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Electrochemical Recycling of Adenosine Triphosphate in Biocatalytic Reaction Cascades

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Cite This: J. Am. Chem. Soc. 2022, 144, 22582−22588
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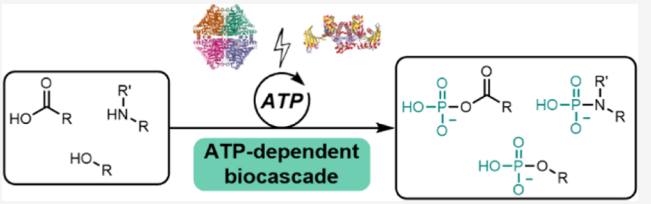
ABSTRACT: Adenosine triphosphate (ATP) provides the driving force necessary for critical biological functions in all living organisms. In synthetic biocatalytic reactions, this cofactor is recycled *in situ* using high-energy stoichiometric reagents, an approach that generates waste and poses challenges with enzyme stability. On the other hand, an electrochemical recycling system would use electrons as a convenient source of energy. We report a method that uses electricity to turn over enzymes for ATP generation in a simplified cellular respiration mimic. The method is simple, robust, and scalable, as well as broadly applicable to complex enzymatic processes including a four-enzyme biocatalytic cascade in the synthesis of the antiviral molnupiravir.



INTRODUCTION

Adenosine triphosphate (ATP) is one of the most ubiquitous cofactors in biology as it provides the energy to drive many fundamental metabolic processes in all living organisms.[1] Many synthetic biochemical systems used for biomass conversion, artificial photosynthesis, and fine chemical or pharmaceutical production also rely on ATP to provide the necessary thermodynamic driving force to access phosphorylated intermediates and necessitate an ATP production or recycling strategy.[2−5] Living organisms use complex processes to produce ATP, such as photosynthesis or cellular respiration (Figure 1A), which are difficult to be reproduced artificially.[6] While simpler cofactor recycling systems have therefore been developed to access the diverse chemistry catalyzed by ATP-dependent enzymes,[7,8] these systems are driven by high-energy stoichiometric sacrificial phosphate donors (*i.e.*, acetyl phosphate or polyphosphate)[8,9] (Figure 1B). These approaches not only compromise the cost, and atom economy, but also potentially impact the enzyme performance.[10,11] Given that electricity is the cheapest alternative energy source and the lowest carbon footprint when produced from renewable sources,[12] here, we report a robust and scalable method to drive the recycling of the ubiquitous cofactor ATP electrochemically (Figure 1C). In this system, pyruvate is oxidized to CO_2 to produce ATP, effectively providing a simplified electrochemical mimic of the cellular respiration process. We demonstrate the versatility and generality of the electrochemical approach in diverse ATP-dependent kinase-catalyzed transformations, including a complex biocatalytic cascade to access a precursor of the COVID-19 antiviral therapeutic molnupiravir.[13]

The inspiration for bioelectrochemical ATP recycling originated from a previously reported recycling system that relies on the oxidative decarboxylative phosphorylation of pyruvate with O_2. That system was catalyzed by the enzyme pyruvate oxidase (PO) to give acetyl phosphate, which, in turn, was converted to ATP and acetate by acetate kinase (AcK) (Scheme 1).[13] However, the use of oxygen as a terminal oxidant for PO has several limitations, specifically, the need to add catalase to avoid the accumulation of H_2O_2, the byproduct of O_2 reduction. Furthermore, using O_2 requires engineering considerations to ensure the efficient mass transfer of O_2 from the gas to liquid phase and to avoid safety issues.[14,15] We therefore envisioned replacing O_2 with electrons from an electric current in the PO/AcK recycling system. The mechanism of PO has been investigated and consists of a complex sequential mechanism that involves the two cofactors thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD). While TPP serves as a nucleophile to activate and decarboxylate pyruvate, FAD serves as a transient electron sink in the reduced form $FADH_2$ before being reoxidized to FAD by O_2.[16] We hypothesized that the two-electron oxidation of $FADH_2$ in PO could be driven electrochemically instead of aerobically.[17−22]

Received: August 22, 2022
Published: November 30, 2022

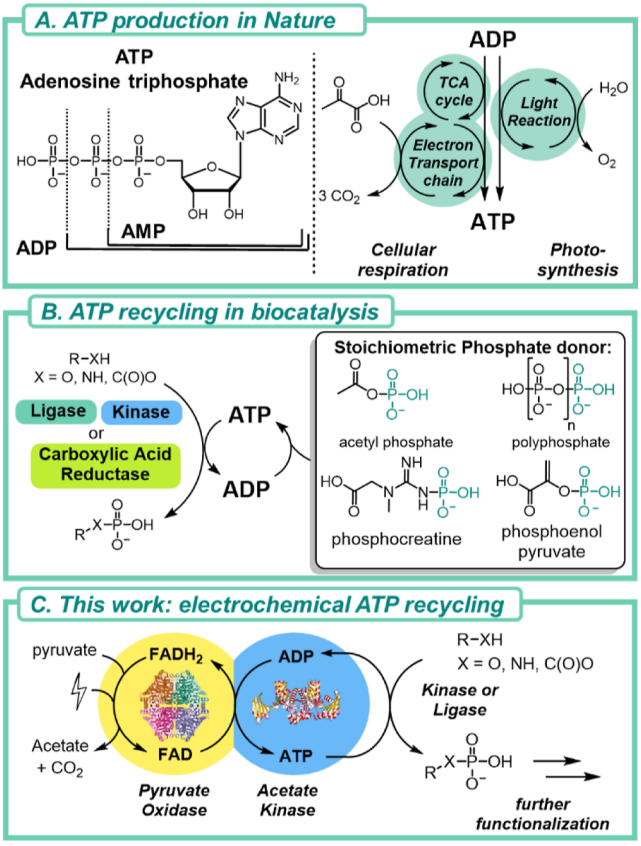
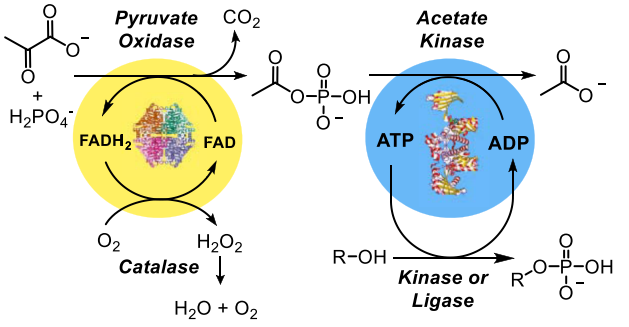


Figure 1. (A) Major pathways for ATP production in nature. (B) ATP recycling systems in biocatalysis and common stoichiometric phosphate donors. (C) This work: bioelectrochemical ATP recycling. The lightning icon represents the electrochemical input.

Scheme 1. Aerobic Recycling of ATP



RESULTS AND DISCUSSION

We initiated this study by exploring methods to turn over PO electrochemically. Direct electron transfer between enzymes and electrodes is not trivial because most enzymes have their active redox cofactor buried in the protein structure.[23,24] The use of a redox mediator that shuttles electrons between the electrode and the enzyme active site can alleviate some limitations.[25,26] Cyclic voltammetry (CV) of PO in the presence of the substrate shows no redox events (Figure 2), confirming that the direct electron transfer between the electrode and the enzyme is not observed under these conditions. The CV curves of redox mediators such as ferrocenemethanol (FcMeOH) show a reversible wave in the presence of pyruvate and phosphate, thereby confirming that the mediator cannot oxidize pyruvate on its own. However, in

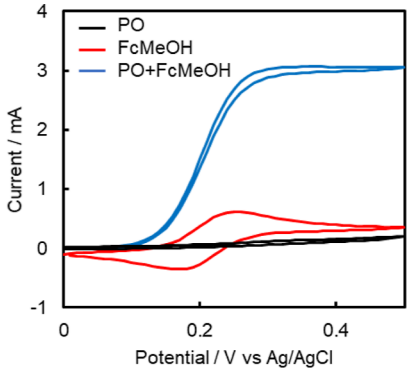


Figure 2. CV curves of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 0.2 mM FcMeOH: (red —). CV curves of 0.5 M sodium pyruvate, 0.5 M KPi, 10 mM $MgCl_2$, 1 mM TPP, and 5 μM PO, without (black —) and with (blue —) 0.2 mM FcMeOH. Scan rate: 5 mV·s^{-1}.

the presence of PO, a catalytic S-shaped curve is observed, indicating the turnover of the enzyme by the mediator (Figure 2). Using the formalism introduced by Saveant *et al.*,[27] the rate constant for the oxidation of PO by the oxidized FcMeOH can be extracted, giving a value of 1.0×10^5 M^{-1} s^{-1}, similar to the previously reported value for FcMeOH (1.5×10^5 M^{-1} s^{-1}).[28] Interestingly, this rate is also similar to that previously measured for the oxidation of PO with molecular O_2 (1.7×10^5 M^{-1} s^{-1}),[16,28] suggesting that the turnover of PO by a redox mediator is possible at competitive rates. When PO is depleted of FAD, we observe a decrease in the oxidation rate (Table 1, entry 11), which suggests that the mediator oxidizes $FADH_2$ and not TPP (Scheme 2). Within the ranges studied, the observed oxidation rate is independent of mediator oxidation potential as similar rates are observed with mediatorswith oxidation potentials ranging from 0.25 to 0.48 V *versus* Ag/AgCl. Similarly, the rate has a very shallow dependance on pH (Table 1). Therefore, the proton-coupled electron transfer between $FADH_2$ and redox mediators is not rate-limiting. Instead, the formation of a precursor complex between the oxidant and the enzyme is likely rate-limiting, as observed with the electrochemical turnover of glucose oxidase.[27]

We therefore propose the following mechanism for the electrochemical recycling of ATP: (1) as expected from the known mechanism of PO,[16] pyruvate undergoes oxidative decarboxylative phosphorylation catalyzed by PO to generate acetyl phosphate, while the FAD cofactor in PO is reduced to $FADH_2$; (2) the FcMeOH mediator is oxidized at the anode; (3) two $[FcMeOH]^+$ oxidize $FADH_2$ in PO to give FAD by two single-electron transfer reactions, thereby producing two FcMeOH and two protons; (4) at the cathode, the two protons are reduced to produce hydrogen, thereby maintaining a constant pH; (5) as in the O_2 system, acetyl phosphate is used by acetate kinase to phosphorylate ADP, thereby generating ATP and acetate; and (6) ATP is available for an ATP-dependent enzyme for further reactions (Scheme 2).

With this analytical data in hand, we began the development of the stoichiometric phosphorylation of ADP to ATP using the electrochemical turnover of PO. First, redox mediators, including ferrocene derivatives, *N*-oxyls, triphenylamines, quinones, and metal complexes, were screened with the HT*e*⁻Chem platform.[29] ATP formation was observed in the presence of several mediators, with the highest conversion

https://doi.org/10.1021/jacs.2c08955
J. Am. Chem. Soc. 2022, 144, 22582−22588

Scheme 2. Proposed Mechanism for the Oxygen-Free Electrochemical Turnover of PO for ATP Recycling

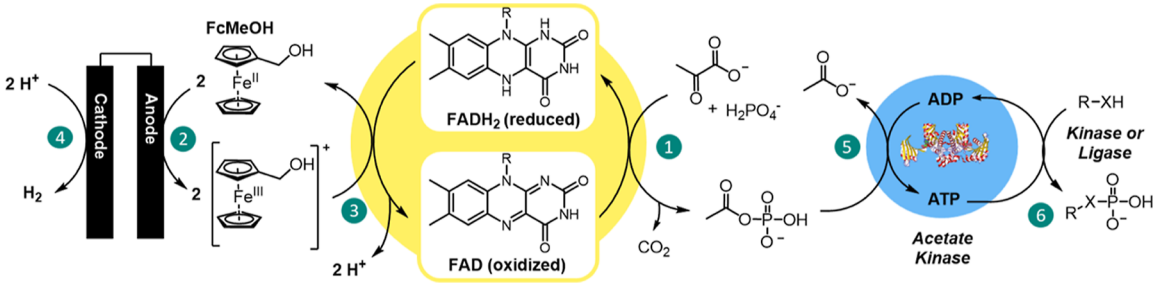
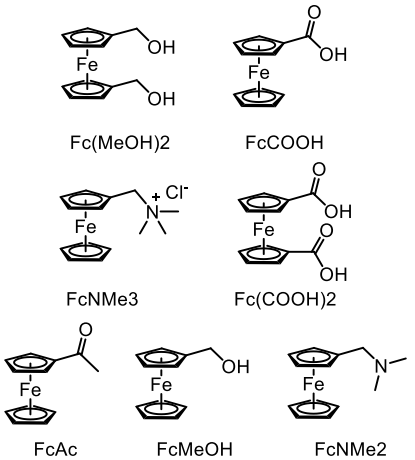


Table 1. Redox Potential (*vs* Ag/AgCl) and Rate Constants for the Different Mediators



mediator	E^0 (V)	pH	k (M^{-1} s^{-1})
$Fc(MeOH)_2$	0.25	6	1.16 (±0.3) × 10^5
FcCOOH	0.31	6	1.52 (±0.1) × 10^5
$FcNMe_2$	0.36	6	2.21 (±0.3) × 10^4
$FcNMe_3$	0.43	6	7.4 (±0.3) × 10^4
$Fc(COOH)_2$	0.45	6	1.79 (±0.1) × 10^5
FcAc	0.48	6	9.00 (±3.4) × 10^5
FcMeOH	0.22	6	1.02 (±0.1) × 10^5
FcMeOH	0.22	6.5	1.19 (±0.1) × 10^5
FcMeOH	0.22	7	1.64 (±0.2) × 10^5
FcMeOH	0.22	7.5	1.7 (±0.2) × 10^5
FcMeOH (0.76:1 FAD/PO)	0.22	6	4.16 (±0.4) × 10^4

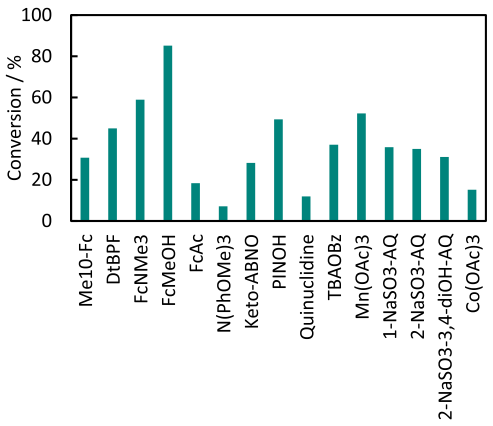
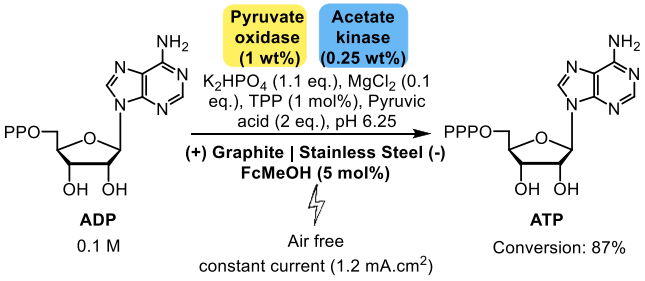


Figure 3. Mediator screening results for the bioelectrochemical stoichiometric ATP formation (see Supporting Information for abbreviations).

Scheme 3. Bioelectrochemical Stoichiometric ATP Formation[a]



[a]P symbolizes PO_3^-.

measured when employing FcMeOH (Figure 3). Upon scale-up, 87% conversion of 8 mmol of ADP to ATP was achieved using FcMeOH as the mediator at 30 mA current at 1.2 mA/cm^2 for 20 h in an undivided cell consisting of graphite rod anodes and stainless steel cathodes under a N_2 atmosphere (Scheme 3). A control reaction in the absence of electrochemical current led to 21% conversion to ATP and 22% adenosine monophosphate (AMP), most likely originating from ADP disproportionation catalyzed by trace amounts of adenylate kinase in the cell lysate.[30] Interestingly, during the mediator screen, some experiments showed lower activity than the adenylate kinase background, which we speculate could come from enzyme deactivation or inhibition by oxidized mediators. In the absence of mediators, a conversion of only 11% was achieved, while the absence of PO led to 10% conversion, thereby highlighting the necessity of both components to efficiently generate ATP and demonstrating that the mediator specifically turns over PO and not AcK (Table 2). When O_2 was used as the oxidant instead of the electrochemical current, a conversion of only 33% was observed (9% AMP), indicating that the conversion seen in the bioelectrochemical method does not originate from either adventitious or electrochemically generated O_2 and that the accumulation of H_2O_2 is deleterious to the reaction, as no catalase was added to remove H_2O_2.

The highest conversions were achieved at a pH of 6.25 (Table 2), close to the midpoint between the optimal pH of 5.7 for PO[31] and 7.4 for AcK.[32] The real-time measurement of the potential of the anode showed a stable voltage over the course of the constant-current reaction at ∼0.35 V *versus* Ag/AgCl (∼1.15 V total cell potential), which is close to the oxidation potential of FcMeOH. When the maximum conversion was achieved, the potential rose sharply by ∼1 V to reach the water oxidation potential (Figure S23). The Faradaic efficiency was constant throughout the reaction at 74% (Figure S22), which is remarkably high compared to other

https://doi.org/10.1021/jacs.2c08955
J. Am. Chem. Soc. 2022, 144, 22582−22588

Table 2. Results for the Bioelectrochemical Stoichiometric ATP Formation under Different Control Conditions and pH

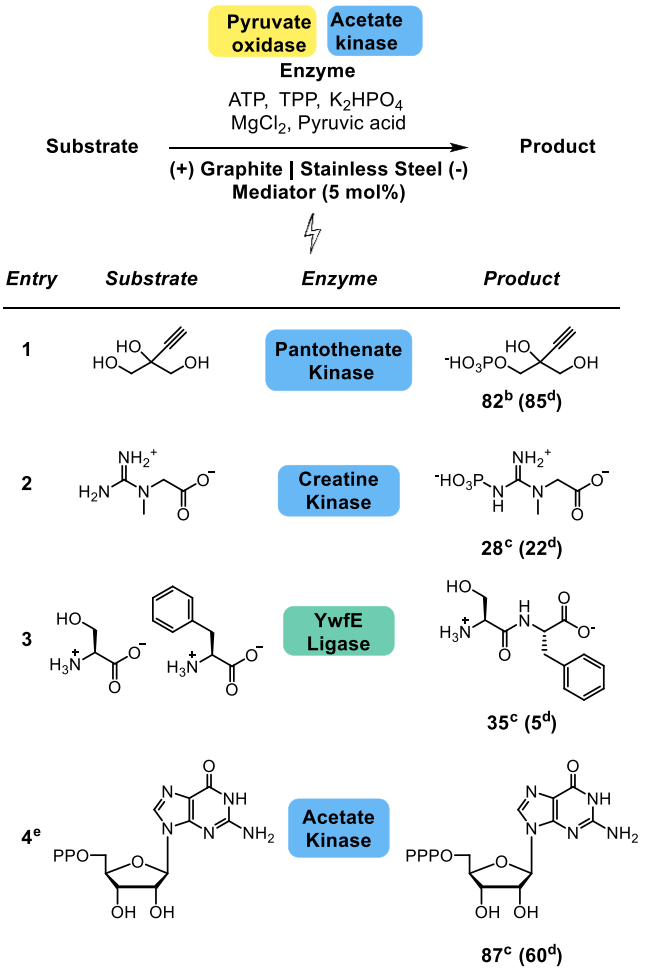
conditions	ATP conversion (%)	AMP conversion (%)
standard	87.3	1.3
no mediator	11.1	12.6
no PO	10.7	14.0
no current	21.5	24.6
with air, no catalase	33.9	9.4
pH 5	0.3	4.9
pH 6	81.7	4.9
pH 7	67.3	9.1
pH 8	64.8	10.3

mediated bioelectrochemical systems.[19] Notably, the electrodes required for this system are inexpensive, earth-abundant, and reusable graphite and stainless steel electrodes, which further contribute to the process sustainability by avoiding the use of precious metals.

We then explored the scope of the bioelectrochemical ATP recycling in preparative biotransformations that rely on an ATP-dependent kinase or ligase enzymes (Scheme 4). First, we investigated the phosphorylation of 2-ethynylglycerol using pantothenate kinase (PanK), a key step in the synthesis of the HIV therapeutic islatravir, which uses a stoichiometric amount of propionyl phosphate to recycle ATP.[10] Under bioelectrochemical conditions and in the presence of stoichiometric phosphate, 2-ethynylglycerol was phosphorylated to produce (*S*)-1-phosphate-2-ethynylglycerol in 82% conversion using *N*,*N*,*N*-trimethyltrimethyl-1-ferrocenylmethanaminium chloride ($FcNMe_3$) as the mediator (Scheme 4, entry 1). Second, we explored the bioelectrochemical phosphorylation of creatine to phosphocreatine using creatine phosphokinase. This transformation is thermodynamically higher by 3 kcal/mol, with ATP as the phosphate source,[33] and is typically used in the reverse direction, with phosphocreatine providing the driving force for ATP formation. We were therefore pleased to observe a 28% yield of phosphocreatine, with FcMeOH as the mediator (Scheme 4, entry 2) for this challenging uphill phosphorylation. Then, we probed ATP-grasp ligases, a promising class of enzymes which catalyze amide couplings between two amino acids and have been used to synthesize complex peptides in the drug discovery space.[5] Under bioelectrochemical conditions, a member of this enzyme family, namely YwfE,[34] formed the L-serine-L-phenylalanine dipeptide in 35% conversion using FcMeOH as the mediator (Scheme 4, entry 3). Finally, we demonstrated that the PO/AcK bioelectrochemical recycling system is not limited to adenine-based nucleotide recycling, as guanosine-5′-diphosphate was successfully phosphorylated into guanosine-5′-triphosphate (GTP) in 87% yield using FcMeOH as the mediator (Scheme 4, entry 4). This further expands the scope of biocatalytic transformations possible with this method as several biological reactions rely solely on GTP hydrolysis as a driving force rather than ATP, the most notable example being protein synthesis in ribosomes.[35] Interestingly, in all cases, the bioelectrochemical recycling system performs either similarly or better than O_2 and catalase as the driving force for PO turnover.

We then investigated the use of this strategy as a cofactor recycling system in a complex ATP-dependent biocatalytic cascade, the second step of the recently reported molnupiravir

Scheme 4. Reaction Scope for the Bioelectrochemical ATP Recycling[a]

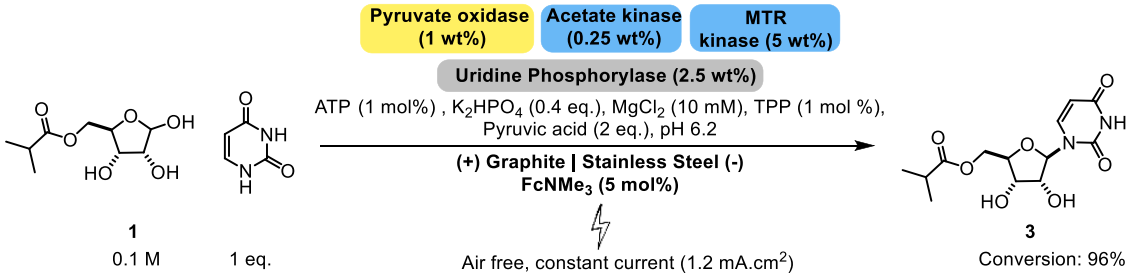


[a]For details on the conditions for individual reactions, see Supporting Information. [b]Mediator: $FcNMe_3$. [c]Mediator: FcMeOH. [d]Conversion for the reaction performed with O_2 and catalase and in the absence of current. [e]No ATP was present in this reaction.

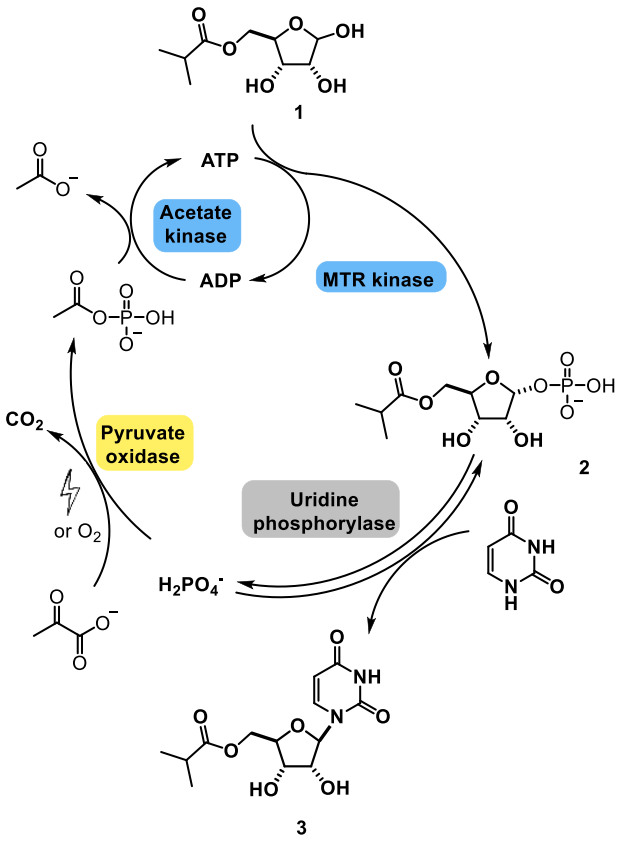
synthesis (Scheme 5).[13] The reaction consists of a four-enzyme biocatalytic cascade that installs uracil on 5-isobutylribose **1** to form the nucleoside 5-isobutyluridine **3** *via* the 5-isobutylribose-1-phosphate intermediate **2** (Scheme 6). A mediator screen showed that $FcNMe_3$ provided higher conversion than other mediators (Figure S24) under bioelectrochemical ATP recycling conditions. Upon scale-up, 96% conversion of 8.2 mmol of **3** was achieved using 30 mA current under anaerobic conditions (Scheme 5). Running the reaction in the absence of the mediator, PO, or electrical current led to the conversion of only 1−2%, confirming the need of all three components for the productive reaction. When the reaction was performed with O_2 instead of an electrochemical current, a conversion of only 12% was observed (Table 3). During electrolysis, the voltage remained constant at ∼0.5 V *versus* Ag/AgCl (∼1.4 V total cell potential) until the maximum conversion was reached, after which the potential rose sharply. Similar to the stoichiometric ADP phosphorylation, the Faradaic efficiency during the reaction was stable at an average of 83% (Figures 4 and S27). Finally, it is crucial to maintain current values below the maximum rate of the downstream enzymatic reactions; otherwise a shortage of inorganic phosphate will occur, leading

https://doi.org/10.1021/jacs.2c08955
J. Am. Chem. Soc. 2022, 144, 22582−22588

Scheme 5. Bioelectrochemical Glycosylation Using Electrochemical ATP Recycling



Scheme 6. Reaction Mechanism for the Bioelectrochemical Glycosylation



to an accumulation of oxidized $FcNMe_3$, rising potential, and enzymatic failure due to overoxidation. Indeed, we observed that the rates of delivery of electron equivalents relative to substrates higher than 0.14 $F{\cdot}mol^{-1}{\cdot}h^{-1}$ showed significant drops in the conversion (Tables S5 and S6 and Figure S25). Similar conversions and efficiencies were observed in batch-type reactors as in flow reactors with continuous parallel plate electrodes at identical current densities (Figure 5).[36] Using a flow cell with a working electrode surface area of 256 cm^2 allowed the execution of the reaction at a 22.5 g scale with respect to **1** at a current of 0.373 A, which reached 94% conversion (Figure S30). The productivity for this reaction is 1.1 g/L/h with an average Faradaic efficiency of 76% (Figure S31) at a current density of 1.5 mA/cm^2.

In conclusion, our bioelectrochemical anaerobic respiration system can be used as the driving force for recycling ATP, an energy source that is ubiquitous in living organisms, instead of high-energy phosphate sources or stoichiometric oxidants. Optimal conversions are achieved if the current is kept

Table 3. Results for the Bioelectrochemical Glycosylation under Different Control Conditions, pH, and Currents

conditions	conversion (%)
standard	96.0
no mediator	2.0
no PO	1.3
no electricity	1.4
under air, no catalase	12.1
pH 4	0.3
pH 5	3.1
pH 6	86.8
pH 7	61.9
pH 8	87.2
2 mA	73.2
2.4 mA	62.2
3 mA	57.6
3.5 mA	50.3
4 mA	49.1
5 mA	39.4

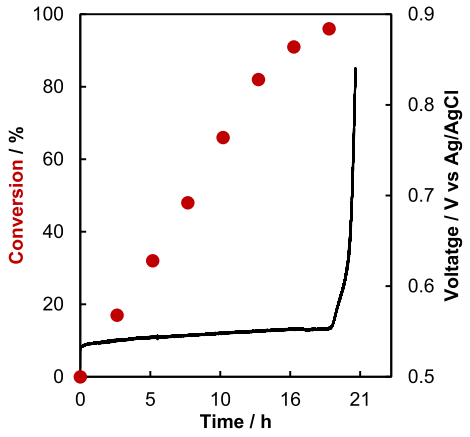


Figure 4. Reaction profile for the 8.2 mmol scale of the bioelectrochemical glycosylation. (Black —) voltage in V *vs* Ag/AgCl, (red ●) uracil conversion in %.

sufficiently high to drive catalysis but sufficiently low to not outpace downstream enzymatic, highlighting the delicate interplay of these multienzyme systems and the corresponding tuneability achievable using electrochemistry. This method was demonstrated on a >20 g scale and was used to successfully drive biocatalytic cascades based on applications of phosphorylation chemistry. Looking forward, we believe that the use of bioelectrochemistry is poised to expand, owing to the wealth and diversity of both aerobic and anaerobic redox biotransformations that could theoretically be driven electrochemically. To that end, achieving a higher performance will be

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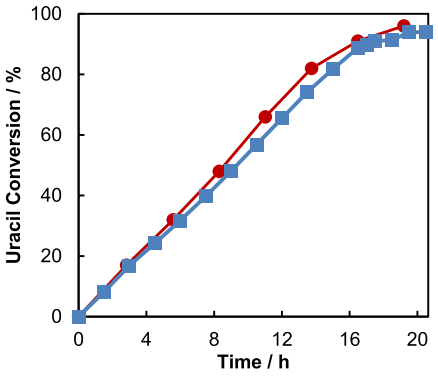

Figure 5. Overlay of the reaction profiles for 8.2 mmol (red ●) and 102 mmol (blue ■) scale of the bioelectrochemical glycosylation processes.

crucial and will require the optimization of the electrochemical cell design as well as improvements in enzymatic activity through directed evolution, opening the door for exciting applications such as green production of commodity chemicals, bioplastics, biofuels, and pharmaceuticals alike.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c08955.

Chemicals, materials, and methods; spectroscopic and electrochemical studies and data analysis; experimental procedures for bioelectrochemical reactions including photographs of reactors; and enzyme acquisition, preparation, and amino acid and DNA sequences (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the following people: Joshua Kolev, Cindy Hong, John McIntosh, Hao Yang, and Christopher Prier for providing enzymes and reagents; Ania Fryszkowska, Rebecca Ruck, François Lévesque, Dan Lehnherr, Patrick Fier, Zachary Dance, Matthew Winston, and Aaron Whittaker for manuscript review. Kate Maldjian is thanked for help with graphics.

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https://doi.org/10.1021/jacs.2c08955
J. Am. Chem. Soc. 2022, 144, 22582−22588