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# Implanted Biofuel Cell Operating in a Living Snail

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## S Supporting Information

**ABSTRACT:** Implantable biofuel cells have been suggested as sustainable micropower sources operating in living organisms, but such bioelectronic systems are still exotic and very challenging to design. Very few examples of abiotic and enzyme-based biofuel cells operating in animals in vivo have been reported. Implantation of biocatalytic electrodes and extraction of electrical power from small living creatures is even more difficult and has not been achieved to date. Here we report on the first implanted biofuel cell continuously operating in a snail and producing electrical power over a long period of time using physiologically produced glucose as a fuel. The “electrified” snail, being a biotechnological living “device”, was able to regenerate glucose consumed by biocatalytic electrodes, upon appropriate feeding and relaxing, and then produce a new “portion” of electrical energy. The snail with the implanted biofuel cell will be able to operate in a natural environment, producing sustainable electrical micropower for activating various bioelectronic devices.

Microbial<sup>1–3</sup> and enzyme-based<sup>3–11</sup> biofuel cells achieved high attention and were rapidly developed in the past decade. While microbial biofuel cells are usually constructed as large-scale biological reactors, enzyme biofuel cells are mostly considered as micropower sources for implantable biomedical devices.<sup>12,13</sup> Despite the fact that implantable biofuel cells operating in vivo were suggested a long time ago,<sup>14</sup> such bioelectronic systems are still exotic and very challenging to design. Toward their ultimate use as implanted devices in a human body extracting power from glucose in blood, model biofuel cells were tested in vitro in human serum solutions,<sup>15,16</sup> but they were never really implanted in a human body and operated in vivo. Very few examples of abiotic biofuel cells (i.e., cells using inorganic catalytic electrodes) operating in animals in vivo have been reported.<sup>17,18</sup> To our best knowledge, only two papers have reported on *enzyme* biofuel cells implanted in animals and operated in vivo, one in the retroperitoneal space of freely moving rats<sup>19</sup> and another in a blood vessel in a rabbit ear.<sup>20</sup> The latter was a partially implanted biofuel cell that used glucose in blood as a biofuel oxidized at the anode, while a gas-diffusion cathode for oxygen reduction was placed outside the body.<sup>20</sup> It should also be noted that these biofuel cells<sup>19,20</sup> used many nonimmobilized materials (cofactors, mediators, etc), and thus, their operation required membranes, capillaries, and other compartmentalization tools.

In addition to future biomedical applications (e.g., powering future generations of implanted medical devices upon harvesting energy from fluids in a human body), another application of implanted biofuel cells is feasible for powering of (bio)sensors continuously monitoring external chemical and physical conditions. This might find important environmental, homeland security, and military applications. For these kinds of applications, the biofuel cells could be implanted in small living creatures, such as snails, worms, insects, etc., thus requiring operation under conditions significantly different from a human body. In this paper, we present the very first example of an implanted membraneless biofuel cell composed of two enzyme-modified biocatalytic electrodes operating in vivo in a well-living and free-moving snail.

The following challenging issues should be addressed in order to achieve sustainable operation of an implanted biofuel cell: (i) The biocatalytic enzymes should be immobilized on electrodes and should not require any cofactors, mediators, etc., that are not immobilized. (ii) Oxygen should not interfere with the anodic biocatalytic oxidation of the biofuel. (iii) The biocatalytic electrodes should be able to operate at low concentrations of the biological fuel and oxygen, particularly under conditions of their slow diffusion and high viscosity of the biological tissue.

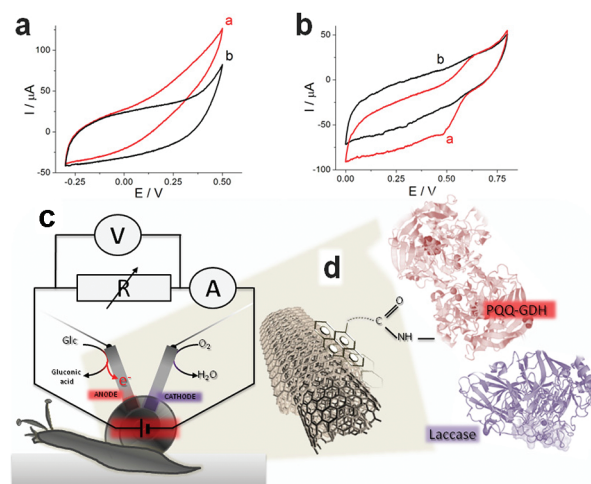
Direct nonmediated electrical “wiring” of enzymes on electrodes is always a challenging problem.<sup>21</sup> Direct electron transfer between the enzyme active centers and the electrode conducting supports is usually much less efficient than mediated electron transport unless very sophisticated multimolecular ensembles are architected on the electrode surfaces.<sup>22,23</sup> To achieve simple-in-preparation and robust-in-operation bioelectrocatalytic electrodes providing efficient nonmediated electrical “wiring” for immobilized enzymes, we applied nanostructured buckypaper composed of compressed multiwalled carbon nanotubes (CNTs).<sup>24–27</sup> The biocatalytic enzymes were linked to the CNTs using a heterobifunctional cross-linker, 1-pyrenebutanoic acid succinimidyl ester (PBSE), which provides covalent binding with amino groups of protein lysine residues through the formation of amide bonds and interacts with CNTs via  $\pi$ – $\pi$  stacking of the polyaromatic pyrenyl moieties.<sup>27</sup> Importantly, this linker results in a random orientation of the enzyme molecules relative to the conducting support because of the large number of amino groups

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differently positioned in the protein structure.<sup>28</sup> In the case of a flat, smooth electrode surface, this would not allow direct electron transport for most of the enzyme active centers, as they would be far away from the conducting support.<sup>28</sup> However, in the case of buckypaper, as a result of its nanostructured composition consisting of densely packed CNTs, enzyme active centers can find a nearby conducting wire regardless of the enzyme orientation, thus potentially allowing efficient electron transport for all (or at least for most) of the enzyme molecules. Selection of the biocatalytic enzymes associated with the buckypaper electrodes is also a very critical issue for an implantable biofuel cell. With the assumption that the biofuel cell should be based on oxygen reduction and glucose oxidation (biofuels other than glucose are potentially possible, but glucose was used in the present study as an example), oxygen-reducing laccase (E.C. 1.10.3.2, from *Trametes versicolor*) was selected for the cathodic reaction as a well-studied biocatalyst that is frequently used in enzyme-biofuel cells<sup>13</sup> and is particularly compatible with the buckypaper electrode.<sup>26</sup> Selection of the enzyme for the anodic reaction was more tricky. NAD<sup>+</sup>-dependent enzymes (e.g., glucose dehydrogenase) require NAD<sup>+</sup> cofactor in a solution, which is not permitted in the implantable biofuel cell.<sup>24</sup> Coimmobilization of NAD<sup>+</sup> cofactors on electrode surfaces always results in highly sophisticated procedures<sup>29</sup> and thus cannot be a good solution for robust implantable biofuel cells. On the other hand, O<sub>2</sub>-dependent oxidases (e.g., glucose oxidase, GOx) would generate H<sub>2</sub>O<sub>2</sub> in the presence of oxygen, but H<sub>2</sub>O<sub>2</sub> is toxic when it is produced in an implanted biofuel cell. Also, the enzyme reaction with oxygen would compete with the direct electron transfer to the electrode, thus inhibiting the current generation. Eventually, a GOx-based biocatalytic anode operating in the presence of oxygen may be possible, but it would require a very sophisticated multimolecular ensemble to achieve kinetically preferential electron transfer to the electrode instead of oxygen.<sup>30</sup> To exclude the problems associated with the NAD<sup>+</sup>- and O<sub>2</sub>-dependent enzymes, we selected pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (PQQ-GDH; E.C. 1.1.5.2) for the biocatalytic oxidation of glucose at the implanted anode. This enzyme does not require soluble cofactors, and its reaction does not interfere with oxygen. However, it has been much less studied for biofuel cell applications, and its direct electrical communication with the buckypaper electrode has not been previously demonstrated. The electrochemical characterization of the enzyme-biocatalytic electrodes and their operation in the biofuel cell implanted into a snail body are described below. The technical details and procedures are collected in the Supporting Information.

The buckypaper electrodes (0.25 cm<sup>2</sup> geometrical area) were modified with PBSE linker and then functionalized with laccase or PQQ-GDH to yield the biocatalytic cathode or anode, respectively (Figure 1d). An assay of the enzyme activity performed in vitro showed that the amounts of immobilized laccase and PQQ-GDH were 0.46 and 0.25 units per electrode, respectively. Cyclic voltammograms (CVs) of the PQQ-GDH- and laccase-modified electrodes were obtained in solutions free of glucose and O<sub>2</sub>, respectively (Figure 1a,b, curves b). The presence of glucose and O<sub>2</sub> in the respective solutions clearly resulted in the formation of bioelectrocatalytic currents (Figure 1a,b, curves a). The anodic current produced by the PQQ-GDH electrode, corresponding to glucose oxidation, was developed at potentials more positive than -0.1 V vs Ag/



**Figure 1.** (a) CVs of the PQQ-GDH anode in the presence (curve a) and absence (curve b) of 20 mM glucose. (b) CVs of the laccase cathode in the presence (curve a) and absence (curve b) of O<sub>2</sub>. All of the CVs were obtained in vitro in a solution composed of 22 mM NaHCO<sub>3</sub>, 40 mg mL<sup>-1</sup> BSA, 6.7 mM MgCl<sub>2</sub>, and 5 mM KCl (pH 7.4) at a scan rate of 1 mV s<sup>-1</sup>. (c) Circuit for the implanted biofuel cell. (d) Coupling of the enzymes with CNTs via the bifunctional linker PBSE.

AgCl, while the cathodic current for the oxygen reduction was produced by the laccase electrode at potentials more negative than 0.6 V vs Ag/AgCl, thus allowing potential difference of ca. 700 mV between the anodic and cathodic reactions. It should be noted that the beginning of the anodic and cathodic bioelectrocatalytic reactions approximately correspond to the potentials of the enzyme active centers (PQQ in GDH<sup>30</sup> and T1 in laccase,<sup>13</sup> respectively), thus confirming the direct nonmediated electrical communication between the active centers and electrode supports. In a control experiment, the bioelectrocatalytic electrodes were connected in a biofuel cell operating in vitro in a solution mimicking the snail hemolymph composition and demonstrated stable electrical output over a long period of time (at least 3 h) without any decay in activity, thus confirming the stability of the enzyme systems, as was previously shown in another related system.<sup>27</sup>

The biocatalytic electrodes were implanted in a snail (*Neohelix albolabris*) (Figure 2). Land snails are terrestrial gastropods (Gastropoda), and they have a main body cavity (the hemocoel) into which the blood (called hemolymph) is pumped by their heart. Oxygenated hemolymph is collected in a mantle cavity, which is modified into an air-breathing organ, and then consequently pumped into a number of open sinuses (they join to form the hemocoel). The tissues and organs are literally bathed in this oxygen-rich hemolymph. Glucose is the major form of carbohydrate found in the hemolymph of most gastropods (ca. 60 μM).<sup>31</sup> The oxygen content in hemolymph varies depending on the physiological conditions, but its concentration is always higher than the glucose concentration.<sup>32</sup> Thus, the oxygen content would not be a limiting factor for operation of an implanted biofuel cell. The implantable electrodes were inserted into the snail through two holes cut in the shell and placed into the hemolymph between the body wall and internal organs (visceral mass).

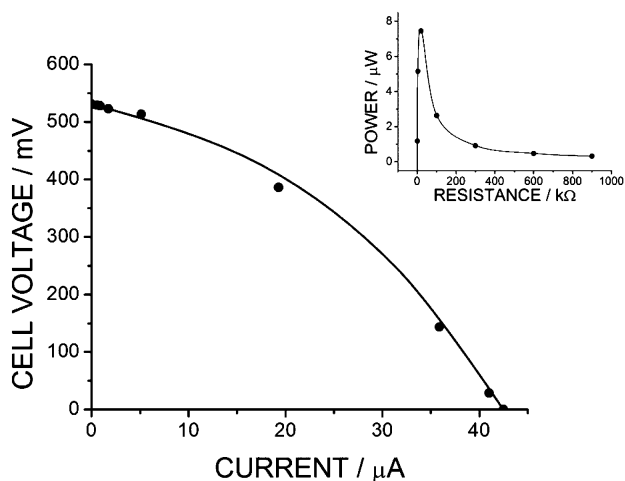
The implanted electrodes performing the bioelectrocatalytic reactions—glucose oxidation at the anode and O<sub>2</sub> reduction at the cathode—were connected in a circuit composed of an





**Figure 2.** Photograph of a snail with implanted biocatalytic electrodes connected with crocodile clips to the external circuitry (close view).

external variable-load resistance (Figure 1c), and the voltage and current were measured during biofuel cell operation in vivo (Figure 3). The open-circuit voltage ( $V_{oc}$ ) and short-circuit

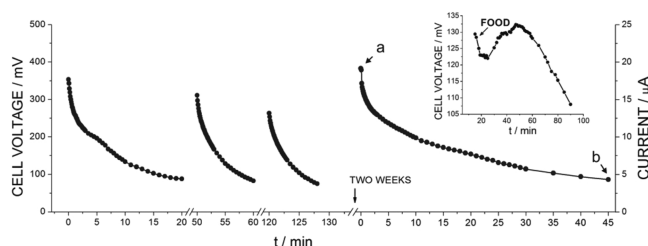


**Figure 3.** Polarization curve of the implanted biofuel cell operated in vivo. Inset: Power generated on a variable-load resistance.

current ( $I_{sc}$ ) achieved in the biofuel cell were 530 mV and 42.5  $\mu\text{A}$  (current density 170  $\mu\text{A cm}^{-2}$  vs the electrode geometrical area), respectively. The maximum power ( $P_{max}$ ) produced by the implanted biofuel cell on the optimum resistance of 20  $\text{k}\Omega$  (equal to the internal resistance of the implanted cell) was 7.45  $\mu\text{W}$  (power density ca. 30  $\mu\text{W cm}^{-2}$ ) (Figure 3 inset). It should be noted that  $I_{sc}$  corresponds to the activity of 0.013 enzyme units, thus suggesting that only ca. 6% of the total enzyme content was electrically wired onto the modified electrodes. The fact that a relatively small fraction of the immobilized enzymes contribute to the current generation gives an opportunity for further improvement of the biofuel cell operation through optimization of the electrical wiring of the enzymes. The electrical output obtained from different snail specimens varied by approximately  $\pm 20\%$  depending on the

individual size and glucose concentration. It should be noted that the electrical output ( $I_{sc}$ ,  $V_{oc}$ ,  $P_{max}$ ) generated by a small snail was comparable to or even higher than the output produced by biofuel cells implanted in animals (rats, rabbits).<sup>19,20</sup> However, a direct comparison with biofuel cells implanted in animals is difficult because of the significant difference in physiological conditions, such as the lower oxygen concentration in blood relative to hemolymph.

The sustainability of the implanted biofuel cell was tested by measuring the voltage and current produced over time on the optimum load resistance of 20  $\text{k}\Omega$  (Figure 4). The electrical



**Figure 4.** Voltage generated by the implanted biofuel cell operated in vivo on a 20  $\text{k}\Omega$  load resistance as a function of time. Inset: Restoring the cell voltage in real time upon feeding the snail.

output decreased rapidly upon cell operation; however, it was effectively restored when the current extraction was interrupted for 30–60 min to allow the snail to rest. The biofuel cell operation was reproducible even after a period of 2 weeks and was not affected by enzyme inactivation and/or biofouling in the biological environment, as expected for the buckypaper electrodes on the basis of their excellent performance in vitro.<sup>27</sup>

The reversible decay in the electrical power generation potentially could occur for two major reasons: (i) consumption of glucose in the close vicinity of the electrode surface, leading to glucose depletion at the electrode surface because of its very slow diffusion in the hemolymph; (ii) total consumption of glucose in the snail's body, if diffusion is not a limiting factor. To clarify the mechanism limiting the electrical output, we analyzed the glucose concentration in the hemolymph. Sampling the hemolymph and measuring the glucose concentration in vitro gave values of 63 and 54  $\mu\text{M}$  glucose before and after extraction of the current for 45 min, respectively (Figure 4, points a and b). The decrease in the glucose concentration in the hemolymph (ca. 14%) is not enough to explain the electrical output decay by almost 80%, and therefore, the local depletion of glucose at the electrode surface plays the dominant role in the current decay. When the current extraction was interrupted (the external circuitry was disconnected) the glucose depletion at the electrode surface was compensated by slow diffusion and the bulk glucose concentration was also restored in the hemolymph through snail metabolic processes, thus allowing a new portion of electrical power to be extracted. Partial restoration of the electrical output in real time was observed upon feeding the snail (Figure 4 inset), but full restoration required more time because of slow metabolic processes and slow glucose diffusion. In another experiment, we applied much higher load resistance (1  $\text{M}\Omega$ ) to limit the extracted current, thus reaching a rate of glucose consumption comparable with its diffusion to the electrode surface. The current of ca. 0.4  $\mu\text{A}$  (power ca. 0.16  $\mu\text{W}$ ) was continuously extracted for 1 h with a decay of less than 10%. Overall, the reversible electrical output decay is

explained by limitations in the glucose mass transport and by exhaustion of the snail rather than by a decrease in the bioelectrocatalytic activity of the electrodes. These limitations are particularly important to keep in mind when small living creatures are considered as a source of electrical power generated by implanted electrodes. Notably, such small species do not have large amounts of the biofuel (glucose) or efficient blood circulation for the biofuel mass transport, thus being different than a mammal.

In conclusion, we have achieved for the first time sustainable generation of electrical power in vivo by implanting electrodes in a snail and demonstrated that metabolically regenerated glucose can “recharge” the living battery for continuous production of electricity. It should be noted that in many previous works *potentially implantable* biofuel cells have been claimed, but the present work has demonstrated an actual *implanted* biofuel cell operating in a small creature living with the bioelectrodes for a long period of time (several months). This opens new perspectives for biofuel cells operating in vivo for many biotechnological applications.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Detailed experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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