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

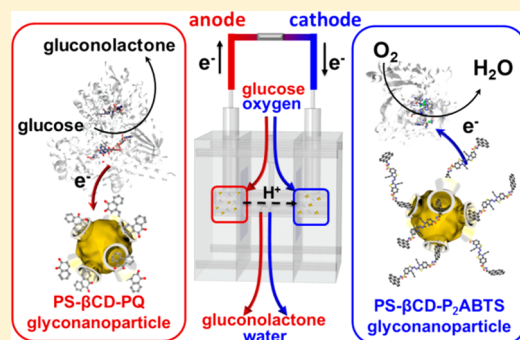
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S Supporting Information

ABSTRACT: Enzymatic biofuel cells are ecofriendly power sources that can deliver μW – 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 β -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 μA ; 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.¹ 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.^{1–4} 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.⁵ Immobilization of the enzyme on the electrode, for example, using carbon nanostructures,⁶ 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.⁷ 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,⁸ encapsulation in polypyrrole films,⁹ or forming a mixed compression with graphite particles² is typically used to stabilize enzymes at electrodes. Such techniques are considered to mitigate catalytic instability and minimize leaching¹⁰ 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 transfer¹¹
77 and reproducibility due to complicated enzyme immobilization
78 procedures.¹² Importantly, Zhu and Zhang¹³ 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).¹⁴

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 synthesis¹⁵ 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 μM – mM 131
concentrations by human metabolism and consumption, but 132
are equally viable for portable applications.¹ 133

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.¹⁶ 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
(P₂ABTS).¹⁵ In both cases, the mediator is encapsulated 144
within a GNP using a guest–host interaction via β -cyclodextrin 145
inclusion complexes. The two separate reactions occurring at 146
the anode and cathode are illustrated in Figure 1. 147 f1

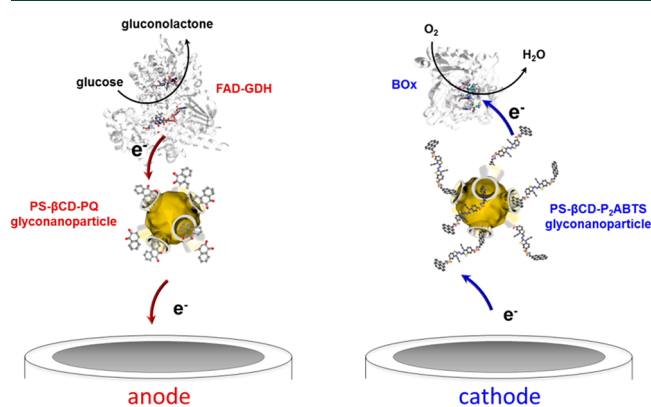


Figure 1. Scheme showing the reactions occurring at the anode (left) and cathode (right)

The redox GNPs were prepared using an amphiphilic β - 148
cyclodextrin (β -CD) polystyrene diblock copolymer based on 149
a click chemistry reaction. The β -CD GNPs are obtained by 150
self-assembly with controlled encapsulation of the mediator in 151
a high yield via the nanoprecipitation technique.¹⁷ Further 152
details of the P₂ABTS GNP preparation, characterization, and 153
use for catalysis within a cathodic half-cell have been published 154
previously by our group.¹⁵ To complement the P₂ABTS 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- β CD-PQ GNPs with 158
approximate PQ concentrations of 34.2 and 68.4 μM (1:1 159
and 1:2, PS- β CD:PQ, mass). These concentrations are much 160
higher than the maximum concentration achieved when 161
dissolving PQ in deionized (DI) H₂O with ultrasonic agitation, 162
of around 5 μM [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 ± 7.0 and 43.9 168
 ± 27.7 nm at 34.2 and 68.4 μM , respectively. This compares to 169
 43.3 ± 18.8 nm for the PS- β CD-P₂ABTS GNPs (SI, Figure 170
S2). Interestingly, increasing the concentration of the guest 171
altered the average GNP diameter, as shown in the size 172

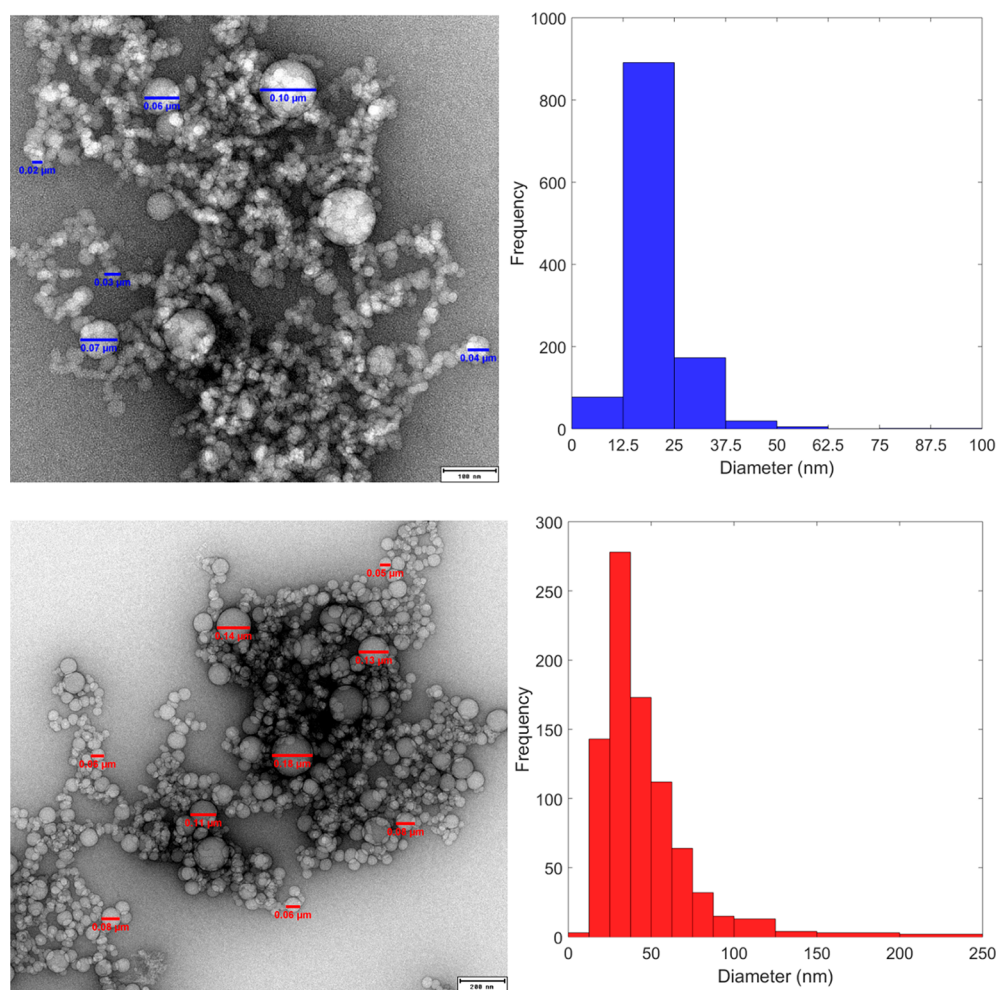


Figure 2. TEM images and histograms showing the typical size distribution of PS- β CD-PQ GNPs.

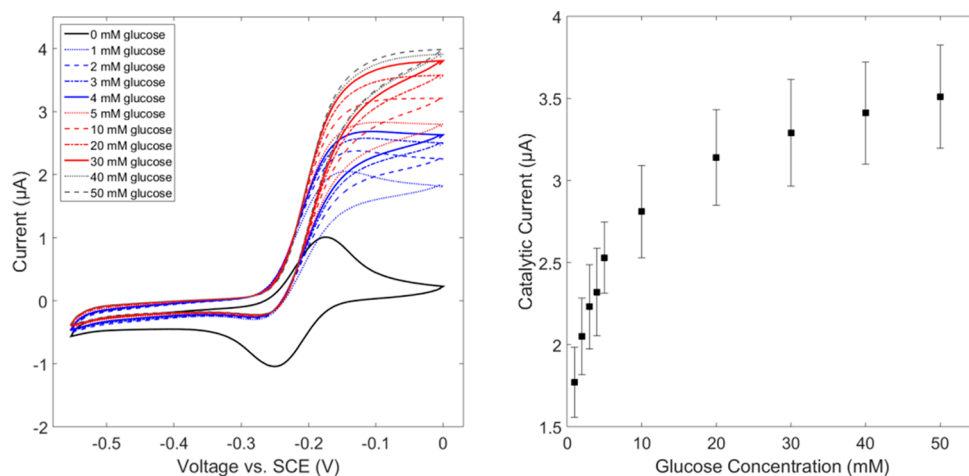


Figure 3. Cyclic voltammograms and glucose dose response for an anode half-cell using GCEs, 2.5 mL of 34.2 μ M PS- β CD-PQ GNPs, and 0.3 mg of FAD-GDH in 25 μ L of pH 7.0 McIlvaine buffer, $v = 5 \text{ mV}\cdot\text{s}^{-1}$.

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- β 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- β CD-P₂ABTS GNPs.¹⁵ 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 H₂O (Figure 3). Furthermore, system 186

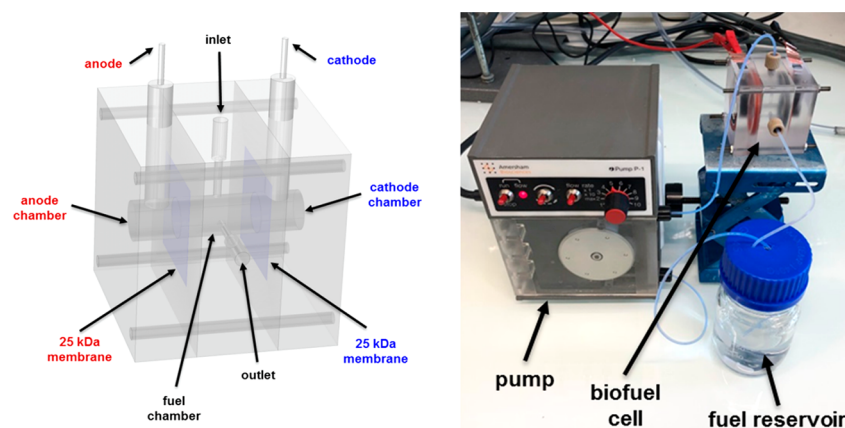


Figure 4. 3D annotated drawing (left) showing the fuel cell components and photograph (right) of the setup when connected.

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_{min} of 1.9 nm ($D_{\text{min}} = 3.9 \text{ nm}$), calculated using the following
201 equation,¹⁸ $R_{\text{min}} = 0.066(Da)^{1/3}$, and the cutoff value is
202 sufficiently smaller than the diameter of PQ (20.0 ± 7.0 and
203 $43.9 \pm 27.7 \text{ nm}$ at 34 and 68 μM , respectively) and P₂ABTS
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.¹⁹ To
208 increase power output, electrodes with increased area (5×40
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 flow²⁰) was used to deliver a
216 continuous stream of pH 7.0 McIlvaine buffer containing
217 ambient O₂ 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 P₂ABTS 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 electrodes¹⁹ 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 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

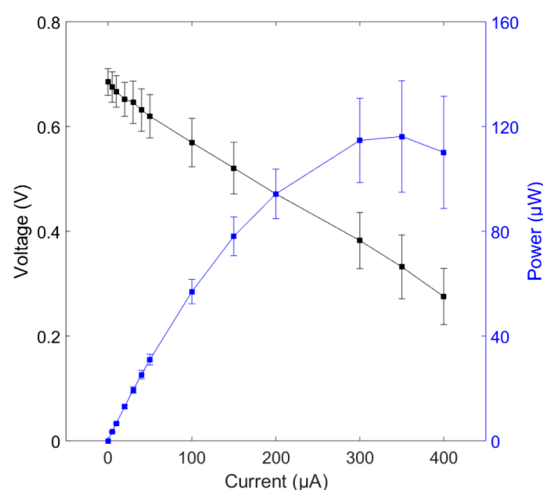


Figure 5. Cell performance ($n = 3$) showing voltage (black, left axis) and power (blue, right axis) as a function of current at a fuel flow rate of $80 \mu\text{L}\cdot\text{min}^{-1}$ (pH 7.0 McIlvaine buffer with 5 mM glucose and ambient O₂), with $120 \mu\text{g}$ of FAD-GDH, $200 \mu\text{g}$ of BOx, and $800 \mu\text{L}$ of each GNP suspension.

voltage after 30 s. A maximum power point of 116.2 ± 21.3 239
 μW 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

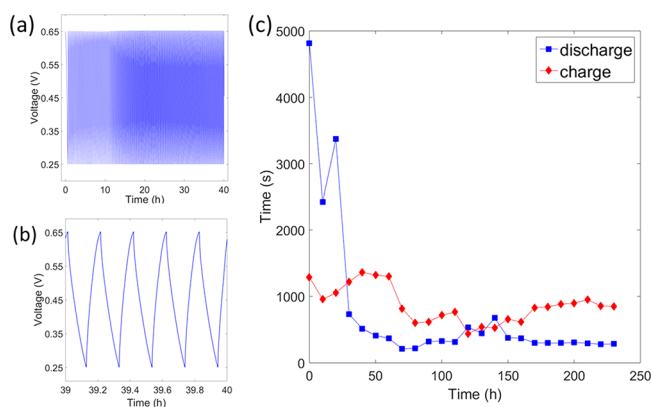


Figure 6. Typical charge–discharge cycles (a), zoomed period between 39 and 40 h (b), and plots of fuel cell discharge (blue, square marker) and charge (red, diamond marker) stability at 10 h intervals (c), all at a $50 \mu\text{A}$ discharge current and fuel flow rate of $80 \mu\text{L}\cdot\text{min}^{-1}$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

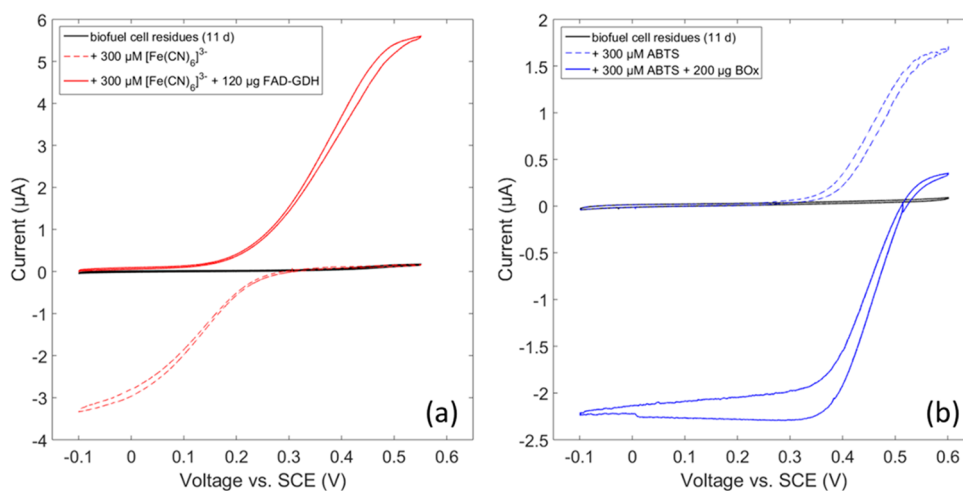


Figure 7. CVs showing response at GCEs for biofuel cell residues (black) with (a) $+300 \mu\text{M} [\text{Fe}(\text{CN})_6]^{3-}$ (red, dashed) and $+120 \mu\text{g}$ of FAD-GDH (red), all in saturated Ar with an Ar blanket, $2 \text{ mV}\cdot\text{s}^{-1}$, and (b) $+300 \mu\text{M}$ ABTS (blue, dashed) and $+200 \mu\text{g}$ of BOx (blue), all in saturated O_2 with an 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 (β -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 μ 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)

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360 Notes

361 The authors declare no competing financial interest.

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