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# Solubilized Enzymatic Fuel Cell (SEFC) for Quasi-Continuous Operation Exploiting Carbohydrate Block Copolymer Glyconanoparticle Mediators

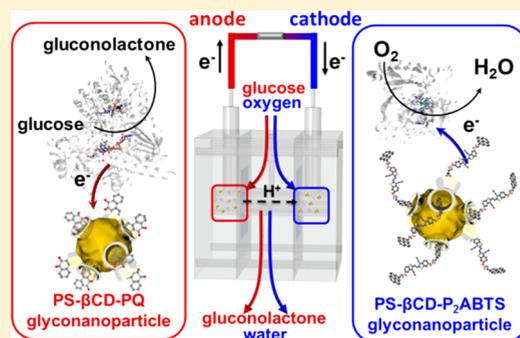
Jules L. Hammond,<sup>†</sup> Andrew J. Gross,<sup>†</sup> Fabien Giroud,<sup>†</sup> Christophe Travelet,<sup>‡</sup> Redouane Borsali,<sup>\*,‡</sup> and Serge Cosnier<sup>\*,†</sup>

<sup>†</sup>Université Grenoble Alpes, CNRS, DCM, 38000 Grenoble, France

<sup>‡</sup>Université Grenoble Alpes, CNRS, CERMAV, 38000 Grenoble, France

**S** Supporting Information

**ABSTRACT:** Enzymatic biofuel cells are ecofriendly power sources that can deliver  $\mu\text{W}$ – $\text{mW}$  outputs from renewable substrates, but their stability is a major issue owing to enzyme fragility. The vast majority of reported biofuel cells can only generate power continuously for relatively short periods of time. Here we report a novel “solubilized enzymatic fuel cell (SEFC)” concept for continuous long-term operation. Avoiding surface immobilization techniques allows biocatalytic activity to be easily restored or replenished. The biofuel cell exploits freely diffusing enzymes and  $\beta$ -cyclodextrin-coated glyconanoparticles with entrapped quinone and thiazoline redox mediators, for mediated glucose and oxygen conversion. The cell was designed with permselective membranes to enable substrate and proton diffusion while trapping the enzymes and glyconanoparticles in separate compartments. The SEFC exhibited a peak power loss of only 26.3% after 7 days of continuous charge–discharge cycling at 50  $\mu\text{A}$ ; thus, SEFCs may be envisaged to power lab-on-a-chip devices for periods of several weeks.



Enzymatic biofuel cells convert chemical energy into electrical energy by electroenzymatic reactions.<sup>1</sup> The use of enzymes provides several advantages compared to conventional noble metal catalysts, including very high specificity toward their substrates, high turnover and activity under mild conditions, and the ability to transform complex organic fuels. Enzymes are also biocompatible and environmentally benign, although intrinsically complex and fragile. Another major drawback of enzymes is their limited stability after immobilization on artificial electrode surfaces. Currently there is an urgent need to develop superior strategies to preserve, optimize, and enhance enzyme stability and activity at electrodes for bioelectrocatalysis under various conditions.

With state-of-the-art prototype biofuel cells already capable of powering small electronic devices such as pacemakers, lactate sensors, and wireless communication systems for short periods of time, their long-term operational stability remains a crucial parameter for their future success.<sup>1–4</sup> One of the key factors determining the lifetime of a biofuel cell is the stability of the enzymes and mediators, presenting pivotal concern for their practical application.<sup>5</sup> Immobilization of the enzyme on the electrode, for example, using carbon nanostructures,<sup>6</sup> often

combined with surface modification chemistry, is considered to be the best way to stabilize the enzyme. However, this method eventually leads to a buildup of inactive enzyme at the surface, making it extremely difficult for the catalytic activity to be restored or replenished.<sup>7</sup> Enzyme immobilization and entrapment methods can also fix the enzymes in inefficient orientations, restricting their degrees of freedom and reducing their activity. Irreversible physical damage to the enzyme upon binding or cross-linking to the electrode is also an issue. Redox mediators are often immobilized together with the enzyme to facilitate electron shuttling to and from the enzyme’s active site (mediated electron transfer (MET)). Covalent attachment of the enzyme followed by entrapment of the mediator by cross-linking,<sup>8</sup> encapsulation in polypyrrole films,<sup>9</sup> or forming a mixed compression with graphite particles<sup>2</sup> is typically used to stabilize enzymes at electrodes. Such techniques are considered to mitigate catalytic instability and minimize leaching<sup>10</sup> but at the expense of reduced activity. High concentrations of 64

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65 immobilized mediators are typically required to maximize  
66 power density, but this can be difficult to realize, expensive,  
67 and unsafe due to toxicity, caused by progressive leaching.

68 The aim of this work is to depart from the traditional  
69 approach of immobilizing the bioelectrocatalytic components  
70 of the fuel cell on the electrodes. Instead, we adopt here a  
71 strategy of utilizing enzymes and redox-mediating glyconano-  
72 particles (GNPs) solubilized (i.e., nonimmobilized) in  
73 solution. Enzymes and mediators in solution can freely diffuse  
74 and rotate for dynamic orientation with each other for effective  
75 electron transfer and bioelectrocatalysis. The use of enzymes in  
76 solution can also avoid issues related to poor mass transfer<sup>11</sup>  
77 and reproducibility due to complicated enzyme immobilization  
78 procedures.<sup>12</sup> Importantly, Zhu and Zhang<sup>13</sup> previously  
79 demonstrated that higher power densities can be obtained at  
80 a nonimmobilized bioanode of a closed biobattery when  
81 compared to an immobilized bioanode.

82 If both the enzymes and mediators are solubilized, as  
83 explored/reported herein, fresh biocatalysts can be introduced  
84 at high concentrations to restore or replenish performance by a  
85 simple exchange of the anode and cathode solutions. This will  
86 further prolong the biofuel cell lifetime and would address the  
87 major problem of conventional biofuel cells whereby the  
88 bioelectrodes with immobilized enzymes must be recon-  
89 structed when the enzyme becomes inactive. The concept of  
90 refueling the fuel solution of a biofuel cell has been previously  
91 demonstrated by Sony Corporation with an immobilized  
92 system. However, the cell voltage collapsed to 0.2 V after 40  
93 fuel exchanges (200 min total operation).<sup>14</sup>

94 Taking into account that redox mediators and substrates  
95 such as glucose are similar in size, it was not possible to  
96 consider a solubilized bioelectrode system that was capable of  
97 retaining redox mediators while still permitting diffusion of  
98 glucose. In this context, the engineering of redox supra-  
99 molecular entities exhibiting a similar size to proteins is a  
100 promising route in the design of bioelectrodes based on a size-  
101 exclusion process. GNP synthesis<sup>15</sup> not only allows hydro-  
102 phobic and insoluble redox-active species to be solubilized but  
103 simultaneously enables size-dependent entrapment within a  
104 fuel cell compartment due to their large size via a size-exclusion  
105 membrane. Replacing classical redox mediators with redox-  
106 active GNPs opens up the new possibility to separate the  
107 anolyte from the catholyte and avoid cross-reaction. Without  
108 size exclusion there would be an inevitable drop in open-circuit  
109 voltage (OCV), close to zero, due to a mixed potential  
110 between the bioanode and biocathode. Furthermore, the use of  
111 GNPs can allow the redox-active anolyte/catholyte or fuel to  
112 be specifically replenished or restored.

113 In summary, we develop here the use of two types of freely  
114 diffusing, solubilized redox glycopolymer nanoparticles for  
115 the electrical wiring of enzymes both at the anode and cathode.  
116 Furthermore, we have engineered a new solubilized enzymatic  
117 fuel cell (SEFC) device that integrates permselective  
118 membranes, allowing us to exploit the size of the GNPs to  
119 prevent mediator leaching into the fuel stream or away from  
120 their respective anode or cathode compartments.

121 The biofuel cell can be envisaged as the combination of two  
122 half-cells that concomitantly undergo oxidation and reduction  
123 reactions at the anode and cathode, respectively. Electrons  
124 produced by oxidation at the anode flow through the external  
125 circuit, delivering power to the load, and are then transferred  
126 to the cathode where they participate in the reduction reaction.  
127 As it is desirable to utilize abundant and low-cost organic

energy sources, we base our system on glucose and oxygen as 128  
the fuel and oxidant, respectively. These substrates have 129  
attracted considerable attention for implantable and wearable 130  
applications, owing to their availability in the body in  $\mu\text{M}$ – $\text{mM}$  131  
concentrations by human metabolism and consumption, but 132  
are equally viable for portable applications.<sup>1</sup> 133

At the anode, we perform catalytic oxidation of glucose with 134  
fungal flavin adenine dinucleotide-dependent glucose dehy- 135  
drogenase (FAD-GDH) from *Aspergillus sp.* using 9,10- 136  
phenanthrenequinone (PQ) as the organic redox mediator. 137  
PQ was recently shown to be an effective mediator in solution 138  
for glucose oxidation with this enzyme compared to other 139  
quinone mediators.<sup>16</sup> For the cathode, we reduce oxygen via a 140  
four-electron process to water using the bilirubin oxidase 141  
(BOX) enzyme from *Myrothecium verrucaria*, mediated by bis- 142  
pyrene-2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) 143  
(P<sub>2</sub>ABTS).<sup>15</sup> In both cases, the mediator is encapsulated 144  
within a GNP using a guest–host interaction via  $\beta$ -cyclodextrin 145  
inclusion complexes. The two separate reactions occurring at 146  
the anode and cathode are illustrated in Figure 1. 147 f1

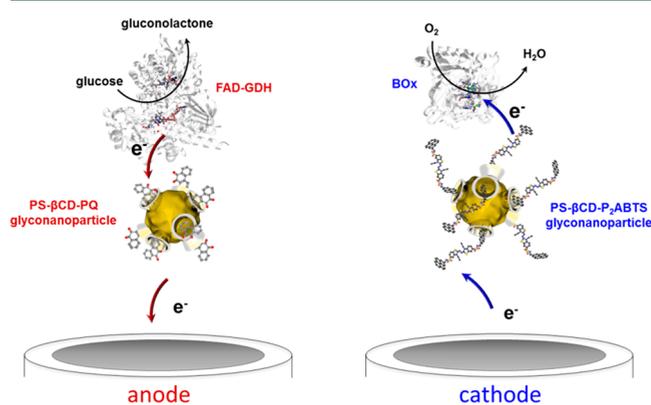


Figure 1. Scheme showing the reactions occurring at the anode (left) and cathode (right)

The redox GNPs were prepared using an amphiphilic  $\beta$ - 148  
cyclodextrin ( $\beta$ -CD) polystyrene diblock copolymer based on 149  
a click chemistry reaction. The  $\beta$ -CD GNPs are obtained by 150  
self-assembly with controlled encapsulation of the mediator in 151  
a high yield via the nanoprecipitation technique.<sup>17</sup> Further 152  
details of the P<sub>2</sub>ABTS GNP preparation, characterization, and 153  
use for catalysis within a cathodic half-cell have been published 154  
previously by our group.<sup>15</sup> To complement the P<sub>2</sub>ABTS GNPs 155  
and complete the aqueous biofuel cell, new GNPs with 156  
entrapped PQ as a redox mediator were developed for the 157  
anodic half-cell. We formed PS- $\beta$ CD-PQ GNPs with 158  
approximate PQ concentrations of 34.2 and 68.4  $\mu\text{M}$  (1:1 159  
and 1:2, PS- $\beta$ CD:PQ, mass). These concentrations are much 160  
higher than the maximum concentration achieved when 161  
dissolving PQ in deionized (DI) H<sub>2</sub>O with ultrasonic agitation, 162  
of around 5  $\mu\text{M}$  [SI, Figure S1]. This demonstrates how 163  
increased quantities of a hydrophobic mediator can be 164  
incorporated in solution for catalysis via host–guest 165  
encapsulation in our GNP system. 166

TEM imaging (Figure 2) of the PQ GNPs showed 167 f2  
nanoparticles with average diameters of  $20.0 \pm 7.0$  and  $43.9$  168  
 $\pm 27.7$  nm at 34.2 and 68.4  $\mu\text{M}$ , respectively. This compares to 169  
 $43.3 \pm 18.8$  nm for the PS- $\beta$ CD-P<sub>2</sub>ABTS GNPs (SI, Figure 170  
S2). Interestingly, increasing the concentration of the guest 171  
altered the average GNP diameter, as shown in the size 172

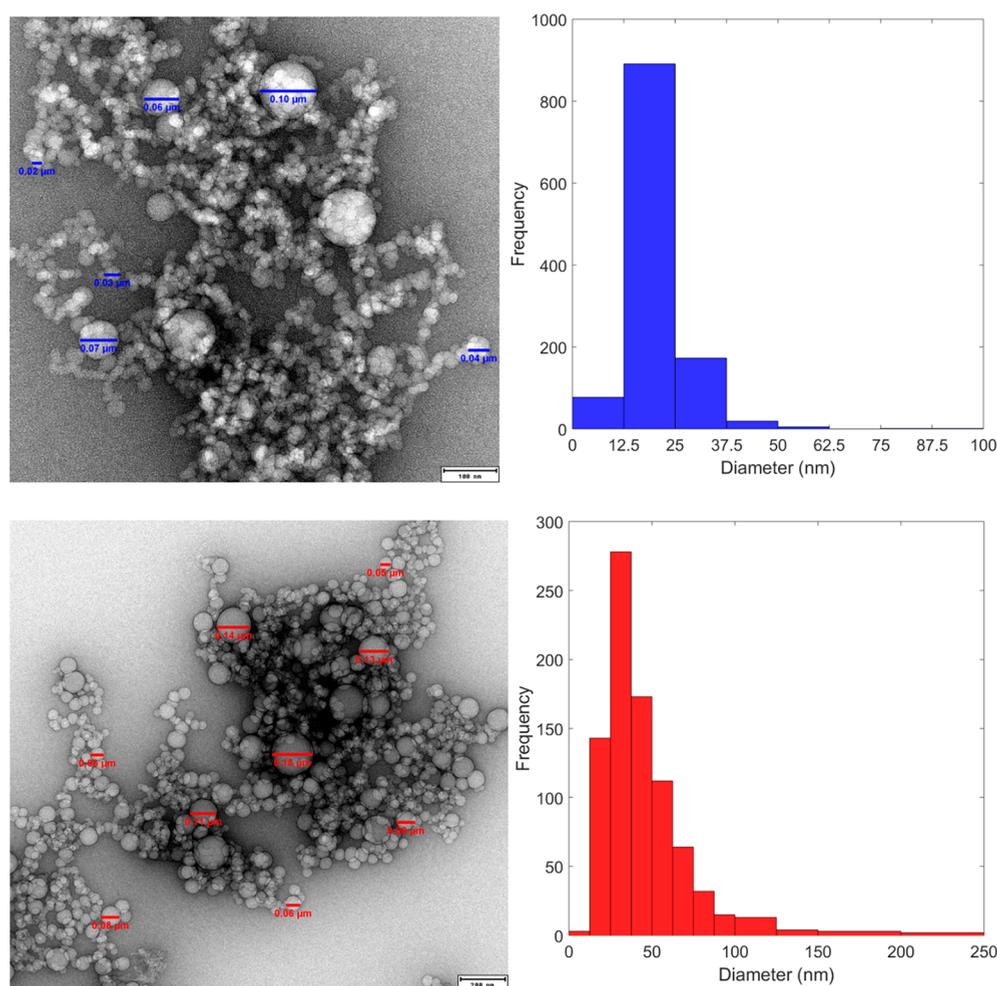


Figure 2. TEM images and histograms showing the typical size distribution of PS- $\beta$ CD-PQ GNPs.

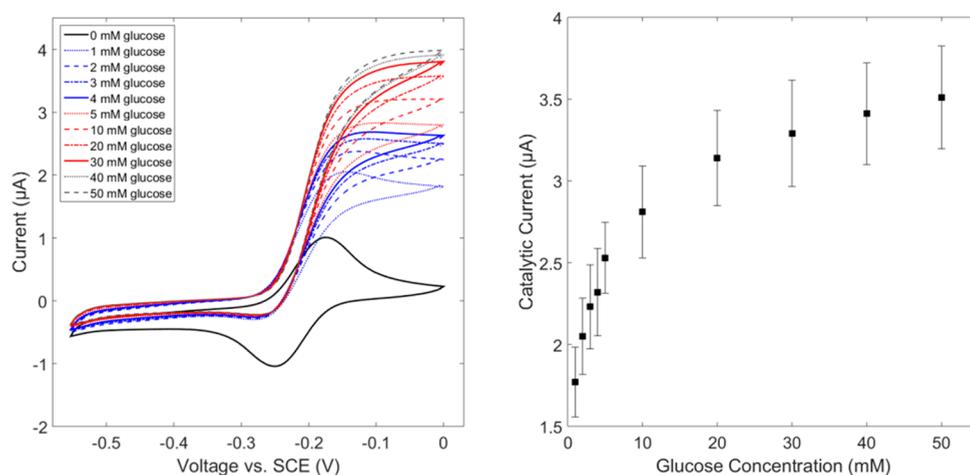


Figure 3. Cyclic voltammograms and glucose dose response for an anode half-cell using GCEs, 2.5 mL of 34.2  $\mu$ M PS- $\beta$ CD-PQ GNPs, and 0.3 mg of FAD-GDH in 25  $\mu$ L of pH 7.0 McIlvaine buffer,  $v = 5 \text{ mV}\cdot\text{s}^{-1}$ .

173 distributions (Figure 2), with this tendency also supported by  
 174 the results of dynamic light scattering (DLS) experiments (SI,  
 175 Figure S3 and Table S1). Importantly, the electroactivity of the  
 176 PS- $\beta$ CD-PQ GNPs is approximately doubled (1:1.91) when  
 177 doubling the concentration of the mediator, as demonstrated  
 178 by the scan-rate dependence shown on cyclic voltammograms  
 179 (SI, Figures S4a,b and Table S2).

TEM images showed no change in the GNPs after 2 months 180  
 or after addition of pH 7.0 McIlvaine buffer (data not shown). 181  
 This is consistent with the apparent stability previously 182  
 observed for our PS- $\beta$ CD-P<sub>2</sub>ABTS GNPs.<sup>15</sup> The electro- 183  
 catalytic activity of the new PQ GNPs was then confirmed at 184  
 glassy carbon electrodes (GCEs) with increasing concen- 185  
 trations of glucose in DI H<sub>2</sub>O (Figure 3). Furthermore, system 186

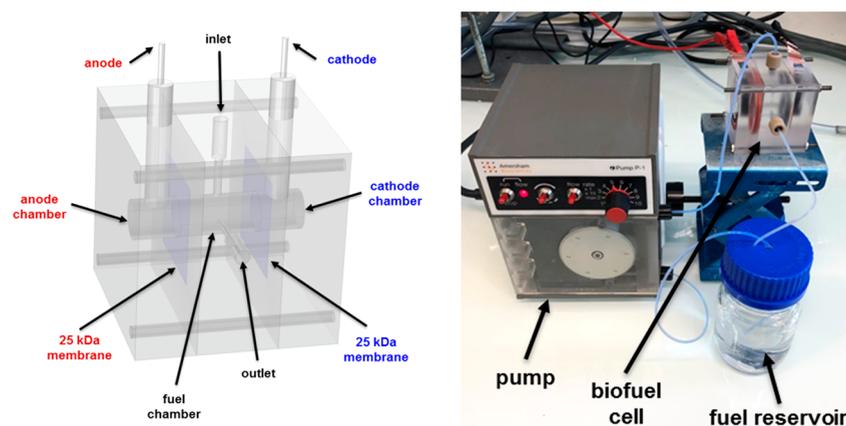


Figure 4. 3D annotated drawing (left) showing the fuel cell components and photograph (right) of the setup when connected.

187 performance was found to be limited by the enzyme mass (120  
188  $\mu\text{g}\cdot\text{mL}^{-1}$ ) in the system (SI, Figure S5). At the higher bound of  
189 a typical blood glucose level (5 mM), the catalytic current was  
190 around  $2.5 \pm 0.2 \mu\text{A}$ .

191 A new fuel cell (Figure 4) was developed specifically for our  
192 system and consists of three main parts. The central chamber  
193 ( $V = 1.5 \text{ mL}$ ) is connected to a peristaltic pump to provide a  
194 constant flow of fuel to the adjacent compartments ( $V = 1 \text{ mL}$ )  
195 that contain the anode and cathode biocatalytic components.  
196 To prevent loss of the enzymes and mediators from their  
197 respective compartments, dialysis membranes with a cutoff  
198 value of 25 kDa are used as separators and sealed with a double  
199 O-ring system. This cutoff value translates to an approximate  
200  $R_{\text{min}}$  of 1.9 nm ( $D_{\text{min}} = 3.9 \text{ nm}$ ), calculated using the following  
201 equation,<sup>18</sup>  $R_{\text{min}} = 0.066(Da)^{1/3}$ , and the cutoff value is  
202 sufficiently smaller than the diameter of PQ ( $20.0 \pm 7.0$  and  
203  $43.9 \pm 27.7 \text{ nm}$  at 34 and 68  $\mu\text{M}$ , respectively) and P<sub>2</sub>ABTS  
204 ( $43.3 \text{ nm} \pm 18.8 \text{ nm}$ ) GNPs and the enzymes (BOx = 66–68  
205 kDa and FAD-GDH = 95–135 kDa). Carbon nanotube  
206 buckypaper offers properties such as high specific surface area,  
207 mechanical flexibility, and high electrical conductivity.<sup>19</sup> To  
208 increase power output, electrodes with increased area ( $5 \times 40$   
209 mm) were fabricated by attaching commercial carbon  
210 nanotube buckypaper to graphite foil current collectors using  
211 carbon paste. To limit evaporation and secure the electrodes in  
212 place, the compartments were sealed with tape and then the  
213 electrodes terminated with conductive adhesive copper tape.

214 A peristaltic pump operating at a flow rate of  $80 \mu\text{L}\cdot\text{min}^{-1}$   
215 (mimicking typical blood vessel flow<sup>20</sup>) was used to deliver a  
216 continuous stream of pH 7.0 McIlvaine buffer containing  
217 ambient O<sub>2</sub> and 5 mM glucose from a 50 mL reservoir. A  
218 volume of  $800 \mu\text{L}$  of PQ GNPs with  $120 \mu\text{g}$  of FAD-GDH and  
219  $800 \mu\text{L}$  of P<sub>2</sub>ABTS GNPs with  $200 \mu\text{g}$  of BOx were injected  
220 into the anode and cathode compartments, respectively.

221 The electrocatalytic behavior of the bioelectrodes was  
222 verified for both half-cells (SI, Figures S6 and S7). Here we  
223 can compare the catalytic performance with our previous work  
224 on buckypaper electrodes<sup>19</sup> with immobilized enzyme and  
225 mediator bioelectrodes (see the SI for further details). Using a  
226 similar but not identical quinone mediator and FAD-GDH  
227 enzyme, we produced a catalytic output of  $0.637 \text{ mA}\cdot\text{cm}^{-2}\cdot$   
228  $\text{mg}^{-1}$ . In contrast, the solubilized system with PQ GNPs and  
229 FAD-GDH reported here achieved  $2.05 \text{ mA}\cdot\text{cm}^{-2}\cdot\text{mg}^{-1}$ .  
230 Likewise, for the biocathode, prepared with immobilized  
231 BOx, we achieved  $1.77 \text{ mA}\cdot\text{cm}^{-2}\cdot\text{mg}^{-1}$ . This compares to a  
232 catalytic output of  $3.79 \text{ mA}\cdot\text{cm}^{-2}\cdot\text{mg}^{-1}$  for the solubilized BOx

system obtained in this work. We therefore clearly observe 233  
better catalytic performances per amount of enzyme catalyst in 234  
this work compared to state-of-the-art buckypaper anodes and 235  
cathodes with immobilized enzymes. 236

Polarization plots (Figure 5) were performed for the biofuel 237  
cell by increasing the discharge current and recording the cell 238

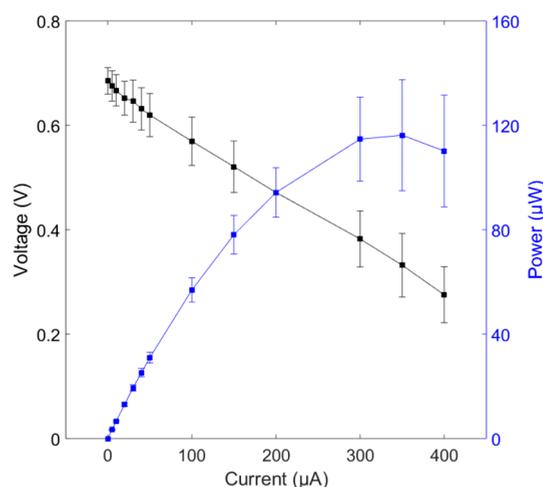
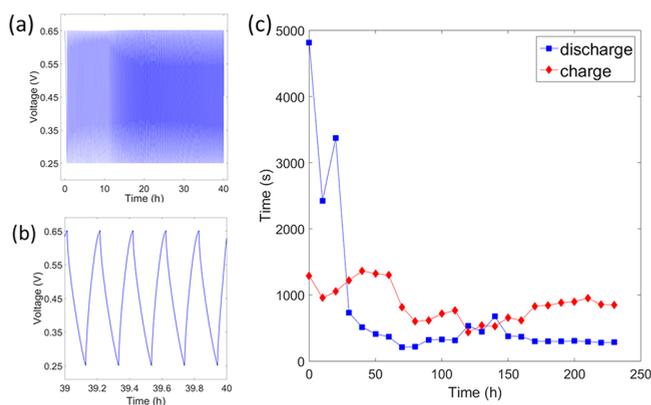


Figure 5. Cell performance ( $n = 3$ ) showing voltage (black, left axis) and power (blue, right axis) as a function of current at a fuel flow rate of  $80 \mu\text{L}\cdot\text{min}^{-1}$  (pH 7.0 McIlvaine buffer with 5 mM glucose and ambient O<sub>2</sub>), with  $120 \mu\text{g}$  of FAD-GDH,  $200 \mu\text{g}$  of BOx, and  $800 \mu\text{L}$  of each GNP suspension.

voltage after 30 s. A maximum power point of  $116.2 \pm 21.3$  239  
 $\mu\text{W}$  was observed at a discharge current of  $350 \mu\text{A}$ . The 240  
observed OCV of the fuel cell was  $0.685 \text{ V} \pm 26 \text{ mV}$ . 241

Operational stability of the fuel cell was tested by performing 242  
constant charge–discharge cycling for extended periods. The 243  
cell is discharged at a current of  $50 \mu\text{A}$  until it reaches 0.25 V 244  
and then is allowed to recover until the voltage reaches 0.65 V. 245  
The glucose oxidation and oxygen reduction reactions are 246  
continuous. The selected current and voltage values provide a 247  
compromise between charge/discharge times, maximizing the 248  
duty cycle of power generation while also preventing damage 249  
to the enzyme. It is important to note that many previous 250  
reports of biofuel cell stability operate the device periodically 251  
for relatively short periods of time. 252

A typical charge–discharge profile is shown in Figure 6a,b. 253  
The charge and discharge times were then calculated at 254



**Figure 6.** Typical charge–discharge cycles (a), zoomed period between 39 and 40 h (b), and plots of fuel cell discharge (blue, square marker) and charge (red, diamond marker) stability at 10 h intervals (c), all at a  $50 \mu\text{A}$  discharge current and fuel flow rate of  $80 \mu\text{L}\cdot\text{min}^{-1}$  (pH 7.0 McIlvaine buffer with 5 mM glucose and ambient  $\text{O}_2$ ) with  $120 \mu\text{g}$  of FAD-GDH,  $200 \mu\text{g}$  of BOx, and  $800 \mu\text{L}$  of each GNP suspension.

255 intervals of 10 h and showed a stabilization of the discharge  
256 time after about 30 h (Figure 6c). After 10 days, the fuel cell  
257 still provided discharge times of around 5 min with charge  
258 times of around 15 min.

259 To provide a further indicator of the fuel cell stability,  
260 discharge tests at the peak discharge current ( $350 \mu\text{A}$ ) were  
261 conducted at 2 and 7 days (typical discharge profiles shown in  
262 SI, Figure S8). This was to provide a further indicator for  
263 degradation of the fuel cell. After 2 days, the mean power was  
264  $115.3 \pm 24.7 \mu\text{W}$  ( $n = 3$ ), which is similar to the values  
265 obtained during initial characterization of the maximum power  
266 point during evaluation of the polarization profiles. At 7 days,  
267 the mean power dropped to  $84.9 \pm 2.9 \mu\text{W}$  ( $n = 5$ ), equating  
268 to a power loss of 26.3%. As a comparison, a power loss of 65%  
269 was reported by Zhu and Zhang after operating the fuel cell for  
270 just 6 rounds of 2 h across a period of 12 days.

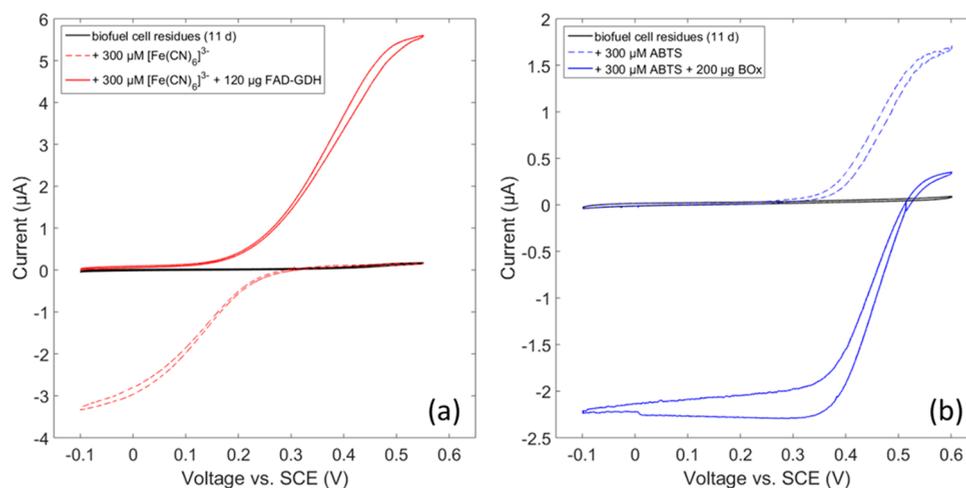
271 Furthermore, one fuel cell that was already subjected to 7  
272 days of operational cycling at a discharge current of  $50 \mu\text{A}$  was  
273 cycled at a higher discharge current of  $350 \mu\text{A}$  for 24 h (SI,

Figures S9–S11). This fuel cell provided relatively stable 274  
operational performance with discharge time falling from 20 to 275  
15 s (–25%) and charge time increasing from 158 to 340 s 276  
(+115%). This demonstrates the ability to operate the biofuel 277  
cell at higher discharge currents over long periods of time 278  
while retaining a reasonable level of charge–discharge time 279  
stability. 280

The recirculating fuel solution was tested for the presence of 281  
any enzymes and mediators after completion of the operational 282  
stability tests and showed no evidence for catalysis (black 283  
lines), as shown by the control experiments (red/blue lines for 284  
anode/cathode, respectively; Figure 7a,b). This confirms that 285  
the biocatalytic elements are retained in their respective 286  
chambers and do not leach into the fuel stream. This is a 287  
testament to the GNPs, which, owing to their markedly larger 288  
sizes compared to classical redox mediators, provide the ability 289  
to constrain them within the anode and cathode compartments 290  
while allowing diffusion of the substrates, glucose, and oxygen. 291  
It is emphasized that redox mediators without nanoparticle 292  
encapsulation would not be retained owing to their smaller 293  
molecular size. A control experiment using classical redox 294  
mediators in place of the GNPs is described further below. 295

To check for the presence of adsorbed biocatalytic 296  
components, discharge profiles were performed following 7 297  
days of operational cycling at  $50 \mu\text{A}$  and after using different 298  
rinse techniques (SI, Figures S12 and S13). Although an OCV 299  
develops (after resting the cell for 2 h between each rinse), 300  
there is negligible power generated and no operational cycling 301  
can be performed, ruling out significant adsorption of the 302  
biocatalysts. The presence of only trace quantities of 303  
biocatalytic components is particularly attractive as the cell 304  
chambers can be effectively refreshed. 305

Finally, a control experiment without PS- $\beta$ CD GNPs to 306  
encapsulate the mediators but instead with solubilized 307  
mediators was carried out. This control test confirmed that 308  
the addition of GNPs in the system helps to efficiently stabilize 309  
and improve performance. Without the GNPs, the perform- 310  
ance was lower and rapidly degraded, with the charge times 311  
steadily increasing to the point that the cell failed to recharge 312  
after 20 h (SI, Figures S14 and S15). This was most likely 313



**Figure 7.** CVs showing response at GCEs for biofuel cell residues (black) with (a)  $+300 \mu\text{M} [\text{Fe}(\text{CN})_6]^{3-}$  (red, dashed) and  $+120 \mu\text{g}$  of FAD-GDH (red), all in saturated Ar with an Ar blanket,  $2 \text{mV}\cdot\text{s}^{-1}$ , and (b)  $+300 \mu\text{M}$  ABTS (blue, dashed) and  $+200 \mu\text{g}$  of BOx (blue), all in saturated  $\text{O}_2$  with an  $\text{O}_2$  blanket,  $2 \text{mV}\cdot\text{s}^{-1}$ .

314 caused by the mediators readily leaching into the fuel flow and  
315 their concentration slowly diminishing.

316 To conclude, we demonstrated an enzymatic biofuel cell  
317 whereby the biocatalytic components are in the aqueous phase  
318 and therefore able to freely diffuse, shifting from the classical  
319 use of immobilized enzymes and mediators. With the use of  
320 GNPs made from the self-assembly of a carbohydrate-based  
321 block copolymer ( $\beta$ -cyclodextrin-*b*-PS), including new GNPs  
322 prepared with phenanthrenequinone, we show how increased  
323 quantities of mediator can be incorporated into the system to  
324 increase catalytic current. With the integration of carbon  
325 nanotube buckypaper electrodes into the diffusion-based  
326 biofuel cell, currents were enhanced by a factor of around  
327 85, relative to GCEs. We have also shown that this novel fuel  
328 cell design significantly extends the operational lifetime, for  
329 example, by preventing leaching of the mediator through a  
330 permselective membrane. After 7 days of operation, the peak  
331 power of the optimized biofuel cell only dropped by 26% to  
332 84.9  $\mu$ W. Further, we clearly show evidence that the use of  
333 enzymes in solution can be a viable approach in biofuel cell  
334 design, contrary to the general consensus in the literature. A  
335 further advantage of this diffusion-based biofuel cell, to be  
336 explored in the future, is the possibility to easily recycle and  
337 refresh the biocatalytic components. It is feasible that SEFCs,  
338 alongside a boost converter, could be used as a green power  
339 source for powering low-power portable lab-on-a-chip  
340 electronics to perform functions such as quasi-continuous  
341 monitoring with data transmission.

## 342 ■ ASSOCIATED CONTENT

### 343 ● Supporting Information

344 The Supporting Information is available free of charge on the  
345 ACS Publications website at DOI: 10.1021/acseenergy-  
346 lett.8b01972.

347 Full details of reagents, materials, and experimental  
348 methods, further optical, electrochemical, and electronic  
349 characterization, results from control tests, as well as  
350 calculations comparing systems with immobilized versus  
351 solubilized biocatalysts (PDF)

## 352 ■ AUTHOR INFORMATION

### 353 Corresponding Authors

354 \*E-mail: redouane.borsali@univ-grenoble-alpes.fr (R.B.).

355 \*E-mail: serge.cosnier@univ-grenoble-alpes.fr (S.C.).

### 356 ORCID

357 Jules L. Hammond: 0000-0002-3308-5421

358 Andrew J. Gross: 0000-0002-7356-7089

359 Fabien Giroud: 0000-0001-6611-2687

### 360 Notes

361 The authors declare no competing financial interest.

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367 Platform for providing facilities (PCN-ICMG).

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