofuel cell devices, despite being much understudied. Gram-positive *Staphylococcus* bacteria

including *S. epidermis*, *S. Aureus*, *S. capitis*, *S. schleiferi*, and *S. hominis*, are a leading cause of medical device related infections (DRIs)^[18,19]. These human commensal bacteria are widely found on the surface of the skin and hands in particular. To prevent infection, sterilisation is a critical step in the preparation of devices introduced into the bloodstream or in contact with sterile tissues in the body. A sterilised medical device is one that is generally accepted as being free from viable microorganisms i.e. theoretically at a level of $\leq 1 \times 10^{-6}$ microorganisms. Some recommendations differentiate heat-resistant and heat-sensitive medical devices. For example, the latter would require a “low level of sterilisation” at a sterility assurance level (SAL) of 10^{-3} rather than the “pharmaceutical sterilisation” SAL of 10^{-6} .^[20] The most widely used and accepted sterilisation technologies for medical devices are heat (steam or dry), ethylene oxide gas, and radiation (gamma, x-ray or electrons) treatments^[21]. Alternative sterilisation methods include, particularly, hydrogen peroxide and ozone treatments.

Heat sterilisation by ‘moist’ (e.g. autoclave) or ‘dry’ heat is typically performed at temperatures of $\geq 120^\circ\text{C}$ for 30 min and $\geq 170^\circ\text{C}$ for longer durations, respectively. Such methods are to be discounted for enzyme-based systems unless appropriately developed thermophilic enzymes are used. Ohsaka and coworkers reported thermostable PQQ glucose dehydrogenase and laccase with activity even at 85°C - 100°C , although power performance was inferior at lower temperatures of 25°C - 40°C that are pertinent to *in vivo* biofuel cells^[22]. Ethylene oxide (EtO) is a commonly used medical sterilisation agent but has major drawbacks. It is extremely flammable and a recognized carcinogen (e.g. by the World Health Organisation, WHO) and requires specialised facilities. The facilities and methods are strictly controlled, yet several large facilities worldwide have been closed or potentially closed in the last few years due to high exposure levels^[23]. EtO could potentially be used for *in vivo* biofuel cells, especially since it is listed as a sterilisation method for enzyme biosensor matrices in several patents^[24-27]. In 2000, Ahmed *et al.*, made an insightful assessment of chemical and gamma irradiation treatments, concluding that the “gold standard dose” for terminal sterilisation of 25 kGy gamma irradiation was well tolerated and provided device sterility for glucose enzyme electrodes^[28]. Martin and coworkers reported an optimal dose of 12 kGy for a laccase biocathode that was sufficient to show absence of implant infection and to “neutralise” (a 5-log reduction) of resistant *Geobacillus stearothermophilus* spores while maintaining around 70% of the initial bioelectrocatalytic activity^[17,29,30]. The same irradiation dose was also used for a glucose oxidase (GOx) bioanode and glucose/O₂ biofuel cell^[6]. Alternative chemical sterilisation approaches include the use of vaporised H₂O₂ (VHP), ozone, and liquid chemical sterilants based on H₂O₂, glutaraldehyde, isopropyl alcohol (IPA), and a mixture of chlorhexidine

digluconate (CHx)/IPA^[27,28,31–33]. VHP methods have now been approved for relatively rapid medical device sterilisation (e.g. 30 to 45 min) but like EtO, requires special handling of a toxic chemical. Initial research on the use of glucose bioelectrodes suggests substantial response degradation with IPA and CHx/IPA and very good “sensocompatibility” for 2% alkanised glutaraldehyde solution (0.5 to 8 h) and 0.6% H₂O₂ (4 days) treatments but not 0.93% H₂O₂ treatments^[28,31]. The sterility was not assessed for the IPA treatments while the glutaraldehyde treatment was found to be more effective than a longer 0.6% H₂O₂ treatment; nevertheless, the glutaraldehyde treatment was not able to reduce bacteria count below 10⁻² spores under the conditions tested against *Bacillus subtilis*^[28].

Chlorhexidine is well known as an effective broad-spectrum antimicrobial agent against Gram-positive bacteria, Gram negative bacteria, fungi and viruses. At low concentrations (0.02% to 0.06%) CHx has bacteriostatic activity. At higher concentrations such as 0.12%, chlorhexidine solutions are known to be bactericidal^[34]. CHx is a cationic molecule (Figure 1) that binds to cell membranes (for example, via negatively charged phospholipids) and disrupts cell function and structure, eventually leading to cell death by cytolysis with release of intracellular components^[34,35]. The antifungal ability is related to the prevention of biofilm formation rather than disruption of the cellular membrane. CHx is widely used in clinical settings for skin disinfection before and during surgery and injections, to sterilise surgical instruments and devices (e.g. catheters), across the field of dentistry from mouthwash to oral gel products to root canal irrigating solutions, and in various drug delivery devices^[34,36–38]. CHx-impregnated devices in animals showed promising anti-infective activity; in one example, chlorhexidine was combined with silver sulfadiazine on Arrowgard Blue® central venous catheters^[39,40]. In 2011, a CHx-coated catheter was cleared for human use^[37]. Later, a CHx-releasing epoxy-based Ti orthopaedic implant displayed potent bactericidal activity against *S. aureus* and was well tolerated *in vivo* in mice, with no signs of toxicity by histological analysis despite high local concentrations of CHx (5 to 10 wt%)^[41]. Hypersensitivity reactions have been reported and serious reactions are rare although do exist. General guidelines are to limit direct exposure of CHx with the body; for example, CHx mouthwashes are only licensed for 30 days’ use at a time in the UK. CHx digluconate solutions or gels (up to 4% - 5%) are listed in the 2021 WHO Model List of Essential Medicines^[42]. The use of CHx as an alternative sterilisation method for bioelectrodes was proposed by Ahmed et al. whose study focused on GOx bioelectrode needles for implantable biosensors^[31]. In this work, mixed solutions of 0.5% w/v chlorhexidine gluconate in 70% IPA were used for 2, 10, and 30-minute treatments. After 2 minutes of “sterilisation”, around 40% of the electrocatalytic current was lost across the glucose

concentration range of 2 to 30 mmol L⁻¹. The response time performance was not negatively impacted and no sterility or antibacterial study data was reported. Furthermore, no data was reported for the use of chlorhexidine without organic solvent.

In the present work, we explore the use of dilute solvent chlorhexidine solutions prepared in water (no organic solvent) as a rapid and convenient disinfection and potential sterilisation treatment for implantable enzymatic bioelectrodes and biofuel cells. Concentration- and time-dependent antimicrobial effects of CHx solutions are considered, as well as the effect of the CHx treatment on bioelectrocatalytic voltammograms at a GOx-based anode and a bilirubin oxidase (BOx)-based cathode. The effect of chlorhexidine treatment on glucose/O₂ biofuel cells in the absence and presence of a permeable cellulose ester dialysis bag are also described. Finally, the first operation of an implanted CHx disinfected glucose/O₂ biofuel cell in a dialysis bag is reported following implantation in the abdomen of a freely-moving rat.

2. Materials and Methods

2.1. Chemicals and materials

Phosphate buffers (monosodium phosphate monohydrate, NaH₂PO₄; disodium hydrogen phosphate heptahydrate, Na₂HPO₄), NaOH, potassium chloride, glucose oxidase (GOx from *Aspergillus niger*, 163.4 U mg⁻¹ solid), catalase (cat) from bovine liver (1600 U mg⁻¹ solid), 1,4-naphthoquinone (1,4-NQ), iron-protoporphyrin IX (FePP), d-(+)-glucose, chlorhexidine digluconate (CHx; Sigma C9394), LB broth medium (Sigma L3022), and sterile physiological solution with 0.9% NaCl (physiodose, 0.154 mol L⁻¹) were purchased from Sigma Aldrich. Distilled water was purified to a minimum resistivity of 15 MΩ cm⁻¹ using a Millipore Ultrapure system. Sterile glucose was from Lavoisier (injectable solution in ampoule 1665 mmol L⁻¹ (G30 %)). Bilirubin oxidase (BOx, 1.96 U mg⁻¹) from *Myrothecium verrucaria* was purchased from Amano. All enzymes were stored at -20 °C when not in use. Commercial grade thin multi-walled carbon nanotubes (MWCNTs, Ø = 9.5 nm; 1.5 µm length, 90% purity, NC7000) were obtained from Nanocyl and used as received without purification. High purity oxygen and argon was obtained from Messer. LB-Agar medium was obtained from VWR Chemicals (Miller, 84684.0500), and sterile purified water was purchased from Aguetant (Otec 600500). Biotech cellulose ester dialysis bags (cut-off = 3.5 - 5 kDa) were from Spectrum Labs and polypropylene SWING-MESH® SMX 3030S Swing Technologies surgical tissue (mesh size = 0.7 × 0.7 mm) from THT-bioscience. Loctite superglue-3 power flex gel was used from Henkel. Freshly-prepared glucose solutions were left to mutarotate overnight to β-D-glucose prior to use.

2.2. Preparation of bioanode and biocathode biopellets

Following our previously reported procedure, MWCNT redox biopellet electrodes ($\varnothing = 6$ mm, *ca.* 1.8 ± 0.2 mm thick) were obtained by soft grinding of the following mixtures^[43]: (i) GOx-1,4-NQ-cat bioanode: 50 μ L distilled H₂O, 7.5 mg of GOx; 5 mg of cat, 2.5 mg of 1,4-NQ, and 17.5 mg of CNTs; (ii) BOx-FePP biocathode: 50 μ L distilled H₂O, 7.5 mg of BOx; 2.5 mg of FePP, and 17.5 mg of CNTs. The resulting homogeneous pastes were then compressed into disks using a manual hydraulic press (Specac, Eurolabo) with a pressure of *ca.* 0.5 ton for 60 s. An Ag-plated multifilament **Cu-coated** wire was connected to the back of the disk with carbon paste (Henkel). Silicone was added to the back and sides to insulate and stabilise the connected disk. Electroactive surface areas are based on the geometric area: 0.28 cm². Redox biopellet electrodes were rinsed with distilled H₂O to remove weakly-adsorbed species prior to use.

2.3. Preparation of 3D printed biofuel cells

Enzymatic biofuel cells for *in vitro* experiments in 0.1 mol L⁻¹ phosphate buffer (PB) (pH 7.4) were prepared by mounting the bioanode and biocathode vis-à-vis on a 3D printed U-shaped polylactic acid (PLA) holder. An Ultimaker 2 Extended and 3D printer filament, PLA (RS PRO, 832-0264), were used for 3D printing. Biopellets connected individually to metal wires (see Section 2.2) were attached to the PLA holder using carbon paste and silicone, ensuring that the wire and the back and sides of the CNT biopellet were insulated. The maximum dimensions of the holder are 12 \times 11 \times 6 mm (height, width, depth) with an inter-electrode spacing of 5 mm. For the case where a dialysis bag was used, the biofuel cell was inserted into the bag prefilled with 100 mmol L⁻¹ glucose in 0.1 mol L⁻¹ PB (pH 7.4) then sealed with Loctite super glue. The final dimensions of the dialysis bag biofuel cell were *ca.* 40 \times 25 \times 10 mm. All biofuel cells were left in a beaker containing 100 mmol L⁻¹ glucose in 0.1 mol L⁻¹ PB (pH 7.4) for 2 h prior to characterisation; in the case that dialysis bags were used, this assured humidification of the bags. All biofuel cells were rinsed with distilled H₂O prior to use.

2.4. Preparation and implantation of 3D printed biofuel cells in a rat

The *in vivo* biofuel cells were prepared as described in Section 2.3. but with the use of an additional sterile surgical porous tissue outer layer (SWING-MESH® SMX 3030 S) that was wrapped around the biofuel cell then sealed by non-absorbable surgical sutures, after pre-filling with 5 mmol L⁻¹ sterile glucose in sterile 0.1 mol L⁻¹ PB (pH 7.4). The final dimensions of the implanted dialysis bag biofuel cell were *ca.* 50 \times 30 \times 20 mm. The preparation of the biofuel cell in the dialysis bag and surgical tissue was performed in a laminar flow hood while the

bioelectrodes and mounting of the biofuel cell on the 3D holder were not performed in a sterile environment.

2.5. Surgical procedure

Male Sprague-Dawley rats aged 3-8 months and weighing 275 to 600 g were used. Experimental protocols were approved by the CELYNE (CEA-042) committee on animals in research (APAFIS#21710_2019080811485194_v2), according to the European Directive 2010/63/UE, and respected the ARRIVE guidelines. The rats were housed in pairs on a 12-h light/dark cycle with controlled temperature ($23 \pm 3^\circ\text{C}$) and hygrometry ($50 \pm 5\%$). Prior to surgery, rats received buprenorphine (Buprecare, Axience, Patin, France, 0.05 mg kg^{-1}) and carprofen (Rimadyl, Zoetis, Malakoff, France, 5 mg kg^{-1}) for pain management (analgesia). Carprofen is a non-steroidal anti-inflammatory agent that is used to relieve pain and inflammation in a variety of species including rodents. The rats were anaesthetised with isoflurane (Iso-Vet, Piramal, Voorshoten, The Netherlands) using 50% O_2 – 50% air (4 to 5% for induction, 1.8% to 2.5% for maintenance). The abdomen and the head of the Sprague-Dawley rat were shaved and cleaned with povidone iodide (Vetedine, Vetoquinol, Tarare, France, 10%). A small incision was subsequently made on the skull, then the rat was placed in dorsal decubitus. A second 6 to 7 cm incision was subsequently prepared through the skin and the abdominal wall. The biofuel cell in the dialysis bag with the surgical tissue was gently placed on the viscera. The abdominal wall and the skin were then sutured independently using resorbable sutures. The conducting metal wires connected to the anode and cathode were tunneled subcutaneously to the neck and exited via the small incision on the skull. The rat was then placed in ventral decubitus. The two metal wires of the anode and cathode were soldered to a 5-pin connector that was glued to the skull using dental cement. The wound was then cleaned with 10% povidone iodide and sutured. After surgery, the rats were given 5 mL of a 5% sterile glucose solution subcutaneously with 0.05 mg kg^{-1} buprenorphine, then left undisturbed and heated with a lamp for 12 h. For analgesic pain management treatment, 0.05 mg kg^{-1} buprenorphine was administered during the first two days, followed by 5 mg kg^{-1} Carprofen for subsequent days. The rat was offered moistened food and fresh fruits, and received a daily subcutaneous injection of 5 mL of glucose 5% with the animal weight and behavior checked each day.

2.6. Chlorhexidine treatment of bioelectrodes and biofuel cells

Electrically-addressable bioelectrodes and biofuel cells were immersed in a beaker containing CH_x (0.2%, 0.5% or 1% w/v) prepared freshly in distilled H₂O (for electrochemical experiments) and sterile distilled H₂O (for microbiology experiments). All electrodes and biofuel cells were rinsed with the distilled or sterile distilled H₂O prior to use.

2.7. Electrochemistry

Electrochemical measurements performed “*in vitro*” were made in 0.1 mol L⁻¹ PB (pH 7.4) in the absence or presence of substrates using a Biologic VMP3 Multi Potentiostat with EC-lab software or Ivium CompactStat potentiostat with Iviumsoft software, respectively. All *in vitro* experiments were performed at 25°C. Half-cell characterisation experiments were performed with a three-electrode cell comprising a biopellet working electrode ($\varnothing = 6$ mm; thickness = 1.8 ± 0.2 cm), a silver-silver chloride reference electrode (Ag/AgCl with saturated KCl), and a Pt wire counter electrode. Electrodes were rinsed with distilled H₂O prior to use. For biocathode characterisation, oxygen and argon saturated solution tests were made after purging the solution with the gas for 15 min; light gas purging was continued during the experiments. Bioelectrodes were stored in fresh buffer solution between experiments at 4°C in the fridge then slowly warmed to room temperature (25°C) prior to characterisation by cyclic voltammetry. Reported catalytic currents were background corrected against the background voltammograms recorded in the absence of glucose (bioanode) or in argon saturated solution (biocathode). Catalytic current densities reported in the text were estimated based on the geometric area: 0.28 cm². *In vitro* biofuel cell experiments were performed by recording a linear sweep polarisation from the open circuit voltage (OCV) to 0.3 V with the anode connected to the counter and reference leads and the cathode connected to the working lead (1 mV s⁻¹). Biofuel cell tests were performed after a stable OCV was reached (*ca.* 15 min).

2.8. Microbiology

In an aseptic laminar flow hood, the electrically-addressable biopellet electrode was inserted in the cellulose ester dialysis membrane bag containing a sterile 5 mmol L⁻¹ glucose solution prepared in sterile 0.1 mol L⁻¹ PB (pH 7.4). The whole bioelectrode in a dialysis bag was immersed, or not, in a bacterial inoculum of *Staphylococcus hominis* (10⁷ bacteria mL⁻¹). After 15 min, the biopellet electrode was rinsed with sterile distilled water and then immersed or not in CH_x solution (see Section 2.5.) prepared in distilled water. Following treatment, the biopellet electrode in a dialysis bag was rinsed with sterile distilled water.

At the same time, in a laminar flow hood, 150 mL of sterile LB broth medium was introduced into an Erlenmeyer flask. The treated or untreated bioelectrode was then immersed in sterile LB solution. Each Erlenmeyer flask was capped and placed at 37°C on a rotary incubator shaker. Samples of the medium were taken after 26 h incubation. 100 µL was subsequently added to a petri dish containing prepared LB-Agar before being spread and placed in an incubator at 37°C overnight for bacteria growth. Bacterial colonies were observed and manually counted. Results are expressed in colony forming units (CFU mL⁻¹). *Staphylococcus hominis* was used as a model gram-positive bacteria of the bacterial genus, *Staphylococcus*, to test the antimicrobial activity. *S. hominis* bacterial samples were adjusted at 10⁷ bacteria mL⁻¹ based on optical absorbance using a spectrometer and confirmed the next day by counting colonies on LB Agar plates from serial dilutions of the *S. hominis* bacterial samples. A cell spreader (Biologix, 65-1001), incubator (Thermo scientific), and incubator shaker (Excella E24, New Brunswick Scientific) were used.

3. Results and Discussion

3.1. Antimicrobial performance of CHx treatments

Initial experiments focused on evaluating the efficacy of various chlorhexidine treatments as a high-level disinfection and potential sterilisation treatment for implantable biofuel cells. The structure of chlorhexidine gluconate is shown in Figure 1A.

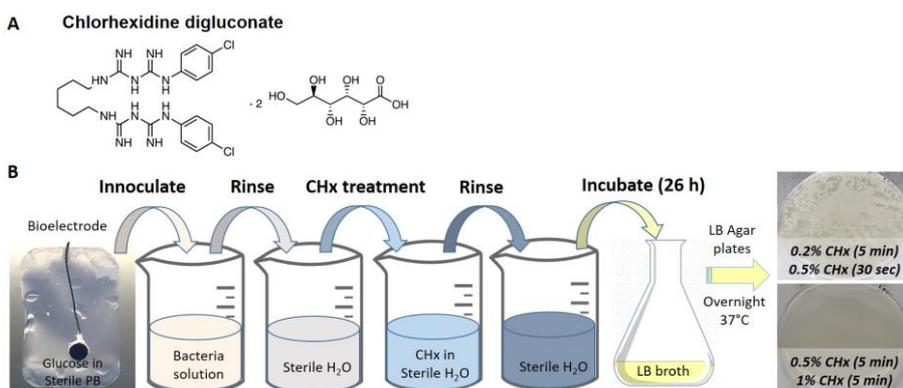


Figure 1: (A) Molecular structure of chlorhexidine digluconate (CHx) and (B) step-by-step representation of the methodology used from the deliberate introduction of bacteria to the bioelectrode, to chlorhexidine treatment, then incubation at 37°C for 26 h, and deposition of

100 μl of LB broth onto an agar plate then incubation overnight at 37°C prior to colony counting.

For the microbiology study, we used a model system that comprised of a redox pellet bioelectrode (biocathode) inserted in a dialysis bag in the absence and presence of 5 mmol L^{-1} glucose solution. A schematic of the colony forming unit assay methodology used, including examples of contaminated and bacteria-free lysogeny broth (LB) agar plates used for counting, are shown in Figure 1B and further detailed in Section 2.8. First, we report the observation (see Table 1) that no bacteria were observed on the agar plates after 26 h at the as-prepared enzymatic bioelectrode that was fabricated in our laboratory under non-sterile conditions then assembled in a dialysis bag with a sterile glucose solution under a laminar flow hood. The control sample prepared without the electrode (LB broth only) under a laminar flow hood in sterile water also showed no bacteria, validating the experiment. The bioelectrode assembled in a dialysis bag then treated with 0.5% CHx in PB for 5 min also showed no presence of bacteria. These findings reveal that our CNT bioelectrode fabrication method was not sensitive to bacterial contamination. On the other hand, the experiment failed to provide any information on the efficacy of the CHx treatment. We also performed two further control experiments with bioelectrodes in dialysis bags prepared (i) without glucose solution or (ii) with nonsterile glucose prepared in PB: no bacteria were observed without glucose solution while an uncountable bacteria film (> 17950 CFU) was observed when nonsterile glucose solution was used. The use of nonsterile glucose solutions in PB should be avoided where possible for the preparation of enzymatic bioelectrodes and biofuel cell systems.

Table 1: Influence of chlorhexidine treatments on cell culture colonies (CFU mL^{-1}) from as-prepared or intentionally bacteria-inoculated bioelectrodes in dialysis bags with sterile glucose.

Sample type ¹	Chemical treatment	Culture Medium ³	No. of colonies CFU mL^{-1} after 26 h incubation
No electrode (LB broth only)	No treatment	LB, 37°C	0
As-prepared bioelectrode	No treatment	LB, 37°C	0
As-prepared bioelectrode	0.5% CHx (5 min)	LB, 37°C	0
<i>S. Hominis</i> inoculated bioelectrode	PB only	LB, 37°C	> 17950 (TNTC)
<i>S. Hominis</i> inoculated bioelectrode	0.2% CHx (5 min)	LB, 37°C	> 17950 (TNTC)

<i>S. Hominis</i> inoculated bioelectrode	0.5% CHx (30 sec)	LB, 37°C	> 17950 (TNTC)
<i>S. Hominis</i> inoculated bioelectrode	0.5% CHx (1 min)	LB, 37°C	180
<i>S. Hominis</i> inoculated bioelectrode	0.5% CHx (5 min)	LB, 37°C	0
<i>S. Hominis</i> inoculated bioelectrode	1% CHx (5 min)	LB, 37°C	0

¹ All bioelectrode samples correspond to biocathodes sealed in dialysis bags containing sterile 5 mmol L⁻¹ glucose in sterile PB (pH 7.4); TNTC corresponds to Too Numerous To Count. ² Bacteria-inoculated refers to bioelectrode samples immersed in the *S. hominis* inoculum (10⁷ bacteria mL⁻¹) for 15 min then rinsed with sterile H₂O. ³ Bioelectrodes were incubated in sterile LB broth at 37°C under shaking, then, after 26 h, sub-samples of the LB broth were spread on LB agar plates and incubated at 37°C.

To obtain reproducible conditions of bacterial contamination, we intentionally contaminated the bioelectrodes by immersing them in an *S. hominis* inoculum (10⁷ bacteria mL⁻¹) for 15 min then, after rinsing with sterile distilled water, subjecting the bioelectrodes to the colony growth assay. Table 1 shows the number of colony forming units after a 26 h incubation for the *S. Hominis* contaminated bioelectrodes that were untreated or treated with dilute chlorhexidine solutions for different durations and at commonly used concentrations. We investigated the use of 0.2%, 0.5% and 1% CHx formulations since bacteriostatic and bactericidal effects have been observed at these concentrations, and with a view to using more diluted solutions where possible. The use of 0.12% to 2% CHx formulations is widespread in dentistry (e.g. for mouthwashes)^[34,35]. Higher concentrations of 0.5% to 5% (and even up to 10 wt%) are employed for skin and surgical tool disinfection and for antibacterial coatings on medical devices^[35–38,41].

For the pre-contaminated bioelectrode that was not treated with CHx, a dense and uncountable accumulation of *Staphylococci* bacteria was observed (see Figure 1B for an image of a representative contaminated agar plate). A similarly strong and uncountable growth of bacteria was also observed when a 0.2% CHx treatment for 5 min or less was used, or if a short duration treatment of 30 sec using 0.5% CHx was used. Such CHx treatments were clearly unsuitable and were not studied further. Previous reports suggested that 0.2% for 60 sec can be bactericidal against some but not all types of *Staphylococcus aureus*^[44]. We did not investigate this further but our results may indicate that the bacteria strain used in our study is more resistant. For the 0.5% CHx for 30 sec treatment, the treatment time in solution without IPA appears to be too short^[45].

S. Hominis contaminated bioelectrodes treated with higher concentrations of CHx for a minimum period of 1 minute were much more effective (Table 1). The use of 0.5% for 1 min proved bacteriostatic after 26 h while a prolonged treatment of 5 min proved bactericidal after the same period. We also tested the use of a 1% solution for 5 min and observed the same bactericidal effect as for 0.5%. These results clearly show the importance of both concentration and time on the antimicrobial behavior of chlorhexidine against *S. hominis*, and most importantly, allowed us to identify the lowest effective treatment of 0.5% CHx for 5 min. This treatment was herein adopted for bioelectrocatalytic and biofuel cell investigations.

3.2. Bioelectrocatalysis at untreated and CHx-treated enzymatic biocathodes and bioanodes

For biocathode construction, the multicopper oxidase (MCO), BOx from *Myrothecium verrucaria* (*Mv*), was used as the biocatalyst for the 4-electron reduction of O₂ to H₂O. We prepared redox biopellets via compression of the enzyme with multiwalled carbon nanotubes and a direct electron transfer (DET) promoting molecule, Fe-protoporphyrin IX (FePP)^[46,47]. Protoporphyrins are a precursor of the natural substrate, bilirubin, that intimately interact with BOx, resulting in favorable enzyme orientation and connection for high potential bioelectrocatalytic O₂ reduction. We previously explored CNT biopellet electrodes for *in vivo* biofuel cell experiments and chose to continue with this general type of high surface carbon nanotube bioelectrode. We note that such redox pellet type “compression” bioelectrodes have enabled high power and high stability (one year) enzymatic biofuel cells in buffers^[47,48].

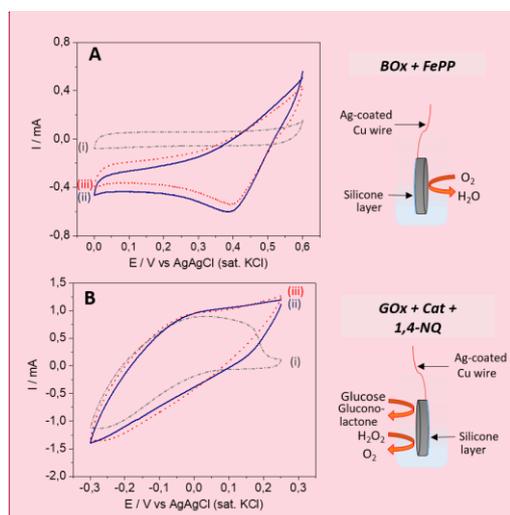


Figure 2: CVs of (A) bioelectrocatalytic O₂ reduction at (i, ii) BOx and (iii) CHx-treated BOx biocathodes in (i) Ar and (ii, iii) O₂ saturated PB (pH 7.4) solutions; (B) bioelectrocatalytic glucose oxidation at (i, ii) GOx-catalase and (iii) CHx-treated GOx-catalase bioanodes in quiescent PB (pH 7.4) solution in (i) the absence and (ii, iii) the presence of 100 mmol L⁻¹ glucose. CVs recorded at room temperature at scan rate = 0.2 mV s⁻¹.

Figure 2A shows representative CVs recorded in Ar and O₂-saturated PB solution at pH 7.4 for the BOx-FePP biocathode, and in O₂-saturated buffer for the CHx-treated BOx-FePP biocathode prepared using the standard chemical treatment (0.5% CHx for 5 min) and without dialysis bags. The CHx-treated biocathode was thoroughly rinsed in distilled H₂O to remove excess CHx. In the presence of argon, no catalytic currents are observed. Only the background capacitive current inherent to MWCNT bioelectrodes is observed. In the presence of oxygen at the untreated biocathode, a well-defined bioelectrocatalytic reduction current appears at an onset potential of *ca.* 0.52 V vs. Ag/AgCl (*sat.* KCl) that is close to the redox potential of the T₁ Cu centre of BOx that is responsible for substrate oxidation and successive electron transfers^[46], confirming effective DET bioelectrocatalytic O₂ reduction. An average catalytic current maximum of 0.59 ± 0.06 mA (2.11 ± 0.21 mA cm⁻²) at 0.36 ± 0.04 V and quasi-plateau of 0.40 ± 0.06 mA (1.43 ± 0.21 mA cm⁻²) at 0.1 V vs. Ag/AgCl (*sat.* KCl) is observed. After CHx treatment using the lowest effective bactericidal treatment of 0.5% for 5 min, the BOx-FePP bioelectrode retains the high potential O₂ reduction peak as well as the current plateau at similar potentials. A high potential catalytic current maximum of 0.50 ± 0.02 mA (1.79 ± 0.07 mA cm⁻²) at 0.38 ± 0.01 V and a low potential catalytic current plateau of 0.34 mA (1.21 mA

Commented [AG1]: 'Ag wire' replaced with 'Ag-coated Cu wire'

cm^{-2}) at 0.1 V are observed, representing a 15% loss in catalysis at both the current maximum and the current plateau. To the best of our knowledge, Cinquin and coworkers are the only group to report the effect of a “sterilisation” treatment on a biofuel cell electrode (biocathode) prepared with immobilised enzymes. A roughly 30% loss in activity was reported after gamma ray treatment at 12 kGy at a laccase pellet biocathode^[29]. Since the catalytic slopes are similar (Figure 2A), reflecting similar enzyme orientation and electron transfer kinetics i.e. bioelectrocatalytic reactivity. The loss of 15% in catalytic current is more likely due to loss of enzyme from the surface or pore blocking effects that restrict the O_2 flux at the electrode. BOx orientation for DET occurs via the positively-charged region near the active site of the enzyme, favored by electrostatic attraction with negatively-charged groups at the electrode surface e.g. immobilised FePP molecules. It was hypothesised that enzyme orientation could be hindered due to interactions between dicationic CHx molecules ($\text{pK}_a = 10.3$ for the first 2 protonations) and negatively-charged porphyrin groups or the enzyme itself (isoelectric point, $\text{pI } 4.1$)^[43,49]. Kano and coworkers carefully unraveled the effect of electrostatics between BOx and COO^- groups at CNTs on the wave shape, onset potential and catalytic current^[50]. The wave shapes before and after CHx treatment are similar in our case, suggesting limited interactions if at all between CHx and biocatalytic components. If there are no strong interactions between CHx and the enzyme then it is difficult to explain why we would have an increased loss of enzyme following CHx treatment. On this basis, we tentatively attribute the losses in catalytic current to changes at the electrode interface, such as pore blocking via adsorbed CHx molecules or compounds, that hinders substrate mass transport. We note that even if the catalytic wave shapes are similar, we do not rule out the possibility that CHx interacts with the enzyme e.g. via a deactivation and/or inhibition mechanism. Importantly, we highlight that the loss of 15% observed here represents a notable improvement compared to the 30% loss in activity following 12 kGy gamma ray treatment. The gamma ray treatment method is also very costly and more time consuming in comparison.

For bioanode development, we prepared a classical GOx-catalase CNT biopellet electrode using 1,4-naphthoquinone (1,4-NQ) as the redox mediator^[47,48]. Catalase is included in the bioelectrode to enzymatically catalyse the decomposition of hydrogen peroxide to water and oxygen^[51]. Hydrogen peroxide can hamper single compartment biofuel cell performance via competing chemical and electrochemical reactions^[52]. Figure 2B shows CVs of the bioanodes recorded in 0 and 100 mmol L^{-1} glucose at the untreated bioanode and the 0.5% CHx-treated bioanode. CVs were recorded at a slow scan rate of 0.2 mV s^{-1} to minimise capacitive currents. In the absence of glucose at the untreated bioanode, we observed the

expected redox activity of the 1,4-NQ mediator at $E_{1/2} = -0.13$ V vs. Ag/AgCl (*sat.* KCl) at a CNT bioanode^[43]. In the presence of glucose, a marked increase in the anodic current appears from 0 V that reaches a near-maximum catalytic current of 0.97 ± 0.02 mA (3.46 ± 0.07 mA cm^{-2}) at 0.25 V vs. Ag/AgCl (*sat.* KCl). While the catalytic current is very good compared to previous biopellets, the current is nevertheless smaller than the 1.8 mA obtained under comparable conditions in previous reports^[46,53]. In these reports, FAD-GDH was used rather than GOx and a higher (saturating) glucose concentration of 150 mmol L⁻¹ was also employed. After CHx treatment, the 1,4-NQ reduction current and catalytic glucose oxidation current remain quite similar. Unexpectedly, a larger catalytic current of 1.13 ± 0.06 mA (4.04 mA cm^{-2}) at 0.25 V is observed after CHx treatment that corresponds to a minor 16.5% increase in catalysis. The very promising resistance and apparent minor performance boost of the bioanode to CHx treatment confirms the compatibility of this chemical method with the GOx-catalase redox pellet bioanode. In previous reports, glucose oxidase bioelectrodes suffered ‘substantial response degradation’ with IPA and CHx/IPA chemical sterilisation, or substantial 50% and 80% activity (sensitivity) losses following 6 kGy and 20 kGy gamma ray treatments^[25,31]. The absence of catalytic current loss following the mild CHx treatment indicates that there are no unfavorable interactions between CHx and biocatalytic components (including reduced quinone mediator species) or limitations in terms of glucose mass transport at this type of anode. The apparent background capacitance at low potentials might tentatively suggest some changes to the electrode interface that facilitate the electrical wiring but this was not investigated further.

3.3. *In vitro* glucose/O₂ biofuel cells in 0.1M mol L⁻¹ PB (pH 7.4): Influence of CHx treatment and dialysis bag

After confirming the bioelectrocatalytic compatibility of the 0.5% CHx (5 min) treatment, we transitioned to the characterisation of the complete enzymatic biofuel cell. The bioanode and biocathode were connected in a 3D-printed biofuel cell configuration (Figure 3) without (i) and with (ii) a dialysis bag (cut-off = 3.5 to 5 kDa) prefilled with 100 mmol L⁻¹ glucose in 0.1 mol L⁻¹ PB (pH 7.4). We employed a 3D holder to fix the electrodes into place to (i) avoid short-circuiting, unwanted contact of the electrode with other interfaces, or limited diffusion, and (ii) maintain a reproducible face-to-face configuration with a controlled inter-electrode spacing. We used PLA which is biocompatible and has been used previously for *in vivo* devices^[54]. Power generation from the glucose/O₂ fuel cells was investigated in oxygen-saturated (≈ 1.1 mmol L⁻¹) PB (pH 7.4) in the presence of 100 mmol L⁻¹ glucose, obtained using linear sweep voltammetry with a slow scan rate (1 mV s⁻¹). The average OCV for the control biofuel cell

without the dialysis bag or CHx treatment was 504 ± 6 mV (Figure 3A). After rinsing with distilled water, CHx treatment, and a further thorough rinse with distilled water, a stable average OCV of 534 ± 9 mV was obtained (Figure 3B). The small increase in OCV appears to correspond to a voltage gain from the anode, based on the half-cell performance. For both the untreated and treated biofuel cells, the maximum power outputs were reached at the same current output of *ca.* 240 μ A. A slightly lower maximum power output of 96 ± 16 μ W for the CHx-treated biofuel (*vs.* 106 ± 9 μ W at the untreated biofuel cell) is linked to the reduced catalytic performance of the limiting biocathode following CHx treatment. The CHx-treated biofuel cell power output also fared less well compared to the untreated biofuel cell once the maximum power output was reached. Under these more “extreme” testing conditions, the current output at the CHx-treated biofuel cell dropped sharply, while at the untreated biofuel cell, a higher catalytic current output was reached as the voltage was swepted *i.e.* from *ca.* 0.4 to 0.3 V. The data clearly shows that the CHx-treatment provokes some small changes. And importantly, that the biofuel cell power performance tolerated excellently the short and convenient 0.5% for 5 min bactericidal treatment. It is noted that we also tested the effect of a longer CHx treatment time of 15 min using the 0.5% CHx solution. The OCV increased to 578 ± 2 mV while the power remained the same as that observed for the 5 min treatment (97 ± 11 μ W). The longer CHx treatment did not have any further negative impact on the biofuel cell power output. The improved OCV indicates a preferential effect for longer CHx treatment times beyond 5 min but this was not evaluated further.

Next, we evaluated the performance of as-prepared and CHx-treated biofuel cells compartmentalised in dialysis bags (Figure 3C and 3D). In previous *in vivo* biofuel cell experiments we compartmentalised bioelectrodes in cellulose acetate dialysis bags with an outer Dacron bag to (i) allow glucose and oxygen diffusion, (ii) to minimise possible leaching of bioelectrode components, and (iii) to prevent direct contact of the biofuel cell with the animal to limit adhesion and inflammation^[4,11]. The dialysis bag biofuel cell set-up is shown in Figure 3 ii with the dialysis bag containing PB (pH 7.4) with 100 mmol L⁻¹ glucose.

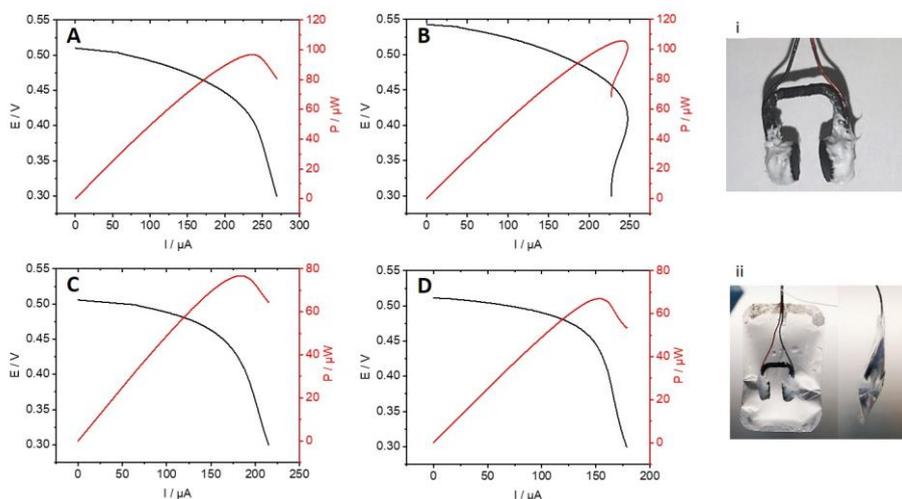


Figure 3: Representative biofuel cell polarisation (black line) and power curves (red line) recorded in oxygen-saturated PB (pH 7.4) in the presence of 100 mmol L⁻¹ glucose for biofuel cell configurations: (A) as-prepared, (B) 0.5% CHx-treated, (C) as-prepared with dialysis bag, and (D) 0.5% CHx-treated with dialysis bag. Polarisation voltammograms were recorded at 25°C at 1 mV s⁻¹.

For the untreated biofuel cell with the dialysis bag (Figure 3C), an initial OCV of 503 ± 3 mV is observed that is similar to the OCV of 504 ± 6 mV exhibited at the untreated biofuel cell without the dialysis bag (Figure 3A). The maximum power output of 80 ± 3 μW (Figure 3C) is however notably smaller than the maximum power output of 106 ± 9 μW obtained without the dialysis bag (Figure 3A). Further interpretation of the polarisation and power curves shows that the characteristic “high current” OCV drop is observed at lower currents when the dialysis bag is present. These results are consistent with the dialysis bag having a significant negative impact on biofuel cell performance, linked to increased mass transport limitations through the dialysis membrane. Finally, after CHx treatment and careful thorough rinsing, the CHx-treated biofuel cell with the dialysis bag was tested. For this biofuel cell configuration (Figure 3D) an equivalent OCV of 506 ± 6 mV was observed compared to the OCV of 503 ± 3 mV for the untreated biofuel cell with the dialysis bag (Figure 3C). The maximum power output for the CHx-treated biofuel cell in the dialysis bag was also very similar at 73 ± 6 μW compared to 80 ± 3 μW for the untreated biofuel cell with the dialysis bag. These results show no significant impact of the 0.5% CHx for 5 min treatment on dialysis bag biofuel cell performance, supporting the attractiveness of the treatment. It is nevertheless emphasized that the dialysis

bag clearly had a significant negative impact on the performance of both untreated and CHx-treated biofuel cells.

We also tested the effect of a longer treatment time of 15 min (0.5% CHx solution) on the dialysis bag biofuel cell. The 15 min treatment negatively affected the maximum power output ($64 \pm 8 \mu\text{W}$) while little change in OCV was exhibited ($487 \pm 32 \text{ mV}$) compared to the 5 min treatment (data not shown). The decreased power performance using a longer treatment time is consistent with a partial blocking of the dialysis bag pores by adsorbed CHx molecules and compounds; CHx is known to be to the formation of precipitates with anions other than gluconate^[55,56]. In control experiments, we observed precipitates prior to washing of the dialysis bag biofuel cell that we tentatively attribute to the formation of sodium and/or phosphate salts. The result observed for the 15 min treatment implies that, for this treatment condition, the washing step was not sufficient to effectively remove the precipitates.

In this study, we also recorded polarisation and power curves under more physiologically-relevant conditions of ambient dissolved oxygen (*ca.* 0.23 mmol L^{-1}) in a 0.01 mol L^{-1} phosphate saline solution (pH 7.4) containing 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, and 5 mmol L^{-1} glucose (Figure S1)^[46]. At an as-prepared biofuel cell without a dialysis bag, an OCV of 420 mV and a maximum power output of $14 \mu\text{W}$ ($50 \mu\text{W cm}^{-2}$) was exhibited. The power output is significantly lower than the $106 \pm 9 \mu\text{W}$ (Figure 3A) observed for biofuel cell tests performed in O_2 -saturated buffer free in the presence of 100 mmol L^{-1} glucose and free of chloride. The significant performance loss is to be expected, especially given the vastly lower substrate concentrations that limit the half-cell reactions, but nevertheless, the conditions are closer to those experienced *in vivo*.

3.3. Short-term operation *in vivo* of a CHx-treated glucose/ O_2 biofuel cell in a rat

After demonstrating CHx-treated biofuel cell performance *in vitro* we proceeded to implantation experiments. The CHx-treated biofuel cell in the dialysis bag was further wrapped in a surgical porous mesh and placed on the abdominal viscera of an anaesthetised rat (Figure 4A i-ii). Here we used a polypropylene material (Swing-Mesh) as opposed to previously-used Dacron® polyester. Both are suitable for abdominal implants to limit adhesion, improve biocompatibility, and avoid direct contact of the biofuel cell/dialysis bag with the animal. The large pores in the Swing-Mesh fabric are compatible for glucose diffusion and hence electrical power generation at the biofuel cell.

The *in vivo* biofuel cell was electrically tunneled and connected via the neck and skull of the rat to an Origaflex potentiostat. Daily subcutaneous injections of 5 mL of 5% glucose were

given to facilitate stabilisation of the body weight as well as biofuel cell function via glucose and O₂ consumption. The overall device geometry was rounded (Figure 4A i) to help avoid injury to the animal and limit local acute inflammations. The OCV was recorded periodically on different days while the rat was freely moving. The OCV here refers to the voltage obtained between the anode and cathode with no external load or external current flow. We cite the article of Xiao, Lojou, Liu and coworkers for further information regarding biofuel cell voltage considerations^[57]. The voltage obtained on day 0, approximately 60 min after implantation, was 0.17 V. The OCV continued to drop over the initial days which is very classic for such implanted biofuel cells. For example, in the work by Cinquin and coworkers, a glucose/O₂ biofuel cell in a rabbit exhibited a strong degradation in OCV from 0.35 to 0.05 V during the first week, before rising up to 0.42 V after 18 days^[6]. For our previous *in vivo* glucose/O₂ biofuel cells, the OCV of the biopellet-based biofuel cell operating in the retroperitoneal space of a rat was close to 0.2 to 0.3 V on day 0 with ~~OCV~~ OCV degradation during the first week. The large drop in OCV from 0.17 to 0.05 V over 4 days is classical and consistent with the literature, previously related to the weak diffusion of substrates and poisoning substances in the early stage of the inflammatory reaction^[4,6]. We can at least rule out hydration restrictions, as suggested previously^[6], since our biofuel cell was already pre-wetted in physiological buffer in the dialysis bag. We note that the OCV data is a limited indicator of the performance and lifetime of the biofuel cell and that artificial buffer-based extracellular fluids at 37°C were not used in our study but would provide a more realistic insight into the large performance decrease between “*in vitro*” and “*in vivo*” conditions. Nevertheless, such artificial buffer solutions are generally limited and do not fully reflect the complex *in vivo* environment. The performance decrease observed *in vivo* vs. *in vitro* is linked to factors including lower substrate concentrations and mass transport limitations through complex environments, the presence of inhibitors and interferences, foreign body responses, and biofouling. Our first results reported here show some progress in surgical procedures and materials as well as some success with the implantation of a biofuel cell a first proof of concept for a successfully implanted biofuel cell after a convenient “soft” and compatible disinfection treatment.

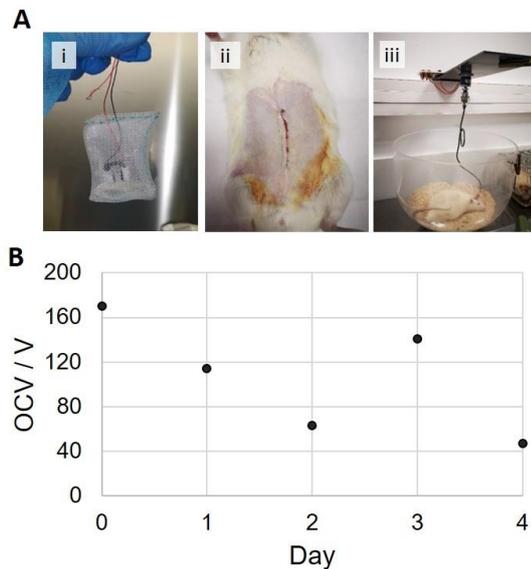


Figure 4: (A) Photographs of the CHx-treated biofuel cell in a dialysis bag with porous polypropylene medical grade surgical tissue (mesh size 0.7×0.7 mm) (i) before implantation, (ii) after implantation and stitching, and (iii) during measurement *in vivo* in the rat. (B) *In vivo* CHx-treated biofuel cell OCV data recorded periodically on the moving rat during 4 days.

Conclusion

Electrical energy generation *in vivo* from substrates present in biological fluids using implanted glucose/ O_2 biofuel cells offers exciting prospects for the battery-less powering of low-power electronics. Nevertheless, current problems linked especially to sterilisation, biocompatibility, and short lifetimes must be addressed and overcome. In this study we set out to make advances in the area of enzymatic biofuel cell sterilisation that has been almost entirely overlooked. Here we report an alternative chemical treatment method with high level disinfection (or sterilisation) for enzymatic biofuel cells that is very simple, convenient and low-cost. The treatment can be performed in any laboratory without requiring Co_{60} radiation treatment or the use of toxic reagents and/or organic solvents. Microbiology cell culture experiments revealed the importance of time and concentration to obtain an effective bactericidal treatment versus pathogenic *Staphylococci* bacteria. Cell cultures also indicate that the CNT biopellets were not susceptible to bacterial growth. Half-cell characterisation and biofuel cell experiments ultimately revealed minimal negative effects of the simple 5 min CHx treatment on bioelectrocatalytic and biofuel cell performance. The impact of dialysis bags on the biofuel

cells was also evaluated *in vitro*, to the best of our knowledge, for the first time. The data shows a clear drop in performance with integration of the external dialysis bag. A further small non-negligible drop in power output due to CHx treatment was observed but only when a longer treatment time of 15 min was used. The decreased performance is linked to an interaction between the dialysis bag and CHx molecules or salts. We developed new surgery protocols, report a new 3D-printed holder design, and provide our first, albeit preliminary, *in vivo* results in a moving rat up to 4 days following the disinfection. In future work, the inflammatory response must be evaluated. Cytotoxic tests as well as further cell cultures with diverse microbes are also required and should be evaluated in a more statistically rigorous manner. Our findings also point to the importance of implementing a highly porous biocompatible membrane to replace the use of dialysis bags. Further goals include reducing the device size and improving electrode geometry such that the loss of raw power output is minimised while device longevity is maximised. Despite the limitations of the study, the presented results are promising with a view to advancing the development of chemical treatments for the sterilization and/or disinfection of enzymatic bioelectrodes and biofuel cells.

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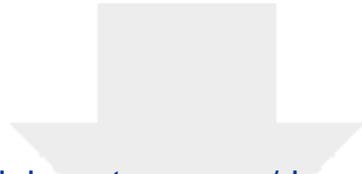
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Highlights

- Soft, convenient bactericidal treatment for enzymatic electrodes and biofuel cells
- Optimised chlorhexidine treatment with good bioelectrocatalytic compatibility
- Dialysis bag has a significant negative impact on biofuel cell power output

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: