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Freestanding biopellet electrodes based on carbon nanotubes and protein compression for direct and mediated bioelectrocatalysis

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Abstract : The fabrication of bioelectrocatalytic protein electrodes by the simple compression of carbon nanotube and protein powders was investigated using a series of proteins including bilirubin oxidase (BOx), bovine serum albumin (BSA), catalase, cytochrome C (Cyt C), diaphorase, FAD-dependent glucose dehydrogenase (FAD-GDH), galactose oxidase (GAOx), glucose oxidase (GOx), horseradish peroxidase (HRP), laccase and urease. The isoelectric points (pI) of the proteins ranged from 3.5 to 12 and the molecular weights ranged from 12.4 to 480 kDa. The compression of diaphorase, laccase, BOx, FAD-GDH, catalase and urease gave mechanically stable biopellets in 0.1 M phosphate buffer pH 7.4. For Cyt C, BSA, GAOx, GOx, and HRP, contact with buffer destabilised the biopellet and induced a fast destruction of the composite. In parallel, stable redox pellets were obtained with differently charged redox mediators: hexaammineruthenium (III) chloride, potassium hexacyanoferrate or 1,4-naphthoquinone. Several parameters were explored to shed new light on the factors that determine biopellet stability. Stable biopellets capable of direct and mediated electron transfer were subsequently elaborated, for O₂ reduction and glucose oxidation, respectively, based on the simplified procedure that does not require stabilising protection membranes or holders on the contrary to existing methods.

Keywords: bioelectrodes, enzyme wiring, isoelectric point, biosensors, biofuel cells, disk electrodes

1. Introduction

The use of carbon nanotubes (CNTs) has aroused great interest in bioelectrochemistry as an interface between biological systems and electrodes owing to their high electrical conductivity, chemical inertia and attractive mechanical properties^{1]}. The unique properties

of CNTs makes them ideal building blocks for the construction of highly conductive scaffolds with high specific surface areas for the development of modified electrodes. Moreover, such electrodes can be directly fabricated from aqueous or non-aqueous dispersions to obtain CNT film or self-supporting electrodes such as pellets^[2] or “buckypaper”^[3]. CNT electrodes are a versatile platform for the immobilisation of redox molecules and biomolecules, ranging from coenzymes to bacteria, for biosensing and biofuel cell applications^[1,4]. CNT electrodes permit the immobilisation and electrical “wiring” of enzymes for direct and mediated electron transfer (DET, MET) electrocatalysis, facilitated by the close proximity and affinity between enzymes and pristine or modified CNTs^[4]. Protein surface immobilisation is carried out by simple adsorption or by surface grafting reactions, for example, *via* activated esters groups such as *N*-hydroxysuccinimide ester^[5,6]. Specific immobilisation has been performed *via* affinity or host-guest interactions involving engineered enzymes or CNTs modified with pyrene derivatives with adamantane, biotin and/or cyclodextrin moieties^[7,8]. The functionalisation of CNT electrodes, such as film and buckypaper electrodes, with enzymes for bioelectrocatalysis is restricted to the immobilisation of a few enzyme monolayer equivalents. The immobilisation of enzymes deep inside strongly hydrophobic and dense CNT networks from aqueous solution represents a major challenge for catalytic applications. The development of CNT bioelectrodes with a high amount of “wired” enzyme in the bulk structure is not straightforward yet highly desirable, for example, due to the possibility to increase the catalytic output and lifetime of enzymatic biofuel cells (EBFCs)^[9,10]. Even if there is a high amount of electrically-connected enzyme this does not necessarily translate into higher currents due to factors such as electrolyte accessibility and mass transport and concentration limitations of the substrate.

Besides the fabrication of bioelectrodes based on CNT films or buckypapers, another original approach consists of mixing and compressing CNT and protein powders to obtain mm-sized solid pellets^[2,11]. The mixture of enzyme and CNTs prior to fabrication ensures enzyme distribution throughout the composite, and thus the amount of enzyme immobilised is significantly higher than that corresponding to a few enzyme monolayers. Besides the possibility to immobilise and electrically wire a higher amount of catalyst, another advantage lies in a “reservoir” effect leading to prolongation of bioelectrode lifetime. Impressive one year stability in buffer solution has been demonstrated using biopellets based on GOx-catalase or NAD-glucose dehydrogenase, for glucose bioanodes, and laccase, for O₂ reduction^[9,10].

CNT pellet bioelectrodes are electrically connected *via* a metal wire glued to the back of the pellet, and the back and side then covered with insulating silicone. The remaining front side of the disk is covered with a cellulose^[11] or chitosan^[12] membrane, organic polymer^[2] or Nafion®^[2,9,10] to improve electrode cohesion, biocompatibility and stability. CNT pellet biocathodes have been prepared with BOx^[11] or laccase^[2,9,12], for O₂ reduction, HRP for H₂O₂ reduction^[13], and GOx^[13], GOx-catalase^[2,9,11,12] or NAD-glucose dehydrogenase^[10], for glucose oxidation.

We describe here a faster and simpler biopellet fabrication strategy to obtain a wide range of freestanding high surface area bioelectrodes, without requiring an additional fixing or stabilisation step (e.g. a sample holder or surface coating with polymer or hydrogel). For example, compared to previous biopellet and CNT film-modified electrodes^[7,11]. We investigate the possibility to obtain by compression physically-stable biopellets using proteins having different molecular weights and isoelectric points, and their potential application for bioelectrocatalysis. The possibility to form redox-active pellets that can be used for different types of mediated bioelectrocatalysis is also reported.

2. Material and methods

2.1. Materials and reagents

All reagents (analytical grade), GOx from *Aspergillus niger* (Type X-S), catalase from bovine liver, laccase from *Trametes versicolor*, Cyt C from *Horse heart*, HRP (Type 2), diaphorase from *Clostridium kluyveri*, GAOx from *Dactylium dendroides*, BSA, urease (type IX from *Canavalia ensiformis*), [Fe(CN)₆]³⁻, [Ru(NH₃)₆]³⁺ and 1,4 naphthoquinone (1,4-NQ) were purchased from Sigma-Aldrich. BOx from *Myrothecium verrucaria* (≥ 1.2 U/mg) from Amano. FAD-GDH from *Aspergillus sp.* from Sekisui Diagnostics (> 900 U/mg). All proteins were commercially sourced and lyophilised according to manufacturer treatments and used as received without further treatment to remove possible impurities. Multiwalled carbon nanotubes (MWCNTs) from Nanocyl (NC7000, $\varnothing = 10$ nm, 1.5 μ m length) and used as received. Single walled carbon nanotubes (SWCNTs) from Thomas Swan. Stability and electrochemical tests were performed in 0.1 M phosphate buffer (PB) pH 7.4 prepared in distilled H₂O (15 M Ω cm⁻¹). High purity O₂ and Ar from Air Liquide. Isoelectric points (pI) and molecular weights were obtained from supplier data sheets, unless otherwise cited.

2.2. Electrode preparation

Following our previously reported procedure, MWCNT biopellets ($\varnothing = 13$ mm, 0.9 ± 0.2 mm thick) were obtained by soft grinding of a mixture of 100 μ L distilled H₂O, 15 mg of protein and 35 mg of CNTs^[2]. 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. MWCNT and SWCNT biopellets ($\varnothing = 6$ mm, 1.8 ± 0.2 mm thick) were obtained as above but using 50 μ L distilled H₂O, 7.5 mg of protein and 17.5 mg of CNTs. For redox biopellets ($\varnothing = 6$ mm), the same as above but with 2.5 mg of mediator. For redox pellets ($\varnothing = 13$ and 6 mm), the same as for the biopellets but without enzyme and using 5 mg or 2.5 mg redox mediator, respectively. A Ag-plated Cu 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 (Fig. 1). The electroactive surface areas are based on the geometric area: 1.33 cm² (13 mm) and 0.28 cm² (6 mm). Redox pellet and biopellet electrodes were rinsed with distilled H₂O to remove weakly-adsorbed species prior to electrochemistry.

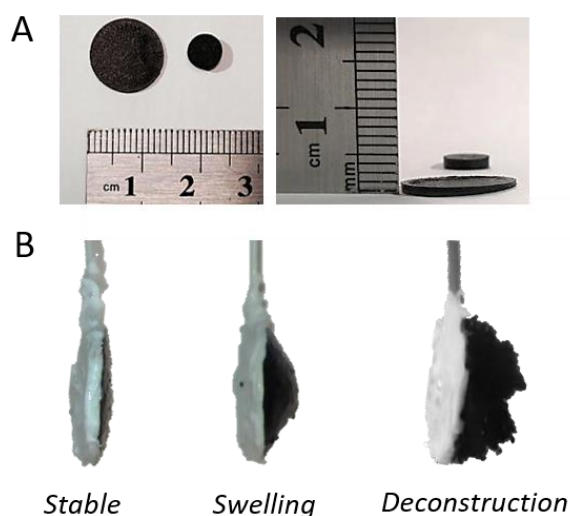


Fig. 1. Photographs of (A) 13 and 6 mm diameter biopellets and (B) physical states of CNT pellets illustrated for 13 mm diameter biopellets.

2.3. Apparatus

Electrochemical measurements were performed using an Ivium CompactStat potentiostat with Iviumsoft software at 25°C in 0.1 M PB pH 7.4 with a three-electrode cell containing a CNT pellet as the working electrode, a Pt wire counter electrode and a Ag/AgCl (sat. KCl)

reference electrode. Static water contact angles were obtained at room temperature by delivering a 5 μ L droplet of distilled water onto the sample surface on a horizontal stage using a Dataphysics OCA 35 system.

3. Results and discussion

Compressed biopellets ($\varnothing = 13$ mm) were elaborated with 11 proteins, including several oxidoreductase enzymes, with a wide range of properties and structures. The list of proteins including their molecular weights and isoelectric points are shown in Table 1. The biopellets containing Cyt C, BSA, GAOx, GOx or HRP, deconstructed and opened immediately upon contact with 0.1 M PB pH 7.4, inducing the release of CNTs and proteins in solution. In contrast, the biopellets with diaphorase, BOx, catalase or urease, gave stable composites with no apparent change in form after immersion in the buffer for at least 6 weeks. With laccase and FAD-GDH, significant pellet swelling was observed but the pellets did not deconstruct during the same period. Fig.1. illustrates the 13 and 6 mm diameter biopellets and the different physical states of pellets after immersion in the buffer. The swelling and ultimate destruction of the biopellets appears to result from a marked penetration of water inside the entangled nanotubes which alters the stress distribution and weakens existing cohesive interactions. Considering only the molecular weights, unstable pellets are observed using proteins with a MW of 12.4 to 160 kDa, while stable pellets are obtained using proteins with a MW of 40 kDa to 480 kDa. No obvious correlation between protein molecular weight and mechanical stability is observed. It is noted that the term mechanical stability refers to the pellet retaining its physical structure without visible loss of CNTs in solution (see Fig. 1B “stable”).

A series of static water contact angle measurements were also performed. The “stable” biopellets exhibited hydrophilic and hydrophobic contact angles with values in the range of 90 to 130 $^{\circ}$ with the exception of laccase, which due to its isoelectric point at 3.5, leads to a contact angle of 73 $^{\circ}$. The “deconstructed” pellets (BSA, Cyt C, GAOx, HRP and GOx), exhibited values varying from 85 to 122 $^{\circ}$. Although no correlation between the contact angles and the biopellet type can be clearly established, it appears that the biopellets in all cases were more wettable (73 to 130 $^{\circ}$) compared to CNT only pellets (132 $^{\circ}$). The integration of protein therefore renders the pellet surface more aqueous permeable.

Table 1: List of proteins used to obtain biopellets including molecular weight (MW), isoelectric point (pI), and stability in 0.1 M PB pH 7.4.

Protein	MW (kDa)	pI	Stability in PB pH 7.4	
			Ø 6 mm	Ø 13 mm
Cyt C	12.4	10-10.5	×	×
Diaphorase ^[14]	40.0	6.8	√	√
HRP ^[15]	44.0	9.1	×	×
BSA	66.4	4.7-4.9	×	×
Laccase ^[16]	67-70	3.5	√	swelling
GAOx ^[17]	68.0	12	×	×
BOx	68.0	4.1	√	√
FAD-GDH	130.0	4.4	√	swelling
GOx	160.0	4.2	×	×
Catalase	250.0	5.4	√	√
Urease	440-480	5.0-5.2	√	√

The effect of the geometrical parameters on the mechanical stability of the biopellets was also investigated. MWCNT biopellets prepared with 6 and 13 mm diameters exhibited the same mechanical stability results except for the 6 mm pellets comprising laccase and FAD-GDH that did not show swelling behaviour, and therefore, showed improved resistance to the buffer. The use of SWCNTs was also investigated to see if it was possible to obtain stable biopellets using CNT powders with different dimensions and properties. HRP- and BOx-SWCNT biopellets ($\varnothing = 6$ mm) were explored and, as observed with the MWCNTs, the HRP composite was unstable while the BOx composite was stable. These results show that the dimensions and type of unmodified CNTs did not have a significant impact on biopellet stability.

The hypothesis that the overall protein charge correlates with biopellet stability at pH 7.4 was examined by considering the pI values of the proteins (Table 1). Proteins like Cyt C (pI 10-10.5), HRP (pI 9.1) and GAOx (pI 12), exhibiting a “strong” overall positive charge did not permit the formation of stable pellets. BSA (pI 4.7-4.9) and GOx (pI 4.2), which have a “strong” overall negative charge, also did not lead to stable biopellets. In contrast, enzymes with “weak” or “weaker” overall negative charge led to stable biopellets, *i.e.* catalase (pI 5.4), diaphorase (pI 6.8) and urease (pI 5.0-5.2). These results, based on 8 different proteins, indicate that strong negative or positive electrostatic interactions between the protein and the

CNTs are not favorable for improved biopellet stability. The significantly higher failure rate for biopellets with “strongly” charged proteins compared to “weakly” charged proteins nevertheless highlights the importance of electrostatics. The instability observed under more charged conditions is consistent with a higher water uptake into these composites, driven by electrostatic gradients between polarised species^[18]. There were three exceptions to the above trend. Stable biopellets were obtained using laccase (pI 3.5), BOx (pI 4.1) and FAD-GDH (pI 4.4) with “strong” negative charge. This result does not rule out the importance of electrostatics but rather highlights the likely importance of other factors. In addition to electrostatic interactions, intermolecular forces may be considered based on hydrophobic, pi-pi stacking and van der Waals interactions between CNTs and amino acids present at the protein surface^[19]. For laccase, biopellet stability may be facilitated by strong hydrophobic interactions between CNTs and a hydrophobic pocket at the enzyme’s surface^[20]. For BOx, intricate interactions with CNTs are known based not only on electrostatics but also hydrophobic and pi-pi stacking interactions^[21]. The formation of stable biopellets with FAD-GDH (pI 4.1) may at first seem surprising given that unstable pellets were observed with GOx (pI 4.2), given that both fungus-derived enzymes have highly similar structures^[22]. A major difference between the two lies in their MW where GOx is significantly larger (*ca.* 23% to 68%, depending on the sizing technique used). The biopellet instability with GOx may be related to its larger size, and consequently larger protein swelling capacity, with increased swelling leading to larger “soft” voids and different stresses and eventual destruction of the composite. Despite this suggestion, it must be noted that the two largest proteins, catalase and urease, both led to stable biopellets and thus larger enzyme size does not always correlate with increased biopellet stability.

To further examine the effect of the overall protein charge on biopellet stability, we prepared pellets with differently charged redox species: $[\text{Fe}(\text{CN})_6]^{3-}$, $[\text{Ru}(\text{NH}_3)_6]^{3+}$ and 1,4 NQ. The exploration of redox molecules allows us to investigate differently charged redox species that do not swell. The resulting redox pellets without enzyme are mechanically stable in the buffer. The charge type did not affect biopellet stability under our conditions on the contrary to the behaviour observed for biopellets showing charge effects. This result reinforces the importance of protein swelling behaviour and, at least in some cases, the protein size on biopellet stability.

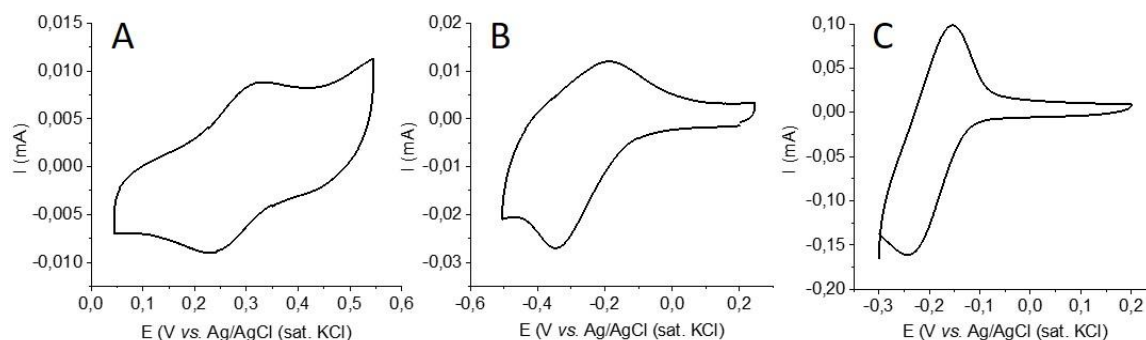


Fig. 2. Cyclic voltammograms of (A, B) 13 mm and (C) 6 mm diameter MWCNT redox pellets comprising (A) $[\text{Fe}(\text{CN})_6]^{3-}$, (B) $[\text{Ru}(\text{NH}_3)_6]^{3+}$, and (C) 1,4-NQ, recorded in 0.1 M PB pH 7.4 at 0.5 mV s^{-1} under Ar.

The pellets exhibit the conventional electrochemical behavior expected for the embedded redox mediators with electrochemically quasi-reversible systems at $E_{1/2} = 0.28 \text{ V}$ ($\Delta E_p = 101 \text{ mV}$), $E_{1/2} = -0.27 \text{ V}$ ($\Delta E_p = 160 \text{ mV}$), and $E_{1/2} = -0.20 \text{ V}$ ($\Delta E_p = 90 \text{ mV}$) for $[\text{Fe}(\text{CN})_6]^{3-}$, $[\text{Ru}(\text{NH}_3)_6]^{3+}$ and 1,4 NQ, respectively (Fig. 2). The electrodes were soaked for 30 min before a stable response was observed. After 6 weeks of immersion in 0.1 M PB pH 7.4, the loss of electroactivity of the immobilised redox mediators was only 10%, 21% and 25% for $[\text{Ru}(\text{NH}_3)_6]^{3+}$, $[\text{Fe}(\text{CN})_6]^{3-}$ and 1,4 NQ, respectively, highlighting good aqueous storage stability for the redox pellets. The loss of electroactivity was calculated based on the average value obtained after integration of the charge of anodic and cathodic peaks. It is noted that the pellet electrodes are affected by non-faradaic capacitance and interfacial resistance owing to the porous carbon nanotube network with a significant electrode thickness of *ca.* 1-2 mm. A scan rate of 0.5 mV s^{-1} was used as a compromise to minimise unwanted capacitive and iR drop effects. A scan rate study performed between 0.2 to 5 mV s^{-1} on a pellet incorporating $[\text{Ru}(\text{NH}_3)_6]^{3+}$ indicates that the embedded redox molecules behave more as a diffusing species than as an adsorbed species, according to Randles Sevcik theory (data now shown).

We finally investigated the formation and activity of bioelectrocatalytic pellets based on DET and MET, with BOx for O_2 reduction (biocathode), and FAD-GDH for glucose oxidation (bioanode), respectively. The possibility to use the BOx biopellet for bioelectrocatalytic O_2 reduction to H_2O is shown in Fig. 3A. A well-defined catalytic wave is observed in O_2 -saturated PB pH 7.4 that reaches a current maximum of 1.2 mA (4.3 mA cm^{-2} at 0.1 V). This compares to *ca.* 2 mA obtained using a ~~non-freestanding~~ porphyrin-modified BOx pellet stabilised with a plastic holder and in a sandwich configuration between a graphite plate and

cellulose membrane^[11]. The BOx pellet reported here is mechanically stable to immersion in the phosphate buffer for at least 8 weeks. Next we turned to a new biopellet anode type based on FAD-GDH which, contrarily to GOx, avoids the formation of H₂O₂ that can degrade glucose EBFC performance^[23]. Effective DET with fungal FAD-GDH is not well established and thus we investigated the formation of a redox biopellet by compression with a redox mediator. With the same CNTs and FAD-GDH enzyme source we previously showed no effective DET^[3]. Unlike the previous GOx and NAD-dehydrogenase pellet bioanodes, the FAD-GDH system does not require catalase (to remove H₂O₂) or NAD cofactor^[10]. 1,4-NQ is a well-known and attractive mediator for FAD-GDH with considerable promise for biofuel cell applications. [Ru(NH₃)₆]³⁺ is a low cost commercially-available redox molecule with excellent stability and an attractive redox potential for FAD-GDH for glucose sensing. The redox FAD-GDH biopellets based on 1,4-NQ or 1,4-NQ were mechanically stable after for at least 6 weeks in 0.1 M PB. The voltammograms of the biopellets were recorded before and after addition of a high glucose concentration (150 mM) (Fig. 3B and 3C).

For the 1,4-NQ biopellet, in the presence of glucose, a marked increase in the anodic current appears from -0.1 V that reaches a maximum current of 1.8 mA (6.43 mA cm⁻² at 0.2 V) corresponding to glucose oxidation to gluconolactone (Fig. 3B). The catalytic curve is well-defined compared to previous biopellets and represents a significant increase in catalytic current compared to previous pellet bioanodes (estimated 0.2 mA cm⁻² at 0.2 V)^[11]. The biopellet also outperformed our previous high performing FAD-GDH buckypaper anodes (5.4 mA cm⁻² at ca. 0.2 V at neutral pH^[3]). MET bioelectrocatalysis was not observed for the FAD-GDH biopellet with [Ru(NH₃)₆]³⁺ (Fig. 3C), consistent with the work of Noew *et al.*^[24].

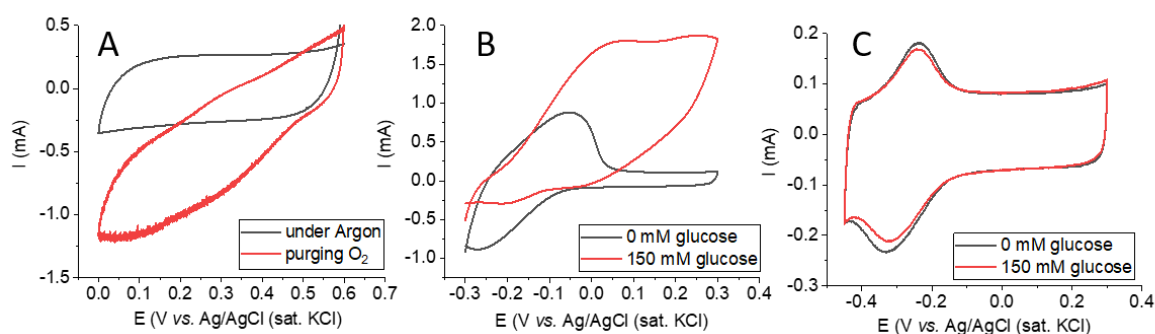


Fig. 3. Cyclic voltammograms of 6 mm diameter MWCNT redox biopellets comprising (A) BOx, (B) 1,4-NQ and FAD-GDH, (C) [Ru(NH₃)₆]³⁺ and FAD-GDH recorded in 0.1 M PB pH 7.4 at 0.5 mV s⁻¹.

Conclusion

The elaboration of a wide range of biopellets by the simple compression of carbon nanotubes and protein powders is a straightforward and fast procedure that can be used for the design of freestanding enzymatic electrodes for biosensors and biofuel cells. The method is extendable for the preparation of redox-active pellets and biopellets. The simplified methodology allowed the formulation of a new pellet bioanode based on FAD-GDH that offered improved catalytic output compared to the equivalent buckypaper design, highlighting the attractiveness of CNT pellet electrodes. Contrary to existing reports, the use of a stabilising membrane, support holder, or cross-linking agents, is not necessary. A protection membrane should be required for implantation but otherwise the biopellets have a large scope of application, for example, for in-vitro and in-field sensor and biofuel cell applications. Furthermore, we shed new light on the importance of swelling and electrostatic interactions on pellet stability that will help as a guideline for ~~with~~ the future development of CNT biopellets and composites. Future work will include assessment of both storage and continuous bioelectrocatalytic activity.

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