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COMMUNICATION

# Biological Anolyte Regeneration System for Redox Flow Batteries

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**Redox flow battery (RFB) electrolyte degradation is a common failure mechanism in RFBs. We report an RFB using genetically engineered, phenazine-producing Escherichia coli to serve as an anolyte regeneration system capable of repairing the degraded/decomposed redox-active phenazines. This work represents a new strategy for improving the stability of RFB systems because, under the influence of genetically engineered microbes, the anolyte species does not display degradation after battery cycling.**

Redox flow batteries (RFBs) represent a promising solution to grid-scale energy storage owing to their scalability provided by the intrinsic decoupling of power output and energy storage.[1] In these systems, charge storage species are solubilized in electrolyte solutions, which flow through an electrochemical cell for charging and discharging.[2] In this context, redox-active small organic molecules (ROMs) have shown promise as charge storage species for RFB applications due to their earth-abundant elemental composition and the ability to tailor the redox potentials, among other physical properties.[3] However, the chemical instability of ROMs limits their practical application in commercial RFB devices. Namely, electrolyte species are subjugated to changes in potential during charging/discharging cycles, resulting in the degradation/decomposition of electrolyte species over time. Previous research has reported ROM degradation pathways in both non-aqueous and aqueous RFBs utilizing various ROM structures, including phenazines, flavins, quinones, pyridiniums, viologens, and TEMPO ((2,2,6,6-tetramethylpiperidin-1-yl)oxyl).[2, 4, 5] The degradation of these species can be inhibited and/or prevented via (1) consideration of the electrolyte, (2) consideration of RFB cycling conditions, and (3) rational design of new robust ROMs with enhanced cycling stability.[6, 7] Although a few research studies have investigated the degradation mechanisms for various groups of ROMs with different substituents, another principal approach is to develop systems capable of RFB electrolyte generation where the electrolytes are either charge-balanced or filtered and then replaced with new cycling molecules (e.g., RFBs based on $V^{2+}/V^{3+}$ and $VO^{2+}/VO_2^+$ couples).[8] Specifically, electrolyte regeneration can be achieved through periodically mixing symmetrical electrolyte solutions in both half-cells, thereby providing the ability to restore capacity[9-11] (note that this approach is not an option with the commonly utilized asymmetric batteries as mixing the anolyte and catholyte induces crossover and short-circuiting). However, the in-situ regeneration of ROMs has not yet been demonstrated. Herein, we demonstrate the design of an RFB system where ROMs are continually regenerated by genetically engineered microorganisms to maintain RFB cycling stability.

Microorganisms secrete different ROMs via several inter- and intra-cellular pathways, including extracellular electron transfer for cellular respiration processes and cell-to-cell signaling mechanisms.[12, 13] The redox-active small organic molecule (ROM) species have been investigated as electron shuttles (e.g., mediators) in various microbial electrochemical technologies, including microbial fuel cells and microbial electrosynthesis,[12] because microbes can communicate with conductive electrode surfaces and/or oxidize/reduce substrates of interest. In this work, we genetically engineered and employed a phenazine-producing *Escherichia coli* strain as an anolyte species regeneration system. Key enzymes for phenazine biosynthesis native to *Pseudomonas aeruginosa* were genetically expressed in the model microorganism *E. coli* to produce redox-active phenazine metabolites. This biosynthetic pathway (Fig. 1A) consists of a seven-gene biosynthetic cluster *phzA-G* responsible for the synthesis of major product, phenazine-1-carboxylic acid (PCA), and two accessory genes *phzM* and *phzS* responsible for pyocyanin (PYO) production. Phenazines are small redox-active nitrogen-containing heterocyclic compounds, which have been recognized for their ability to engage in redox cycling processes.[14, 15] Given their small sizes and similar physiological properties, phenazines can facilitate electron transfer and transfer content from inside the cell to the extracellular environment.[16] The minor differences in functional groups surrounding the phenazine core structure impact the redox activity of each species. We introduced the core biosynthetic genes encoding

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Electronic Supplementary Information (ESI) available: [Experimental methods and details, mass spectrometry data of cellular supernatant, redox flow battery testing results for the PCA/FcN setup without the genetically engineered *E. coli* cells in the anolyte tank, and redox flow battery testing results for the PCA/FcN without PCA in the anolyte tank]. See DOI: 10.1039/x0xx00000x

various terminal-modifying enzymes in *E. coli*, resulting in the production of various phenazine metabolites. The phenazine biosynthetic pathway begins with chorismic acid, which yields PCA via the biosynthetic enzymes *phzA-G*. In the following steps, involving enzyme *phzM* and monooxygenase *phzS*, PCA is converted to PYO. These biological pathways allow for degradation products of the phenazines to be regenerated via the bacteria. Researchers have previously shown phenazines as ROMs with stable cycling.[17] This work demonstrates a significant proof-of-concept RFB cell setup utilizing the genetically engineered, phenazine-producing *E. coli* as an anolyte regeneration system with the ability to repair the degraded/decomposed phenazines, producing a longer-lasting battery with more stable phenazine-based electrolyte species.

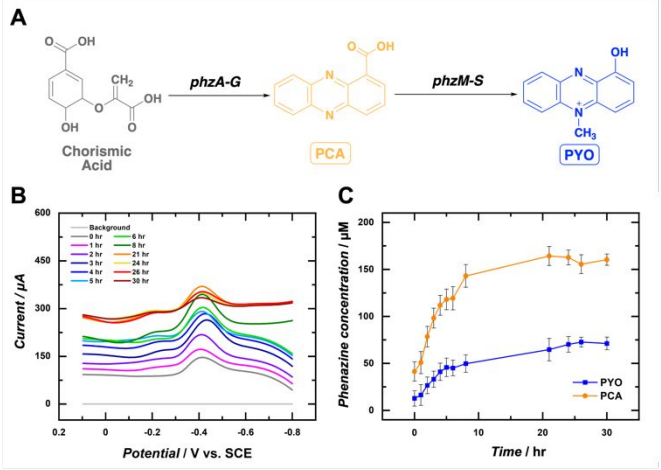


**Fig. 1** Time-dependent electrochemical data to monitor phenazine production in engineered *E. coli phzAG-SM* strain. (A) Phenazine biosynthetic pathway from *P. aeruginosa* was introduced into the model microorganism *E. coli*. The genetically engineered, phenazine-producing E. *coli* strain was constructed via DNA manipulations, nucleotide sequencing, plasmid recombination operation, and transformations. The arrows in the schematic denote the enzymes acting as catalysts for the shown conversion reactions, namely *phzA-G* for the conversion of chorismic acid to phenazine-1-carboxylic acid (PCA), and *phzS-M* for the conversion of PCA to pyocyanin (PYO). The major phenazine product produced by the genetically engineered *E. coli phzAG-SM* is PCA. (B) Time-dependent square wave voltammetry data showing phenazine production in engineered *E. coli phzAG-SM*. The minor peak around -0.209 V vs. SCE is due to PYO redox, while the major peak and PCA around -0.398 V vs. SCE is due to PCA redox. (C) Phenazine production of PCA and PYO in engineered *E. coli phzAG-SM* strain over time was determined using square wave voltammetry data and calibration curves (not shown). For electrochemical measurements, shown are the average of 4 replicate measurements, and the data in (b) shows the average values with error bars from n=4 experiments. A 20 mM MOPS buffer (pH 7) + 10 mM MgCl2 + 100 mM Glucose was used as electrolyte solution (background) in electrochemical tests. A frequency of 15 Hz was used in square wave voltammetric studies.

First, the production of phenazine metabolites in the engineered *E. coli phzAG-SM* strain was electrochemically evaluated (Fig. 1). Fig. 1B shows time-dependent square-wave voltammetry data, over 30 hours, obtained at different times of cell growth after cell immobilization on the carbon paper electrode surface in a 20 mM MOPS + 10 mM $MgCl_2$ + 100 mM glucose buffer (pH 7). The shown current-potential voltammetric curves are averages of four replicate experiments with PYO and PCA showing their redox potential signatures at -0.209 V and -0.398 V vs. SCE, respectively.[13, 18] Measurements were taken every hour for the first 6 hours, followed by every 2 hours, followed by 13 hours, and finally followed by 3 hours of cellular and biofilm growth on the electrode surfaces. The voltammetric data shows increasing peak currents for PCA and PYO over time corresponding to phenazine production by the engineered *E. coli* cells. This cellular phenazine production plateaus after 21 hours of cell immobilization and growth on the electrode surfaces. Using the square-wave voltammetric data and our obtained calibration curves for PYO and PCA (calibration data not shown), the phenazine production for PYO and PCA produced by the engineered *E. coli phzAG-SM* cells was quantitatively determined (Fig. 1C). The determined PYO and PCA concentrations fall in the µM concentration range as previously determined to be produced in *P. aeruginosa*.[13, 18] However, the types of phenazines and the relative µM quantities differ in *E. coli*. Specifically, *P. aeruginosa* produces PYO in larger micromolar amounts than PCA. However, correlated to oxygen reactivity, PYO is highly toxic to *E. coli* cells under oxic (e.g., oxygen-containing) conditions. Namely, PYO toxicity has been attributed to its ability to rapidly reduce molecular oxygen, generating superoxide species, which produce hydrogen peroxide via enzymatic or abiotic dismutation redox processes. Therefore, it is likely that the engineered *E. coli* cells metabolically regulate PYO production to avoid the toxicity effects of this phenazine metabolite. It should also be noted that the carbon electrodes with immobilized *E. coli* cells show a distinct increase in capacitance, indicative of biofilm formation on the electrode surfaces (Fig. 1B). Previous research works have suggested that increased capacitive currents result from an increase of the active biomass in the bioelectrochemical system due to accumulation of cellular biofilm on the electrode surface.[19-21] The identity of PCA and PYO metabolites produced by *E. coli* was confirmed using mass spectrometry (MS) analysis of the cell supernatant samples (Fig. S1, ESI).

The *E. coli phzAG-SM* strain not only excretes its own phenazine metabolites, but the bacteria also intakes phenazine species present in the solution that are consequently repaired through microbial metabolism, thus increasing the phenazine production rates. Time-dependent cyclic voltammetry (CV) experiments (Fig. 2) established evidence of the possibility of using the genetically engineered *E. coli phzAG-SM* to provide a feedstock of phenazine species for RFBs. Namely, the CV data show increases in the current peak magnitude indicative of increasing PCA concentrations during 1 hour of testing. Voltammetric studies were performed utilizing PCA in the anolyte tank as this is the major phenazine product secreted by the genetically engineered *E. coli*. Experiments with the second phenazine product, PYO, were also attempted, which demonstrated decreasing PYO amounts in solution over time, resulting from the toxic effects exhibited by PYO on *E. coli* growth rates over a longer time period, which our research group has reported on previously.[22, 23] The data in Fig. 2 shows that the engineered, phenazine-producing *E. coli* cells can tolerate higher concentrations (greater than or equal to 250 µM) of exogenously added PCA, which would result in toxicity effects and cell death in non-engineered *E. coli* cells, as described in previous research work.[22, 23] It is important to note that the microbes are supplied with glucose to perform metabolism and are resistant to the extreme environments present during RFB cycling (e.g., high salt concentrations). Nonetheless, the voltammetric data in Fig. 1 indicate that the genetically engineered microorganisms, capable of producing phenazine metabolites, have long-term stability because the *E. coli* cells have biosynthetic and repair pathways, resulting in the generation and re-generation of phenazine species.

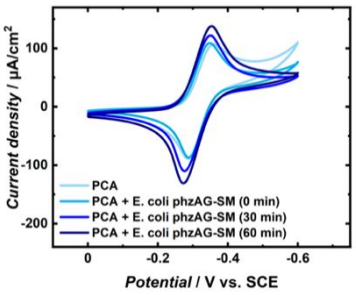


**Fig. 2** Time-dependent cyclic voltammetric responses of phenazine ROMs, namely PCA, generated from genetically modified *E. coli phzAG-SM* microbes immobilized on AvCarb carbon paper electrodes. Enhancement in the current density correlates to an increase in the phenazine concentrations in solution over time.

A zero-gap RFB cell was modified to function as an aqueous microbial-based RFB, employing PCA as the major naturally occurring phenazine metabolite as the anolyte with (ferrocenylmethyl)trimethylammonium (FcN) as a well-characterized aqueous-based RFB catholyte species (Fig. S1 and Fig. S2, ESI). Given the low PCA concentration tolerance of the microbes, a 500 µM PCA in a 20 mM MOPS (pH 7) + 10 mM $MgCl_2$ + 200 mM glucose buffer was used for redox-active species in the anolyte tank. A 1000 µM FcN solution was utilized in the catholyte tank. Fig. 3 shows the RFB cycling data for the PCA/*E. coli phzAG-SM*/FcN system. The charge and discharge currents applied were 0.2 mA and –0.1 mA, respectively. A low current was necessary to completely charge and discharge the anolyte and catholyte species. Higher charging and discharging current rates (1 mA and –0.5 mA, respectively) were attempted. However, the results indicated that the electrolyte species were not completely charging and/or accessing the charged states, typically displayed by a color change (Fig. S3, ESI). The genetically engineered *E. coli* can survive a battery experiment for a total of 10 battery cycles, and repair damaged anolyte species in the process. The pre- and post-cycling cyclic voltammograms for PCA (Fig. 3A) and FcN (Fig. 3B) show how the electrolyte species change over the course of the battery cycling. These results demonstrate that the PCA anolyte does not degrade throughout the battery cycling experiment. Although the pre- and post-cycling PCA anolyte CV results show a slight decrease in the peak current after cycling, new phenazine-related peaks are not observed (Fig. 3A). The potential shift of the PCA peak is due to a pH change in the solutions throughout the experiment (pH of PCA anolyte was 7.03 pre-cycling and 6 post-cycling), which has been observed previously with phenazine species.[13] The pre-cycling FcN catholyte CV retained its characteristic peak after cycling, as evidenced by the post-cycling data (Fig. 3B). This RFB cell was cycled for 10 cycles (Fig. 3C) with a reasonable capacity and efficiency retention (Fig. 3D-E). The cycling data show (1) a Coulombic efficiency of 70%, and (2) an average of 91% capacity retention per cycle and 51% total capacity retention through 10 charge/discharge cycles (Fig. 3E).

Control experiments were performed with (1) 500 µM PCA only (Fig. S4, ESI), and (2) genetically engineered *E. coli* only (Fig. S5, ESI) in the anolyte tank. The results in Fig. S4 (ESI) indicate that the PCA anolyte degrades throughout the battery cycling experiment when *E. coli phzAG-SM* cells are not present. The peak current of the post-cycling PCA anolyte CV does not only show a decrease in the concentration of PCA anolyte species but also the formation of a second CV peak in the post-cycling CV analysis, indicating that PCA undergoes a degradation mechanism. The structural identity of the secondary redox species in CV data remains elusive as evidenced by post-cycling MS analysis (Fig. S6, ESI). MS data of the post-cycling PCA anolyte with *E. coli phzAG-SM* shows a higher relative abundance of PCA compared to the anolyte without engineered cells. Compared to the results in Fig. 3C-E, the cycling data for this control experiment with 500 µM PCA only in the anolyte tank show comparable Coulombic efficiency of 71%, and higher total capacity retention of 59% through 10 charge/discharge cycles with a 94% average capacity retention per cycle (Fig. S4C-E, ESI). Although the anolyte containing *E. coli phzAG-SM* cells can regenerate PCA, the RFB electrochemical performance with regard to the Coulombic efficiency and capacity retention is partly reduced. Specifically, in addition to phenazines, the engineered *E. coli phzAG-SM* are metabolically secreting a wide range of other biological metabolites, which can polymerize on the RFB carbon-based electrodes, causing electrode fouling that leads to reduced capacity retention. Fig. S5 (ESI) shows pre- and post-cycling CVs and RFB cycling data of a system without the PCA anolyte to show that there is negligible electrochemical influence from the MOPS buffer solution and the *E. coli phzAG-SM* cells. There is a minor peak in the cyclic voltammogram of the buffer solution resulting from nanomolar-micromolar concentrations of PCA produced by the engineered microbes. In comparison to the data in Fig. 3 and Fig. S5 (ESI), the cycling data for this control experiment with *E. coli phzAG-SM* cells only demonstrate lower Coulombic efficiency of 61%, and lower total capacity retention of 50% through 10 charge/discharge cycles with an average of 92% capacity retention per cycle (Fig. S5C-E, ESI). While PCA does not boast the best potential for an anolyte and is not the most stable anolyte species for an efficient and productive battery, these results are extremely promising given that under the influence of genetically engineered phenazine-producing *E. coli* cells, the phenazine anolyte species does not show degradation after battery cycling while also demonstrating a reasonable capacity and efficiency retention throughout 10 charge/discharge cycles.

In summary, this work demonstrates the possibility of using genetically engineered phenazine-producing *E. coli* as an anolyte regeneration system with the ability to repair degraded/decomposed phenazines in an aqueous-based RFB system for ten charge/discharge cycles. To the best of our knowledge, this is the first RFB system using phenazine-producing microbes as an anolyte regeneration factory. The inclusion of microbes in RFBs inherently presents multiple challenges, including the salinity of the supporting electrolyte, high concentration of radicals in solution, and typically hypoxic environments, which do not include typical microbe substrate sources. Future characterization of the post-cycling anolyte and catholyte, as well as an understanding of how the buffer interacts with the ROM species during electrochemical cycling will be needed. Although future work on further engineering and optimization of this microbial-based RFB system is necessary, the RFB concept presented here is an important milestone toward the rational design of RFBs with improved charge storage capabilities.

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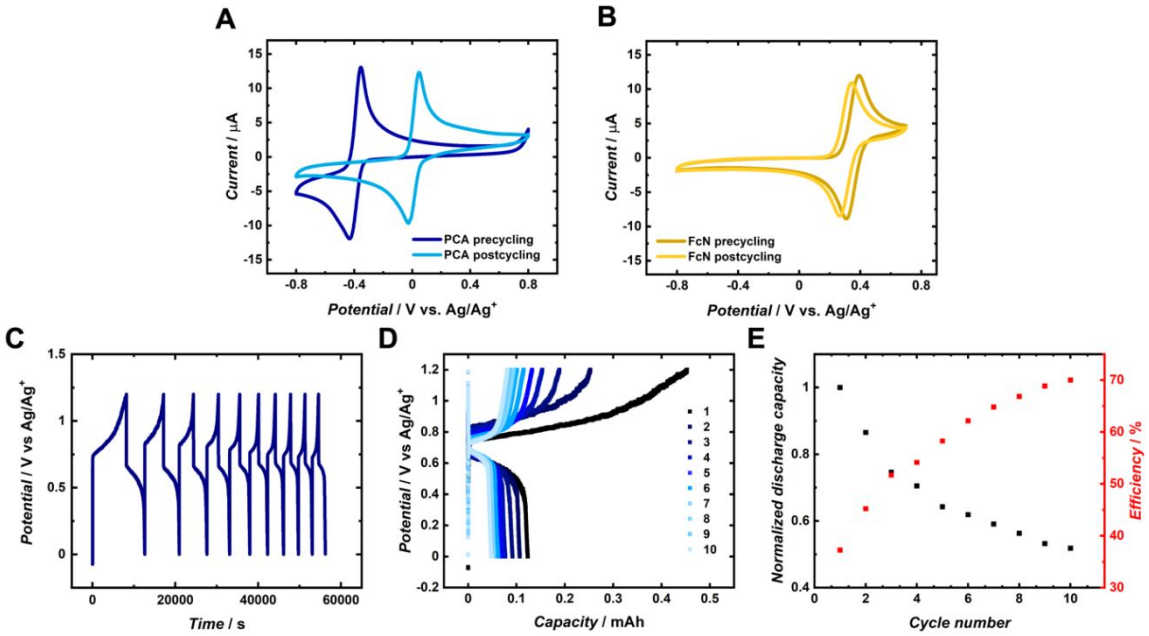


**Fig. 3** Results for a redox flow battery (RFB) system with 500 µM phenazine-1-carboxylic acid (PCA) with *E. coli phzAG-SM* cells as the anolyte and 1000 µM (ferrocenylmethyl)trimethylammonium (FcN) as the catholyte, in 20 mM MOPS buffer (pH 7) + 10 mM $MgCl_2$ + 200 mM glucose. Pre- and post-battery cycling cyclic voltammograms for (A) PCA anolyte and (B) FcN catholyte solutions. (C) RFB cycling data and (D) charge/discharge curves, for 10 cycles. (E) Normalized discharge capacity for the PCA/FcN RFB cell and cell cycling Coulombic efficiency.

## Author Contributions

S.D.M. and O.S. conceived the study, designed tests, and planned experiments. O.S. performed genetic engineering of cells and prepared cell cultures, and performed microbe testing, electrochemical measurements, data analysis, and characterization. Z.R. modified the RFB cell design and performed initial studies with pyocyanin as the anolyte. E.C. and K.N.P. completed RFB cycling experiments. E.C. and O.S. performed pre-/post-cycling analyses. All authors contributed to writing and editing the manuscript.

## Conflicts of interest

There are no conflicts to declare.

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