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A copper-specific microbial fuel cell biosensor based on riboflavin biosynthesis of engineered *Escherichia coli*

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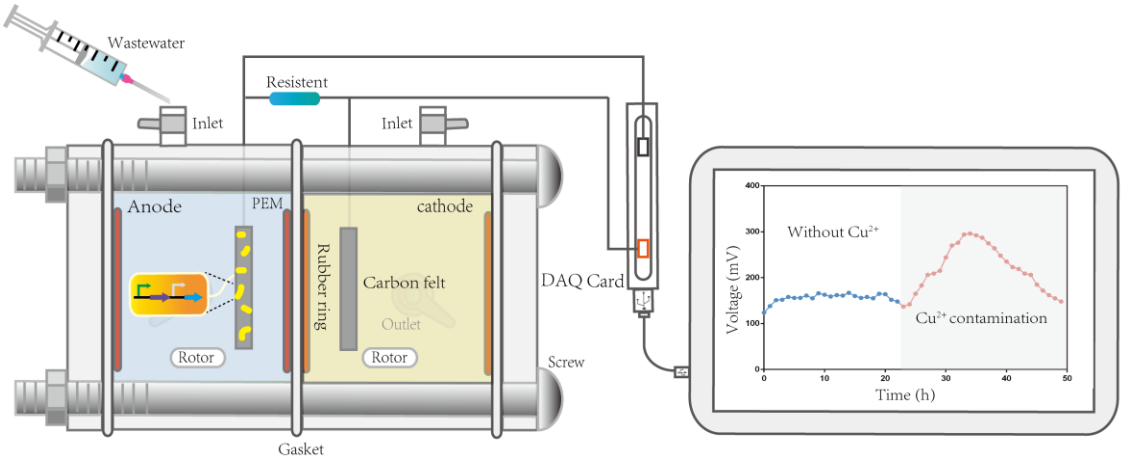
ABSTRCT: Copper pollution poses a serious threat to the aquatic environment; however, *in situ* analytical methods for copper monitoring are still scarce. In the current study, *E. coli* Rosetta was genetically modified to express *OprF* and *ribB* with promoter P_{t7} and P_{cusC}, respectively, which could synthesize porin and senses Cu^{2+} to produce riboflavin. The cell membrane permeability of this engineered strain was increased and its riboflavin production (1.45-3.56 μM) was positively correlated to Cu^{2+} (0-0.5 mM). The biosynthetic strain was then employed in microbial fuel cell (MFC) based biosensor. Under optimal operating parameters of pH 7.1 and 37°C, the maximum voltage (248, 295, 333, 352 and 407 mV) of the constructed MFC biosensor showed a linear correlation with Cu^{2+} concentration (0.1, 0.2, 0.3, 0.4, 0.5 mM, respectively) (R^2 = 0.977). The continuous mode testing demonstrated that the MFC biosensor specifically senses Cu^{2+} with calculated detection limit of 28 μM, which conforms to the common Cu^{2+} safety standard (32 μM). The results obtained with developed biosensor system were consistent with the existing analytical methods such as colorimetry, FAAS, and ICP-OES. In conclusion, this MFC-based biosensor overcomes the signal conversion and transmission problems of conventional approaches, providing a fast and economic analytical alternative for *in situ* monitoring of Cu^{2+} in water.

Graphical Abstract



KEYWORDS: MFC; Cu^{2+} biosensor; *cusC* promoter; riboflavin; cell permeability

1 INTRODUCTION

Copper is one of common heavy metals (HMs) that is considered a priority pollutants (C.-Y. Hsieh, Tsai, Ryan, & Pancorbo, 2004). Currently, the copper release from agricultural, industrial and mining wastes into the aquatic environment has posed a serious threat to the stability of ecosystem (Rehman et al., 2019). The lack of effective warning tools for copper pollution has resulted in many negative impacts. For example, contaminated soil in the Jiuhuashan mountains in China resulted in accumulation of more than 40 mg kg^{-1} copper ions in food crops, posing potential health hazards to the residents (F. Wu, Liu, Xia, Shen, & Chen, 2011), while copper pollution at the El Salvador mine in Chile highlighted its drastic effects on marine invertebrates, fish, and algae in the Chañaral Bay (Medina et al., 2005). Although trace amounts of copper play an important role in physiological processes, high doses of copper exposure disrupt the endocrine and immune systems and lead to oxidative stress, cirrhosis, kidney dysfunction, and serious neurodegenerative diseases (G.

Georgopoulos, 2001; Uriuadams & Keen, 2005). Consequently, it is urgent to develop an effective and environmentally friendly copper *in-situ* analysis method as an implementation tool for water quality assessment, so as to provide a direct basis for targeted elimination of copper pollution.

Conventional detection methods for copper include anodic stripping voltammetry, UV–vis spectroscopy, fluorescence spectroscopy, colorimetric method, flame atomic absorption spectrometry (FAAS), X-ray fluorescence spectrometry (XFS), and inductively coupled plasma optical emission spectrometry (ICP-OES) (Awual, 2015). Among them, anodic stripping voltammetry and UV–vis spectroscopy are not sensitive. XFS is a quantitative analysis of fluorescence intensity emitted by atoms excited by radiation energy (Sitko et al., 2015). FAAS and ICP-OES measure HM concentrations by observing their characteristic peaks that form in the vapor phase (Bansod, Kumar, Thakur, Rana, & Singh, 2017; Losev, Buyko, Trofimchuk, & Zuy, 2015). These methods can be applied to comprehensively detect heavy metals and have simultaneously high sensitivity and accuracy. Whereas, they often require expensive equipment, laborious operation, professional technicians, and complex pretreatment processes (Gumpu, Sethuraman, Krishnan, & Rayappan, 2015). Moreover, due to bulky equipment and complex detection processes, they are not suitable for real-time measurement and *in situ* analysis.

Recently, microbial fuel cell (MFC)-based biosensors have been widely developed as a novel alternative for pollutant detection with advantages of miniaturization, easy operation and low cost (Su, Jia, Hou, & Lei, 2011). Based on the relationship between substrate concentration and corresponding voltage, target analytes have been extended to various parameters such as biochemical oxygen

demand (BOD)(M.-C. Hsieh & Chung, 2014), microbial activity (Tront, Fortner, Plötze, Hughes, & Puzrin, 2008), and chemical toxicants (Chouler, Cruz-Izquierdo, Rengaraj, Scott, & Di Lorenzo, 2018; Shen, Lefebvre, Tan, & Ng, 2012). However, this technique for specific substrate monitoring is still under-utilized. As a *in situ* sensing tool with low power consumption, synthetic biology-mediated MFC biosensor has shown great potential in system design and engineering applications in the field of electrochemistry (Khan et al., 2020; Webster et al., 2014; West, Jain, & Gralnick, 2017; Yang et al., 2015). For instance, a study characterized a circuit-engineered strain in which extracellular electron transfer (EET) responds specifically to trimethylamine N-oxide (TMAO) (West et al., 2017). By combining different promoters and voltage output elements to design "AND" and "OR" circuit logic gates, modular genetic elements can be used to customize biosensor for analyzing various or multiple target pollutants. Accordingly, the combination of synthetic biology and engineering will further expand the application potential of MFC biosensor to detect specific substrates.

Bacterial biosensors connected to metal-responsive regulatory units have been widely used to monitor HMs (Leth et al., 2002). Among these biosensors, the two-component system (TCS) *cus* is a well-known sensor kinase system that responds to extracellular Cu^{2+} stimulation and controls differential expression of target genes (Munson, Lam, Outten, & O'Halloran, 2000). It had elucidated that EET efficiency and electricity output of the MFC system can be facilitated by riboflavin (Marsili et al., 2008). The porin OprF from *Pseudomonas aeruginosa* is a demonstrated approach to improve cell membrane permeability (Yang-Chun Yong et al., 2013). By recombining promoter of *cusC* (P_{cusC}), T7 promoter (P_{t7}), riboflavin synthesis gene (*ribB*), and porin synthesis gene (*OprF*) in *E. coli* Rosetta, a biosynthetic MFC was

constructed for specifically monitoring Cu^{2+}. The riboflavin synthesis level and cell membrane permeability of recombinant Rosetta was investigated, and the performance (continuity, sensitivity, and specificity) of the constructed MFC was further calibrated under various operational parameters. Based on the linear relationship between voltage generated by MFC and external Cu^{2+}, an improved portable biosensor device was developed and compared with current methods.

2 MATERIALS AND METHODS

2.1 Plasmid construction and culture conditions

Biobrick compatible restriction site sequence was introduced into pET-23b (+) plasmid by Blunting Kination Ligation Kit (Takara, Japan) and named pLZ01 for standard part assembly (Figure S1). Coding sequences of genes were obtained from NCBI, IGEM and BioCyc databases. *gfp* and P_{t7} from our laboratory storage, terminator (BBa_B0014) from 2017 DNA Distribution Kit Plates, *OprF* from *P. aeruginosa* PAO1, and *ribB* and P_{cusC} (Ravikumar, Pham, Lee, Yoo, & Hong, 2012) from *E. coli* DH5α were amplified using the primers listed in Table S1 and were then cloned into pLZ01. Subsequently, *ribB* (or *gfp*) was inserted downstream of P_{cusC}, while *OprF* was fused to P_{t7}. The module P_{cusC}-*ribB* and P_{T7}-*OprF* was then assembled together, and terminator (BBa_B0014) was introduced between the two parts. The constructed plasmids were first transformed into *E. coli* DH5α and screened on LB plates containing 50 μg mL^{-1} ampicillin. Plasmids were extracted for PCR verification, double-enzyme digestion validation, and sequencing confirmation (BGI, China). Commercial *E. coli* Rosetta (Rosetta) was used as a chassis microorganism for expression and characterization of all engineered strains (Bane, Velasquez, & Robinson, 2007). LB medium was used for the molecular construction and strain activation, while M9 basic medium (1×M9 medium, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.4% glucose was employed for formal experiments. Ampicillin and chloramphenicol were added to the culture

medium at a final concentration of 100 μg mL^{-1} to maintain antibiotic pressure. For MFC operation, all strains were first inoculated in 5 mL LB medium overnight at 37°C with shaking; next, the cultures were centrifuged at 5000 g for 10 min. Cell pellets were washed and suspended in M9 medium, and OD_{600} was adjusted to be approximately 1.0. The initial cell culture was inoculated at a ratio of 1:100 in the anode chamber of the MFC containing 250 mL M9 medium. $CuSO_4$ was used to simulate Cu^{2+} when performing biosensor experiments.

2.2 Evaluation of the Cu^{2+} dependence of P_{cusC}

The *E. coli* Rosetta harboring plasmid pLZ01-P_{cusC}-*gfp* (reporter Rosetta) was grown overnight at 37°C in an orbital shaker at 160 rpm, and the culture was inoculated in M9 medium (1:100) containing different concentrations of Cu^{2+} (0, 0.05, 0.2, 0.5, 1, 1.2 mM) for 4 h. Subsequently, the GFP fluorescence intensity (excitation wavelength: 490 nm; emission wavelength: 510 nm) and OD_{600} of reporter Rosetta was determined by microplate reader (Thermo Scientific, USA). The mean GFP/cell was calculated by dividing the raw fluorescence intensity by OD_{600}. To visualize GFP expression of induced cells with Cu^{2+}, the cultures were centrifuged (5000 g, 5 min, 4°C) after 4 h incubation and resuspended with PBS buffer (10 mM, pH 7.4). The fluorescence imaging was performed using 40× objective on a fluorescence microscope (Olympus, Japan). For flow cytometry analysis, a total of 20,000 cells were counted using FITC voltage of 400 V on a flow cytometer (Biorad, USA) after 4 h induction with different Cu^{2+} and the data were analyzed by Flowjo 7.6.

2.3 Analysis of riboflavin production

Riboflavin concentration in the MFC was determined by high-performance liquid chromatography (HPLC) and microplate reader (Thermo Scientific, USA) (Y. C. Yong et al., 2013). To quantitatively measure riboflavin production, 200 μM of aqueous standard riboflavin solution was prepared in distilled water and then diluted in linear gradient from 0.1 μM to 100 μM. The

MFC sample was centrifuged (10000 g, 10 min, 4 °C) and filtered through a 0.22 μm membrane before analysis. Two hundred microliters of the supernatant was added to a black 96-well plate for fluorescence assay using a microplate reader (excitation wavelength: 450 nm; emission wavelength: 525 nm). The supernatant (10 μL) was then analyzed by HPLC (Agilent, USA) using an Agilent analytical C18 column (4.6 mm × 105 mm) with a mobile phase of 50% methanol and 50% ddH_2O at a flow rate of 1.0 mL min^{-1}. The HPLC profile was monitored at 270 nm, with a peak time of about 3 min.

2.4 Determination of membrane permeability

To analyze the effect of *OprF* expression on Rosetta, the membrane permeability of bacteria was measured using NPN (1-N-phenylnaphthylamine) uptake and ONPG (o-nitrophenyl-β-galactoside) hydrolysis assays (Jing, Yan, Zhi, Hao, & Chang, 2012; Z. X. Zhou, Wei, Guan, Zheng, & Zhong, 2010). NPN is a fluorescent reagent indicating cell membrane permeability. The enhancement of NPN fluorescence intensity demonstrates the increase of cell membrane permeability. ONPG can be hydrolyzed by intracellular β-galactosidase. Thus, the amount of ONPG hydrolysis product will reflect the level of substrate diffusion through the cell membrane. Bacteria were cultured overnight and then diluted 100 folds in fresh LB medium for 5 h at 37°C, with shaking at 160 rpm. The culture was centrifuged (8000 g, 5 min, 4°C) and washed three times with PBS buffer (10 mM, pH 7.4). For the NPN uptake assay, 2 μL NPN (3 mM in alcohol) was mixed with 1 mL of cell suspension, and a microplate reader was used to measure the resulting fluorescence emission (405 nm; 355 nm excitation wavelength). In the ONPG hydrolysis assay, 15 μL cell suspension was mixed with 135 μL ONPG solution (1.5 mM in 10 mM PBS, pH 7.4), and absorbance at 405 nm was detected by microplate reader (Thermo Scientific, USA).

2.5 MFC establishment

A modular two-compartment MFC was employed to construct the Cu^{2+} biosensor. Each single chamber of the MFC reactor was composed of a polymethyl methacrylate (PMMA) cube (12×12×12 cm), which contains a cylinder with a volume of approximately 300 mL (5 cm high and 4 cm radius). The anode and cathode chambers were separated from each other by proton exchange membranes (PEM, Nafion117, DuPont, USA) and fixed with flat screws and nuts. PEM was pretreated with 3% H_2O_2 and 0.5 M H_2SO_4 to remove impurities (Biffinger, Byrd, Dudley, & Ringeisen, 2008). Carbon felt with 28 cm^2 (1×2×4 cm) surface area was soaked for 12 h each with 1 M NaOH and 1 M HCI sequentially, and then washed with dH_2O until the pH was neutral (Bond & Lovley, 2003). Before assembly, the MFC reactor, titanium wire, stirring rod and pretreatment carbon felt were sterilized by autoclaving at 121 °C for 15 min. Temperature probes were sterilized with 75% ethanol and inserted into MFC chambers to control system temperature. $K_3[Fe(CN)_6]$ solution (250 mL of 100 mM) in 50 mM phosphate buffer (pH 7.0) and M9 medium were added into the cathode and anode chambers, respectively (Kong et al., 2010). Bacteria was inoculated into the anode chamber and different amount of $CuSO_4$ were added. To reduce mass transfer resistance, the speed of the stirring rod was maintained at 160 rpm. Carbon felt was used as anode and cathode as electrodes meanwhile titanium wire was applied as external circuit to connect electrodes. The voltage across 1000 Ω external resistance was recorded at 10 min intervals by a data acquisition apparatus. The current density and power density were then calculated according to Ohm's law. To evaluate electron transfer mechanism, cyclic voltammetry (CV) tests were carried out on the anode solutions by using Ag/AgCl (3 M KCl) was the reference electrode and platinum wire as the counter electrode. A potential range of -0.9 to 0.9 V at a scan rate of 5 mV s^{-1} was used and three repeats analyses were performed using a potentiostat (CHI604E, Shanghai, China).

2.6 Relative expression analysis

A bacterial RNA extraction kit (Omega Bio-tek, USA) was used to isolate total RNA from logarithmic phase cultures according to the manufacturer's instructions. Subsequently, cDNA was synthesized by using PrimeScript™ RT reagent Kit (Takara, Japan). Quantitative analyses of target gene expression were performed using TB Green™ Premix Ex Taq™ II (Takara, Japan) with 16S rRNA gene as a reference gene. Primers used to amplify the small parts of the gene are also listed in Table S1. To reduce relative quantitative error, the data were analyzed using the $\log_2^{2^{\wedge}-\Delta\Delta Ct}$ method (Bustin & Mueller, 2005).

2.7 SEM analysis

Scanning electron microscopy (SEM) was used to analyze the formation of anode biofilms. Slices sized 0.5 × 0.5 cm were cut from carbon felt, placed in a 1.5 ml tube, and fixed in 2.5% glutaraldehyde buffer overnight at 4 °C. Subsequently, slices were washed three times with PBS and dehydrated using progressively increasing concentrations of ethanol (30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%). Samples were then freeze-dried and coated by sputtering gold, followed by SEM observation (S-3400, HITACHI, Japan).

2.8 Characterization of the MFC biosensor

To verify the response performance and specificity of the constructed MFC biosensor under different environmental parameters, the voltage generated at different operating temperatures (27°C, 30°C, 37°C, 45°C, 55°C), pH values (5.01, 6.00, 7.14, 8.03, 9.12, 10.01, 11.02), and HMs (0.2 mM Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Fe^{3+}, and Cr^{6+}) was recorded and analyzed. The response time of MFC biosensor is defined as the period required for the Cu^{2+} sensing Rosetta generated higher voltage than baseline, while the recovery time is the period required for the voltage restored to baseline. For the test of practical application ability, the MFC rector was assembled in a developed

portable device (Figure S2) (Zhengjun Chen et al., 2016). The collected wastewaters supplemented with 0.2% glucose was suppled in anode and the Cu2+ concentration of wastewater can be obtained by the portable biosensor.

Meanwhile, control MFC inoculated with wild-type Rosetta was applied to deduct the background differences of mixed samples (Webster et al., 2014). Chemical oxygen demand (COD), dissolved oxygen (DO), NH^{4+}-N and several common heavy metals (Al-Saydeh, El-Naas, & Zaidi, 2017) of the analyzed wastewaters were determined using reported methods (X. Yu et al., 2016; L. Zhou & Boyd, 2016) (Table S2). Next, DDTC-based colorimetry (Uddin, Abdus Salam, & Hossain, 2013), FAAS, and ICP-OES were performed to detect the Cu^{2+} of samples, and the results were compared with that of the constructed biosensor.

3 RESULTS AND DISCUSSION

3.1 Engineering design of Cu^{2+} sentinel strain

The novel gene circuit of engineered *E. coli* Rosetta (sentinel Rosetta) that enable sensing $Cu^{2+}$and generating responsive electricity comprises the following two modules: *ribB* modulated by cognate P_{cusC} and *OprF* controlled by P_{t7} (Figure 1a). P_{cusC} is a Cu^{2+}-dependent induction promoter from the *cus* system (Munson et al., 2000) while *ribB* is derived from the *rib*ABDGHE gene cluster. CusS senses Cu^{2+} in the external environment and relays the signal to CusR, which modulates P_{cusC} to transcribe *ribB*, thereby increasing riboflavin synthesis. *OprF* under the control of P_{t7} encodes a large channel protein that promotes riboflavin diffusion from the outer membrane to the anode electrode. The MFC inoculated with sentinel Rosetta produced limited voltage output without Cu^{2+} stimulation (Figure 1b), but its electricity generation dramatically increased when the *cus* TCS system sensed extracellular Cu^{2+} and activated P_{cusC} to synthesize additional riboflavin (Figure 1c).

The efficiency of EET significantly affects the electricity power generation of electrogens in MFC systems (Logan, 2009; Rabaey & Verstraete, 2005). Riboflavin (vitamin B2), an electron shuttle that can improve MFC performance, is synthesized by GTP through a series of enzymatic reactions (Fischer & Bacher, 2008). Overexpressing *ribB* in *E. coli* leads to the release of more riboflavin (Pedrolli et al., 2015), but engineered Rosetta (R-ribB) with enhanced riboflavin production (8.23 μM) through constitutive expression of *ribB* driven by P_{t7} cannot elevate voltage output of MFC (Figure S3). This is due to the fact that the compact cell membrane of *E. coli* limits the diffusion of riboflavin across the cell membrane (Jing et al., 2012). To overcome the permeable barrier, an aquaporin (OprF) from *Pseudomonas aeruginosa* PAO1, which has the known largest pore diameter (Sugawara, Steiert, Rouhani, & Nikaido, 1996), was introduced to the R-ribB to increase the shuttling efficiency of riboflavin. The results demonstrate co-expressing *ribB* and *OprF* in *E. coli* Rosetta (R-ribB+OprF) significantly improved voltage output of MFC (Figure S3). Therefore, we hypothesized that the riboflavin synthesis level of Cu^{2+} sentinel Rosetta was positively correlated with extracellular Cu^{2+}, and that the voltage output of MFC would also change accordingly.

3.2 Effect of Cu^{2+} on sentinel Rosetta

By fusing P_{cusC} and *gfp* in plasmid pLZ01, the fluorescence changes of reporter Rosetta harboring the above recombinant plasmid revealed this promoter can precisely respond to Cu^{2+} ranging from 0 mM to 1 mM (Figure 2a). This result was in accordance with data showing that the relative fluorescence density of BL21 harboring P_{cusC}-*rfp* plasmid increased 6 fold at 1 mM of Cu^{2+} (Ravikumar, Ganesh, Yoo, & Hong, 2012). Likewise, flow cytometry also substantiated that the number of fluorescent cells was positively correlated with Cu^{2+} concentration (Figure S4). Fluorescence microscopy showed the reporter Rosetta producing distinct green fluorescence when exposed to 0.5 mM Cu^{2+} (Figure 2b). These results corroborated the ability of P_{cusC} to up-regulate

the expression of downstream genes when induced by Cu^{2+}. Subsequently, *ribB* was fused to P_{cusC} and assembled upstream of the constructed module P_{t7}-*OprF* in pLZ01 for constructing a Cu^{2+}-responsive electrogen. The Cu^{2+} sentinel Rosetta produced riboflavin (1.45, 1.84, 2.35, 2.89, 3.56 μM) in a dose-dependent response to Cu^{2+} concentration (0, 0.1, 0.2, 0.3 and 0.5 mM) within 84 h incubation. We note, however, increasing Cu^{2+} to 0.7 mM suspends cell metabolism activity and reduces maximum riboflavin production to 2.76 μM (data not shown). Furthermore, the background riboflavin produced by untreated sentinel Rosetta was slightly higher than WT Rosetta (1.28 μM) due to leaky production of P_{cusC} (Figure 2c). Whole-cell β-galactosidase analysis showed that Rosetta harboring *OprF* exhibited an approximately 4-fold increase in substrate diffusion rate, which essentially agrees with a previous study showing that *OprF* expression increased cell membrane permeability of BL21 by 3.2-fold (Yang-Chun Yong et al., 2013)(Figure 2d). NPN analysis also demonstrated that OprF Rosetta generated higher fluorescence intensity than control strain (Figure S5).

Metal-induced promoter/operator regions such as *mer*-, *znt*-, and *ars*-operons have been widely applied to construct metal biosensors (Y. Zhou et al., 2016). P_{copA} is a *mer*-like induced activation promoter with maximum induction coefficient of only 3-fold after 10 μM Cu^{2+} induction (Kang et al., 2018). In contrast, the quantitative limit of P_{cusC} was shown to be 26 μM, and its highest detected concentration can reach 1 mM (Pedrolli et al., 2015). Electron shuttles such as riboflavin, phenazine-1-carboxamide, and melanin are key factors that mediate EET efficiency and impact voltage output of the MFC system (Boon et al., 2008; Marsili et al., 2008; Turick, Tisa, & Caccavo Jr, 2002). It has been authenticated that 75% of EET to graphite electrode was dependent on the flavin electron shuttle in *S. oneidensis* (Kotloski & Gralnick, 2013). The concentration of riboflavin is positively correlated with voltage generation of electrogen (Yang et al., 2015). Thus, riboflavin can be applied as an ideal mediator for output of electricity signal in sentinel Rosetta. As mentioned before, the outer membrane of *E. coli*, covered in a dense lipolypolysaccharide (LPS) layer, restrains

riboflavin diffusion and limits EET efficiency. Previous research indicated that the porin OprF from *P. aeruginosa* is a non-specific hydrophilic channel protein with an exclusion limit about 5-fold greater than that of general porins of *E. coli* (Bellido, Martin, Siehnel, & Hancock, 1992). Hence introducing the channel protein OprF could improve the signal output and system sensitivity of the Cu^{2+} bioelectrical sensor. The relative expression level of *ribB* and *OprF* in sentinel Rosetta was determined by qRT-PCR, which suggested that the transcription strength of *OprF* increased about 9 times over control, while the expression of *ribB* was related to Cu^{2+} (Figure S6).

3.3 Relationship of Cu^{2+} and voltage output of MFC

The Cu^{2+} sentinel *E. coli* was inoculated into a dual chamber MFC with 300 mL working volume, and its voltage yield increased with addition of copper sulfate in the range of 0-0.5 mM (Figure 3a). Further increasing Cu^{2+} to 0.7 mM reduced the maximum voltage of MFC to 387 mV, indicating that cell growth was inhibited by exposure to Cu^{2+} above 0.5 mM. Voltage generated by the recombinant Rosetta increased beyond that of the control strain after 5 h post-inoculation, and peak voltage appeared at around 10 h, followed by a voltage drop as the carbon source dried up. According to the fitted curve obtained, a significant linear relationship exists between Cu^{2+} concentration and maximum voltage (R^2=0.9772) (Figure 3b). The response time of MFC biosensor was essentially maintained around 5 h, and the toxicity of high-dose Cu^{2+} (0.7 mM) to this engineered Rosetta also exhibited a delay in the response time (about 7 h). Moreover, the amendment of Cu^{2+} concentrations ranging from 0-0.5 mM resulted in an increase of recovery time from 6 h to 20 h, indicating a linear correlation with R^2=0.9526 (Figure 3c).

The Cu-specific biosensor *P. fluorescens* DF57-Cu15 identified by Tn5::luxAB transposon mutagenesis cassette exhibited a linear increase in bioluminescence with Cu concentrations ranging from 0.08 to 8 μM (Nybroe, Brandt, Ibrahim, Tom-Petersen, & Holm, 2008). In the constructed soil bacterium *Achromobacter* sp. AO22 that harnessed P_{copA} to CopR to express lacZ, β-galactosidase

activity was linearly correlated with 0.1-3 mM Cu^{2+} (Ng, Palombo, & Bhave, 2012). Nevertheless, the above output signals of Cu^{2+} biosensors are not suitable for *in situ* monitoring due to unstable fluorescence emission or enzyme activity, which compromise their capacity in practical applications. Similar to current research, an As^{3+} special bioelectricity sensor employing recombinant *S. oneidensis* MR-1 bearing P_{ars}-arsR to drive *mtrB* exhibited reliable current alternation ranging 10 to 40 μA in response to 0 to 100 μM As^{3+} (Webster et al., 2014), demonstrating the application potential of engineered strain in electrochemical biosensor. Another study indicates that increasing Cu^{2+} to 0.69 mM dramatically inhibited the voltage generation of soil organisms in a MFC system, indicating the toxicity of high dose Cu^{2+} to edaphon (Deng, Jiang, Zhou, Shen, & Zhong, 2015). The higher voltage generated by the MFC; the longer time required to restore the baseline. It is consistent with previous research that showed recovery time from 300 mV voltage is about 50 h, while voltage of 100 mV only requires 20 h (D. Wu et al., 2013). CV analysis also indicated that sentinel Rosetta induced by 0.5 mM Cu^{2+} generated more redox substances in anode solution (Figure S7). In summary, these data demonstrate that the amount of Cu^{2+} in the medium can alter the riboflavin generation of bioelectricity-sensing Rosetta and directly relates to electricity production of MFC.

3.4 Optimizing operational parameters of the constructed MFC biosensor

To obtain the best operating parameters for practical application, we tested the electricity generation of constructed MFC that employed biosynthetic Rosetta at different environmental parameters: temperature, pH, and various HMs. To test temperatures, the pH of the medium was fixed at 7.1, and results revealed that the optimal temperature of the biosensor was 37°C with maximum voltage of 245.75 ± 14.06 mV. When the temperature continued to rise to 45°C, voltage generation was dramatically suppressed due to inhibition of bacteria growth (Figure 4a). Subsequently, the specificity of the copper-sensitive Rosetta was investigated by adding different

metals ions (0.2 mM). The voltage variation results showed the constructed MFC sensor (operating temperature was fixed at 37°C and the pH of the medium was fixed at 7.1) was highly responsive to Cu^{2+} and not sensitive to other divalent metals, such as Zn^{2+}, Cd^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Fe^{3+}, Cr^{6+} (Figure 4b). In regard to pH interference, the constructed MFC biosensor (operating temperature was fixed at 37°C) produced the highest voltage (320.94 ± 8.51 mV) at pH 9.12, which was 12.7% and 29.2% higher than that in pH 7.14 and pH 8.03, respectively (Figure 4c). It is intriguing to note that the above result corresponded to the improved riboflavin synthesis levels of recombinant *E. coli* under alkaline conditions (Figure 4d).

The sensing mechanism of the bioelectrical sensor system was based on the response regulation of cells to pollutants; thus, environmental parameters such as pH, temperature or heavy metals significantly affected its voltage output and signal stability. The influence of temperature on MFC voltage output has been described in a study that inoculated a MFC with domestic wastewater, yielding a power density (70 mW m^{-3}) at 30 °C that was 1.3-fold higher than that at 22 °C (43 mW m^{-3}) (Min, Roman, & Angelidaki, 2008). The HM interference test demonstrated the design of sentinel Rosetta targeting Cu^{2+} was the antecedent to fabricating specific bioelectricity sensors. These selected heavy metals are common heavy metals in wastewater (Fu & Wang, 2011) (Z. Chen et al., 2016). As we observe that many regions that suffer from copper contamination are also threatened by antinomy, the concentration of heavy metals in Copper containing wastewater was lower than influence concentration (Fawell & Nieuwenhuijsen, 2003) (Markku J Lehtola et al., 2004; Martin & Calvert, 2003) (Lagos, Maggi, Peters, & Reveco, 1999). On the contrary, if multiple heavy metal contamination is present, the MFC sensor discussed in this study may be used as a multi-analyte sensor. Although more work is needed to solve the problems involving Ni^{2+} and Cr^{6+} interferences within the concentration

range in real time, we believe that the common physiological response of our test strain and control strain is the basis of our proposed control method. The concentration of riboflavin in *S. oneidensis* MR-1 increased from 0.16 μM to 0.37 μM at pH range of 6 to 9, and the MFC voltage also increased from 200 mV to 500 mV, agreeing with what was observed in recombinant Rosetta (Y. C. Yong et al., 2013). It should be noted that the riboflavin content of WT *E. coli* also increased at alkaline pH, but its effect on voltage was almost negligible (data not shown). This also implied that the low permeability of *E. coli* may render it a poor exoelectrogen. Regarding the specific mechanism of pH affecting bacterial riboflavin synthesis, it still requires elucidation through further research. Finally, despite the MFC system generated the highest voltage signal at pH 9, copper precipitation owing to alkaline conditions will affect the bioavailability of the recombinant Rosetta. Therefore, a temperature of 37°C and a pH of 7 were considered as optimized operating parameters for subsequent studies.

3.5 Evaluate the accuracy and stability of the MFC based Cu^{2+} biosensor

Further studies were executed to mimic on spot monitoring by verifying the performance of MFC biosensor under continuous mode. As indicated in the batch experiment, the maximum recovery time of MFC bearing engineered Rosetta was about 20 h within the linear range, thus the cycle interval of continuous operation was set at 24 h. After each cycle, fresh M9 medium and $K_3[Fe(CN)_6]$ were supplied to replace the consumed anode and cathode media, respectively. Scanning electron microscopy (SEM) revealed that a large amount of exoelectrogens were attached to the anode carbon felt after 12 h operation of MFC (Figure S8), so additional inoculation of *E. coli* was not required during the cycles. In the continuous mode, a

linear relationship also can be observed between Cu^{2+} (0-0.5 mM) and maximum voltage (255 mV-405 mV), while increasing Cu^{2+} up to 0.6 mM reduced the electricity generation of MFC (Figure 5a, b). Moreover, continuous addition of 0.2 mM Cu^{2+} within 5 test cycles induced the MFC biosensor to produce a relatively stable voltage signal, with an average maximum voltage of 296.34 ± 9.01 mV (Figure 5d). Compared with batch MFC, the response time and recovery time of MFC in the continuous mode were both decreased, except for in the first cycle. It was speculated that the biofilm formed on the carbon felt accelerated the growth rate of bacteria (Figure 5c, 5f).

A similar result indicated that p-nitrophenol (PNP) at 15-45 mg is linearly correlated to voltage (75-125 mV) generated by MFC harboring *Pseudomonas monteilii* LZU-3 over 5 continuous cycles (Zhengjun Chen et al., 2016). The lowest Cu^{2+} concentration that produce a detectable voltage compare to the background, i.e., the detection limit of the constructed MFC biosensor was calculated by using the average background value plus 3-fold standard deviation was 28.5 μM, which was comparable to the recommended Cu^{2+} limit of 31.5 μM (2 mg L^{-1}) in drinking water (Tapiero, Townsend, & Tew, 2003). Also, the relationship between recovery time and voltage was in accordance with results that showed the recovery time of MFC biosensor shortened with declining voltage (D. Yu, Bai, Zhai, Wang, & Dong, 2017). After running 5 cycles continuously, the detection performance of the constructed biosensor was still reliable, demonstrating its good long-term stability and data repeatability. When measuring 1.03 ± 0.08 g COD L^{-1} d^{-1} in continuous mode, an MFC designed to quickly detect COD exhibited a stable voltage generation with 45 mV maximum voltage (Liu, Liu, Zhang, Xing, & Su, 2011). Although recombinant strain relies on mediated electron transfer, the formation of biofilms is beneficial to the stability of the system and the efficiency of electron transmission (Choi & Chae, 2013). In this study, we had mimicked the continuous monitoring of Cu^{2+} at laboratory scale by replacing the anode and cathode solutions of

MFC biosensor at fixed time (Figure 5). A continuous flow MFC bioreactor had been developed for automatic continuous testing (Webster et al., 2014). Unlike this batch system, the reactor is continuously sampled through the pump system and provided with a growing medium through another inlet. Therefore, it is easy to improve this batch system for continuous monitoring.

3.6 Practical application and cross-validation of analytical methods

The potential of the Cu^{2+}-specific MFC biosensor for onsite monitoring was evaluated by testing three types of actual wastewater in continuous mode and cross-verifying with existing analytical methods. The results showed that the response of constructed MFC biosensor to 0.005, 0.123 and 0.281 mM Cu^{2+} (measured by ICP-OES) generated voltage outputs of 253, 293 and 340 mV, respectively (Figure S9) (background differences are eliminated according to method description). Cross-verification also indicated that there was no significant difference in the determination of Cu^{2+} by colorimetry, FAAS, ICP-OES, and MFC biosensor (Figure 6). The current analytical approaches for water quality monitoring generally require expensive laboratory instruments, elaborate sample preparation and well-trained analysts, limiting their wide use in practical application. Environmental samples usually need to be digested to obtain dissolved Cu^{2+}, so the measured concentration does not accurately reflect the bioavailability of metal ions (Hakkila et al., 2004). By contrast, the MFC biosensor is a cost-effective method to detect wastewater containing Cu^{2+}. Table S3 summarizes the comparison between the MFC biosensor and conventional Cu^{2+} methods in detection range, response time, and system cost. The data suggest that it could provide a real-time monitoring system with direct toxicity assessment and low maintenance cost. Besides, MFC biosensor with lower Cu^{2+} detection limit has been reported (Table S3). Therefore, it is possible to increase the sensitivity of the

constructed MFC biosensor by optimizing the reactor structure, operating conditions or further genetic modification.

We need to emphasize on the 5 hours' response time required by first-generation biosensor system which is comparatively longer than existing detection approaches, whereas considering that the current monitoring programs for environmental risk are limited to filed monitoring and do not support continuous sampling on a daily basis, thus the response time of the biosensor device to the target pollutant is reasonable and adequate. Further, improvements in the MFC configuration could readily shorten the response time of this prototype system. For instance, Sivasankaran Ayyaru et al. reduced the response time of MFC based BOD biosensor using double MEAs-MFC to replace the single MEA-MFC from 200 min to 80 min by optimizing the feeding rate to 0.65 cm^3 min^{-1} (Ayyaru & Dharmalingam, 2014). As MirellaDi Lorenzo described, a 75% smaller MFC reactor only require 40 min to generate steady response signal, while 4 h required for the original biosensor (Di Lorenzo, Curtis, Head, & Scott, 2009). The signal of MFC biosensor is not only sensitive to Cu^{2+}, but also affected by the characteristics of the stream to be monitored. A pertinent issue is the electrical current recorded by the MFC sensor will be misestimated due to a sudden change in organic strength of wastewater (Chang et al., 2004; Di Lorenzo et al., 2009). The electrical signals of ideally constructed MFC biosensor should only respond to Cu^{2+} and not be affected by non-toxic events. Tan et al. demonstrated that the addition of acetate and glucose could improve robustness of MFC biosensor against organic strength fluctuations for Cu^{2+} detection (Tan et al., 2018). Another approach is to reduce the impact of environmental fluctuations by using control groups (Webster et al., 2014). In this study, therefore, wastewater samples were

supplemented with 2% glucose and a control reactor inoculated with wild type Rosetta was set up to evaluate the background signal of wastewaters.

Albeit *S. oneidensis* MR-1 or *P. aeruginosa* PAO1 are most common model microorganisms in electrochemical studies (Rahimnejad, Adhami, Darvari, Zirepour, & Oh, 2015), their high background signals potentially affect the sensitivity of MFC biosensor. Thus, *E. coli* Rosetta, a poor exoelectrogen, was employed in this study to verify the design concept. The results illustrated that the P_{cusC}-driven MFC biosensor can specifically sense Cu^{2+} and meets the information requirements of water quality supervision. Whereas, the optimal temperature of 37°C for Rosetta strain does not match the ambient temperature, so the next work of this research is to seek an appropriate chassis microorganism. Alternatively, genetic modification to knock out the endogenous redox medium of *S. oneidensis* MR-1 (optimum temperature 30 °C) may also reduce its background signal, making it an ideal host for MFC biosensor (Okamoto, Nakamura, & Hashimoto, 2011).

The level of copper in surface and groundwater is generally low, while high levels of copper can be occasionally observed in some polluted wastewaters (Gokcekus et al., 2003; Markku J. Lehtola et al., 2004). The biosensor has a wide detection range, so it can be applied to different pollution sites. But the operating pH and temperature of MFC reactor need to be kept constant in field application. Therefore, we believe that the biosensor developed in this study is more suitable for drinking water monitoring in water distribution centers. Some studies have also s indicate copper levels in drinking water ranging from 0.005 to 30 mg L^{-1} (WHO, 2004), meeting the upper detection limit (31.5 mg L^{-1}) of this biosensor. One the other hand, a number of developing countries still lack water supply facilities or infrastructure to provide safe drinking water (Cosgrove & Loucks, 2015; Daud et al., 2017; Marin, 2009). This constructed biosensor provides a simple and convenient

copper monitoring method for water environment early warning in underdeveloped areas.

In short, although further exploration is clearly needed to optimize the field application capability of the Cu^{2+} prototype biosensor, the substantial progress obtained in this study lays a foundation for the specific detection of Cu^{2+} by MFC system in water environment.

4 CONCLUSIONS

A copper responsive exoelectrogen was constructed by corporately expressing *ribB* and *oprF* with the promoters P_{cusC} and P_{t7}, respectively in *E. coli* Rosetta. In the presence of Cu^{2+}, P_{cusC} was activated and promoted the synthesis of riboflavin. With the assistance of *OprF* encoded porin, riboflavin was released into the extracellular membrane and increased the voltage production of MFC. MFC biosensor was then constructed by employing a copper-specific recombinant exoelectrogen. A linear relationship between voltage generation of MFC biosensor and Cu^{2+} level (0.1-0.5 mM) was then established, and its specificity and repeatability were tested. The detectable limit of this constructed MFC biosensor in water was 28.5 μM，which conforms to the recommended safety standard of Cu^{2+} in drinking water (31 μM). Therefore, the biosensor could be used as an early warning device to ensure the safety of drinking water for residents in cities or remote areas. In conclusion, this study provides an economical, sensitive and practical technology for the detection of Cu^{2+} in drinking water, and its commercial application prospect remains to be further explored.

AUTHOR COMTRIBUTIONS

Tuoyu Zhou： Writing – Original Draft, Visualization, Conceptualization, Investigation. **Rong Li：** Investigation, Formal Analysis**. Shuting Zhang：** Investigation, Data Curation. **Shuai Zhao:**

Resources, Investigation. **Monika Sharma and Apurva kakade:** Writing- Reviewing. **Saurabh Kulshrestha:** Conception and Editing. **Aman Khan:** Software, Methodology. **Huawen Han:** Conceptualization, Methodology. **Yongyan Niu:** Resources**,** Investigation**. Xiangkai Li:** Writing-Reviewing and Editing, Supervision, Project Administration, Funding Acquisition.

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURES

Figure 1. Schematic illustration of signal-responsive regulatory units in Cu^{2+} sentinel *E. coli*. **(a)** Genetic circuit of expressed plasmid that harnessed P_{cusC} to regulate *ribB* and P_{t7} to express *OprF*. **(b)** Without the presence of Cu^{2+}, the engineered *E. coli* secreted limited riboflavin and the voltage of MFC remained in a low state. **(c)** P_{cusC} sensed Cu^{2+} and yielded extra riboflavin, thus increasing the electron transfer from bacteria to anode electrode.

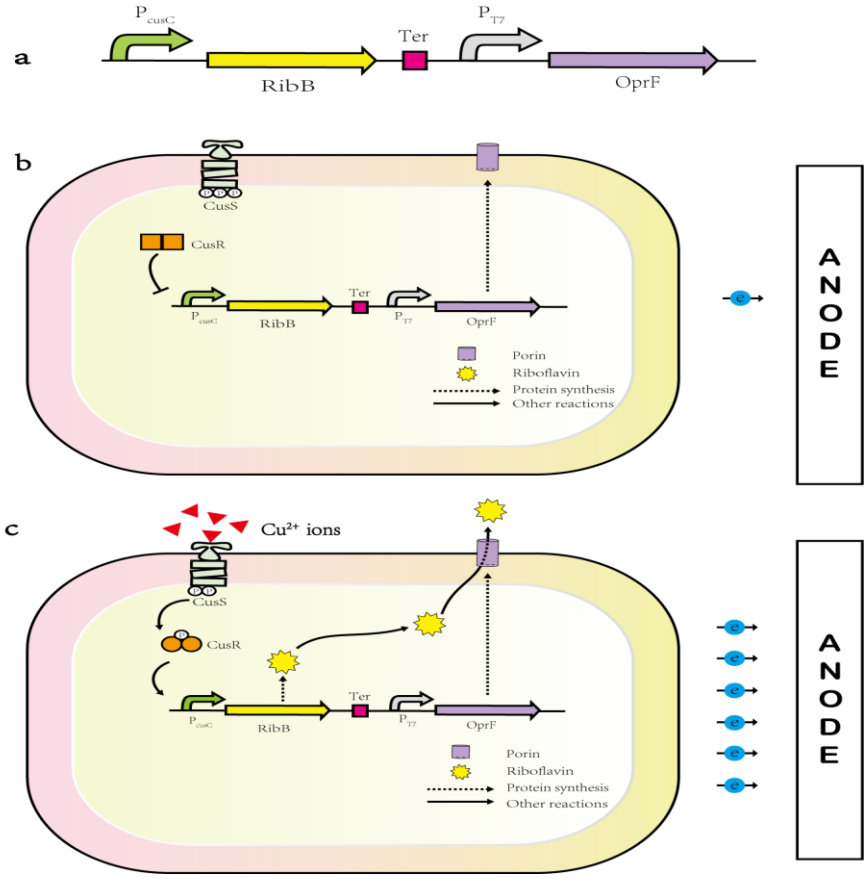

Figure 2. (a) *E. coli* Rosetta containing reporter plasmid pLZ01-P_{cusC}-*gfp* was incubated with various $CuSO_4$ for 4h to determine the concentration dependence of P_{cusC} to Cu^{2+}. **(b)** Fluorescence microscopy image of above reporter Rosetta and control exposed to 0.5 mM Cu^{2+}. Time-course kinetics of **(c)** riboflavin yield and **(d)** membrane permeability in sentinel Rosetta induced by different Cu^{2+}. Error bars represent the standard deviation of three independent replications.

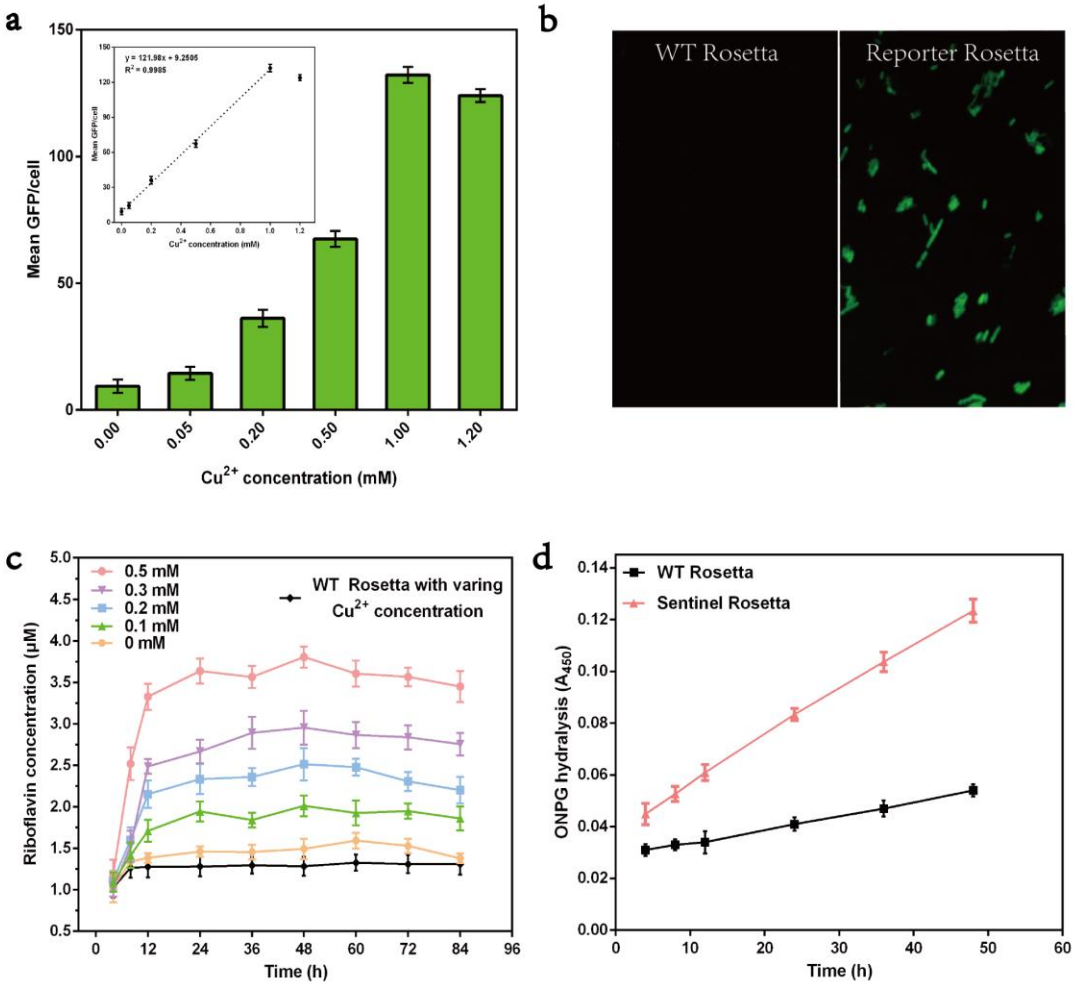

Figure 3. Influence of Cu^{2+} on electricity yield of sentinel Rosetta in the MFC system. **(a)** Time course of voltage generation with different Cu^{2+} treatment. The correlation between **(b)** maximum voltages and Cu^{2+} concentrations. The **(c)** response time **(d)** and recovery time of MFC were analyzed at different Cu^{2+}. Error bars represent the standard deviation of three technical replications.

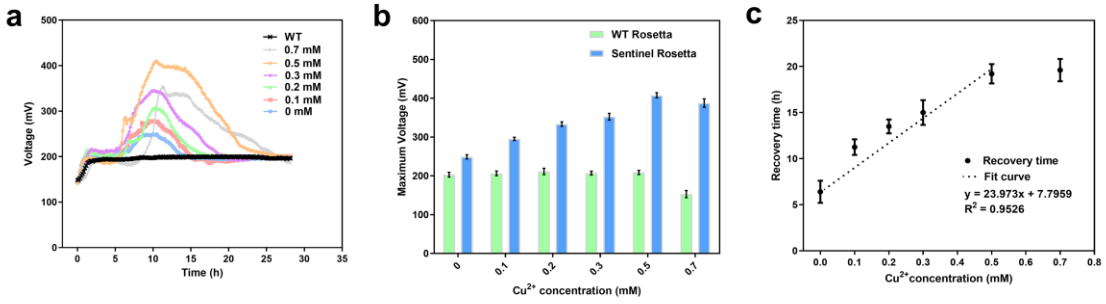

Figure 4. MFC voltage generation under varying conditions. Effects of different **(a)** temperatures, **(b)** heavy metals and **(c)** pH on voltage generation. **(d)** riboflavin production of sentinel Rosetta under different pH. Error bars represent the standard deviation of triplicate independent tests.

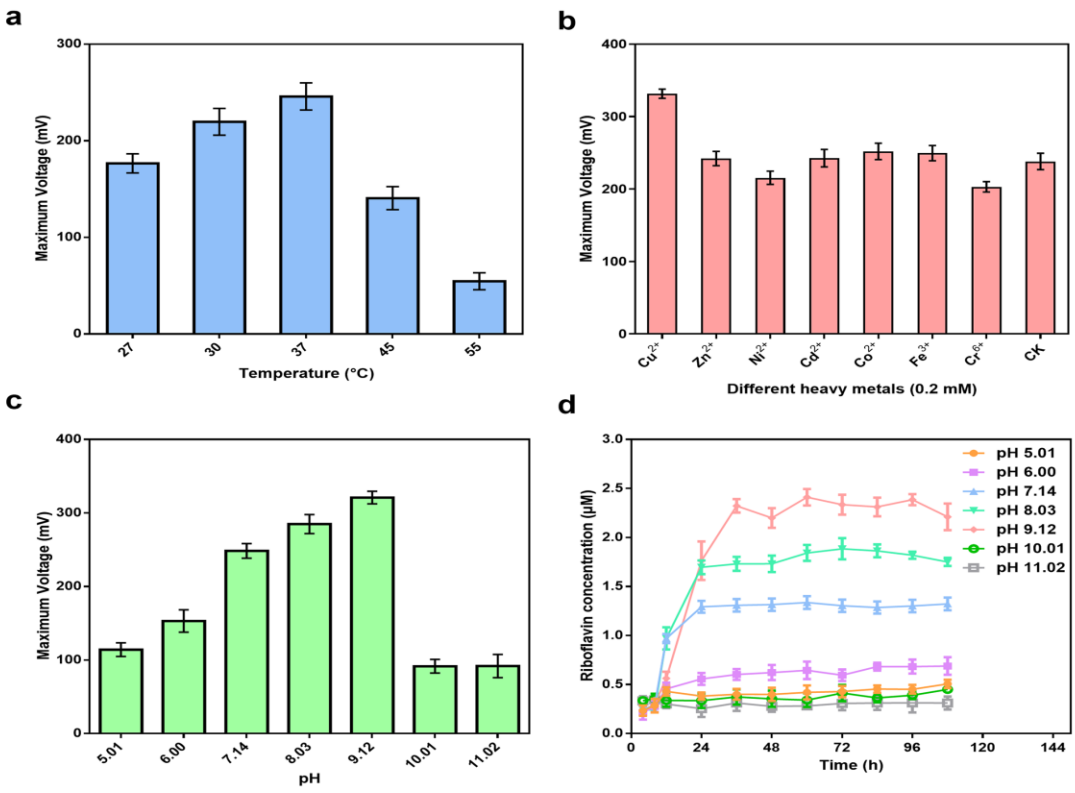

Figure 5. The performance of MFC biosensor in continuous mode. **(a)** Influence of various Cu^{2+} on voltage generation of MFC; linear relationship between **(b)** maximum voltage, **(c)** response time, recovery time, and Cu^{2+} concentration. **(d)** Voltage output in the MFC with 0.2 mM Cu^{2+} over 5 cycles. (**e**) Maximum voltage, **(f)** response time and recovery time of MFC biosensor. The arrows indicate the time point to simultaneously replace both catholyte and anolyte solutions. Error bars represent the standard deviations of triplicated independent tests.

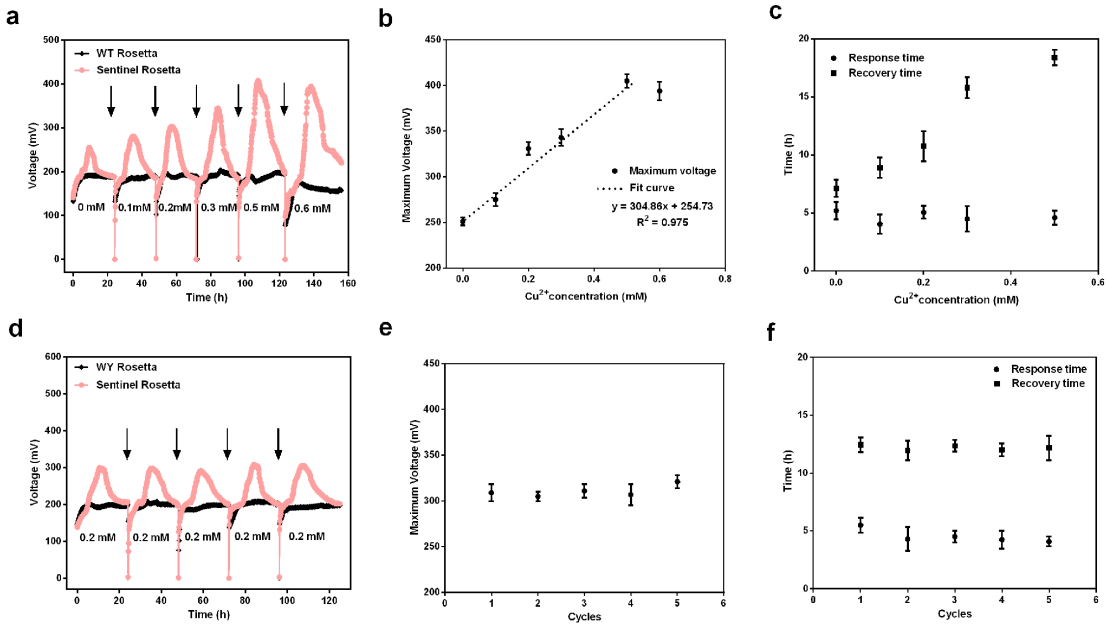

Figure 6. Three wastewater samples were cross-verified by employing colorimetry, ICP-OES, FAAS, and biosensor. DW: Domestic wastewater; PW: printing and dyeing wastewater; EW: electroplating effluent.