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Bio-electrocatalytic Alkene Reduction Using Ene-Reductases with Methyl Viologen as Electron Mediator

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Asymmetric hydrogenation of alkene moieties is important for the synthesis of chiral molecules, but achieving high stereoselectivity remains a challenge. Biocatalysis using ene-reductases (EReds) offers a viable solution. However, the need for NAD(P)H cofactors limits large-scale applications. Here, we explored an electrochemical alternative for recycling flavin-containing EReds using methyl viologen as a mediator. For this, we built a bio-electrocatalytic setup with an H-type glass reactor cell, proton exchange membrane, and carbon cloth electrode. Experimental results confirm the mediator's electrochemical reduction and enzymatic consumption. Optimization showed increased product concentration at longer reaction times with better reproducibility within 4–6 h. We tested two enzymes, Pentaerythritol Tetranitrate Reductase (PETNR) and the Thermostable Old Yellow Enzyme (TOYE), using different alkene substrates. TOYE showed higher productivity for the reduction of 2-cyclohexen-1-one (1.20 mM h^{-1}), 2-methyl-2-cyclohexen-1-one (1.40 mM h^{-1}) and 2-methyl-2-pentanal (0.40 mM h^{-1}), with enantiomeric excesses ranging from 11% to 99%. PETNR outperformed TOYE in terms of enantioselectivity for the reduction of 2-methyl-2-pentanal (ee 59% ± 7% (*S*)). Notably, TOYE achieved promising results also in reducing ketoisophorone, a challenging substrate, with similar enantiomeric excess compared to published values using NADH.

Introduction

Asymmetric hydrogenation of alkenes plays a crucial role in the synthesis of active pharmaceutical ingredients (APIs), flavor and fragrances, and fine chemicals.[1] Examples include Tipranavir,[2] Sitagliptin,[3] and Pregabalin.[4] However, one main challenge with these hydrogenation reactions is reaching high stereoselectivity.[1] In this context, biocatalytic methods using ene-reductases (EReds) are especially promising.[5] For instance, the so-called Thermostable Old Yellow Enzyme (TOYE) was reported to be stable up to 70 °C, a property that is of interest for applicability in the industry.[6] EReds from the Old Yellow Enzymes (OYE) family contain the flavin mononucleotide (FMN) cofactor to catalyze the asymmetric reduction of an activated alkene substrate. In nature, the catalytic mechanism consists in a double-displacement (ping-pong) reaction, in which the FMN cofactor must first be reduced at the expense of a molecule of nicotinamide adenine dinucleotide or its phosphate analogue (NADH or NADPH). After the reduction step of FMN, NAD^+ (or $NADP^+$) is released and the activated alkene substrate can enter the active site and be subsequently reduced.[5a,b]

Using EReds on a large-scale is currently limited by the need for a reducing agent like the NAD(P)H cofactor, because adding this in stoichiometric amounts is prohibitively expensive. This can be solved by applying NAD(P)H in catalytic amount and recycling it *in situ* with D-glucose dehydrogenase/D-glucose or formate dehydrogenase/formate (the so-called coupled-enzyme approach).[7] Recycling of NAD(P)H or flavin cofactors can also be done using biomimetic,[8] photocatalytic,[9] and electrochemical methods.[10]

Biomimetics are synthetic analogs of the natural NAD(P)H cofactor, designed to be structurally simpler. They are typically used in stoichiometric or suprastoichiometric amounts,[11] though engineered enzymes or artificial metalloenzymes can be employed to recycle biomimetics in catalytic quantities.[12] Photocatalytic methods use a photovoltaic material to transfer electrons to the NAD(P)H for recycling.[9] Theoretically, for electrochemistry, the sole requisite is an electrode with optimal electrical conductivity to facilitate the regeneration of the reduced state of the enzymes.[13] The electrochemical system is also simpler, facilitating product purification. The drawback is that while $NAD(P)^+$ can be directly reduced by carbon or metal electrodes, this can give undesired dimerization and over-reduction due to intermediate radical formation (Scheme 1).

These side reactions can cause rapid deactivation of $NAD(P)^+$, lowering the overall efficiency. One solution is replacing the NAD(P)H with another molecule that can reduce the FMN.[8b,12a] Recently, methyl viologen dichloride (MV^{2+}) in its reduced radical form ($MV^{+\bullet}$) was shown to be a promising

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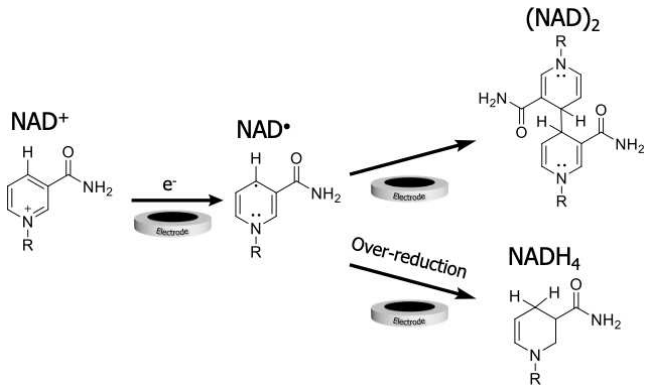
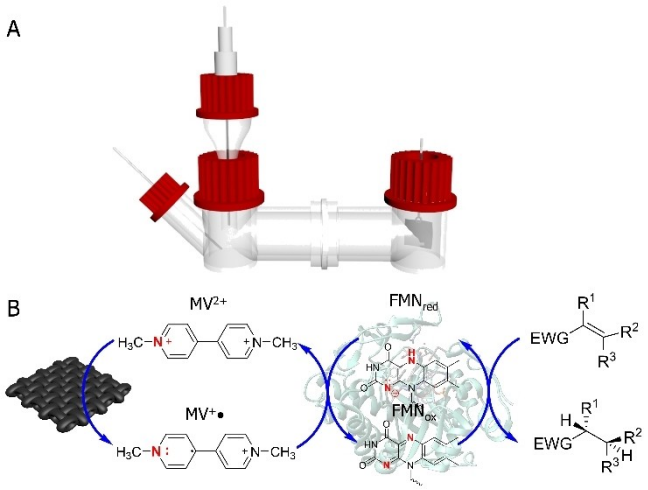


Scheme 1. The direct electrochemical reduction of NAD^+ to NADH proceeds via the $NAD^•$, but this radical can also dimerize to $(NAD)_2$. Over-reduction to $NADH_4$, which cannot be accepted by EReds, is also observed. The adenine dinucleotide substituents are denoted as "R" groups for clarity.



Scheme 2. The system using MV^{2+} as a mediator molecule for bio-electrocatalytic reaction. (a) The design of the electrochemical cell. (b) Schematic of the bio-electrocatalytic process.

candidate for this reaction thanks to its similarity to NAD(P)H and lower redox potential compared to FMN.[14] Importantly, EReds can accept reduced methyl viologen as the hydrogen donor, facilitating the transfer of both electrons and hydrogen to the FMN bound in the active site.[9] The MV^{2+} cation is then readily reduced again at the cathode to give the methyl viologen radical $MV^{+•}$, with no need for additional catalysts, and no side products formation.[15]

Here, we combine the formal biocatalytic hydrogenation reaction using an ERed with the electrochemical regeneration of methyl viologen, thereby closing the catalytic cycle without using the NAD(P)H cofactor. We then demonstrate the system's validity in bio-electrocatalytic asymmetric reduction of activated alkenes using two EReds, namely TOYE[6] and Pentaerythritol Tetranitrate Reductase (PETNR).[16]

Results and Discussion

Design and Construction of the Asymmetric Hydrogenation Bio-Electrocatalytic System

To maintain a stable cathodic reduction process in bio-electrocatalytic reactions, we employed an H-type glass reactor cell as a standard reaction system. A proton exchange membrane separated the two chambers, and a flexible carbon cloth electrode was chosen as the working electrode to enhance the surface area. Under constant potential conditions, TOYE[6] and PETNR[16] served as the formal hydrogen (i.e., H^- and H^+ added to the flavin cofactor) acceptor enzymes, thereby catalyzing the reduction of C=C bonds. The substrate's conversion rate and enantiomeric excess demonstrated the significant potential of this system in bio-electrocatalytic asymmetric hydrogenation.

To segregate the reaction chambers into cathodic and anodic sections, we built an H-type glass reactor setup (Scheme 2A). The two chambers were separated with an activated Nafion® 117 proton exchange membrane. In the anodic part, platinum, acting as the counter electrode, generated protons. These protons crossed the membrane to the cathodic chamber.

Upon introducing the substrate into the cathodic chamber, the reaction process run as shown in Scheme 2B. MV^{2+} is reduced at the cathode, generating the oxygen-sensitive radical $MV^{+•}$. This radical serves as the electron mediator, enabling the transfer of electrons from the electrode to the FMN within the enzyme. Simultaneously, protons formally originating from the anode in the adjacent chamber reach the active site along with the substrate, enabling asymmetric hydrogenation within the enzyme scaffold.

Electrochemical Analysis of the Asymmetric Hydrogenation System

The reduction of MV^{2+} under negative potential occurs in two distinct steps.[17] Initially, MV^{2+} is reduced to the $MV^{+•}$ radical under a lower negative potential. Then, as the potential increases, the $MV^{+•}$ radical is reduced further to MV^0. Notably, EReds exclusively recognize $MV^{+•}$ as the mediator molecule, inducing asymmetric hydrogenation. To validate the capability of $MV^{+•}$ in facilitating electron transfer to enzymes, we ran cyclic voltammetry (CV) experiments on a solution containing MV^{2+}, PETNR as enzyme, and 2-cyclohexen-1-one (**1**) as substrate. In Figure 1, the baseline CV response (brown line) represents 1 mM MV^{2+} in 100 mM pH = 7.4 KPi buffer, indicating a turnover potential of approximately −0.8 V vs Ag/AgCl reference electrode. This signifies the lowest potential suitable for MV^{2+} reduction on the electrodes. We added 0.1 M KCl to enhance the electron transfer through the electrolyte. Upon introducing 10 μM of the substrate **1** into the solution (Figure 1, green line), the curve shape remained unchanged, suggesting that $MV^{+•}$ cannot reduce the substrate without the presence of the enzyme. In another experiment, when 10 μM PETNR enzyme were mixed to the MV^{2+} solution (Figure 1, orange line), the

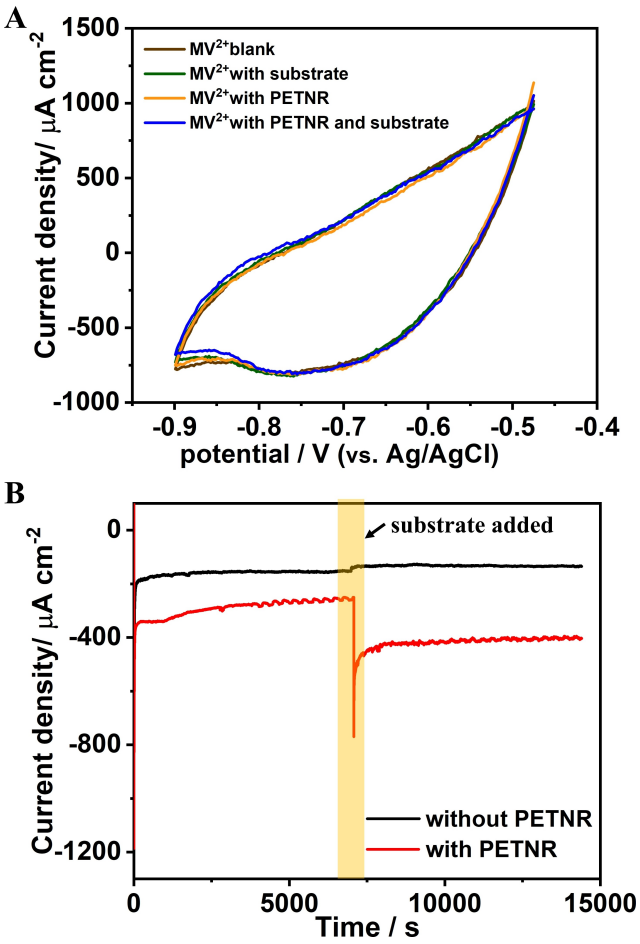


Figure 1. (a) Cyclic voltammograms (CV) response of MV^{2+} reduction on the carbon cloth electrode in the h-type bottle (scan rate: 10 mV/s). The CV response of 1 mM MV^{2+} in 100 mM pH = 7.4 KPi buffer with 0.1 M KCl as the electrolyte (Brown). The CV response of MV^{2+} in buffer with 10 μM of 2-cyclohexen-1-one (Green). The CV response of MV^{2+} in buffer with 10 μM PETNR enzyme (Orange). CV response of MV^{2+} in buffer with 10 μM PETNR enzyme and 10 mM 2-cyclohexen-1-one (Blue). (b) Chronoamperometry (at −0.9 V vs Ag/AgCl) response of 1 mM MV^{2+} by adding 10 μM PETNR enzyme and 10 mM 2-cyclohexen-1-one during the electrochemical catalytic process.

reduction peak of MV^{2+} also did not change. The CV curve retained its shape after adding PETNR and substrates into this system, indicating the stable reduction of MV^{2+} in this configuration. The lack of an apparent oxidation peak is due to the difficult desorption of the product from the electrode.

We then ran a series of chronoamperometry (CA) experiments to assess the functioning of the bioelectrocatalytic system (Figure 1B). The CA analysis was initiated with 1 mM MV^{2+} under a constant potential of −0.9 V vs Ag/AgCl, resulting in a gradual decrease in current and a corresponding darkening of the solution. Upon introducing 10 μM PETNR into the cathodic chamber, the current increased slightly, indicating that $MV^{+\bullet}$ reacted with PETNR. Once the current value stabilized, 10 mM substrate was introduced into the system, leading to a significant decrease in current, reflecting the consumption of $MV^{+\bullet}$ by the substrate-enzyme complex.

The comprehensive analysis, combining CA results with substrate conversion rate assessments using gas chromatography (GC) in Figure S1, supports our conclusion that MV^{2+} functions as an effective mediator molecule in bio-electrocatalytic reactions.

Optimization of Bio-Electrocatalytic System

To determine the optimal reaction condition, we varied the reaction time from 1 to 6 h (Figure 2). In theory, one could prolong the reaction until all substrates are consumed. However, triplicate reactions showed that the standard deviation values increased significantly after 4 h due to the non-specific absorption on the electrode surface and the decreased activity of the enzyme over longer reaction times. Consequently, we selected 4 h as the optimal reaction time for our subsequent experiments under reproducible reaction conditions.

Bio-Electrocatalytic Hydrogenation of Different Substrates

To assess the effectiveness and the synthetic applicability of our bio-electrocatalytic system, we studied four alkene substrates and two ERed enzymes, as detailed in Table 1. All substrates had both a carbonyl group and a C=C double bond. Since the ERed specifically reduces the latter, our objective was to confirm that the substrate underwent catalysis by the enzyme and not by other species. Comparing the product yields of PETNR and TOYE, we noticed that TOYE outperforms PETNR in terms of product amounts for the reduction of 2-cyclohexen-1-one (**1**, 1.20 mM h^{-1}; TOF ≈ 2 min^{-1}), 2-methyl-2-cyclohexen-1-one (**3**, 1.40 mM h^{-1}; TOF ≈ 2.3 min^{-1}) and 2-methyl-2-pentenal (**4**, 0.40 mM h^{-1}; TOF ≈ 0.7 min^{-1}). This compelling evidence suggests that TOYE exhibits superior catalytic efficiency within

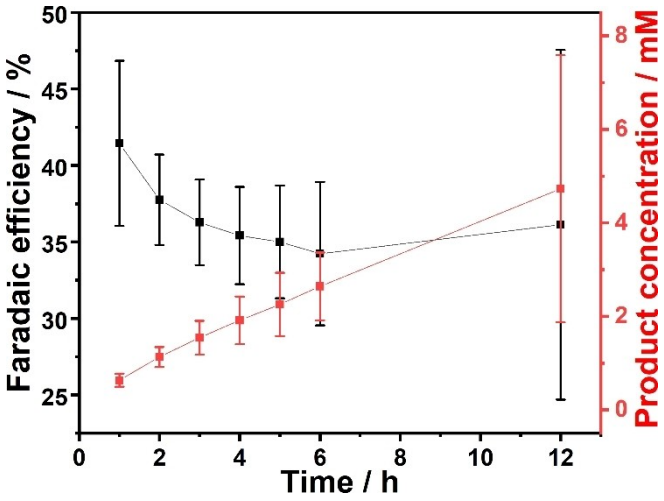
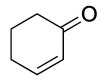
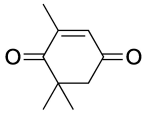
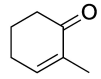
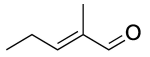


Figure 2. The concentration of cyclohexanone product obtained in the biocatalytic reduction of 2-cyclohexen-1-one (**1**) using the MV^{2+}/$MV^{+\bullet}$ regeneration system and the faradaic efficiency of this system. The reaction solution contained 10 μM PETNR, 1 mM MV^{2+} in 0.1 M pH = 7.4 KPi buffer with 0.1 M KCl. The concentration of **1** was 10 mM. Error bars represent the standard deviation of three independent experiments.

Table 1. Bio-electrocatalytic hydrogenation of different substrates with PETNR and TOYE.

Substrate	PETNR		TOYE	
	Yield rate ($mM^{-1}h^{-1}$)	ee_p (%)	Yield rate ($mM^{-1}h^{-1}$)	ee_p (%)
 2-Cyclohexen-1-one (**1**)	0.48 ± 0.13	n.a.	1.20 ± 0.50	n.a.
 Ketoisophorone (**2**)	2.31 ± 0.17	*rac*	1.19 ± 0.42	17 ± 8 (*R*)
 2-Methyl-2-cyclohexen-1-one (**3**)	0.55 ± 0.02	75 ± 10 (*R*)	1.40 ± 0.07	98 ± 1 (*R*)
 2-Methyl-2-pentenal (**4**)	0.28 ± 0.11	59 ± 7 (*S*)	0.40 ± 0.14	11 ± 1 (*S*)

this system. In the reaction for 12 h with substrate **1**, the calculated TON for the mediator MV^{2+} was ca. 5. This data indicates that future studies should aim to reduce the amount of mediator or, preferably, promote direct electron transfer from the electrode to the enzyme without a mediator. In theory, the mediator could also be recycled and reused after the reaction, although efficient separation of used enzyme and product must be addressed first.

Detailed literature data for the reduction of these substrates using a catalytic amount of $NADP^+$, which is recycled in situ, are available for PETNR.[16] These data show that conducting the reaction with PETNR (2 μM) at a substrate concentration of 5 mM and with $NADP^+$ (6 μM) recycled in situ by D-glucose and D-glucose dehydrogenase resulted in productivities of 1.16 $mM\,h^{-1}$ for **1** (4-hour reaction time; TOF ≈ 9.6 min^{-1}), 0.10 $mM\,h^{-1}$ for **3** (48-hour reaction time, TOF ≈ 0.9 min^{-1}), and 0.08 $mM\,h^{-1}$ for **4** (48-hour reaction time; TOF ≈ 0.9 min^{-1}). The highest calculated TON for the recycling of $NADP^+$ was 825. Data reported for TOYE (6 μM) with suprastoichiometric $NADP^+$ (10% molar excess; 6 mM) resulted in productivities of 0.16 $mM\,h^{-1}$ for **2** (24-hour reaction; TOF ≈ 0.3 min^{-1}) and 0.17 $mM\,h^{-1}$ for **4** (24-hour reaction; TOF ≈ 0.3 min^{-1}).[6] Our data shows that the bioelectrochemical conversion of the activated alkenes exhibits similar or even superior productivity compared to conventional biotransformations using the NADP cofactor and established cofactor-recycling systems.

Among the substrates tested, ketoisophorone (**2**) gave the highest product yield, with 2.31 $mM\,h^{-1}$ and 1.19 $mM\,h^{-1}$ for PETNR and TOYE, respectively. However, the reduction of **2** also exhibited the poorest stereoselectivity with both PETNR (racemic product) and TOYE (ee 17% ± 8% (*R*)). Furthermore, in control reactions performed without enzymes and MV^{2+}, the product yield of **2** was approximately 1.26 $mM\,h^{-1}$. Therefore, a background reduction reaction of **2** might occur directly on the electrodes. Considering both the background reaction and the stereoselectivity for the reduction of **2** catalyzed by PETNR with NADH, as reported elsewhere[6] we conclude that the background reaction significantly influences the PETNR system. However, this is not the case in the reduction of **2** catalyzed by the TOYE system that gave an ee of 17% ± 8% (*R*). In fact, an enantiomeric excess of 26% was reported for the reduction of TOYE in presence of NADH.[6] The discrepancy between no enantioselectivity with PETNR vs 17% ± 8% *ee* with TOYE can be attributed to a higher stability of the latter. Overall, the reduction of 2-methyl-2-cyclohexen-1-one (**3**) exhibited perfect stereoselectivity using the TOYE system (ee 98% ± 1% (*R*)). In the case of the reduction of 2-methyl-2-pentenal (**4**), our system yielded around 0.28 $mM\,h^{-1}$ and 0.40 $mM\,h^{-1}$ of saturated aldehyde product for the reactions catalyzed by PETNR and TOYE, respectively. PETNR exhibited good stereoselectivity towards formation of the (*S*)-aldehyde compared to TOYE with ee of 59% ± 7% and 11% ± 1%, respectively. These findings provide valuable insights into the selectivity and efficiency of our bio-electrocatalytic system.

Conclusions

We have successfully constructed a bio-electrocatalytic hydrogenation system, utilizing MV^{2+} as mediator molecule. The use of readily available commercial carbon cloth electrodes and methyl viologen molecules facilitates the accessibility of this system. Assembly of the h-type reaction bottle and addition of ene-reductase into the system resulted in efficient bio-electrocatalysis and demonstrated acceptable stereoselectivity under constant reducing potential conditions. This simple system holds significant potential for bio-electrocatalytic reactions, with the ability to adapt to different enzymes and substrates. However, certain challenges remain, including addressing issues related to the electrochemical reduction of the produced compounds, ensuring stability of the mediator molecules, and achieving full orthogonality between enzyme-catalyzed alkene substrate reduction and electrochemical regeneration of the reduced form of the mediator. We hope that our results will encourage other researchers to use similar bio-electrocatalytic systems.

Supporting Information Summary

The authors have cited additional references within the Supporting Information.[7b,8b,10b,18]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Biocatalysis · Electrocatalysis · Ene-reductases · Asymmetric hydrogenation · Cofactor regeneration

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