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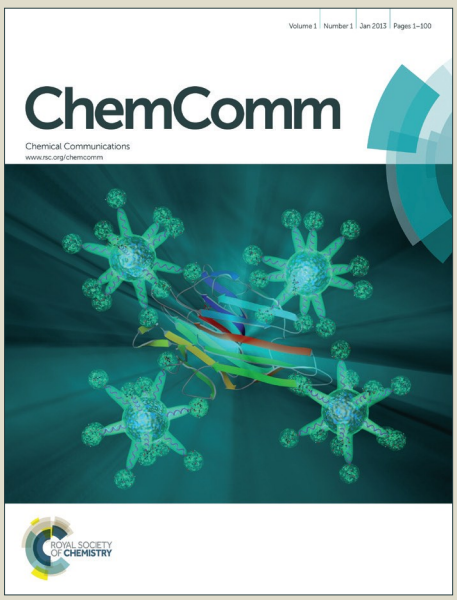

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Improving mediated electron transport in anodic bioelectrocatalysis

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A novel design of microbial fuel cell is realized by constructing bio-cocatalyst beads immobilized with riboflavin-secreting Escherichia coli and decoupling them from anodic biocatalyst. Microbial fuel cell loaded with these bio-cocatalyst beads shows significantly enhanced performance without occupying active electrode surface area.

Shewanella oneidensis MR-1, a facultative anaerobe, has been widely used as a model anode biocatalyst in microbial fuel cells (MFCs) due to its easiness of cultivation, adaptability to aerobic and anaerobic environment and both respiratory and electron transfer versatility. Proposed mechanisms for *S. oneidensis* extracellular electron transport (EET) include: (i) mediated electron transport (MET) through electron shuttles; (ii) direct electron transport (DET) through physical contact between outer-membrane c-type cytochromes (*c*-Cyts) or bacterial appendages and the electrode surface. Although *S. oneidensis* possesses versatile EET mechanisms, their efficiencies are relatively low and could be further improved considering the fact that it exhibits an excess of substrate oxidation ability.[1] *S. oneidensis* MR-1 is straight rod shaped bacterium[2] and consequently its cell surface is incapable of achieving complete contact with any two-dimensional (2D) electrode within MFC anodic chamber, which is partly responsible for the slow kinetics of EET to anode. A reasonable solution to this problem is to add exogenous artificial electron shuttles (AES) into the anolyte, which accept electrons from terminal *c*-Cyts of bacterial EET chain that cannot touch the anode directly, move to anode surface and release electrons to the anode. Exogenous AES such as neutral red,[3] anthraquinone-2,6-disulfonate (AQDS),[4] humid acids,[5] methyl viologen,[6] porphrins[7] or phenazine[8] are usually utilized. However, they are generally costly and toxic[9,10] to anodic electricigens, causing difficulties to practical applications of MET-type MFCs. *S. oneidensis* MR-1 is capable of utilizing self-secreted flavins like riboflavin (RF) as endogenous electron shuttles to accelerate EET more efficiently than exogenous AES.[1,11] Moreover, RF is vitamin B_2 and accordingly anodic electricigens have a better tolerance to RF than other toxic AES. Thus, increasing RF concentration in the anolyte is proposed to improve MFC performance.

There are two rational strategies for achieving that goal: (i) *Shewanella* strains could be genetically engineered to enhance their ability to secrete RF. However, only a narrow range of carbon sources such as formate, lactate, pyruvate and amino acids[12,13] can be used as electron donors by *Shewanella* strains under strictly anaerobic conditions. Metabolizing the limited categories of fuels by engineered *Shewanella* strains to not only generate electricity but also increase the yield of self-secreted RF will undoubtedly lower the coulombic efficiency and consequently reduce energy harvesting from biomass by MFCs. Recently, *S. oneidensis* MR-1 has been engineered to gain the ability to utilize glucose, which is a platform chemical in biorefinery and cannot be metabolized by *Shewanella* strains under strictly anaerobic conditions,[14,15,16] as carbon source to generate electricity in MFCs.[17] Thus, there exists a theoretical possibility that this recombinant glucose-utilizing *S. oneidensis* MR-1 strain could be further engineered to increase its RF secretion. However, the fact that this recombinant *S. oneidensis* MR-1 strain cannot survive without external electron acceptors like fumarate or electrode under anaerobic condition[17] indicates that anode surface area will limit the amount of the recombinant riboflavin-secreting strain and eventually limit the concentration of RF in the anolyte; (ii) *S. oneidensis* MR-1 could be co-cultivated in combination with riboflavin-producing microbes in MFCs. Recent studies have shown that the interactions between microbes in mixed culture systems are complicated and consequently their electrochemical performances are quite unpredictable:[18,19] a synergistic effect with enhanced electricity generation;[20,21] a food-web relationship with similar power output;[22] and a negative effect with reduced electricity production[20,23], compared to a pure culture of anodic electricigen. Ideally, to achieve the maximum electrical output, the anode surface ought to be completely occupied by

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electricigens. However, the presence of nonelectricigen within the anode microbial community in MFCs will inevitably reduce the effective anode area available for electricigen accumulation on the electrode, which might partly account for differing MFC performances in mixed culture systems.

Escherichia coli, an easily grown facultative anaerobe capable of utilizing both nonfermentable and fermentable carbon sources,[9] can be cultivated under strictly anaerobic condition without external electron acceptors. Herein, we engineer *E. coli* BL21 (DE3), a widely used strain for protein expression, to enhance its anaerobic RF secretion. The recombinant *E. coli* cells are then immobilized in alginate beads, placed at the bottom of MFC reactor without any occupation of anode surface and metabolize glucose to secrete RF, as a bio-cocatalyst, to improve the electricity generation by *S. oneidensis* MR-1, as a biocatalyst, at the anode. In principle, these bio-cocatalyst beads can be loaded into the continuous flow reactor for wastewater treatment, saving the cost of adding exogenous AES and in particular, the amount of bio-cocatalyst beads used can be adjusted according to the flavinogenic activity, hydraulic retention time (HRT), reactor volume, etc. Recently, it has been reported that flavins promote EET of *Enterococcus faecalis*,[24] three methanotrophic bacteria,[25] alkaliphilic Bacteria consortium,[26] and even *Geobacter sulfurreducens*[27] as either electron shuttles or redox cofactors. It suggests that our riboflavin-secreting bio-cocatalyst beads, given that the immobilized bio-cocatalyst avoids direct contact with anodic electricigen and consequently unpredictable interactions between nonelectricigen and electricigen at the anode, can be applied in combination with diverse biocatalysts besides *S. oneidensis* MR-1 in MFCs in the future.

The five-gene biosynthetic pathway of RF was constructed by cloning *ribA, ribB, ribD, ribC* and *ribE*[28] genes from *E.coli K-12* genome into pQLinkNMut vector, a derivative of pQLinkN vector for protein coexpression,[29] under the control of P_{tac} promoter, resulting in a ~9.6k plasmid of pQLinkNMut-RibABDCE, which was then electroporated into *E. coli* BL21 (DE3) electrocompetent cells (see details in the ESI, Fig. S1 and Table S1). To examine the anaerobic flavinogenic activity of bio-cocatalyst beads, 39 g of beads (9 g recombinant *E. coli* cell pellets, wet weight) were placed into an anaerobic culture bottle containing 100 ml anolyte and incubated anaerobically at 30 °C. Samples (100µl) were collected at 12 h intervals and analyzed by high-performance liquid chromatography (HPLC). As shown in Fig. 1, the RF concentration in anolyte can reach up to 82.0±0.8 µM (n=3) in the first batch (168 h). Although RF was known as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD),[28] both of which could also be used as electron shuttles to enhance EET,[11,30] neither of them was detected within the anolyte by HPLC analysis (data not shown). Thus, the contribution of bio-cocatalyst to MFC performance was attributed solely to the increase of RF concentration. To evaluate the reusability of these bio-cocatalyst beads, experiments of repeated batch operation were performed for 8 cycles. RF secretion by bio-cocatalyst beads gradually decreased from the first cycle to the eighth cycle. After 8 reuse cycles, approximately 42% of

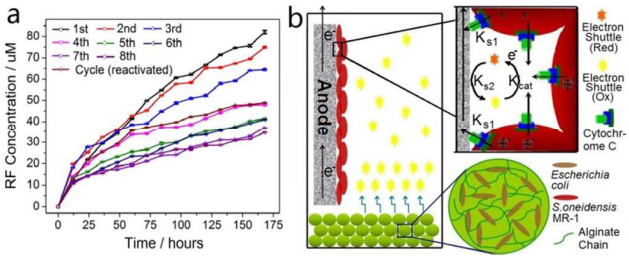

Fig. 1 (a) Anaerobic flavinogenic activity measurements. 39 g of beads (9 g recombinant E. coli cell pellets, wet weight) in 100 ml anolyte were incubated anaerobically at 30 °C. After completion of 8 batches, the bio-cocatalyst beads were reactivated by replacing anolyte with 100 ml fresh LB broth (supplemented with 100 µ g/ml ampicillin, 1 mM IPTG and 0.1 M $CaCl_2$) and incubated at 37 °C for 24 h. All measurements were performed in triplicates. (b) Schematic of the mechanism of MFC anodic electrocatalyzed process enhanced by the bio-cocatalyst beads.

flavinogenic activity of bio-cocatalyst beads still remained (Fig. 1a). To test the ability to recover their flavinogenic activity, these beads were reactivated by 100 ml fresh LB broth supplemented with 100µg/ml ampicillin, 1 mM IPTG and 0.1 M $CaCl_2$. After incubated at 37 °C for 24 hr, the restored activity of bio-cocatalyst beads was approximately 60% of original flavinogenic activity (Fig. 1a).

Dual chamber reactors with the *S. oneidensis* MR-1 bio-anode, Potassium ferricyanide cathode and 2kΩ load resistor were used to evaluate MFC performances, except as noted. The anode was made from carbon cloth, which was made more hydrophilic for better electricigen adhesion by oxygen plasma treatment.[31] After a stable output of MFC was obtained (about 500h after inoculation), the number of living microbes at the O_2 plasma treated anodic carbon cloth was determined at $15.89\pm0.35\times10^6$ colony forming units per square centimeter (CFU/cm^2), compared to $2.61\pm0.08\times10^6$ CFU/cm^2 at the anodic carbon cloth without O_2 plasma treatment. Then, 39 g of bio-cocatalyst beads were loaded into this MFC anodic chamber containing 100 ml fresh anolyte. After another 160h, the stable output of MFC was obtained again. At this point, 100µl of sample was taken for HPLC assay, which shows that RF concentration in MFC anodic chamber containing bio-cocatalyst beads accumulated to 81.3±1.6µM (n=3) and the MFC was used for electrochemical measurements. Given that flavins were capable of increasing the amount of microbes colonizing the anode,[1] the *S. oneidensis* MR-1 bio-anode incubated with bio-cocatalyst beads under the same condition mentioned above was transferred into a MFC reactor without bio-cocatalyst beads as the control. The polarization and power output curves (Fig. 2a and Fig. S2, ESI) show that although the open circuit potential (OCP) of the MFC with O_2 plasma treated anode containing bio-cocatalyst beads (799.6±0.4mV) is only slightly higher than that of the control MFC (785.8±0.3mV), the former MFC delivers a maximum power density of 1079.6 ± 5.1mW/m^2, corresponding to a current density of 2722.9 ± 9.3mA/m^2 at a cell potential of 396.5±0.5mV, which is 9.6-fold as much as that of the control MFC (112.2 ± 3.0 mW/m^2), obtained at a current density of 210.8 ± 5.4mA/m^2 with a cell potential of 532.5±0.6mV. The maximum current output of the MFC

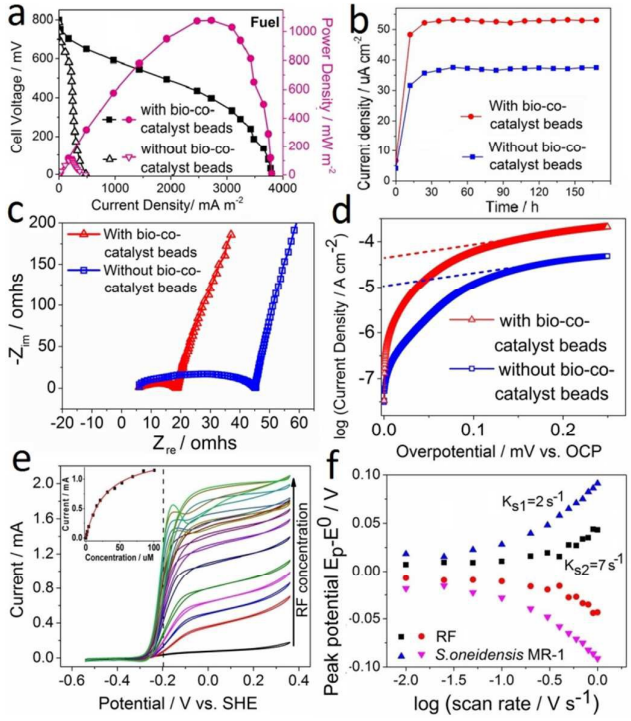

Fig. 2 (a) Power output and polarization curves of MFCs with O_2 plasma treated carbon cloth anodes. All measurements were performed in triplicates. (b) Discharge performance of MFCs with and without bio-co-catalyst beads. (c) EIS of S. oneidensis MR-1 bio-anode in anodic chamber. (d) Tafel plots recorded at the scan rate of 0.2 mV/s from η =0 to 0.25 V versus OCP. (e) Cyclic voltammograms (1 mV/s) from MR-1 biofilm residing on the O_2 plasma treated carbon cloth anode in increasing levels of RF. Inset: Variation of the magnitude of the catalytic current, measured at -0.2 V, with RF concentrations (background value at 0µM RF was subtracted), which were fit to the Michaelis-Menten kinetics. (f) Peak potential separation versus log(scan rate) for MR-1 biofilm and RF on the carbon cloth. The data were fit to a k_{s1} of $2s^{-1}$ and a k_{s2} of $7s^{-1}$, respectively, according to the Laviron theory.

containing biococatalyst beads is 3804.3±9.4mA/m^2, which is almost 8-fold higher than that of the control MFC (477.4±5.2 mA/m^2). The role of bio-cocatalyst beads in promoting performance of the MFC using anode without O_2 plasma treatment was also examined. The polarization and power output curves reveal (Fig. S3, ESI) that the maximum power density of the MFC without plasma treatment containing bio-cocatalyst is 434.2±3.4mW/m^2, which is 9.9-fold as much as that of the control MFC (43.7±1.0mW/m^2). Thus, MFCs loaded with bio-cocatalyst beads have much higher activity, regardless of the amount of electricigen at the anode. The discharge performance evaluated by measuring the output current profiles at 2kΩ load resistor showed that the MFC containing bio-cocatalyst beads delivered a steady-state current density of 52.7±0.5µA/cm^2, which was higher than that of control MFC (37.1±0.5µA/cm^2, n=3). Electrochemical impedance spectroscopy (EIS) measurements were performed and the Nyquist plots (Fig. 2c) show semicircles over the high frequency range, followed by straight lines. A Randle equivalent circuit was used to fit the impedance data, in which the interfacial charge transfer resistance (R_{ct}) was extracted.[32,33] For *S. oneidensis* MR-1 bio-anode in the MFC containing bio-cocatalyst beads, R_{ct} greatly declines compared to that in the MFC without bio-cocatalyst. After connecting a 1 kΩ load resistor, the Coulombic efficiency of the MFC containing bio-cocatalyst beads is calculated at 10.3%, which is higher than that (7.6%) of the control MFC. The low charge transfer resistance may reduce energy loss during electron transport and could increase the coulombic efficiency of the MFC containing bio-cocatalyst. The Tafel plots (Fig. 2d) show that the exchange current density of MFC loaded with bio-cocatalyst beads, determined by extrapolation of Tafel slopes back to η=0 (corresponds to the OCP of the bio-anode *versus* the reference electrode),[34] is 4-fold as much as that of the control MFC. It is speculated that RFs are reduced by out-membrane *c*-Cyts, move *via* diffusion to the anode and oxidized by the electrode.[35] The bio-cocatalyst beads are capable of raising RF concentration in the anolyte and thus increase RF flux between *c*-Cyts in the biofilm and the anode. Accordingly, the concentration of reduced RFs close to the anode surface is increased, which leads to the enhanced current density (-*j*).

To further explore the underlying mechanism of *S. oneidensis* EET kinetics enhanced by RF-secreting bio-cocatalyst, the electrocatalytical response of *S. oneidensis* MR-1 bio-anode to the presence of varying RF concentrations was analyzed. After anolyte in MFC was replaced with the fresh one, RF was added into the anodic chamber and cyclic voltammograms of *S. oneidensis* MR-1 biofilm residing on the O_2 plasma treated carbon cloth anode in 0, 1.2, 2.3, 3.2, 5.4, 11.8, 17.1, 22.4, 33, 43.6, 54.2, 70.1, 86.0 and 101.9µM RF were recorded respectively (Fig. 2e). The magnitude of catalytic current was measured at -0.2 V to minimize the interference of nonspecific current and maximize the contribution to the catalytic current from RF.[1,11] The catalytic current demonstrated saturating behavior with the increase of RF concentration. The data were fit to the Michaelis-Menten equation to yield an apparent Michaelis constant (K_m) of 30.4±1.5µM which represents an average of *c*-Cyts in the electroactive *S. oneidensis* MR-1 biofilm. The cyclic voltammograms of *S. oneidensis* MR-1 biofilm at the anodic carbon cloth without O_2 plasma treatment in the presence of increasing levels of additional RF were also examined (Fig. S4, ESI). After fitting, these data yielded an apparent K_m of 30.5±2.1µM. K_m is known as a physical constant characteristic of enzyme-catalyzed reactions and for the *c*-Cyts catalyzed reduction of RF (eqn(1)), K_m is defined as (eqn(2)).

$$c\text{-Cyts} + RF_{Ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} c\text{-Cyts}\ RF_{Ox} \xrightarrow{k_2} RF_{Red} + c\text{-Cyts} \quad (1)$$

$$K_m = (K_{-1} + K_2)/K_1 \quad (2)$$

Although the reaction rate *j* captured by electrochemical measurement corresponds to the concentration of reduced RF at the surface of electrode rather than that in the vicinity of *c*-Cyts in the biofilm, according to the model developed by Torres *et al.*[35], transfer of RF to the electrode is accomplished by diffusion obeying Fick's law and consequently *j* is proportional to the concentration of reduced RF in the vicinity of outer-membrane *c*-Cyts saturated with RF. Accordingly, K_m obtained by fitting data on variation of current with added RF concentrations is identical to that obtained by

variation of reduced RF concentration in the vicinity of *c*-Cyts with added RF concentration and this method has the superiority that the reactivity of outer-membrane *c*-Cyts in the electroactive biofilm with RF can be easily determined in real time by electrochemical measurement. Thus, K_m could be used to estimate the turnover number (K_{cat}) of *c*-Cyts in the biofilm. As K_{cat} / K_m values with RF for purified MtrC and OmcA are 1.2×10^5 and 5.9×10^5 $M^{-1}s^{-1}$ respectively,[36] the estimated K_{cat} ranges from 3.7 to 17.9 s^{-1}. Although this K_{cat} window is at least one order of magnitude slower than that (from 617 to 1045s^{-1}) estimated from data obtained by measuring relative fluorescence unit changes of cells cultivated in flasks,[37] the electron transfer rate constant between *c*-Cyts in the biofilm and RF (from 7.4 to 35.8s^{-1}) is still higher than that (k_{s1}=2s^{-1}) between *c*-Cyts in the biofilm and the carbon cloth anode (Fig. 2f), given that each RF accepted 2 electrons from *c*-Cyts at a time. *In vitro* studies have observed that *c*-Cyts redox kinetics were at least two orders of magnitude faster than that *in vivo*.[1,36,38] As an explanation for this inconsistency, transporting electrons from the cell interior to the external electrode directly through *c*-Cyts resulted in cation accumulation within the cell, which produced the liquid junction potential to retard the electron export.[38] RF was assumed to have the capability of coupling H^+ export with electron transport, which could alleviate this retardation and accelerate electron transfer. Furthermore, the interfacial electron transfer rate constant (K_{s2}) between RF and carbon cloth electrode determined was 7s^{-1} (Fig. 2f), which was faster than that (k_{s1}=2) between *c*-Cyts in the biofilm and the electrode. Therefore, we propose that electron export to electrode *via* RF shuttles has a faster kinetics than that *via* direct contact (Fig. 1b).

In conclusion, by immobilizing engineered RF-secreting *E.coli* cells as bio-cocatalyst beads, we present a novel strategy of using bio-cocatalyst beads to significantly enhance the performance of MFCs, which has the following potential advantages: It can adjust RF concentration to desired level without occupying any anode surface area; the beads are reusable and easy to recycle. Since there is no direct contact with anodic electricigen in the decoupled design, they also have the potential to be applied together with other biocatalysts besides *S. oneidensis* MR-1.

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