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Riboflavin with H_2-Driven or Electrochemical Recycling: A Cheap Cofactor System for Supporting Biocatalytic Alkene Reduction

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This study shows that the organic cofactor riboflavin provides a cheap and atom-efficient source of reducing equivalents to sustain biocatalytic alkene reductions by ene-reductase enzymes, when coupled with a H_2-driven or electrochemical recycling system. We employ the robust NiFe hydrogenase, *Escherichia coli* Hyd1, for H_2-driven riboflavin reduction and show the feasibility of unmediated electrochemical recycling of reduced riboflavin at a simple carbon electrode. We show that H_2-driven reduction of riboflavin can be extended to continuous flow with a packed bed reactor comprising of Hyd1 immobilized on a carbon support. These findings demonstrate that there is scope for replacing the expensive nicotinamide cofactors—NADH or NADPH with riboflavin, for applications of ene-reductases in biotechnology in either batch or continuous flow and in electrosynthesis.

1. Introduction

It is well-established that flavin mononucleotide (FMN) in its reduced hydroquinone form ($FMNH_2$) is able to replace the expensive nicotinamide cofactor, NADH, as a source of reducing equivalents for "old yellow enzyme" (OYE)-type ene-reductase enzymes which catalyze the reduction of a variety of activated alkene compounds (Scheme 1A).[1–4] Reduced flavin has been recycled in biotechnology by stoichiometric reduction using artificial cofactor analogues such as BNAH (1-benzyl-1,4-dihydronicotinamide), by photoreduction or by direct electrochemical reduction.[2,5–9] Observations that FMN can also be reduced by nickel-iron (NiFe) hydrogenase enzymes (Scheme 1C) have opened up opportunities for H_2-driven biocatalysis.[1,4,10] Electrons released during H_2 oxidation at the active site of robust NiFe hydrogenase 1 (Hyd1) from *Escherichia coli* (*E. coli*) are channeled to the surface of the protein via an iron-sulfur cluster relay, where they become available for the reduction of non-native substrates such as flavins.[1] Coupling hydrogenase with ene-reductase or nitro-reductase in the presence of H_2 and FMN provides a platform for more sustainable biocatalytic alkene reductions and nitro reductions. This compares favorably to the standard way of operating these enzymes with NAD(P)H and the carbon-intensive glucose dehydrogenase/ glucose cofactor recycling system.[11]

It has also been shown that Hyd1 from *E. coli* gives good activity for the reduction of flavin adenine dinucleotide (FAD).[1] Herein, we show that the scope of Hyd1 for flavin reductions can be further extended to riboflavin (RF) which is significantly cheaper than FMN and FAD and which is already produced at > 9000 ton per annum scale, by fermentation, as the nutritional supplement vitamin B2.[12] RF has a similar reduction potential to FMN (around −0.2 V versus SHE), as well as similar structure, differing only in the absence of a phosphate group (Scheme 1B, see Supporting Information, S.2.1, for reported reduction potentials and cost comparisons).[13–14] RF has been demonstrated as a source of reducing equivalents for ene-reductases using a light-driven recycling system in which oxidized RF in an electronically excited state is reduced by an organic sacrificial reductant, ethylene-diamine tetra-acetic acid.[2] In this study, we establish feasibility for the application of Hyd1 in atom-efficient H_2-driven recycling of RF (Scheme 1C) for supplying ene-reductase enzymes, and we also explore feasibility for an unmediated electrochemical recycling system at a carbon electrode (Scheme 1D).

The growing application of biocatalysis in selective chemical transformations has led to interest in translation of batch processes into continuous flow, using packed bed reactors. There are a number of examples of flow processes which unite NAD(P)H dependent catalysis with a cofactor recycling system such as formate dehydrogenase/formate.[15,16] Hyd1 is readily immobilized on a carbon support, and has been exploited in flow as part of an NADH recycling cascade for NADH-dependent biocatalysis with alcohol dehydrogenases.[17] Here, we also explored whether immobilized Hyd1 on carbon packed into a flow cartridge[18] can be used for H_2-driven RF reduction which would underpin extension of the use of this cost-efficient

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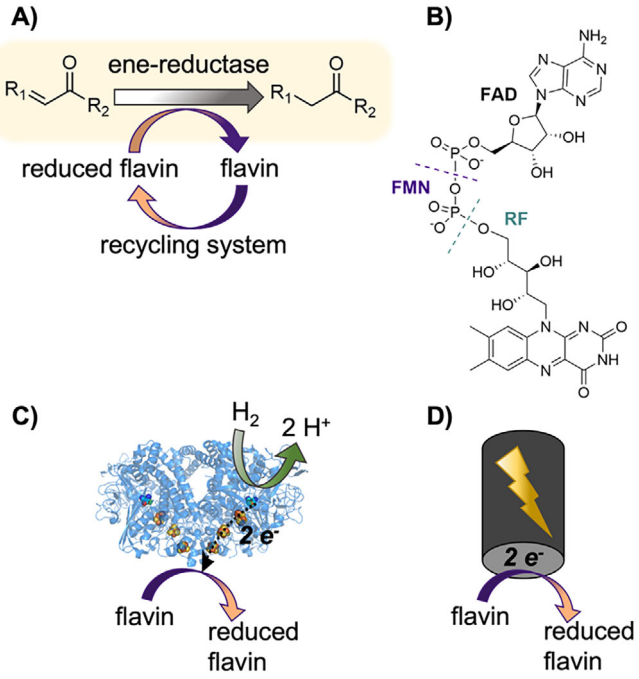


Scheme 1. (A) Biocatalytic selective C=C reduction using an ene-reductase and flavin recycling system. (B) Flavin chemical structures (RF: riboflavin, smallest flavin with the ribitol chain ending in OH; FMN: flavin mononucleotide, same structure as RF with a phosphate group at the end of the ribitol; and FAD: flavin adenine dinucleotide, an adenine is joined via a ribose and phosphate to the FMN structure). (C) H_2-driven reduction of flavins can be carried out using hydrogenase (Hyd1) enzyme. (D) Flavins can be electrochemically reduced.

cofactor for continuous processes. Together, these findings set the scene for riboflavin as a cheap cofactor for biotechnology in batch as well as in continuous flow.

2. Results and Discussion

2.1. Demonstration of RF Recycling by Hyd1 and Applicability as Reductant for Ene-Reductases

First, we performed activity assays to investigate whether Hyd1 can reduce RF and to compare the rate to FMN reduction by Hyd1. Hyd1 was added to a cuvette containing a H_2-saturated solution of flavin and the cuvette headspace (sealed using a Suba-seal) was kept under a flow of H_2 gas during the assays. For both FMN and RF, spectral changes in the UV–vis region indicate reduction of the flavin, with clear isosbestic points indicative of clean reduction to the hydroquinone form of the respective flavin (Figure 1A,B).[14] The initial activities (moles of flavin per minute per mole of enzyme) for reduction of FMN and RF by Hyd1/H_2 were very similar within the small experimental error range calculated from a duplicate of the experiment with RF.

Encouraged by this result, we then used Hyd1/H_2 to prepare samples of reduced RF and FMN, followed by removing the Hyd1 (using a size-exclusion filter). The reduced flavin was then mixed with the commercial ene-reductase enzyme ENE-108 (Johnson Matthey), and an alkene substrate, 4-phenyl-3-buten-2-one, **1** (Figure 1C,D). Pleasingly, initial activity values were identical for ENE-108 fed with reduced RF or with reduced FMN (i.e., the value for FMN matched with the average value from duplicate assays run for RF) showing that ENE-108 can take up electrons effectively from reduced RF.

It is also useful to compare the reaction rates for the reduction and oxidation steps: here, ENE-108 was found to give a lower rate for oxidation of reduced flavin coupled to alkene reduction, compared to Hyd1-catalysed flavin reduction, suggesting that when combining the two systems, a significant excess of ENE-108 to Hyd1 must be used so that the reaction is not severely rate limited by the alkene reduction step. We also carried out activity assays under the same conditions to measure the rate of NADH oxidation by ENE-108 or ENE-103. The results gave initial activities of 1.7 ± 0.3 min^{-1} for each ene-reductase, from assays carried out in triplicate (see Supporting Information, S.2.4). This rate is 13–25 times faster than was observed with reduced RF or FMN. This is not surprising since NADH is the native electron donor for these enzymes and the conditions may not be optimized in the case of the RF reduction.

Additionally, to investigate suitable RF concentrations for the combined Hyd1/ene-reductase reactions, assays measuring the initial rate of RF reduction (with Hyd1 and H_2) were carried out for a series of RF concentrations, from 0 to 0.3 mM. The solubility of RF in an aqueous solution is significantly lower than FMN, with the limit around 0.3 mM for RF. These assays showed the initial rate increased linearly until around 0.1 mM RF, with a smaller increase up to 0.2 mM where activity levels off (see Supporting Information, S.2.2, for assay results and comparison to assays run with FMN and FAD).

2.2. Ene-Reductase with Hyd1/H_2 Recycling of Reduced RF

Next the H_2-driven RF recycling using Hyd1 was coupled with ene-reductase for selective alkene reduction (Table 1). These reactions were all carried out using 1 bar H_2, with multiple reactions run simultaneously in plastic tubes inside a pressure vessel (details of reaction set-up and conditions can be found in the Methods section). The alkene reduction was initially investigated using substrate **1**, for the selective reduction of the C=C bond over the C=O bond to give 4-phenyl-2-butanone, **2**, as determined by GC analysis, comparing to reference standards. A 0.1 mM concentration of RF was chosen for the initial reactions since this was indicated to provide suitable activities by the assays discussed earlier. Two commercial ene-reductases were compared for their activity with substrate **1**; ENE-108 gave approximately 3 times higher conversion after 24 h than ENE-103 and 1.5 times higher initial turnover frequency (TOF), measured after 2 h (Table 1, entries 1 and 2). A similarly faster rate with ENE-108 was measured in UV–vis activity assays, monitoring the rate of re-oxidation of reduced RF, in the presence of **1** (see Supporting Information, S.2.3). When the reaction using ENE-108 was intensified by increasing the substrate concentration from 1 to 10 mM while also increasing the enzyme concentrations, it was found to be necessary to increase the RF concentration from 0.1 to 0.2 mM to maintain complete conversion to **2** in 24 h (Table 1, entries 3 and 4).

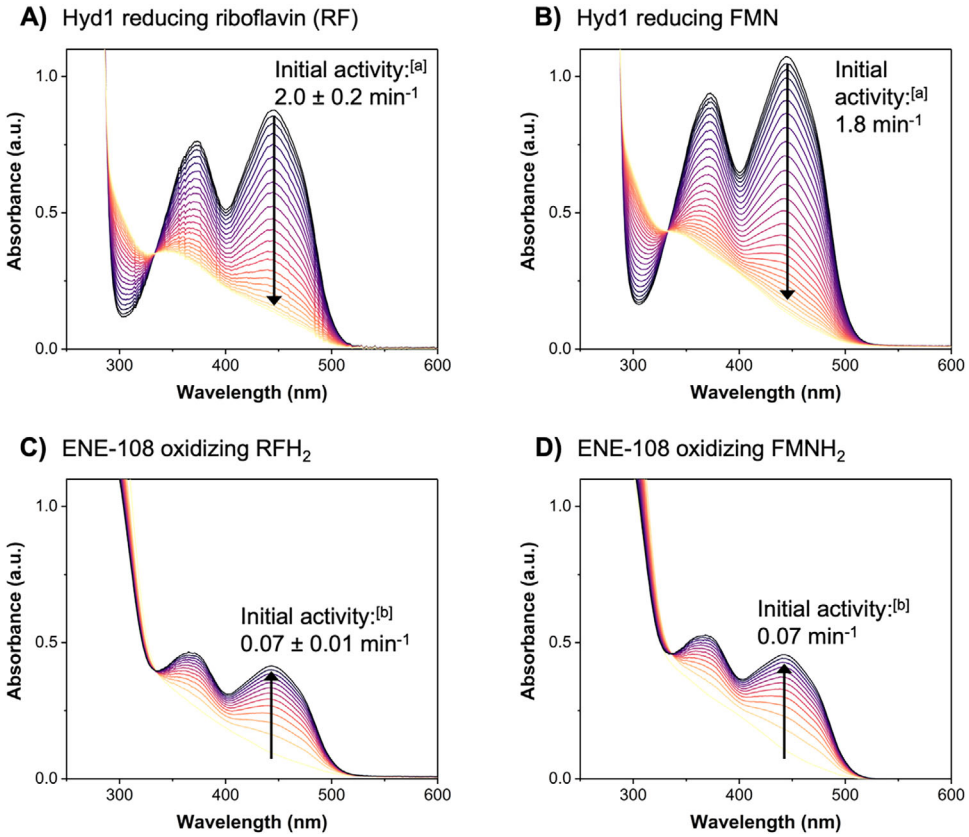


Figure 1. Testing RF as a cheaper alternative to FMN as a biological cofactor, where Hyd1, under H_2, can reduce the flavin cofactor, and subsequently the reduced cofactor can be used by an ene-reductase for the biocatalytic selective reduction of an alkene to alkane. UV–vis spectra (showing scans recorded every 5 mins) of flavin solutions containing: Hyd1/H_2 with (A) RF, (B) FMN, or ene-reductase (ENE-108) with alkene substrate (4-phenyl-3-buten-2-one) with (C) RFH_2, (D) $FMNH_2$. [a] Hyd1 initial activity: mol flavin per minute per mol Hyd1. [b] ENE-108 initial activity: mol flavin per minute per mol ENE-108. See Methods section for assay procedures.

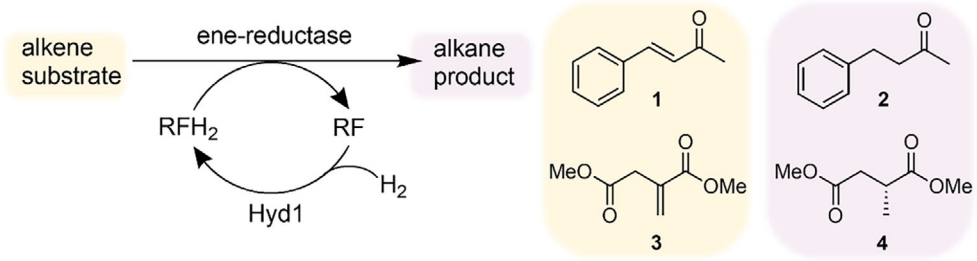
Entry 5 of Table 1 shows the results of an experiment using ENE-108, in which the concentration of substrate **1** was further increased to 20 mM, and the ENE-108 concentration was doubled, while keeping the concentration of Hyd1 the same as in entry 4 where the substrate concentration was 10 mM. An increase in the initial TOF measured at 2 h suggests that the reaction rate was limited by the ene-reductase in entry 4. Despite the faster initial TOF, the conversion appeared to slow over time, reaching 60% after 24 h and showing only a small increase to 66% after 44 h (Table 1, entry 5). To investigate a hypothesis that substrate or product might be affecting the activity of the enzymes over time, assays were carried out to study the effect of **1** and **2** on Hyd1. First, the reduction rate of RF was measured, using Hyd1 under H_2, after adding specific amounts of **1** or **2** (see S.2.5). These assays were then repeated on the same samples after 24-h incubation periods. There was no significant difference between the rates obtained in any of these assays (or compared to the control which had no DMSO, **1** or **2**), showing that Hyd1 is not affected by the alkene substrate or build-up of **2** even in reactions at high concentration. Therefore, product inhibition is more likely to occur at the ene-reductase than at Hyd1.

Another substrate was then tested using the H_2-driven RF recycling system with ENE-108: dimethyl itaconate, **3**, which upon reduction of the C=C bond gives rise to a chiral product, dimethyl methylsuccinate, **4**. Chiral-GC analysis showed ENE-108 was enantioselective (>99% *e.e.*) for the (*R*)-enantiomer of **4**. An initial test at 1 mM of **3**, under the same conditions as used for **1**, found ENE-108 was less active for this substrate, with the reaction having a lower initial TOF value (measured after 2 h) and lower conversion after 24 h (Table 1, entry 6 compared to entry 2). However interestingly, under conditions in which 20 mM **3** was introduced, it was possible to achieve 96 ± 5% conversion after 44–48 h, thereby giving a Hyd1 total turnover number (TTN) of 4104 ± 224 and RF turnover number (TN) of 95.8 ± 5.2 (Table 1, entry 7). This again suggests there is product inhibition by **2** in the reduction of **1** but minimal effect on reduction of **3**. For these two substrates, reasonable total turnover numbers and RF turnover numbers were reached, demonstrating the feasibility of using RF as a cofactor with ene-reductases.

2.3. Translation of Hyd1/H_2 Reduction of RF into a Packed Bed Flow Column

Having established successful biocatalytic RF reduction in batch, operation in single-pass continuous flow was next investigated in a packed bed reactor housed within an anaerobic glove box.

Table 1. H_2-driven RF recycling for the biocatalytic reduction of **1** or **3**.[a]



Entry	[Hyd1] (mg mL^{-1})	ENE-	[ENE] (mg mL^{-1})	Alkene	[Alkene] (mM)	[RF] (MM)	Conv. (%)[b]	e.e. (%)[c]	Hyd1 TOF (min^{-1})[d]	Hyd1 TTN[e]	RF TN[e]
1[f]	0.05	103	0.5	**1**	1	0.1	29 ± 14	n/a	1.0 ± 0.2	620 ± 308	2.9 ± 1.4
2[f]	0.05	108	0.5	**1**	1	0.1	99	n/a	1.5	2114	9.9
3	0.5	108	5	**1**	10	0.1	36	n/a	1.6	769	35.9
4	0.5	108	5	**1**	10	0.2	>99 ± <1	n/a	2.6 ± 0.1	2138 ± 0.5	49.9 ± <0.1
5	0.5	108	10	**1**	20	0.2	60 (66)[g]	n/a	4.7	2563	59.8
6	0.05	108	0.5	**3**	1	0.1	32 (55)[g]	>99	0.6	689	3.2
7	0.5	108	10	**3**	20	0.2	66 ± 13 (96 ± 5)[h]	>99	2.2 ± 0.5	2817 ± 546 (4104 ± 224)[h]	65.7 ± 12.7 (95.8 ± 5.2)

[a] Reaction conditions: RF (0.1 or 0.2 mM), Hyd1 (0.05 or 0.5 mg mL^{-1}), ene-reductase (0.5 to 10 mg mL^{-1}), 4-phenyl-3-buten-2-one, **1**, or dimethyl itaconate, **3**, in DMSO (1 vol%), pH 7, 50 mM potassium phosphate, H_2 (1 bar), room temperature (20–25 °C). See Methods section for reaction procedures.
[b] GC conversion to **2** or **4** at 24 h.
[c] Enantioselectivity for (*R*)-enantiomer of **4** calculated from chiral-GC analysis at final reaction time point.
[d] Hyd1 turnover frequency (mol **2** or **4** per mol Hyd1 per minute) calculated after 2 h.
[e] Hyd1 total turnover number (mol **2** or **4** per mol Hyd1) and RF turnover number (mol **2** or **4** per mol RF) calculated after 24 h.
[f] Acetonitrile (1 vol%) co-solvent used instead of DMSO.
[g] GC conversion to **2** or **4** at 44 h.
[h] These results in brackets were an average of three experiments: two measured at 44 h and one at 48 h.

Hyd1 immobilized on activated charcoal was incorporated into a packed bed catalyst cartridge incorporated into the H-cube hydrogenation reactor (ThalesNano) with in-built H_2 generation via water electrolysis (Figure 2A). The reaction mixture contained RF (0.2 mM, in pH 7, 100 mM potassium phosphate buffer) and was first purged with N_2 to remove trace O_2 which would cause re-oxidation of RF. Pure buffer flushed with H_2 was flowed through the system for 2 h to saturate the solution with H_2. The effluent was pumped through an in-line flow UV–vis, with peak wavelength data plotted at 60-min intervals to measure the concentration of oxidized RF remaining (Figure 2B) via its UV–vis absorbance. Addition of RF from a stock solution to the reagent reservoir caused a transient concentration spike in the effluent. Absorption from oxidized flavin detected in the effluent then dropped to zero indicating full single-pass conversion to reduced riboflavin.

Full reduction of RF was maintained over 24 h of measurement. In order to confirm that loss of absorption from oxidized RF indeed corresponds to complete reduction of the flavin, the 20-h sample was re-oxidized in air, and gave rise to the UV–vis trace shown in the inset to Figure 2B. This confirms the expected 0.2 mM concentration of RF in solution, that is, showing that RF was not lost by adsorption on the support.

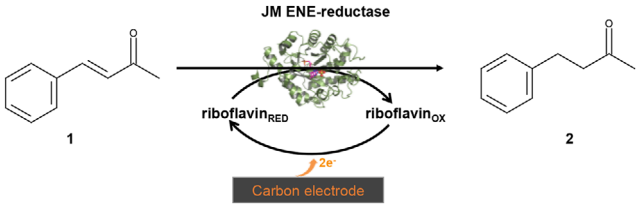
The fact that full reduction of RF could be achieved in single pass is highly promising for future flow applications of biocatalysis requiring reduced RF as cofactor.

2.4. Demonstration of Ene-Reductase with Electrochemical Recycling of Reduced RF

Electrochemical reduction of RF at a pyrolytic graphite "edge" electrode shows a reversible response indicative of both surface-adsorbed and solution species under diffusion control, consistent with a degree of pi-stacking of RF on the carbon surface (see Figure S7). To determine whether a bulk solution of RF could be reduced at mild potential at a high surface area carbon felt electrode, a fixed potential of −0.356 V versus SHE was applied to a carbon felt electrode in contact with a 10 mL, stirred solution of 0.1 mM RF (see Supporting Information, S.2.8, for details). Aliquots (200 µL) were removed at 5-min intervals for UV–vis spectrophotometric analysis and subsequently returned to the electrochemical cell. Spectra showed that RF was fully reduced within 20 min, consistent with the measure of charge passed from integration of the current-time trace (see Figure S8).

Encouraged by this, electrosynthesis experiments were then performed to investigate whether electrochemically reduced RF retained its biological activity for supply of reducing equivalents to ene-reductase (see Methods section). In an initial test (Table 2, entry 1), the electrochemical cell solution contained 1 mM of substrate **1**, RF (0.1 mM), ENE-108 (0.5 mg mL^{-1}) and 10 vol% DMSO as a co-solvent in aqueous buffer. The electrode was poised at −0.40 V versus SHE. The concentration of the product, **2**, was determined by GC analysis after a 24-h reaction period.

Table 2. Electrochemical-driven RF recycling for the biocatalytic reduction of **1**.[a]



Entry	[ENE-108] (mg mL^{-1})	[1] (mM)	[RF] (mM)	Reaction Time (h)	GC Conv.(%)[b]	Faradaic Efficiency (%)[c]
1	0.5	1	0.1	24	60	79
2[d]	0.5	5	0.1	24	68	96
3	0.5	10	0.1	24	58	~100
4[e]	0.5	10	0.1	120	>99	~100
5[f]	0.5	20	0.1	3	63	86

[a] Unless otherwise stated the reaction conditions were as follows: RF (0.1 mM), ene-reductase, ENE-108, (0.5 mg mL^{-1}), 4-phenyl-3-buten-2-one, **1**, in DMSO (10 vol%), pH 7, 50 mM potassium phosphate, room temperature (20–25 °C), fixed potential −0.40 V versus SHE, stirred continuously (400 rpm). See Methods section and Supporting Information, Section S.2.8, for procedure.
[b] GC conversion to **2**.
[c] Faradaic efficiency: GC conversion per 2 e$^-$ equivalents passed.
[d] A more negative electrode potential of −0.42 V versus SHE was used.
[e] Dosing experiment adding substrate **1** at hourly intervals: starting at 2 mM with 2% DMSO up to 10 mM with 10% DMSO.
[f] Performed in electrochemical flow cell with a carbon felt working electrode (4 mm x 5 mm), ENE-108 (1.0 mg mL^{-1}), **1** (20 mM), DMSO (30 vol%).

Integration of the current over time was used to determine the charge passed, and hence a Faradaic efficiency (moles of product **2** per 2 moles of electrons consumed). The reaction conditions were intensified by increasing concentration of **1** to 5 mM and 10 mM (entries 2 and 3 in Table 2) with the RF concentration maintained at 0.1 mM. After 24 h, the conversion was similar in entries 1–3, despite the increase in substrate concentration, but Faradaic efficiency increased with substrate concentration.

A dosing experiment was then performed (Table 2, entry 4) where initially a 2 mM concentration of **1** was added with 2 vol% DMSO and then over the next 48 h the concentration of **1** was increased up to 10 mM (in 2 mM increments) which gave a final concentration of 10 vol% DMSO. This reaction was left to run for 120 h and achieved >99% conversion of **1** to **2**, with 100% Faradaic efficiency.

2.5. Flow Electrochemistry for Recycling of Reduced RF to Drive Alkene Reduction

One of the possible limiting factors affecting conversion in the electrochemical cell was stirring inefficiencies. To overcome this issue the reaction was performed in continuous flow using a home-built electrochemical flow cell machined from polyether ether ketone (PEEK) (Figure 3A shows a diagram of the set-up, full details in Supporting Information, S.2.9). A 12.7 mL reaction solution comprising **1** (20 mM), RF (0.1 mM) and ENE-108 (1.0 mg mL^{-1}) was recirculated continuously through the electrochemical flow cell (internal cell volume, 180 µL) whilst a potential of −0.40 V versus SHE was applied to the 5 × 10 mm^2 carbon felt working electrode. Figure 3B shows the current-time trace (left hand axis). Integrating the current/time curve gives the charge passed which was used to calculate the 2 e$^-$ equivalents passed at each time point (right hand axis). Determination of conversion to **2** by GC after approximately 3 h gave a Faradaic efficiency of 86% (Table 2, entry 5).

3. Conclusion

We have demonstrated that the cheap flavin cofactor, riboflavin, vitamin B2, can be reduced readily by Hyd1 under mild H_2 conditions. In its reduced form, riboflavin serves as an effective source of reducing equivalents for commercial OYE-type ene-reductases, ENE-103, and ENE-108, with a total turnover number for Hyd1 of approximately 4000 and RF cofactor turnover number of about 100 measured in these demonstration reactions. We also show that H_2-driven reduction of RF can be translated into a flow hydrogenation reactor by immobilizing Hyd1 on a carbon support, with full, single-pass conversion to reduced RF sustained over 24 h. Electrochemical reduction of RF is also shown as a viable approach for recycling the reduced flavin cofactor, without the need for an additional enzyme. This shows that electricity could serve as a clean source of electrons for driving flavin-dependent biocatalysis. Together these findings establish RF as a cost-effective cofactor for applied biotechnology.

4. Methods

4.1. General Reagents and Enzymes

Most commercial reagents were purchased from Merck and used as received, without any further purification. The exceptions were dimethyl itaconate and dimethyl (*R*)-(+)-methylsuccinate which were purchased from Fluorochem and ChemCruz,

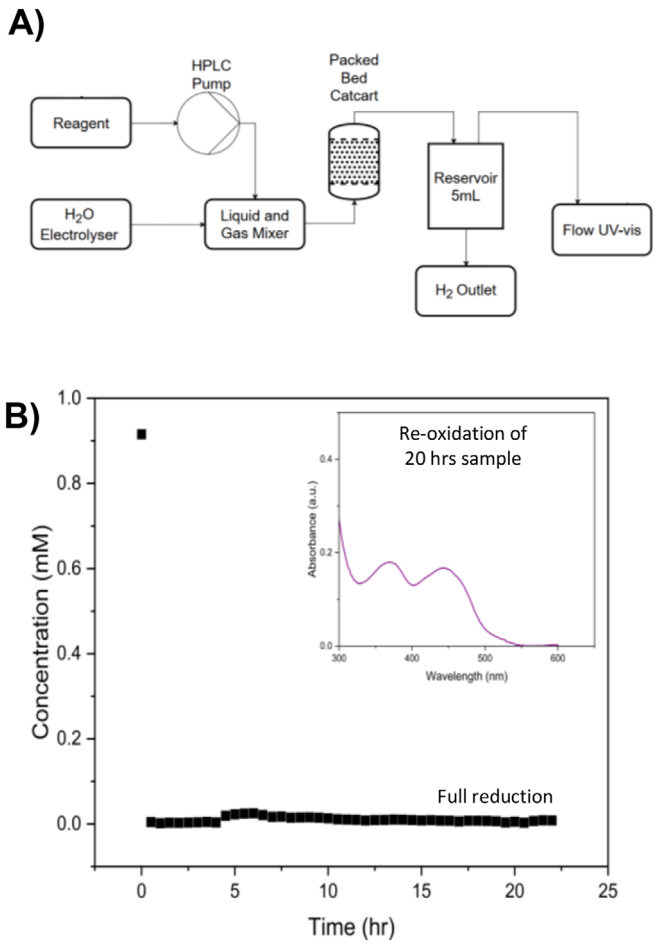


Figure 2. Single pass biocatalytic RF reduction in continuous flow at room temperature, using 0.1 mL min^{-1} liquid and 3 mL min^{-1} H_2 gas flow in an H-cube flow reactor. RF (0.2 mM) was reduced by pre-immobilized Hyd1 on activated charcoal (0.45 mg Hyd1 on 30 mg activated charcoal) at pH 7, 100 mM potassium phosphate buffer, over 24 h. (A) Diagram of flow set-up including commercial H-cube flow reactor and Uniqsis inline UV–vis. (B) Concentration time graph from inline UV–vis data, showing change in oxidized riboflavin concentration over time. Inset shows re-oxidized RF 20-h sample to confirm presence of RF in effluent.

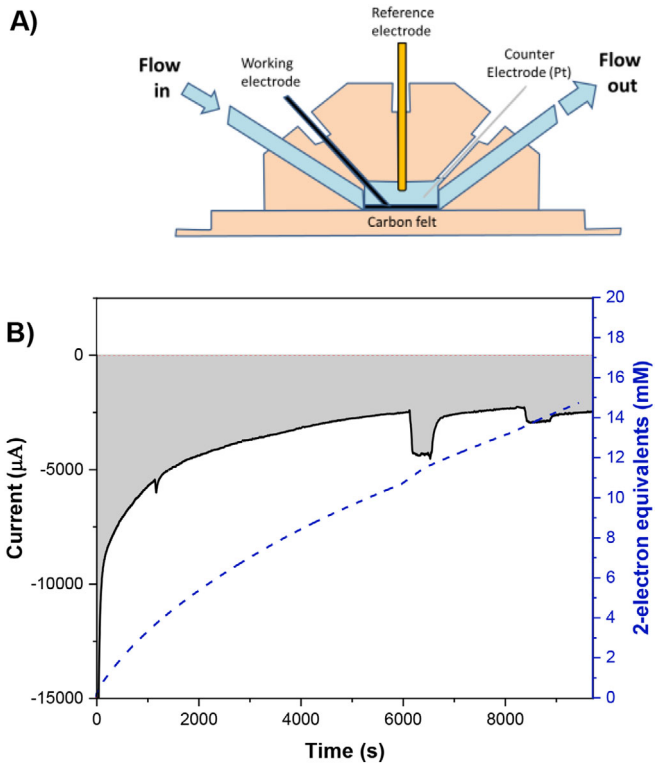


Figure 3. Electrochemical RF recycling coupled with ene-reductase in a flow cell. (A) Diagram of home-built electrochemical flow cell. (B) Current-time trace (left hand axis) during reduction of 12.7 mL of substrate 1 (20 mM) performed in continuous flow using RF (0.1 mM), and ENE-108 (1.0 mg mL^{-1}) with the working electrode poised at −0.40 V versus SHE. Area of shaded region shows the area integrated to calculate the charge passed which was used to calculate the '2 e^- equivalents' (mM, blue, right hand axis).

respectively. All aqueous solutions were prepared with deionized Milli-Q water (Millipore, 18 MΩcm). *E. coli* Hyd1, was prepared following published procedures.[10] Molecular weight for over-expressed Hyd1 is 107 kDa per monomer of small and large subunit (PDB: 4GD3, without cytochrome). The ene-reductases (ENE-103 and ENE-108, molecular weight: 37780 and 37,460 Da, respectively) used for the alkene reduction were received in lyophilized form from Johnson Matthey, Cambridge, and used without further purification. Activities for reduction of 4-phenyl-3-buten-2-one with NADH were measured as 1.72 and 1.74 mol NADH/min/mol ENE, for ENE-103 and ENE-108 (see Supporting Information, S2.4).

4.2. General Method Considerations

To minimize any competing oxidation reactions, all reactions, including UV–vis assays and electrochemical reactions, were carried out in a glovebox, filled with a N_2 atmosphere (O_2 < 5 ppm). When handling aqueous flavin solutions (FMN, FAD, or RF), including while the alkene reduction reaction was carried out, aluminum foil was used to cover solutions to protect from photodecomposition.[19] Before Hyd1 was used in UV–vis activity assays or in alkene reduction reactions with ene-reductase, the Hyd1 was first "pre-activated" under H_2. A typical procedure for this involved pipetting the desired amount of Hyd1 into a 0.5 mL plastic tube (StarLabs), piercing the lid of the plastic tube with a needle and inserting into a Büchi Tinyclave pressure vessel which was then filled with H_2 (to 2.5 bar and vented x3 and then filled to 1 bar) and left for 16–20 h.

4.3. Flavin Reduction UV–Vis Activity Assay

Assays were performed using a Cary 60 UV–vis spectrophotometer (Agilent) and a quartz cuvette (1 cm path length). A Peltier accessory was used to control the temperature of the cuvette which was set to 25 °C for all assays. The settings on the Scan software were set to record a spectrum from 800 to 200 nm every 30 s during an assay. A background spectrum using pH 7, 50 mM potassium phosphate was recorded before beginning the experiment. A 0.103 mM solution of flavin (FMN or RF) in pH

7, 50 mM potassium phosphate was prepared and 880 µL of this solution was added to the quartz UV-vis cuvette. A Suba-seal was used to seal the UV–vis cuvette, and two needles were added through the top of the Suba-seal. A H_2 gas line and an outlet line were attached to the needles and the inlet needle was moved below the surface of the flavin solution to sparge the solution with H_2 for a minimum of 15 min. Meanwhile at pH 7, 50 mM potassium phosphate buffer was used to make the volume of the Hyd1 solution up to 20 µL (thus, making the flavin concentration in each assay experiment as 0.1 mM and Hyd1 concentration as 0.05 mg mL^{-1}). After the sparging of the flavin solution was complete, both needles were lifted out of the solution, so they were now in the cuvette headspace. The UV–vis was set to start scanning every 30 s before the Hyd1 solution was added to the cuvette, using a needle and syringe to do so, using 200 µL of flavin solution to aid transfer. The flavin reduction rate was then measured by the decrease in absorbance over time, using the data from the linear section of the graph. Molar adsorption coefficients used to calculate Hyd1 initial activity values: 11.0 mM^{-1} cm^{-1} at 444 nm for RF[20] and 12.5 mM cm^{-1} at 445 nm for FMN.[14]

4.4. Oxidation of Reduced Flavin UV–Vis Activity Assay

The same UV–vis settings as detailed for the flavin reduction activity assays were used. Reduced-flavin solutions were prepared as per the conditions in flavin reduction activity assays (does not need to be carried out in a UV–vis cuvette, can also prepare multiple solutions at the same time by preparing in StarLabs 0.5 mL plastic tubes which are then put under H_2). After reducing the flavin, the Hyd1 enzyme was removed using a 50 kDa size exclusion filter (centrifuging at 12,000 rpm for 10 min). The filtered reduced-flavin solution was then added to the quartz cuvette for the UV–vis assay. A stock solution of ene-reductase was prepared at pH 7, 50 mM potassium phosphate (45 mg mL^{-1}). The alkene substrate 4-phenyl-3-buten-2-one, **1**, (as a 100 mM stock solution in DMSO) was added to the solution (making a 1 mM assay concentration with 1 vol% DMSO) and mixed thoroughly using a pipette. The ene-reductase was added (making a 0.5 mg mL^{-1} assay concentration) and the solution was mixed using a pipette. The reduced-flavin oxidation rate was then measured by the increase in absorbance, at 444 nm or 445 nm for RF or FMN respectively, over time, using the data from the linear section of the graph.

4.5. Alkene Reduction Using Ene-Reductase and Hyd1/H_2 Recycling of Reduced RF

A 0.124 mM solution of RF at pH 7, 50 mM potassium phosphate was prepared and sparged with H_2 for at least 15 min. If extra buffer was needed to make the desired total Hyd1 concentrations, then that was added to the plastic tubes containing "pre-activated" Hyd1. Then 350 µL of H_2-saturated RF (making a 0.1 mM reaction concentration; initial stock concentration was adapted as necessary for reactions at 0.2 mM RF) was added and the plastic tubes were placed back into a Büchi Tinyclave pressure vessel, filled with H_2 (to 2.5 bar, vented x3, and then filled to 1 bar) and then placed on a Stuart mini see-saw rocker set to 30 oscillations/min. Typically, the reactions were left for 1–1.5 h before the vessel was vented and ene-reductase (from a stock solution prepared at pH 7, 50 mM potassium phosphate) followed by alkene (in acetonitrile or DMSO) were added to the plastic tubes, and solutions were mixed using a pipette. These were then put back into a Büchi Tinyclave pressure vessel, filled with H_2 (to 2.5 bar, vented x3, and then filled to 1 bar) and placed back on the rocker set to 30 oscillations/min. The reactions were analyzed by GC (as detailed in S.1.1 in the Supporting Information).

4.6. Flow Set-Up for Hyd1/H_2 Recycling of RF

Mesh 100 activated charcoal were purchased from Merck. For the immobilized catalyst, 0.45 mg Hyd1 was immobilized on 30 mg activated charcoal suspended in 200 µL pH 7, 100 mM potassium phosphate buffer by leaving for 1 h at 4 °C. The catalyst was separated by centrifugation at 12,000 g for 2 min, then the supernatant was removed and analyzed via UV–vis. This showed a negligible protein absorbance (0.005 a.u.) at 280 nm, indicating that >99% of the enzyme had been successfully immobilized. The buffer was purged with N_2 overnight before addition to the glovebox and the system was run with H_2 and buffer for 2 h prior to RF addition and start of the reaction to saturate the buffer with H_2. The reaction was run single-pass at room temperature and pressure, with 3 mL min^{-1} H_2 and 0.1 mL min^{-1} liquid flow rate used to push the RF solution through the packed bed using 1 SF-10 peristaltic pump (Vapourtec) and a H-cube mini reactor (Thales Nano) with chemically resistant PEEK fittings (Figure 2A). A flow UV–vis spectrophotometer (Uniqsis) was used in line to monitor column effluent.

4.7. Electrochemical Reduction of RF and Coupling to Ene-Reductase

Electrochemical work was all carried out in an anaerobic glove box under N_2 (5 ppm O_2), with glass cells wrapped in aluminum foil to exclude the possibility of photochemical side reactions involving RF. A three-electrode set-up was used in all cases. Bulk reduction of RF (see Supporting Information, S.2.8) was carried out in a stirred glass cell using a PalmSens4 potentiostat and the associated PSTrace software. The working electrode was carbon felt, the reference was a Saturated Calomel Electrode (SCE), and the Pt wire counter electrode was contained in KCl solution and separated from the main cell by a frit. Potentials (E) were converted to volts (V) versus the Standard Hydrogen Electrode (SHE) using the conversion $E_{SHE} = E_{SCE} + 0.242$ V. An electrochemical flow experiment was carried out using an Autolab PGStat30 potentiostat and the associated Nova 1.1 software, again using a carbon felt working electrode and separated Pt counter electrode, within a home-built cell (Figure 3A, and see Supporting

Information, S.2.9). In this case the reference electrode was a leak-free Ag/AgCl electrode (PalmSens BV Netherlands), and potentials were corrected to V versus SHE by comparison of the reference electrode potential to a SCE and application of the appropriate conversion.

Supporting Information

The authors have cited additional references within the Supporting Information.[21–23]

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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 Supporting Information of this article.

Keywords: Biocatalytic electrosynthesis · Biocatalytic hydrogenation · Cofactor recycling · Flavin recycling · Riboflavin

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