

Electro enzymatic NADPH regeneration using the thioredoxin-based reaction cascade

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ABSTRACT

NADPH-dependent oxidoreductases are central to biocatalysis and industrial biotransformations, including alcohol dehydrogenases, imine reductases, monooxygenases and ene-reductases. However, their application is often limited by the requirement of an efficient NADPH regeneration system. While NADH can be efficiently be regenerated by formate dehydrogenase on industrial scale, NADPH regeneration systems suffer from lower activities, sacrificial substrates and increased waste stream formation due to side products. The TR/Trx1 cascade from *Thermus thermophilus* enables *in situ* recycling of NADPH and is highly stable under alkaline conditions, elevated temperatures, and organic solvents, however produces oxidised dithiothreitol as an unavoidable side-product. To address this limitation an electrochemical approach was chosen to directly reduce NADP⁺ to NADPH in an electro enzymatic setup. In this work, we present the characterisation and application of a TR/Trx1-based electroenzymatic NADPH regeneration system immobilised on carbon felt electrodes. The enzymes were embedded in an agarose- or alginate-based immobilisation matrix, enabling operation in a bio electrochemical cuvette setup. An e-cuvette setup is developed for spectroelectrochemical measurements in a three-electrode system. NADPH regeneration was detected at only -450 mV vs. Ag/AgCl. A proof-of-concept reaction demonstrated functional coupling of electroenzymatic NADPH synthesis and downstream biocatalysis with an NADPH-dependent alcohol dehydrogenase.

1. Introduction

Today, enzymatic catalysis is an indispensable part of the pharmaceutical and chemical industries, as well as environmental applications [1–5]. Enzymes lower the activation energy of reactions by stabilising transition states, which allows to run reactions under milder conditions (ambient temperatures, neutral pH, atmospheric pressure) with often high chemo-, regio- and stereoselectivity [4]. Hence, enzymatic processes can be considered as sustainable alternatives to traditional chemical processes [6]. Oxidoreductases (EC 1) catalyse redox reactions, transferring electrons between molecules [7,8]. These enzymes are of particular interest to the chemical and pharmaceutical industries as they can facilitate highly selective transformations, such as the production of chiral products for agrochemicals and pharmaceuticals, which are difficult to achieve through chemical means [6,7]. However, oxidoreductases often depend on cofactors such as nicotinamide nucleotides (NAD(P)H), flavins (FADH₂, FMN), or coenzyme A for catalytic activity

[8]. Since these cofactors are costly and continuously consumed during catalysis, efficient cofactor regeneration systems are essential, particularly for industrial-scale biocatalytic processes [6]. NADPH-dependent oxidoreductases play a central role in biocatalysis, particularly in asymmetric redox reactions [7]. Industrially relevant examples of such enzymes include alcohol dehydrogenases (ADHs) [9], ene reductases (EREDs) [10], imine reductases (IREDs) [11] and monooxygenases (MO) [12]. To run these reactions, a variety of cofactor regeneration strategies have been developed, including enzymatic, electrochemical, photochemical, and hydrogen-driven systems [13]. A few examples for enzymatic cofactor regeneration systems are: formate dehydrogenase (NADH) [14], glucose dehydrogenase (NADH/NADPH) [15] and phosphite dehydrogenase (NADH) [16]. However, the majority of these systems are only suitable to regenerate NADH and not the even more expensive NADPH. One example of an enzymatic NADPH regeneration system is the TR/Trx1 reaction cascade, which consists of thioredoxin 1 (Trx1) and thioredoxin reductase (TR) derived from *Thermus*

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thermophilus (*T. thermophilus*) [17]. This cascade has previously demonstrated efficient NADPH regeneration activity and has been applied as an *in situ* cofactor regeneration system for different oxidoreductase-catalysed reactions [12]. In the cascade, DTT is used to reduce Trx1. This then reduces TR and catalyses the reduction of NADP^+ (Scheme 1 A). A major advantage of the TR/Trx1 system is its exceptional stability under harsh reaction conditions, including alkaline pH, elevated temperatures, and the presence of organic solvents [12]. However, like many enzymatic cofactor regeneration systems, the TR/Trx1 cascade produces side-products during catalysis. Dithiothreitol (DTT), which serves as the reducing equivalent in the reaction, is oxidised to *trans*-4,5-Dihydroxy-1,2-dithian, generating an unavoidable waste stream. In addition, DTT is considered as a toxic small molecule due to its reducing power. Similar issues are observed in other enzymatic regeneration systems, which produce by-products such as glucono- δ -lactone (glucose dehydrogenase), CO_2 (formate dehydrogenase), or phosphate (phosphite dehydrogenase) [16,18,19]. The formation of these by-products contributes to increased waste generation and consequently to a higher environmental factor (E Factor) of the process [20–22]. Therefore, alternative approaches such as electroenzymatic or hydrogen-driven cofactor regeneration systems have gained increasing attention as strategies for developing greener biocatalytic processes [23].

Bioelectrochemical systems (BES) offer several advantages over conventional enzymatic regeneration systems, including the elimination of sacrificial substrates, lower raw material consumption, reduced by-product formation, and improved atom efficiency. In addition, fewer side-products simplify downstream processing and contribute to more sustainable bioprocessing. Since direct electrochemical NADPH regeneration often results in the formation of non-bioactive 1,6-NADPH isomers, electro enzymatic approaches represent an attractive alternative. By combining the high efficiency of electrochemical systems with the selectivity of enzymatic catalysis, more energy- and resource-efficient regeneration processes can be developed.

In this work, we present the characterisation of the TR/Trx1 system in a BES for electroenzymatic NADPH recycling. Our work was motivated by the findings of Hamill et al., who demonstrated the direct electrochemical characterisation of a thioredoxin reductase derived from *Thermoplasma acidophilum* [24]. In their study, TR was immobilised on a pyrolytic graphite edge electrode, and direct electron transfer (DET) between the enzyme and electrode was investigated using cyclic voltammetry.

In electroenzymatic systems, both the electrode material and the immobilisation strategy strongly influence catalytic performance. In the present work, we selected an inexpensive carbon felt working electrode and compared enzyme immobilisation using either an alginate [25]- or agarose-based matrix [26,27]. We aim on investigating the electro enzymatic NADPH synthesis using the TR/Trx1 cascade (Scheme 1 B and C) and present a proof of concept for mediated electron transfer with Trx1 as mediator (MET). The proof-of-concept reaction is based on the electro enzymatic synthesis of NADPH from NADP^+ followed by the addition of NADPH-dependent ADH to examine the generation of the biologically active 1,4-NADPH and measure product formation.

2. Methods

2.1. Chemicals and hardware

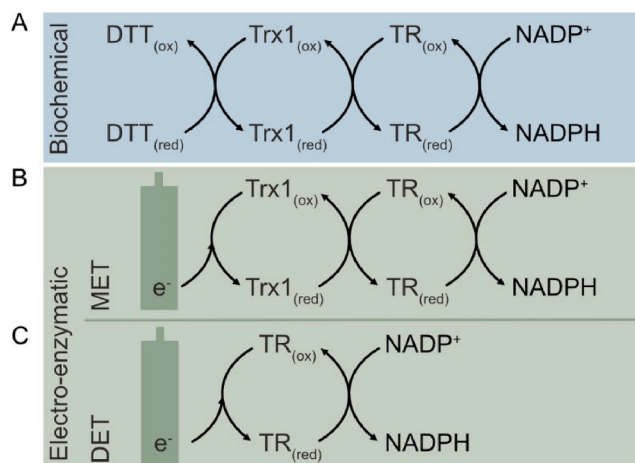
Chemicals were purchased from Carl Roth and Sigma-Aldrich. Photometric enzyme assays were conducted with an Agilent Cary 60 UV-Vis photometer and all electrochemical measurements were performed with a Gamry 1010 B. All 3-D printed objects were printed with a Prusa i3 MK3S with PLA filament.

2.2. Protein expression and purification

Thioredoxin reductase (UniProt Q72HD8) and thioredoxin 1 (UniProt Q72HU9) are located in a pET52b(+) expression vector and expressed with *Escherichia coli* (*E. coli* BL21(DE3) (NEB). For purification, all constructs included a C-terminal His10-tag (Table SI 1). Both proteins are produced according to the protocol by Loderer et al. [17] A 20 ml lysogenic broth (LB) preculture (shaking flask with baffles) with 100 $\mu\text{g}/\text{ml}$ ampicillin (Amp) grown overnight at 37 °C and 180 rpm was used to inoculate two 500 ml LB (+Amp) main cultures (shaking flask with baffles), which was grown to an OD_{600} of 1 at 37 °C and 130 rpm. For TR, the culture was cooled on ice for 10 min, then induced with 0.1 mM IPTG and grown for 20 h at 20 °C at 130 rpm. Trx1 was also induced with 0.1 mM of IPTG and grown for 20 h at 37 °C. Cells were harvested through centrifugation (4000 $\times$ g, 4 °C, 10 min) and stored at -20 °C for further use. For cell lysis, frozen cell pellets were thawed and resuspended in 15 ml lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 8.0), DNase I (Sigma-Aldrich), lysozyme (Sigma-Aldrich), MgCl_2 and a protease inhibitor tablet (cOmplete™ EDTA-free, Roche) were added and the suspensions incubated in an orbital mixer for 60 min at 10 °C. Mechanical lysis was performed with sonication (2 $\times$ 5 min, 80% amplitude). Lysates were centrifuged (20.000 $\times$ g, 4 °C, 60 min) and the supernatant filtered (0.45 μm) before performing IMAC purification (HisTrap™HP 1 ml, Cytiva). Proteins were eluted with elution buffer (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0). Following IMAC purification, a buffer exchange (50 mM TrisHCl, 300 mM NaCl, pH 7.5) was performed with a desalting column (HiTrap™ desalting, 2 $\times$ 5 ml, Cytiva), the final fractions combined and stored in 20% glycerol at -80 °C for subsequent experiments. ADH from *Lactobacillus brevis* (LbADH) was produced as described elsewhere [12]. Protein quantification was performed using Bradford assay with a BSA calibration curve and SDS-PAGE analysis using Mini-Protein TGX Stain-Free Gels (4–20% gradient). SDS-PAGE gels were analysed using ImageJ software.

2.3. Enzyme activity assays

Enzyme activity assays were performed as described before [12]. These assays were performed with 0.002 mM Trx1, 0.0004 mM TR, 20 mM DTT and 1 mM NADP^+ in different buffer systems at pH 8.0. The absorbance at 340 nm was measured for 5 min to indicate the generation of NADPH.



Scheme 1. Comparison between biochemical and proposed electro-enzymatic reduction of NADPH with the TR/Trx1 cascade. **A** Biochemical reduction using DTT as reduction equivalent. **B** Mediated electron transfer with Trx1 acting as an electrochemical mediator for electron shuttling. **C** Direct electron transfer from the electrode to TR for direct NADPH regeneration.

2.4. Enzyme immobilisation and electrode preparation

Carbon felt electrodes (CFE) were cut into 1 cm² pieces (projected area 2 cm²) and connected with a titan wire. The connection was secured using a conductive glue (Conduct-C (Sigma-Aldrich)) and the electrode dried overnight. For alginate immobilisation a 1.5% alginate solution was immersed on the electrode and precipitated with 200 mM CaCl₂ solution for 20 min. For agarose immobilisation, agarose powder (2%) was added to 50 mM phosphate buffer pH 8.0 and heated in a microwave to dissolve. At 60 °C TR and Trx1 were added to the soluble agarose and mixed briefly. TR and Trx1 were added with a concentration of 0.008 mM and 0.02 mM respectively. Prior to enzyme immobilisation the electrode was weighed. Then the electrode was put onto a microscope slide and the melted agarose spread on it. To ensure a smooth surface a second microscope slide was put on the electrode (Figure SI 1). As soon as the agarose gel was set, the electrode was cut out and weight again to determine the amount of bound agarose, hence the amount of theoretically bound enzyme.

2.5. Cuvette setup and spectroelectrochemical measurements

Following the concept of Gemünde et al. we established a spectroelectrochemical system, to measure simultaneously absorbance and electrochemical data in cuvettes [28]. For this purpose a 3D-printed cuvette cap (Figure SI 2) was designed, to facilitate a three-electrode setup inside of a standard 3 ml cuvette (Fig. 1). Platinum wire was used as the counter electrode and an Ag/AgCl reference electrode (Pine Research) was used as a reference electrode. The e-cuvette system was degassed with N₂ prior and during the experiments. All electrodes were incubated in phosphate buffer (100 mM, pH 8.0) for 10 min prior to the CV measurements to wash out non-bound protein. Bradford assay with a BSA calibration curve was performed to measure the amount of non-bound protein. Potentiostatic measurements at variable potentials against the Ag/AgCl reference electrode were performed to characterise electron transfer (ET) behaviour. The Faradaic efficiency (FE) is defined as the fraction of the total charge that is actually used to produce the desired chemical reaction, here NADPH in an electrochemical process (1). Amount of NADPH formed (n_{NADPH}), number of electrons transferred (z), Faradaic constant (F) and amount of transferred charge (Q) are used to calculate FE.

$$\text{FE} [-] = \frac{n_{\text{NADPH}} [\text{mol}] \cdot z [-] \cdot F \left[\frac{\text{C}}{\text{mol}} \right]}{Q [\text{C}]} \quad (1)$$

2.6. Proof of concept reaction

A one-pot two-step (1pot2step) process was performed as a proof of

concept for NADPH synthesis and downstream usage. In the first step electroenzymatic NADP⁺ to NADPH formation was performed and photometrically detected in the cuvette set-up for 3 h at −450 mV vs. Ag/AgCl. In the second step, 0.01 mM LbADH and 10 mM acetophenone (ACP) was added to the reaction. The enzymatic conversion of acetophenone to 1-phenylethanol (1-PEA) was run in 1 ml for 24 h at 30 °C and 900 rpm.

2.7. Analytical methods

ACP and 1-PEA were extracted from the reaction medium with ethyl acetate and dried with anhydrous MgSO₄. ACP and 1-PEA were analysed and identified by GC-MS (Agilent). The GC method for the detection of ACP and 1-PEA was: 1 min hold at 40 °C, then 35 °C/min till 250 °C with a 4 min hold. Helium was used as carrier gas and the GC is equipped with an Agilent 122-7032 DB-WAX GC Column (30, 0.25 mm, 0.25). The split ratio was 100:1 with an inlet heater at 240 °C. The MS is run in Scan mode with a mass range of 50–550, a threshold of 150 and a scan speed of 1562 u/s.

3. Results and discussion

3.1. Expression and biochemical activity

TR and Trx1 from *T. thermophilus* were expressed in *E. coli* BL21 (DE3). Both proteins were isolated from the soluble protein fraction after cell disruption using IMAC with a following buffer exchange. SDS-PAGE confirmed the presence of both proteins at their expected sizes (Figure SI 3). A purity of 87% and 91% was achieved for TR and Trx1, respectively. To confirm the biochemical activity of the protein-enzyme cascade, a reductive assay with DTT as a reduction equivalent was performed. Different buffer systems at a pH of 8.0 and a concentration of 100 mM were tested to identify the most suitable chloride-free buffer for subsequent electrochemical measurements (Fig. 2). Control experiments without Trx1 or TR revealed the functionality of the complete cascade. The phosphate and HEPES buffers show the highest specific activities of (1.1 ± 0.1) U/mg and (1.00 ± 0.05) U/mg, respectively. These results are comparable to the values reported elsewhere [12]. All following electrochemical experiments are performed in 100 mM phosphate buffer pH 8.0.

3.2. Immobilisation and electrochemical characterisation

Carbon felt was used as the electrode material for protein immobilisation. We started by comparing the thermal stability of the alginate and agarose matrix on the CFE. Since the activity of the TR/Trx1 reaction cascade increases with rising temperatures, the prepared alginate and agarose electrodes were heated to 40 °C [17]. The alginate matrix

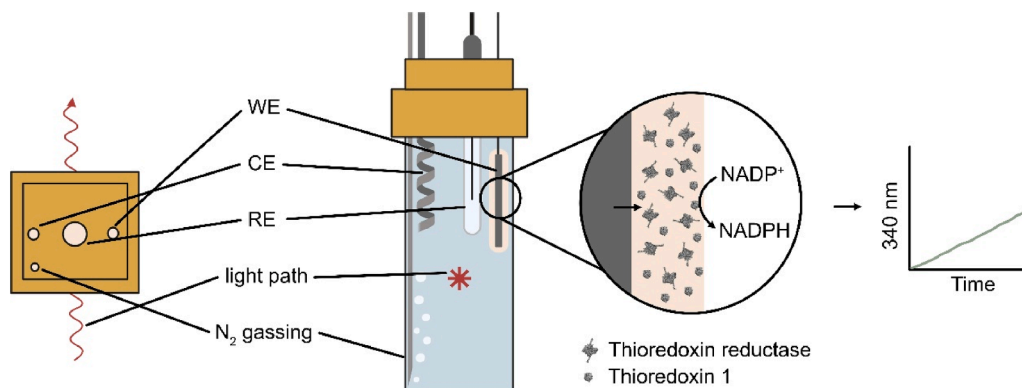


Fig. 1. Spectroelectrochemical e-cuvette for online measurement of NADPH regeneration. WE – working electrode (carbon felt with immobilised TR/Trx1), CE – counter electrode (platinum wire), RE – reference electrode (Ag/AgCl).

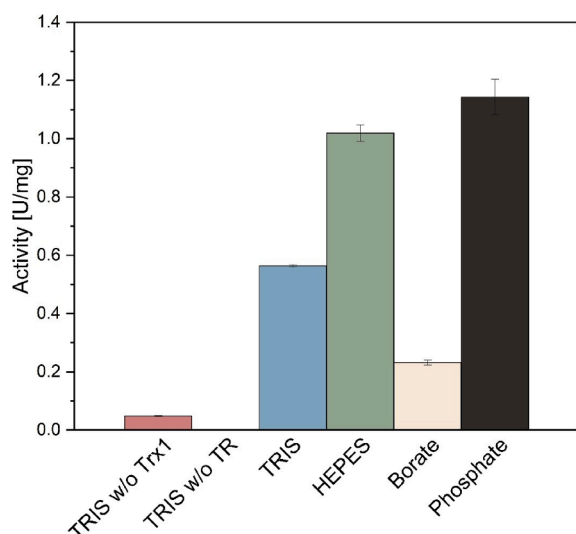


Fig. 2. Buffer screening with the Trx1/TR cascade using different chloride-free buffer systems at pH 8.0 with a concentration of 100 mM. Specific activity [U/mg] for NADPH regeneration using DTT as reduction equivalent. Conditions: TRIS buffer without (w/o) Trx1 (red), TRIS buffer without (w/o) TR (yellow), TRIS buffer (blue), HEPES buffer (green), borate buffer (sand) and phosphate buffer (black). $n = 3$.

was found to be unstable due to gel matrix leakage (Figure SI 4), therefore further experiments were continued only with the agarose CFE (aCFE). Agarose is highly porous and stable at different temperatures and pH levels. This makes it a good matrix for immobilising proteins on CFE [29]. Next, protein bleeding from the TR/Trx1 immobilised aCFEs (TR/Trx1aCFE) was investigated by testing different agarose gel concentrations (Figure SI 5). No significant difference was detected between 1–2% agarose gels. About 80% of the total protein mass was still immobilised in TR/Trx1aCFE after 24 h. The observed protein loss is likely caused by the relatively small size of TR/Trx1 compared to the pore size of the agarose matrix, allowing the proteins to diffuse out of the gel. This limitation could potentially be reduced by introducing affinity tags to enhance protein retention within the agarose matrix [30].

Activity assays were performed in three set-ups, to analyse the influence of enzyme immobilisation on the activity of the cascade and investigate possible ET behaviour (Fig. 3). All measurements were performed in the e-cuvette and the absorption at 340 nm followed, to detect NADP^+ to NADPH conversion. In set-up I, TR and Trx1 were incubated with NADP^+ and DTT in solution, to confirm the biochemical activity prior immobilisation. Set-up II included the TR/Trx1aCFE, which was incubated with NADP^+ and DTT in solution to investigate the biochemical activity post immobilisation. In set-up III, TR/Trx1aCFE was incubated with NADP^+ in solution and an electric potential of -450 mV vs. Ag/AgCl was applied to the working electrode, to detect possible ET activity.

In solution, no NADPH formation could be detected without the addition of DTT as a reduction equivalent (Fig. 3A). Among immobilisation the reaction velocity decreased half, from (16.1 ± 0.9) $\mu\text{M}/\text{min}$ to (7.1 ± 0.6) $\mu\text{M}/\text{min}$. The specific activity decreases from 1.1 U/mg (in solution) to 0.88 U/mg (immobilised) corresponding to a ΔA_{spec} of 77% (based on the molecular mass of TR). This could be a result of either the un-oriented random immobilisation of TR and Trx1 on the aCFE, which causes only a fraction of the proteins to be accessible by the DTT and NADP^+ (Fig. 3A and B). The reaction is now limited by mass transfer of the substrate into the agarose matrix. The same behaviour was observed for a cytochrome P450 immobilised on a indium tin oxide electrode [31]. Other reasons are enzyme inactivation among immobilisation due to conformational changes or the loss of protein due to diffusion out of the agarose matrix. NADPH formation was detected, when a potential of

-450 mV vs. Ag/AgCl was applied to the e-cuvette with a reaction velocity of (0.06 ± 0.02) $\mu\text{M}/\text{min}$ (Fig. 3C). This is the first indication that ET between TR/Trx1 and the CFE is feasible, and that this cofactor regeneration process could be powered by an electrical current. Without a potential being applied, a slight increase in absorbance at 340 nm could be detected (0.01 ± 0.01) $\mu\text{M}/\text{min}$. This is most likely due to protein leaking from the aCFE as TR contains a FAD (flavine-group) in its active side, which absorbs at 340 nm [32].

To further characterise the ET behaviour of the TR/Trx1 cascade, we performed chronoamperometric measurements at varying electrochemical potentials within the range of -200 mV to -600 mV vs. Ag/AgCl (Fig. 4). In addition, we included a negative control without immobilised protein, as well as electrodes with TR solely immobilised on the aCFE (TRaCFE). With the TRaCFE possible DET between the electrode and TR can be detected. For each condition the amount of NADPH formed in 45 min was calculated from the absorbance increase at 340 nm.

Within the tested range, potentials from -300 mV to -500 mV yielded NADPH formation with the TR/Trx1aCFE that exceeded that in the control experiment without an applied potential. The highest NADPH concentration was achieved at -450 mV vs. Ag/AgCl, at 2.6 ± 0.2 μM . No significant NADPH formation could be achieved in the control experiments, as well as with the TRaCFE. This led us to hypothesise that Trx1 acts as an electron shuttle between the electrode and TR, functioning as an electrochemical mediator within the investigated system. Following this hypothesis electrons are transferred to Trx1, the disulphide bond (C31, C34, Figure SI 6) is reduced and subsequently Trx1 reduces TR (Scheme 1 B). This results in the conversion of NADP^+ to NADPH.

This study represents the first demonstration of an electrochemical drive for the TR/Trx1 cascade. The optimal applied potential of -450 mV vs. Ag/AgCl corresponds to approximately -253 mV vs. the standard hydrogen electrode (SHE). This value lies above (less negative than) the standard redox potential of the free $\text{NADP}^+/\text{NADPH}$ couple ($E^\circ = -320$ mV vs. SHE, corresponding to approximately -517 mV vs. Ag/AgCl), indicating that NADPH formation in this system is not governed by direct electrochemical reduction of free NADP^+ . At pH 8.0 the formal potential shifts to about -350 mV vs. SHE. At the high concentration of the oxidised NADP^+ the Nernst potential of the $\text{NADP}^+/\text{NADPH}$ couple is about -274 mV vs. SHE. However, direct electrochemical reduction of NADP^+ is kinetically strongly hindered and typically requires a large overpotential, so a potential close to the equilibrium value is not sufficient to drive it at a measurable rate [33]. Instead, the measured optimal potential of -253 mV vs. SHE falls within the range of redox potentials reported for the FAD/FADH₂ and active-site disulphide/dithiol couples of TRs from other organisms (approximately -240 to -300 mV vs. SHE) and for the active-site disulphide couple of Trx1 from other organisms (-230 to -270 mV vs. SHE) (Table 1). This agreement supports a mechanism in which the applied potential drives reduction of the protein-bound redox centres of the Trx1 rather than the free nicotinamide cofactor. Potentials more positive than -300 mV vs. Ag/AgCl do not provide sufficient reducing power to efficiently reduce the disulphide bond in Trx1. In contrast, applying potentials more negative than -500 mV vs. Ag/AgCl may promote undesired and unspecific side reactions at the electrode surface, resulting in lower amounts of detected NADPH. The disulphide bond of Trx1 exhibits a redox potential of $E^\circ = -230$ to -270 mV vs. SHE, corresponding to approximately $E^\circ = -427$ to 467 mV vs. Ag/AgCl (Fig. 5, Table 1) [34,35]. This is in agreement with the electrochemical results presented here and indicates that only a small overpotential is required for efficient electron transfer within the electrochemical set-up. Trx1's disulphide couple sits right in reach of the applied potential, so electrons flow from the electrode surface to Trx1, converting its disulphide into the reduced form. Reduced Trx1 then reduces the disulphide pair of TR, reducing the bound FAD cofactor. For comparison, DTT, which was used as the reducing agent in the biochemical activity assays,

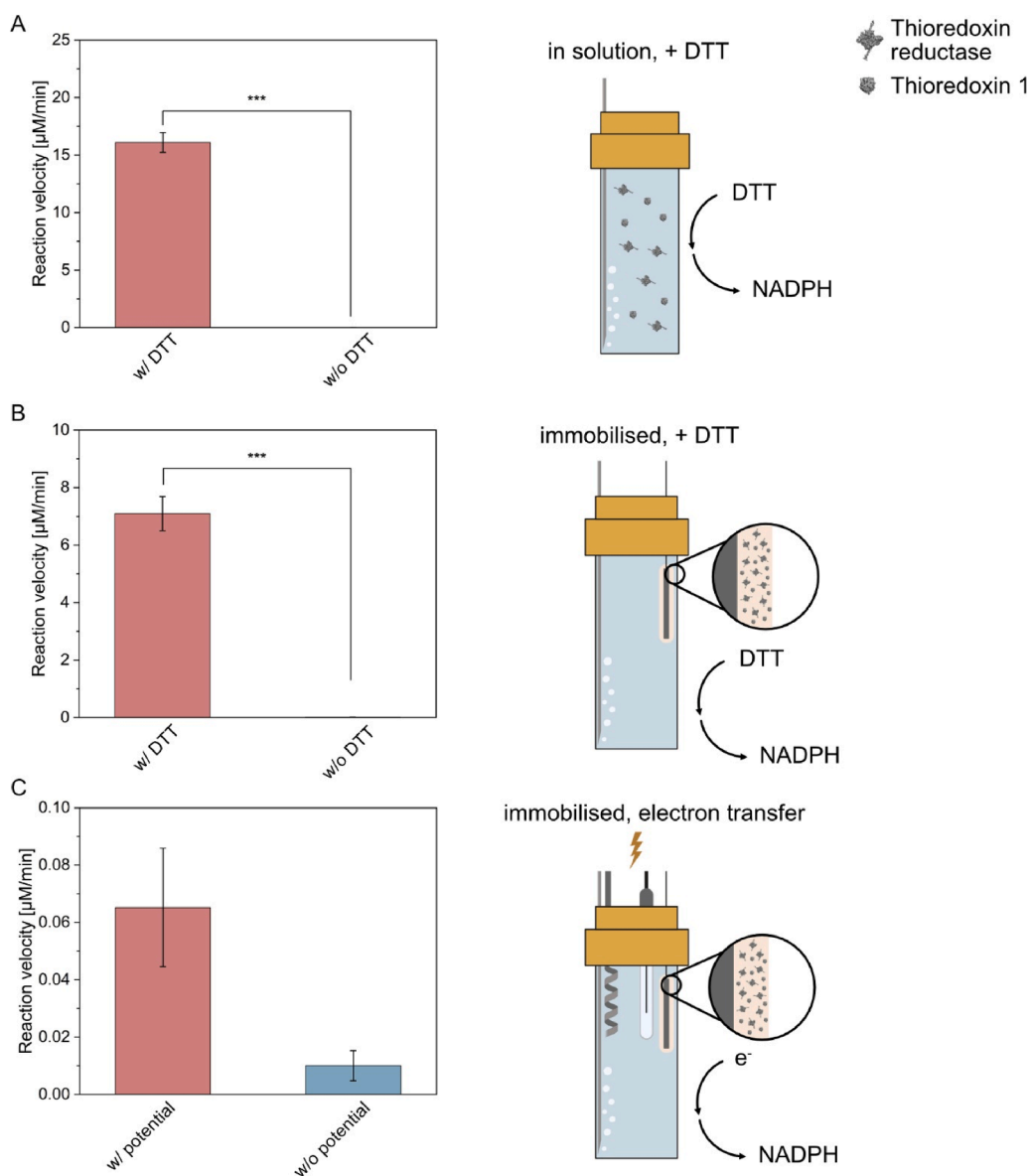


Fig. 3. TR/Trx1 activity assay in three different set-ups with free and immobilised TR and Trx1. **A** Biochemical activity assay with DTT (w/ DTT), NADP^+ and TR/Trx1 in solution (0.0004 mM TR, 0.002 mM Trx1). Negative control without DTT (w/o DTT). **B** Biochemical activity assay with DTT and NADP^+ in solution and immobilised TR/Trx1 (0.008 mM TR, 0.02 mM Trx1). Negative control without DTT (w/o DTT). **C** Electro enzymatic activity assay with NADP^+ in solution and an applied voltage of -450 mV vs. Ag/AgCl for the detection of ET between TR/Trx1 and the aCFE (0.008 mM TR, 0.02 mM Trx1). Negative control without an applied potential (w/o potential). One-way ANOVA with pairwise comparison using Tukey's HSD post-hoc test $p \leq 0.001$. $n = 3$.

has a redox potential of $E^0 = -330$ mV, corresponding to approximately $E^0 = -530$ mV vs. Ag/AgCl at pH 7.0 [36]. The significantly more negative potential of DTT highlights its strong reducing capability. The FAD/ FADH_2 couple in TR from *E. coli* has a calculated $E^0 = -263$ mV vs. SHE, corresponding to approximately $E^0 = -463$ mV vs. Ag/AgCl [37, 38]. The TR from *Thermatoga maritima* (*T. maritima*) has a reported $E^0 = -230$ mV vs. SHE, corresponding to approximately $E^0 = -427$ mV vs. Ag/AgCl [39]. No NADPH formation was detected in experiments using the TRaCFE, indicating that the active site of TR cannot be efficiently accessed by electrons delivered from the CFE. These findings suggest that DET between the electrode surface and TR is not achievable with the immobilisation strategy employed in this study (Scheme 1 C). A likely explanation is the structural organisation of the enzyme itself. The catalytically active FAD cofactor is embedded within the protein matrix, while the surrounding amino acid residues form a rather insulating outer layer around the active site. In the enzyme's mechanism, electrons are transferred to the FAD via a disulphide pair (C150, C147) near the active

site, which itself exchanges the electrons with Trx1 (Fig. 5). However, this disulphide pair is also buried within the protein and DET from the electrode was not possible in the shown experiment. The closest distance from Flavin moiety of FAD to the proteins surface is in the range of 5–8 Å. As a result, the electrode-derived electrons are unable to effectively penetrate the protein shell and reach the buried redox centre. Consequently, electron transfer to the active site is hindered, preventing electrochemical reduction of the enzyme and subsequent NADPH formation. To achieve efficient DET between TR and the electrode surface, alternative immobilisation strategies or targeted protein engineering approaches may be required. Modifying the enzyme structure to position redox-active sites closer to the protein surface, or introducing surface residues that facilitate electron hopping, could improve electron transfer efficiency [40,41]. In addition, optimised immobilisation methods that provide a more favourable enzyme orientation at the electrode interface may help to reduce the electron-transfer distance between the electrode and the disulphide pair [24]. Such approaches enhance electron transfer

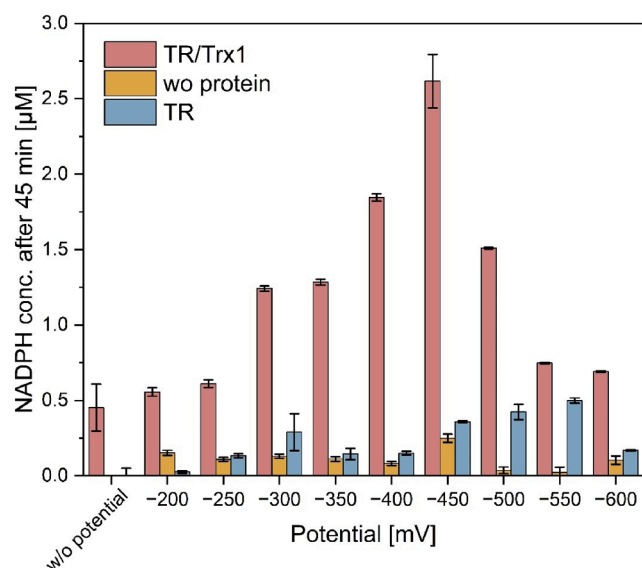


Fig. 4. Characterisation of NADPH formation in e-cuvettes with varying potentials (vs. Ag/AgCl). NADPH concentration after 45 min of catalysis is shown for TR/Trx1 (red), negative control without protein (w/o protein) (yellow) and TR only (blue) is shown as a function of the electrochemical potential. All aCFE were 2% agarose gels. $n = 3$.

between TR and the electrode surface and thereby promote efficient DET [42–44].

Compared to the total amount of NADP^+ present in the system, only a 0.26% yield was achieved during the 45 min of electroenzymatic catalysis. To identify potential limiting factors within the setup, the counter electrode was redesigned and replaced with a larger one. The platinum foil counter electrode was adjusted to match the size of the aCFE, ensuring that the overall reaction is no longer restricted by the limited surface area of the counter electrode or by the rate of the anodic reaction (Figure SI 7). However, no increase in yield was observed across the tested potential range. These results indicate that, under the investigated conditions, the reaction is not limited by the size of the counter

electrode. Other possible limitations of the system include diffusion limitations, protein leakage, and electron transfer limitations. Diffusion limitations may arise from the transport of substrates through the agarose matrix to reach the immobilised enzyme, as well as from the diffusion of the generated product back out of the matrix for photometric detection. Since NADPH is relatively small compared to the pore size of the agarose matrix, diffusion effects are expected to be relatively minor in the presented system. Nevertheless, local substrate depletion near the electrode surface and accumulation of reaction products within the matrix may still occur. These effects can reduce the overall reaction rate and lead to delayed response times. As previously demonstrated (Figure SI 5), protein leakage represents another significant drawback of the system. The leakage of Trx1 and TR from the aCFE decreases both catalytic productivity and long-term operational stability. One possible strategy to improve protein retention within the aCFE is the use of affinity tags, such as carbohydrate-binding modules (CBM-tags), which can strongly and selectively bind to the agarose matrix, thereby enhancing enzyme immobilisation and reducing protein loss [30]. Electron transfer limitations represent another important factor affecting the efficiency of the system. Electron transfer between the electrode, Trx1, and TR is hindered by the intrinsically insulating nature of the enzymes themselves, as well as by the non-conductive agarose matrix. As a result, proteins located further away from the electrode surface may not efficiently participate in the catalytic process. Consequently, only a small fraction of the immobilised proteins is likely to remain electrochemically active, leading to reduced current generation and lower product formation. One possible strategy to overcome this bottleneck is the development of a conductive agarose matrix [47]. Introducing conductive properties into the gel could facilitate electron transport throughout the matrix and enable a larger proportion of immobilised proteins to actively participate in catalysis.

To gain a deeper understanding of the electrochemical efficiency of the cofactor regeneration system, the FE of the TRTrx1aCFE was determined (Formula 1 and Figure SI 8). Therefore, a higher FE indicates that fewer competing side reactions occur within the system. The obtained results show that the FE decreases with increasing applied potentials. At the previously identified optimal potential for NADPH regeneration, a FE of 11% was achieved. These findings suggest that, although NADPH formation is favoured at this potential, a significant

Table 1

E^0 of relevant redox couples converted to $E^{0'}$ vs. Ag/AgCl.

Redox couple	Host/System	$E^{0'}$ vs. SHE [mV]	$E^{0'}$ vs. Ag/AgCl [mV]	Reference
Free $\text{NADP}^+/\text{NADPH}$	Standard biochemical value, pH 7	−320	−517	Berg, Tymoczko, Stryer (2019) <i>Biochemistry</i> (8th ed.) [45]
Trx1 disulphide	Different species	−230, −270	−427 to −467	Krause et al. (1991) [46] Watson et al. (2003) [35]
TR active site disulphide	<i>E. coli</i>	−263, −280	−460	O'Donnell et al. (1983) [37] Lennon et al. (1996) [38]
TR	<i>T. maritima</i>	−230	−427	Valiauga et al. (2020) [39]

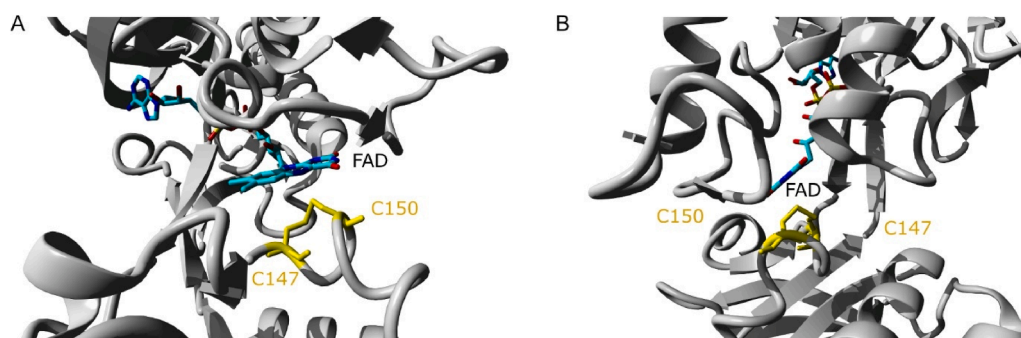


Fig. 5. Alpha fold model of the TR from *T. thermophilus* in A front and B side view (AF-Q72HD8-F1). The cofactor FAD is labelled and represented elemental colours. The reactive disulphide pair C147/C150 is represented in yellow.

proportion of electrons is still consumed by side reactions rather than being utilised for productive cofactor regeneration. Compared to other studies investigating electroenzymatic NADPH regeneration, the FE obtained in this work is relatively low (Table 2). The cuvette system is ideally suited to screening purposes. However, side reactions at the counter electrode cannot be entirely ruled out and a small amount of oxygen may still enter the system, influencing the FE. Further studies should therefore focus on optimising the reactor. Previous studies commonly employed immobilised ferredoxin-NADP⁺ reductase (FNR) on various electrode materials to regenerate NADPH either via MET [48, 49] using viologens or through DET [50,51]. In these systems, electrode potentials of −660 mV or lower were typically required to achieve efficient cofactor regeneration. In contrast, the system presented in this study enables NADPH regeneration at less negative electrode potentials. Operating at milder potentials reduces the required energy input and therefore represents a potential advantage in terms of energy efficiency and overall process sustainability, despite the comparatively lower FE observed.

3.3. Proof-of-concept

As a proof-of-concept, the TR/Trx1-aCFE system was applied for NADPH synthesis in a one-pot two-step (1pot2step) electro enzymatic process (Fig. 6). In the first step, the e-cuvette setup was used to electrochemically regenerate NADPH from externally supplied NADP⁺. In the second step, the NADPH-dependent *Lactobacillus brevis* ADH (LbADH) and the substrate ACP were added to catalyse the reduction of ACP to 1-PEA. A two-step approach was chosen because the e-cuvette setup operates as an undivided electrochemical cell and is not completely gas-tight. Under these conditions, 1-PEA could be re-oxidised at the counter electrode and both substrate and product can evaporate, making simultaneous substrate conversion during electrochemical NADPH synthesis impractical. Separating the process into two sequential steps therefore enabled more controlled substrate handling and improved the feasibility of the proof-of-concept reaction.

Continuous NADPH formation was observed during the photometric measurements (Figure SI 9), indicating ongoing electroenzymatic cofactor regeneration throughout the experiment. After 3 h of electroenzymatic catalysis, a theoretical NADPH amount of 9 μM was calculated based on the change in absorbance, corresponding to a theoretical conversion of 0.9%. Following the subsequent LbADH-catalysed reaction, the formation of 1-PEA was confirmed by GC-MS analysis (Figure SI 10, 11). After 24 h of enzymatic catalysis, 1-PEA was detected, demonstrating the successful coupling of electrochemical NADPH synthesis with downstream biocatalytic product formation. No product was detected in the aCFE negative control.

It should be noted that this experiment represents only a limited proof of concept. Because electrochemical NADPH synthesis and the LbADH-catalysed reduction were performed sequentially rather than simultaneously, NADPH was consumed only after electrolysis had ended, and no continuous NADPH recycling was demonstrated. The low theoretical conversion of 0.9% and the stoichiometric rather than

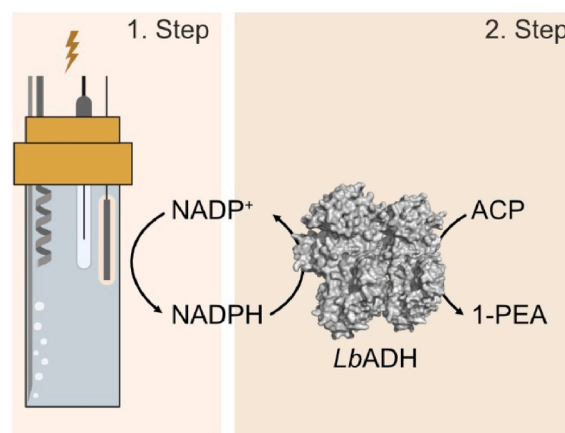


Fig. 6. Schematic overview of the 1pot2step electro enzymatic process as a proof of concept for NADPH regeneration. The first step includes electroenzymatic regeneration of NADPH in the e-cuvette set-up. The second step compromised the LbADH catalysed reaction of ACP to 1-PEA.

catalytic use of the cofactor further show that the system still needs to be developed into a gas-tight, compartmentalised or otherwise integrated setup. Only such a setup would allow true *in situ* cofactor regeneration coupled to simultaneous substrate conversion.

4. Conclusion

In conclusion, this work demonstrates the successful immobilisation of enzymes on carbon felt electrodes and highlights the advantages of agarose as an immobilisation matrix, showing superior stability compared to the commonly reported alginate-based systems. Furthermore, MET for NADPH regeneration within the TR/Trx1 cascade was demonstrated for the first time, representing an important step towards efficient electroenzymatic cofactor, in particular NADPH, recycling. The results support the hypothesis that Trx1 acts as an electrochemical mediator, enabling electron transfer from the electrode surface to the enzyme system. In addition, an optimal applied potential (−450 mV vs. Ag/AgCl) for maximum NADPH formation was identified, providing valuable insight into the electrochemical behaviour of the cascade. A proof-of-concept reaction further confirmed the feasibility of coupling electrochemical NADPH regeneration with enzymatic catalysis under operational conditions. Particularly noteworthy is that the process operates at comparatively mild, less negative potentials than many literature-reported electrochemical catalytic systems, which often require significantly more reducing conditions. This reduced overpotential is advantageous in terms of energy efficiency, selectivity, and compatibility with sensitive biocatalytic systems, underlining the potential of this approach for sustainable applications in biocatalysis and industrial synthesis. Further research could characterise the electron transfer in the cascade directly and determine the proteins redox potentials to identify the underlying electron transfer mechanism.

Table 2

Comparison of experimental configurations and performance metrics for electro enzymatic NADPH regeneration.

Enzyme/Protein	Immobilisation strategy	DET/MET (+Mediator)	Potential vs. Ag/AgCl [mV]	Electrode material	FE [%]	Reference
FNR	Free enzymes	MET (Methyl viologen mediator)	−750	Graphite rod	51 ± 4 (bias-free, <i>in vitro</i>)	Bachar et al. (2025) [48]
FNR	Viologen-modified hydrogel (V ²⁺ -PVA)	MET (Redox hydrogel)	−772	Glassy carbon	92 ± 6	Castañeda-Losada et al. (2021) [49]
FNR	Oxidised multi-walled carbon nanotubes	DET	−700	Carbon paper	74.8	Housseini et al. (2025) [50]
FNR	Adsorption	DET	−660	Indium tin oxide (ITO)	Not stated	Siritanaratkul et al. (2017) [51]
TR/Trx1	Agarose matrix	MET	−450	Carbon felt	11 ± 6	This study

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used DeepL Write and Claude in order to refine wording and grammar checking. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Anna-Lena Drommershausen: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jonas Gail:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Christoph Loderer:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Dirk Holtmann:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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Data availability

Data will be made available on request.

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