Bioelectrocatalytic Cofactor Regeneration Coupled to CO₂ Fixation in a Redox-Active Hydrogel for Stereoselective C–C Bond Formation

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Abstract: The sustainable capture and conversion of carbon dioxide (CO₂) is key to achieving a circular carbon economy. Bioelectrocatalysis, which aims at using renewable energies to power the highly specific, direct transformation of CO₂ into value-added products, holds promise to achieve this goal. However, the functional integration of CO₂-fixing enzymes onto electrode materials for the electrolyseshis of stereochemically complex molecules remains to be demonstrated. Here, we show the electricity-driven regio- and stereoselective incorporation of CO₂ into crotonyl-CoA by an NADPH-dependent enzymatic reductive carboxylation. Co-immobilization of a ferredoxin NADP⁺: reductase and crotonyl-CoA carboxylase/reductase within a 2,2'-viologen-modified hydrogel enabled iterative NADPH recycling and stereoselective formation of (2S)-ethylmalonyl-CoA, a prospective intermediate towards multi-carbon products from CO₂, with 92 ± 6 % faradaic efficiency and at a rate of 1.6 ± 0.4 μmolcm⁻²h⁻¹. This approach paves the way for realizing even more complex bioelectrocatalytic cascades in the future.

Introduction

A main goal of carbon capture and utilization technologies is to combine renewable energy with the reduction of CO₂ as building block for the synthesis of added-value chemicals, which will allow the transition towards a circular carbon economy. In spite of recent achievements of the electrocatalytic reduction of CO₂ into small commodity chemicals, which will allow the transition towards a circular carbon economy. In spite of recent achievements of the electrocatalytic reduction of CO₂ into small commodity chemicals, which will allow the transition towards a circular carbon economy.

Using redox-active hydrogels for the immobilization and wiring of the biocatalyst, Minteer and co-workers reported the bioelectrochemical regeneration of NADH, which was applied for the synthesis of polyhydroxybutyrate, methanol and propanol, and more recently chiral β-hydroxy nitriles in a biphasic bioelectrocatalytically driven enzymatic cascade. In comparison to direct electron transfer, the mediated wiring in redox active hydrogels with a high load of enzymes has been shown to enhance the synthetic output of biohybrid systems.

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Overall, while NAD(P)H recycling and multistep enzyme reactions, using both direct and mediated electron transfer, have been shown as proof of concept for the synthesis of small molecules that are otherwise inaccessible through electrocatalytic approaches, the direct bioelectrocatalytic CO₂ fixation for synthesis of structurally demanding molecules remains to be demonstrated. Here we show that redox-active hydrogel films can be specifically designed to wire and accommodate enzymes for bioelectrocatalytic NADPH regeneration coupled to the synthesis of complex molecules from CO₂ via regioselective and stereoselective C–C bond formation. We co-immobilized the enzymes ferredoxin NADP⁺ reductase (FNR) and NADPH-dependent crotonyl-CoA carboxylase/reductase (Ccr) within a viologen-based redox hydrogel. Electrons transferred to the FNR through a mediated electron transfer mechanism allowed the bioelectrocatalytic reduction of NADP⁺, thus continuously supplying Ccr with the reduced cofactor to drive the synthesis of (2S)-ethylmalonyl-CoA (C_{26}H_{42}N_{7}O_{19}P_{3}S, 882 g·mol⁻¹) by enzymatic reductive carboxylation of crotonyl-CoA.

**Results and Discussion**

**Bioelectrocatalytic NADP⁺ Reduction**

Although redox-active hydrogels were previously used for the regeneration of NADH⁹,¹⁰ their application has not yet been demonstrated for the recycling of NADPH. We use a recently reported 2,2’-viologen modified polyvinyl alcohol (V₂⁺-PVA)¹³,¹⁴ as low-potential redox polymer for the immobilization of FNR from *Synechococcus* sp. directing electrons towards the reduction of NADP⁺. The bioelectrocatalytic film (Figure 1A), formed by drop casting a mixture of V₂⁺-PVA and FNR onto a glassy carbon electrode, was first characterized with cyclic voltammetry (CV). The redox potential of V₂⁺-PVA was determined at ~466 mV vs. SHE being 110 mV more negative than the standard redox potential of the NADP⁺/NADPH pair (~355 mV at pH 8)¹⁵ thus providing sufficient driving force for the electron transfer to FNR (Figure 1B). The stability of the film was tested by performing continuous CVs where over 95% of the cathodic and anodic peak current densities under non-turnover conditions were kept after 500 cycles corresponding to 1.7 h of continuous cycling (Figure S5A).

![Figure 1. Bioelectrocatalytic NADPH generation. A) Schematic representation of NADP⁺ reduction in films of FNR (PDB 2B5O) immobilized in a viologen-based redox hydrogel (V₂⁺-PVA/FNR) on a carbon-based electrode surface. B) Cyclic voltammetry of FNR immobilized within the redox polymer on a glassy carbon electrode (0.07 cm² area) with V₂⁺-PVA/FNR 129/19 mgc m⁻² in absence (black) and in presence (red) of 9.5 mM NADP⁺ at 2 mVs⁻¹. C) NADPH detection by means of UV/Vis spectroscopy from the electrolyte after bulk electrolysis of V₂⁺-PVA/FNR 21/15 µg cm⁻² on a 1 cm² Toray paper modified with active and denatured FNR. Background (black), temperature-denatured FNR (dashed gray line), directly deposited FNR (15 µg cm⁻²) without hydrogel (solid gray line), active FNR in the redox active hydrogel (red). D) Catalytic currents for NADP⁺ reduction as a function of the film thickness (expressed as the amount of deposited polymer and enzyme). All measurements were done with stirring in Tris-HCl (100 mM), KCl (100 mM), pH 8 under argon atmosphere.](angewandte.org)
The electrocatalytic properties of the modified electrodes are defined by the electroactive film thickness\cite{16} and therefore a uniform film morphology is desired.\cite{17} The electroactive film thickness distribution was quantified using an electroanalytical method based on linear sweep voltammetry.\cite{18} The relative standard deviation in thickness of a thin film of V\textsuperscript{2+}-PVA/FNR (8/1.1 \(\mu\)g cm\textsuperscript{-2}) was 80\% (Figure S6) which reflects a significantly higher degree of homogeneity in comparison to previously reported viologen modified polymer films.\cite{17}

In the presence of NAD\textsuperscript{+}, the electrodes modified with V\textsuperscript{2+}-PVA/film generate cathodic catalytic current with a half-wave potential (\(-442\) mV vs. SHE) near the formal redox potential of the viologen (Figure 1B). This indicates that the viologen moiety mediates the electron transfer between the electrode and the FNR. A maximum in catalytic current of 290 \(\mu\)A cm\textsuperscript{-2} was obtained for electrodes modified with a ratio of V\textsuperscript{2+}-PVA to FNR of 129/19 \(\mu\)g cm\textsuperscript{-2} in the presence of 9.5 mM NAD\textsuperscript{+} (Figure 1B). The bioelectrocatalytic NAD\textsuperscript{+} reduction can also be operated in presence of oxygen (Figure S8 and Table S2). While the rate for NADPH production remained significant, the competing \(\text{O}_2\) reduction by the redox-active polymer\cite{19,20,21} strongly decreased the faradaic efficiency. Therefore, the bioelectrocatalytic NAD\textsuperscript{+} reduction was carried out under inert atmosphere in analogy to previously reported electrochemical NAD(P)H regenerating systems.\cite{10,22–24}

The effect of the film thickness on the catalytic current was tested by modulating the total amount of polymer and enzyme used for electrode modification while maintaining their previously optimized ratio constant\cite{19} (Figure 1D and Figure S8B). The catalytic current for NAD\textsuperscript{+} reduction increases with polymer and enzyme amount up to a total loading of 60 \(\mu\)g cm\textsuperscript{-2} and is mostly independent of loading for values above 100 \(\mu\)g cm\textsuperscript{-2}. According to theoretical kinetic models of thin electrocatalytic films\cite{16,25} the initial linear regime can be assigned to current limited by catalyst loading while the thickness-independent currents are due to electron transfer and/or catalysis limitations within the film. For this reason, the maximum bioelectrocatalytic output of the system in terms of both catalytic current and average turnover frequency is reached at the transition between the two regimes at an amount of enzyme and polymer around 100 \(\mu\)g cm\textsuperscript{-2}.

The apparent Michaelis–Menten constant (\(K_M\)) of 2.4 \pm 0.3 mM was obtained when fitting the steady-state catalytic current response (at \(-595\) mV vs. SHE) to the NAD\textsuperscript{+} concentration (Figure S2A,B) using the approximate analytical expression for a reaction within a uniform biocatalytic film limited by the substrate amount\cite{19} (see SI 6). The obtained value is two orders of magnitude higher than those reported for other FNRs in solution (7–30 \(\mu\)M).\cite{15} Substrate depletion within hydrogel films under catalytic turnover is generally the reason for increased values of the apparent Michaelis constant.\cite{20} The V\textsuperscript{2+}-PVA/FNR-modified electrode also reduces NAD\textsuperscript{+} with a similar \(K_M\) (3.6 \pm 0.4 mM) but at about three-fold lower catalytic currents (Figure S22C,D), highlighting the substrate specificity of the FNR for NAD\textsuperscript{+}.

The bioelectrocatalytic reductive system was further scaled up by drop-casting V\textsuperscript{2+}-PVA/FNR onto a Toray paper electrode of 1.0 cm\textsuperscript{2} geometrical area. The film incorporation onto a high-surface-area electrode showed stability comparable to the one on glassy carbon electrodes (Figure S9B). This high film stability was confirmed by monitoring the absorbance of the electrolyte before and after cycling. We detected the absorbance of the polymer corresponding to 22\% of the initially deposited amount on the electrode. Most of the lost polymer was released before and early into the cycling, and the remaining polymer (78\%) was highly stable as shown by the absence of further loss between 250 and 500 cycles (Figure S9C and Table S3). Addition of NAD\textsuperscript{+} resulted in an increased cathodic catalytic current (Figure S9A) and generation of NADPH, as demonstrated spectrophotometrically by the appearance of a strong absorbance with a maximum at 340 nm in the UV/Vis spectrum of the bulk of the electrolyte (Figure 1C). In contrast, no catalytic current or NADPH production was detected when FNR was directly deposited on the bare electrode without polymer (Figure S10), when using polymer only (Figure S7A), or when temperature-denatured FNR was immobilized (Figure S7B–E), confirming that the catalytic cofactor reduction takes place via a redox hydrogel-mediated and enzyme-catalyzed process.

At optimal film compositions (V\textsuperscript{2+}-PVA/FNR 21/15 \(\mu\)g cm\textsuperscript{-2}, Figure S9D,E) the NADPH production rate was 1.9 \pm 0.2 \(\mu\)moles cm\textsuperscript{-2} h\textsuperscript{-1}. The turnover frequency (TOF) of the system, calculated as moles of NADPH produced per mole of total amount of FNR per second, was 1.2 \pm 0.1 s\textsuperscript{-1} with 10 mM NAD\textsuperscript{+} at pH 8 (see SI 5). This is three times higher than values obtained with FNR working through a direct electron transfer mechanism when directly immobilized on an ITO electrode (0.4 s\textsuperscript{-1})\cite{15} which highlights the benefits of improved electron transfer to the enzyme when immobilized in the redox hydrogel. The apparent \(K_M\) value was 4.4 \pm 0.9 mM, which was comparable to values achieved with the glassy carbon electrode (Figure S22E,F). The faradaic efficiency of the system was 98 \pm 3\%, as quantified by the charge consumed during 30 min of electrolysis and the amount of NADPH formed (Figure S11). Taking into account the 220 mV of overpotential used for NADPH formation, we obtain an energy efficiency of 82\% for the cathodic half-cell.\cite{27}

To test whether the system was able to fuel NADPH-dependent enzymes, we next coupled glutamate dehydrogenase (GLDH), as well as alcohol dehydrogenase (ADH) to the system after bulk electrolysis. Either enzyme was able to consume the electrochemically formed NADPH to completion (Figure S12), demonstrating that our setup generates the functional NADPH cofactor with high specificity and could be in principle used to operate different NADPH-dependent enzymes.
Enoyl-CoA carboxylase/reductase (Ecr) enzymes belong to a new class of NADPH-dependent reductive carboxylases which catalyze the stereoselective fixation of CO\textsubscript{2} into diverse enoyl-CoAs yielding (2\textsuperscript{S})-alkylmalonyl-CoA as product.\textsuperscript{28,29} Members of this family belong to the fastest CO\textsubscript{2}-fixing enzymes described to date and have been implemented in vitro and light-powered cyclic multi-enzyme cascades for the continuous fixation of CO\textsubscript{2} into organic acids and complex natural products.\textsuperscript{30,31} Although these systems have already demonstrated that they provide a promising route for the production of complex chemicals directly from CO\textsubscript{2}, they are bound to the supply of external chemical energy or to biological energy systems (i.e., photosynthetic membranes).

Thus, an important step towards harnessing the unique capabilities of Ecrs is a setup that would allow to continuously provide these enzymes with reducing equivalents from renewable electricity, which would dramatically expand their application potential ex vivo. To realize this goal, we aimed at coupling the Ecr homologue crotonyl-CoA carboxylase/reductase (Cc) from \textit{Methylobacterium extorquens} to the developed bioelectrochemical NADP\textsuperscript{+} reduction platform to continuously incorporate CO\textsubscript{2} into crotonyl-CoA (Figure 2A). We co-immobilized Ccr on a glassy carbon electrode in the viologen-modified hydrogel (V\textsubscript{2+}-PVA/FNR/Ccr) in a 1:1 mass ratio with FNR. CO\textsubscript{2} was supplied to the reaction through the addition of HCO\textsubscript{3}\textsuperscript{-} and carbonic anhydrase in the electrolyte. The system was first characterized through cyclic voltammetry.

An increase in catalytic current was observed after the sequential addition of crotonyl-CoA to the electrolyte containing NADP\textsuperscript{+} and CO\textsubscript{2} (Figure 2B), which was attributed to enhanced local cofactor recycling of the NADP\textsuperscript{+} formed by Ccr during catalysis. The matching potentials of the redox active polymer and of the catalytic wave indicated a viologen-mediated catalytic process. When temperature-inactivated FNR was immobilized in the film, no catalytic current was observed (Figure S13A,B) and no product was detected, demonstrating that Ccr activity was directly driven by FNR-based NADPH regeneration.

Co-immobilizing enzymes of a multistep catalytic process locally concentrates the biocatalysts in a small volume. The small distances between the catalysts can provide an advantage by allowing the fast diffusive mass transport of intermediates and cofactors between active sites, leading to higher reaction rates\textsuperscript{32} and thus enhanced electrocatalytic properties.\textsuperscript{7} Such confinement effect was observed when comparing a system with Ccr co-immobilized with FNR in the hydrogel,
versus Ccr freely diffusing in solution (Figure S13C,D). In spite of adding a 21-fold higher amount of Ccr when the second enzyme was present freely diffusing in solution, the steady-state catalytic currents were established faster and the absolute values for the catalytic currents were more than double for the co-immobilized Ccr. Confinement also affected overall productivity; while the co-immobilized system showed 30% yield after one hour of electrolysis, only 3% yield was reached with Ccr in solution.

The amount of ethylmalonyl-CoA generated after one hour of electrolysis was used to determine the faradic efficiency for the CO₂ fixation as 92 ± 6% (Figure S14). Taking into account the 220 mV of overpotential used for (2S)-ethylmalonyl-CoA formation, we obtain an energy efficiency of 77% for the reductive carboxylation (using the NADP⁺/NADPH redox potential as reference). A product rate of formation of 1.6 ± 0.4 μmol cm⁻² h⁻¹ and a TOF of 1.2 ± 0.3 s⁻¹ were calculated for the generation of ethylmalonyl-CoA starting from 1 mM crotonyl-CoA. Using a previously established enzyme assay the stereochemistry of the product was confirmed as (2S)-ethylmalonyl-CoA, while the (2R)-diastereomer was below the detection limit (Figure S15).

Prolonged operation of the system was tested by multiple additions of crotonyl-CoA (Figure 2C). The biocatalytic film maintained substantial activity for over 40 hours of continuous electrolysis, although we observed slow enzyme deactivation or film degradation over the course of the experiment (Figure S17). These experiments also showed that over time the product spectrum was shifted from ethylmalonyl-CoA (Figure S18B). The use of crotonyl-CoA in the low mM range leads to a decrease of one order of magnitude in the apparent Kₘ for NADP⁺ (20 μM), adding to the benefits associated with the co-immobilization of the enzymes as a confined cofactor regeneration system. The apparent Kₘ for crotonyl-CoA is 1.5 mM when using NADP⁺ at concentrations of 1 mM (Figure S18B).

Aiming to optimize the total turnover number (TTN) for NADP⁺, we performed full electrolysis in presence of 2 mM crotonyl-CoA (a concentration above the apparent Kₘ) and low NADP⁺ concentrations (2–200 μM, Figure S19). The unreacted substrate and products were quantified using HPLC–MS after terminating the electrosynthesis (Table S4). In spite of yielding only 12% product, the system could be operated at NADP⁺ concentrations as low as 2 μM NADP⁺ leading to a TTN of 117, demonstrating cofactor use at catalytic amounts. Increasing the NADP⁺ concentration to 20 μM significantly increased the yield to 61% while reaching a TTN of 61 after 7 hours of reaction. A further increase in the NADP⁺ concentration up to 200 μM did not strongly impact product yield despite higher reaction rates. Low cofactor concentrations are generally targeted to reach high TTNs and thus for economically competitive cofactor regeneration.[34] When using fragile and complex substrates or products, the total duration of the electrosynthetic process is also an important criterion. In our system, the hydrolysis rate of the substrate (Figure S20), the long-term stability of the film (Figure S17), and the cofactor stability[35,36] impose a maximum reaction time of about one day. This requires high reaction rates that can in principle be achieved with NADP⁺ concentrations as low as 20 μM without compromising the TTN excessively.

Finally, we scaled up the system into a two-compartment cell (H-Cell with 2 mL volume on the cathode compartment) using a 1.0 cm² Toray paper electrode (Figure S21) with 4.2 mM of crotonyl-CoA (8.4 μmol, 7 mg). Aiming to secure a maximized conversion of this valuable substrate into ethylmalonyl-CoA, we used 500 μM of the NADP⁺ cofactor. The reaction was completed after 25 h with an analytical yield of 57% and 91% faradaic efficiency according HPLC–MS. After using preparative HPLC, we obtained the pure product with an overall yield of 37%.

**Conclusion**

In this study, we established a bioelectrocatalytic system for NADPH recycling via mediated electron transfer through an immobilized FNR in a viologen-based redox hydrogel of adjusted reduction potential. We showed that the produced NADPH could be further used by co-immobilized Ccr for the reductive fixation of CO₂ into crotonyl-CoA. Together, the co-immobilized enzymes constitute an electrically driven cofactor regeneration and coupled CO₂ fixation system for stereoselective (2S)-ethylmalonyl-CoA formation at high rates. Our system provides the proof-of-principle for the electro-biocatalyzed CO₂-fixation into structurally complex substrates with high regio- and stereocontrol during C–C bond formation. Altogether, the biobattery system fosters the role of bioelectrochemical CO₂ fixation and represents an important step towards synthetic applications of NADPH-dependent enzymes. Further extension of the system through additional enzymes might allow the realization of more complex reaction cascades and pave the way for the development of advanced electrochemically driven synthetic C₁ chemistry platforms.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · carbon dioxide · CO₂ reduction · cofactor recycling · redox polymers

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Electrified CO₂ fixation switched on! In a bioelectrocatalytic approach an NADPH recycling module and a C–C bond formation module were co-immobilized within a viologen-based redox hydrogel. This enabled the regio- and stereoselective incorporation of CO₂ into crotonyl-CoA, yielding the most complex product known in bioelectrocatalytic CO₂ conversion so far.